Muscle Abnormalities in cancer patients: Exploring the biological characteristics of skeletal muscle of cancer patients

by

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Abstract

Computed tomography-derived muscle measures of sarcopenia and low muscle radiodensity have been associated with poor outcomes in cancer patients, however, the biological characteristics of the muscle in this population are not well understood. This research was conducted to first evaluate muscle measures of fiber size, fiber type, fat content and differentially expressed genes in males and female cancer patients. Secondly, the association between muscle radiodensity and fat content and distribution in rectus abdominis of cancer patients was determined. Lastly, the prognostic significance of biological features of skeletal muscle of cancer patients was assessed. To determine the biological features of muscle of cancer patients, *rectus abdominis* biopsies were collected from cancer patients undergoing open abdominal surgery scheduled as part of their clinical care. Clinical features, patient demographics, CT-derived muscle features, morphological features and muscle gene expression data of a large sample size provides a point of reference for these measures in the literature which are poorly described to date given published poor quality studies that commonly are characterized by sampling bias, discrepancies between sample collection and processing techniques, and failure to acknowledge factors that influence muscle biology. In our patient cohort (n=190), sexual dimorphism was evident in CT-derived muscle features and genes associated with muscle catabolism/anabolism. In the second study, it was hypothesized and also demonstrated that a negative association exists between muscle radiodensity and muscle triglyceride content [r = -0.409, $p< 0.001$ (N=75)]. Also, wide variation in muscle radiodensity was observed across the *rectus abdominis* (coefficient of variance = 3 to 61%). This is an important finding since, in the literature, a single abdominal CT image is used to define muscle radiodensity and then used to compare with biological features of the muscle. Also, the heterogeneous distribution of lipids in muscle compartments was observed suggesting that

different and potentially multiple mechanisms contribute to fat infiltration in muscle. Prognostic significance of fatty acid composition of skeletal muscle phospholipids was determined (n=35), to reveal that depletion of arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in muscle phospholipids are risk factors for death in cancer patients [(HR, 0.29, 95%CI, 0.09-0.9), (HR, 0.23, 95%CI, 0.06-0.81) and (HR 0.23, 95%CI, 0.06 to 0.83), respectively]. Significantly lower content of ARA and a trend of lower EPA and DHA in skeletal muscle phospholipids were observed in patients who died within one year of surgery compared to who lived longer. It is concluded that there is a wide variation in the distribution of fat across the muscle and low muscle radiodensity might not be only because of fatty infiltrations. Other factors contributing to the radiodensity of muscle need to be explored. Also, the skeletal muscle phospholipid composition data we assembled suggests that alterations in membrane fatty acids can be prognostic in cancer patients. Further studies are required to confirm if the changes in fatty acid composition are systemic or selective and to explore the mechanisms involved.

Preface

All the work presented in the present dissertation was conducted at University of Alberta. Human skeletal muscle biopsies were collected after obtaining written informed consent from patients and this was approved by the Health Research Ethics Board of Alberta (HREBA)-Cancer Committee. Post-biopsy profiling of samples and collection of patient information was conducted under ethics protocol ETH-21709: *The Molecular Profile of Cancer Cachexia*. This work was funded by the Canadian Institutes of Health Research (259704 and 142427).

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Chapter 5. This chapter is written in a manuscript format and part of this chapter will be prepared for submission to the Journal of Lipid Research. I was responsible for performing the fatty acid analysis, statistical analysis, and drafting the manuscript. Liquid chromatography-mass spectrometry was conducted by Irma Rivas Serna. Dr Vera C. Mazurak, Dr. Vickie E. Baracos and Dr. Michael T. Clandinin provided critical input. Abha Dunichand-Hoedl assisted with CT image analysis.

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List of Abbreviations

ALA	Alpha-linolenic acid
ARA	Arachidonic acid
ATP	Adenosine Triphosphate
BMI	Body mass index
CL	Cardiolipin
CT	Computed Tomography
DEXA	Dual-energy X-ray absorptiometry
DGLA	Dihomo-gamma-linolenic acid
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid
EMCL	Extramyocellular lipids
EPA	Eicosapentaenoic acid
FAP	Fibro/adipogenic progenitor
GC	Gas Chromatography
GLA	Gamma-linolenic-acid
HPLC	High performance liquid chromatography
HR	Hazard Ratio
HU	Hounsfield Unit
IMAT	Intermuscular adipose tissue
IMCL	Intramyocellular lipids

L3	Lumbar 3
LA	Linoleic acid
LC-MS	Liquid chromatography–mass spectrometry
MRI	Magnetic resonance imaging
MUFA	Monounsaturated fatty acids
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PL	Phospholipids
PPAR	Peroxisome proliferator-activated receptor
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
SAT	Subcutaneous adipose tissue
SCD	Stearoyl CoA desaturase
SFA	Saturated fatty acids
SM	Sphingomylein
SMI	Skeletal muscle index
SREBP	Sterol regulatory element binding proteins
TG	Triglyceride
TLC	Thin layer chromatography
UCP3	Uncoupling protein 3
VAT	Visceral adipose tissue

Chapter 1: Literature Review

1.1 Skeletal Muscle in health

Skeletal muscle is the largest organ in the human body comprising 25-50% of total body weight in normal weight individuals (Abe et al., 2018). Skeletal muscle function is classically defined as the ability to perform muscular contractions and generating external mechanical force to enable physical activities of daily living and exercise. From a metabolic perspective, the role of skeletal muscle includes serving as storage for amino acids and carbohydrates, and consumption of the majority of oxygen and fuel used during physical activity. Of relevance to disease prevention and health, reduced muscle mass and altered composition of muscle impair the body's ability to respond to stress and chronic illness (Wolfe, 2006). Robust skeletal muscle mass and composition is essential for maintaining whole-body homeostasis and health (Ebner et al., 2015; Wolfe, 2006).

1.2 Body Composition Analysis

As the importance of skeletal muscle in many normal functions of the body was recognized, techniques to quantify muscle mass and composition were developed. Amount of muscle can be quantified by urinary creatinine 24 h excretion, ultrasonography, whole-body K counting by neutron activation, computed tomography (CT), magnetic resonance imaging (MRI), magnetic resonance spectroscopy and dual-energy X-ray absorptiometry. Development and validation of methods for evaluating human body composition were first conducted over the life span and in ageing and obesity (Baumgartner et al., 1998; Ross et al., 2000; Steiner et al., 2002). From this body of literature, a relationship between various tissues and health outcome was established. For example, visceral and subcutaneous adipose tissue accumulation is associated with diabetes (Goodpaster et al., 1997; Goodpaster et al., 2003). Intramuscular lipid content is associated with

obesity (Goodpaster et al., 2000b). Type-2 diabetes and frailty are associated with a decrease in skeletal muscle mass (Evans, 1995; Park et al., 2009).

1.2.1 Computed tomography imaging

Computed tomography (CT) is regarded as the gold standard method in the evaluation of human body composition. Use of CT scan for the purpose of body composition analysis in healthy individuals is not possible due to the exposure to high radiation dose (Duren et al., 2008; Heymsfield et al., 1997). In patient populations for whom CT scans are performed for diagnostic and treatment planning purpose such as oncological population, CT images can be opportunistically used for body composition analysis and research (Mourtzakis et al., 2008).

CT employs X-rays that are attenuated by body tissues. The radiodensity of tissues is represented in the image as a linear grey scale (Figure 1-1A). Each pixel in the two dimensional CT image has a specific number that corresponds to a specific location within the patient. The numerical value of each pixel within the matrix corresponds to a specific level of grey within the image. These values are called Hounsfield Units (HU). Hounsfield Unit is normally defined on a linear coefficient attenuation scale where 0 equals the HU of water and -1000 equals the HU of air (Sjöström, 1991). Depending on the physical property (density) and electron mass ratio, attenuation characteristics of different tissues differ and this makes CT capable of distinguishing tissues in the body. With the increase in density of tissue, there is an increase in the linear attenuation coefficient.

Fat and muscle are discriminated in CT imaging anatomically, as well as by their widely different radiodensity values. Segmentation of specific tissues is based on their anatomical features and then demarcated and quantified using predefined Hounsfield units for specific tissues in

conjunction with manual correction. Attenuation values used for adipose tissue segmentation are: -150 to -50 for visceral adipose tissue (VAT), and -190 to -30 for intermuscular adipose tissue (IMAT) and subcutaneous adipose tissue (SAT) (Heymsfield, 1997; Miller et al., 1998) (Figure 1-1B). Cross-sectional areas of each tissue are subsequently calculated by multiplying the number of pixels by the surface area of the pixel for a given tissue (Heymsfield et al., 1997). In the case of skeletal muscle, HU range from 0 to 100 HU was originally used for quantification (Mitsiopoulos et al., 1998). Mean radiodensity of 30 HU is considered as the lower boundary for normal muscle (Goodpaster et al., 2000b; Lee et al., 2005) and is based on two standard deviations below the mean attenuation of muscles of young healthy persons (Goodpaster et al., 2000b). Low muscle radiodensity is defined in the range from 0 to +29 HU by authors of some studies (Goodpaster et al., 2000b; Lee et al., 2005) while other authors included -29 to +30 HU. In cancer research, generally accepted attenuation range for the muscle in cancer research is from -29 HU to 150 HU (Heymsfield et al., 1997; Mourtzakis et al., 2008).

Mitsiopoulos et al. (1998) established CT imaging as a reference method for skeletal muscle measurement. CT estimates of skeletal muscle in leg and arm of 119 cadavers were highly correlated with corresponding cadaver values ($r=0.99$, standard error of estimates = 3.8 cm^2, $p<0.001$) (Mitsiopoulos et al., 1998). Diagnostic CT images usually do not encompass the whole body, thus there was a need to determine landmarks that represent whole-body composition. Shen et al. (2004) examined the relationship between single cross-sectional abdominal magnetic resonance images (MRI) and the total volume of skeletal muscle and adipose tissue in 328 healthy adults to reveal that the highest correlation between skeletal muscle area and skeletal muscle volume was located at the 3rd lumbar vertebra (L3).

1.2.2 Skeletal muscle radiodensity

Computed tomography (CT), proton magnetic resonance spectroscopy (1H-MRS), and magnetic resonance imaging (MRI) have provided non-invasive methods to explore and measure deposition of fat in and around muscles (Schrauwen-Hinderling et al., 2006). These techniques can be used to measure lipid content over a larger muscle area as compared to histological methods. CT scans are becoming common approaches used for measurement of lipids in skeletal muscles. In the early nineties, researchers used CT imaging to describe muscle composition and reported an association between reduced skeletal muscle radiodensity and reduced muscular strength in patients with neurological disease, muscular dystrophy and diabetes (Kelley et al., 1991; Liu et al., 1993; Nordal et al., 1988). Recently, muscle radiodensity have been studied in cancer populations and is emerging as an important factor in determining clinical outcomes and survival.

To illustrate the variation in muscle radiodensity observed in two individuals, subject 1 (male, age 72) and subject 2 (male, age 77), an example is provided (Figure 1-2). Subject 2 with low radiodensity has a lower proportion of cross-sectional area of normal muscle as compared to subject 1 (13% vs 83%).

1.3 Skeletal Muscle lipid infiltration

Fat infiltration in the skeletal muscle, also known as myosteatosis, is the commonly associated with ageing, obesity, type-2 diabetes, renal disease, genetic muscle disorders, spinal cord injury, muscle injuries and recently with cancer (Cheema et al., 2010; Elliott et al., 2006; Freda et al., 2008; Goodpaster et al., 2000b; Goodpaster et al., 2005; Gorgey et al., 2007; Goutallier et al., 1994; Kelley et al., 2002). In one of the largest longitudinal studies in ageing, Delmonico et al. (2009) reported increased fat infiltration in 1678 elderly individuals with an age range of 70 to

79 years. The accumulation of fat in skeletal muscle, measured by CT scans, was a consistent characteristic of ageing irrespective of body weight change or change in subcutaneous adipose tissue. In males, an increase in fat in muscle ranged from 36% to 75% and in females, it ranged from 17 to 50%. In elderly, increased fat infiltration has been associated with loss of muscle strength (Goodpaster et al., 2001), poor function and a greater risk of mobility restriction (Visser et al., 2005). Fat infiltration has also been associated with insulin resistance and poor functional capacity across a number of disease states. In the Health ABC Study population, fat infiltration in thigh muscle was positively associated with higher levels of serum inflammatory markers (Beasley et al., 2009).

1.3.1 Location of lipid deposition in skeletal muscle

There are different depots of lipids in the muscle with varying health implications. Intermuscular lipid is located between the muscle groups and beneath the fascia. Extramyocellular lipid (EMCL) is located between the muscle fibers and intramyocellular lipid (IMCL) is present inside muscle fibers. Intermuscular lipid and extramyocellular lipid are deposited as adipose tissues whereas intramyocellular lipid deposited as microscopic lipid droplets (Figure 1-3). IMCL is a dynamic pool of lipids in muscle fibers and an important substrate for exercise in endurance athletes (Daemen et al., 2018). Excess IMCL deposition has been associated with insulin resistance, obesity, inflammation and muscle dysfunction (reviewed by Hamrick et al., 2016). Insulin resistance has been associated with predominant deposition of IMCL near the cell membrane and with large lipid droplet size (Daemen et al., 2018; Goodpaster et al., 2000b) and lipid droplet number (Tarnopolsky et al., 2006). Intermuscular lipids and EMCL accumulate as adipocytes around the muscle fibers and can originate from stem cell populations residing in skeletal muscle (Jia et al., 2018; Uezumi et al., 2014). EMCL has been reported in a variety of

muscle groups, including soleus, tibialis anterior and vastus lateralis (Larson-Meyer et al., 2008) and is noted for its non-uniform distribution across the length of a muscle (Wendling et al., 1996). Even in healthy muscle, EMCL is clustered in certain areas (Hwang et al., 2001; Vermathen et al., 2004), notably in the vascularized areas of the intermuscular connective tissue (Yudkin et al., 2005).

1.3.2 Mechanisms of skeletal muscle lipid infiltration

An increasing number of studies are reporting an association between skeletal muscle lipid infiltration and clinical outcomes in ageing and pathological conditions (Choi et al., 2016; Delmonico et al., 2009; Gueugneau et al., 2015; Miljkovic et al., 2016; Reinders et al., 2016). Several mechanisms may contribute to the accumulation of lipids within skeletal muscle (reviewed by Hamrick, 2016). As mentioned above, lipids accumulate in different compartments in skeletal muscle: Intermuscular, EMCL and IMCL. Different mechanisms can be responsible for these lipid depots. IMCL is stored within skeletal muscle fibers in discrete organelles termed lipid droplets, which are located near endoplasmic reticulum (site of lipid esterification) and mitochondria (site of lipid oxidation). Triglycerides (TG) are the major (60%) component of the lipid droplets along with cholesterol esters and other neutral lipids (Watt et al., 2012). Lipid droplets in muscle fibers usually serve as a lipid reservoir to provide energy and provide building blocks for membrane synthesis (Walther et al., 2012). Under conditions of increased energy demands (fasting or low-intensity exercise), TG turnover increases in lipid droplets without an increase in TG content, suggesting that lipolysis and lipid oxidation is matched by lipid uptake and lipogenesis (Guo et al., 2000). Accumulation of IMCL can be a result of an imbalance between lipid uptake, lipogenesis, lipolysis and lipid oxidation. Both animal and human studies in obesity and diabetic conditions suggest an increase in lipid uptake (Bonen et al., 2004; Luiken et al., 2001; Zhang et al., 2010) and

lower rates of lipid oxidation (Zhang et al., 2010). The increase in triglyceride synthesis rate and decreased oxidative capability could lead to IMCL accumulation (Zhang et al., 2010). The oxidative capability of muscle fibers is dependent on its mitochondrial function and/or content. Mitochondrial dysfunction has been associated with loss of muscle mass (Julienne et al., 2012; Wang et al., 2012) and increased IMCL in ageing (Peterson et al., 2012), obesity and diabetes (Montgomery et al., 2015). Peroxisome proliferator-activated receptor gamma, CCAAT/enhancer binding proteins and sterol regulatory element binding protein 1c isoform are the major transcriptional factors that regulate lipid metabolism including lipogenesis and lipolysis in skeletal muscle and are associated with excess? IMCL in animal models of high-fat diet induced diabetes (Chabowski et al., 2012; Ye et al., 2001).

Another pathway for lipid accumulation is adipogenesis. At the cellular level, muscle fibers are surrounded by several stem cell populations. The most well-defined stem cell population in skeletal muscle are satellite cells (reviewed by Dumont et al., 2015). Satellite cells are mainly responsible for myogenesis during the process of muscle regeneration (reviewed by Karalaki et al., 2009). Whether satellite cells can adopt and adipogenic fate is under debate (Scarda et al., 2010; Starkey et al., 2011). Other stem cell populations in skeletal muscle are fibro/adipogenic progenitors, multipotent mesenchymal progenitors and PW1+ interstitial cells. These are stem cells populations that can potentially contribute to adipogenesis within skeletal muscle (Pannerec et al., 2013; Uezumi et al., 2014). Depending on several environmental and cellular factors these multipotent progenitors can either develop into adipocytes (adipogenesis) or muscle fibers (myogenesis) (Pisani et al., 2010; Uezumi et al., 2014). In ageing, obesity and diabetes, adipogenic differentiation of skeletal muscle stem cells have been reported (Vettor et al., 2009).

1.4 Skeletal muscle in cancer: sarcopenia, low radiodensity and survival

Sarcopenia is an age-related condition defined as progressive loss of skeletal muscle mass and strength (Cruz-Jentoft et al., 2010). In older adults, reduced muscle mass and function are associated with mortality and physical disability (Arango-Lopera et al., 2013; Fielding et al., 2011; Landi et al., 2013). Loss of muscle is also a secondary feature of many chronic illnesses, chronic obstructive pulmonary disease (COPD), chronic renal failure, liver cirrhosis, HIV/AIDS, rheumatoid arthritis, diabetes and cancer (Cruz-Jentoft et al., 2010; Kim et al., 2017; Kim et al., 2010; Leenders et al., 2013; Park et al., 2009; Sayer et al., 2007). In surgical patients, low muscle mass has been recognized as a prognostic factor for both postoperative complications and death (Joglekar et al., 2015).

In cancer populations, sarcopenia is prevalent in 40% of patients at the time of diagnosis (Pamoukdjian et al., 2018; Prado et al., 2009, 2008; Tan et al., 2009). The observations that skeletal muscle abnormalities exist in cancer patients has fueled interest in determining the association of such abnormalities with clinical outcomes. Low muscle mass in cancer patients has been associated with shorter survival, chemotherapy toxicity, physical impairment, poor surgical outcome and poor quality of life (reviewed by Bozzetti, 2017; Kamarajah et al., 2019; Mintziras et al., 2018). Sarcopenia in lung and gastrointestinal cancer patients, defined by sex-specific cut-offs for CT derived skeletal mass index (SMI), was reported to be independently prognostic of survival (Martin et al., 2013; Prado et al., 2008). A recent study reported a two-fold higher risk for all-cause mortality (hazard ratio of 2.15, 95% CI 1.59–2.92) in colorectal cancer patients with muscle loss of 11% from the baseline compared to muscle loss of 5.7% (Brown et al., 2018). An association between reduced survival and sarcopenia has also been reported in advanced gastric cancer (Lee et al., 2018), oesophagal cancer (Järvinen et al., 2018), pancreatic (Tan et al., 2009),

biliary tract (Cho et al., 2017; Limpawattana et al., 2018) and adrenocortical carcinoma (B. S. Miller et al., 2012). Sarcopenia in patients with cancer has also been associated with surgical complications and toxicity from anti-cancer therapies (Bozzetti, 2017; Kamarajah et al., 2019)

Pathological infiltration of fat in the skeletal muscle, termed myosteatosis, is another abnormality of skeletal muscle that has been studied in ageing, obesity and insulin resistance and recently reported in cancer patients (Antoun et al., 2013; Goodpaster et al., 2000b; Hamrick et al., 2016; Martin et al., 2013). Low muscle radiodensity has been associated with triglyceride content in healthy and diabetic individuals (Goodpaster et al., 2000a). Based on this observation, low muscle radiodensity is interpreted as myosteatosis (Malietzis et al., 2016; O'Brien et al., 2018; Reinders et al., 2016; Stretch et al., 2018) Low muscle radiodensity was revealed in cancer patients in 2011 and 2013 with the emergence of CT imaging as a gold standard for body composition (Antoun et al., 2013; Martin et al., 2013; Sabel et al., 2011).

Low muscle radiodensity in cancer patients gained the interest of researchers as it has been shown to have prognostic value. Sabel et al. (2011) reported that psoas muscle radiodensity was significantly associated with disease-free survival ($p=0.04$) and distant disease-free survival ($p=0.0002$) in stage III melanoma patients. Psoas muscle radiodensity was reported as a predictor of surgical complications including infections, longer hospital stays and bleeding complications (Sabel et al., 2011). Independent of BMI, cancer patients who concurrently have weight loss, muscle wasting and low muscle radiodensity are likely to die 20 months sooner compared to patients with none of these conditions (Martin et al., 2013). When stratified in two groups based on median value of 21.4 months, patients with low muscle radiodensity had median survival of 14 months compared to patients with high muscle radiodensity with median overall survival of 29 months (Antoun et al., 2013). These findings are further supported by recent reports of an

association with presence of low muscle radiodensity and the development of major postoperative complications in cancer (Boer et al., 2016; Margadant et al., 2016). A recent observational study with large sample size has confirmed the prognostic value of low muscle radiodensity in non-small cell lung cancer (Sjoblom et al., 2016). Low muscle radiodensity in non-small lung cancer was independently prognostic for survival when adjusted for established prognostic factors, including skeletal muscle mass (Sjoblom et al., 2016). In a cohort of 763 colorectal cancer patients, low muscle radiodensity (defined by sex-specific cut-offs by Martin et al 2013) has been significantly associated with systemic inflammation quantified by neutrophil to lymphocyte ratio and albumin level (Malietzis et al., 2016). Therefore, both low muscle mass and radiodensity are associated with poor outcomes in cancer patients. However, the biology of skeletal muscle in cancer patients remains poorly characterized.

1.5 Biological characteristics of skeletal muscle in cancer

A large number of studies have reported an association between body composition, specifically sarcopenia and low muscle radiodensity, and poor survival, chemotherapy toxicity, increased risk of post-operative complications and systemic inflammation in cancer patients. These findings led to investigations of skeletal muscle at the biological level to determine mediators and pathways influencing skeletal muscle mass in patients with cancer (Fearon et al., 2012; Mueller et al., 2016). The majority of the reports that identified anabolic and catabolic pathways involved in skeletal muscle loss are from animal studies but studies on human samples are relatively scarce. Within human studies, the emphasis has been on muscle atrophy and not fat infiltration.

To our knowledge, there are 60 reports including characteristics of skeletal muscle biopsies in cancer patients. Out of 60 studies, 57 studies reported pathways and mediators associated with skeletal muscle mass and only three studies reported low muscle radiodensity in the muscle of

cancer patients. Studies on human skeletal muscle samples reported alterations in muscle morphology, increased inflammatory markers, increased proteolysis, autophagy, and the identification of differentially expressed genes in patients with low skeletal muscle mass (Johns et al., 2014; Narasimhan et al., 2017, 2018; Op den Kamp et al., 2012; Stretch et al., 2018). Studies in the 1970s were the first reports showing an increase in activity of lysosomal enzymes, decreased rate of protein synthesis and decreased capacity of glucose assimilation in rectus abdominis muscle of cancer patients as compared to controls (Lundholm et al., 1976; Scherstén et al., 1978). Most of the studies reported alterations in protein synthesis, protein degradation, morphology and apoptosis in muscle of cancer patients based on weight loss but very few compared muscle tissue characteristics with body composition. Only recently CT-derived measures of skeletal muscle mass have been used to investigate an association with biological features of the muscle in cancer patients (Johns et al., 2014; MacDonald et al., 2015; Narasimhan et al., 2017, 2018). Johns et al, (2014) reported smaller muscle fiber diameter and lower DNA content in the *rectus abdominis* of gastrointestinal and pancreatic cancer patients with sarcopenia (defined by sex-specific skeletal muscle index cut-offs of Prado et al, 2008) as compared to patients with high muscle mass. A recent retrospective cohort study reported genes related to lipid metabolism, glucocorticoid signalling and signal transduction that were significantly associated with sarcopenia and weight loss (Johns et al., 2017).

While pathways associated with muscle atrophy are well described, research on the biological characteristics of low radiodensity muscle in cancer is in an early phase with only three studies on human biopsies appearing in the literature so far. In the first report of fat in muscle of cancer patients, Stephens et al, (2011) evaluated rectus abdominis muscle by electron microscopy and reported an increase in intramyocellular lipid droplet numbers with increase in weight loss in

the muscles of 19 patients with upper gastrointestinal cancer as compared to 6 controls. No association between number of droplets and CT-derived measure of skeletal mass was observed (Stephens et al., 2011). In a recent study, transcriptomic analysis of rectus abdominis muscle of cancer patients revealed 772 differentially abundant transcripts associated with low muscle radiodensity (defined by muscle radiodensity <30HU) (Stretch et al., 2018). Differentially expressed genes were associated with cell death and survival, cellular function and maintenance and cell morphology (Stretch et al., 2018). Oxidative phosphorylation was the most significant canonical pathway associated with muscle radiodensity, with all 18 differentially abundant genes encoding protein complexes in the electron transport chain showing lower expression in the muscle. The results of this study suggests that the biological processes of muscle atrophy and low muscle radiodensity are independent based on gene signatures (Stretch et al., 2018). Another study reported a weak positive correlation between muscle radiodensity at L3 region and muscle protein content of rectus abdominis ($r=0.406$, $p=0.021$) (Ramage et al., 2018). Of note, muscle radiodensity is usually measured at one slice of lumbar 3 CT image composed of various muscles but a biopsy represents a very small portion of a rectus abdominis which might explain the weak correlation.

Interest in investigating skeletal muscle in cancer patients is increasing. The majority of studies including muscle biopsies from cancer patients were published in the last 10 years. Biopsies are collected during cancer surgery or percutaneously with local anaesthesia (Narasimhan et al., 2017; Op den Kamp et al., 2015; Stretch et al., 2018). Tissue handling and manipulation during biopsy collection and sample preparation can induce changes in the tissue that affect the interpretation of results (Chatterjee, 2014). In a recent study, gene expression patterns reflecting inflammation, atrophy and carbohydrate metabolism were compared in muscle collected at start

and end of the surgery. Gene expression of cathepsin-L (autophagy), Forkhead Box O1(atrophy), F-box protein 32 (atrophy) and tumour necrosis factor-α (inflammation) in rectus abdominis and vastus lateralis muscle were elevated by 7.5 to 16.5 fold at the end of surgery compared to the start of surgery (Varadhan et al., 2018). While the effects of age and sex are well established, comorbidities, medications, cancer type, chemotherapy and tumor stage can also have an influence on muscle metabolism (Edwards et al., 2014; Salvatore et al., 2014; Stephens et al., 2012).

1.6 Membrane phospholipids

Biological membranes are highly organized and dynamic structures whose composition determines its biophysical and physiological properties. Alterations in lipid composition of these membranes affects lipid-protein and receptor interactions thus influencing cell signaling and function (Watson, 2015). Biological membranes consist of a bilayer, mainly comprised of lipid molecules in addition to protein and carbohydrates (Lombard, 2014; Singer et al., 1972). The major types of lipids found in biological membranes are phospholipids, glycolipids, and cholesterol. Phospholipids make up 80-85% of biological membrane structure (Dufourc et al., 1992). Phospholipids consist of two fatty acid chains linked to glycerol and a phosphate group (Figure 1-4). The amphipathic nature of phospholipids naturally leads to bilayer formations with hydrophilic heads pointing outward and hydrophobic tails pointing inward. Two fatty acid chains attach to the first and second carbons of the glycerol molecule denoted as sn1 and sn2 positions, respectively. The head group varies between phospholipids, resulting in different phospholipid species (Hazel et al., 1990). Phosphate head-groups can be choline, ethanolamine, serine or inositol (Hazel et al., 1990). Four major types of phospholipids are phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylinositol (PI). PC and PE are the most abundant phospholipids in mammalian biological membranes, making up 45-55% and 15-25% of the total

membrane phospholipids, respectively (Mitchell et al., 2007; Vance, 2015). Lipid composition of mammalian cell membrane is shown in Table 1-1. Composition of phospholipids in the membrane confers its structure and fluidity. PE are ordered- crystalline-phase lipids and can pack closely in membranes, while PC are liquid-crystalline-phase lipids, and do not pack close in the membrane. A balance of phospholipid species within membrane is needed for optimal membrane fluidity. Cardiolipin (CL) is a phospholipid with two phosphorus and four fatty acid chains with linoleic acid accounting for 85% of fatty acids (Ritov et al., 2006). Sphingomyelin (SM) is comprised of a sphingosine backbone, choline head group, and 2 fatty acyl chains (R. E. Brown, 1998). Alterations in lipid composition of membrane change its fluidity and which effects folding and function of proteins embedded in the membrane (Vance, 2015).

1.6.1 Membrane Fatty acids

Biological membranes generally consist of fatty acid chains between 14 to 24 carbons in length, which may be saturated or unsaturated (Sato et al., 1988). Fatty acids with no double bonds are termed as saturated fatty acids (SFA). Unsaturated fatty acids are classified as monounsaturated fatty acids (MUFA, with one double bond) and polyunsaturated fatty acids (PUFA, with more than 1 double bond). Polyunsaturated fatty acids are further sub-classified as omega-3 fatty acids and omega-6 fatty acids depending on the position of first double bond relative to methyl end of the chain. For example, α-linolenic acid (ALA, 18:3n-3) has 18 carbons chain and 3 double bonds and first double bond is 3 carbons from the methyl end (omega) and is an omega- 3 fatty acid. Omega-3 fatty acids include ALA, docosapentaenoic acid (DPA, 22:5n-3), eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3). Omega-6 fatty acids include linoleic acid (LA, 18:2n-6), gamma-linolenic-acid (GLA, 18:3*n*-6), dihomo-gamma-linolenic acid (DGLA, 20:3*n*-6), and arachidonic acid (ARA, 20:4*n*-6). Humans cannot synthesize LA and ALA thus

these fatty acids are referred to as essential fatty acids and must be obtained from the diet (Spector et al., 2015). Humans can metabolize these fatty acids through series of desaturation and elongation steps and form longer and more unsaturated PUFAs (EPA, DHA, and ARA) However, conversion of essential fatty acids to long-chain PUFAs is inefficient as conversion rate is 1%-10% (Gerster, 1998).

In phospholipids, two fatty acid chains are linked to glycerol and triglycerides are comprised of three fatty acids esterified to a glycerol backbone. Triglycerides are mainly comprised of saturated and monounsaturated fatty acids with little to no polyunsaturated fatty acids (Table 1-1A and B). PUFAs with 20 to 22 carbon chains are mainly found in phospholipids at sn2 position. (Figure 1-4) (Stillwell, 2016).

1.6.2 Biological significance of fatty acids

The fatty acid composition of phospholipids determines the physicochemical properties of the membrane and influence membrane fluidity, permeability, plasticity and occurrence of microdomains (Abbott et al., 2010; Feller et al., 2005; Stillwell et al., 2003). Most commonly found fatty acids in membranes are palmitic acid (16:0), stearic acid (18:0), palmitoleic acid (16:1), oleic acid (18:1), LA, ARA, and DHA (Kuksis, 1978). Alterations in quality and quantity of different fatty acids in membrane regulate protein function and protein-lipid interactions affecting physiological and pathological responses (Lee, 2004; McIntosh et al., 2006). For example, increased incorporation of DHA in plasma membrane of mesenchymal stem cells has been shown to promote osteogenic differentiation with enhanced activity of protein kinase B (Levental et al., 2017). Increase in SFAs in red blood cells has led to reduce membrane fluidity and has been associated with diabetes (Pilon, 2016). This has been further supported by two large longitudinal

studies that reported individuals with highest SFAs in phospholipids of red blood cells were more likely to develop type-2 diabetes (Kröger et al., 2015, 2011).

The effects of ARA, EPA and DHA are predominantly dependent on incorporation into phospholipids (Calder, 2012). EPA and DHA compete with ARA for same enzymes during oxylipin synthesis (Calder, 2009). PUFAs are generally attached at sn2 position of phospholipids and are liberated by an enzyme, phospholipase A2 (Figure 1-4). Liberated fatty acids, ARA, EPA and DHA act as precursors for oxylipin production such as prostaglandins, thromboxanes and leukotrienes (Nakamura & Nara, 2004; Zamaria, 2004). Oxylipins are biological mediators of inflammation and metabolism, and are synthesized by enzymatic oxygenation of omega-6 and omega-3 fatty acids. ARA is a precursor of pro-inflammatory oxylipins of 2 and 4 series whereas EPA and DHA are precursors of anti-inflammatory oxylipins of 3 and 5 series. Saturated fatty acids also play role in inducing an inflammatory response. Studies have reported the role of saturated fatty acids in inducing cyclooxygenase (COX) which is a catalyst for the conversion of ARA to pro-inflammatory prostaglandins (Lee et al., 2004). Also, saturated fatty acids induce nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a key inflammatory factor (Lee et al., 2004). PUFAs from diet or membrane phospholipids can affect signalling pathways and interact with nuclear receptors thus influencing gene expression (Ntambi et al., 2001). Several studies demonstrated the direct role of PUFAs in modification of key transcription factors of lipid metabolism. Sterol regulatory element binding proteins (SREBPs), liver X receptor and peroxisome proliferator-activated receptor (PPARs) have been reported to be regulated by PUFAs (Nakamura, Cheon, et al., 2004). In animal studies, PUFAs suppressed SREBP-1c activity which resulted in downregulation of key lipogenic genes stearoyl CoA desaturase (SCD), fatty acid synthase and delta-6 desaturase (Clarke et al., 1976; Dobrosotskaya et al., 2002; Nara et al.,

2002; Tabor et al., 1999). PUFAs also act as a ligand for transcription factor PPARα and increase the activity of fatty oxidation enzymes in mitochondria and peroxisomes (Dallongeville et al., 2001; Nakamura, Cheon, et al., 2004). At the cellular level, fatty acids can influence gene expressions, metabolism and produce bioactive mediators that can lead to alterations at the tissue level and eventually at the whole body level.

1.7 Fatty acids in skeletal muscle

Phospholipid fatty acid composition can influence skeletal muscle regeneration, muscle atrophy, TG content of muscle, insulin signalling and expression of genes related to metabolism of carbohydrates and amino acids (Jeromson et al., 2017; Montgomery et al., 2017). There is extensive literature on the role of membrane fatty acids of skeletal muscle on insulin sensitivity. Significant alterations in skeletal muscle membrane phospholipid and triglyceride fatty acid composition have been reported in models of metabolic dysfunction (S. M. Park et al., 2017), high-fat diet induced obesity and insulin resistance (Montgomery et al., 2017). Insulin resistance of skeletal muscle developed through high-fat feeding containing saturated fatty acids which is prevented when omega-3 fatty acids were substituted in high-fat diet (Storlien et al., 1987; Wong et al., 1984). Since these first observations, several studies followed to show that improved insulin action was associated with an increased percentage of omega-3 fatty acids, DPA and DHA, acids in muscle membrane phospholipids. A number of studies with different study populations confirmed that higher proportions of SFA and lower proportions of polyunsaturated fatty acids in membrane phospholipids were related to insulin resistance in skeletal muscle (Borkman et al., 1993; Pan et al., 1995; Vessby et al., 1994). Also, TG content in skeletal muscle of obese patients contained more SFA compared to people with normal weight. Total SFA was positively associated with insulin resistance in skeletal muscle of obese people (Manco et al., 2000).

Alterations in phospholipid fatty acid composition of skeletal muscle have also been reported in models of Duchenne Muscular Dystrophy. Lower abundance of DHA, total omega-3 fatty acids and higher abundance of LA, total omega-6 fatty acids has been reported in skeletal muscle of dystrophin knockout mice (Tuazon et al., 2012). Linoleic acid and DHA were positively correlated with creatine kinase activity and muscle strength. Muscle injury has also been associated with changes in fatty acid composition and fatty acid related cellular processes. Using an established muscle trauma model, an injury was induced to muscle of left hind leg of mice. Muscle tissue from the injured leg showed a higher content of monounsaturated fatty acids and a lower content of polyunsaturated fatty acids compared with muscle tissue from control animals (Werner et al., 2018).

Few studies reported association between changes in specific phospholipid species of skeletal muscle and metabolic dysfunction. Clore et al. (2000) reported changes in fatty acid composition of PC in skeletal muscle of human are associated with insulin resistance. After administration of nicotinic acid, an insulin resistance-inducing agent, an increase in 16:0 and a decrease in 18:0, omega-3 fatty acids and total PUFAs were observed. DPA and total PUFA content of PC in skeletal muscle were negatively correlated ($r= -0.65$, $p<0.01$) with insulin concentration (Clore et al., 2000). Skeletal muscle PC and PE depicted significantly different fatty acid composition. PC mainly incorporated C16:0, C18:1 and C18:2, comprise 70% of total fatty acids whereas PE was mainly composed of C18:0, ARA and DHA (combined total of nearly 45% of total fatty acids) (Clore et al., 2000). In a recent animal study, high-fat feeding led to increased total muscle TG content with consistent decrease in DHA-containing PC species and ARA-containing PE species across the five mouse strains (Montgomery et al., 2017). Also, positional distribution of fatty acids within a phospholipid class and tissues can vary. In retina tissue, there

is abundant PC and PE with DHA at both sn-1/2 positions whereas in pulmonary surfactant there is abundant PC with C16:0 at both positions. This observation indicates that positional distribution and pattern of fatty acids in phospholipids are linked to biological roles (Nakanishi et al., 2010). Alterations in distribution of fatty acids in sn1 and sn2 positions can also affect fluidity of biological membranes (Saiz et al., 2001).

All of the work on demonstrating mechanisms by which membrane fatty acids affect skeletal muscle function has been conducted *in vitro* or in experimental systems. An *in vitro* study reported an association between alteration in membrane phospholipid composition of C2C12 muscle cells and increase in TG accumulation, membrane fragility and increased apoptosis (da Costa et al., 2004). In a recent study, alterations in membrane phospholipid fatty acid composition have been shown to have an effect on membrane-associated proteome (Jeromson et al., 2017). C2C12 cell lines, mouse skeletal muscle myoblasts, with higher EPA and DHA in membranes showed an alteration in membrane proteins associated with protein folding, oxidative metabolism and ribosome formation. Increasing EPA and DHA in membranes supports protein synthesis and reduces protein breakdown (Jeromson et al., 2017). Polyunsaturated fatty acids, EPA and DHA, have been shown to modulate sarcolemma ion channels and increase nerve conduction and thus improve contractile activity of the muscle (Lewis et al., 2015; McGlory et al., 2014). Arachidonic acid derived lipid metabolites, oxylipins, have an influence on protein turnover and muscle growth (Veliça et al., 2010). On the other hand, omega-3 fatty acids influence myogenesis by altering membrane lipid composition which promotes myoblast fusion. Omega-3 fatty acids are precursor of pro-inflammatory prostaglandins that promote an environment of muscle growth and regeneration (Endres et al., 1989; Oh et al., 2010; Rieu et al., 2009; Williams-Bey et al., 2014). As

no muscle biopsies were collected molecular mechanism and role of membrane fatty acid alteration in muscular strength in unknown.

In animals and humans, remodelling of membrane phospholipids of skeletal muscle and cardiac muscle have detrimental effects on mitochondrial function and has been associated with muscle wasting and muscle weakness (Mitsuhashi et al., 2011; Shaikh et al., 2014). In the only study in muscle of patients with cancer investigating skeletal muscle lipidomics, a decrease in cardiolipin and an increase in PC of skeletal muscle mitochondria phospholipids has been reported to be associated with decreased oxidative phosphorylation activity in mitochondria of wasting muscle, increased expression of mitochondrial uncoupling protein 3 (UCP3) and increased apoptosis (Antunes et al., 2014). These factors can reduce the ability of mitochondria to produce ATP and thus increase deposition of lipids in the skeletal muscle. Adiposity, measured as percentage body fat by hydrodensitometry, and fasting plasma insulin levels have also been positively associated with PUFAs (ARA, DGLA and DHA) in the phospholipid fraction of skeletal muscle (Antunes et al., 2014). Fatty acid composition of skeletal muscle in cancer patients has not been characterized in literature.

1.8 Fatty acids in cancer

Several studies reported changes in MUFAs, SFAs and PUFAs (omega-3 and omega-6) in various bio-specimens from patients with different cancer types (Table 1-2). Mostly, erythrocytes and plasma phospholipids were analyzed to determine membrane fatty acid composition. High SFAs (16:0, 18:0), MUFAs (16:1, 18:1) and low PUFAs (LA, ALA, ARA, EPA and DHA) were commonly reported in plasma and erythrocyte phospholipids of cancer patients as compared to healthy controls (Baró et al., 1998; de Castro et al., 2014; Macášek et al., 2012; McClinton et al., 1991; Mikirova et al., 2004; Mosconi et al., 1989; Okuno et al., 2013; Pratt et al., 2002; Zuijdgeest-

van Leeuwen et al., 2002). In contrast, some studies reported higher LA (Jolanta et al., 2018; Rahrovani et al., 2018b) and ARA (Amézaga et al., 2018; Jurczyszyn et al., 2014) in erythrocyte or plasma phospholipids in cancer patients as compared to healthy controls. Alterations in fatty acid compositions were independent of caloric or total fat intake (Amézaga et al., 2018; Pratt et al., 2002; Rahrovani et al., 2018a). While alterations in fatty acid composition in biospecimen of cance patients is reported compared to healthy individuals, these alterations has also been associated with clinical outcomes in variety of cancer types.

High EPA and DHA content in plasma phospholipids has been reported to be indicator of better prognosis in pancreatic cancer patients (Macášek et al., 2012). Also, lower proportion of ARA and a lower unsaturation index (sum of products of proportion of fatty acids and number of double bonds) in serum phospholipids has been observed in colorectal cancer patients who had disease progression as compared to patients who were disease free after one year follow-up (Jolanta et al., 2018). Murphy et al. (2012) reported lower concentration of ARA, EPA and DHA in plasma phospholipid of advanced cancer stage patients as compared to early stage patients. Within the group of advanced stage patients, lower LA, stearic acid, omega-6 fatty acids and total PUFAs were observed in patients who did not complete chemotherapy treatment due to toxicity or disease progression as compared to patients who completed the treatment (Murphy et al., 2012). A comparison of plasma phospholipid fatty acids between patients who were <8 months from death and patients who did not complete chemotherapy revealed similar levels of EPA and DHA but all other fatty acids were relatively higher (Murphy et al., 2012). Lower concentration of all plasma phospholipid fatty acids were reported in patients closest to death as compared to patients farthest from death (less than or more than median survival of 238 days) (Murphy et al., 2010b). Cut-points for plasma phospholipid fatty acids, defined by optimal stratification, were used to

classify patients as higher versus lower risk of death. Patients with plasma phospholipid LA, ALA, ARA EPA and DHA above the cut-points lived 1.4 to 2.3 folds longer than patients below the cut-points (Murphy et al., 2010b). A meta-analysis of three prospective and four case-control studies with over 60,000 participants revealed an inverse association between omega-3 fatty acids in the biospecimen (serum/plasma/erythrocyte) and colorectal cancer risk (B. Yang et al., 2014). Specifically, lower EPA and DHA levels were associated with higher cancer risk (Odds Ratio 0.78 95%CI: 0.64, 0.96 and 0.68 95%CI: 0.54, 0.84, respectively) (B. Yang et al., 2014).

There are number of potential ways by which alterations in fatty acid composition in biospecimen might influence survival of cancer patients. Fatty acids, specifically EPA and DHA, have been reported to have anticancer properties. *In vitro* studies in human colon cancer cells and studies in animal models of cancer suggest omega-3 fatty acids exert anticancer properties by influencing proliferation and apoptosis in the tumor cells (reviewed by Calviello et al., 2007). These findings are further supported by intervention studies in humans. Supplementation of EPA (2g/day) for 3 months in patients with colon cancer have shown to substantially reduced abnormal proliferation and apoptosis of colonic mucosa cells compared to no supplementation (Courtney et al., 2007). Omega-3 fatty acids potentially affect carcinogenesis by specific mechanisms including, cyclooxygenase 2 (COX-2) dependent synthesis of prostaglandins. Prostaglandin E2, ARA derived prostaglandin, plays a critical role in the early stages of colorectal carcinogenesis (Hull et al., 2004). EPA compete with ARA for COX-2 and leads to a reduction of '2-series' prostaglandins in favour of 'three-series' prostaglandins (Hawcroft et al., 2010). Prostaglandin E3 is reported to have anti-tumorigenic activity against human lung cancer cells *in vitro* (P. Yang et al., 2004). DHA is also reported to bind to substrate channel of COX-2 and inhibits its activity (Vecchio et al., 2010). Omega-3 fatty acids also alter the membrane dynamics and cell surface receptor function

thus influencing proliferation and apoptosis by signal transduction (reviewed by Cockbain et al., 2012) . Additionally, omega-3 fatty acids are known to have adjuvating effect on anti-cancer drugs thus enhancing efficacy of these drugs in different human tumor cells (Gabriella Calviello et al., 2005). Several clinical trials suggest improvement in chemotherapy tolerability with omega-3 fatty acid supplementation (reviewed by Morland et al., 2016). Finally, omega-3 fatty acids can exert beneficial effects in different stages of cancer management by modulating inflammatory pathways and generating lipid mediators critical for the resolution of inflammation (resolvins, protectins, and maresins) (reviewed by Miccadei et al., 2016).

Additionally, phospholipid fatty acid composition in biospecimens of cancer patients is associated with weight loss and body composition. In lung cancer patients, the plasma phospholipid concentrations of EPA and total omega-3 fatty acids were significantly lower in weight losing patients as compared to weight-stable patients (Zuijdgeest-van Leeuwen et al., 2002). Also, a significant positive correlation has been reported between weight gain and EPA content in plasma phospholipids of cancer patients ($r=0.86$, $p=0.006$) (Pratt et al., 2002). In sarcopenic lung cancer patients, defined by SMI cut-off of $<55.4 cm^2/m^2$ for males and $<38.9 cm^2/m^2$ in females, lower concentrations of EPA, DHA and omega-3 fatty acids in plasma phospholipid was reported (Murphy et al., 2010a). As body composition is associated with prognosis in cancer patients, fatty acids can may influence survival through their role in body composition.

1.9 Conclusion

Sarcopenia and low muscle radiodensity are the features of body composition associated with poor prognosis in cancer patients and other chronic disorders. CT imaging can be used opportunistically in cancer patients for the diagnosis of sarcopenia and to measure muscle

radiodensity. An increasing number of researchers are collecting muscle biopsies to understand the pathophysiology that affects skeletal muscle composition in cancer patients. The research on biological characterisation of muscle biopsies of cancer patients is still at an early phase. Within the studies available, most reported biological characteristics, mediators and pathways associated with muscle loss and only 3 studies to date have reported features of muscle biopsies with fat infiltration in cancer patients. As muscle radiodensity is emerging as an important predictor of clinical outcomes in cancer population, there is need to understand biology and mechanisms involved in development of this feature.

Membrane phospholipid fatty acid composition of blood of cancer patients has been shown to be associated with body composition, disease progression and survival in number of studies. Specifically, high EPA and DHA content of blood phospholipid have been reported to have protective effect in cancer patients. In skeletal muscle, high saturated fatty acids and low polyunsaturated fatty acids have been associated with muscle loss, insulin resistance, mitochondrial dysfunction and triglyceride infiltration. However, fatty acid composition skeletal muscle phospholipids in cancer patients is not determined.

Table 1-1A. Fatty acid composition of each phospholipid species and triglyceride is unique in mammalian tissue.

	Percentage of total lipids in membrane*	SFA**	MUFA**	PUFA**	Location
PC	45-55	48%	11%	36%	Outer monolayer (66%)
PE	15-25	55%	8%	35%	Inter monolayer (20%)
PI	10-15	47%	3%	45%	Inner monolayer
PS	5-10	51%	3%	43%	Inner monolayer
SM	5-10	-	-	-	Outer monolayer
CL	2-5	11%	28%	61%	Mitochondrial membrane
CHL	10-20	44%	31%	30%	Inner and outer monolayer
TG	-	34%	31%	13%	Lipid droplets in cells, adipocytes

*Data are compiled from several sources. Adapted from Vance 2015 and Stillwell 2013.

** Fatty acid composition of main lipids of rat liver. Adapted from Getz et al. 1961 and Pfleger et al. 1968

Abbrevations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; CL, cardiolipin; CHL, cholesterol; TG, triglyceride; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 1-1B. Fatty acid composition of phospholipids and triglycerides in human skeletal muscle.

	Human Skeletal Muscle	
% fatty acid*	TG	PL
C16:0	23.6	22.9
C18:0	5.2	13.8
C16:1	6.3	0.8
C18:1	49.0	12.7
C18:2n-6	10.2	30.7
C18:3n-3	0.9	0.5
C20:3n-6	0.1	1.2
C20:4n-6	0.4	11.6
C20:5n-3	ND	1.4
C22:5n-3	0.3	1.6
C22:6n-3	0.4	2.4

*Data are compiled from Andersson et al. 1998, 2000, 2002.

Table 1-2. Articles reporting changes in fatty acid composition of tissues in cancer patients

				Changes in cases as compared to controls		
Author	Cases/cancer type (N, age in years)	Control type (N, age in years)	Biospecimen	MUFAs	SFAs	PUFAs
Amezaga 2018	Breast, Colon, Lung, Prostate, Ovarian, Lymphoma (54, 59)	Healthy (37, 42)	RBC PL	high 18:1	low 16:0 and 18:0	high 18:2n-6 and 20:3n-6
Jurczyszyn 2014	Multiple myeloma (43, 61)	Healthy (21, 52)	RBC PL	high 16:1 and low 18:1	low 18:0	high 20:3n-6 and 20:4n-6, low 20:5n-3, 22:6n-3 and 22:5n-3
Okuno 2013	Colorectal (61, 65)	Non-Malignant (42, 65)	RBC PL	high 24:1	high 16:0 (trending) and low 20:0	low 18:2n-6, 20:5n-3
			Plasma PL	low 20:1, high 16:1 (trending)	high 16:0 and low 24:0	low 20:5n-3
			Adipose tissue TG			high 20:4n-6
Zuijdgeest-van Leeuwen 2002	Pancreatic (15, 64)	Healthy (45, 59)	Plasma PL	high 18:1	high 16:0	low 18:2n-6, 20:5n-3 and 22:5n-3
	Lung (22, 66)				high 16:0	
	Oesophageal (35, 63)				high 16:0	low 18:2n-6 and high 22:6n-3
McClinton 1991	Bladder (98, 67)	Controls (477, NR)	Plasma PL	high 18:1	high 16:0 and 18:0	low 18:2n-6, 20:4n-6, 20:5n-3, 22:5n-3 and 22:6n-3
Murphy 2012	Lung, stage 3 or 4 (12, NR)	Cancer, stage1 or 2 (38, NR)	Plasma PL		low 16:0 and 18:0	low 18:2n-6, 20:4n-6, 20:5n-3 and 22:6n-3

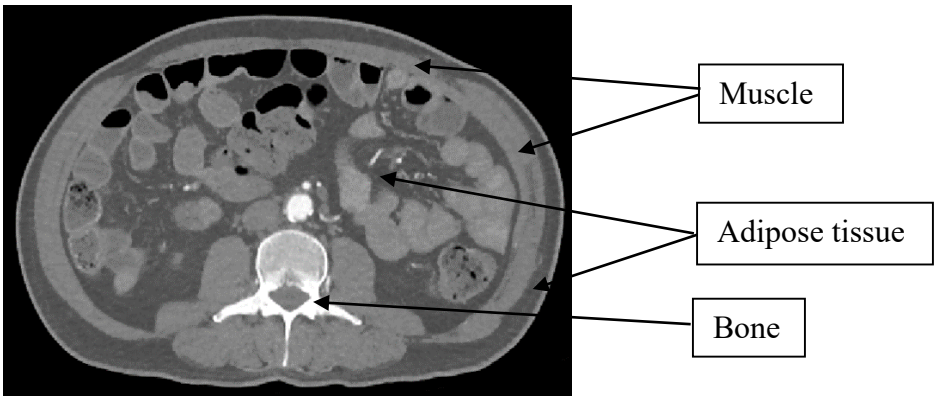
Jolanta 2018	Colorectal cancer (67, 63)	Healthy (16, 48)	Serum PL	high 16:1	high 18:0	high 18:2n-6 and low 18:3n-3, 20:4n-6, 20:5n-3 and 22:6n-3
	Proximal (18, NR)					high 18:2n-6 and low 20:4n-6, 20:5n-3 and 22:6n-3
	Distal (17, NR)					high 18:2n-6 and low 20:4n-6 and 22:6n-3
	Rectum (17, NR)					low 22:6n-3
Baro 1998	Colorectal cancer (17, 63)	Non-malignant (12, 63)	Plasma PL	ND	high 16:0	low 18:2n-6
			Plasma TG	high 18:1	high 18:0	low 18:2n-6, 20:3n6, 18:3n-3
			RBC PL		high 18:0	
Mosconi 1989	GI (12, 55)	Healthy (8, NR)	Plasma PL	high 18:1	high 16:0	
			RBC PL	high 18:1		
Murphy 2010	Lung, sarcopenia (25, NR)	Cancer, non-sarcopenic (16, NR)	Plasma PL			low 20:5n-3 and 22:6n-3
Mikirova 2004	Breast, prostate, liver, pancreas, colon and lung (255, NR)	Non-malignant (2800, NR) and healthy (34, NR)	RBC PL	high 18:1	low 18:0	low 20:5n-3 and 22:6n-3 relative to 18:3n-3
Cottet 2013	Colorectal (328, 58)	Non-malignant (619, 59)	RBC PL	high 18:1 associated with higher risk in advance cancer	no association	high 20:3n-6 doubled the risk and low 20:5n-3 and 22:6n-3 associated with high risk in advance cancer

Castro 2014	Adenocarcinoma (20, 69)	Healthy controls (55, 60)	RBC Total FA	high 18:1	high 18:0	low 20:4n-6
	Squamous cell carcinoma (30, 67)			high 18:1	high 18:0	low 20:4n-6
	Small cell lung cancer (15, 65)			no difference	no difference	low 18:2n-6, 20:4n-6
Rahrovani 2018 a, b	Basal cell carcinoma (40, 57)	Non-malignant (40, 54)	RBC PL	high 18:1	low 16:0 and high 18:0	high 18:2n-6 and 20:4n-6
Macasek 2012	Pancreatic (84, 64)	Non-malignant (68, 59)	Plasma PL	high 16:1 and 18:1	high 16:0 and low 18:0	low 18:2n-6, 18:3n-3, 20:5n-3
			Plasma TG	high 18:1	low 18:0	low 18:3n-3 and 20:5n-3
Pratt 2002	Pancreatic, prostate, lung, rectum, breast, kidney (23, NR)	Healthy (6, NR)	Plasma PL			low 18:2n-6 and 18:3n-3
			Neutrophil PL		low 16:0	high 20:4n-6

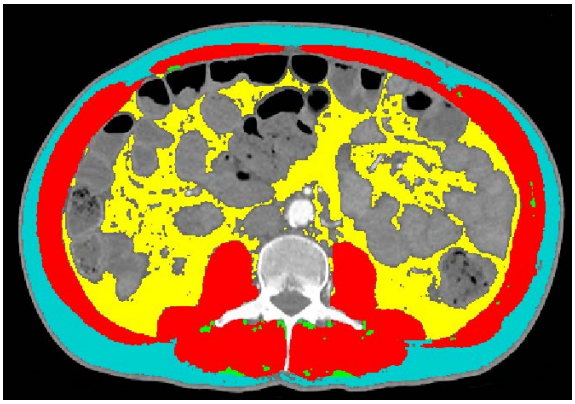
PL, Phospholipids; TG, Triglyceride; RBC, Red Blood Cells; ND, Not Detected; NR, Not Reported.

FIGURES

A



B



Skeletal Muscle: -29 to +150 HU	
Visceral adipose tissue: -150 to -50 HU	
Intermuscular adipose tissue: -190 to -30 HU	
Subcutaneous adipose tissue: -190 to -30 HU	
HU: Hounsfield Units	

Figure 1-1. Computed tomograpgy images at the 3rd lumbar vertebra. A) Tissues are represented in the CT image as linear gray scale representing the mean radiation attenuation value of each pixel. Muscles have higher density than adipose tissues, thus have higher attenuation coefficient. Variations in attenuations is directly visualized as the levels of gray within the image. B) CT image analysed to determine areas of different tissues using imaging software SliceOmatic. Skeletal muscle is annotated in red and is defined within range of -29 HU to +150 HU. Subcutaneous and intermuscular adipose tissue are shaded light blue and green, respectively and are defined using range of -190 HU to -30 HU. Visceral adipose tissue is shaded yellow and is defined within range of -150 HU to -50 HU.

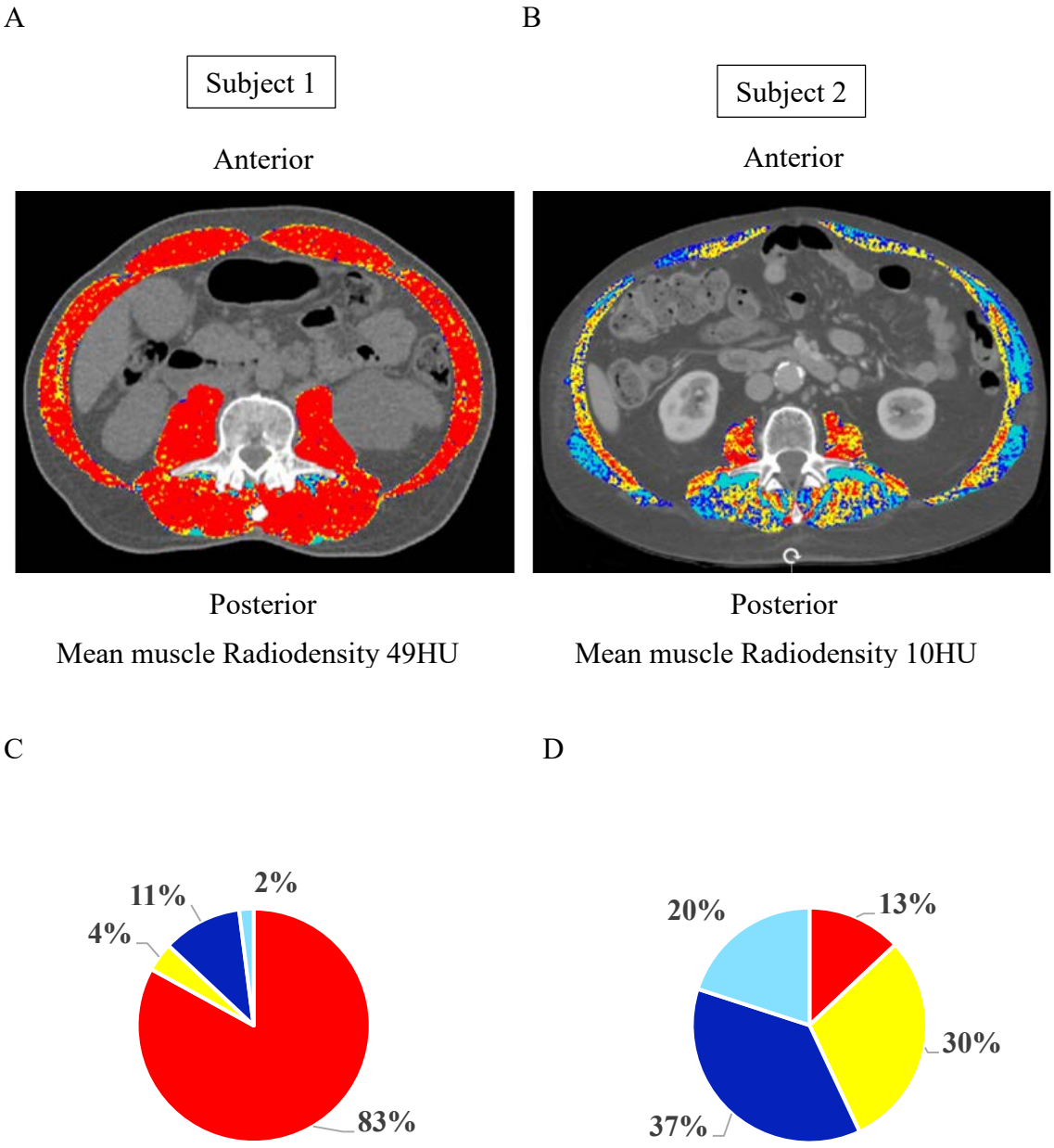


Figure 1-2. Muscle radiodensity map of abdominal muscles. Subject 1 (A, C) show visible fat within the fascia surrounding skeletal muscle (intermuscular fat, light blue) making up 2% of total tissue area. Exclusive of the intermuscular fat, the mean overall radiodensity is 49 HU with 83% of the total muscle cross-sectional area falling into the normal radiodensity range for muscle [red]. Subject 2 (B, D) exhibits extensive visible regions of intermuscular fat infiltration (light blue) comprising 20% of total area. Exclusive of the visible fat infiltration, low overall mean radiodensity of 10 HU. In this subject, only 13% [annotated in red] of the total tissue cross-sectional area falls within the normal range of muscle radiodensity. HU, Hounsfield Unit.

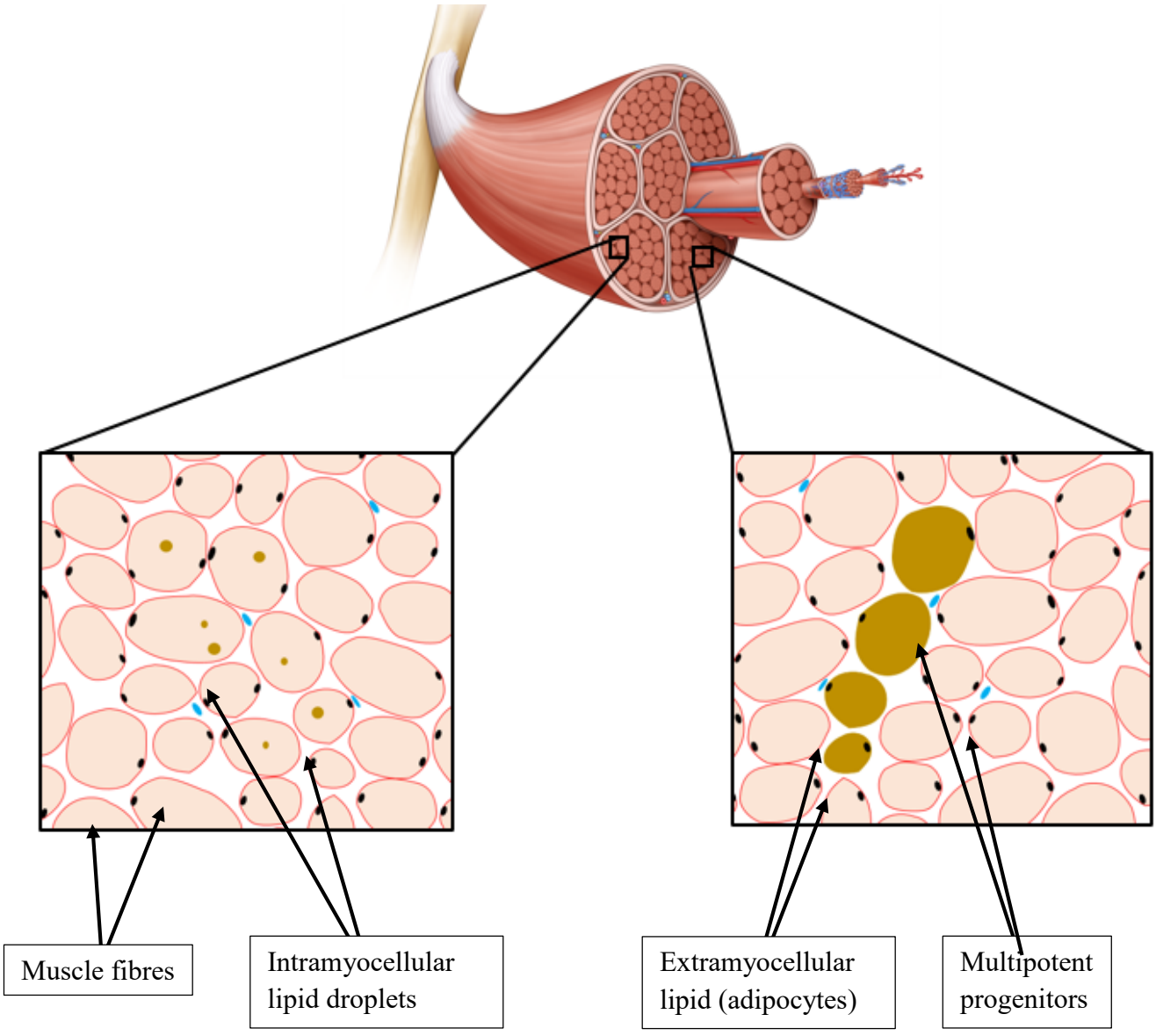


Figure 1-3. Intramyocellular and extramyocellular lipids in skeletal muscle. IMCL are present inside muscle fibres as lipid droplets whereas EMCL are present outside muscle fibres as adipocytes.

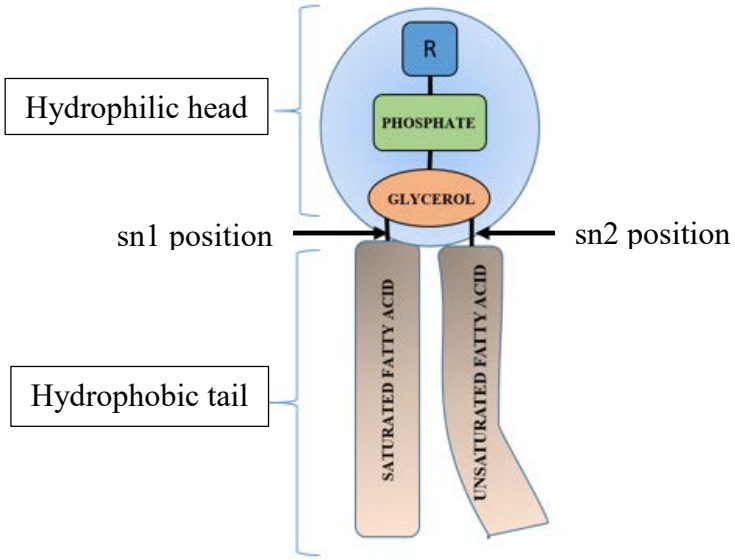


Figure 1-4. Phospholipid structure. Phospholipids are comprised of two fatty acids, a glycerol molecule and a phosphate head group. The phosphate head group is hydrophilic and the fatty acid tail is hydrophobic. Saturated fatty acids preferably attached to sn1 position of the glycerol backbone whereas unsaturated fatty acids attach to sn2 position. R in the structure represent different phosphate groups, for example choline, ethanolamine, inositol and serine.

Chapter 2: Research plan

2.1 Rationale

Biological characteristics of sarcopenia and low muscle radiodensity and how these features develop in cancer patients are not well understood. An increasing number of researchers are collecting skeletal muscle biopsies from cancer patients to understand the biological features that confers poor prognosis. Muscle biopsies are collected either during open surgery, laparoscopy or needle biopsy techniques. As the interest in collecting and exploring muscle biopsies in cancer patients is increasing a review of existing literature is required to determine the quality of studies and sources of variations. Literature review can provide recommendations regarding factors that influence interpretation of results for future studies.

Recently, skeletal muscle radiodensity has been of increasing attention as in cancer patients it is associated with poor prognosis. Based on a study in diabetes and healthy individuals by Goodpaster et al., (2000), low muscle radiodensity is interpreted as myosteatosis in cancer patients. In cancer studies, radiodensity of muscles at 3rd lumbar vertebra (L3) is generally reported and different ranges of HU is used to determine radiodensity than used in Goodpaster's study. If these differences in methodologies influence the association between radiodensity and fat content of the muscle in oncological settings remain unknown. Additionally, non-uniform distribution of lipids across the length of a muscle might lead to variation in muscle radiodensity when measured in different CT image slices of same patient. This is important to determine as two studies that compared biological features of muscle of cancer patients with muscle radiodensity (measured at L3) did not find significant or strong associations (Stephens et al. 2011; Ramage et al. 2018). The distribution of lipids in muscle may provide insight into possible mechanisms involved in myosteatosis.

Phospholipid fatty acids regulate the biophysical properties of proteins, provide substrates for second messengers and intracellular signals to alter gene expression, while providing precursors for biological messengers in the form of oxylipins (Gabbs et al. 2015). Membrane-derived signals function in numerous cell types (ie.,tumor, muscle, immune cells, adipocytes). In cancer, low PUFAs in blood phospholipids have been associated with poor survival in cancer patients. Survival benefit associated with these fatty acids could be due to a) effects on the tumor (directly), b) modification of the effect of chemotherapy on the tumor, and c) modification of immune/inflammatory responses in the tumor bearing host. However, no study reported phospholipid fatty acid composition of skeletal muscle in cancer patients. Considering the relationship between mortality and skeletal muscle mass/fat infiltration; and also, role of fatty acids in skeletal muscle biology it is would seem important to determine relationship between fatty acid composition of muscle and prognosis in cancer patients.

2.2 Research objectives and hypotheses

2.2.1 Clinical and biological characterization of skeletal muscle biopsies of surgical cancer patients

Objectives:

i. To conduct a state-of-the-science review of the literature on muscle biopsy in cancer patients to identify sources of variations within the studies.
ii. To provide recommendations of components to consider when evaluating and reporting the results of muscle biopsies from cancer patients.
iii. To evaluate sources of variation in the muscle biopsy material to better understand the risk of sampling bias.

iv. To determine the possible consequences of sexual dimorphism and age as confounders using a relatively well-powered sample.

2.2.2 Association between muscle radiodensity and muscle triglyceride content in cancer patients

Objectives:

i. To determine the association between muscle radiodensity and the triglyceride content of rectus abdominis muscle biopsies obtained from cancer patients.
ii. To determine the variation in radiodensity across the length and breadth of skeletal muscle.
iii. To investigate the distribution of lipid within the intramyocellular and extramyocellular compartments.

Hypothesis:

It was hypothesized that muscle radiodensity is associated with triglyceride content of the muscle. There would be wide variation in radiodensity across the skeletal muscle. Lipid would be heterogeneously distributed in IMCL and EMCL compartments.

This objective was investigated in chapter 4.

2.2.3 Association between membrane fatty acid composition of skeletal muscle and survival in cancer patients

Objectives:

i. To profile the fatty acids of skeletal muscle phospholipids in cancer patients who lived longer compared to patients with shorter survival.

ii. To investigate the relationship between the fatty acid composition of phospholipid in skeletal muscle and survival in cancer patients.

iii. To determine if the fatty acid composition of muscle was associated with skeletal muscle depletion.

Hypothesis:

It was hypothesized that patients with longer survival would have higher polyunsaturated fatty acids (ARA, EPA and DHA) in phospholipids of skeletal muscle compared to patients with shorter survival and these polyunsaturated fatty acids would be associated with better prognosis in cancer patients. This objective was investigated in chapter 5

Chapter 3: Clinical and biological characterization of skeletal muscle tissue biopsies of surgical cancer patients

3.1 Introduction

Several radiologically–defined features of skeletal muscle have been associated with clinical outcomes in patients with cancer. Reduced muscle mass (i.e. sarcopenia), loss of muscle mass over time and reduced muscle radiodensity, are related to mortality, shorter progression – free survival, chemotherapy toxicity and complications of cancer surgery (Kazemi-Bajestani et al., 2016; Lieffers et al., 2012; Martin et al., 2016; Carla M.M. Prado et al., 2009). In light of the associations between muscle and outcomes, researchers are increasingly investigating the pathophysiology of muscle abnormalities (Miyamoto et al., 2016; Mueller et al., 2016; Stretch et al., 2018) and attempting to relate the findings to the much broader base of knowledge that exists from research in animal models. Muscle may be obtained from cancer patients by percutaneous biopsy as well as intraoperatively during cancer surgery. Clinical data aligned with the biopsy provides a comprehensive approach to understand cancer cachexia from the vantage point of muscle wasting. Evaluation of human muscle contributes significantly to the understanding of molecular mechanisms in a variety of primary pathologies of skeletal muscle (Joyce et al., 2012; Lacomis, 2004).

Biopsy and tissue manipulation techniques can induce changes in the muscle that alter enzyme activity, metabolite concentrations and protein metabolism (Chatterjee, 2014; Hers et al., 1966; Varadhan et al., 2018). Also, patient characteristics such as age, sex, cancer type, comorbidities, medications (including chemotherapy) taken at the time of biopsy collection are known factors that influence muscle metabolism (Batchelor et al., 1997; Edwards et al., 2014; Jackson et al., 2014; Salvatore et al., 2014; Stephens et al., 2012). These methodological issues

pose limitations in the reliability, interpretation and comparability of the findings on muscle biopsies in patients with cancer. Therefore our first aim was to conduct a state-of-the-science review of the literature on muscle biopsy in cancer patients. This type of review retains many features of a systematic review except that studies are not excluded on the basis of a quality assessment, and thus presents a broader search of the literature. An associated aim was to provide recommendations of components to consider when evaluating and reporting results of muscle biopsies from cancer patients.

The second aim of this study was to evaluate sources of variation in the muscle biopsy material to better understand the risk of sampling bias, to determine variance and effect size to enable sample size calculations, and to determine the possible consequences of sexual dimorphism and age as confounders using a relatively well-powered sample (n=190). Our research group has experience in the radiological characterization of muscle (Martin, 2016; Mourtzakis et al., 2008; Xiao et al., 2018) and skeletal muscle morphology, cell biology and biochemistry (Johns et al., 2017; Narasimhan et al., 2017, 2018; Stretch, 2018). Our collaborative effort with hepatopancreatobiliary cancer surgeons has enabled muscle biobanking and exploration of muscle biology within large populations. We have published studies on muscle expression of mRNA, microRNA, and alternative splice variants (Narasimhan, 2017, 2018; Stretch et al., 2013), alongside specific and precise measures of muscle mass, radiodensity and muscle loss.

3.2 Materials and methods

3.2.1 Literature review

A state-of-the-science review is a broad search of the literature that includes all studies in a particular area (Grant et al., 2009). Our review protocol follows the Preferred Reporting Items

for Systematic Reviews and Meta-Analyses (PRISMA) guidelines to reduce bias (Figure 3-1) (Liberati et al., 2009). Articles indexed in SCOPUS from January 1, 1900 to August 16, 2018 were queried to capture reports on skeletal muscle biopsies from cancer patients. Search terms included adult humans, malignant disease [(cancer) OR (neoplasm) OR (carcinoma) OR (tumor) OR (malignant) OR (metastasis)], skeletal muscle [(skeletal muscle) OR (muscle mass) OR (lean body mass) OR (rectus abdominis) OR (cachexia) OR terms for other specific muscle] and biopsy. Review articles and studies on experimental models, laboratory animals, non-cancer populations or those not employing muscle biopsies were excluded. Bibliographies of identified articles were hand searched to find additional relevant publications. There were no exclusion criteria regarding number of patients and type of study (retrospective, prospective or cross-sectional). Data were extracted from the result sections, tables and figures of each article. As we did not aggregate the data, no additional data was contributed from the investigators.

Two reviewers independently assessed each of the included studies, disagreements were resolved by consensus. A score for study quality was given using assessment tools provided by the National Heart, Lung and Blood Institute (NIH -U.S. Department of Health & Human Services) for cross-sectional, cohort, case-control, randomized control trials and before-after studies. The Newcastle-Ottawa scale modified for cross-sectional studies was used to give a bias score based on the a) representativeness, b) size and c) non-respondent report (Modesti et al., 2016).

3.2.2 Computed tomography image analysis

Digital axial CT scans performed pre-operatively and used to plan surgery were used to quantify skeletal muscle cross-sectional area (CSA, cm^2) as in our prior work (Mourtzakis, 2008; Carla MM M Prado et al., 2008). Measures with CT have excellent precision (precision error values of ~1.5%) (Mitsiopoulos et al., 1998). Briefly, images at the 3rd lumbar vertebra (L3) were

analyzed for total L3-CSA within a specified Hounsfield Unit (HU) range (-29 to +150) using Slice-O-Matic software (v.4.3, Tomovision, Magog, Canada). Muscle area was normalized for stature and reported as skeletal muscle index (SMI, cm^2/m^2). Mean radiodensity (HU) was also reported. Adipose tissue CSA at L3 was calculated in a HU range of −150 to −50 and −190 to −30, for visceral and subcutaneous adipose tissue, respectively (Mitsiopoulos, 1998). The distribution of SMI of the patients providing biopsy for this study were compared to a previously described large cohort of oncology patients (n=1,473) to confirm that the population sampled is representative of muscle mass distribution and mean values for our population (Figure 3-2). Sarcopenia was classified according to previously reported (Caan et al., 2017; Xiao, 2018) sex- and body mass index (BMI)- specific criteria: for BMI <30 kg/m^2 - SMI <52.3 cm^2/m^2 for men and <38.6 cm^2/ m^2 for women, and BMI ≥30 kg/ m^2- SMI <54.3 cm^2/m^2 for men and <46.6 cm^2/m^2 for women.

3.2.3 Rectus abdominis biological characterization

3.2.4 Subjects and acquisition of muscle samples

The study was approved by the Health Research Ethics Board of Alberta-Cancer. Patients undergoing elective abdominal surgery were consecutively approached to participate in tumor and tissue banking at a hepatopancreatobiliary surgical service in Alberta, Canada. Three percent of approached patients declined participation. Patients provided written informed consent for muscle biopsy and tissue banking. Release of n=190 samples from the bank for analysis, as well as patient information (demographic, clinical and operative data) from medical records, was performed under the auspices of Protocol ETH-21709: *The Molecular Profile of Cancer Cachexia*. Patients consent freely to muscle biopsy from the site of incision at the time of surgery, as this entails little if any

incremental discomfort or risk, as the surgery is inherently invasive. All patients were either diagnosed as having cancer or were suspected of having cancer due to their symptoms and radiological assessments such as CT imaging.

The study cohort and conditions for acquisition of muscle samples have been described previously (Stretch, 2013). Briefly, *rectus abdominis* (0.5 – 3 g) samples were collected during open abdominal surgery scheduled as part of their clinical care. Upper abdominal transverse incision was performed, muscle biopsy was obtained at opening by sharp dissection, without the use of electrocautery.

3.2.5 Processing of Muscle Biopsy

From each biopsy, several analysis were performed, each with specific preparation procedures. In the operating room, visible adipose and connective tissue was removed from the biopsy and it was cut into two pieces; one piece to be used for analysis of gene expression, and myosin heavy chain by electrophoresis was immediately frozen in liquid nitrogen in the operating room prior to being transported to the lab for storage in liquid nitrogen until analysis. The other piece of the biopsy to be used for microscopy was transported on ice to the laboratory within 20 to 30 minutes. For morphological preservation, isopentane (2-Methylbutane, C_5H_{12}) was cooled at -160 °C in liquid nitrogen for 20 minutes or until the appearance of a thick frozen layer at the bottom of the container. A piece of muscle was oriented for transverse section and delicately placed on aluminum foil. Tissue was submerged in isopentane for 20 seconds, aluminum foil was turned upside down to allow full exposure of the muscle section. After submersion, tissue was wrapped and left in liquid nitrogen for 5 minutes. Information about surgery date, time, and sample reception was documented.

3.2.6 Immunofluorescence: Fiber types, laminin/dystrophin and nuclear stain

Muscle serial sections (10μm) were cryosectioned (cryostat Leica model CM300) transversely at -22°C and stored at -80°C until staining. Myosin Heavy Chain (MyHC) I, IID and IIA were determined as previously described (Gallo et al., 2006). Primary and secondary antibodies are described in the Appendix A. After the secondary antibody application, a nuclear stain (4',6-diamidino-2-phenylindole, DAPI) was added for 2 minutes and washed. Slides (Apex^{TM} superior adhesive slides, Leica biosystems) were mounted, covered and let dry for twelve hours. Images for tissue sections were acquired using a 20X/0.85 oil lens with a spinning disk confocal microscope (Quorum Wave FX Spinning Disc Confocal System – Quorum technologies). Individual Z-stacked images were assembled to create a composite image of a whole tissue cross-section. Tissue images were capture and analyzed with Volocity 6.3 software [PerkinElmer, Waltham, MA, USA]. A software script was established to identify muscle fibres types (I, I/IIA, IIA, IIA/D and D) using intensity of the MyHC stains and quantified automatically by the software. Mean muscle fiber area (μm^2) was calculated by the detection of membrane (laminin/dystrophin antibody) fluorescence of muscle fibers in a cross-section. Percentage of fibers with centralized nuclei was manually assessed by selecting muscle fibers with mispositioned nuclei (clearly separated from sarcolemma, equidistant or not) in a tissue cross-section.

3.2.7 Electrophoretic Analysis of Myosin heavy chain (MyHC) Isoform Content

Semi-quantitative MyHC isoform analyses were completed on frozen rectus abdominis using Western blotting as previously described (Gallo, 2006; Martins et al., 2012; Putman et al., 2007). All three of the adult MyHC isoforms (I, IIA and IID) were clearly visible on all gels and reliably quantified in at least triplicate by integrated densitometry (Syngene ChemiGenius, GeneTools, Syngene).

3.2.8 Triglyceride (TG) content analysis

A piece of biopsy [50 mg] was ground using a frozen pestle and mortar without letting the tissue thaw. Ground tissue was homogenized in a 1.6 ml calcium chloride [CaCl2; 0.025%] solution with glass beads [0.5 mm diameter; FastPrep ®-24, MP Biomedicals, Santa Ana, CA, USA] in 20 sec intervals for 1 min. Samples were placed on ice for 15 sec between each homogenization interval. A modified Folch method was used to extract lipids using chloroform/methanol (2:1, vol/vol) as previously described (Murphy et al., 2010; Pratt et al., 2001). The TG fraction was isolated on G-plates and the TG band was identified and scraped. An internal standard C15:0 [10.2 mg/100 ml hexane] was added, followed by saponification and methylation. Samples were analyzed using gas liquid chromatography (flame-ionisation detector) on a Varian 3900 [Varian Instruments, Georgetown, ON, Canada]. Quantity of fatty acids within the TG fraction was calculated by comparison with the known concentration of the internal standard and sum of all fatty acids was reported as total TG.

Gene expression: Microarray

Microarray was conducted as previously described (Stretch, 2013). The data have been deposited in the U.S. National Center for Biotechnology Information (NCBI) Gene Expression Omnibus25 and are accessible through GEO series accession number GSE41726.

3.2.9 Statistical analysis

Statistical analyses were conducted in IBM® SPSS ® software, version 24. A test for normal distribution was applied to the continuous variables. Descriptive statistics were reported as mean ± standard deviation. Comparisons between groups were conducted using independent t-test or Mann-Whitney U according to the variable normal distribution and chi-square test for categorical variables. Statistical significance was considered at p values less than 0.05 (two-sided).

3.3 Results

3.3.1 Literature review

A total of 59 articles reporting analysis of skeletal muscle in cancer populations were reviewed. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Liberati, 2009) flow diagram of our search strategy is shown in Figure 3-1.

3.3.2 Study Quality and Design

Table 3-1 includes all of the extracted data as well as scores for sampling bias (Newcastle-Ottawa scale) and study quality assessments (NIH). In general the study quality rated as low for the majority of studies (Table 3-1). Applying the Newcastle-Ottawa criteria for sampling bias revealed the majority of studies had a high risk of sampling bias with 58% of studies lacking representativeness, 96% lacking sample size justification and no study mentioned non-respondent rate (% of population approached who declined participation). Muscles biopsied were rectus abdominis (n=40), quadriceps (n=20), tibialis anterior (n=1), gastrocnemius (n=1), pectoralis major (n=1), sternocleidomastoid (n=1), serratus anterior (n=1), diaphragm (n=1) and latissimus dorsi (n=1), and in seven studies more than one muscle was collected. Four studies reported evaluation of rectus abdominis from cancer patients and quadriceps for non-cancer controls, and four studies reported biopsied muscle from 2 or 3 different muscles.

Gastrointestinal cancers were the most common diagnoses; 31/59 studies included patients of exclusively one cancer type: colorectal, pancreatic, gastric, breast or prostate. Inclusion of patients with two or more cancer types was reported in 27/59 studies. Cancer stage or presence of metastasis was described in 39/59 studies. Combined data from two or more cancer stages was reported in 38/59 studies.

The majority of studies were cross-sectional (Appendix B). For investigation of patients with cancer cachexia, weight loss was considered as the main reference for classification. In 36 studies weight loss was graded with varying cut points (e.g. 5%, 10% or 15%). Time frame of weight loss was not specified in 16 of these studies (Table 3-1). Percentage weight loss ranged from 5 to 22% in weight-losing groups (Appendix B). Measures of body composition were included in 25 studies, however these measures were used to assess muscle mass or rate of muscle wasting over time in only 7 studies (Appendix B).

Total sample size in each study was generally limited (mean, n=26; median n=18; range 1 – 134). Seventy-six percent of studies included n≤30 cancer patients; 48/59 studies included a non-cancer control group, sample size ranging from n=3 to 41. Fifty-two studies included men and women, 5 studies only men, 1 study only women and 2 studies did not specify the sex of their patients. For those studies including both sexes, 50 had an imbalance between treatment groups in the % of male and female patients, and only 3 studies matched the number of male and female participants. When reporting the results, almost all of the studies (98%) presented aggregate data from men and women.

When a non-cancer control group was employed in the study , the majority of studies included control groups that went under surgical procedures (i.e. Cholelithiasis and cholecystitis, ovarian cyst, inguinal hernia, laparocele, abdominal aorta aneurysm, hemangioma of liver, gallstones, chronic pancreatitis), or healthy volunteers (Appendix B). No study defined the criteria used to select healthy volunteers. Table 3-1 highlights the features of the cancer groups compared to control groups. More than 54% of the studies included cancer patients with an average age of ≥65 years, and for studies involving non-cancer patients as controls, 26% included patients with an average age of ≥65 years.

Most (33/59) reports failed to mention comorbidities as a component of their exclusion criteria or patient's demographics. Commonly excluded diagnoses were: diabetes, chronic obstructive pulmonary disease (COPD), liver failure, renal failure, chronic hepatitis, autoimmune diseases and inflammatory bowel disease. Use of medications (e.g. corticosteroids, anabolic/catabolic agents and/or beta blockers) was described in 17 studies as clinical characteristics or exclusion criteria. Prior exposure to antineoplastic drugs was reported in 14/59 studies. Inclusion of patients naïve to chemotherapy or radiotherapy was stated in 6/59 studies, two studies acknowledged the inclusion of some patients with one or fewer cycles of chemotherapy that concluded 4 weeks previous to biopsy collection.

3.3.3 Technical Considerations: Biobanking protocol and tissue manipulation

Abdominal and thoracic muscle biopsies were collected during a surgical procedure in 43 studies, with collection at the start of surgery being explicitly stated in 31 studies (Table 3-2). Presence or absence of tissue cauterization was specified in 29/43 studies. Percutaneous procedure (needle biopsy) was the main method for collection of muscles of the lower limb (n=19 studies), open muscle biopsy technique was reported in 1 study and in 1 study the collection method was unspecified. For both surgical and percutaneous biopsies, removal of blood traces and/or fat/fibrotic tissue after collection was mentioned in 7/59 studies (Table 3-2).

Information provided on biopsy manipulation was limited and mainly focused on freezing and storage procedures. In 43/59 studies, immediate freezing in liquid nitrogen was reported. In only one study was it explicitly stated that freezing was done in the operating room versus a laboratory facility. The most common temperatures for sample storage were between -70 and -80°C; storage details were not mentioned in 11/59 studies. Details on time between biopsy and transportation to laboratory facilities and waiting periods were not reported in any study.

3.3.4 Rectus abdominis biological characterization: Study population

Demographics and clinical data from 190 patients are provided in Table 3-3. Nearly all patients (97%) who were approached consented to intraoperative biopsy, as this entails little, if any, incremental discomfort as the surgery is inherently invasive. Therefore there was no selection bias inherent in the cohort. Typical of hepato-pancreatic-biliary case load, 88% of cancers were gastrointestinal, with the largest proportions being colorectal and pancreatic cancer. Surgical procedures included, hepatectomy, liver metastasectomy, pancreatectomy, Whipple procedure, bile duct resection, cholecystectomy, colectomy, and gastrectomy. Metastasis was present in 50% of the patients. Most of the patients were naive to chemotherapeutic agents, 23% had exposure to chemotherapy within 2 to 4 weeks prior to the surgical procedure. The majority of patients were classified as overweight. Diabetes type II and hypertension were the most common comorbidities. Most commonly used medications reported among the population were analgesics, anti-inflammatory, statins, glucose-lowering drugs, anti-hypertensives, anti-reflux and thyroid hormone replacement (Table 3-4).

3.3.5 CT image analysis

Muscle L3-CSA, SMI and muscle radiodensity of rectus abdominis and total muscle are shown in Table 3-5. Sarcopenia was present in 56% of the patients, 60% (n=97) of males and 49% (n=42) of females. Weight history was available for 45 patients. Fifty six percent of patients experienced weight loss (11±12% in 5±12 months), and 60% of weight losing patients were sarcopenic. Out of 44% (n=20) weight stable patients, 70% were sarcopenic.

3.3.6 Sex differences

In light of the fact that most of the papers in the literature review included samples of mixed sex of varying proportions, we examined all of the biopsy features for sex differences. Sexual dimorphism was prominent in **L3-CSA** total lumbar muscle and RA, muscle radiodensity of RA and total muscle (Table 3-5), mean fibre cross-sectional area (Table 3-6), and in expression of genes associated with muscle growth, apoptosis and inflammation (Table 3-7). Proportions of fiber types using both quantification methods, MyHC isoforms and individual fiber types, were not different between male and female patients (Table 3-6).

For centralized nuclei assessment the mean % of fibers with centralized nuclei was 12±9% (4 to 36%) and 10±8% (3 to 27%) in males and females, respectively. No differences were found between men and women (p=0.39) with a combined mean value of 11±8%.

3.3.7 Rectus abdominis: proportion of fiber types and muscle fiber area

Electrophoretic Analysis of Myosin heavy chain (MyHC) Isoforms confirmed MyHC I and MyCH IIA to be present at similar proportions, while MyHC IID was less abundant (Table 3-6A). MyHC type IIA was the most abundant isotype, followed by MyHC type I and IID (Table 3-6B). In addition, 15.5% of the fibers were identified as hybrids, which is the sum of MyHC type I/IIA and IIA/D. For individual fiber types, type I fibers comprised the greatest proportion (46.4%) followed by fiber type IIA (36.1%) and hybrid type IIA/D (15%). Presence of fiber type IID, as well as hybrid type I/IIA, was minimal (1.8 and 0.7%). Mean muscle fiber area (μm^2) was calculated by the detection of membrane (laminin/dystrophin antibody) fluorescence on 1069±771 muscle fibers per biopsy (Table 3-6C). Mean muscle fiber area was determined in total and per fiber type, which includes collective results of MyHC isoforms and individual fiber types (Table 3-6C). Mean fiber area of MyHC type I was smaller than MyHC type IIA and IID. For individual

fiber types, type I and type I/IIA were smaller compared to type IIA, IIA/D and IID. Type IID had the largest mean fiber area compared to the other individual fiber types.

3.3.8 Age effects

Comparison of older (74±4 years, n=13) and younger (50±6 years, n=13) males revealed no differences between groups with respect to mean muscle fiber area (total, individual fiber types or MyHC isoforms), % of individual fiber types or % of MyHC isoforms. Age effect was evaluated in males (n=26) by comparing mean values of a younger group versus an older group. No significant differences were found in relation to % of MyHC isoform content.

3.3.9 Skeletal muscle gene expression for genes associated with cancer cachexia

Differences in genes encoding proteins commonly explored in cancer-muscle wasting are summarized in Table 3-7 (also see Appendix C). Atrophy, autophagy, apoptosis, muscle growth, and inflammation genes were selected based on reviewed literature on muscle atrophy in cancer (M Bossola et al., 2001; Maurizio Bossola et al., 2003; Higuchi et al., 2000; Jagoe et al., 2002; Johns, 2017; Khal et al., 2005; Noguchi et al., 1998; Stephens et al., 2010; A. Williams et al., 1999). Sexual dimorphism exists in pathways related to skeletal muscle anabolism and catabolism illustrating the need for caution when generalizing results from only one sex or discussing results from a mixed group of cancer patients.

3.4 Discussion

There is a perceived need to understand the human biology of cancer-associated muscle atrophy and to frame it in the context of our larger understanding of experimental findings (Argilés et al., 2014; Baracos et al., 2018; Egerman et al., 2014; Johns, 2017; Mueller, 2016). The emergent

literature on human muscle biopsies has been generated with that intent, but has a number of substantial limitations within the study design as well as procedures for collection and preparation of the biopsy material. At the same time there is substantial opportunity for collaboration between cancer surgeons and researchers to obtain intraoperative biopsies with a high rate of patient consent and the additional capability to describe the muscles of these patients with precise radiological metrics. Agreement to a set of standardized procedures and reporting, will enhance the consistency, reliability and comparability of future research in this area. Evaluation of human rectus abdominis muscle presents the expected variation in several measures that may be of interest for emerging studies in this area.

3.4.1 Study Quality and Design

The quality of the studies reporting on biopsy material to characterize varying features of muscle biology was uniformly low. Quality assessment tools revealed several inconsistencies in sample selection strategies, study design, data collection and analysis in the existing literature. Bias assessment of sample selection exposed a clear absence of sample representativeness in 59% of studies and lack of sample size justification in 96% of studies. In 75% of the studies reviewed, samples from a relatively small number of participants (n=≤30) were evaluated without accounting for age or sex variation.

The majority of published studies use weight loss (versus weight stability) to define cachexia. This approach is limited by not accounting for the characteristics of muscle (muscle mass or change in muscle over time), which are the clinically relevant features related to cancer outcomes. Indeed weight stable patients may well be losing muscle over time (Roeland et al., 2017) and they can also be profoundly sarcopenic (Martin, 2016; Carla MM M Prado, 2008). Weight loss was the most commonly used criteria for cancer cachexia assessment, however, application

of this measure alone poses major concerns in misclassification and unintended exclusion of cachectic patients. Many studies were published prior to the widespread use of CT images to quantify muscle, as well as prior to the publication of the international cachexia consensus, which defines muscle mass as a diagnostic criterion for cachexia (Acharyya et al., 2005; Agustsson et al., 2011; Banduseela et al., 2007; M Bossola, 2001; Maurizio Bossola et al., 2006; Maurizio Bossola, 2003; Busquets et al., 2007; Eley et al., 2008; Higuchi, 2000; Jagoe, 2002; Khal, 2005; Lundholm et al., 1976; Noguchi, 1998; Rhoads et al., 2010; Schmitt et al., 2007; Shaw et al., 1991; I. J. Smith et al., 2011; Stephens, 2010; Weber et al., 2007, 2009; A. Williams, 1999; S. Zampieri et al., 2010, 2010; Sandra Zampieri et al., 2009; Zeiderman et al., 1991). The premise of using weight loss when muscle is being evaluated is erroneous. Muscle wasting can be experienced by patients with less than 5% weight loss (Roeland, 2017). Also, the arbitrary selection of weight loss percentage and timeframe in different studies complicates the comparison of results between studies. In the cohort of patients we evaluated, 70% of weight stable patients and 60% of weight-losing patients were sarcopenic. Therefore, assessment of muscle mass is essential, this can be easily achieved through the secondary analysis of CT images used to plan the surgery (Caan, 2017; Mourtzakis, 2008; Xiao, 2018).

Some authors reported mortality-defined cutpoints to define sarcopenia according to age and sex of a reference population (Martin et al., 2015; Carla MM M Prado, 2008) and these have been secondarily used by other authors (Rier et al., 2016). Caution should be used in applying these cutpoints to define sarcopenia in patients undergoing muscle biopsy, these may not necessarily reflect the population from which biopsies are evaluated (Rier, 2016). Here we suggest using CT to quantify muscle features for the overall population from which the biopsy sampling is done. In this way, patients providing biopsy for our study are clearly representative of the entire

L3 SMI distribution of our regional population (Alberta, Canada) (Figure 3-2). This representation eliminates the possibility of sampling bias. It also allows each patients' skeletal muscle index to be ranked within the population distribution overall as well as compared to values available for healthy young individuals (Derstine et al., 2018).

Age and sex differences exist at the level of muscle function, biochemistry/metabolism and mass (Esfandiari et al., 2014; Jackson, 2014; Stephens, 2012).The majority of studies reported combined data from both sexes without acknowledging sexual dimorphisms. Age was generally not accounted for. In the first forty years of life, muscle mass is relatively stable in both men and women; then it begins to decline, however, the rate of loss is slower in women than in men (G. I. Smith et al., 2016). In our sample, differences between men and women were observed for muscle fiber area, SMI and muscle radiodensity. Sexual dimorphism in gene expression was not limited to a particular pathway or function but was identified in growth (AKT1, FOXO1, MSTN, PAX7, TGFα1), apoptosis (CASP9) and inflammation (TNF, STAT3). In relation to the age effect, we did not find any significant differences in mean muscle fiber area and proportion of fiber types when comparing young versus old male cancer patients; this could be potentially explained by the narrow age range in our study. Differences between young (18 to 48 years) and older (66 to 99 years) participants (Marzani et al., 2005) has been reported for fiber type distribution in rectus abdominis and vastus lateralis. Therefore, age differences and sexual dimorphism must be acknowledged when comparing, reporting and interpreting muscle characteristics.

Here we present many characteristics of human rectus abdominis muscle. We obtained a detailed analysis of its radiological features, for the first time. Our analysis of fibre type is multidimensional and confirms the mixed fibre distribution of the rectus abdominis. A prior study in cancer patients with upper gastrointestinal malignancies, reported mean values of 48% and 55%

for MyHC type I and IIa , respectively (Johns et al., 2014) . Muscle gene expression and triglyceride content levels as presented here are new information about rectus abdominis. Future work on rectus abdominis can be usefully planned, using this base of information. The majority of evidence to date (Table 3-1) on muscle from cancer patients is coming from rectus abdominis. Due to the unique characteristics of each muscle type, we suggest that future researchers identify candidate muscles for intensive research using the principle that the muscle(s) most often transected in cancer surgeries would be the greatest resource. This can be decided in function of the common surgical approaches. Thus over time a large base of evidence may be obtained from vastus lateralis (for example) from thoracic cancer surgeries.

A key component of case-control studies is to provide details of the control group relative to the research question. However, this is rarely done in the literature that we reviewed (Narasimhan, 2017, 2018). Detailed clinical characterization of non-cancer controls is usually missing, and assumption of a healthier status of the control group when compared to cancer patients is common. In many cases the comparator group is a non-cancer surgical patient population, however, there is no documentation provided around diagnosis or medications. Presumably healthy volunteers could have underlying co-morbid conditions or be taking medications that impact skeletal muscle. Comorbidities and use of medications were not generally mentioned either for patients undergoing non-cancer surgery or "healthy" volunteers recruited outside the clinical setting. Approximately 60% of people diagnosed with malignancy are 65 years and older (Edwards, 2014). Prevalence of comorbidity in cancer population range from 30-50% depending on type of cancer (Xiao, 2018) and a patient with history of cancer has on average three comorbidities (Garman et al., 2003; Seo et al., 2004). Diabetes and hypertension were the most common conditions in our patient population but cardiovascular disorders and

mental health problems are also prevalent in the cancer population (Edwards, 2014; Xiao, 2018). These chronic conditions and medications taken to control them can independently affect muscle physiology (Bouchi et al., 2017; D'Souza et al., 2013; Henriksen et al., 2017; Langen et al., 2013; Larsen et al., 2016; Leenders et al., 2013; Marquis et al., 2002; Salvatore, 2014; Vuong et al., 2010; Wang et al., 2016; Wüst et al., 2007) (Table 3-4). COX inhibitors, statins, biguanides, proton pump inhibitors and thyroid hormones were the most common medications prescribed in our patient population apart from those prescribed during cancer treatment. These classes of drugs have known effects on muscle protein synthesis (Burd et al., 2010; Standley et al., 2013; T. A. Trappe et al., 2002; Todd A Trappe et al., 2013) and catabolism (Bodine et al., 2015; Mammucari et al., 2007; Sandri et al., 2004; Stitt et al., 2004), atrophy pathways (Cetrone et al., 2014), insulin sensitivity (Malin et al., 2014) and mitochondria function (Wessels et al., 2014). Therefore, it is important that for both the cancer group and "control" groups have a detailed medical history that captures diagnosis of other conditions and medications. In addition to drugs prescribed for management of comorbid conditions, antineoplastic treatment previous to tissue biopsy is also a relevant event that may impact interpretation of results as the long lasting effects in the muscle are unknown (Schiessel et al., 2018).

3.4.2 Technical considerations

We suggest recommendations for minimum procedures to follow in biobanking practices, tissue manipulation and patient characterization to enhance the consistency, reliability and comparability of future research (Table 3-8). Acknowledgement of differences between muscle groups is essential when comparing and interpreting results. RA is commonly collected in patients with gastrointestinal disease due to its practicality in relation to the surgical incision while maintaining patient burden to the essential minimum. Its broad extension in the abdominal area

enables for collection of muscle tissue from a variety of locations (Pedersen et al., 2014); however, no one has demonstrated how homogeneous the RA is in relation to the biopsy site. On the other hand, quadriceps or tibialis anterior are collected in healthy volunteers serving as controls as there is no justification for surgical intervention. Importantly, physiological variations between muscle groups exist (Johnson et al., 1973; Miller et al., 1993), which strongly suggest that studies collecting different muscles must avoid comparing or combining data of more than one muscle.

Most researchers did not report on surgical procedures and muscle biopsy collection, transport and processing of the samples, each of which can impact on the morphological and molecular profile of the biopsy (Chatterjee, 2014; Srinivasan et al., 2002; Truong et al., 2017). Collecting abdominal muscle biopsies at the start of the surgical procedure and avoidance of electrocautery, is strongly recommended to reduce variations associated with the surgical trauma, variable duration of surgery and intra-operative effect of anesthetics (Chatterjee, 2014; Essen et al., 1992; Lattermann et al., 2002; Luo et al., 1998; Ruel et al., 2003; Varadhan, 2018). Skeletal muscle collected at the start and end of a surgery expresses differences in genes associated with inflammation, growth differentiation and transcription factors (Ruel, 2003). For percutaneous biopsies, the Bergstrom protocol is a well-developed method with several adjustments to improve the quality of the muscle biopsies (Shanely et al., 2014; Tarnopolsky et al., 2011). Procedures followed after biopsy collection must also be detailed as sample preservation and storage impacts on muscle integrity and potentially interpretation of the results. Lastly, the numbers of medical conditions and drugs taken by patients in this sample are important and all of these and their different combinations may have an impact on specific aspects of muscle biology. As much as possible, we recommend to annotate the presence of comorbidities and medications in patients consenting to biopsy.

Overall, the literature review reveals a high risk of sampling bias, poorly characterised patient populations. These features make reliable comparison between studies and aggregation of data challenging. Muscle biopsy preparation and biobanking practices are also variable between studies. Data from an unbiased sample of 190 patients presents a variety of measures of interest on rectus abdominis to provide a point of reference for researchers exploring biological characteristics of this muscle. Continued collaboration between researchers and cancer surgeons would enable a more complete understanding of mechanisms of cancer-associated muscle atrophy.

Table 3-1. Original articles reporting muscle biopsy collection in patients with cancer: assessment of bias and study quality, population characteristics weight loss or cancer cachexia classification criteria.

Author	Bias ǂ	Quality ǂ	Muscle	Cancer site	Cancer Stage	Cancer population		Control group		Patient weight loss or cachexia criteria
						n (% male)	Age (years) mean ± SD	n (%male)	Age (years) mean ± SD	
Acharyya 2005 [47]	1/3	3/12	RA	Gastric	NR	27(NR)	NR	14(NR)	NR	N/A
Agustsson 2011 [61]	1/3	3/12	RA	Pancreas Other GI	NR	Pancreas 13 (30) Other GI 8 (37)	Pancreas: 70 ± 2 Other: 68 ± 3	Benign: 8 (37) Pancreatitis : 8 (63)	Benign: 53 ± 4 Pancreatitis 52 ± 3	NR
Aversa 2016 [107]	1/3	6/12	RA	Colorectal Pancreas Gastric	1-4	All: 29 (59) WS = 14 WL =15	68 ± 10.7	11 (63)	63 ± 13.2	5% WL (6 months)
Bonetto 2013 [108]	1/3	3/12	RA	Gastric	1-4	16(NR)	64 ± 11	6 (NR)	62 ± 17.4	>5% WL
Bossola 2006 [48]	1/3	5/12	RA	Gastric	1-4	16 (50)	60.8 ± 11.2	5 (60)	65.6 ± 7.5	WL Mild: 0–5%. WL Moderate: 6–10%. WL Severe: >10%
Bossola 2001[39]	1/3	4/12	RA	Gastric	NR	20 (55)	61 ± 79.6	10 (60)	62 ± 45.8	WL Mild: 0–5%. Moderate 6–10%. Severe: >10%
Bossola 2003 [41]	1/3	5/12	RA	Gastric	NR	23 (61)	59.5 ± 16.1	14 (64)	61.2 ± 12.3	>10% WL

Busquets 2007 [49]	0/3	3/12	RA	Esophageal Gastric Pancreas	1-4	16(NR)	66 ± 10	11 (NR)	66 ± 10.2	>5% WL (1 month)
DeJong 2005 [109]	0/3	4/12	RA	Pancreas	1-4	16 (63)	66 ± 8	11 (81)	67 ± 13.2	N/A
D'Orlando 2014 [110]	1/3	6/12	RA	Gastric	1-4	38 (66)	68.1 ± 11.6	12 (58)	64.2 ± 11.6	>5% WL (6 months)
Eley 2008 [53]	1/3	3/12	RA	Esophageal Gastric	1-4	15 (87)	66 (49 – 83) *	9 (10)	56 (41 – 86) *	N
Johns 2017 [22]	2/3	9/12	RA	Esophageal Gastric Lung and Other	1-4	134 (51)	65 ± 13	N/A	N/A	WL >5% >10% >15% and SMI with any degree of WL (>2%)
Johns 2014 [70]	0/3	5/12	RA	Upper GI Pancreas	NR	41 (73)	65 ± 12.8	N/A	N/A	>5% WL (6 months) and low muscularity with 2% WL
Khal 2005 [42]	0/3	1/12	RA	Pancreas Colorectal	NR	All: 18 (67) WS = 5 (60) WL = 13 (69)	WS:79.8 ± 2.2 WL:70.6 ± 8.2	10 (80)	69.6 ± 7.3	WL Moderate: 1-11%. WL Severe: >11%
Lundholm 1976 [62]	1/3	3/12	RA	Esophageal Gastric Pancreas Colorectal Kidney and others	NR	43 (44)	♂:62 ± 13.1 ♀: 63 ± 9.7	55 (51)	56 ± 14.8	N/A

Marzetti 2017 [111]	1/3	5/12	RA	Gastric	1-4	All: 18 (94) WS = 9 (100) WL =9 (89)	WS:70.6 ± 8.63 WL:66.8 ± 12.5	9 (88)	57.4 ± 15.9	>5% WL (6 months)
Narasimhan 2017 [21]	2/3	8/12	RA	Pancreas Colorectal	1-4	22 (41)	64.9 ± 10	20 (45)	63.6 ± 7.9	>5% WL (6 months) or BMI of <20 with WL >2% and sarcopenia
Narasimhan 2018 [20]	1/3	5/12	RA	Pancreas Colorectal	1-4	All: 40 (43) WS =19 (47) WL = 21 (40)	WS:64 ± 8 WL: 66 ± 11	N/A	N/A	WL >5% >10% >15% and sarcopenic (SMI) with any degree of WL (>2%)
Noguchi 1998 [36]	0/3	3/12	RA	Esophageal Gastric Colorectal	1-4	10 (90)	56 (50 to 63)*	N/A	N/A	N/A
Pessina 2010 [112]	1/3	6/12	RA	Gastric	1-3	30 (57)	63.8 ± 2.8	8 (62)	64.2 ± 2.6	N/A
Prokopchuk 2016 [127]	0/3	4/12	RA	Pancreas	1-4	All: 25 (32) NC=13 (38) CC=12 (25)	NC:67 (36-87) CC:70 (52-83)*	Benign=15 (80) Pancreatitis=9 (45)	Benign: 67 (32-73) Pancreatitis: 49.5 (40-75)*	>10% WL (6 months)
Ramage 2018 [135]	1/3	3/12	RA	Esophageal Gastric Pancreas	1-4	32 (81)	64.5 (43-83)	N/A	N/A	>5% WL of pre-illness

Rhoads 2009 [54]	1/3	6/12	RA	Gastric	1-4	All: 14 (57) WS = 6 (66) WL = 8 (50)	64.2 ± 3.8	10 (60)	63.9 ± 2.8	NR
Schmitt 2007 [50]	0/3	2/12	RA	Pancreas	2, 4	All: 16 (63) NC= 8 (37) CC = 8 (88)	NC: 62 ± 8.5 CC:53 ± 11.3	N/A	N/A	>10% WL (6 months)
Skorokhod 2012 [113]	0/3	1/12	RA	Pancreas	2-4	All: 23 (61) WS = 13 (69) WL = 10 (50)	WS: 66 (51-69) WL: 65 (57-74)	N/A	N/A	>10% WL of pre-illness
Smith 2010 [60]	0/3	4/12	RA	Gastric	1-4	15 (67)	66 ± 11.6	15 (80)	57 ± 19.3	>5% WL
Stephens 2011 [114]	0/3	2/12	RA	Esophageal Gastric Pancreas Rectal	2-4	19 (58)	67 ± 10	6 (33)	53 ± 8	>10% WL (6 months)
Stephens 2015 [115]	0/3	3/12	RA	Esophageal Gastric Pancreas and other	1-4	All: 92 (72) NC = 41 (82) CC = 51(63)	All: 65 ± 10 NC:68 ± 9 CC:63 ± 9	15 (53)	56 ± 17	>5% WL
Stretch 2013 [23]	0/3	4/12	RA	Liver Bile duct GI tract Pancreas and other	NR	134 (51)	♂:59 ± 13 ♀:63 ± 13	N/A	N/A	N/A
Sun 2012 [116]	0/3	5/12	RA	Gastric	1-4	102 (71)	62.13 ± 6.54	29 (72)	61.8 ± 6.4	>10% WL
Taskin 2014 [117]	0/3	1/12	RA	Colorectal Pancreas Gastric and other	NR	All: 14 (50) NC= 8 (37) CC = 6 (66)	NC: 68 ± 5 CC: 70 ± 15	5 (40)	77 ± 5	>10% WL (6 months) weight stable <5%

Williams 1999 [68]	0/3	2/12	RA	Colorectal	NR	6 (66)	67 (53-76) *	6 (83)	54(22-92) *	N/A
Zeiderman 1991 [63]	0/3	5/12	RA	Esophageal Gastric Colorectal Pancreas	NR	30 (70)	Hospital diet: 67 ± 9.5 3 days intervention: 72 ± 3.2 7 days intervention: 67 ± 6.3	N/A	N/A	> 5 kg WL (3 months)
Zampieri 2010 [57]	0/3	3/12	RA, QF	Colorectal	NR	14 (36)	65.1 ± 10.3	Myopathy: 13 (38) Healthy: = 19(NR)	Myopathy: 64.3 ± 6.3 Healthy: 30.1 ± 13.3	N/A
Zampieri 2009 [55]	0/3	1/12	RA, QF	Colorectal	2-3	10 (30)	65.1 ± 10.3	10(NR)	22.7 ± 2.6	N/A
Zampieri 2010 [56]	1/3	3/12	RA, QF	Colorectal	2-3	11 (36)	65.1 ± 10.3	7 (0)	44.5 ± 18.3	N/A
Aversa 2012 [118]	1/3	3/12	RA, **SA**	NSCLC Gastric	1-4	39 (74)	Lung: 66 ± 9 Gastric :65 ± 10	10 (50)	Abdominal: 63 ± 10 Thoracic	NR

									:65 ± 12	
MacDonald 2015 [119]	0/3	2/12	RA, QF	Esophageal Gastric	1-4	All: 14 (57) WS = 6 (66) WL = 8 (50)	WS: 62.5 (57.0-70.3)** WL: 63.4 (61.5-66.3)**	7 (42)	52.1 (51.5-53.1)**	>5% WL
Shaw 1991 [58]	0/3	6/14	RA, SCM	Colorectal Pancreas Head & neck thyroid and other	NR	All: 43 (42) WS = 25 (48) WL = 18 (66)	WS:61 ± 20 WL: 64 ± 12.7	18 (33)	57 ± 16.9	>15% WL of pre-illness
Stephens 2010 [35]	1/3	3/12	RA, VL, DIAPH	Esophageal Gastric Pancreas	NR	18 (66) WL	67 ± 8.4	3 (66)	45 ± 3.4	>5% WL
Brzeszczynska 2016 [132]	0/3	2/12	QF	Esophageal Gastric Pancreas	2-3	All: 28 (75) NC = 18 (72) CC = 10 (80)	NC: 67 ± 10.5 CC: 65 ± 8.1	Middle age 20 (60) Elderly: 21 (52)	Middle-age: 61 ± 7 Elderly: 79 ± 3.6	>5% WL of pre-illness
Ebhardt 2017 [133]	0/3	1/12	QF	Esophageal Gastric Pancreas	NR	All: 19 (79) NC = 14 (85) CC = 5 (60)	Non- CC: 66.3 ± 10.2 CC:64 ± 4.1	Non-sarcopenic 10 (60) Sarcopenic 8 (50)	Non-sarcopenic: 77.4 ± 2.3 Sarcopenic: 80.3 ± 3.9	>5% WL of pre-illness
Gallagher 2012 [134]	1/3	7/14	QF	Esophageal Gastric Pancreas	1-3	12 (83)	65	6 (66)	58	NR

Christensen 2016 [120]	N/A	13/14	VL	Testicular germ cell	NR	8 (100)	33.4 ± 7.5	Control =9 (100) Ref=13 (100)	Control: 37.8 ± 7.6 Reference group:32. 1 ± 6.3	N/A
Christensen 2014 [121]	N/A	13/14	VL	Testicular germ cell	NR	15 (100)	Intervention: 34.4 ± 7.6 Control: 35.8 ± 8.9	19 (100)	31.5 ± 6.0	N/A
Lamboley 2017 [130]	1/3	3/12	VL	Prostate	2	8 (100)	68 ± 5.6	14 (100)	71 ± 3.7	N/A
Nilsen 2016 [122]	N/A	9/14	VL	Prostate	NR	12 (100)	67 ± 7	11 (100)	64 ± 6	N/A
Op den Kamp 2015 [123]	0/3	6/12	VL	NSCLC	3-4	All: 26 (65) Pre-CC = 10 (80) CC= 16 (56)	Pre-CC: 62.4 ± 10.4 CC: 59.8 ± 8.2	22 (59)	61.4 ± 7.0	>5% WL (6 months)
Op den Kamp 2012 [124]	0/3	3/12	VL	NSCLC	1-3	16 (93)	65.9 ± 7.5	10 (70)	63.7 ± 5.6	10% WL (6 months)
Op den Kamp 2013 [125]	0/3	5/12	VL	NSCLC	3-4	All: 26 (65) Pre-CC = 10 (80) CC= 16 (56)	Pre-CC: 62.4 ± 10.4 CC:59.8 ± 8.2	22 (59)	61.4 ± 7.02	5% WL (6 months) 2% WL with BMI <20 or sarcopenia
Phillips 2013 [126]	0/3	4/14	VL	Colorectal	Early	8 (50)	62.5 ± 23.4	8 (50)	70.7 ± 4.5	N/A

Puig-Vilanova 2014 [128]	1/3	3/12	VL	Lung	1-4	10 (100)	65 ± 9	Healthy = 10 (100) COPD = 16 (100)	65 ± 11 64 ± 9	Fat Free Mass Index: <18.5kg/m^2
Weber 2007 [52]	0/3	3/12	VL	Gastric Pancreas Leukemia	NR	17 (53)	52.5 ± 6.5	27 (52)	57.9 ± 12.4	>10% WL (6 months)
Weber 2009 [59]	0/3	2/12	VL	GI tract (not defined)	NR	19 (52)	58 ± 9	19 (53)	56 ± 7	>10% WL (6 months)
Williams 2012 [129]	0/3	5/12	VL	Colorectal	Early	13 (46)	66 ± 10.8	8 (50)	71 ± 5.6	N/A
Banduseela 2007 [51]	N/A	N/A	TA	NSCLC	NR	1 (100)	63	6 (50)	Healthy: 49±7 Myopathy: 60±18	NR
Higuchi 2000 [38]	N/A	N/A	Gastroc	Gastric	NR	1 (100)	54	N/A	N/A	N/A
Jagoe 2002 [40]	0/3	1/12	LD	Lung	3-4	36 (75)	64.1 ± 9	10 (40)	51.3 ± 15.1	Any % WL (6 months)
Bohlen 2018 [131]	0/3	4/12	PM	Breast	1-4	14 (0)	56.5 ± 17.2	6 (0)	44.2 ± 7.4	N/A

Values reported as mean ± standard deviation (SD) unless indicated Otherwise *median (range) and ** median (interquartile range). ǂ Modified Newcastle-Ottawa Scale ǂ ǂ Quality Assessment score-high score means high quality. NIH-NHLBI: National Heart Lung and Blood Institute; RA: Rectus Abdominis; TA: Tibialis anterior; QF: Quadriceps Femoris; VA: Vastus Lateralis; PM: Pectoralis Major; SA: Serratus Anterior; LD: Latissimus Dorsi; Gastroc: Gastrocnemius; SCM: Sternocleidomastoid; DIAPH: Diaphragm; GI: Gastrointestinal; NSCLC: Non-small cell Lung

carcinoma; N/A: Not applicable; NR: Not reported. WS: Weight stable WL: Weight loss. NC: non-cachexia .BMI: Body Mass Index. SMI: Skeletal Muscle Index.

Table 3-2. Biopsy collection and handling procedures across the studies

Author	Biopsy collection (collected in start or end of surgery)	Cauterized	Blood traces, fat or connective tissue removed	Sample handling and storage conditions
Acharyya 2005	NR	NR	NR	NR
Agustsson 2011	Initial phase of surgery	NR	NR	Incubated in vitro
Aversa 2016	Initial phase of surgery	No	NR	Immediately frozen, stored at -80°C
Aversa 2012	Initial phase of surgery	No	NR	Immediately frozen, stored at -70°C
Banduseela 2007	Percutaneous biopsy (local anaesthesia)	N/A	Yes (fat, connective tissue)	Immediately frozen, stored at -80°C
Bohlen 2018	NR	N/A	NR	Stored in RNA stabilization solution at -4°C overnight and then stored at -80°C
Bonetto 2013	Initial phase of surgery	No	NR	Immediately frozen, stored at -80°C
Bossola 2006	Initial phase of surgery	No	NR	Immediately frozen, stored at -70°C
Bossola 2001	Initial phase of surgery	No	NR	Immediately frozen, stored at -70°C
Bossola 2003	Initial phase of surgery	No	NR	Immediately frozen, stored at -70°C
Brzeszczyn ska 2016	Initial phase of surgery	No	Yes (blood)	Immediately frozen, stored at -80°C
Busquets 2007	Initial phase of surgery	No	NR	Immediately frozen, stored at -80°C
Christensen 2016	Percutaneous biopsy (local anaesthesia)	N/A	NR	Immediately frozen, stored at -80°C
Christensen 2014	Percutaneous biopsy (local anaesthesia)	N/A	NR	Immediately frozen, stored at -80°C
DeJong 2005	Initial phase of surgery	No	NR	Immediately frozen, stored at -70°C
D'Orlando 2014	Initial phase of surgery	No	NR	Immediately frozen, stored at -80°C
Ebhardt 2017	Percutaneous biopsy (local anaesthesia)	N/A	Yes (blood)	Immediately frozen, stored at -80°C
Eley 2008	Initial phase of surgery	No	NR	Immediately frozen, stored at -70°C

Gallagher 2012	Percutaneous biopsy (local anaesthesia)	N/A	Yes (blood)	Immediately frozen, stored at -80°C
Higuchi 2000	NR	N/A	NR	NR
Jagoe 2002	Initial phase of surgery	No	NR	Immediately frozen, stored at -80°C
Johns 2017	Initial phase of surgery	No	NR	Immediately frozen, stored in liquid nitrogen
Johns 2014	Initial phase of surgery	NR	Yes (blood)	Immediately frozen, stored at -80°C
Khal 2005	NR	No	NR	Immediately frozen, stored at -70°C
Lamboley 2017	Percutaneous biopsy (local anaesthesia)	N/A	Yes (blood)	Immediately and stored in liquid nitrogen
Lundholm 1976	Initial phase of surgery	NR	NR	Muscle fibre isolation on fresh tissue
MacDonald 2015	Initial phase of surgery and Percutaneous biopsy (local anaesthesia)	NR	NR	Immediately frozen in liquid nitrogen, storage temperature NR
Marzetti 2017	Initial phase of surgery	No	NR	Immediately frozen, stored at -80°C
Narasimhan 2017	Initial phase of surgery	No	NR	Immediately frozen, stored at -80°C
Narasimhan 2018	Initial phase of surgery	No	NR	Immediately frozen, stored at -80°C
Nilsen 2016	Percutaneous biopsy (local anaesthesia)	N/A	Yes (fat)	Frozen by immersion in isopentane, stored at -80°C
Noguchi 1998	Initial phase of surgery	NR	NR	Immediately frozen in situ, stored at -70°C
Op den Kamp 2015	Percutaneous biopsy (local anaesthesia)	N/A	NR	Immediately frozen, stored at -70°C
Op den Kamp 2012	Percutaneous biopsy (local anaesthesia)	N/A	NR	Immediately frozen, stored at -80°C
Op den Kamp 2013	Percutaneous biopsy (local anaesthesia)	N/A	NR	Frozen by immersion in isopentane, stored in -80°C
Pessina 2010	Initial phase of surgery	No	NR	Immediately frozen, stored at -70°C
Phillips 2013	Percutaneous biopsy (local anaesthesia)	N/A	NR	Immunoblotting in fresh tissue
Prokopchuk 2016	NR	NR	NR	Immediately frozen and stored at -80°C
Puig-Vilanova 2014	Open muscle biopsy technique	N/A	NR	NR

Ramage 2018	NR	NR	NR	Immediately frozen, stored at -80°C
Rhoads 2009	Initial phase of surgery	No	NR	Immediately frozen, stored at -70°C
Schmitt 2007	NR	NR	NR	Immediately frozen in liquid nitrogen, storage temperature NR
Shaw 1991	NR	NR	NR	Snap-frozen in liquid nitrogen, thawed after 48 hrs
Skorokhod 2012	Initial phase of surgery	NR	NR	Immediately frozen in liquid nitrogen, storage temperature NR
Smith 2011	Initial phase of surgery	No	NR	Immediately frozen and stored at -80°C
Stephens 2011	Initial phase of surgery	NR	NR	Fixation for microscopy
Stephens 2010	Rectus abdominis – NR Quadriceps - Percutaneous biopsy (local anaesthesia)	NR	NR	Immediately frozen
Stephens 2015	Initial phase of surgery	NR	Yes (blood)	Immediately frozen, stored at -80°C
Stretch 2013	Initial phase of surgery	No	NR	Immediately frozen and stored in liquid nitrogen.
Sun 2012	Initial phase of surgery	NR	NR	Immediately frozen and stored at -80°C
Taskin 2014	NR	NR	NR	Transferred to lab on ice cold buffer, stored at -20°C
Weber 2007	Percutaneous biopsy (local anaesthesia)	N/A	NR	Immediately frozen, stored at -80°C
Weber 2009	Percutaneous biopsy (local anaesthesia)	N/A	NR	Immediately frozen, stored at -70°C
Williams 2012	Percutaneous biopsy (local anaesthesia)	N/A	NR	NR
Williams 1999	Initial phase of surgery	No	NR	Immediately frozen, stored at -70°C
Zampieri 2010	Rectus abdominis – NR Quadriceps - Percutaneous biopsy (local anaesthesia)	NR	NR	Immediately frozen and stored in liquid nitrogen.
Zampieri 2009	Rectus abdominis – NR Quadriceps - Percutaneous biopsy (local anaesthesia)	NR	NR	Immediately frozen and stored in liquid nitrogen.
Zampieri 2010	Rectus abdominis - NR Quadriceps - Percutaneous biopsy (local anaesthesia)	NR	NR	Immediately frozen and stored in liquid nitrogen.
Zeiderman 1991	Initial phase of surgery	NR	NR	Incubation

NR: NR; N/A: Not applicable

Table 3-3. Patient characteristics

Age, mean years ± SD (Min-Max)	**61 ± 12 (19-87)**	**65 ± 12 (21-87)**	**0.049**
Tumour type, % (n):			0.006
Colorectal	45 (55)	26 (18)	
Pancreas	23 (28)	31 (21)	
Other gastro-intestinal	25 (31)	22 (15)	
Other[b]	6 (8)	20 (14)	
Presence of metastasis, % (n)	56 (68)	40 (27)	0.03
Chemotherapy exposure within 4 weeks prior to muscle biopsy, % (n):	23 (28)	22 (15)	0.9
Patients with weight lost, % (n)	56 (14)[#]	55 (11)[##]	0.9
Sarcopenia, % (n)	60 (61)*	50 (23)**	0.2
BMI (kg/m^2), mean ± SD	27 ± 5	28 ± 7	0.7
BMI classification, % (n):			0.1
Underweight	1 (1)	1 (1)	
Normal	26 (32)	26 (18)	
Overweight	39 (48)	28 (19)	
Obesity I	17 (21)	6 (4)	
Obesity II	5 (6)	10 (7)	
Obesity III	2 (2)	4 (3)	
Missing BMI	10 (12)	24 (16)	
Comorbidities, % (n):			
Diabetes type II	12 (15)	18 (12)	0.3
Hypertension	24 (29)	29 (20)	0.4
Cardiovascular disease	15 (18)	7 (5)	0.1
Dyslipidemia	7 (9)	7 (5)	0.9
History of smoking habit, % (n)	28 (34)	24 (16)	0.3
Computed tomography, body composition analysis, mean ± SD:			
Subcutaneous adipose tissue (cm^2)	166.4±91.5’	251.1±134.4”	<0.001
Visceral adipose tissue (cm^2)	174.8±105.1’	111.9±65.7”	<0.001
Muscle biopsy triglyceride content (µg/mg), mean ± SD:	13.2±14.8[^]	29.5±21.7[^^]	<0.001

[a]Other gastro-intestinal: Stomach, small intestine, liver, intrahepatic bile duct, gallbladder, biliary tract and appendix.
[b]Other: adrenal gland, skin, kidney, mesothelium, lymphoma, melanoma, chronic lymphocytic leukemia, prostate, ovary, uterus, head and neck.
BMI: Body mass index
Patients with weight loss information: [#] n=25 / [##] n=20
Patients with sarcopenia information: * n=102 / ** n=46
CT Adipose tissue information: ‘n=98 / ‘n=44
Patients with muscle biopsy triglyceride content: ^ n=69 / ^^ n=19
Differences between males and females were analyzed by independent t-test (continuous variables) and chi-square test (categorical variables).

Table 3-4. Most common medications prescribed and potential effects on skeletal muscle

Class of drug	Medication	% (n)	Common use	Possible implications to skeletal muscle
Cyclooxygenase inhibitors	Aspirin, Acetaminophen	15 (29)	Pain, fever, inflammation, prevention of cardiovascular disease	Influence muscle prostaglandin synthesis, muscle protein metabolism, and cellular processes regulating muscle protein synthesis. (Burd, 2010; Liu et al., 2016; Standley, 2013; Todd A Trappe, 2013)
HMG-CoA reductase inhibitors	Rosuvastatin, Simvastatin, Atorvastatin	13 (24)	lipid-lowering	Association with myalgia and related symptoms. Associated to mitochondrial oxidative stress. (Bouitbir et al., 2016; Diaz et al., 2015)
Biguanide	Metformin	8 (16)	Type 2 diabetes, suppressor of hepatic gluconeogenesis	Mitochondrial dysfunction in skeletal muscle. Sensitizes muscle to insulin; increases glucose disposal in skeletal muscle. (Diaz, 2015; Elsaid et al., 2017; Malin, 2014; Wessels, 2014)
Proton pump inhibitors	Omeprazole, Pantoprazole	8 (16)	Gastroesophageal reflux, erosive esophagitis	Concomitant administration with atorvastatin and dexamethasone is associated to increase risk of myopathy (Elazzazy et al., 2012)
Hormones	Levothyroxine	7 (13)	Thyroid hormone (T4) deficiency	Influences myogenesis, associated with sarcopenia and myopathy (Bloise et al., 2018; Salvatore, 2014)
Angiotensin converting enzyme inhibitor	Ramipril	7 (13)	Hypertension, congestive heart failure	Associated with larger muscle cross sectional area and muscle remodeling, associated with cancer cachexia (Burks et al., 2011; Delafontaine et al., 2016; Di Bari et al., 2004; Penafuerte et al., 2016)
Thiazide diuretic	Hydrochloro-thiazide	6 (12)	Hypertension, diuretic by reducing sodium reabsorption	None reported or reviewed
Calcium channel blockers	Amlodipine	5 (9)	Hypertension, calcium channel blocker	None reported and reviewed (Godfraind, 2017)
Opioid	Oxycodone	3 (5)	Pain	Hypogonadism and testosterone depletion in men (Vuong, 2010)
Alpha-adrenergic blocker	Tamsulosin	3 (5)	Muscle relaxer of prostate and bladder	None reported or reviewed
Xanthine oxidase inhibitor	Allopurinol	3 (5)	Gout prevention, decrease blood uric acid levels	Prevents skeletal muscle atrophy (Derbre et al., 2012)
Anticoagulant	Warfarin	3 (5)	Anticoagulant	None reported or reviewed

Percentage of patients prescribed this medication out of a total of 190 patients who had a medical history available with information provided on current medication use.

Table 3-5. Computed tomography defined muscle composition at L3 for rectus abdominis and total skeletal muscle in cancer patients, stratified by sex and age

Sex	Age stratum		Rectus Abdominis	Total Lumbar Muscle	Lumbar skeletal muscle index	Rectus Abdominis	Total Lumbar Muscle
		N	L3-CSA (cm^2)		cm^2/m^2	Radiodensity (Hounsfield Units)	
Male	<50	17	15.9 ± 3.8 (9.8-23.4)	188.7 ± 29.1 (123.6-238.2)	58.2 ± 8.9 (42.8-73.3)	36.2 ± 12.3 (7.6-54.8)	39.6 ± 10.5 (15.4-55.3)
	50-60	34	13.6 ± 3.9 (6.6-24.5)	156.2 ± 27.5 (107.2-228.9)	50.6 ± 8.2 (37.1-66.5)	30.9 ± 12.2 (4.4-50.0)	36.5 ± 8.9 (13.8-50.5)
	60-70	23	13.3 ± 3.3 (5.7-19.4)	158.4 ± 20.7 (109.0-192.5)	50.8 ± 6.6 (36.4-60.8)	28.0 ± 12.3 (-10.8-44.3)	33.8 ± 10.1 (7.1-54.4)
	70-80	23	11.5 ± 2.6 (6.0-17.6)	141.4 ± 23.0 (94.6-187.2)	46.6 ± 6.0 (35.6-59.1)	20.0 ± 11.3 (-2.0-44.6)	28.9 ± 7.7 (10.0-42.6)
	>80	4	9.8 ± 4.2 (6.2-15.2)	139.0 ± 16.4 (122.8-160.9)	46.1 ± 7.1 (40.1-56.3)	21.5 ± 8.3 (12.3-30.4)	27.5 ± 3.0 (24.8-31.5)
Female	<50	3	9.3 ± 3.2 (5.9-12.2)	114.9 ± 14.8 (97.8-124.4)	43.8 ± 1.6 (42.9-45.7)	32.0 ± 5.7 (26.6-38.0)	45.1 ± 5.3 (40.5-50.9)
	50-60	11	7.0 ± 2.4 (3.8-10.9)	101.5 ± 16.8 (67.5-125.4)	38.3 ± 6.8 (23.9-46.4)	22.7 ± 13 (4.2-41.1)	35.4 ± 7.6 (20.9-46.1)
	60-70	15	8.7 ± 3.7 (2.8-16.9)	102 ± 16.6 (66.2-122.7)	39.2 ± 7.0 (27.7-52.8)	19.1 ± 10.3 (2.5-39.1)	29.0 ± 7.1 (18.2-39.6)
	70-80	16	6.7 ± 2.3 (1.4-10.9)	101.0 ± 13.8 (79.0-127.3)	40.5 ± 4.8 (33.8-49.7)	13.1 ± 10.0 (-7.7-30.9)	28.9 ± 7.0 (15.0-38.9)
	>80	3	7.7 ± 3.1 (4.2-10.0)	92.8 ± 14.8 (77.9-107.5)	41.1 ± 8.1 (32.9-49.1)	12.2 ± 19.8 (-10.1-27.6)	22.9 ± 4.1 (18.2-25.3)
Total male		101	13.6 ± 3.8 (5.7-24.5)	158.2 ± 29 (94.6-238.2)	50.8 ± 8.3 (35.6-73.3)	28.2 ± 12.9 (-10.8-54.8)	34.3 ± 9.7 (7.1-55.3)
Total female		48	7.6 ± 2.9 (1.4-16.9)	101.7 ± 15.4 (66.2-127.3)	39.8 ± 6 (23.9-52.8)	18.2 ± 12 (-10.1-41.1)	31 ± 8.3 (15-50.9)

Values reported in mean ± SD (range). L3, 3rd Lumbar vertebra. CSA, Cross-seccional area.

Table 3-6. Rectus abdominis myosin heavy chain content and mean muscle fiber area of cancer patients

	All	Male	Female	P value
A. MyHc content by electrophoresis[a] (% ± SD N= 40 M / n=8 F)				
MyHC I (%)	39.3 ± 11.1	39.1 ± 10.3	40.6 ± 15.6	0.73
MyHC IIA (%)	38.4 ± 11.1	37.5 ± 10.0	42.6 ± 15.7	0.24
MyHC IID (%)	22.3 ± 8.9	23.4 ± 8.6	16.8 ± 9.1	0.06
B. MyHc content by immunohistochemistry[a] (% ± SD N= 20 M / n=10 F)				
MyHC isoforms (%):				
MyHC type I	47.1 ± 13.0	47.0 ± 12.6	47.3 ± 14.6	0.91
MyHC type IIA	51.8 ± 13.4	52.4 ± 12.6	50.5 ± 15.6	0.53
MyHC type IID	16.7 ± 14.3	19.2 ± 13.7	11.8 ± 15.1	0.19
All Hybrids[b]	15.5 ± 13.5	18.5 ± 13.5	9.6 ± 12.2	0.08
Individual fiber types (%):				
Fiber type I	46.4 ± 12.9	48.9 ± 9.4	46.2 ± 14.2	0.32
Fiber type I/IIA	0.7 ± 1.0	0.6 ± 0.9	1.2 ± 1.6	0.15
Fiber type IIA	36.1 ± 9.5	35.7 ± 9.4	40.7 ± 9.6	0.71
Fiber type IIA/D	15.0 ± 13.7	13.1 ± 12.4	8.5 ± 12.9	0.39
Fiber type IID	1.8 ± 4.6	1.7 ± 3.7	3.4 ± 7.3	0.32
C. Mean muscle fiber area (μm^2) (% ± SD N= 20 M / n=10 F)				
All Fibers	3236 ± 1390	3784 ± 1285	2139 ± 854	<0.05
MyHC isoforms (μm^2):				
MyHC type I	2323 ± 944	2591 ± 970	1786 ± 635	<0.05
MyHC type IIA	4009 ± 1937	4848 ± 1725	2331 ± 1054	<0.05
MyHC type IID	4026 ± 2060	4722 ± 1895	2461 ± 1546	<0.05
Individual fiber types (μm^2):				
Fiber type I	2325 ± 941	2591 ± 970	1795 ± 633	<0.05
Fiber type I/IIA	2253 ± 1209	2726.6 ± 1181	1306 ± 528	<0.05
Fiber type IIA	3940 ± 1970	4760 ± 1820	2299 ± 1012	<0.05
Fiber type IIA/D	4012 ± 2055	4833.5 ± 1841	2266 ± 1268	<0.05
Fiber type IID	5243 ± 2407	5323 ± 2553	4729 ± 1524	0.75

MyHC: Myosin heavy chain

[a] There were no differences in age, BMI, metastasis, chemotherapy exposure, comorbidities nor smoking history between men and women

[b] All hybrids refer to fibers of mixed myosin heavy chain isoforms MyHC type I/IIA and MyHC type I

Table 3-7. Skeletal muscle gene expression for genes associated with cancer cachexia in cancer patients[a]

Biological function	Gene symbol	Gene name	Agilent transcript ID [Refseq RNA ID]	Female (n=64)	Male (n=69)	p-value
Atrophy	FOXO1	Forkhead Box O1	A_24_P22079	1.53 ± 1.04	1.11 ± 0.68	0.005
Autophagy	BECN1	Beclin 1	A_23_P433071 [NM_003766]	0.91 ± 0.27	1.03 ± 0.3	0.05
			A_23_P89410 [NM_003766]	1.00 ± 0.27	1.11 ± 0.33	0.05
	CTSL2	Cathepsin L2	A_23_P146456 [NM_001333]	1.31 ± 0.57	0.99 ± 0.44	<0.0001
Apoptosis	CASP8	Caspase 8	A_23_P209389 [NM_033355]	0.97 ± 0.32	1.09 ± 0.38	0.08
	CASP9	Caspase 9	A_23_P97309 [NM_001229]	0.95 ± 0.19	1.06 ± 0.25	0.008
			A_24_P111342 [NM_001229]	0.97 ± 0.22	1.08 ± 0.31	0.03
Muscle growth	AKT1	V-Akt Murine Thymoma Viral Oncogene Homolog 1	A_23_P2960 [NM_005163]	1.23 ± 0.52	1.04 ± 0.35	0.03
	DMD	Dystrophin	A_24_P342388 [NM_004019]	1.34 ± 0.67	0.94 ± 0.29	<0.0001
			A_24_P185854 [NM_004010]	1.11 ± 0.27	0.94 ± 0.23	<0.0001
			A_24_P34186 [NM_004010]	1.19 ± 0.55	0.97 ± 0.39	0.01
			A_32_P199796 [NM_004023]	1.27 ± 0.66	0.98 ± 0.42	0.005
	MSTN	Myostatin	A_23_P165727 [NM_005259]	1.71 ± 2.43	2.74 ± 3.74	0.02
	PAX7	Paired Box 7	A_23_P126225 [NM_013945]	0.99 ± 0.49	1.08 ± 0.39	0.05
			A_23_P500985 [NM_013945]	0.96 ± 0.45	1.03 ± 0.33	0.09
	PPARGC1A	Peroxisome Proliferator-Activated Receptor Gamma, Coactivator 1 Alpha	A_24_P303052 [NM_013261]	1.22 ± 0.77	1.00 ± 0.51	0.07

	SMAD3	SMAD Family Member 3	A_23_P48936 [NM_005902]	1.14 ± 0.42	1.00 ± 0.28	0.07
	TGFB1	Transforming Growth Factor, Beta 1	A_24_P79054 [NM_000660]	1.42 ± 1.47	1.06 ± 0.54	0.01
Inflammation	JAK1	Janus Kinase 1	A_24_P410678 [NM_002227]	0.92 ± 0.37	1.15 ± 0.43	0.001
	JAK2	Janus Kinase 2	A_23_P123608 [NM_004972]	1.21 ± 0.48	1.06 ± 0.45	0.03
	JAK3	Janus Kinase 3	A_23_P329112 [NM_000215]	1.03 ± 0.46	1.19 ± 0.57	0.09
	STAT3	Signal Transducer And Activator Of Transcription 3	A_23_P107206[NM_213662]	1.21 ± 1.02	0.53 ± 0.35	0.02
	STAT5A	Signal Transducer And Activator Of Transcription 5A	A_23_P207367 [NM_003152]	1.12 ± 1.01	0.32 ± 0.34	0.03
			A_24_P173088 [NM_003152]	1.19 ± 1.00	0.47 ± 0.45	0.005
	TNF	Tumor Necrosis Factor	A_24_P50759 [NM_000594]	0.99 ± 0.35	1.15 ± 0.44	0.03

Values (unitless) reported as mean ± standard deviation

[a] Cancer type (p=0.003) and metastasis presence (p=0.002) were different between men and women. There were no differences in age, BMI, chemotherapy exposure, comorbidities nor smoking history between men and women.

Table 3-8. Summary of recommendations for muscle biopsy processing and population characterization

A) Biobanking protocols and tissue manipulation:

- For intraoperative muscle biopsies, collect at the start of the surgical procedure and avoid cauterization.
- Avoid or report the use of foreign substances (e.g. use of saline-moistened gauzes)
- Report waiting periods between surgical/needle removal, transportation to other facilities and freezing; include the use or not of crushed ice during the waiting process.
- Report any removal of blood traces or unrelated tissue from the muscle biopsy.
- If muscle is "immediately frozen" clarify the location, time and other relevant details (e.g. RNA stabilizer solution) of this action after the surgical removal.
- Sample storage recommended $\leq$-70°C; however, the temperature selection will depend on the molecules of interest and/or experimental techniques.

B) Cancer population characterization.

- Clearly state the patient selection method and possible limitations.
- Report information on metastatic status or tumour classification.
- Report comorbidities and medications.
- Report past or current exposure of antineoplastic treatments.

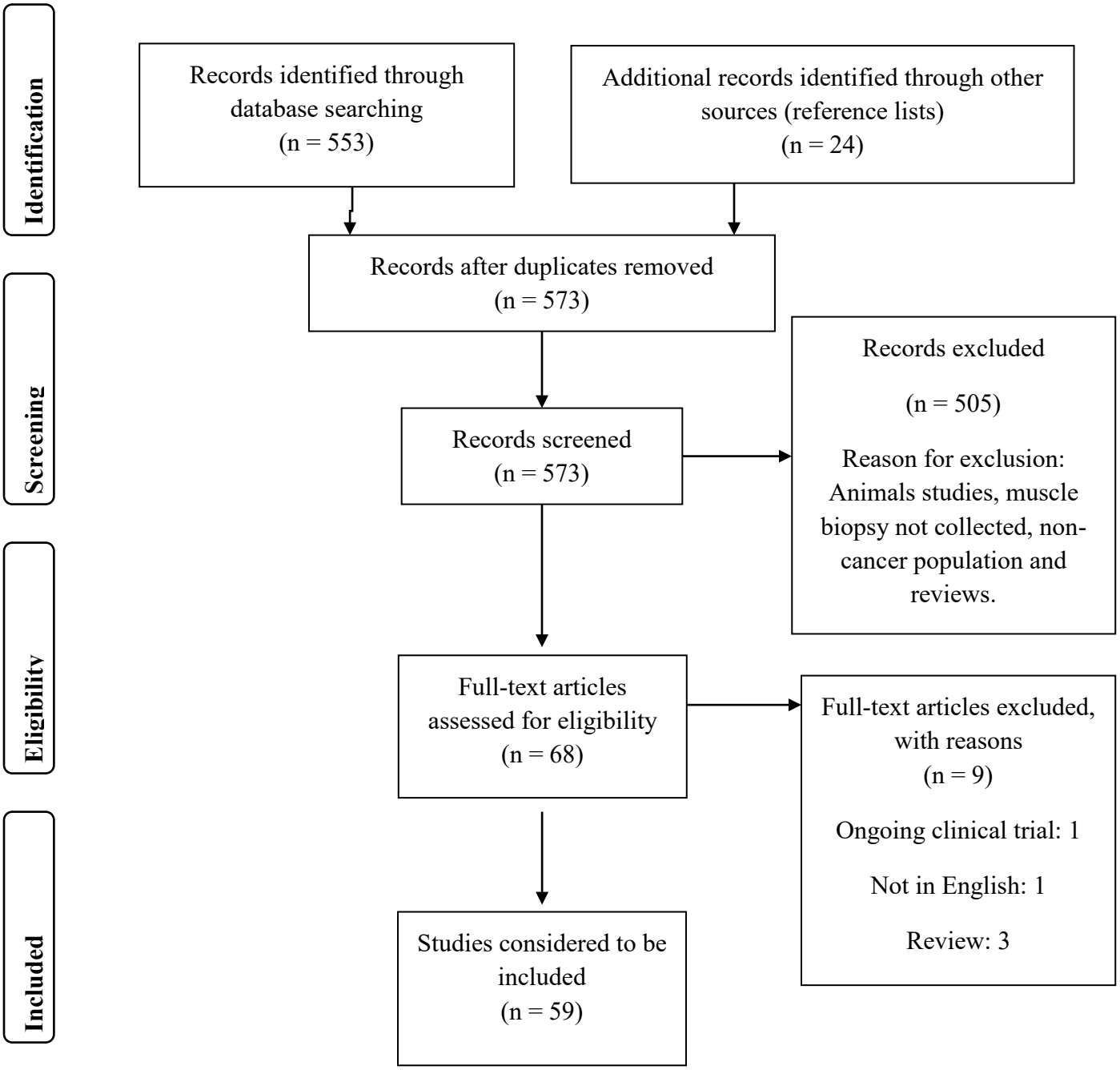
C) Inclusion of control groups

- Provide a clear characterization of the control group.
- Report comorbidities and medications.
- Match age and sex with study population. Provide justification for case-matching criteria
- Collect same muscle in control and study populations.

D) Classification and results

- Avoid mixing the results of two or more muscle groups or comparing one muscle group with a different muscle group (e.g. rectus abdominis versus quadriceps).
- Acknowledge sexual dimorphism in skeletal muscle by reporting results based in male and females, include mean and standard deviation.
- Classification of cancer cachexia should include both, body composition analysis (muscle mass values) and weight loss

Figure 3-1. Flow chart of search. PRISMA diagram for the identification, screening, eligibility and inclusion of papers (January 1st 1990 – August 16th 2018) from SCOPUS. All articles included investigated cancer, skeletal muscle and muscle biopsies. Excluded records: review articles and ongoing clinical trials



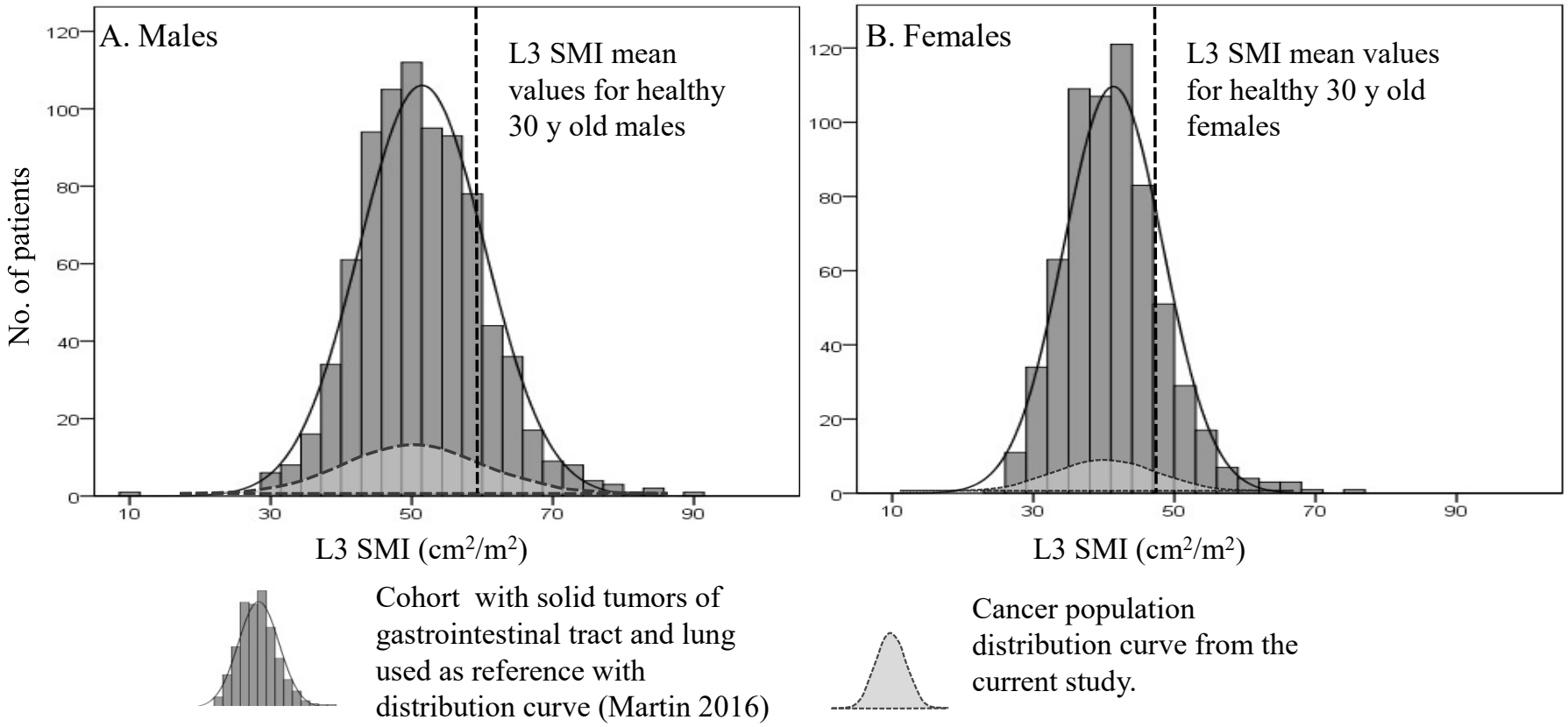


Figure 3-2: Figure represents overlap of L3 SMI distributions for male (A) and female (B) patients of current cancer population (small, light gray distribution) and a cancer cohort with solid tumors of gastrointestinal tract and lung (big, dark gray distribution) (Martin 2016 and Kazemi-Bajestani 2015). A. L3 SMI mean ± standard deviation values are 50.8±8.3 cm2/m2 and 51.5±8.9 cm2/m2 for the current population and Martin et al. gastrointestinal and lung cancer cohort, respectively. B. L3 SMI mean values are 39.8±6 cm2/m2 and 41.3±7 cm2/m2 for the current population and Martin et al. gastrointestinal and lung cancer cohort, respectively. L3 SMI mean values for healthy 30 y old kidney donor candidates (dotted line) are placed at 60.9 and 47.7 cm2/m2 for males and females, respectively (Derstine 2018).

Chapter 4: Association between muscle radiodensity and muscle triglyceride content in cancer patients

4.1 Introduction

Computed tomography (CT) imaging has recently revealed that a reduced level of muscle radiodensity is associated with shorter survival and systemic inflammation in cancer patients (Hayashi et al., 2016; Malietzis et al., 2016; Martin et al., 2013; Rier et al., 2017; Rollins et al., 2016; van Vugt et al., 2018). Radiodensity is measured in Hounsfield Units (HU), which is a linear transformation of the attenuation coefficient, where the radiodensity of distilled water at standard pressure and temperature is defined as 0 HU and the radiodensity of air at standard temperature and pressure is defined as -1000 HU (Heymsfield et al., 1997; Larson-Meyer et al., 2008). Reduced muscle radiodensity has also been associated with insulin resistance (B H Goodpaster et al., 1997; Maltais et al., 2018), mitochondrial dysfunction (Simoneau et al., 1995), decrease in contractile force of the muscle(Nordal et al., 1988), low aerobic capacity (B H Goodpaster, 1997) and impaired lipolytic response (Kelley et al., 1999).

Based on a study by Goodpaster et al., in which the relationship of mid-thigh muscle radiodensity and triglyceride (TG) content in healthy and diabetic patients was reported, it has been assumed that low muscle radiodensity is related to high TG content of RA (Bret H Goodpaster, Kelley, et al., 2000). However, this relationship between radiodensity and TG content must be re-examined in cancer patients for a number of reasons. First, body composition studies in cancer patients are typically done using CT images acquired in the lumbar region (axial images at the 3rd lumbar vertebra (L3). The physicochemical properties of lumbar muscles may differ substantially from muscles of the limb. Second, in the report from Goodpaster and coworkers, muscle radiodensity was reported as mean radiodensity from all pixels within the range of 0-100 HU at mid-thigh cross-section (Bret H

Goodpaster, 2000), whereas in the oncology setting, standardized measures of muscle between -29 and 150 HU are widely used and typically the muscles imaged are in the lumbar region (Aubrey et al., 2014).

The presence of high levels of lipid in skeletal muscle have been associated with aging (Marcus RL, Addison O, Kidde JP, Dibble LE, 2010; Reinders et al., 2016), frailty (Idoate et al., 2015), chronic back pain (Goubert et al., 2018; Teichtahl et al., 2015), diabetes (Bret H Goodpaster, Thaete, et al., 2000; Bret H Goodpaster et al., 2004; Hilton et al., 2008; Kelley et al., 2002), obesity (Bret H Goodpaster, 2004; Hilton, 2008; Khan et al., 2015), chronic obstructive pulmonary disease (Robles et al., 2015) and cancer (Stephens et al., 2011). Lipid content has been reported in variety of muscle groups, including soleus (Jacob et al., 1999; Perseghin et al., 1999; Sinha et al., 2002), tibialis anterior (Jacob, 1999; Sinha, 2002), vastus lateralis (B H Goodpaster et al., 2001; Pan et al., 1997) and mid-thigh (B H Goodpaster, 1997; Bret H Goodpaster et al., 2003; Bret H Goodpaster, 2000) and is noted for its non-uniform distribution across the length of a muscle (Wendling et al., 1996). Even in healthy muscle, lipid depots are clustered in certain areas (Hwang et al., 2001; Vermathen et al., 2004), notably in the vascularized areas of the intermuscular connective tissue (Yudkin et al., 2005). On this basis, we suspected that lipids would not be uniformly distributed in the muscles of cancer patients and there would be variation in muscle radiodensity within the same muscle group.

Skeletal muscles encompass lipid depots found deep in the fascia and within muscles, referred to as extramyocellular lipid (EMCL) as well as lipid droplets inside muscle fibres, referred to as intramyocellular lipid (IMCL). IMCL is associated with insulin resistance, inflammation and functional deficit in skeletal muscle (Coen et al., 2012; Rivas et al., 2016). EMCL can originate from adipogenic differentiation of stem cell populations of skeletal muscle (Uezumi et al., 2014). In cancer patients, it is not known whether lipids are located inside muscle fibres as IMCL or adjacent to the

muscle fibres within adipocytes as EMCL. The etiology of each lipid depot is different therefore it is important to know the location of lipid deposition in muscles.

A number of studies have involved the study of *rectus abdominis* (RA) biopsies in cancer patients to delineate biological features associated with low muscle mass and radiodensity (Johns et al., 2014; Narasimhan et al., 2018; C. Stretch et al., 2013; Cynthia Stretch et al., 2018). The primary aim of this study was to investigate the association between muscle radiodensity and TG content in the RA of cancer patients. As lipids are not uniformly distributed across the length of the muscle, our collateral aim was to gain an appreciation of the variation in muscle radiodensity within the RA. Finally, we sought to understand how lipid content was distributed in the intramyocellular and extramyocellular compartments.

4.2 Materials and Methods

4.2.1 Ethics statement

The study was approved by the Health Research Ethics Board of Alberta-Cancer. Patients undergoing elective abdominal surgery were consecutively approached to participate in tumor and tissue banking at a hepatopancreatobiliary surgical service in Alberta, Canada. Three percent of approached patients declined participation. Patients provided written informed consent for muscle biopsy and tissue banking. Release of 75 samples from the bank for analysis, as well as patient information (demographic, clinical and operative data) from medical records, was performed under the auspices of Protocol ETH-21709: *The Molecular Profile of Cancer Cachexia*.

4.2.2 Subjects and muscle biopsies

The study cohort and conditions for acquisition of muscle samples have been described previously (C. Stretch, 2013). Briefly, RA biopsies (0.5 – 3 g) were collected from cancer patients

(>18 years old) undergoing open abdominal surgery scheduled as part of their clinical care. Biopsies were collected at the start of surgery using sharp dissection and without the use of electrocautery. Samples were processed under sterile conditions. Visible adipose and connective tissues were trimmed. One piece of biopsy was immediately frozen in liquid nitrogen and stored at -80°C. Another piece was frozen in isopentane cooled at -160°C in liquid nitrogen and stored at -80°C for cryostat sectioning and ORO staining and morphological analysis. Diagnosis was confirmed before including patients in current study. Age and cancer type were abstracted from medical charts.

4.2.3 CT Image Analysis

Pre-operative CT scans completed with a spiral CT scanner for initial cancer staging and surgical planning were used to quantify skeletal muscle area and radiodensity (Mourtzakis et al., 2008; Prado et al., 2008). Images were analysed using SliceOmatic® V4.2 software with CT image parameters that include: contrast, 5mm slice thickness, 120 kVP, and 290 mA. Total skeletal muscle area (cm^2) was evaluated on an axial single image at the 3^{rd} lumbar vertebra (L3) using Hounsfield unit (HU) thresholds of -29 to 150 for skeletal muscle (Mourtzakis, 2008). The sum of skeletal cross-sectional muscle areas was normalized for stature (m^2) and reported as skeletal muscle index (SMI) (cm^2m^2). Muscle radiodensity was assessed as the mean value for the full range of -29 HU to +150 HU. Mean lumbar muscle radiodensity (HU) is reported for the entire muscle area (quadratus lumborum, psoas, erector spinae, external obliques, transverse abdominis, internal obliques, and RA) and RA separate. Mean time period between CT image and biopsy collection was 64 days (ranged from 8 to 135 days). To examine the variation in muscle radiodensity within RA, a series of ten slices at 5 mm interval from each other were analysed for a subset of 19 patients. To examine the variation within same slice of RA, muscle radiodensity was measured at 10 different regions of interest in the single image slice in a subset of 13 patients. Ten different regions of interest were selected in the single

image slice. The area near the boundary of the muscles was avoided to exclude subcutaneous, visceral adipose tissue and intramuscular fat area.

4.2.4 TG content of rectus abdominis

Biopsy [≈50 mg] was ground using a frozen pestle and mortar without letting the muscle tissue thaw. Ground tissue was homogenized in a 1.6 ml calcium chloride [$CaCl_2$; 0.025%] solution with glass beads [0.5 mm diameter; FastPrep ®-24, MP Biomedicals, Santa Ana, CA, USA] in 20-sec intervals for 1 min total. Samples were placed on ice for at least 15 sec between each homogenization interval. A modified Folch method was used to extract lipids using chloroform/methanol (2:1, vol/vol) as previously described (Murphy et al., 2010; Pratt et al., 2001). The TG fraction was isolated on G-plates and the TG band was identified and scraped from G-plates. An internal standard C15:0 [10.2 mg/100 ml hexane] was added, followed by saponification and methylation. Fatty acid (FA) composition was determined using gas chromatography-flame-ionisation detector analysis on a Varian 3900 [Varian Instruments, Georgetown, ON, Canada]. Fatty acids were separated between 6 and 24 carbon chain lengths and identified using a fatty acid standard of known composition [GLC-82 and GLC-502, Nu-Chek Prep, USA]. Quantity of fatty acids within the TG fractions was calculated by comparison with the known concentration of the internal standard and sum of all fatty acids was reported as total TG. TG content of each muscle biopsy was analysed in duplicates. The coefficient of variance was <5%.

4.2.5 Immunofluorescence: Oil red O

Tissues were cryosectioned transversely [10 μm thick] and stained for neutral lipid content using ORO as previously described (Koopman et al., 2001). Primary and secondary antibodies are described in the supplementary materials (Appendix A). Laminin and dystrophin staining were used to define muscle cell membranes, aiding in lipid location. Briefly, sections were fixed with 10%

formalin, followed by incubation with primary antibodies, laminin (1:200) and dystrophin (1:25) and secondary antibody, AlexaFluor 488. Sections were immersed in ORO for 30 mins at room temperature. Muscle sections were visualized with a spinning disk confocal microscope (Quorum Wave FX Spinning Disc Confocal System – Quorum technologies). An EM-CCD (Electron Multiplying-Charge Couple Device) cooled camera [Hamamatsu; Quorum Technologies, Guelph, 80 ON, Canada]. Volocity 6.3 software [PerkinElmer, Waltham, MA, USA] was used to capture and analyse all images. Visualization and quantification were done as previously described (Biltz et al., 2017; Mehlem et al., 2013). Z-stacked images captured tissue section using the 20X/0.85 oil lens and were assembled together and plane-merged to create a composite image that enabled the visualization of a whole and clear tissue cross section. A software script was established to identify muscle fibres using intensity of the laminin/dystrophin stain. Neutral lipids were quantified by establishing thresholds for the intensity of ORO staining and calculating the number of red pixels in relation to μm^2 of the section analysed. Once thresholds were set, quantitation then proceeded by Volocity software. The total area stained with ORO was reported as a percentage of the total area of tissue analysed. Intramyocellular and extramyocellular lipids were defined based on whether ORO stain was present inside or outside muscle fibre boundaries delimited by laminin/dystrophin staining.

4.2.6 Immunofluorescence: Fiber types

Muscle serial sections (10μm) were cryosectioned (cryostat Leica model CM300) transversely at -22°C and stored at -80°C until staining. Myosin Heavy Chain (MyHC) I, IID and IIA were determined as previously described (Gallo et al., 2006). Primary and secondary antibodies are described in the Appendix A. After the secondary antibody application, a nuclear stain (4',6-diamidino-2-phenylindole, DAPI) was added for 2 minutes and washed. Slides (Apex^{TM} superior adhesive slides, Leica Biosystems) were mounted, covered and let dry for twelve hours. Images for

tissue sections were acquired using a 20X/0.85 oil lens with a spinning disk confocal microscope (Quorum Wave FX Spinning Disc Confocal System – Quorum technologies). Individual Z-stacked images were assembled to create a composite image of a whole tissue cross-section. Tissue images were captured and analyzed with Volocity 6.3 software [PerkinElmer, Waltham, MA, USA]. A software script was established to identify muscle fibres types (I, I/IIA, IIA, IIA/D and D) using intensity of the MyHC stains and quantified automatically by the software.

4.2.7 Statistical analysis

Descriptive statistics were reported as mean±standard deviation. Categorical data were presented as counts with percentages. Shapiro-Wilk test was used to test normality. TG data was not normally distributed so non-parametric tests were used for analysis. Comparisons between groups were conducted using Mann-Whitney U and chi-square test for categorical variables. For the association between TG content of RA and muscle radiodensity, Spearman's rho (non-parametric test) was used because TG content data was not normally distributed. Statistical significance was reported when p-value <0.05. Correlation coefficients are referred to as high (>0.5), moderate (0.3 - 0.5) and low (<0.3). All statistical analyses were performed using IBM® SPSS ® software, version 20 (Chicago, IL, USA) for Windows.

4.3 Results

4.3.1 Patient characteristics

Patient characteristics are shown in Table 4-1. The study population consisted of 59 (77%) males and 17 (23%) females with a mean age of 63.2±10.9 years and mean BMI of 25.0±10.6 kg/m^2. There was significant difference in mean skeletal muscle index (SMI), muscle radiodensity, cross-sectional area of subcutaneous adipose tissue and TG content of RA between males and females (Table 4-1). Colorectal cancer was the most common cancer type, comprising 44% of the population followed

by pancreatic cancer (29%). Almost a quarter of the patients had metastatic disease. Hypertension (37%) was the most common comorbidity followed by diabetes (17%) and cardiovascular disease (CVD) (17%).

4.3.2 TG content of muscle was associated with *rectus abdominis* and total muscle radiodensity at L3.

Radiodensity of RA at L3, total lumbar muscle radiodensity at L3, and TG content of RA biopsy were analysed for 75 patients. Radiodensity of RA at L3 ranged from -10.8 to 50.6 HU, total lumbar muscle radiodensity at L3 ranged from 7.1 to 54.4 HU, and TG content of RA ranged from 0.7 to 88.7µg/mg.

RA radiodensity was moderately negatively associated with TG content of the muscle [r = -0.409, $p< 0.001$ (N=75)] (Figure 4-1A). Similarly, there was a moderate negative association between TG content and total lumbar muscle radiodensity [r = -0.372 $p< 0.001$ (N=75)] (Figure 4-1B). These results suggest that both mean muscle radiodensity at L3 and RA are associated with TG content of the muscle in cancer patients. An association between TG content and percentage area of ORO was observed (r=0.49, p=0.02). No significant association was found between percentage ORO area in the muscle section and muscle radiodensity (r= -0.29, p=0.21).

4.3.3 Variation in muscle radiodensity within *rectus abdominis*

Analysis of RA at 10 slices and 10 regions is illustrated in Figure 4-2A and 4-2B. The variation in RA radiodensity within ten distinct slices at 5 mm interval is illustrated in Figure 4-3A. Percentage coefficient of variance within subject for 10 slices analysis ranged from 3 to 55% (Figure 4-3A). The narrowest intra-subject range of RA radiodensity in 10 slices was 4 HU and highest was 17 HU. The variation in RA radiodensity within 10 regions in the same CT image slice is illustrated in Figure 4-

3B. Percentage coefficient of variance within subject for 10 regions analysis ranged from 6 to 61% (Figure 4-3B). The narrowest intra-subject range of RA radiodensity in 10 regions was 10 HU and highest was 70 HU.

4.3.4 Location of neutral lipid in *rectus abdominis* muscle

A variable pattern of neutral lipids was observed in the muscle sections by the use of ORO staining (Figure 4-4). Fibres with >50% of total area stained with neutral lipids were observed (Figure 4-4A). While this was generally uniformly distributed across each fibre, some regions showed focal deposition of lipid droplets near cell membrane (Figure 4-4B). Perivascular deposition of adipocytes was also exhibited in muscle sections (Figure 4-4C).

Total area stained for neutral lipids in muscle section was on average 13% (4 to 30%). Out of the total lipid area, mean proportion of IMCL and EMCL area were 46% and 54%, respectively. Even though almost equal proportions of IMCL and EMCL overall were observed, the ranges of percentage area were wide. IMCL area ranged from 22 to 76%; likewise EMCL area range 24 to 78% (Figure 4-5). Figure 4-6 illustrates variation in IMCL and EMCL in muscle sections of four cancer patients. Subject 1 and 2 with similar percentage of neutral lipid area and TG content of RA showed variation in lipid distribution pattern. Subject 1 had higher IMCL (62%) and lower EMCL (38%) and subject 2 had similar percentage area of IMCL and EMCL, 56% and 44% respectively. Similarly, subjects 3 and 4 with similar percentage of neutral lipid area and TG content of RA showed variation in lipid distribution pattern of IMCL and EMCL depots. These results show that distribution of IMCL and EMCL is heterogeneous even though TG content and total area of neutral lipids were similar. There was no association between percentage area of IMCL and percentage of fibre types.

4.4 Discussion

CT imaging is commonly used by our research group (Aubrey, 2014; Esfandiari et al., 2014; Martin, 2013; C. Stretch, 2013; Cynthia Stretch, 2018; Xiao et al., 2018) and others (Hayashi, 2016; Malietzis, 2016; Rollins, 2016; van Vugt, 2018) to investigate associations between muscle radiodensity and clinical outcomes in cancer patients. Low muscle radiodensity in people with cancer is assumed to reflect lipid infiltration, however, we confirm the association between muscle radiodensity and TG content of RA in cancer patients. This is the first study to report an association between RA/total lumbar radiodensity at L3 and TG content of biopsies in cancer patients. We also found wide variation in RA radiodensity within the same slice of CT image and also across the length of RA. Furthermore, neutral lipid staining revealed heterogeneous distribution of IMCL and EMCL.

Nagao *et al* first observed an association between muscle radiodensity and accumulation of fat in infants while investigating techniques for diagnosis of neuromuscular disorders (Nagao et al., 1987). It has been observed that people with obesity and Duchenne muscular dystrophy exhibit changes in lipid content of leg and thigh muscles (B H Goodpaster et al., 2000; Jones et al., 1983). We demonstrated that RA radiodensity is indicative of its TG content measured biochemically. This is in line with Goodpaster's observation of a negative association between mid-thigh muscle radiodensity and TG content of vastus lateralis biopsy in healthy volunteers and diabetic patients (Bret H Goodpaster, 2000). However, that study reported stronger association (r= -0.580) as compared to our observation (r= -0.401). In prior study, all subjects had muscle radiodensity greater than 25 HU with TG content in range of 4 and 26 μg/mg. Also, healthy and diabetic individuals were between 25 and 49 years of age. In our study, more than half of the patients had RA radiodensity less than <25 HU with wider TG content range of 0.7 and 88μg/mg. This could reflect older age of cancer patients (average age of 63 years). We also observed that total lumbar muscle radiodensity at L3, most

commonly analysed CT image cross-section for radiodensity, is also indicative of total TG content of the muscle. The latter findings should be interpreted with some caution because total muscle radiodensity values at L3 are mixtures of radiodensity values of different muscle groups (i.e., quadratus lumborum, psoas, erector spinae, external obliques, transverse abdominis, internal obliques, and RA) and TG content was analysed for RA only as this is the most common biopsied muscle in cancer patient (Johns, 2014; Narasimhan, 2018; C. Stretch, 2013).

The second important purpose of this study was to assess the variability of radiodensity within RA. Analysis of radiodensity across the length and breadth of RA revealed wide variation within the same subject confirming non-uniform distribution of lipids in the muscle (B H Goodpaster, 2000; Vermathen, 2004; Vidt et al., 2016). Generally, research studies in oncology settings followed the lead of Shen *et al.* to use a single CT image at L3 as muscle area at this level strongly correlates with whole body volume of muscle (Mourtzakis, 2008; Wei Shen, Mark Punyanitya, ZiMian Wang, Dympna Gallagher, Marie-Pierre St.-Onge, Jeanine Albu, Steven B. Heymsfield et al., 2004). Muscle radiodensity is also measured at L3 but it does not represent spatial patterning and volume distribution of lipid in the whole muscle.

An increasing number of investigators are collecting RA biopsies to study biological features of muscles of cancer patients (Johns, 2014; Narasimhan, 2018; C. Stretch, 2013; Cynthia Stretch, 2018). In practice, RA biopsies collected during surgery will vary depending on the site of incision. Thus, biopsy is possibly from a different area of the abdomen than the area of muscle radiodensity evaluation. Also, the biopsy is small relative to the area being evaluated for radiodensity. As pattern of lipid deposition is heterogeneous, this can have significant effect on the results. This may explain the moderate correlation between RA radiodensity at L3 or total lumbar muscle radiodensity at L3 and TG content of the biopsy in our study. As only 22% and 18% of variance in radiodensity of RA at L3

and total muscle radiodensity at L3, respectively, is explained by TG content of the biopsy. This also explains why there was no significant correlation between neutral lipid content measured by ORO staining and muscle radiodensity, in contrast to Goodpaster et al (Bret H Goodpaster, 2000). A recent study did not find an association between muscle radiodensity and muscle protein content in gastrointestinal cancer patients, again explained by a relatively small muscle biopsy compared to the area analysed for muscle radiodensity (Ramage et al., 2018). Changes in muscle protein and extracellular water content might also affect muscle radiodensity (Bret H Goodpaster, 2000). Variation in radiodensity and uneven distribution of lipid depots in the muscle suggest that when investigating muscle at tissue level and fatty infiltration is primary outcome, precise quantification of amount of lipid is helpful.

In current study, we characterized location of lipids in and around the muscle fibres. Heterogeneous distribution of EMCL and IMCL was observed in the muscle sections. Wide variation in the distribution of lipids within and between muscle fibres suggest that pathways associated with both IMCL and EMCL need to be resolved to delve into the mechanism of fatty infiltration. IMCL is a dynamic pool of lipids in muscle fibre and an important substrate for exercise in endurance athletes (Daemen et al., 2018). Excess IMCL deposition has been associated with insulin resistance, obesity, inflammation and muscle dysfunction (Hamrick et al., 2016). Insulin resistance has been associated with predominant deposition of IMCL near cell membrane, large lipid droplet size (Daemen, 2018; Bret H Goodpaster, 2000), and lipid droplet number (Tarnopolsky et al., 2006). EMCL is an accumulation of adipocytes around the muscle fibres and can originate from stem cell populations residing in skeletal muscle (Hamrick, 2016). The most well-defined stem cell population in skeletal muscle are satellite cells (Dumont et al., 2015). However, whether satellite cells adopt adipogenic fate is under debate (Scarda et al., 2010; Starkey et al., 2011). Fibro/adipogenic progenitors (FAPS),

multipotent mesenchymal progenitors and PW1+ interstitial cells are potential stem cells populations that can contribute to adipocyte development in skeletal muscle (Pannerec et al., 2013; Uezumi, 2014). In the current study, isolation of multipotent progenitors from muscle biopsies was carried out but only a few number of adipogenic progentitors were isolated (Appendix D). Of note, only one study reported isolation of multipotent progenitors in human muscle (Pisani et al., 2010). However, several studies indicate role of FAPs in fatty infiltration in injured muscle (Jia et al., 2018; Joe et al., 2010; Uezumi, 2014). Further studies are required to investigate role of FAPs in fatty infiltration in muscle of cancer patients.

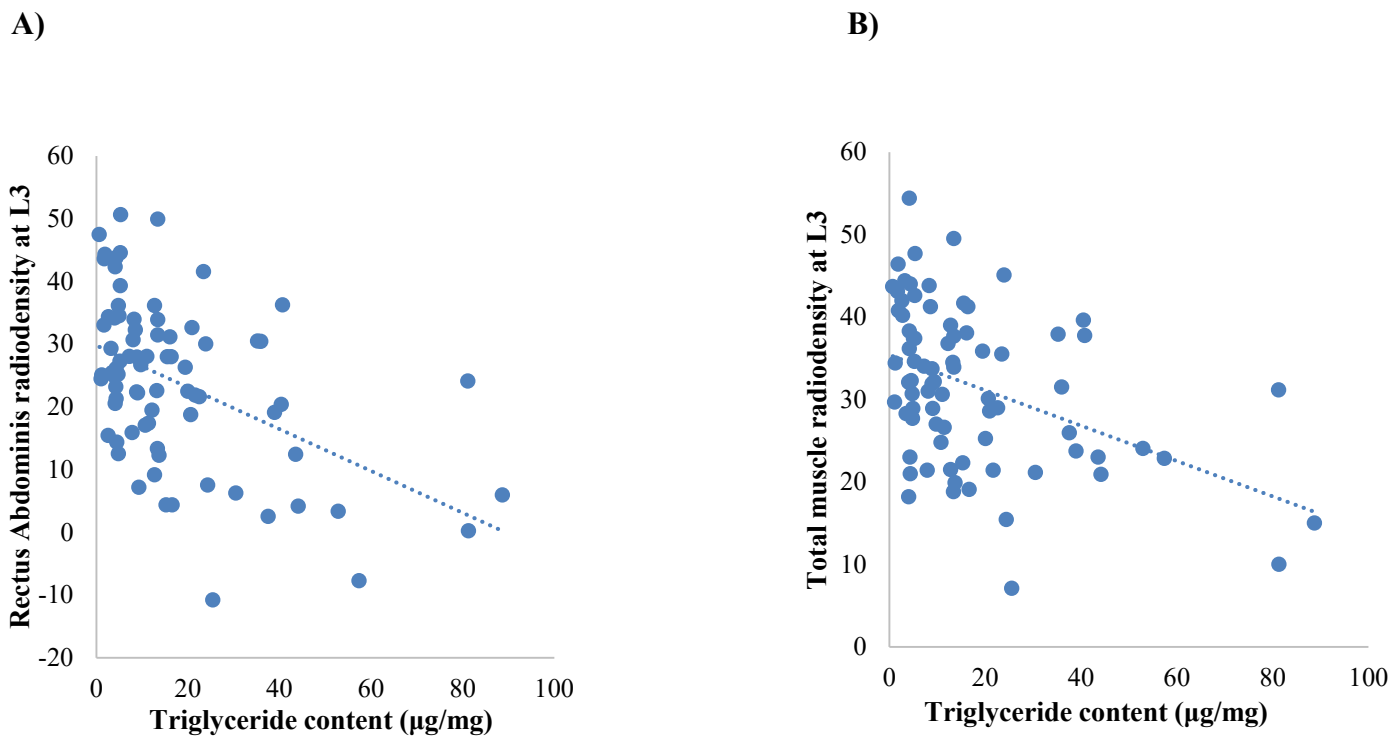
CT imaging is an important non-invasive and opportunistic tool in studying lipid infiltration in the muscle in cancer patients. The present study supports the claim made by several studies that low muscle radiodensity is indicative of increased lipid content. However, when investigating muscle characteristics at tissue level, biochemical TG extraction and histological ORO staining are more comprehensive and are better representative of amount and location of lipid depots. In cancer patients, more work is required to understand mechanisms that contribute to muscle lipid accumulation and its effects on muscle physiology.

Table 4-1. Characteristics of patients with cancer – Values are mean ± SD, except for categorical variable, where numbers in each category are shown. Skeletal Muscle Index (SMI) was measured by normalizing cross-section muscle area for height and muscle radiodensity was measured as the average Hounsfield units of the total skeletal muscle area on a single cross-sectional computed tomography (CT) image at the level of the third lumbar vertebra (L3).

	Males (n=58)	Female (n=17)	All Patients (n=75)	p-value
Age (years), mean±SD	62.6±11.4	66.2±7.0	63.2±10.9	NS
Tumor type, N (%)				
Colorectal	26(45)	7(41)	33(44)	NS
Pancreatic	18(31)	4(31)	22(29)	NS
Other Gastro-intestinal[a]	15(25)	5(29)	20(27)	NS
Presence of metastasis, N (%)	12(20)	8(47)	20(26)	NS
BMI (kg/m^2)[b]**, mean±SD**	24.9±10.1	25.4±12.7	25.0±10.6	0.793
CT-image measures at L3				
Skeletal Muscle Index (cm^2/m^2)	49.4±8.1	41.0±6.3	47.9±8.7	0.001
Muscle radiodensity (HU)	33.2±9.3	26.7±8.3	31.9±9.3	0.009
Subcutaneous adipose tissue (cm^2)	186.7±102.2	308.8±176.6	205.9±131.3	0.003
Visceral adipose tissue (cm^2)	196.7±92.6	165.5±70.1	173.6±96.1	NS
Comorbidities, N (%)				
Diabetes type II	9(13)	4(24)	13(17)	NS
Hypertension	20(29)	8(47)	28(37)	NS
Cardiovascular disease	12(18)	1(6)	13(17)	NS
Dyslipidemia	9(15)	4(24)	13(17)	NS
Smoking habit, N (%)	8(14)	3(18)	11(15)	NS

[a] Small bowel, bile duct, liver, appendix, stomach, gall bladder.

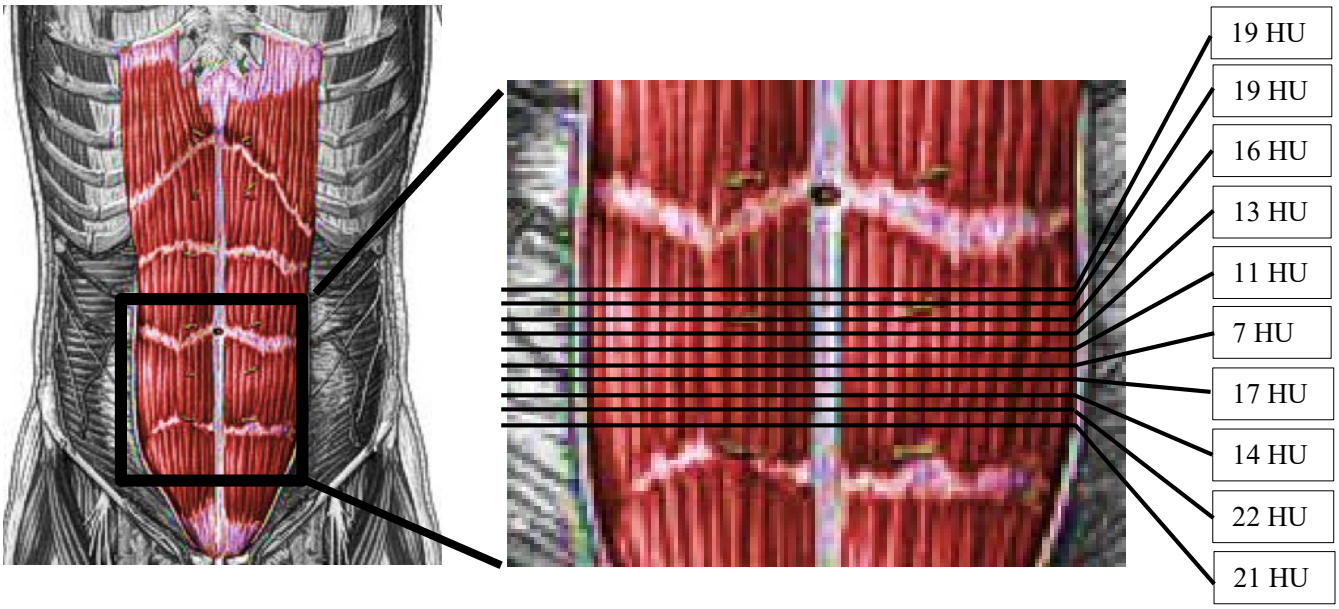
[b] Missing data: BMI n=9



	Mean	Range
TG content of RA	17 µg/mg	0.7 to 88.7 µg/mg
RA muscle radiodensity at L3	24 HU	-11 to 51 HU
Total muscle radiodensity at L3	32 HU	7.1 to 54.4 HU

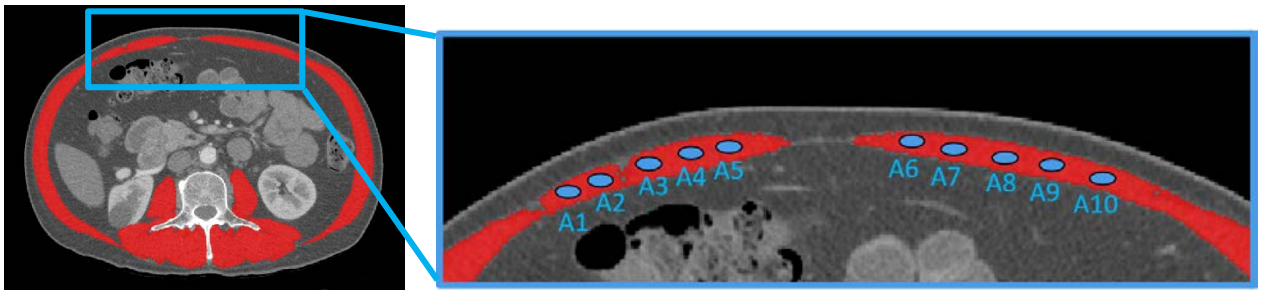
Figure 4-1. Association between muscle radiodensity and triglyceride content of *rectus abdominis*. A) Association between *rectus abdominis* (RA) radiodensity at 3rd lumbar (L3) region and total triglyceride content. r = -0.409, $p < 0.001$ (N=75). Muscle radiodensity of RA was determined at L3 slice of CT images obtained from medical records of the patients. **B)** Association between total lumbar muscle radiodensity at L3 and total triglyceride content measured in rectus abdominis biopsy. r = -0.372, $p < 0.001$ (N=75). Total muscle radiodensity was analysed at L3 slice of CT images. TG content was analysed by biochemical extraction followed by quantitative gas chromatography. Spearman's rho analysis was used to determine the associations. In males, correlation between TG content of RA and muscle radiodensity of RA or at L3 was weak but significant, r = -0.230, $p < 0.048$ and r = -0.285, $p < 0.030$, respectively. Similarly, in females, weak significant association was observed between TG content of RA and total muscle radiodensity at L3 (r = -0.201, $p < 0.04$) but strong significant association was observed between TG content and muscle radiodensity of RA (r = -0.581, $p < 0.014$).

A) Illustration of variability in RA radiodensity in ten repeated measures (5mm interval)



Muscle radiodensity at each image slice in Hounsfield units (HU)

B) Illustration of within-image variability of RA radiodensity in ten regional measures.

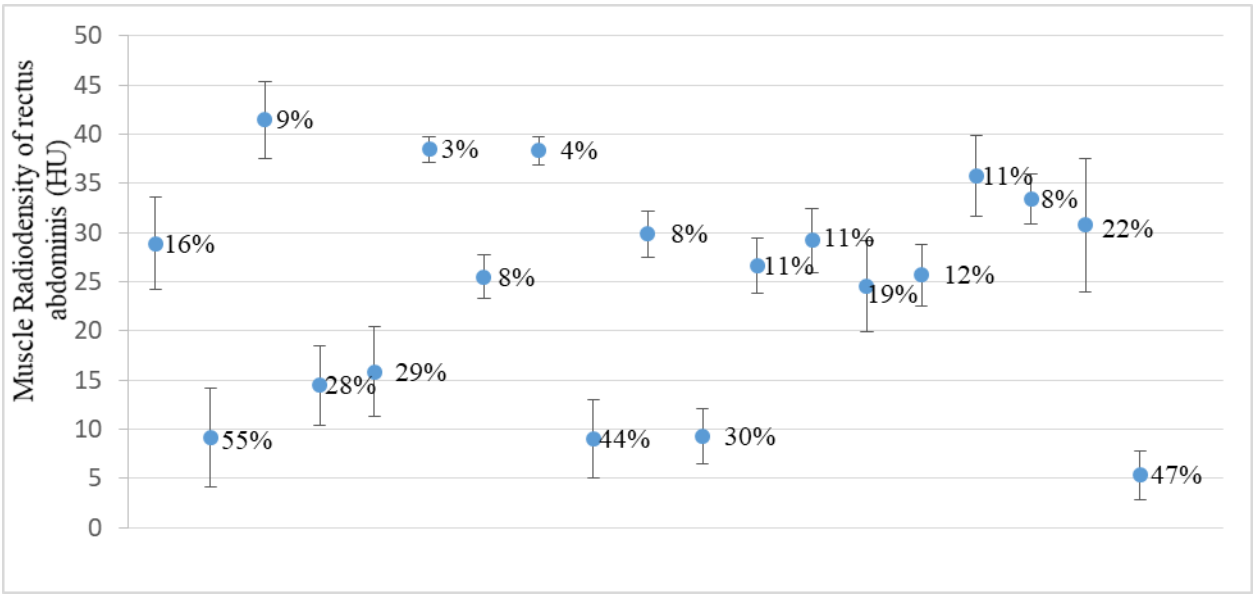


Region of analysis	Radiodensity (HU)
A1	6
A2	27
A3	-5
A4	8
A5	24
A6	30
A7	49
A8	44

A9	41
A10	43
Mean rectus abdominis	24

Figure 4-2. Illustration of variability in radiodensity of rectus abdominis A) Variation of rectus abdominis radiodensity in ten repeated measures (5mm intervals). Zoomed image of rectus abdominis illustrates 10 slices of rectus abdominis and muscle radiodensity at each slice. **B)** Within-image variability of rectus abdominis radiodensity in ten regional measures. Each patient's computed tomography (CT) image at 3rd lumbar was analysed. Red color shows the muscle area. Regions of analysis in rectus abdominis were manually selected and are shown in blue circles in the zoomed CT image (A1-A10). Table shows values of radiodensity at each region of analysis.

A) Percentage coefficient of variability in radiodensity across 10 images of RA



B) Percentage coefficient of variability in radiodensity in 10 regions within the same CT-image of RA

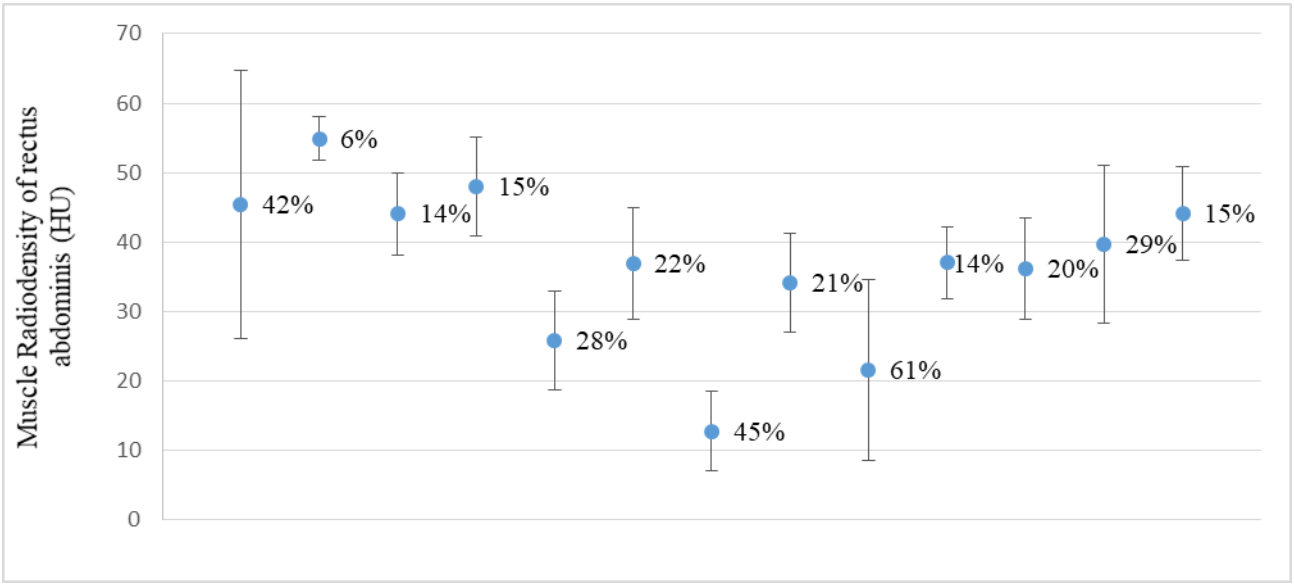
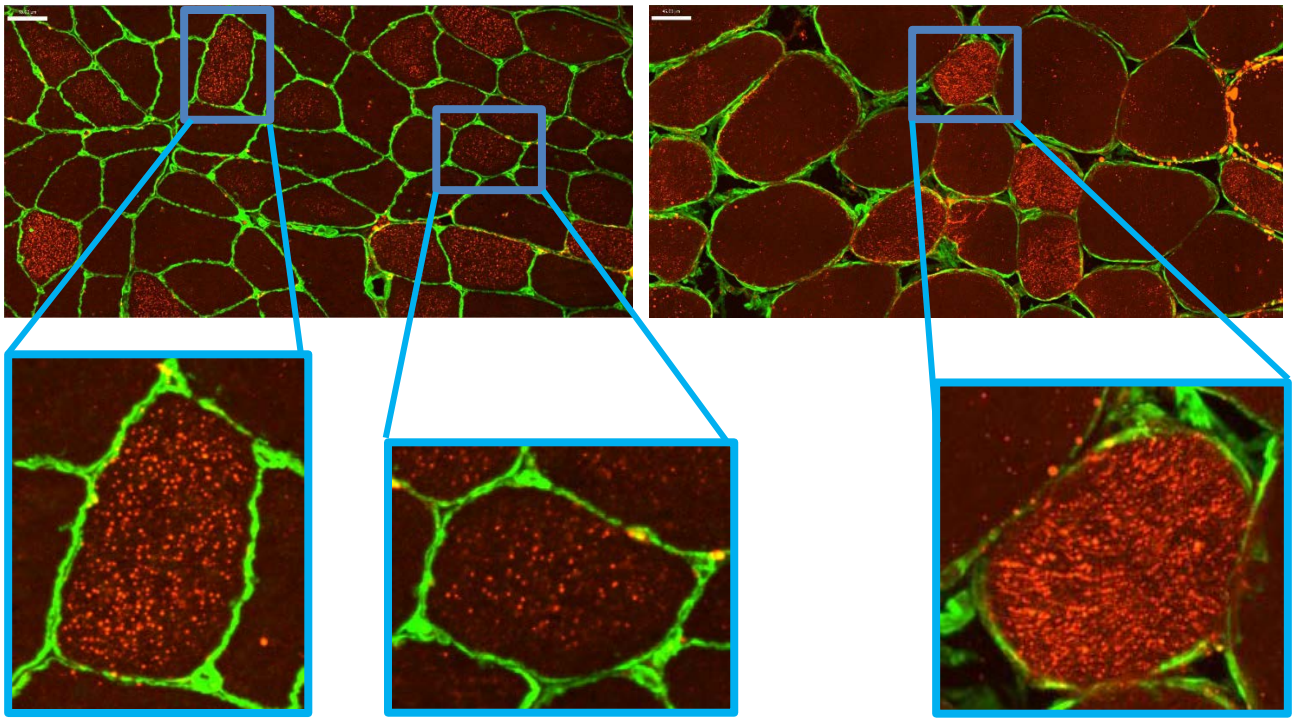


Figure 4-3. Variation in mean rectus abdominis radiodensity across the muscle A) Percentage coefficient of variation in mean rectus abdominis radiodensity of 10 distinct slices with an interval of 5 mm in 19 patients. Each point in the figure represents a patient. Male to female in this subset of patients was 15/4. Coefficient of variation widely varied in males and females with a range of 3 to 47% and 11 to 55%, respectively **B)** Percentage coefficient of variation in muscle radiodensity in 10 regions within the same computed tomography image slice of rectus abdominis in 13 patients. Each point in the figure represents a patient. Male to female in this subset of patients was 11/2. Coefficient of variation widely varied in males and females with a range of 6 to 61% and 20 to 42%, respectively

A) Variation in neutral lipids in muscle fibres.

i) ii)



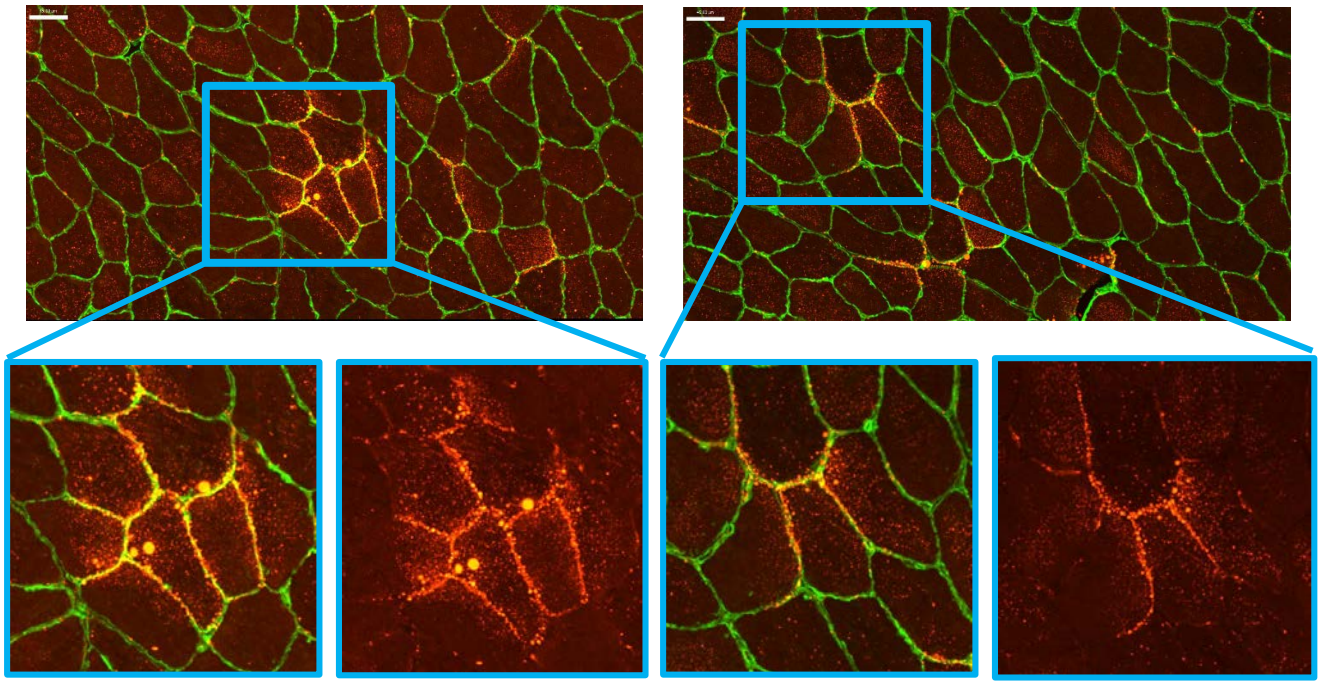
Neutral lipid area in the muscle fiber = 23%

Neutral lipid area in the muscle fiber = 4%

Neutral lipid area in the muscle fiber = 56%

B) Neutral lipids near cell membrane

i) ii)



With laminin and ORO | ORO only | With laminin and ORO | ORO only

C) Adipocytes in muscle

i) ii)

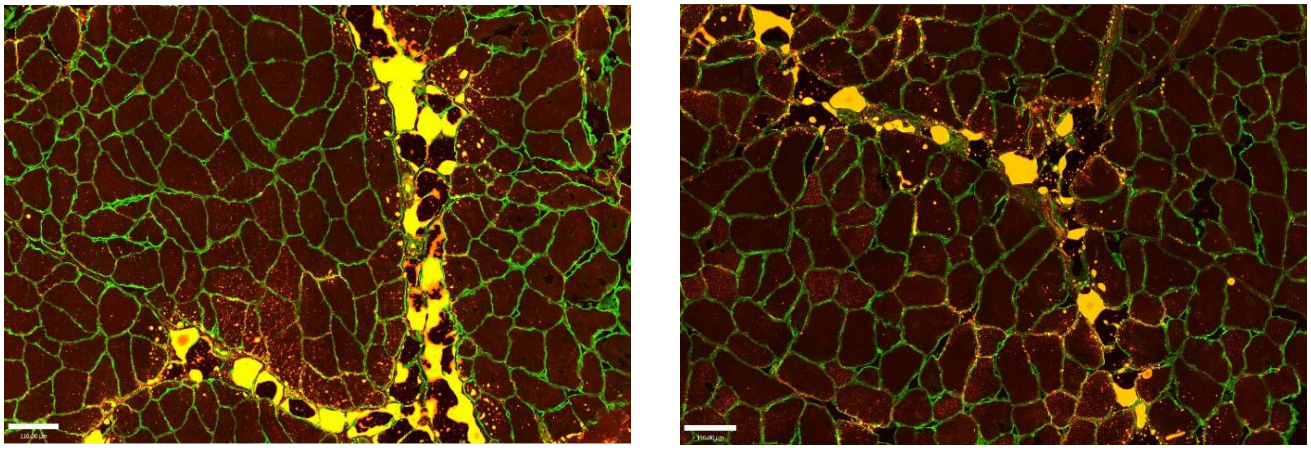
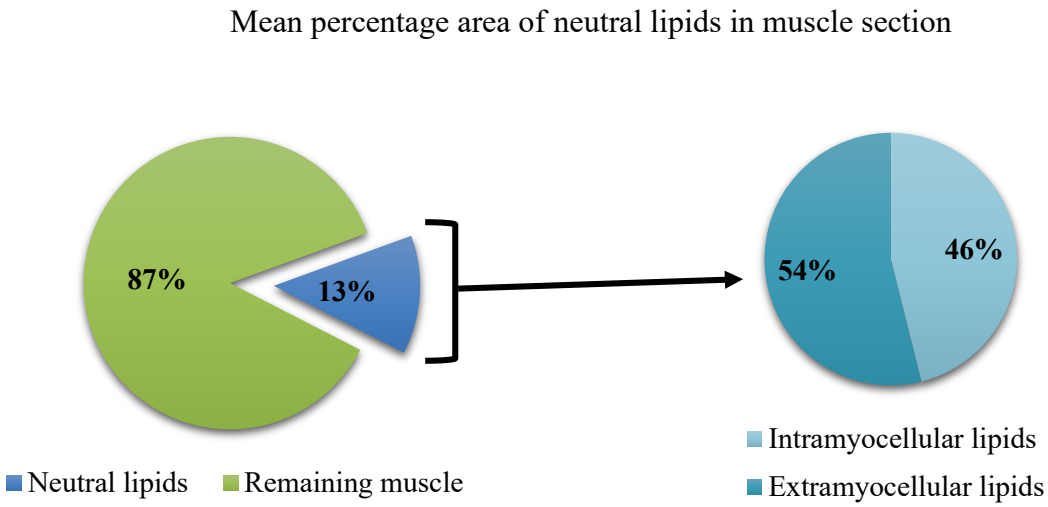


Figure 4-4. Neutral lipid staining revealed different lipid distribution patterns in muscle fibres of cancer patients. A) Variation in lipid deposition inside muscle fibre. i) and ii) are muscle sections of two patients stained with laminin and dystrophin (green) for cell membrane and oil red O (bright red) for neutral lipids. Zoomed images show variation in percent area of neutral lipids within fibres. Scale bars, 45µm. **B)** Lipid droplets in the area near the cell membrane. i) and ii) are muscle sections of two patients stained with laminin and dystrophin (green) for cell membrane and oil red O (bright red) for neutral lipids. Zoomed images show deposition of lipid droplets in the area around the cell membrane. Scale bars, 45µm. **C)** Adipocytes in the perivascular area of the muscle. i) and ii) are muscle sections of two patients stained with laminin and dystrophin (green) for cell membrane and ORO (bright red) for neutral lipids. Scale bars, 140µm.

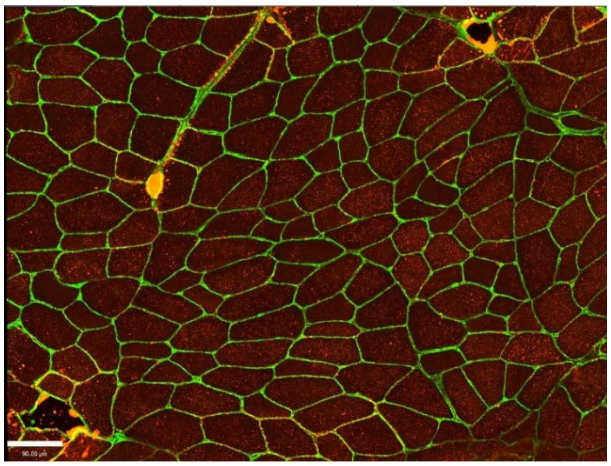


	Mean±SD	Range (Min-Max)
Percentage of total area with neutral lipids	**13.1±7.1**	**3.8-29.7**
- Percentage of area with intramyocellular lipids	46.1±16.9	22.2-76.5
- Percentage of area with extramyocellular lipid	53.9±16.9	23.5-77.8

Figure 4-5. Mean percentage area of neutral lipids in muscle section analysed using oil red O staining in 22 patients. Total area stained for neutral lipids in muscle section was on average 13% (4 to 30%). Out of the total lipid area, the mean proportion of intramyocellular lipid and extramyocellular lipid area were 46% and 54%, respectively. There were no significant difference in percentage neutral lipid area between males and females.

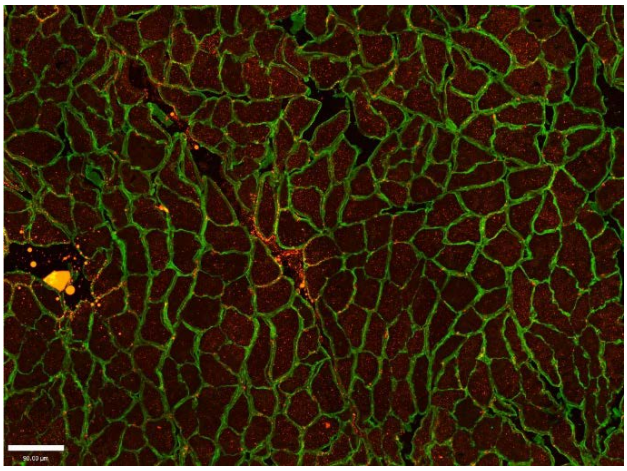
Subject 1

Percent neutral lipid area = 24%
Percent intramyocellular lipid area = 62%
Percent extramyocellular area = 38%
TG content of RA = 41µg/mg



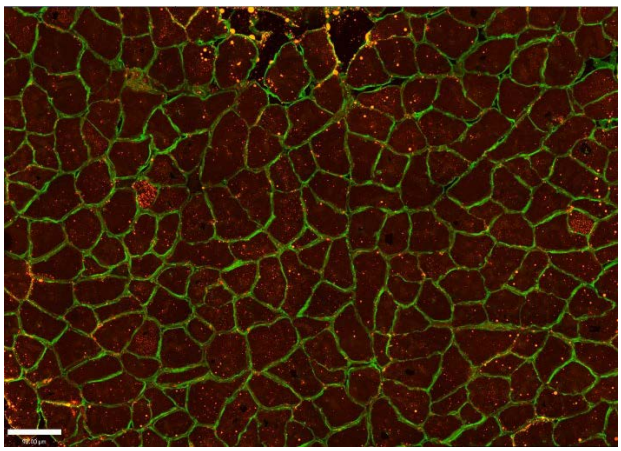
Subject 2

Percent neutral lipid area = 29%
Percent intramyocellular lipid area = 56%
Percent extramyocellular area = 44%
TG content of RA = 40µg/mg



Subject 3

Percent neutral lipid area = 9%
Percent intramyocellular lipid area = 23%
Percent extramyocellular area = 77%
TG content of RA = 38µg/mg



Subject 4

Percent neutral lipid area = 9%
Percent intramyocellular lipid area = 32%
Percent extramyocellular area = 68%
TG content of RA = 37µg/mg

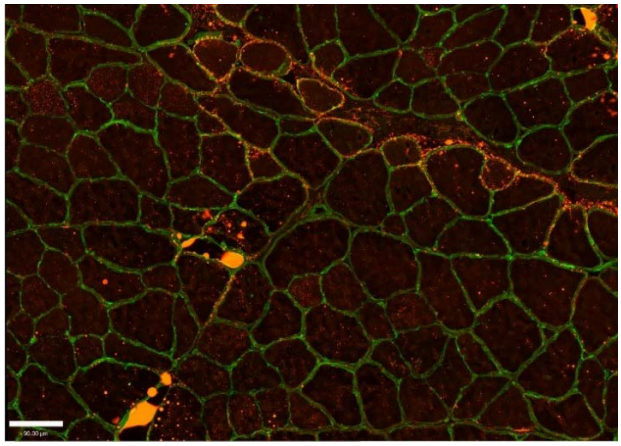


Figure 4-6. Variation in intramyocellular lipid (IMCL) and extramyocellular lipid (EMCL) percentage area. Subject 1 and 2 similar neutral lipid area but different IMCL:EMCL area. Similarly, subject 3 and 4 have similar neutral lipid area but different IMCL:EMCL area. Scale bars, 90µm.

Chapter 5: Association between skeletal muscle phospholipid fatty acid composition and survival of surgical patients with gastrointestinal cancer

5.1 Introduction

Fatty acids influence cancer progression in many ways. Fatty acids incorporated into cellular membranes of tumors and immune cells are implicated in production of lipid mediators and influencing gene expressions as well as activating signal transduction molecules that influence carcinogenesis (reviewed by Calder, 2009, 2015). Experimental studies of cancer have shown that arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) regulate proliferation, apoptosis, cytotoxicity, metastasis and immune cell functions (reviewed by Catala, 2017). EPA and DHA have also been reported to improve efficacy of anticancer treatments, chemotherapy tolerability, and inflammatory profile of the host while attenuating skeletal muscle mass depletion (reviewed by Miccadei et al., 2016; Morland et al., 2016). Collectively, these properties of fatty acids may influence survival of cancer patients.

Alterations in phospholipid fatty acid composition of adipose tissue, erythrocytes, neutrophils and body fluids (plasma and serum) have been reported in patients with lung, colorectal, pancreatic cancers (de Castro et al., 2014; Murphy et al., 2012; Murphy et al., 2010; Wilke et al., 2010; Okuno et al., 2013; Pratt, 2002). Specifically, decreases in ARA, EPA and DHA have been reported in cancer patients, independent of changes in caloric and fat intake (de Castro, 2014; Jolanta et al., 2018; McClinton et al., 1991; Mikirova et al., 2004). Higher contents of palmitoleic (16:1) and oleic acid (18:1) and a lower content of ARA and linoleic acid (18:2n-6) have been reported in phospholipids of erythrocytes in lung adenocarcinoma and small cell lung cancer patients compared to healthy controls (de Castro, 2014). Alterations in plasma phospholipid fatty acids are associated with prognosis as shorter survival has been observed in lung, pancreatic

and colorectal cancer patients who have lower levels of polyunsaturated fatty acids (Macášek et al., 2012; Murphy, 2010). Murphy et al. (2010) reported 59% lower ARA, 26% lower EPA, and 40% lower DHA (statistically significant) in cancer patients who survived fewer than the median of 238 days compared to who survived longer. Plasma phospholipid EPA and DHA have been associated with better prognosis (survival more than 100 days) in pancreatic cancer patients (Macášek, 2012). A recent study reported lower ARA and unsaturation index of serum phospholipids in colorectal patients who had disease progression after one year follow up compared to individuals without tumor progression (Jolanta, 2018). Overall, measures in plasma suggest a depletion polyunsaturated fatty acids is associated with survival in cancer patients.

EPA and DHA have been reported to attenuate skeletal muscle mass depletion in people with cancer suggesting the role of these fatty acids in improving prognosis by maintaining skeletal mass (Murphy et al., 2011a; Ryan et al., 2009; van der Meij et al., 2010; Weed et al., 2011). EPA and DHA were lower in plasma phospholipids in patients with sarcopenia (Murphy et al., 2010a). Lung cancer patients who maintained or gained skeletal muscle during cancer treatment exhibited the highest concentrations of EPA and DHA in plasma phospholipids (Murphy et al., 2010a). Muscle loss was attenuated in a group of lung cancer patients undergoing chemotherapy who received omega-3 fatty acid supplementation compared to patients receiving standard of care or an isocaloric control supplement (Murphy et al., 2011b; van der Meij, 2010), although the incorporation of these fatty acids into the muscle membrane was not assessed. Dietary EPA and DHA intake and supplementation have also been reported to improve the prognosis of patients with gastrointestinal cancer (Shirai et al., 2017; Song et al., 2017), however, these studies have assessed blood and not muscle directly.

There are no studies that reported alterations in the fatty acid composition of membrane lipids of skeletal muscle in cancer patients. Based on the observations in blood phospholipids, ARA, EPA and DHA in skeletal muscle phospholipid are candidate fatty acids that are likely to associate with survival of cancer patients, however, alterations in monounsaturated and saturated fatty acids, while less explored, must also be evaluated in skeletal muscle phospholipids. Given the association between mortality, plasma fatty acids and skeletal muscle, the aim of the study was to investigate the relationship between the fatty acid composition of phospholipids in skeletal muscle and survival in cancer patients. It was hypothesized that patients with more polyunsaturated fatty acids would be associated with better prognosis in gastrointestinal cancer patients. A secondary objective was to determine if the fatty acid composition of muscle was associated with skeletal muscle depletion.

5.2 Materials and methods

5.2.1 Ethics statement

The study was approved by the Health Research Ethics Board of Alberta-Cancer. Patients undergoing elective abdominal surgery were consecutively approached to participate in tumor and tissue banking at a hepatopancreatobiliary surgical service in Alberta, Canada. Three percent of approached patients declined participation. Patients provided written informed consent for muscle biopsy and tissue banking. The release of 35 samples from the bank for analysis, as well as patient information (demographic, clinical and operative data) from medical records, was performed under the auspices of Protocol ETH-21709: *The Molecular Profile of Cancer Cachexia*.

5.2.2 Subjects and muscle biopsies

Rectus abdominis biopsies of 35 patients undergoing surgery for colorectal, pancreatic and other gastrointestinal tumors were studied. All patients were either previously diagnosed to have

cancer or were diagnosed after surgery. The study cohort and conditions for acquisition of muscle samples have been described previously in Chapter 3. The characteristics of the patients are shown in Table 5-1.

5.2.3 CT image Analysis

CT scans completed with a spiral CT scanner for initial cancer staging and routine diagnostic purposes were used to quantify muscle area, cross section area, adipose tissue area and mean skeletal muscle radiodensity (Mourtzakis et al., 2008). CT scans completed before surgery were analysed using SliceOmatic® V4.2 software with CT image parameters that include: contrast, 5mm slice thickness, 120 kVP, and 290 mA. Total skeletal muscle area (cm^2) was evaluated on a single image at the third lumbar vertebrae (L3) using Hounsfield unit (HU) thresholds of -29 to 150 for skeletal muscle. Total skeletal muscle area was normalized for stature (m^2) and reported as skeletal muscle index (SMI) (cm^2m^2). Mean muscle radiodensity (HU) is reported for the entire muscle area (i.e., quadratus lumborum, psoas, erector spinae, external obliques, transverse abdominis, internal obliques, and rectus abdominis). Adipose tissue cross-sectional areas were calculated by using standard HU thresholds of −50 HU to -150 HU for visceral adipose tissue and −190 to −30 HU for subcutaneous adipose tissue. Visceral and subcutaneous adipose tissues cross-sectional areas were summed for total adipose tissue area.

The mean time period between CT image and biopsy collection was 64 days (±51). Change in muscle mass was determined in a subset of patients (n = 26) who had an additional postoperative CT image taken within 270 d of their initial CT image as part of standard of care for cancer diagnosis. The mean time period between CT images was 134 days (±114). To allow for comparison between individuals, the change in muscle was expressed as percentage change from

the initial CT image and divided by the number of days elapsed between the 2 CT images. The daily rate of change was multiplied by 100 to form a standard unit expressed as % change/100 d.

5.2.4 Analysis of phospholipids by Gas chromatography

The biopsy [≈50mg] was ground using a frozen pestle and mortar without letting the muscle tissue thaw. Ground tissue was homogenized in calcium chloride [$CaCl_2$; 0.025%] solution. A modified Folch method was used to extract lipids from muscle (Murphy, 2010b; Pratt, 2002). Lipids were extracted using chloroform/methanol (2:1, vol/vol). The PL fraction was isolated and scraped from G-plates. An internal standard, C17:0, was added for PL fatty acid quantification followed by methylation. PL fatty acid composition was analysed by gas chromatography-flame-ionisation detector in a Varian 3900 gas chromatography [Varian Instruments, Georgetown, ON, Canada] as previously described. The quantity of fatty acids within the PL fraction was calculated by comparison with the known concentration of the internal standard and sum of all fatty acids was reported as total PL. The peak area of each fatty acid was normalized against the sum of peak areas of all fatty acids to determine the relative proportions.

5.2.5 Statistical analysis

For survival analysis, univariate Cox proportional hazard model was used to determine if amounts of fatty acids were associated with number of days of survival. As the sample size was small, fatty acids that were associated with number of days of survival with p-value less than 0.2 were selected to determine cut-points using bivariate Cox regression analysis. Optimum stratification was used to find the most significant P value to define the cut-points associated with survival. Number of days of survival were defined as days to death from the date of surgery or as number of days between date of surgery and date of data collections (for patients with censored survival time).The Kaplan–Meier method was used to establish the effect of amount of

phospholipid fatty acids on number of days of survival. Log-rank tests were used to compare the survival curves of each variable ($p<0.05$). Variables were entered into a univariate Cox proportional hazards model with 95% confidence intervals.

Statistical significance was reported when p-value <0.05. Data are reported as mean ± SD. Levels of significance are P values <0.05. All statistical analyses were performed using SPSS 20.0 (Chicago, IL, USA) for Windows.

5.3 Results

Univariate Cox regression analysis revealed that palmitoleic acid (16:1), linoleic acid (18:2n-6), DGLA, ARA, EPA, DPA and DHA had p-value <0.2 (Table 5-2). These fatty acids were analysed using bivariate Cox regression model to determine association with survival. Optimal stratification was used to determine cut-points. Table 5-3 represents fatty acid with statistically significant cut-points. Median survival was approximately 20 to 70% longer for the patients with the amount of DGLA, ARA, EPA and DHA in muscle phospholipid above specific cut-points compared to patients below (Table 5-3). In the patients with quantity of DGLA, ARA, EPA and DHA in skeletal muscle phospholipids below the cut-point value, 63% patients died whereas above the cut-points 10 to 25% patients died. In contrast, patients with the quantity of palmitoleic acid above cut-point in muscle phospholipids had 20% fewer median days of survival and percentage of deaths (events) 69% whereas below the cut-points percentage of deaths 29% (Table 5-3). Bivariate Cox regression analysis showed DGLA, ARA, EPA and DHA were protective for survival and palmitoleic acid and nervonic acid were risk factors. BMI, age, tumor site and metastasis did not influence survival but sex was significant predictor of survival (HR 2.7(1.1-7.2), p=0.04). The protective effect remained significant for DGLA [HR 0.09 (0.01-0.68),

p=0.02], ARA [HR 0.28 (0.10-0.90), p=0.03], EPA [HR 0.25 (0.07-0.91), p=0.03] and DHA [HR 0.24 (0.01-0.87), p=0.03] when adjusted for sex.

The survival curves for patients with muscle phospholipid fatty acid content below and above cut-points for each fatty acid in muscle phospholipids is shown in Figure 5-1. The survival distributions for patients below and above the cut-points were statistically significantly different ($p<0.05$). To represent most biologically active fatty acids, ARA, EPA and DHA, were combined to present their effect on survival. In the group with skeletal muscle ARA, EPA and DHA below the cut-points, 11 out of 17 patients died compared to only 1 out of 9 patient in the group above cut-points.

Patients with ARA, EPA and DHA below the cut-point in muscle phospholipids lost muscle at a rate of -9.2±8.2% per 100 days and patients above cut-point lost -1.2±4.4% muscle per 100 days (Figure 5-2.). The difference in the rate of muscle loss was statistically not significant. When patients with low ARA, EPA and DHA quantity in muscle phospholipids were compared with high ARA, EPA and DHA no significant differences were observed in patient characteristics and CT-derived muscle features.

5.4 Discussion

This is the first study to investigate the fatty acid composition of skeletal muscle phospholipids in cancer patients. The main goal of this study was to determine the association between fatty acid composition in skeletal muscle phospholipids and survival. The most significant finding is having a lower mean absolute amount of ARA, EPA and DHA in skeletal muscle phospholipids is associated with poor prognosis in cancer patients. .

In cohort of patients with DGLA, ARA, EPA and DHA below cut-points, 40% more patients died as compared to those with fatty acids above the cut-points. Noticeably, in the group of patients with ARA, EPA and DHA above the cut-points, only one patient died, but after 600 days of surgery. In contrast, over half of patients died within one year of surgery in the group of patients with ARA, EPA and DHA amounts below the cut-points. Two studies have previously reported an association between levels of ARA, EPA and DHA of plasma phospholipid and survival. Lower contents of ARA, EPA and DHA were reported in plasma of cancer patients who lived <238 days as compared to >238 days, which was the median survival in that study population (Murphy, 2010). Also, longer survival of the pancreatic cancer patients has been associated with higher contents of EPA and DHA, but not ARA, in blood plasma (Macášek, 2012). Lower proportions of ARA, EPA and DHA in plasma and erythrocyte phospholipids have been reported in bladder, lung, colorectal and pancreatic cancer as compared to healthy or non-malignant populations (Jolanta, 2018; Macášek, 2012; McClinton, 1991; Murphy, 2012, 2010) and here we show a similar finding for muscle.

Low muscle mass is an independent prognostic factor in cancer patients. Muscle loss and plasma phospholipid fatty acid depletion have each been separately reported to be predictors of survival in cancer (Antoun et al., 2010; Lieffers et al., 2012; Martin et al., 2013; Murphy, 2010b). Also, the positive association between muscularity and concentration of EPA and DHA in plasma phospholipids has been observed (Murphy et al., 2010a). Based on this, it was hypothesised that there will be a negative association between polyunsaturated fatty acids and skeletal muscle index, however, this was not observed. In patients with ARA, EPA and DHA contents below the cut-points all the patients lost muscle at mean rate of -9.2±8.2%/100 days (n=12) whereas in patients above the cut-points, the rate of muscle change was -1.2±4.4%/100 days (n=6). This observation

is in line with the data of Murphy et al. (2010b) who observed maximal muscle loss in a subset of lung cancer patients with the lowest concentration of EPA and DHA in plasma. In older adults, low levels of EPA and total omega-3 fatty acids are reported to be associated with low muscle mass and muscle function (Reinders et al., 2015; ter Borg et al., 2019). In a trial of EPA and DHA supplementation in older adults, an increase in thigh muscle volume over a 6-month follow–up was reported compared to the control group who were provided corn oil. Supplementation with EPA and DHA compared to isocaloric isonitrogenous control supplement in pancreatic cancer patients has been shown to improve muscle mass (Fearon et al., 2003). Plasma phospholipid EPA levels after eight weeks of supplementation were strongly correlated with an increase in muscle mass. A positive relationship was reported between the increase in plasma EPA concentration after fish oil supplementation and the rate of muscle gain in lung cancer patients (Murphy, 2011b). While a handful of studies that reported an association between changes in plasma phospholipid fatty acid composition after omega-3 supplementation and an increase in muscle mass (Fearon, 2003; Murphy, 2011b). No study has reported alterations in the fatty acid composition of muscle phospholipids and its association with muscle mass in cancer patients.

Membrane phospholipid fatty acids regulate the biophysical properties of proteins, provide substrates for second messengers and intracellular signals to alter gene expression. Evidence suggests that EPA and DHA in membrane phospholipids can have a protective effect against cancer through several actions including protein kinase activation, enhancing cell apoptosis and modulating inflammation (Kansal et al., 2014; Turk et al., 2013; Yang et al., 2013). The association between dietary/plasma ARA and the risk of cancer is highly controversial (Sakai et al., 2012). ARA and its metabolites play role in muscle growth (Korotkova et al., 2014). ARA is converted into 2 series eicosanoids known as prostaglandins F2alpha (PGF2α) and prostaglandin E2 (PGE2).

PGF2α and PGE2 have been shown to activate the major anabolic pathway in muscle, phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signalling pathway and induce myotube hypertrophy (Trappe et al., 2001, 2002). Suppression of PGF2α in the muscle of experimental models has shown to inhibit muscle recovery after disuse atrophy (You et al., 2010). EPA and DHA in skeletal muscle membranes have been suggested to influence membrane properties that may influence anabolic signalling, particularly through key regulators of muscle protein synthesis (Kamolrat et al., 2013).

In a preclinical cancer model of myosteatosis, our research group has previously reported lower amounts of EPA and DHA in muscle phospholipids compared to healthy animals (Almasud et al., 2017). Low levels of EPA and DHA in muscle phospholipids were associated with increased expression of lipogenic and adipogenic transcriptional factors and mitochondrial dysfunction, indicating role of altered membrane composition in myosteatosis. In our patient cohort, lower proportions of polyunsaturated fatty acids were observed in PE and PC fraction of muscle phospholipids in patients with high TG content compared to low TG content in the muscle (Appendix F).

Diet affects cell membrane fatty acid composition in humans (Abbott et al., 2012; Andersson et al., 2002). In healthy adults, the fatty acid composition of skeletal muscle reflected the fatty acid composition of diet after three months of dietary intervention (Andersson, 2002). In the present study, dietary records were not available for the patients, therefore, the effect of diet on skeletal muscle composition was not determined. It is well established that diets consumed by Canadians contain proteins in the form of meat and egg which are rich sources of linoleic acid and ARA (Simopoulos, 2000, 2016). In gastrointestinal and lung cancer patients, meat contributes the largest fraction (13 to 16%) of total energy (Hutton et al., 2006; Prado et al., 2012). Moreover,

Pratt et al. (2002) and Amezega et al. (2018) reported alterations in plasma and erythrocyte fatty acid composition in cancer patients compared to healthy controls regardless of total calorie and total fat intake. There are few studies that reported lower levels of ARA in cancer patients compared to healthy controls but results are inconsistent (de Castro, 2014; Jolanta, 2018; McClinton, 1991; Murphy, 2012). Given the western dietary composition and food choices during cancer trajectory, the low levels of ARA observed is not likely to be related to intake. Both, linoleic acid and ARA, are important structural components of cell membrane and can influence membrane fluidity, receptor behaviour and gene expression (Das, 2006). ARA obtained from the diet or biosynthetic pathway, is incorporated into membrane phospholipids by the enzymes, arachidonoyl-CoA synthetase and lysophospholipid acyltransferase (Yamashita et al., 2014). ARA is attached to phosphate head group preferably at the sn2 position and is released by one or various phospholipase A2 enzyme for oxylipin synthesis (Shearer et al., 2018). The depletion of ARA observed in total phospholipids could be due to the increased action of phospholipase A2. The sn2 position prefers a polyunsaturated fatty acid, however, when one is not available it compensate by an increase in saturated and/or monounsaturated fatty acids thus affecting membrane fluidity (Holman et al., 1995, 1989).

In conclusion, patients with ARA, EPA and DHA amounts in muscle phospholipids that were below the cut-points had shortened survival and had noticeable, but not significant, decreases in muscle mass. No data are available on the fatty acid composition of skeletal muscle membranes and its relationship with skeletal muscle mass. The present study is a first step in establishing alterations in skeletal muscle fatty acid composition and how it might affect muscle mass and survival in cancer. This work provides a rationale for conducting mechanistic studies to examine

depletion of bioactive fatty acids and also provides the basis to conduct intervention studies designed to limit fatty acid depletion in cancer patients.

Table 5-1. Characteristics of patients.

Characteristic	All patients
N	35
Age (years)	64.4±10.8
BMI (kg/m2)	27.1±7.1
Male:Female	24:11
Cancer types, N (%)	
Colorectal	16 (46)
Pancreatric	12 (34)
Other Gastro-intestinal[a]	7 (20)
CT-Image measures at L3[b]	
Skeletal muscle Index (cm2/m2)	45.5±9.2
Muscle radiodensity (HU)	30.1±9.2
Subcutaneous adipose tissue (cm2)	205.8±138.2
Visceral adipose tissue (cm2)	175.1±88.8
Comorbidities, N (%)	
Diabetes type II	10 (28)
Cardiovascular disease	12 (34)
Dyslipidemia	9 (26)
Smoking habit, N (%)	14 (40)

Values are mean±SD.

[a] Other GI cancer includes liver, gastric and gall bladder cancer.

[b] CT measurements available for 33 patients.

Table 5-2. Univariate Cox regression analysis for each fatty acid of skeletal muscle phospholipid. Fatty acids with p-value <0.2 were selected to determine cut-points.

	Univariate Cox regression analysis (continuous variable)	
	p-value	**HR(95% CI)**
Palmitic acid (16:0)	0.866	1.00(0.99-1.00)
Palmtoleic acid (16:1)	0.078	1.05(0.99-1.11)
Stearic acid (18:0)	0.506	0.99(0.99-1.00)
Oleic acid (18:1)	0.341	1.00(0.99-1.01)
LA (18:2n-6)	0.175	0.99(0.99-1.00)
GLA (18:3n-6)	0.791	1.02(0.87-1.18)
ALA (18:3n-3)	0.739	0.96(0.78-1.18)
DGLA (20:3n-6)	0.107	0.96(0.92-1.00)
ARA (20:4n-6)	0.089	0.99(0.98-1.00)
EPA (20:5n-3)	0.061	0.91(0.82-1.00)
DPA (22:5n-3)	0.090	0.96(0.92-1.00)
DHA (22:6n-3)	0.089	0.96(0.93-1.00)
Total MUFAs	0.197	1.00(0.99-1.01)
Total omega-3	0.078	0.98(0.97-1.00)
Total omega-6	0.123	0.99(0.99-1.00)
Total SFA	0.767	1.00(0.99-1.00)
UI	0.134	0.98(0.97-1.00)

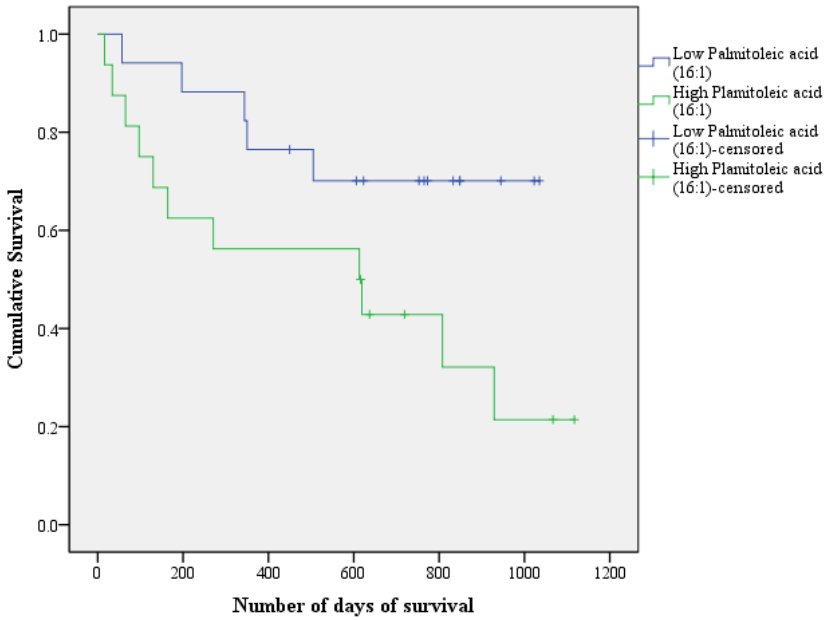
ALA, alpha linolenic acid; ARA, arachidonic acid; DGLA, Dihomo-gamma-linolenic acid; DHA, Docosahexaenoic acid; EPA, Eicosapentaenoic acid; GLA, gamma linolenic acid; LA, linoleic acid; MUFA, Monounsaturated fatty acids; SFA, Saturated fatty acids; UI, unsaturation index.

Table 5-3. Percentages of deaths, the median number of days of survival and odds ratio for univariate analysis to determine the effect of skeletal muscle phospholipid fatty acids on the survival of cancer patients.

	Cut-points[a] (ng/mg)	Below cut-point			Above cut-point			Bivariate Cox regression analysis	
		No. of patients, N	Event, N (%)	Survival time (median)	No. of patients, N	Event, N (%)	Survival time (median)	HR (95%CI)	p-value
Palmitoleic acid (16:1)	20.5	17	5 (29)	642	16	11 (69)	493	2.9(1.01-8.3)	0.049
DGLA (20:3n-6)	27.5	23	15 (65)	450	10	1 (10)	765	0.09(0.01-0.69)	0.021
ARA (20:4n-6)	136.9	19	12 (63)	478	14	4 (28)	742	0.29(0.09-0.9)	0.033
EPA (20:5n-3)	7.2	20	13 (65)	480	13	3 (23)	719	0.23(0.06-0.8)	0.022
DHA (22:6n-3)	25.2	21	13 (62)	477	12	3 (25)	806	0.23(0.07-0.83)	0.024
Omega-3	53	18	11 (61)	505	15	5 (33)	719	0.36(0.13-1.05)	0.062
Omega-6	696.8	18	12 (67)	609	15	4 (27)	719	0.3(0.1-0.95)	0.040
UI	120.7	18	11 (61)	505	15	5 (33)	719	0.35(0.12-1.03)	0.055

ARA, arachidonic acid; DGLA, Dihomo-gamma-linolenic acid; DHA, Docosahexaenoic acid; EPA, Eicosapentaenoic acid; UI, unsaturation index. [a] Cut-points were defined by optimum stratification. BMI, tumor site, metastasis and age were not significant predictors of number of days of survival.

A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Number of days of survival
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High Plamitoleic acid (16:1)
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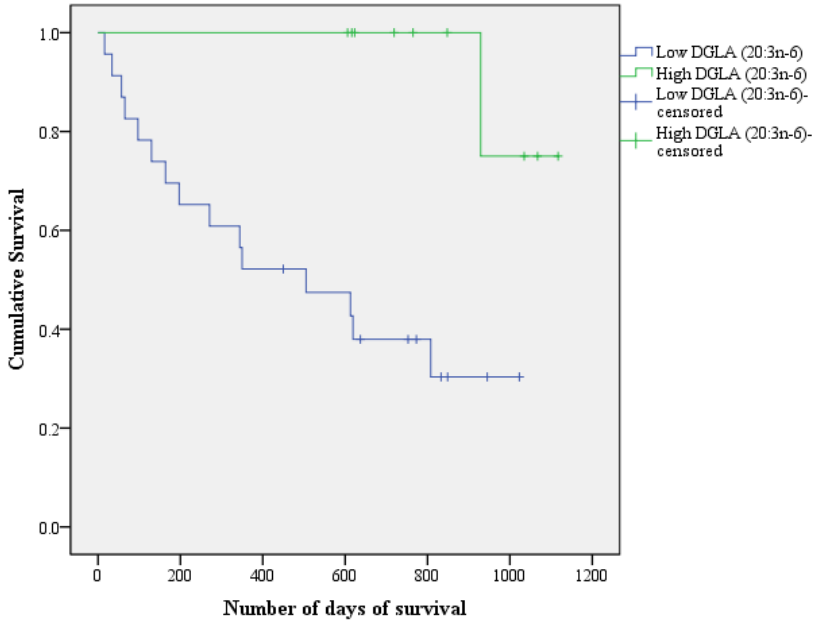
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Number of days of survival
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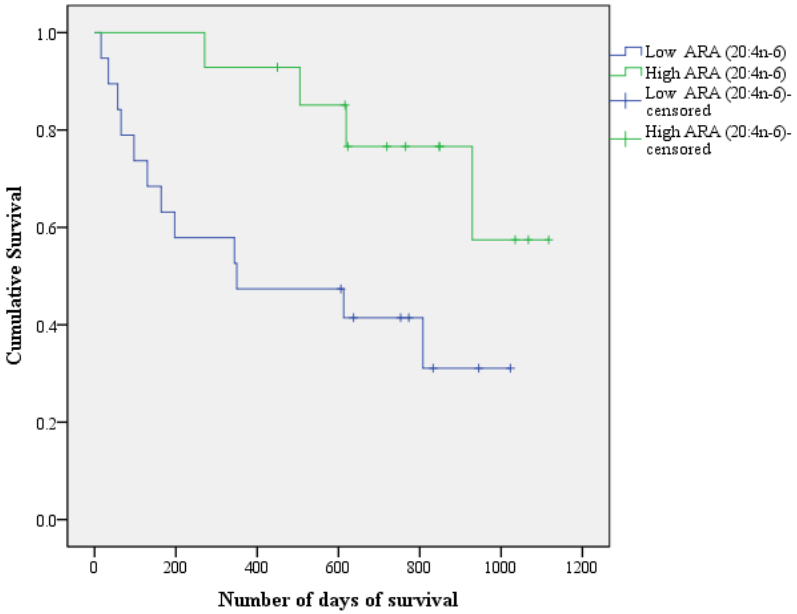
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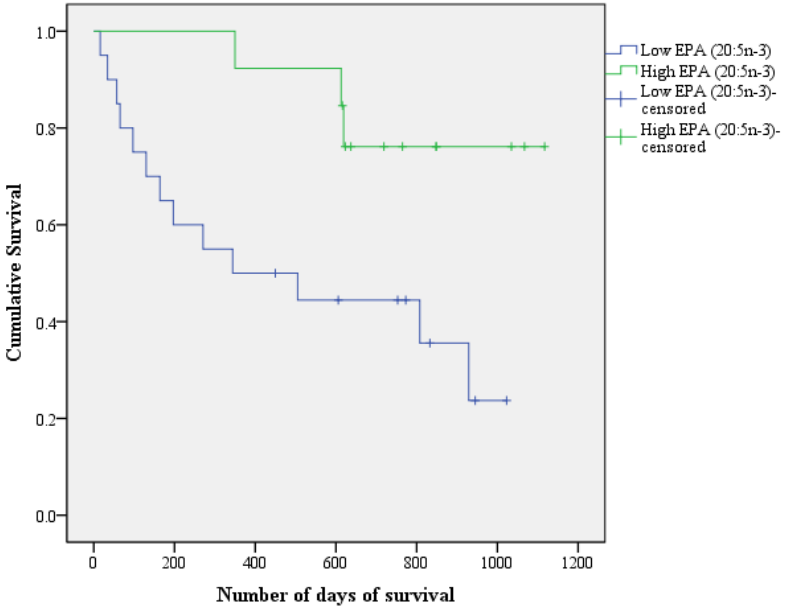
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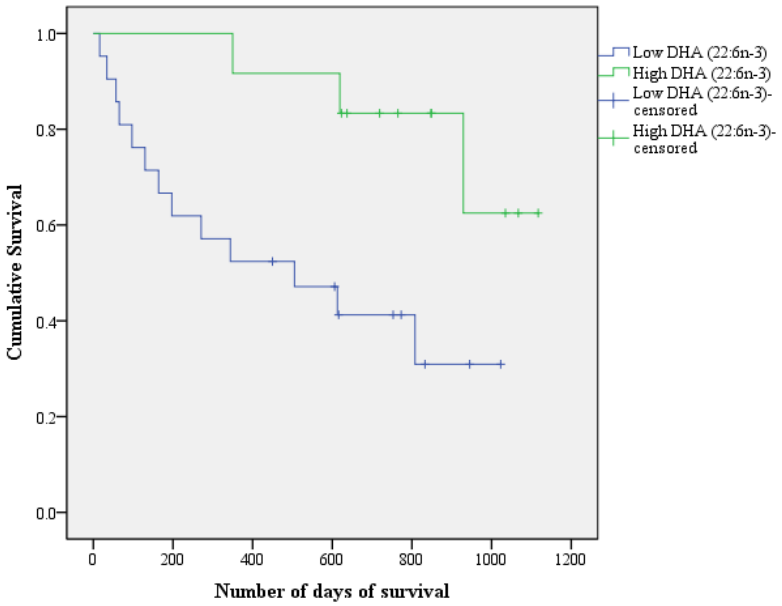
E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Number of days of survival
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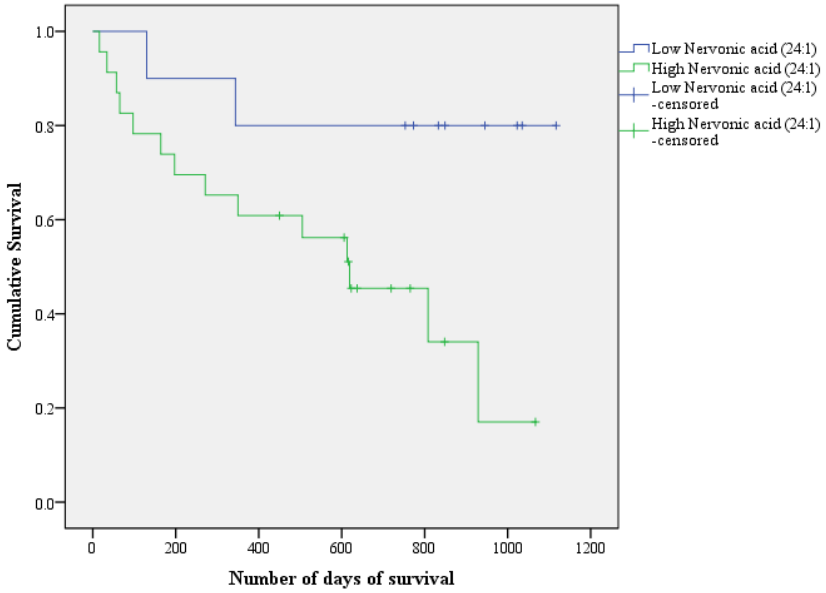
F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1000
1200
Number of days of survival
Low Nervonic acid (24:1)
High Nervonic acid (24:1)
Low Nervonic acid (24:1) -censored
High Nervonic acid (24:1) -censored

G

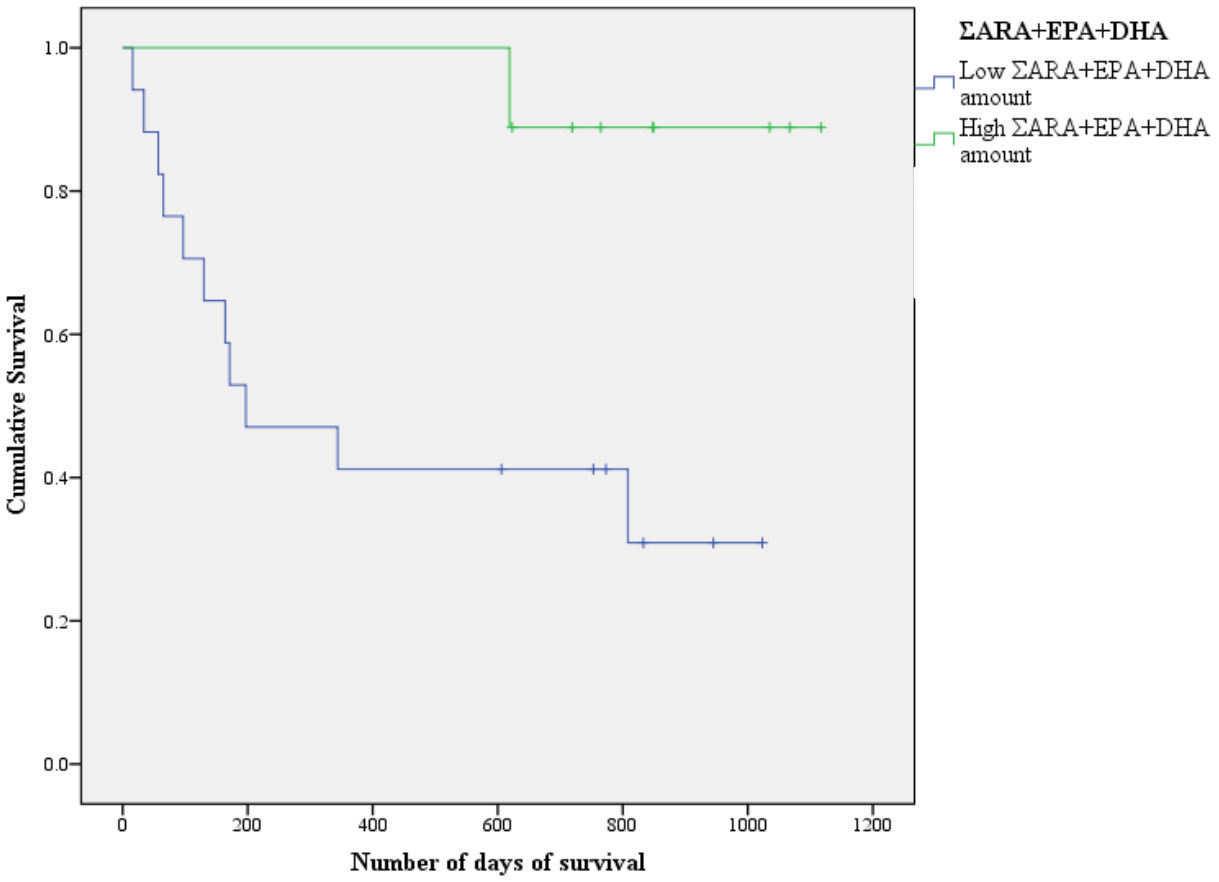

Figure 5-1. Kaplein-Meier survival curve for patients with low versus high fatty acid content in skeletal muscle phospholipids. A-F) represent survival distribution of surgical patients with gastrointestinal cancer based on fatty acid cut-points associated with increased mortality risk obtained by optimum stratification. G) represents survival distribution of patients with ARA, EPA and DHA above and below the cut-points. Log-rank tests were used to compare the survival curves of each variable ($p<0.05$).

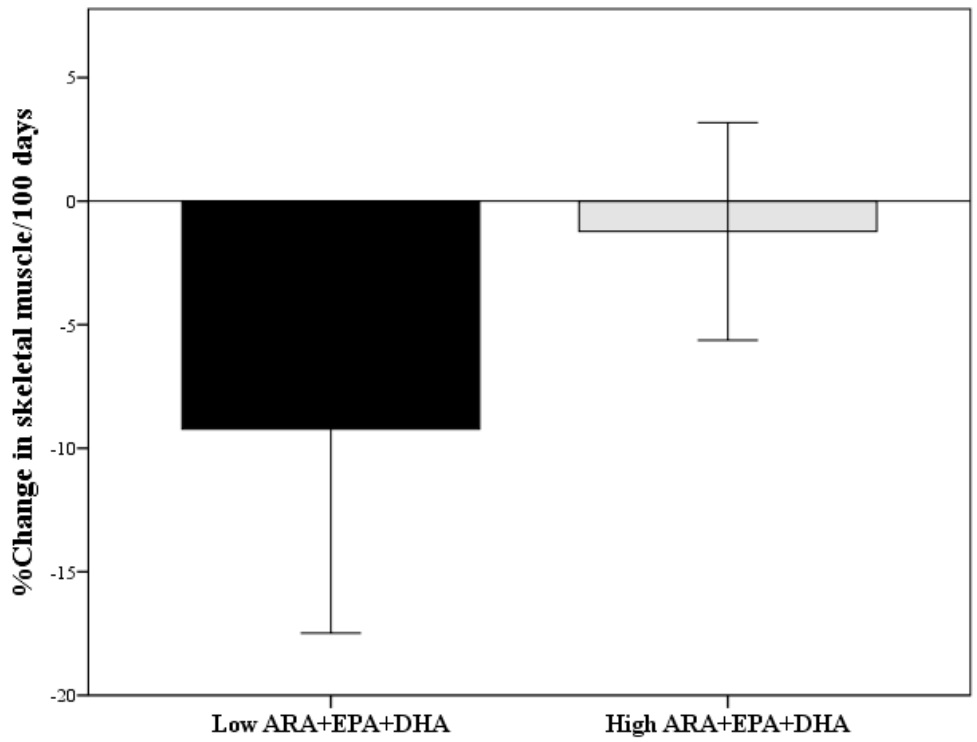

Figure 5-2. Mean rate of change in skeletal muscle in patients with ARA, EPA and DHA in skeletal muscle phospholipids below and above cut-points. Low ARA+EPA+DHA, n=12; High ARA+DHA+EPA, n=9. Bars represent mean±SD. The difference is not statistically significant (p=0.213).

Chapter 6: Final Discussion

6.1 Introduction

Skeletal muscle, as one of the major body compartments, plays an important role in mediating human metabolism. Sarcopenia and low muscle radiodensity are independent prognostic factors for cancer survival (Antoun et al., 2013; Malietzis et al., 2016; Martin et al., 2013). Biological features of muscles in cancer are widely studied in experimental models and research investigating human muscle biopsies has recently gained momentum. Therefore, this research was conducted to first identify potential sources of variation within the study design and biopsy collection procedures in studies on human muscle biopsies. The data collected provides a reference range for a variety of biological and clinical features of *rectus abdominis* from an unbiased sample. Secondly, the association between muscle radiodensity and fat content and distribution in *rectus abdominis* of cancer patients was determined. Lastly, the prognostic significance of fatty acid composition of skeletal muscle membrane phospholipids in cancer patients was determined. This discussion summarizes key findings of studies presented in the previous chapters and makes recommendations for future research.

6.2 Clinical and biological variations in muscle biopsy studies in the cancer population

In Chapter 3, clinical and biological variations in studies investigating muscle biopsy in cancer patients were determined. A state-of-the-science review was conducted, retaining features of a systematic review, and recommendations were provided to evaluate and report results of muscle biopsies from cancer patients. Sources of variation in an unbiased sample of 190 muscle biopsies from cancer patients were determined. Using quality assessment tools, it was determined that the quality of studies in this area is uniformly poor. Sampling bias was identified in more than half of the studies in the existing literature. Moreover, discrepancies between sample collection, and processing techniques were common in the reviewed studies. Age and sex differences in muscle features are well established;

however, most of the studies on muscle biopsies in cancer have failed to acknowledge this difference. In our patient cohort, sexual dimorphism was evident in CT-derived muscle features and in the genes associated with muscle catabolism/anabolism. Clinical features, patient demographics, CT-derived muscle features, morphological features and muscle gene expression data of a large sample size are presented in this study to provide a point of reference for future studies.

Previous studies on human muscle biopsies in cancer have inconsistencies in reporting methods and results that make it difficult to compare the findings. In the current study, the list of recommendations for biopsy processing and study population characterization is provided based on the identified potential sources of variation. As the studies collecting muscle biopsies are focused on investigating biological differences in the muscle of cancer patients as compared to controls, the effect of the biopsy collection protocol on muscle biochemistry must be considered. Cancer type, stage, medications and comorbidities can influence muscle biology; thus these characteristics of patients need to be reported. Also, whatever control groups need to be clearly defined with details about comorbidities and medications. Another point to consider in future studies is to use a measure of muscle instead of weight loss. Comparison of biological features of muscle between the groups stratified based on weight loss is unreasonable as weight change can be because of change in either fat or muscle or both (Fearon et al., 2011; Mourtzakis et al., 2008). In our patient cohort, exploration of weight loss data revealed that 70% weight-stable and 60% weight-losing patients were sarcopenic. Therefore, classification of patients based on weight change can potentially exclude muscle losing patients. Lastly, to understand the muscle biology of cancer patients, biopsies must be collected from large populations. This can be achieved by establishing muscle biopsy biobanks that can be accessed by researchers exploring mechanisms of muscle loss or fat infiltration in cancer populations. Collaboration between researchers and surgeons will help improve the rate of patient consent to obtain

intraoperative biopsies, which will provide a large sample size to investigate cancer type and stage-specific changes in muscle characteristics.

6.3 Association between muscle radiodensity and muscle triglyceride content in cancer patients

The study presented in Chapter 4 aimed to investigate the association between muscle radiodensity and muscle triglyceride content in a cohort of cancer patients. Variation in muscle radiodensity across the length and breadth of *rectus abdominis* and distribution of lipids in IMCL and EMCL compartments were also determined. It was hypothesized and also demonstrated that a negative association exists between muscle radiodensity and muscle triglyceride content. Given that fat infiltration is spatially non-uniform across the muscle volume (Biltz et al., 2017), it was also hypothesized that muscle radiodensity would widely vary across different regions and slices of CT-images. In line with the hypothesis, the percentage coefficient of variance ranges from 3 to 61% within the subject for regions or slices of CT-images. This wide variation in radiodensity is an important finding since, previous studies only used a single abdominal CT image to define muscle radiodensity and compare with biological features of the muscle (Ramage et al., 2018; Stephens et al., 2011). Heterogeneous distribution of lipids in muscle compartments was observed suggesting that different and potentially multiple mechanisms contribute to fat infiltration in muscle.

An increasing number of studies have reported myosteatosis, defined as low muscle radiodensity, as a prognostic factor in cancer patients (Malietzis, 2016; Rollins et al., 2016; Stretch et al., 2018; van Dijk et al., 2017). Myosteatosis refers to fat accumulation in skeletal muscle (Miljkovic et al., 2010). A moderate negative association between radiodensity and TG content along with wide variation in radiodensity across the muscle presents important points to consider when interpreting experimental results. Our finding that muscle radiodensity, as determined by CT imaging, is only

partly (22%) due to TG content of the muscle is in line with the observations reported by Goodpaster et al. (Goodpaster et al., 2000). Other factors that can contribute to lack of strength of association between muscle radiodensity and TG content are discussed in Chapter 4 and include relatively small biopsy size compared to the area of the CT image analyzed to determine radiodensity. Muscle fibrosis is another factor that is not well explored in cancer patients, and its association with muscle radiodensity is largely unknown. The first report of muscle fibrosis in cancer patients was recently published and reported that muscle fibrosis was associated with metastasis and poor survival (Judge et al., 2018). Further studies are warranted to determine if muscle fibrosis contributes to changes in muscle radiodensity.

Both IMCL and EMCL have been associated with metabolic diseases, but different mechanisms contribute towards the deposition of fat in a specific compartment (Chapter 1). Our results suggest that pathways involved in both EMCL and IMCL deposition need to be further explored to understand the pathophysiology of fat infiltration in the muscles of cancer patients. EMCL is mainly due to the deposition of adipocytes that developed from stem cells around muscle fibers. Mononuclear cells were isolated from cancer patient muscle biopsies to quantify multipotent progenitors that have the potential to develop as adipogenic or myogenic progenitors (Pisani et al., 2010). A very low number of multipotent progenitors and fewer adipogenic/myogenic progenitors in the muscle were found (Appendix D). Alterations in lipid metabolism in muscle including lipogenesis, lipolysis, and lipid oxidation and lipid uptake can contribute to increase in IMCL. Additional variation in IMCL may be introduced by fiber type composition of skeletal muscle as type I fibers have been reported to have three-fold more IMCL TG content than type II fibers (Essén et al., 1975; Howald et al., 1985). Our study did not find an association between IMCL and percentage fiber types in *rectus abdominis*, which is a mixed fiber type muscle (Johns et al., 2014).

6.4 Skeletal muscle phospholipid fatty acid composition and survival of cancer patients

Chapter 5 focused on determining the association of fatty acid composition of skeletal muscle phospholipids and survival in cancer patients. It was hypothesized that low PUFA content in skeletal muscle phospholipids of cancer patients will be associated with shorter survival. Patients who died within one year of surgery had a lower content of ARA in skeletal muscle phospholipids. We were also interested in investigating if low PUFA content associates with CT-derived muscle features and hypothesized that low PUFA content of muscle will have an adverse effect on muscle mass. As opposed to our hypothesis, no significant association was observed between the fatty acid composition of skeletal muscle phospholipids and CT-derived muscle features. In this study, no association between skeletal muscle index/radiodensity and survival was observed. It should be noted that studies which identified skeletal muscle index and radiodensity as independent prognostic factors in cancer were conducted in large patient cohorts (Malietzis, 2016; Martin, 2013; Rollins, 2016; van Dijk, 2017).

The fatty acid composition of diet influences membrane phospholipid composition (Abbott et al., 2012). Foods rich in ARA, such as meat and eggs, compose a large proportion of energy intake in Canadian and cancer population (Hutton et al., 2006; Prado et al., 2012; Simopoulos, 2000, 2016). Thus, depletion of ARA because of low dietary intake is unlikely whereas intake of EPA and DHA is generally low in a typical western diet and in cancer patients. Depletion of ARA in the membrane could either because of impaired incorporation into phospholipids or increased release by phospholipase A2 or both (Pérez-Chacón et al., 2009). In a variety of cancer types, dysregulation of phospholipase A2 is observed resulting in release of ARA from membrane phospholipids (Nakanishi et al., 2006). Free ARA is rapidly metabolized by cyclooxygenase, epoxygenase and lipooxygenase enzymes to yield oxylipins which act as second messenger and influence a broad range of biological processes including inflammation, apoptosis and tumorigenesis (Shearer et al., 2018). Of note,

increased levels of ARA derived plasma oxylipins have been reported in colorectal, pancreatic, lung and prostate cancer (Liu et al., 2014; Rodríguez-Blanco et al., 2014; Zhang et al., 2017). In colorectal cancer, ARA derived oxylipins increase further as the disease progresses (Zhang, 2017). Free ARA may also exert signalling functions as an inducer of apoptosis in the C2C12 derived myotubes (Spector et al., 2004). Furthermore, the product of ARA, lysophosphatidic acid and its metabolite, platelet-activating factor have also been reported to affect inflammation and promote tumorigenesis (Fonteh et al., 1992; Tou, 1989). Metabolism of lysophospholipids may influence ARA content of the membrane as they are needed for ARA incorporation into the membrane (Balsinde, 2002).

A trend of lower EPA and DHA content in muscle phospholipids was also observed in patients who survived less than a year. Similar to ARA, EPA and DHA are released from membrane phospholipids by specific phospholipase A2 and used for the synthesis of oxylipins. It has been suggested that EPA and DHA not only regulate the production of omega-3 oxylipins but also effect the abundance of omega-6 oxylipins (Shearer, 2018). As omega-3 and omega-6 derived oxylipins might have opposing roles in the regulation of inflammation, tumorgenesis or apoptosis, future studies are required to determine their role in cancer progression and survival.

6.5 Considerations for future studies

Future studies attempting to understand mechanisms that regulate fatty infiltration and its link to muscle dysfunction should consider precise quantification of the amount and distribution of fat in the muscle of cancer patients. In experimental models, quantification of fat in whole muscle by histological and biochemical methods have been suggested (Biltz, 2017), but this is not feasible in human muscle biopsies because the amount of tissue available for analysis is very limited. CT-derived muscle radiodensity is an important non-invasive measure that is useful to characterize the muscles of cancer patients. In previous studies, low radiodensity was interpreted as the presence of myosteatosis

but without measuring the fat content of muscle (Malietzis, 2016; Montano-Loza et al., 2016; O'Brien et al., 2018). In our study, while muscle radiodensity varied from 3% to 61% and TG content varied from 0.1 to 5%. Given the wide variation in radiodensity measures, when fatty infiltration is the primary outcome, and its mechanisms are being explored, biochemical quantification of fat should be conducted.

Fatty muscle in cancer represents a pathological condition that requires further investigations to understand the mechanisms involved. Attempts to isolate multipotent progenitors from the muscle of cancer patients to understand the origin of EMCL in cancer patients has been reported in only one study (Pisani, 2010). Our attempts to isolate enough of these cells for additional analysis using flow cytometry was not successful. However, several recent studies suggest a potential role of fibro/adipogenic progenitors (FAPs) in fat infiltration after muscle injury or muscle dysfunction (Jia et al., 2018; Shirasawa et al., 2017; Uezumi et al., 2014). The relation between these progenitors and EMCL in cancer needs to be explored as it has been suggested that fibro/adipogenic progenitor differentiation into adipocytes is modulated by the presence of disease and the muscle environment (Jia, 2018). Also, FAPs has been reported to contribute to skeletal muscle fibrosis, an under-explored feature of muscles of cancer patients (Uezumi, 2014).

Research in our lab has shown an increase in neutral lipid in fibers and a decrease in EPA and DHA content of phospholipids in the muscle of preclinical cancer model of myosteatosis (Almasud et al., 2017). Feeding diets containing fish oil increased EPA and DHA level in muscle membranes and decreased the expression of transcription factors involved in adipogenesis/lipogenesis and increased mitochondrial enzymes activity compared with the control group. Also, fish oil supplementation in cancer patients has been reported to be associated with the loss of intramuscular adipose tissue, but biopsies were not collected to investigate if this association was because of increased incorporation of

EPA and DHA in membrane phospholipids of muscle (Murphy et al., 2011). In our cancer patient cohort, a lower proportion of polyunsaturated fatty acid in phospholipid fractions of muscles with high TG content compared to low TG content was observed (Appendix F). The sample size was small, and a potential sampling bias was identified. Based on observations from an experimental model, future large sample size studies are required to understand the relationship between the fatty acid composition of skeletal muscle membranes and fat infiltration in cancer patients.

In Chapter 3, it was identified that previous studies investigating features of muscle biopsies of cancer patients had low sample sizes, patients with different cancer types and cancer stages that might increase the risk of bias. In our study, the results of different cancer types and stages were reported together as collecting biopsies from a single tumor type is challenging. Collaborations among researchers investigating the muscle biology of cancer patients and biobanking of collected biopsies are recommended in future studies to improve sample size and report cancer type and stage-specific results.

In Chapter 5, it was reported that ARA content was low in skeletal muscle phospholipids of patients who died within one year as compared to those who lived longer. Similarly, a recent study reported lower serum phospholipid ARA in patients with disease progression compared to disease-free patients after one year follow up (Jolanta et al., 2018). A trend of lower EPA and DHA amount in skeletal muscle of cancer patients who lived shorter compared to individuals who lived longer was also observed. Several studies reported lower levels of EPA and DHA in plasma/erythrocyte phospholipid of cancer patients (Table 1-2). Future studies need to consider dietary intake of study populations to identify if changes in fatty acid composition are because of differences in diet. Studying fatty acid composition of plasma, skeletal muscle, adipose tissue and tumor collected from same patients could be an area of interest for further investigation to determine if depletion of bioactive fatty

acids is systemic or selective. Further studies are also required to determine if there is increased release of ARA/EPA/DHA from phospholipids, leading synthesis of oxylipins. It is important to note, that some ARA-derived oxylipins have anti-inflammatory and anticancer activity while others might aggravate inflammation and promote tumorigenesis (Gabbs et al., 2015).

The method used to quantify fatty acids in skeletal muscle phospholipids represents fatty acid of all the phospholipid in the cell, including organelles such as mitochondria. In general, membrane phospholipid and fatty acid composition not only varies between different cell types but also between organelles of the same cell that can alter the cell functions. For example, a decrease in CL and increase in PC in skeletal muscle mitochondria of an experimental model of cancer led to the dysfunction of this organelle (Antunes et al., 2014). Mitochondrial dysfunction has been reported to contribute to cancer-related muscle atrophy and fat infiltration (Almasud, 2017; Antunes, 2014). We attempted to isolate cardiolipin from skeletal muscle, but quantities were not detectable using thin layer chromatography. Future studies should consider isolating mitochondria to investigate alterations in the lipid composition of membranes in this organelle. Also, the fatty acid composition of individual phospholipid fractions (PC, PE, PI, PS, SM, CL) should be reported as an increase of specific fatty acids in one phospholipid fraction can be masked by the decrease in another. In our study, the majority of alterations in fatty acid composition were observed in PC, and no differences in PE and PI was observed.

6.6 Conclusion

Skeletal muscle radiodensity is associated with the fat content of the muscle in cancer patients; however, the relationship is moderate in strength. This observation is in line with the study in diabetic and obese population (Goodpaster, 2000). Additionally, fat is heterogeneously distributed across the muscle and in IMCL/EMCL compartments of the muscle. Considering the variation reported in muscle

radiodensity and fat distribution in muscle, this work can help guide researchers in designing studies exploring factors and mechanisms contributing to muscle radiodensity and interpreting results. Understanding the mechanisms involved in fat infiltration in the muscle of cancer patients will enhance our understanding of aberrations of metabolic and biological pathways in cancer. This study also suggests an association between the fatty acid composition of skeletal muscle phospholipids and survival of cancer patients. Depletion of bioactive fatty acids, ARA, EPA, DHA, seem to place patients at risk for shorter survival. This is a new and remarkable observation but is in line with literature reporting depletion of bioactive fatty acids in plasma and erythrocytes of cancer patients and their association with survival. Further studies are required to determine if the depletion of fatty acids in cancer is systemic or selective and also, the mechanisms involved in the depletion.

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Appendix A: Antibody information used for immunofluorescence and Oil red O staining experiments: muscle fiber types, laminin/dystrophin, Oil red O and nuclear stain.

Primary Antibody	Dilution factor-primary	Supplier	Secondary antibody	Dilution factor-secondary	Supplier (Code)	Isotype	Species
Laminin	1:200	SIGMA	AlexaFluor® 647	1:400	Fisher	IgG	Rabbit
Dystrophin	1:25	Abcam				IgG	Rabbit
MyHC I (BAD5)	1:400	American Type Culture Collection	AlexaFluor® 568	1:200	Fisher	IgG2b	Mouse
MyCH IID (6H1)	1:400	American Type Culture Collection	AlexaFluor® 488	1:400	Fisher	IgM	Mouse
MyCH IIA (SC71)	1:400	American Type Culture Collection	AlexaFluor® 405	1:200	Fisher	IgG1	Mouse
DAPI	300 nM	BD Biosciences					

Antibody information used for immunofluorescence experiments: Oil red O							
Primary Antibody	Dilution factor-primary	Supplier	Secondary antibody	Dilution factor-secondary	Supplier (Code)	Isotype	Species
Laminin	1:200	SIGMA	AlexaFluor® 488	1:400	Fisher	IgG	Rabbit
Dystrophin	1:25	Abcam				IgG	Rabbit
DAPI	300 nM	BD Biosciences					
Wash buffer - Phosphate-buffered saline							

Appendix B: Complete extraction table of the reviewed articles in relevance of muscle biopsy collection in cancer patients

Section 1 - Quality assessment, study design, muscle type, surgery type, cancer type and stage.

Section 2 – Sample size, male/female ratio, age, control group type

Section 3 – Body composition and weight loss

Section 4 – Treatment history, comorbidities and medications

Section 1- Quality assessment, study design, muscle type, surgery type, cancer type and stage.

Author	Year	BIAS - Modified Newcastle-Ottawa scale - sample bias score (max. 3 points)	QUALITY - Quality Assessment score (NIH)	Study design	Muscle	Type of surgical procedure	Cancer type	Cancer stage
Acharyya	2005	1	3 out of 12	Cross-sectional	RA	NR	Gastric	NR
Agustsson	2011	1	3 out of 12	Cross-sectional	RA	Open surgery	Pancreatic and other (gastric, colon, ampulla of Vater, bile duct, small intestine and gallbladder)	NR

Aversa	2016	1	6 out of 12	Cross-sectional	RA	Open surgery	Colorectal, pancreatic and gastric	Stage 1-4
Aversa	2012	1	3 out of 12	Cross-sectional	RA and SA	Open surgery	NSCLC and gastric	Stage 1-4
Banduseela	2007	N/A	N/A	Case study	TA	N/A	NSCLC	NR
Bohlen	2018	0	4 out of 12	Cross-sectional	PM	Open surgery	Breast	Stage 1-4
Bonetto	2013	1	3 out of 12	Cross-sectional	RA	Open surgery	Gastric	Stage 1-4
Bossola	2006	1	5 out of 12	Cross-sectional	RA	Open surgery	Gastric	Stage 1-4
Bossola	2001	1	4 out of 12	Cross-sectional	RA	Open surgery	Gastric	NR
Bossola	2003	1	5 out of 12	Cross-sectional	RA	Open surgery	Gastric	NR
Brzeszczynska	2016	0	2 out of 12	Cross-sectional	QF	Open surgery	Oesophageal, gastric and pancreatic	Stage 2-3
Busquets	2007	0	3 out of 12	Cross-sectional	RA	Open surgery	Oesophageal, gastric and pancreatic	Stage 1-4
Christensen	2016	N/A	13 out of 14	Randomized controlled trial	VL	N/A	Testicular germ cell	NR
Christensen	2014	N/A	13 out of 14	Randomized controlled trial	VL	N/A	Testicular germ cell	NR
DeJong	2005	0	4 out of 12	Cross-sectional	RA	Open surgery	Pancreatic	Stage 1-4
D'Orlando	2014	1	6 out of 12	Cross-sectional	RA	Open surgery	Gastric	Stage 1-4
Ebhardt	2017	0	1 out of 12	Cross-sectional	QF	N/A	Oesophageal, gastric and pancreatic	NR

Eley	2008	1	3 out of 12	Cross-sectional	RA	Open surgery	Oesophageal and gastric	Stage 1-4
Gallagher	2012	1	7 out of 14	Longitudinal	QF	N/A	Oesophageal, gastric and pancreatic	Stage1-3
Higuchi	2000	N/A	N/A	Case study	Gastroc	N/A	Gastric	NR
Jagoe	2002	0	1 out of 12	Cross-sectional	LD	Open surgery	Lung	Stage3-4
Johns	2017	2	9 out of 12	Cross-sectional	RA	Open surgery	Oesophageal, gastric and pancreatic, lung and other (not defined)	Stage 1-4
Johns	2014	0	5 out of 12	Cross-sectional	RA	Open surgery	Upper GI tract and pancreatic	NR
Khal	2005	0	1 out of 12	Cross-sectional	RA	Open surgery	Pancreatic and colorectal	NR
Lamboley	2017	1	3 out of 12	Cross-sectional	VL	N/A	Prostate	Stage 2
Lundholm	1976	1	3 out of 12	Cross-sectional	RA	Open surgery	Oesophageal, gastric, pancreatic, choledochal, colorectal and liver, gall-bladder, renal and ovary	NR
MacDonald	2015	0	2 out of 12	Cross-sectional	RA and QF	Open surgery	Oesophageal and gastric	Stage 1-4
Marzetti	2017	1	5 out of 12	Cross-sectional	RA	Open surgery	Gastric	Stage 1-4

Narasimhan	2017	2	8 out of 12	Cross-sectional	RA	Open surgery	Pancreatic and colorectal	Stage 1-4
Narasimhan	2018	1	5 out of 12	Cross-sectional	RA	Open surgery	Pancreatic and colorectal	Stage 1-4
Nilsen	2016	N/A	9 out of 14	Randomized controlled trial	VL	N/A	Prostate	NR
Noguchi	1998	0	3 out of 12	Cross-sectional	RA	Open surgery	Oesophageal, gastric and colorectal	Stage 1-4
Op den Kamp	2015	0	6 out of 12	Cross-sectional	VL	NR	NSCLC	Stage 3-4
Op den Kamp	2012	0	3 out of 12	Cross-sectional	VL	NR	NSCLC	Stage 1-3
Op den Kamp	2013	0	5 out of 12	Cross-sectional	VL	NR	NSCLC	Stage 3-4
Pessina	2010	1	6 out of 12	Cross-sectional	RA	Open surgery	Gastric	Stage 1-3
Phillips	2013	0	4 out of 14	Longitudinal	VL	Open surgery	Colorectal	Early stage
Prokopchuk	2016	0	4 out of 12	Cross-sectional	RA	Open surgery	Pancreas	Stage 1-4
Puig-Vilanova	2014	1	3 out of 12	Cross-sectional	VL	NR	Lung	Stage 1-4
Ramage	2018	1	3 out of 12	Cross-sectional	RA	Open surgery	Oesophageal, gastric and pancreatic	Stage 1-4
Rhoads	2009	1	6 out of 12	Cross-sectional	RA	Open surgery	Gastric	Stage 1-4
Schmitt	2007	0	2 out of 12	Cross-sectional	RA	Open surgery	Pancreatic	Stage 2 and 4

Shaw	1991	0	6 out of 14	Non-randomized trial	RA and SCM	Open surgery	Colorectal and pancreatic, head and neck, thyroid, melanoma and sarcoma	NR
Skorokhod	2012	0	1 out of 12	Cross-sectional	RA	Open surgery	Pancreatic	Stage 2-4
Smith	2010	0	4 out of 12	Cross-sectional	RA	Open surgery	Gastric	Stage 1-4
Stephens	2011	0	2 out of 12	Cross-sectional	RA	Open surgery	Oesophageal, gastric, pancreatic, bile duct and rectal	Stage 2-4
Stephens	2010	1	3 out of 12	Cross-sectional	RA, VL and DIAPH	Open surgery	Oesophageal, gastric and pancreatic	NR
Stephens	2015	0	3 out of 12	Longitudinal	RA	Open surgery	Oesophageal, gastric, small-bowel, pancreatic and bile duct	Stage 1-4
Stretch	2013	0	4 out of 12	Cross-sectional	RA	Open surgery	Liver/Bile duct, GI tract, pancreatic, ovary/uterus, head & neck, skin and kidney	NR
Sun	2012	0	5 out of 12	Cross-sectional	RA	Open surgery	Gastric	Stage 1-4

Taskin	2014	0	1 out of 12	Cross-sectional	RA	Open surgery	Colorectal, pancreatic and gastric and lymphoma	NR
Weber	2007	0	3 out of 12	Cross-sectional	VL	N/A	Gastric, pancreatic and leukemia	NR
Weber	2009	0	2 out of 12	Cross-sectional	VL	N/A	GI tract (malignancy types not defined)	NR
Williams	2012	0	5 out of 12	Longitudinal	VL	N/A	Colorectal	Early stage
Williams	1999	0	2 out of 12	Cross-sectional	RA	Open surgery	Colorectal	NR
Zampieri	2010	0	3 out of 12	Cross-sectional	RA (cancer group) / QF (control group)	Open surgery and Laparoscopy	Colorectal	NR
Zampieri	2009	0	1 out of 12	Cross-sectional	RA (cancer group)/ QF or RA (control group)	Laparoscopy	Colorectal	Stage 2-3

Zampieri	2010	1	3 out of 12	Cross-sectional	RA (cancer group)/ QF or RA (control group)	Laparoscopy	Colorectal	Stage 2-3
Zeiderman	1991	0	5 out of 12	Longitudinal	RA	Open surgery	Oesophageal, gastric, colorectal and pancreatic	NR

Values reported as mean ± standard deviation (SD) unless indicated otherwise, *median (range) and ** median (interquartile range). NIH: National, Heart, Lung and Blood Institute; RA: Rectus Abdominis; TA: Tibialis anterior; QF: Quadriceps Femoris; VA: Vastus Lateralis; PM: Pectoralis Major; SA: Serratus Anterior; LD: Latissimus Dorsi; Gastroc: Gastrocnemius; SCM:Sternocleidomastoid;DIAPH:Diaphragm; GI: Gastrointestinal; NSCLC: Non-small cell lung carcinoma; N/A: Not applicable; NR: Not reported. WS: Weight stable, WL: Weight loss. NC: non-cachexia. CC: Cancer cachexia. BMI: BMI. SMI: Skeletal Muscle Index. . BIA: Bio-electrical impedance analysis. DXA: dual-energy x-ray absorptiometry. CT: Computed Tomography.

Section 2 – Sample size, male/female ratio, age, control group type

Author	Year	Population sample size, n (male%)	Age (years), mean±SD	Control group type	Control group sample size, n (male %)	Control group age (years), mean±SD
Acharyya	2005	27(NR)	NR	Healthy	14 (NR)	NR
Agustsson	2011	Pancreas=13 (30) / Other=8 (37)	Pancreas:70±2 / Other: 68±3	Benign disease (age and sex matched)	Benign=8 (37) / Chronic pancreatitis= 8 (63)	53±4 / Chronic pancreatitis:52±3
Aversa	2016	29 (59), WS = 14 / WL =15	68±10.7	Benign disease	11 (63)	63±13.2
Aversa	2012	39 (74)	Lung:66+9 /Gastric:65+10	Benign disease, weight stable	10 (50)	Abdominal: 63+10 / Thoracic:65+12
Banduseela	2007	1 (100)	63	Muscle disease (acute quadriplegic myopathy, hereditary motor and sensory neuropathy of demyelinating, amyotrophic lateral sclerosis) and healthy	6 (50)	Healthy ♂: 42 and 56 / Myopathy ♂: 75 / Myopathy ♀: 30, 74 and 61
Bohlen	2018	14 (0)	56.5 ± 17.2	Prophylactic mastectomy or breast reconstruction surgery	6 (0)	44.2 ± 7.4
Bonetto	2013	16(NR)	64±11	Benign disease	6 (NR)	62±17.4
Bossola	2006	16 (50)	60.8±11.2	Benign disease	5 (60)	65.6±7.5
Bossola	2001	20 (55)	61±79.6	Benign disease, weight stable	10 (60)	62+45.8
Bossola	2003	23 (61)	59.5+16.1	Benign disease, weight stable	14 (64)	61.2+12.3

Brzeszczynska	2016	28 (75), NC = 18 (72) / CC = 10 (80)	NC: 67±10.5 / CC: 65±8.1	Healthy elderly and healthy middle age	Elderly = 21 (52), Middle age = 20 (60)	Middle-age: 61±7 / Elderly: 79±3.6
Busquets	2007	16(NR)	66±10	Benign disease, weight stable	11 (NR)	66±10.2
Christensen	2016	8 (100)	33.4±7.5	Malignant and healthy	Control =9 (100), Ref=13 (100)	Control:37.8±7.6 / Reference group:32.1±6.3
Christensen	2014	15 (100)	Intervention: 34.4±7.6 / Control: 35.8±8.9	Healthy (age matched)	19 (100)	31.5±6.0
DeJong	2005	16 (63)	66±8	Benign disease, weight stable	11 (81)	67±13.2
D'Orlando	2014	38 (66)	68.1±11.6	Benign disease	12 (58)	64.2±11.6
Ebhardt	2017	19 (79), NC = 14 (85) / CC = 5 (60)	Non- CC: 66.3±10.2 / CC:64±4.1	Healthy elderly: sarcopenic and non-sarcopenic	Non-sarcopenic = 10 (60), Sarcopenic = 8 (50)	Non-sarcopenic: 77.4±2.3 / Sarcopenic: 80.3±3.9
Eley	2008	15 (87)	66 (49 – 83) *	Hernia, weight stable	9 (10)	56 (41 – 86) *
Gallagher	2012	12 (83)	65	Benign disease, weight stable	6 (66)	58
Higuchi	2000	1 (100)	54	N/A	N/A	N/A
Jagoe	2002	36 (75)	64.1±9	Benign disease (Thoracotomy)	10 (40)	51.3±15.1
Johns	2017	134 (51)	65±13	N/A	N/A	N/A
Johns	2014	41 (73)	65 ± 12.8	Malignant, Weight stable	N/A	N/A
Khal	2005	18 (67), WS = 5 (60) / WL = 13 (69)	WS:79.8±2.2 / WL:70.6±8.2,	Hernia, liver cyst and gall stones, weight stable	10 (80)	69.6±7.3
Lamboley	2017	8 (100)	68± 5.6	Healthy (age and physical activity matched)	14 (100)	71±3.7

Lundholm	1976	43 (44)	Male:62± 13.1 /Female: 63± 9.7	Uncomplicated gall bladder disease or peptic ulcer	55 (51)	56± 14.8
MacDonald	2015	14 (57), WS = 6 (66) / WL = 8 (50)	WS: 62.5 (57.0-70.3)** / WL: 63.4 (61.5-66.3)**	Healthy	7 (42)	52.1 (51.5-53.1)**
Marzetti	2017	18 (94), WS = 9 (100) / WL =9 (89)	WS:70.6±8.63 /WL:66.8±12.5	Benign disease, weight stable (age-matched)	9 (88)	57.4±15.9
Narasimhan	2017	22 (41)	64.9 ±10	Malignant, Weight stable	20 (45)	63.6±7.9
Narasimhan	2018	40 (43), WS =19 (47) / WL = 21 (40)	WS:64±8 /WL: 66±11	N/A	N/A	N/A
Nilsen	2016	12 (100)	67±7	Prostate cancer without physical training	11 (100)	64 ±6
Noguchi	1998	10 (90)	56 (50 to 63)*	N/A	N/A	N/A
Op den Kamp	2015	26 (65), Pre-CC = 10 (80) / CC= 16 (56),	Pre-CC:62.4±10.4 / CC:59.8±8.2	Healthy	22 (59)	61.4±7.0
Op den Kamp	2012	16 (93)	65.9±7.5	Healthy	10 (70)	63.7±5.6
Op den Kamp	2013	26 (65), Pre-CC = 10 (80) / CC= 16 (56),	Pre-CC:62.4±10.4 / CC:59.8±8.2	Healthy	22 (59)	61.4±7.02
Pessina	2010	30 (57)	63.8+2.8	Benign disease, weight stable (age-matched)	8 (62)	64.2+2.6
Phillips	2013	8 (50)	62.5±23.4	Healthy	8 (50)	70.7±4.5

Prokopchuk	2016	25 (32), NC=13 (38) / CC=12 (25)	NC:67 (36-87) / CC:70 (52-83)*	Diverticular disease, seous cystadenoma of pancreas, nodular hyperplasia of liver, gallstones, liver rupture and chronic pancreatitis.	Benign=15 (80) / Chronic pancreatitis=9 (45)	Benign:67 (32-73) / Chronic pancreatitis: 49.5 (40-75)*
Puig-Vilanova	2014	10 (100)	65±9	Healthy and COPD cachexia	Healthy = 10 (100) / COPD = 16 (100)	65±11 and 64±9
Ramage	2018	32 (81)	64.5 (43-83)	N/A	N/A	N/A
Rhoads	2009	14 (57), WS = 6 (66) / WL = 8 (50)	64.2±3.8	Benign disease	10 (60)	63.9±2.8
Schmitt	2007	16 (63), NC= 8 (37) / CC = 8 (88)	NC: 62±8.5 / CC:53±11.3	N/A	N/A	N/A
Shaw	1991	43 (42), WS = 25 (48) / WL = 18 (66)	WS:61±20 / WL: 64±12.7	Benign disease, weight stable	18 (33)	57±16.9
Skorokhod	2012	23 (61), WS = 13 (69) / WL = 10 (50)	WS: 66 (51-69) /WL: 65 (57-74)	N/A	N/A	N/A
Smith	2010	15 (67)	66±11.6	Cholelithiasis, weight stable	15 (80)	57±19.3
Stephens	2011	19 (58)	67±10	Benign disease, weight stable	6 (33)	53±8
Stephens	2010	18 (66), WL	67±8.4	Benign disease	3 (66)	45±3.4
Stephens	2015	92 (72), NC = 41 (82) /CC = 51(63),	All: 65±10 / NC:68±9/ CC:63±9	Hernia and cholecystectomy	15 (53)	56±17

Stretch	2013	134 (51)	♂:59±13 / ♀:63±13	N/A	N/A	N/A
Sun	2012	102 (71)	62.13±6.54	Benign disease, (age and sex matched)	29 (72)	61.8±6.4
Taskin	2014	14 (50), NC= 8 (37) / CC = 6 (66)	NC: 68±5 / CC: 70±15	Benign disease	5 (40)	77±5
Weber	2007	17 (53)	52.5±6.5	Healthy	27 (52)	57.9±12.4
Weber	2009	19 (52)	58±9	Healthy, weight stable (age, sex and height matched)	19 (53)	56±7
Williams	2012	13 (46)	66±10.8	Healthy (age and sex matched)	8 (50)	71±5.6
Williams	1999	6 (66)	67 (53-76) *	Benign disease	6 (83)	54(22-92) *
Zampieri	2010	14 (36)	65.1 ±10.3	Muscle disease (Polymyositis and dermatomyositis) and healthy	Muscle disease= 13 (38) / Healthy = 19	Healthy: 30.1±13.3 / Myopathy: 64.3±6.3
Zampieri	2009	10 (30)	65.1 ± 10.3	Healthy	10 (NR)	22.7±2.6
Zampieri	2010	11 (36)	65.1 ± 10.3	Benign disease, weight stable	7 (0)	44.5±18.3
Zeiderman	1991	30 (70), Hospital diet = 10 (70) / 3 days intervention= 10 (70) / 7 days intervention= 10 (70)	Hospital diet: 67±9.5 /3 days intervention:72±3.2 / 7 days intervention: 67±6.3	N/A	N/A	N/A

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Gastrointestinal; NSCLC: Non-small cell lung carcinoma; N/A: Not applicable; NR: Not reported. WS: Weight stable, WL: Weight loss. NC: non-cachexia. CC: Cancer cachexia. BMI: BMI. SMI: Skeletal Muscle Index. . BIA: Bio-electrical impedance analysis. DXA: dual-energy x-ray absorptiometry. CT: Computed Tomography.

Section 3 – Body composition and weight loss

Author	Year	Patient weight loss or cancer cachexia criteria	Body composition analysis	Body composition used for muscle mass assessment	Cancer Patients, weight loss % (mean±SD)	Controls, weight loss % (mean±SD)
Acharyya	2005	N/A	No	No	NR	NR
Agustsson	2011	NR	No	No	Pancreas:6±3 / Other:3±3	2±2 / Chronic pancreatitis:6±3
Aversa	2016	5% WL over 6 months	Anthropometic and BIA	Yes	13.2±7.7,0.6±1.1	0
Aversa	2012	NR	No	No	Lung:3.7±4.6, Gastric: 5.6±5.9	0
Banduseela	2007	NR	No	No	N/A	N/A
Bohlen	2018	N/A	No	No	N/A	N/A
Bonetto	2013	>5% WL	Anthropometric	No	5.3±4.8	0.5±0.09
Bossola	2006	WL Mild: 0–5%. WL Moderate: 6–10%. WL Severe: >10%	Anthropometric	No	6±2	0.5±0.1
Bossola	2001	WL Mild: 0–5%. Moderate: 6–10%. Severe: >10%	No	No	5.6±21.9	0.8±1.2
Bossola	2003	>10% WL	No	No	5.2±4.7	0.7±0.3
Brzeszczynska	2016	>5% WL of pre-illness	DXA	Yes	NC:6.5±9.3, CC:9.3±7.8	NR
Busquets	2007	>5% WL over 1 month	No	No	5.1±4.4	1.4±2.6
Christensen	2016	N/A	No	No	N/A	N/A

Christensen	2014	N/A	DXA	Yes	N/A	N/A
DeJong	2005	N/A	No	No	18.4±14.8	2.2±6.3
D'Orlando	2014	>5% WL over 6 months	No	No	4.2±4.1	1.4±0.6
Ebhardt	2017	>5% WL of pre-illness	DXA	Yes	Non-sarcopenic:2.3±3.2, Sarcopenic: 15.9±6.8	NR
Eley	2008	NR	No	No	7.8 (0-27.5) *	0
Gallagher	2012	NR	No	No	Baseline:7.3±2.7, follow up:13.8±2.7	0
Higuchi	2000	N/A	No	No	N/A	N/A
Jagoe	2002	Any % WL over 6 months	No	No	2.9±6.6	2.7±9.8 (gain)
Johns	2017	WL >5%, >10%, >15% and SMI with any degree of WL (>2%)	CT	Yes	6±9	6±9
Johns	2014	>5% WL over past 6 months (in absence of simple starvation) and low muscularity with 2% WL	CT	Yes	> 5% WL: 12±4, > 10% 15±7, low muscularity: 6±5, Low muscularity +2% WL: 11±4	> 5% WL: 0±1, > 10% WL:2±1, low muscularity: 5±3, Low muscularity +2% WL:2±1
Khal	2005	WL Moderate: 1-11%. WL Severe: >11%	No	No	14±9, 0	0
Lamboley	2017	N/A	No	No	N/A	N/A
Lundholm	1976	N/A	No	No	N/A	N/A
MacDonald	2015	>5% WL	CT	Yes	10 (7.6-12.1) **	0
Marzetti	2017	>5% WL over 6 months	No	No	WS: 1.8±0.8, WL:7±0.8,	0.1±0.1

Narasimhan	2017	>5% pre-illness WL within 6 months or BMI of <20 with WL >2% and sarcopenia	CT	Yes	11.4±6.6	NR
Narasimhan	2018	WL >5%, >10%, >15% and sarcopenic (Skeletal Muscle Index) with any degree of WL (>2%)	CT	Yes	11.4±6.5	N/A
Nilsen	2016	N/A	No	No	N/A	N/A
Noguchi	1998	N/A	No	No	N/A	N/A
Op den Kamp	2015	>5% WL over 6 months	DXA	Yes	Pre-CC: 1.7±1.4, CC:12±5.5	0
Op den Kamp	2012	10% WL over 6 months	DXA	Yes	3.1±4.4	0.6 ±2 (gain)
Op den Kamp	2013	5% WL over 6 months, 2% WL with BMI 20 or sarcopenic (Skeletal Muscle Index)	DXA	Yes	Pre-CC: 1.7±1.4, CC:12±5.5	0
Pessina	2010	N/A	No	No	10±3.1	0
Phillips	2013	N/A	DXA	Yes	N/A	N/A
Prokopchuk	2016	>10% WL 6 months previous to surgery	CT	Yes	NC:4.9 (0-9.1), CC:14.3 (8.6-26.7)	0 (0-6) / Chronic pancreatitis:3.7 (0-29.5)
Puig-Vilanova	2014	Fat Free Mass Index: <18.5kg/m2	Anthropometric	Yes	1-8 kg	N/A
Ramage	2018	>5% WL of pre-illness	CT	Yes	3.7 (-25 to +10.9)*	N/A
Rhoads	2009	NR	Anthropometric	No	11±4	0

Schmitt	2007	>10% WL in the last 6 months	No	No	NC:5.2±3.9, CC:12.4±2.8	N/A
Shaw	1991	>15% WL of pre-illness	No	No	WS:1.6±3, WL:18±4.2	2±2.1
Skorokhod	2012	>10% WL of pre-illness	No	No	WS:2 (0-5.5) *, WL:13.9 (10-19.2)	N/A
Smith	2010	>5% WL	No	No	1.2±1.9	0.5±0.73
Stephens	2011	>10% WL in 6 months	No	No	6±7.1	0.3±1.4 (gain)
Stephens	2010	>5% WL	Anthropometric	No	8.9±6.7	0
Stephens	2015	>5% WL	No	No	NC:0.8±3.0, CC:13.9±8.6	0
Stretch	2013	N/A	CT	Yes	N/A	N/A
Sun	2012	>10% WL	No	No	N/A	N/A
Taskin	2014	>10% WL in 6 months, weight stable <5%	No	No	12.15 (10.6-14)*	NR
Weber	2007	>10% WL in 6 months	BIA	Yes	22.3±115.4	NR
Weber	2009	>10% WL in 6 months	BIA	Yes	>10	NR
Williams	2012	N/A	DXA	Yes	N/A	N/A
Williams	1999	N/A	No	No	N/A	N/A
Zampieri	2010	N/A	No	No	N/A	N/A
Zampieri	2009	N/A	No	No	N/A	N/A
Zampieri	2010	N/A	No	No	N/A	N/A
Zeiderman	1991	> 5 kg WL over 3 months	Anthropometric	Yes	Hospital diet = 16.4±8.8 Intervention 1 = 19.7±13.5, Intervention 2 = 19.2±10.1	N/A

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Section 4 – Treatment history, comorbidities and medications

Author	Year	Anti-neoplastic treatment exposure previous to biopsy	Comorbidity history	Medication history	Medication used as an exclusion criteria	Comorbidities used as an exclusion criteria
Acharyya	2005	NR	NR	NR	NR	NR
Agustsson	2011	NR	NR	NR	NR	Diabetes
Aversa	2016	NR	NR	NR	NR	Liver failure, diabetes, metabolic acidosis, acute and chronic renal failure, sepsis, AIDS, inflammatory bowel diseases, acute and chronic hepatitis, autoimmune disorders and chronic obstructive pulmonary disease.
Aversa	2012	NR	NR	NR	NR	Acute and chronic renal failure, liver failure, diabetes, metabolic acidosis, sepsis, AIDS, inflammatory bowel disease, autoimmune disorders, acute and chronic hepatitis, and chronic obstructive pulmonary disease.
Banduseela	2007	NR	Type 2 diabetes	Corticostero -ids	NR	NR
Bohlen	2018	Naïve, except six patients undergoing chemotherapy	NR	NR	NR	NR
Bonetto	2013	NR	NR	NR	NR	Liver failure, diabetes, acute or chronic renal failure, metabolic acidosis, AIDS, inflammatory bowel disease, autoimmune disorders, sepsis, chronic obstructive

						pulmonary disease, chronic heart failure, hepatitis, hyperthyroidism.
Bossola	2006	NR	NR	NR	NR	Acute or chronic renal failure, liver failure, diabetes, metabolic acidosis, sepsis, AIDS, inflammatory bowel disease, autoimmune disorders, chronic heart failure, acute and chronic hepatitis, hyperthyroidism, and chronic obstructive pulmonary disease.
Bossola	2001	NR	NR	NR	NR	Acute or chronic renal failure, liver failure, diabetes, metabolic acidosis, sepsis, AIDS, inflammatory bowel disease, autoimmune disorders, chronic heart failure, and hyperthyroidism.
Bossola	2003	NR	NR	NR	NR	Acute or chronic renal failure, liver failure, diabetes, metabolic acidosis, sepsis, AIDS, inflammatory bowel disease, autoimmune disorders, chronic heart failure, acute and chronic hepatitis, hyperthyroidism, and chronic obstructive pulmonary disease.
Brzeszczynska	2016	NR	NR	NR	NR	NR
Busquets	2007	Naïve	NR	NR	Corticosteroid or ß-blocking medication. Prior exposure to chemotherapy or radiotherapy.	Endocrine disease

Christensen	2016	Chemotherapy	NR	NR	NR	Cardiomyopathy, coronary heart disease,diabetes mellitus and chronic obstructive pulmonary disease.
Christensen	2014	Chemotherapy	NR	NR	NR	Cardiomyopathy, coronary heart disease,diabetes mellitus and chronic obstructive pulmonary disease.
DeJong	2005	NR	NR	No corticosteroids or beta blockers	Corticosteroids, beta blockers	Endocrine disease
D'Orlando	2014	NR	NR	No	Chronic corticosteroid treatment	Renal failure, diabetes mellitus, sepsis, HIV infection, inflammatory bowel disease, congestive heart failure, acute or chronic hepatitis, thyroid disorders, chronic obstructive pulmonary disease
Ebhardt	2017	Naïve	NR	NR	NR	NR
Eley	2008	Naïve	NR	No	NR	NR
Gallagher	2012	Naïve, except five patients with previous chemotherapy exposure, but no treatment within 4 weeks before surgery.	NR	No under anabolic or catabolic agents	Anabolic/catabolic agents	Uncontrolled diabetes or thyroid disorders.
Higuchi	2000	Naïve	NR	NR	NR	NR
Jagoe	2002	NR	NR	NR	NR	NR
Johns	2017	NR		NR	Corticosteroids.	Cognitive impairment and infection
Johns	2014	NR	NR	NR	NR	NR
Khal	2005	NR	NR	NR	NR	NR
Lamboley	2017	Naïve	NR	NR	NR	Cardiovascular disease and type 1 diabetes.

Lundholm	1976	NR	NR	NR	NR	NR
MacDonald	2015	No chemotherapy exposure within 4 weeks before surgery	NR	NR	NR	NR
Marzetti	2017	NR	NR	NR	Corticosteroids.	Acute or chronic renal failure, liver failure, heart failure, chronic obstructive pulmonary disease, diabetes mellitus, thyroid disorders, metabolic acidosis, sepsis, HIV infection, inflammatory bowel disease, acute or chronic hepatitis, autoimmune disorders
Narasimhan	2017	Naive, except one patient under chemotherapy	NR	NR	NR	NR
Narasimhan	2018	Naïve	NR	NR	NR	NR
Nilsen	2016	Radiotherapy and Androgen deprivation therapy	NR	NR	Osteoporosis medication	NR
Noguchi	1998	NR	NR	NR	NR	NR
Op den Kamp	2015	NR	NR	NR	Corticosteroids or hormonal therapy	Chronic obstructive pulmonary disease, cardiac failure, severe endocrine, hepatic or renal disorders, other malignancies in the last 3 years, and chronic inflammatory diseases, or acute infection.
Op den Kamp	2012	NR	NR	NR	NR	NR
Op den Kamp	2013	NR	NR	NR	Hormones or continual oral corticosteroids,	Chronic obstructive pulmonary disease, Congestive Heart Failure and active infectious disease, other malignant disease.

					antitumor therapy	
Pessina	2010	NR	NR	NR	NR	Acute or chronic renal failure, liver failure, diabetes, metabolic acidosis, sepsis, AIDS, inflammatory bowel disease, auto-immune disorders, chronic heart failure, acute and chronic hepatitis, hyperthyroidism, and chronic obstructive pulmonary disease.
Phillips	2013	Naïve	NR	NR	NR	Metastasis, metabolic, respiratory or cardiovascular disorders.
Prokopchuk	2016	NR	NR	NR	NR	NR
Puig-Vilanova	2014	NR	NR	NR	NR	NR
Ramage	2018	NR	NR	No under anabolic or catabolic agents	NR	NR
Rhoads	2009	NR	NR	NR	NR	Acute or chronic renal failure, liver failure, diabetes, metabolic acidosis, sepsis, AIDS, inflammatory bowel disease, autoimmune disorders, chronic heart failure, acute and chronic hepatitis, hyperthyroidism, and chronic obstructive pulmonary disease.
Schmitt	2007	NR	NR	Diabetes treatment (unclear) for 3 patients	NR	NR
Shaw	1991	NR	NR	NR	Intotropic and antiarrhythmic agents	Hemodynamic treatments
Skorokhod	2012	NR	NR	NR	NR	NR

Smith	2010	NR	NR	Corticosteroids excluded	NR	Renal failure, diabetes, sepsis, acquired immunodeficiency syndrome (AIDS), inflammatory bowel disease, chronic heart failure, acute and chronic hepatitis, hyperthyroidism, chronic pulmonary disease, and corticosteroid treatment.
Stephens	2011	Naive, except 3 patients that finished chemotheraphy cycles before the surgery	NR	NR	Anabolic/catabolic agents	Metastatic disease, undergoing palliative surgery, uncontrolled diabetes or known thyroid disorders.
Stephens	2010	NR	NR	NR	NR	NR
Stephens	2015	No chemotherapy exposure within 4 weeks before surgery	No subjects had uncontrolled diabetes or thyroid disorders.	No subjects were knowingly taking anabolic/catabolic agents	NR	NR
Stretch	2013	NR	NR	NR	NR	NR
Sun	2012	NR	NR	NR	NR	Acute or chronic renal failure, liver failure, diabetes, metabolic acidosis, sepsis, AIDS, inflammatory bowel disease, autoimmune disorders, chronic heart failure, and hyperthyroidism.
Taskin	2014	NR	NR	NR	NR	NR
Weber	2007	Chemotherapy and Radiotherapy	NR	NR	NR	NR
Weber	2009	NR	NR	NR	NR	Severe heart failure, severe pulmonary disease, pregnancy, or myocardial infarction within the preceding two weeks. cardiovascular, metabolic,

						respiratory, renal, hepatic, neurological, psychiatric or inflammatory disease, cancer (type of cancer other than the aforementioned type admitted to the group of cachectic patients).
Williams	2012	Naïve	NR	NR	NR	Metabolic, respiratory, or cardiovascular disorders or other contraindications to a healthy status.
Williams	1999	NR	NR	NR	NR	NR
Zampieri	2010	Naïve	NR	NR	NR	Patients under chemotherapy treatment or taking drugs known to induce myopathy as main side effect.
Zampieri	2009	Naïve	NR	NR	NR	Patients under chemotherapy treatment or taking drugs known to induce myopathy as main side effect.
Zampieri	2010	Naïve	NR	NR	NR	Patients reporting fatigue, weaknes or asymptomatic for muscle pain.
Zeiderman	1991	NR	NR	NR	NR	NR

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Appendix C: Skeletal muscle gene expression for genes associated with cancer cachexia in cancer patients

Biological function	Gene symbol	Gene name	Agilent transcript ID [Refseq RNA ID]	Female (n=64)	Male (n=69)	p-value
Atrophy	FBXO32	F-box protein 32	A_23_P82814 [NM_058229]	1.08 ± 0.59	1.06 ± 0.6	0.9
			A_24_P218259 [NM_058229]	1.18 ± 0.54	1.10 ± 0.57	0.4
			A_24_P918147 [NM_058229]	0.99 ± 0.48	1.07 ± 0.53	0.3
	FOXO1	forkhead box O1	A_23_P151426 [NM_002015]	1.43 ± 1.11	1.17 ± 0.66	0.6
			A_24_P22079	1.53 ± 1.04	1.11 ± 0.68	0.005
	FOXO3	forkhead box O3	A_23_P345575 [NM_001455]	1.10 ± 0.53	1.03 ± 0.51	0.4
			A_32_P102062 [NM_001455]	1.16 ± 0.59	1.05 ± 0.54	0.3
	TRIM63	tripartite motif containing 63	A_23_P114983 [NM_032588]	1.03 ± 0.36	1.10 ± 0.41	0.4
	UCP1	uncoupling protein 1	A_23_P30091 [NM_021833]	1.21 ± 0.85	1.28 ± 1.35	1
Autophagy	BECN1	beclin 1	A_23_P433071 [NM_003766]	0.91 ± 0.27	1.03 ± 0.3	0.05
			A_23_P89410 [NM_003766]	1.00 ± 0.27	1.11 ± 0.33	0.05
			A_24_P313597 [NM_003766]	0.98 ± 0.18	1.07 ± 0.27	0.1
	CTSB	cathepsin B	A_23_P215944 [NM_147780]	1.04 ± 0.5	1.19 ± 0.5	0.1

			A_24_P303770 [NM_147780]	1.08 ± 0.4	1.1 ± 0.38	0.7
			A_24_P397928 [NM_147780]	1.08 ± 0.46	1.09 ± 0.55	0.9
	CTSC	cathepsin C	A_23_P1552 [NM_001814]	1.10 ± 0.51	1.00 ± 0.43	0.4
			A_24_P115762 [NM_148170]	1.12 ± 0.48	1.06 ± 0.54	0.3
	CTSH	cathepsin H	A_23_P14774 [NM_004390]	1.05 ± 0.33	1.02 ± 0.34	0.5
	CTSL1	cathepsin L1	A_23_P94533 [NM_001912]	1.05 ± 0.29	1.04 ± 0.36	0.8
	CTSL2	cathepsin L2	A_23_P146456 [NM_001333]	1.31 ± 0.57	0.99 ± 0.44	<0.0001
	CTSS	cathepsin S	A_23_P46141 [NM_004079]	1.58 ± 2.69	1.27 ± 1.48	0.2
			A_24_P242646 [NM_004079]	1.10 ± 0.44	1.14 ± 0.48	0.6
	HIF1A	hypoxia inducible factor 1, alpha subunit	A_24_P56388 [NM_181054]	1.06 ± 0.53	1.08 ± 0.55	0.9
Apoptosis	APAF1	apoptotic peptidase activating factor 1	A_23_P36611 [NM_181861]	1.04 ± 0.37	1.08 ± 0.42	0.6
	BAX	BCL2-associated X protein	A_23_P208706 [NM_138764]	1.04 ± 0.31	1.06 ± 0.36	0.7
			A_23_P346311 [NM_138764]	1.11 ± 0.43	1.01 ± 0.3	0.2
			A_23_P346309 [NM_138763]	2.81 ± 4.72	2.65 ± 4.53	0.7
	BCL2	B-cell CLL/lymphoma 2	A_23_P352266 [NM_000633]	1.09 ± 0.48	1.22 ± 0.61	0.3
	CASP3	caspase 3	A_23_P92410 [NM_004346]	1.07 ± 0.29	1.11 ± 0.51	0.9

	CASP8	caspase 8	A_23_P209389 [NM_033355]	0.97 ± 0.32	1.09 ± 0.38	0.08
			A_24_P157087 [NM_033355]	1.07 ± 0.41	1.1 ± 0.65	0.8
			A_24_P148499 [NM_033358]	1.68 ± 2.08	1.25 ± 0.79	0.1
	CASP9	caspase 9	A_23_P97309 [NM_001229]	0.95 ± 0.19	1.06 ± 0.25	0.008
			A_24_P111342 [NM_001229]	0.97 ± 0.22	1.08 ± 0.31	0.03
	FASLG	Fas ligand	A_23_P369815 [NM_000639]	1.31 ± 0.83	1.25 ± 0.94	0.4
			A_24_P54220 [NM_000639]	1.45 ± 1.45	1.25 ± 1.13	0.4
Muscle growth	AKT1	v-akt murine thymoma viral oncogene homolog 1	A_23_P2960 [NM_005163]	1.23 ± 0.52	1.04 ± 0.35	0.03
	DMD	dystrophin	A_23_P321860 [NM_004019]	1.04 ± 0.44	1.25 ± 1.11	0.1
			A_24_P342388 [NM_004019]	1.34 ± 0.67	0.94 ± 0.29	<0.0001
			A_24_P185854 [NM_004010]	1.11 ± 0.27	0.94 ± 0.23	<0.0001
			A_24_P34186 [NM_004010]	1.19 ± 0.55	0.97 ± 0.39	0.01
			A_32_P199796 [NM_004023]	1.27 ± 0.66	0.98 ± 0.42	0.005
	IGF1	insulin-like growth factor 1	A_23_P13907 [NM_000618]	1.15 ± 0.95	1.34 ± 1.35	0.1
			A_24_P304419 [NM_000618]	1.14 ± 0.9	1.32 ± 1.36	0.2

		A_24_P304423 [NM_000618]	1.19 ± 1.08	1.42 ± 1.76	0.2
		A_24_P398572 [NM_000618]	1.86 ± 2.57	2.23 ± 2.66	0.2
IGF1R	insulin-like growth factor 1 receptor	A_23_P205986 [NM_000875]	1.06 ± 0.41	1.04 ± 0.37	0.9
		A_23_P417282 [NM_000875]	1.07 ± 0.3	1.00 ± 0.27	0.2
MAPK14	mitogen-activated protein kinase 14	A_23_P426292 [NM_001315]	1.03 ± 0.19	1.03 ± 0.19	1
		A_24_P283288 [NM_139013]	1.03 ± 0.31	1.08 ± 0.37	0.5
		A_24_P397566 [NM_139013]	1.1 ± 0.37	1.01 ± 0.28	0.2
MSTN	myostatin	A_23_P165727 [NM_005259]	1.71 ± 2.43	2.74 ± 3.74	0.02
MTOR	mechanistic target of rapamycin	A_23_P34606 [NM_004958]	1.04 ± 0.25	1.04 ± 0.26	0.9
MYOD1	myogenic differentiation 1	A_24_P30257 [NM_002478]	1.12 ± 0.70	1.24 ± 0.79	0.4
MYOG	myogenin	A_23_P160438 [NM_002479]	1.73 ± 2.56	1.38 ± 1.33	0.7
		A_24_P311036 [NM_002479]	1.74 ± 2.53	1.43 ± 1.57	0.7
PAX7	paired box 7	A_23_P126225 [NM_013945]	0.99 ± 0.49	1.08 ± 0.39	0.05
		A_23_P500985 [NM_013945]	0.96 ± 0.45	1.03 ± 0.33	0.09
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	A_23_P300033 [NM_006206]	1.01 ± 0.32	0.98 ± 0.31	0.6

	PPARGC1A	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	A_23_P18447 [NM_013261]	1.11 ± 0.58	1.05 ± 0.46	0.6
			A_24_P303052 [NM_013261]	1.22 ± 0.77	1.00 ± 0.51	0.07
	SMAD2	SMAD family member 2	A_23_P15937 [NM_001003652]	1.01 ± 0.19	1.05 ± 0.15	0.1
			A_24_P202527 [NM_001003652]	1.01 ± 0.23	1.00 ± 0.18	0.9
			A_32_P109002 [NM_001003652]	0.98 ± 0.44	1.05 ± 0.42	0.2
			A_32_P12580 [NM_001003652]	1.06 ± 0.29	1.06 ± 0.27	1
	SMAD3	SMAD family member 3	A_23_P48936 [NM_005902]	1.14 ± 0.42	1.00 ± 0.28	0.07
	TGFB1	transforming growth factor, beta 1	A_24_P79054 [NM_000660]	1.42 ± 1.47	1.06 ± 0.54	0.01
Inflammation	IFNG	interferon, gamma	A_23_P151294 [NM_000619]	1.04 ± 0.32	1.09 ± 0.34	0.3
	IL1A	interleukin 1, alpha	A_23_P72096 [NM_000575]	1.02 ± 0.3	1.07 ± 0.34	0.4
	IL1B	interleukin 1, beta	A_23_P79518 [NM_000576]	5.74 ± 16.9	5.60 ± 23.8	0.6
	IL6	interleukin 6	A_23_P71037 [NM_000600]	19.9 ± 63.8	26.6 ± 100	0.6
	IL6R	interleukin 6 receptor	A_24_P379413 [NM_000565]	1.35 ± 1.20	1.33 ± 1.13	0.9
	JAK1	Janus kinase 1	A_23_P97005 [NM_002227]	1.09 ± 0.37	1.05 ± 0.32	0.7
			A_24_P410678 [NM_002227]	0.92 ± 0.37	1.15 ± 0.43	0.001

	JAK2	Janus kinase 2	A_23_P123608 [NM_004972]	1.21 ± 0.48	1.06 ± 0.45	0.03
	JAK3	Janus kinase 3	A_23_P329112 [NM_000215]	1.03 ± 0.46	1.19 ± 0.57	0.09
			A_24_P308096 [NM_000215]	1.12 ± 0.56	1.11 ± 0.52	1
			A_24_P59667 [NM_000215]	1.28 ± 0.74	1.33 ± 1.32	0.4
	NFKB1	Nuclear factor kappa B subunit 1	A_23_P30024 [NM_003998]	1.07 ± 0.22	1.03 ± 0.24	0.2
	STAT3	signal transducer and activator of transcription 3	A_23_P100795 [NM_213662]	1.11 ± 1.05	0.44 ± 0.44	0.4
			A_23_P107206 [NM_213662]	1.21 ± 1.02	0.53 ± 0.35	0.02
			A_24_P116805 [NM_213662]	1.17 ± 1.00	0.50 ± 0.31	0.0
	STAT5A	signal transducer and activator of transcription 5A	A_23_P207367 [NM_003152]	1.12 ± 1.01	0.32 ± 0.34	0.03
			A_24_P173088 [NM_003152]	1.19 ± 1.00	0.47 ± 0.45	0.005
	STAT5B	signal transducer and activator of transcription 5B	A_23_P100788 [NM_012448]	1.05 ± 1.00	0.36 ± 0.36	0.3
	TNF	tumor necrosis factor	A_23_P376488 [NM_000594]	1.27 ± 0.93	1.44 ± 1.21	0.4
			A_24_P50759 [NM_000594]	0.99 ± 0.35	1.15 ± 0.44	0.03
	TNFSF12	tumor necrosis factor (ligand) superfamily, member 12	A_24_P245298 [NM_003809]	1.01 ± 0.24	1.05 ± 0.32	0.6

Values (unitless) reported as mean ± standard deviation

Appendix D: Isolation of mononuclear cells and multipotent progenitors from skeletal muscle

The goal was to have ability to isolate both mononuclear and multipotent progenitors from a muscle biopsy. Considering previous available methods for mononuclear cell isolation (McKay et al., 2010) and multipotent progenitors (Pisani et al., 2010).

Under a sterile flow hood, a 150 mg piece of the muscle biopsy was weighed and minced in 3mL of a collagenase-dispase digestion solution (10 mg/mL collagenase I, Gibco/Invitrogen + 2.4 U/mL Dispase II, Gibco/Invitrogen + 0.5 M calcium chloride; PH adjusted to 7.4) made in sterile culture medium, until tissue is minced into tiny pieces, followed by 10 min incubation at 37℃ (5% CO2). After 10 min, collagenase-dispase solution (1.5 mL) was added to the mixture, agitated by trituration and incubated for another 10 min at 37℃ (5% CO2). Digestion reaction was stopped by adding 15 mL of pre-warmed (room temperature) sterile wash medium containing 20% FBS and 1% antibiotics, and filtered through 70µm nylon mesh into a 50 mL conical tube, vortexed for 10 sec, followed by centrifugation at 800 x g for 10 min at room temperature. Decant supernatants and add 1 mL of PBS, Use 20µL to count cells under a haemocytometer. The remainder of the cell suspension was aliquoted evenly into pre-conditioned tubes such that each tube would contain at least 500,000 cells.

The tubes were spun again at 800xg for 5 minutes, and the supernatant was removed. The appropriate concentration of the labeled, cell surface primary antibodies were added to their respective tubes and incubated in the fridge for 30-45 minutes. Suspensions were washed with PBS solution, centrifuged again at 800xg for 5 minutes, and the supernatant was decanted. All wells used for analysis were fixed in 100 µL of 1% paraformaldehyde for 10 minutes at room temperature. After being washed, wells with cell surface antigens only were then refrigerated until cells could be acquired using flow cytometry analysis. All other cell pellets were permeabilized with 0.5% Triton-X in PBS for 15

minutes at room temperature, washed, and incubated with primary, intracellular antigens for 30-45 minutes. Incubation was again conducted if wells required secondary antibody. All remaining wells were then refrigerated until analysis. Wells used for the identification of cells within the multipotent progenitor and satellite cell populations used the antibodies CD34/PEcy7, CD56/BV510, and CD15/PerCP5.5 (eBiosciences, 12-0349-42, 17-0567-42, and 11-0159-42, respectively). Flow cytometry was conducted using the BD Fortessa flow analyzer (BD Biosciences), and programs for analysis included BD FacsDiva and FCS Express 4.

Table D1: Multipotent progenitors isolated from muscle of cancer patients

Patient ID	Sex	Age	g of tissue	Total number of cells isolated	Absolute number of Multipotent per 100000 of cells isolated	Absolute number of Myogenic per 100000 of cells isolated	Absolute number of Adipogenic per 100000 of cells isolated	Absolute number of Multipotent per mg of wet tissue	Absolute number of Myogenic per mg of wet tissue	Absolute number of Adipogenic per mg of wet tissue
Bio 4	F	68	0.37	83680000	0.34	12.34	0.95	0.77	28.22	2.17
Bio 5	F	67	0.15	53200000	0	43.70	0	0	154.99	0
Bio 7	F	67	0.33	13700000	0	56.96	0	0	23.36	0
Bio 11	F	86	0.16	27800000	1.38	33.83	0.69	2.44	59.90	1.22
Bio 12	F	69	0.16	12900000	2.42	32.36	9.98	0.98	13.11	4.04
Bio 16	M	39	0.16	4400000	0	5.04	0	0	1.43	0
Bio 17	M	71	0.15	7000000	0	194.54	0	0	93.27	0
Bio 22	M	70	0.27	12400000	0	1.32	2.59	0	0.61	1.20
Bio 23	M	73	0.16	21000000	0	0	0	0	0	0
Bio 26	M	53	0.16	33400000	0	1.63	0.81	0	3.47	1.73

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Pisani, D. F., Clement, N., Loubat, A., Plaisant, M., Sacconi, S., Kurzenne, J.-Y., … Dechesne, C. A. (2010). Hierarchization of Myogenic and Adipogenic Progenitors Within Human Skeletal Muscle. *STEM CELLS*, *28*(12), 2182–2194. https://doi.org/10.1002/stem.537

Appendix E: Proportion of Phosphatidylinositol (PI) species in skeletal muscle of cancer patients.

	Survival >1year		Survival <1year		
	Mean	SD	Mean	SD	p-value
PI 34:1	1.7	0.6	1.8	0.5	NS
PI 36:1	2.9	1.6	2.7	0.9	NS
PI 36:2	8.4	2.2	8.8	2.2	NS
PI 36:3	1.5	0.5	1.5	0.5	NS
PI 38:2	1.8	0.5	2.1	0.7	NS
PI 38:3	16.8	2.9	17.9	3.1	NS
PI 38:4	55.5	6.5	54.2	5.7	NS
PI 38:5	3.3	2.5	3.2	1.2	NS
PI 40:4	1.3	0.3	1.3	0.3	NS
PI 40:5	1.7	0.6	1.7	0.8	NS
PI 40:6	1.2	0.6	1.2	0.8	NS

Appendix F: Low polyunsaturated fatty acids and high monounsaturated fatty acids in membrane lipids of skeletal muscle with high triglyceride content in cancer patients.

Introduction

Abnormal lipid accumulation in muscle is termed myosteatosis. Myosteatosis occurs in many conditions such as aging[1,2], obesity[3], type 2 diabetes[4] and muscle weakness[5–7]. Myosteatosis can be detected radiologically through computed tomography (CT) imaging as muscle with low radiodensity. Low muscle radiodensity is associated with poor survival in every cancer type in which it has been evaluated [8–14]. In healthy adults, cancer and diabetic patients, muscle radiodensity is inversely associated with triglyceride content [15].

Phospholipid lipid species have important structural and metabolic functions in the cell.[16] These lipid species are major components of cell membrane and contain precursors of second messengers. The phospholipid bilayer is the most important component of cell membranes and organelles whereas a monolayer of phospholipid surrounds lipid droplets in muscle. Changes in phospholipid composition, including fatty acid carbon chain length and number of double bonds influence ability of lipids to pack against one another thus altering membrane structure and fluidity which in turn effects cell signaling [17,18].

In an animal model of chemotherapy induced myosteatosis, a reduction in triglyceride content and concurrent incorporation of very long chain polyunsaturated fatty acids in phospholipids of the muscle was observed when rats were fed EPA and DHA in the diet[19]. These observations led the speculation that there are differences in fatty acid composition of phospholipids in muscle of cancer patients with high triglyceride content. To detect and identify characteristic phospholipids species, phospholipid fatty acid composition of muscles were identified as being low or high in TG content in

people with cancer. Total phospholipids and major phospholipid classes; phosphatidylcholine (PC) and phosphatidylethanolamine (PE), phosphatidylinositol (PI) and sphingomyelin (SM) were analyzed. PI holds a central role in cell signaling and regulation[20,21] and SM is most abundant phosphosphingolipid in human muscle [22]. The objective of the present study is to identify and characterize phospholipid species associated with myosteatosis in muscle from cancer patients.

Materials and methods

Ethics statement

The study was approved by the Health Research Ethics Board of Alberta-Cancer. Patients undergoing elective abdominal surgery were consecutively approached to participate in tumor and tissue banking at a hepatopancreatobiliary surgical service in Alberta, Canada. Three percent of approached patients declined participation. Patients provided written informed consent for muscle biopsy and tissue banking. Release of n=34 samples from the bank for analysis, as well as patient information (demographic, clinical and operative data) from medical records, was performed under the auspices of Protocol ETH-21709: *The Molecular Profile of Cancer Cachexia*.

Subjects and muscle biopsies

Skeletal muscle biopsies of 34 patients undergoing surgery for abdominal tumors were studied. All patients were either previously diagnosed to have cancer or were diagnosed after surgery. The study cohort and conditions for acquisition of muscle samples have been described previously [23]. Briefly, *rectus abdominis* biopsies (0.5 – 3 g) were collected from adult (>18 yrs) patients who were undergoing open abdominal surgery scheduled as part of their clinical care. Biopsies were collected at the start of surgery using sharp dissection and without the use of electrocautery. Samples were processed under sterile conditions. Visible adipose and connective tissue were trimmed. One piece of biopsy was immediately frozen in liquid nitrogen and stored at -80°C. Another piece was frozen in

isopentane cooled at -160°C in liquid nitrogen and stored at -80°C for cryostat sectioning and Oil Red O analysis. At the time of surgery, patients were either previously diagnosed with gastrointestinal cancer or were suspected of having cancer due to their symptoms and radiological assessments using CT imaging. Age, cancer type, metastasis information and comorbidities were abstracted from medical charts.

CT image Analysis

CT scans completed with a spiral CT scanner for initial cancer staging and routine diagnostic purposes were used to quantify skeletal muscle radiodensity.[24,25] Cross-sectional imaging using CT or magnetic resonance imaging is suggested as the preferred method for analyzing muscle mass in patients with cancer.[26] CT scans completed before surgery were analysed. Images were analyzed using SliceOmatic® V4.2 software with CT image parameters that include: contrast, 5mm slice thickness, 120 kVP, and 290 mA. An intraobserver coefficient of variation of 1.3% was required, which is consistent with other reports in the literature.[24,25,27] Total skeletal muscle area (cm^2) was evaluated on a single image at the third lumbar vertebrae (L3) using Hounsfield unit (HU) thresholds of -29 to 150 for skeletal muscle. The sum of skeletal cross-sectional muscle areas were normalized for stature (m^2) and reported as skeletal muscle index (SMI) (cm^2m^2). Muscle radiodensity was assessed for the full range of -29 HU to +150 HU. Mean muscle radiodensity (HU) is reported for the entire muscle area (quadratus lumborum, psoas, erector spinae, external obliques, transverse abdominis, internal obliques, and rectus abdominis.

Analysis of triglycerides and total phospholipids by Gas chromatography

The biopsy [≈50 mg] was ground using a frozen pestle and mortar without letting the muscle tissue thaw. Ground tissue was homogenized in calcium chloride [$CaCl_2$; 0.025%] solution. A

modified Folch method was used to extract lipids from muscle.[28] Lipids were extracted using chloroform/methanol (2:1, vol/vol).[29,30] The TG and PL fraction were isolated and scraped from G-plates.[30] Internal standard, C15:0, was added for TG and quantification followed by saponification and methylation.[30] Half of the PL amount was also methylated and used to determine fatty acid composition. TG and total PL fatty acid composition was analysed by gas chromatography-flame-ionisation detector in a Varian 3900 gas chromatography [Varian Instruments, Georgetown, ON, Canada] as previously described.[8] Quantity of fatty acids within the TG fraction was calculated by comparison with the known concentration of the internal standard and sum of all fatty acids was reported as total TG. In PL, the peak area of each fatty acid was normalized against the sum of peak areas of all fatty acids to determine the relative proportions.

Separation of phospholipid species by High-performance liquid chromatography (HPLC)

Phospholipid extracts were separated by normal phase chromatography with an Agilent Zorbax RX-Sil column (3.0 x 100 mm, 1.8 μm particle size) using an Agilent 1260 Infinity LC system (Santa Clara, CA) [31]. For phosphatidylcholine (PC) and phosphatidylethanolamine (PE), the mobile phase was composed of 75% acetonitrile 25% water/methanol (50/50, v/v) with 5 mM ammonium acetate and 0.01% (v/v) acetic acid. The total LC run time was 9 min at a flow rate of 0.5 μL/min. For sphingomyelin (SM) and phosphatidylinositol (PI), the mobile phase A was composed of acetonitrile-methanol-acetic acid (97:2:1, v/v) with 5mM ammonium acetate and and mobile phase B consisted of methanol-acetic acid (99:1, v/v) with 5mM ammonium acetate (Merrill et al., 2005). A gradient elution consisting of an increase of mobile phase B from 10 to 90 min for over 9 min was used with a total LC run of 14 min for SM at a flow rate of 0.5 μL/min and an increase of mobile phase B from 0 to

95% over 12 min was used with a total run of 13 min for PI (with 2 min post-run) at a flow rate of 0.5 µL/min.

Analysis of phospholipid species by Mass spectrometry (MS).

The HPLC (Agilent Technologies 1260 Infintiy) was coupled to a triple quad MS (Agilent Technologies 6430 Triple Quad LC/MS) for analysis of molecular species of individual phospholipids [31]. Protonated or deprotonated gas-phase ions of various phospholipid species were obtained using electrospray ionization, with the electrospray needle held at 4500 V. The MS was operated in multiple reaction monitoring (MRM) in positive or negative mode. A library of theoretical precursor ions was generated for PC, PE, SM and PI varying the length of fatty acid carbon chain. Phospholipids were fragmented at PC m/z 184, SM m/z 184, PI m/z 241 and PE was fragmented for a neutral loss of 141 amu. The collision energy varies from 18 to 50 eV. LC-MRM-MS analysis was performed in positive ion mode with transitions to detect all PE, PC, PI and SM species containing two fatty acids chains 12-24 carbons in length.

A second method determined the fatty acid (FA) composition of PE(36:0); PE(36:2); PE(38:6); PE(40:6); PE(40:7); PI (34:2); PI (36:2); PI (36:3); PI (36:4); PI (38:3); PI (38:4); PI (40:5); PI(40:6); PC(36:0); PC(38:6); phospholipid species in negative mode using the transitions of $[M+OAc]^- \rightarrow [FA-H]^-$ for PC; $[M-H]^- \rightarrow$[FA-H]- for PE and PI using different collision energies from first method. A library of the probable fatty acid carbon chains present in these phospholipid species was developed and used as product ions. Fatty acid were found around the mass region of m/z 200-330 according to the deprotonated fatty acid species between 12:0 and 22:6 carboxyl acid length chain scanned. To confirm the presence of these fatty acid only the peak on the MRM chromatogram was used in the RT obtained in first method.

Statistical analysis

To compare lipid profiles of muscle with high and low TG content, patients were divided into three groups based on tertile cut-offs, low TG (<13.6µg/mg), mid TG (13.6 to 26.4 µm/mg) and high TG (>26.4µm/mg). The low and high TG groups were compared with respect to phospholipid composition. Continuous data are presented as mean and standard deviation (SD). Categorical data are presented as counts with percentages. The peak area of each species was normalized against the sum of all peak areas within that class to determine the relative abundances of each PL species (expressed as percent of total PE, PC, PI and SM). For comparison between muscle of cancer patients with high TG and low TG content, Mann-Whitney's test was used for continous data and Fisher's exact test was used for categorical data. Statistical significance was reported when p value <0.05. All statistical analyses were performed using SPSS 20.0 (Chicago, IL, USA) for Windows. All graphs are generated using GraphPad Prism7 software (GraphPad Software, La Jolla, CA).

Results

Patient characteristics and body composition

The lowest and the highest tertiles of TG content were compared to evaluate differences in patient characteristics (Table 1). There were no significant differences in age nor BMI between those with low TG and high TG muscle. There were more males in the group of patients with low TG content in muscle as compared to group with high TG content muscle ($p<0.05$). Pancreatic and colorectal cancer were the most common diagnosis, comprising two-thirds of the patients in each group. Eighty two percent of patients with high TG muscle had tumor metastasis compared to thirty-six percent with low TG muscle. CT-derived measurements were compared to investigate difference in body composition between two groups. Patients with high TG content in muscle had significantly lower

muscle radiodensity and greater adiposity (subcutaneous adipose tissue and intramuscular adipose tissue) compared to those with low TG muscle. There was no significant difference in skeletal mass of patients between the two groups.

Lipid species and TG content

Relative abundance of many PE, PI, PC and SM species were different between muscles with high TG compared to low TG content (Fig 1A-D). For example, polyunsaturated PL species; PE (38:6), PE (40:6), PE (40:7), PI (40:6), PI (40:5), PI (38:4) and PC (38:6) were less abundant in muscle with high TG content (Fig 1A-C). PI (36:3) and PI (36:4) were the only polyunsaturated fatty acid species that were more abundant in high TG muscle as compared to low TG muscle (Fig 1B). Species with single bond [(PI (36:1)] and with double bond [PE (36:2), PI (34:2), PI (36:2)] were more abundant in high TG muscle (Fig 1A and 1B). Saturated fatty acid species PE (36:0) and PC (36:0) were also lower in abundance ($p<0.05$) in high TG muscle (Fig 1A and 1C). Sphingomyelin species SM (32:1), SM (34:0), SM (34:2) and SM (40:0) were significantly higher and SM (40:3) significantly lower in high TG muscle compared to low TG muscle (Fig 1D).

Unsaturation indices and carbon chain length

Polyunsaturated fatty acids species within PE and PI were less abundant ($p<0.01$) and mono/polyunsaturated fatty acid species more abundant ($p<0.05$) in high TG muscle compared to low TG muscle (Fig 2A-B). Proportion of PE species containing saturated fatty acid was also lower in high TG muscle whereas saturated fatty acid SM was significantly higher in high TG muscle (Fig 2D). There was no significant difference in fatty acid composition of total PLs (Supplement Table S1). Unsaturation indices of PE and PI were lower ($p<0.05$) in high TG muscle (Fig 3).

By examining the differences in fatty acid chain length, significantly lower proportion ($p<0.05$) of very long fatty acid chain PE (40 carbons), PI (40 carbons) and PC (38 carbons) species was observed in the high TG muscle compared to the low TG muscle (Fig 4A-C). In PE and PI species, there was lower proportion of 36 carbons chain length ($p<0.05$) in high TG muscle and similar proportion of shorter fatty acid chains between the groups. In SM species, C32 and C34 were higher ($p<0.05$) in high TG muscle compared to low TG.

Fatty acid composition

To determine identities of fatty acids, analysis were performed for each PE, PI and PC species that were differentially abundant between muscles with high TG compared to low TG (Supplement Table S2). Species PE (40:6), PE (40:7), PI (40:6), and PC (38:6) had DHA as the most prominent species. Muscles with high TG content had less abundant ($p<0.01$) DHA-containing PE (40:6) and PI (40:6). PI species containing arachidonic acid (C20:4) and docosapentaenoic acid (DPA, C22:5) were significantly less abundant in high TG muscle (Table 2). Any species containing a MUFA (C18:1 or C20:1) was more abundant in muscle with high TG regardless of the fatty acid in the alternate position (Table 2).

There was significant difference in distribution of males and females between the groups. To address this, analysis was performed using data from male patients only. Male patients were stratified based on TG content of the muscles and phospholipid composition was compared between muscle with high TG content and low TG content. A similar trend in differences in phospholipid composition of muscle was observed in male cancer patients as compared to male and female patients combined (Supplement Table S3).

Discussion

The significance of this study of cancer patients resides in novel data showing that phospholipids at the molecular-species level differ in muscle infiltrated with TG as compared muscle with low TG content. PE and PI species containing PUFAs and SFAs were less abundant and species with MUFAs were more abundant in muscle with high TG content. Specifically, DHA (c22:6n-3) and stearic acid (c18:0) containing species were less abundant and oleic acid (c18:1n-9) containing species more abundant in muscle with high TG content as compared to low TG content in cancer patients. PE and PI species containing PUFAs between 38 and 40 carbons and their unsaturation indices were higher in muscle with low TG content as compared to muscles with high TG content.

PE and PC are major structural phospholipids in membranes and dictate membrane biophysical properties [32]. PC is mainly present in the outer layer of the lipid bilayer whereas PE is present mainly in inner layer. PI plays small structural role but has a crucial role in cell signaling and function [20]. In the present study, there was no difference in fatty acid composition of total phospholipids and the majority of differences were observed in PE and PI. PE and PI exhibit higher polyunsaturated fatty acid species concurrent with lower poly/monounsaturated species in the muscle with high TG content. Also, lower abundance of long chain fatty acids containing species (40 carbons) and lower unsaturation indices of PE and PI were observed in muscle with high TG content. Changes in carbon chain length and degree of unsaturation of fatty acids attached to phospholipids alter physiochemical properties of cell membranes that include fluidity, asymmetry, plasticity, organization and occurrence of microdomains [33,34]. This in turn alters localization and function of membrane protein involved in the regulation of cellular processes [33].

Studies using muscle biopsies from people with obesity and diabetes have reported association between PUFAs in membranes and prevention of triglyceride accumulation [35,36]. Animal studies

reported an inverse relationship between muscle TG content and insulin sensitivity [35]. Lower concentration of LC-PUFAs in skeletal muscle phospholipids has been associated with decreased insulin sensitivity [37,38]. On the other hand a decrease in PUFA content of PC and not PE was associated with insulin resistance [39,40]. In the present study, lower proportion of PC (38:6) in muscle was observed in patients with high TG content. There was no difference in other PC species and in total polyunsaturated PC species. However, a lower proportion of total polyunsaturated PE and PI species was observed in muscle with high TG content. Furthermore, we found higher proportion of C18:1 (oleic acid) containing PE and PI species. Oleic acid has been shown to prevent and reverse insulin resistance in diabetes [41,42]. In vitro studies using hepg2 cell lines, a human liver cancer cell line, reported hepatic steatosis when treated with oleic acid [43]. However, in skeletal muscle cells, oleic acid treatment increased rate of fatty acid oxidation [44]. Interestingly, in the present study, there was no difference in number of cancer patients with diabetes as comorbidity between groups. These observations suggest that mechanisms other that insulin resistance might be involved in TG accumulation. Alterations in fatty acid composition of membranes had also been reported in lipidosis. Hepatic lipidosis and polyunsaturated fatty acid lipidosis in brain have been associated with reduced levels of polyunsaturated fatty acid with concurrent increase in monounsaturated fatty acid, specifically oleic acid [45].

Increased stearoyl-coenzyme A desaturase 1 (SCD1) activity can be another possible mechanism contributing to TG accumulation. SCD1 catalyses the rate-limiting step in the synthesis of mono-unsaturated fatty acids [46]. In the present study, high abundance of monounsaturated fatty acid-containing and low abundance of saturated fatty acid containing PE and PI species was observed. The imbalance between monounsaturated and saturated species indicate increased activity of stearoyl-coenzyme A desaturase 1 (SCD1) in muscle with high TG content. Higher abundance of SM (34:2) in

muscle with high TG content can also be explained by increased activity of SCD1. Because the sphingosine backbone of SM consists of a C18:1 molecule, the species C34:2 likely also contains palmitoleic acid [47]. SCD1 is a unique enzyme that appears to influence lipid bilayer fluidity [48], lipid metabolism [49] and fatty acid oxidation[50].

DHA containing PE (40:6) and PI (40:6) were lower in muscle containing high level of TG content. Upon incorporation into membranes, DHA has been shown to alter the activity of many proteins. Membrane model studies report that DHA acyl chains produced the highest level of protein kinase C (PKC) activity when incorporated into PE but not into PC [32]. PKC has been reported play important role in adipocyte differentiation and lipid metabolism in liver and skeletal muscle [51]. DHA down-regulates the expression of genes involved in fatty acid metabolism and lipid synthesis, such as fatty acid desaturase 2 and sterol-regulatory element binding transcription factor 1 in the liver and 2,4-dienoyl CoA reductase 2 in the muscle of experimental models [52]. These results were further supported by rat study that reported prevention and reversal of TG accumulation in muscles of tumor-bearing rats when fed EPA and DHA supplemented diets[19]. EPA and DHA supplemented diet down-regulated potent activators of adipogenesis; peroxisome proliferator-activated receptor (PPAR)γ and CCAAT/enhancer-binding protein (C/EBPβ, δ) in muscles of tumor-bearing rats [19]. Study also confirmed incorporation of EPA and DHA in phospholipids of skeletal muscle of rats in supplemented group (unpublished data). In humans, there is one clinical trial that showed reduction in intermuscular adipose tissue in advanced non-small cell lung cancer patients when supplemented with long chain polyunsaturated fatty acids, EPA and DHA, as compared to control group over the same time period.[53] The present study reported differences in composition of major phospholipid species; PE, PI, PC and SM in muscles of cancer patients with high TG content compared to low TG content. These

observations warrant intervention studies aimed to change phospholipid composition that might decrease TG content of the muscle in cancer patients.

The present is the first human study reporting differences in phospholipid species composition of muscle containing a high level of TG as compared to muscle with low TG content in cancer patients. Both total phospholipid fatty acid composition and individual phospholipid species composition were explored. Several limitations of the current study warrant mention. First, the study was cross-sectional by design, thus results do not allow for differentiation of causation and effect. This is difficult to overcome in humans as it is difficult to collect biopsy at several time points. Second, there was imbalance between the proportion of males and females in high TG and low TG content groups. However, it was addressed by performing comparisons using data from male patients only. A similar but weaker trend in differences in phospholipid composition of muscle was observed in male cancer patients, probably because of smaller sample size, as compared to male and female patients combined. Third, phospholipid composition differences represent muscle fibers, stem cells, immune cells and cell organelles. This makes it difficult to derive hypothesis about possible mechanisms that associates TG content and phospholipid composition. Analysis of isolated cells of interest will help understanding relationship between phospholipid composition and TG content.

In conclusion, we have shown PUFA and saturated fatty acid containing phospholipids to be less abundant concurrent monounsaturated fatty acid containing phospholipids more abundant in muscle with high TG content in cancer patients. Particularly, DHA-containing PE and PI were less abundant and oleic acid-containing PE and PI were more abundant. The underlying mechanisms for high TG content may be explained by altered phospholipid composition and lipid signaling. However, longitudinal studies are necessary to determine causality in the hypothesis we have generated here.

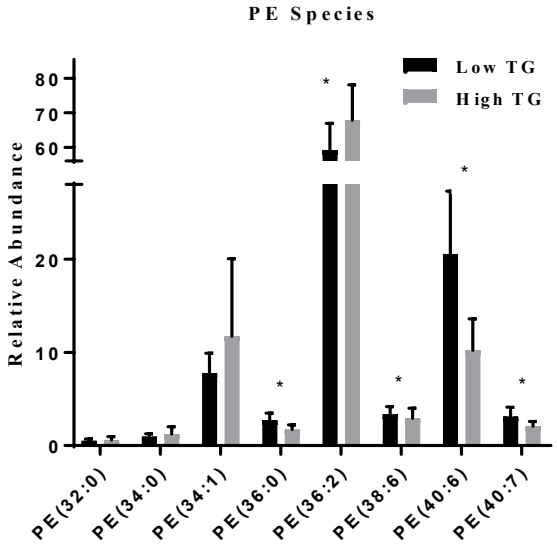
Future studies should focus on lipid signaling pathways and transcriptional factors that are involved in phospholipid and TG metabolism.

Table 1 – Characteristics of cancer patients with respect to high and low triglyceride content of the muscle.

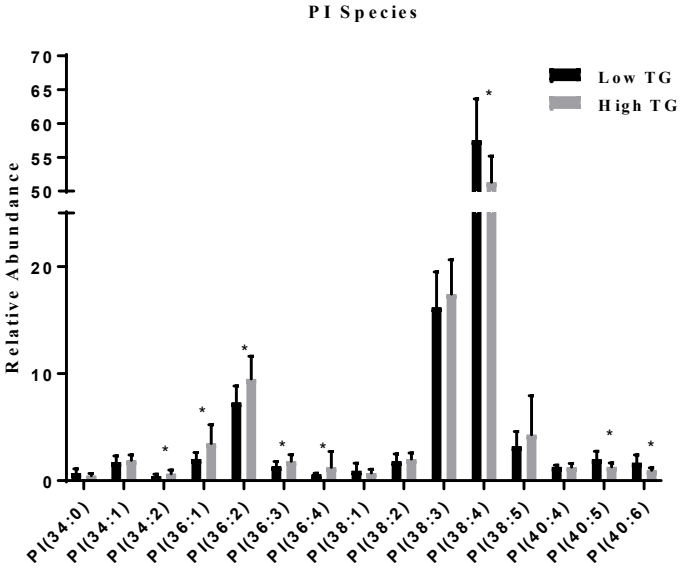
	All patients (n=34)	Low TG (n=11)	Mid TG (n=12)	High TG (n=11)	p-value[a]
Age (years), mean±SD	64.3±11.0	63.9±12.1	60.0±12.1	69.4±6.5	NS
Sex (M/F)	24/10	10/1	10/2	4/7	0.01
Tumor type, % (n)					
Colorectal	41 (14)	18 (2)	42 (5)	63 (7)	NS
Pancreatic	23 (8)	45 (5)	16 (2)	9 (1)	NS
Other Gastro-intestinal	29 (10)	27 (3)	42 (5)	18 (2)	NS
Other	6 (2)	9 (1)	0 (0)	9 (1)	NS
Presence of metastasis, % (n)	58 (20)	36 (4)	58 (7)	82 (9)	NS
BMI (kg/m2), mean±SD	27.5±6.9	26.3±5.2	26.8±6.0	29.5±9.3	NS
CT-image measures at L3, mean±SD					
Skeletal Muscle Index (cm^2/m^2)	46.1±8.9	48.7±6.3	46.8±10.7	42.8±8.9	NS
Muscle radiodensity (HU)	30.2±9.4	36.3±7.2	29.1±9.2	24.9±8.7	0.004
SAT (cm^2)	208.4±139.7	138.6±56.6	216.6±132.9	270.6±178.1	0.03
VAT(cm^2)	177.7±89.0	179.2±116.2	164.5±87.0	188.0±63.3	NS
IMAT (cm^2)	10.6±9.4	6.9±5.6	8.2±4.2	16.5±12.8	0.005
Triglyceride content (µg/mg), mean±SD	27.1±22.4	9.7±2.8	18.4±3.5	53.9±20.5	<0.001
Comorbidities, % (n)	56 (19)	45 (5)	66 (8)	63 (7)	NS
Diabetes type II	29 (10)	27 (3)	25 (3)	36 (4)	NS
Arthritis	3 (1)	9 (1)	0 (0)	0 (0)	NS
CVD	35 (12)	18 (2)	50 (6)	36 (4)	NS
Dyslipidemia	26 (9)	36 (4)	25 (3)	18 (2)	NS
Smoking habit, % (n)	41 (14)	36 (4)	50 (6)	36 (4)	NS

[a] Comparing patients with high TG content and low TG content in the muscle. (Mann-Whitney test for continuous data and Fisher's exact test for categorical data, Significance level $p<0.05$)

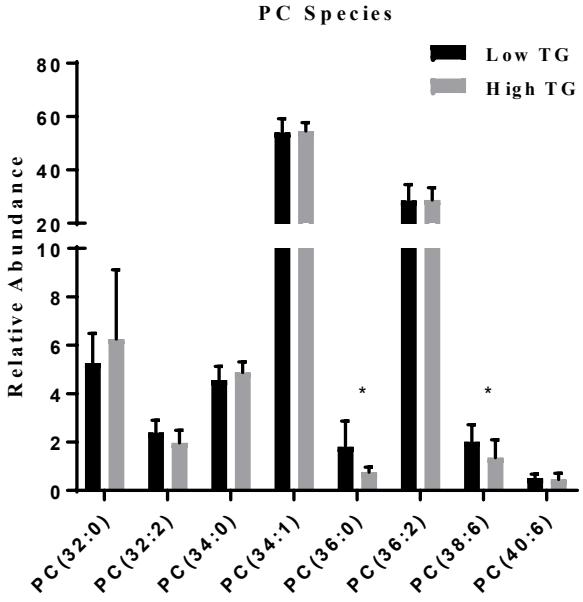
A


PE Species
Low TG
High TG
Relative Abundance
80
70
60
20
10
0
PE(32:0)
PE(34:0)
PE(34:1)
PE(36:0)
PE(36:2)
PE(38:6)
PE(40:6)
PE(40:7)

B


PI Species
Low TG
High TG
Relative Abundance
70
65
60
55
50
20
10
0
PI(34:0)
PI(34:1)
PI(34:2)
PI(36:1)
PI(36:2)
PI(36:3)
PI(36:4)
PI(38:1)
PI(38:2)
PI(38:3)
PI(38:4)
PI(38:5)
PI(40:4)
PI(40:5)
PI(40:6)

C


PC Species
Low TG
High TG
Relative Abundance
80
60
40
20
10
8
6
4
2
0
*
*
PC(32:0)
PC(32:2)
PC(34:0)
PC(34:1)
PC(36:0)
PC(36:2)
PC(38:6)
PC(40:6)

D

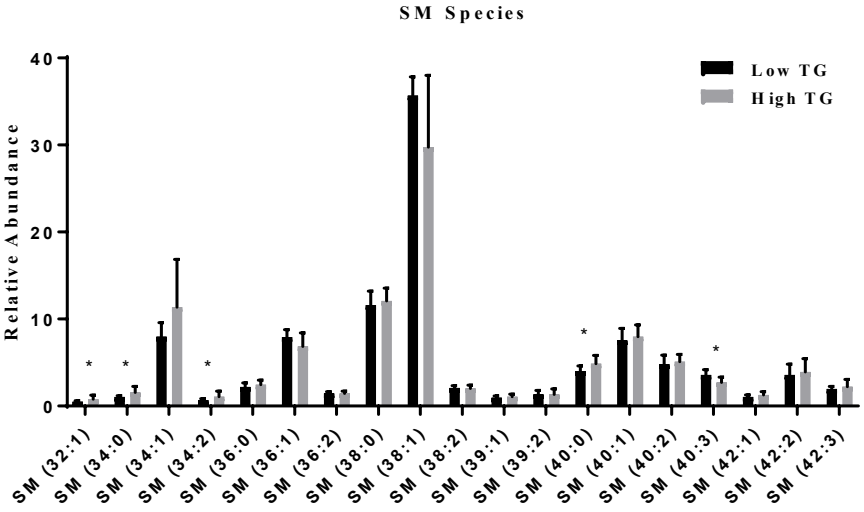
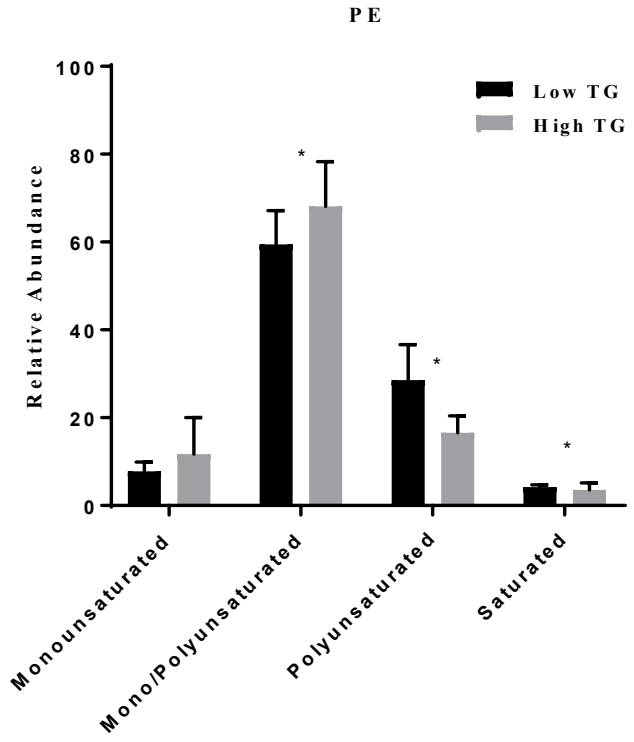
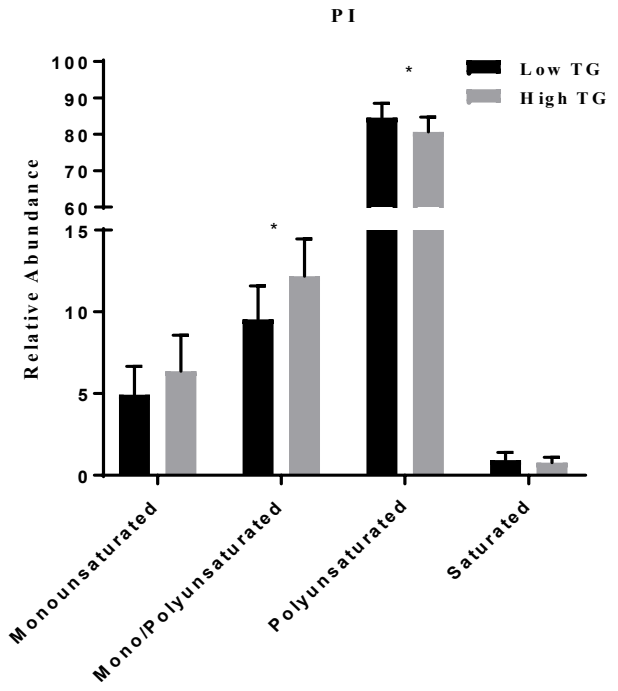

SM Species
Low TG
High TG
Relative Abundance
40
30
20
10
0
*
*
*
*
*
SM (32:1)
SM (34:0)
SM (34:1)
SM (34:2)
SM (36:0)
SM (36:1)
SM (36:2)
SM (38:0)
SM (38:1)
SM (38:2)
SM (39:1)
SM (39:2)
SM (40:0)
SM (40:1)
SM (40:2)
SM (40:3)
SM (42:1)
SM (42:2)
SM (42:3)

Figure 1 – PE (A), PI (B), PC (C) and SM (D) species were analyzed by LC-MS. Relative abundance are expressed as the percent of total PE, PI, PC or SM peak areas. Data represent mean±SD.

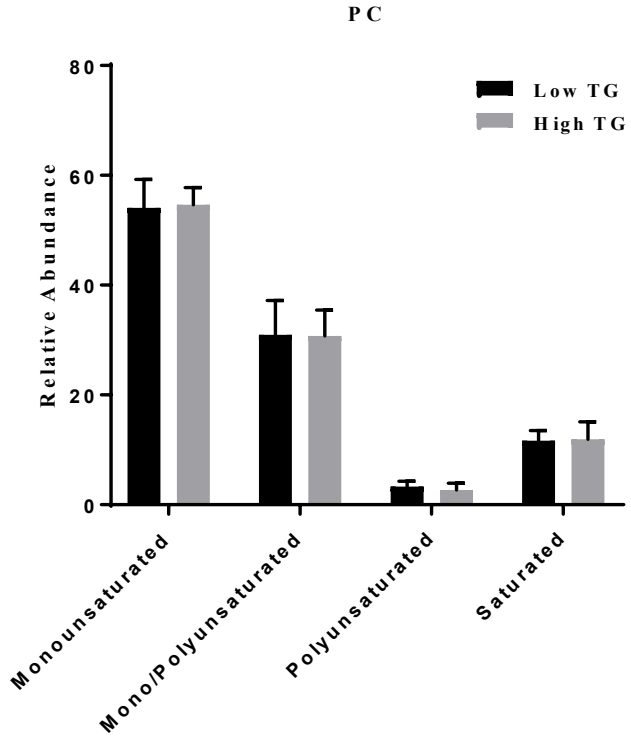
A



B


PI
Low TG
High TG
Relative Abundance
100
90
80
70
60
15
10
5
0
*
*
Monounsaturated
Mono/Polyunsaturated
Polyunsaturated
Saturated

C


PC
Low TG
High TG
Relative Abundance
80
60
40
20
0
Monounsaturated
Mono/Polyunsaturated
Polyunsaturated
Saturated

D

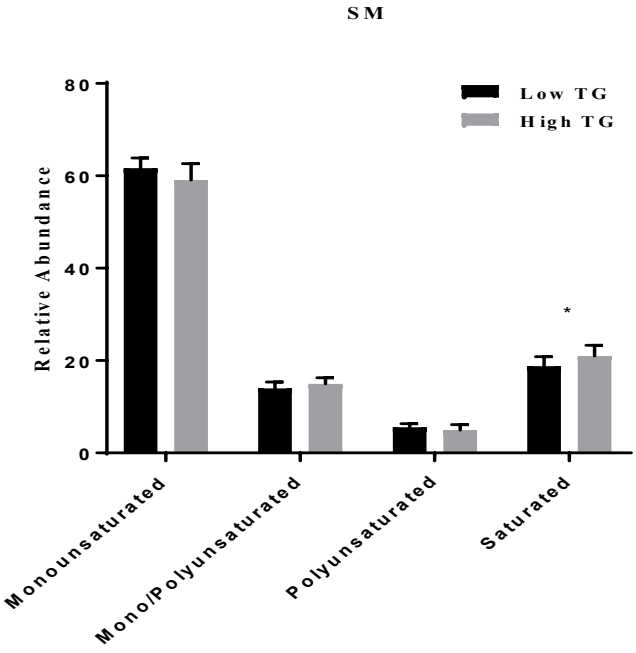


Figure 2 Muscle with high amount of TG are more abundant in mono/polyunsaturated fatty acid species and less abundant in PUFA containing PE and PI. A) PE, B) PI, C) PC and D) SM. Analysed by LC/MS. Total monounsaturated, mono/polyunsaturated, polyunsaturated and saturated species were calculated by adding all species with one, two, more than two and no double bonds, respectively. Data is represented as mean±SD.

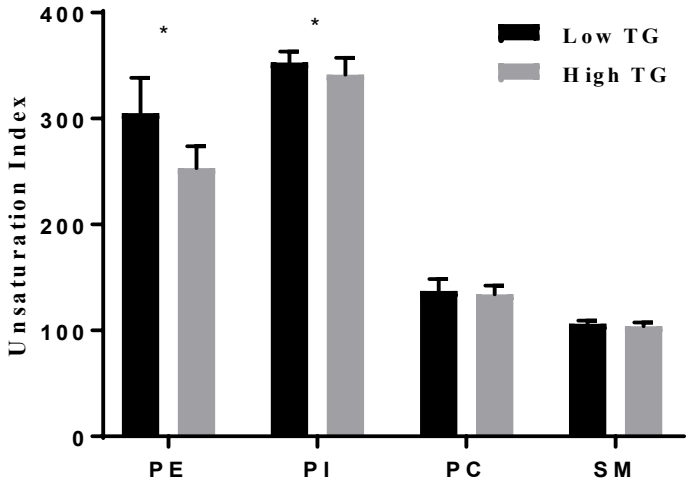
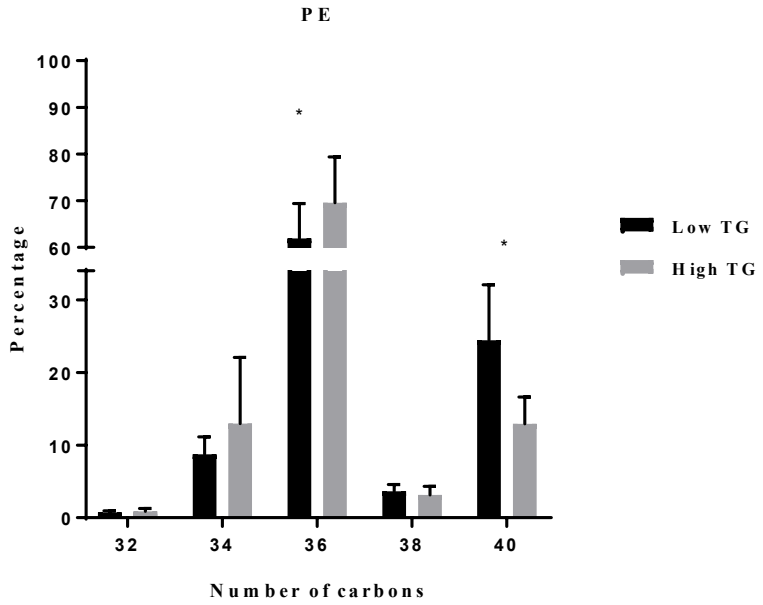
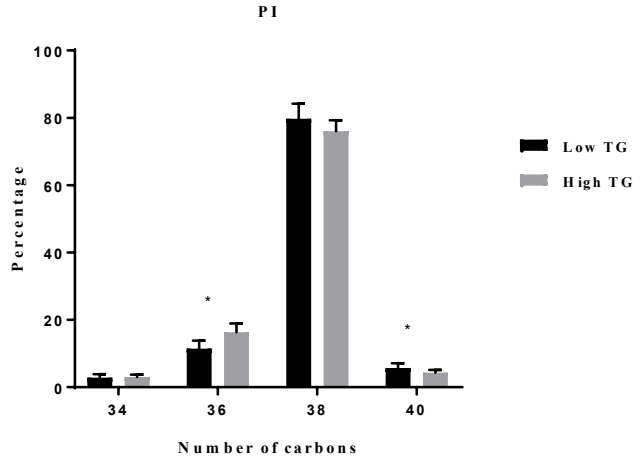


Figure 3– Unsaturation index of PE and PI were lower in high TG group. Unsaturation index (UI) was calculated by formula UI = ∑(number of double bonds) x (relative abundance) for each species in a specific phospholipid class.

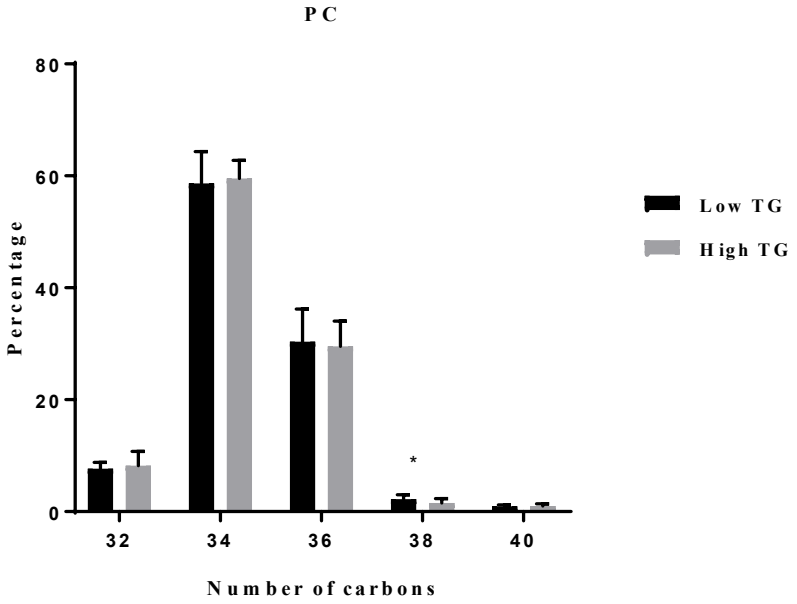
A


PE
100
90
80
70
60
30
20
10
0
Percentage
*
*
32
34
36
38
40
Number of carbons
Low TG
High TG

B


PI
100
80
60
40
20
0
Percentage
*
*
34
36
38
40
Number of carbons
Low TG
High TG

C


PC
80
60
40
20
0
Percentage
*
32
34
36
38
40
Number of carbons
Low TG
High TG

D

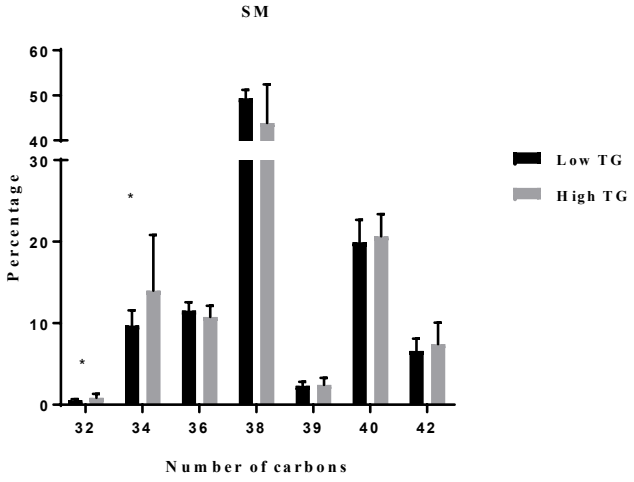

SM
60
50
40
30
20
10
0
Percentage
*
*
32
34
36
38
39
40
42
Number of carbons
Low TG
High TG

Figure 4 – Differences in fatty acid chain lengths of phospholipids when comparing high TG content muscle with low TG content. In PE (A) and PI (B), species with 36 carbon chain length were higher and species with 40 carbon chain length were lower in high TG group. In PC (C), species with 38 carbon chain length were lower in high TG group. In SM (D), Species with 32 and 34 carbon chain lengths were higher in high TG group.

Table 2 – Fatty acids with 4, 5 and 6 double bonds were less abundant in muscle with high TG content. The only exception was when paired with a monounsaturated fatty acid. All PL species containing monounsaturated fatty acid, regardless of the accompanying fatty acid were more abundant in muscle with high TG content.

	Low TG Mean (%)±SD	High TG Mean (%)±SD	p value
PE (40:6)			
20:1/20:5	2.5±1.9	9.2±11.4	0.01
18:0/22:6	81.6±6.1	66.9±17.0	<0.01
PI (36:2)			
18:1/18:1	16.6±5.4	31.5±22.3	<0.01
18:0/18:2	83.4±5.4	68.5±22.3	<0.01
PI (36:3)			
18:1/18:2	77.5±9.4	85.5±6.8	<0.05
18:0/18:3	15.1±9.9	8.2±4.6	<0.05
PI (36:4)			
18:1/18:3	7.6±6.7	13.4±9.3	<0.05
PI (38:4)			
18:1/20:3	1.5±0.5	5.6±9.8	<0.01
18:0/20:4	98.3±0.6	92.1±16.3	<0.01
PI (40:5)			
18:1/22:4	8.3±5.1	17.1±14.7	<0.05
20:1/20:4	1.3±1.2	3.4±3.3	<0.05
18:0/22:5	87.5±7.9	71.2±22.3	0.01
PI (40:6)			
18:0/22:6	83.4±9.7	72.5±18.0	<0.05

Bold text in the table denotes species that are less abundant in muscle with high TG content

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Appendix G: Potential role of omega-3 fatty acids on the myogenic program of satellite cells

Introduction

Skeletal muscles comprise 40–50% of the total body mass in an adult human and include a broad range of muscle types. Skeletal muscle mass and muscle fiber size change in response to physiological and pathological conditions. Muscle loss occurs as a normal process in aging, and is also characteristic of catabolic hormonal stimulation or disease, such as cancer cachexia, diabetes, renal failure, denervation, motor neuron disease and heart failure. Skeletal muscle depletion is associated with reduced muscle function and performance[1], decreased quality of life and shorter length of survival in older adults and in people with cancer.[2-4] Poor clinical outcomes associated with muscle loss present compelling reasons to develop strategies to reverse or slow muscle loss. Development of effective therapies requires an understanding of contributing mechanisms and factors required to reverse muscle loss. An emerging area of research is the potential dysregulation of myogenic stem cells as contributors to muscle loss.

Importance of nutritional status in regulating muscle protein synthesis and muscle mass is well accepted but limited literature exists regarding the role of nutrients specifically on satellite cells.[5] Dietary protein and amino acids of sufficient quality and quantity are key nutrients required for muscle health. Recent studies have provided evidence that protein supplementation combined with exercise may accelerate satellite cell proliferation.[6-8] Within the last decade, nutritional compounds such as resveratrol, epigallocatechin gallate (catechins in green tea), β-hydroxy-β-methylbutyrate (HMB, leucine metabolite) have been reported to improve satellite cell proliferation, especially in fast muscles in experimental models.[9-11] It is suggested that resveratrol and epigallocatechin gallate buffer high levels of reactive oxygen species and reduce oxidative stress in aging muscle, thus favoring satellite

cell differentiation and proliferation.[12] HMB reduces protein catabolism and muscle loss during disease and/or disuse.[13]

Polyunsaturated omega-3 fatty acids (n-3 PUFA) are a family of essential fatty acids with many biological activities. There are three major dietary n-3 fatty acids: α-linolenic acid (ALA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). Omega-3 fatty acids comprise cell membrane phospholipids, contributing structural and functional characteristics by altering signaling platforms for membrane proteins and lipids.[14-17] In obese adolescents, supplementation with omega-3 fatty acids increased proportion of EPA and DHA in the muscle while improving glucose tolerance and insulin sensitivity.[18] Providing EPA and DHA in the form of fish oils in human diets has been reported to enhance anabolic potential and reduce muscle loss (reviewed by Ewaschuk).[19] While several mechanisms may be contributing to disease associated muscle atrophy, little is known about impact of omega-3 fatty acids on differentiation and proliferation of satellite cells. This paper reviews studies examining the effect of ALA, EPA and DHA on myogenic regulation of satellite cell proliferation and differentiation. Possible mechanisms by which omega-3 fatty acids modulate the myogenic lineage of satellite cells are also explored. Currently, there are no studies exploring these questions in humans therefore the emphasis is on studies performed in experimental systems.

Role of satellite cells (SC) in myogenesis

Satellite cells are a heterogeneous collection of quiescent muscle stem cells that reside within adult myofibers, between the basement membrane and sarcolemma.[20] Normally, in adult muscles, satellite cells are in a quiescent state and express Pax7.[9,21,22] These mesodermal-derived multipotent stem cells are capable of self-renewing proliferation, and are responsible for muscle growth and

regeneration. In response to muscle damage, satellite cells are activated to proliferate, differentiate and fuse with existing muscle fibers. Pax7-positive daughter cells either differentiate by migrating through the sarcolemma and fusing with existing muscle fibers during the growth of existing muscle fiber or have the capacity to fully regenerate new myotubes (Figure 1).[23,24] Commitment of satellite cells to a myogenic lineage is indicated by expression of specific myogenic transcriptional regulatory factors (MRFs), which include but are not limited to myogenic differentiation 1 protein (MyoD), myogenic factor 5 (Myf5) and myogenic regulatory factor 4 (MRF4).[25] On activation, committed satellite cells upregulate MyoD and enter the cell cycle to proliferate as myoblasts and differentiate by downregulating Pax7 and upregulating myogenin and MRF4 (Figure 1).[26] Thus the expression of Pax7, MyoD and myogenin identifies whether satellite cells are in a quiescent or committed state ($Pax7^+/MyoD^-/myogenin^-$), proliferation state ($Pax7^+/MyoD^+/myogenin^-$) or differentiation state ($Pax7^-/MyoD^+/myogenin^+$).[27] Measuring three MRFs concurrently enables status of satellite cells as activated, proliferating or differentiating to be determined.

Myogenic transcriptional regulatory factor expression is also affected by extracellular matrix molecules such as heparan sulfate proteoglycans (HSPG). Syndecan-4 and glypican-1 are most studied HSPGs in relation to myogenesis. Syndecan-4 is required for activation and proliferation of satellite cells and regulates MyoD and MRF4 expression.[28] Syndecan-4 also serves as a marker for quiescent, activated and subset of satellite cells.[29] In contrast, knockdown of glypican-1 inhibited mouse muscle cell differentiation and delayed or decreased myogenin expression.[30] Glypican-1 plays a primary role in satellite cell differentiation by sequestering FGF2 (fibroblast growth factor), a potent inhibitor of differentiation.[30,31] These data suggest a regulatory role of syndecan-4 and glypican-1 in satellite cell function and expression of transcriptional regulatory factors during satellite cell myogenesis.

Effect of omega-3 fatty acids on satellite cell function

Low plasma levels of EPA and DHA have been associated with skeletal muscle depletion and impaired muscle function in older adults and in people with type-2 diabetes, obesity and cancer.[32-34] A cross-sectional and retrospective cohort study reported an increase in grip strength of men and women with each additional portion of fatty fish per week, independent of adult height and age.[35] Low level of plasma omega-3 fatty acids was related to a decline in physical performance in middle-aged and older adults in the large InCHIANTI cohort study.[36] Supplementation with EPA has been reported to enhance muscle protein synthesis, increase muscle mass and improve function in elderly individuals.[37] Human studies have reported a relationship between EPA and DHA supplementation and the preservation or gain of muscle mass in patients with cancer, who would otherwise experience muscle loss.[38,39] EPA and DHA can reduce contributing factors, such as acute phase response, pro-inflammatory cytokines, and insulin resistance, which mediate muscle loss.[40] Few studies have examined the effect of EPA and DHA on myogenic potential of satellite cells. The studies that do exist have been performed in a variety of experimental models (Table 1).

a) Omega-3 fatty acids can affect inflammatory pathways

Inflammation plays a significant role in muscle damage and loss. Inflammatory processes that disrupt muscle homeostasis and promote injury have been an area of intense research. Cellular mediators, such as interlukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) are key regulators of skeletal muscle cell responses to injury. Disruption of TGF-β by introducing the dominant-negative mutant receptor (i.e., truncated type II TGF-β receptor transfected into C2C12 cells) results in inhibition of myogenic differentiation and suppression of MyoD expression.[41] Low physiological concentrations of TNF-α appear to activate myogenesis, whereas sustained high levels of TNF-α in chronic inflammatory disease have been associated with impaired myogenic differentiation.[42] TNF-α induces muscle loss by inhibiting myogenic

differentiation and by stimulating apoptosis of differentiated myotubes.[43,44] Also, TNF-α is associated with increased activation of NF-κB, proinflammatory signaling pathway, and high expression of atrogenes such as Atrogin-1 and MuRF-1 leading to protein degradation.[45] In response to various stimuli, immune cells secrete lipid mediators, prostaglandins (PGs) and leukotrienes (LTs) that may influence myogenesis and are potent mediators of pain and inflammation.[46] Resolvins, maresins and protectins are eicosanoids derived from EPA and DHA that act as anti-inflammatory agents, however their impact on myogenic progenitors requires further investigation.[47]

C2C12 is a well-established murine skeletal muscle cell line extensively used in satellite cell research. It is a subclone of the murine myoblast cell line established by Yaffe and Saxel (1977) and produced by Blau et al. (1985).[48,49] C2C12 myoblasts exhibit the majority of features and proteins as satellite cell-derived myoblasts.[50,51] C2C12 has been extensively used as a model for skeletal muscle proliferation, differentiation, cell-based therapies, and other research related to muscle development. The effects of TNF-α and EPA treatment on skeletal muscle cells during differentiation from myoblasts to myotubes has been studied in C2C12 cells (Table 1).[52] C2C12 cells were differentiated in the presence or absence of TNF-α (20 ng/ml) with or without EPA (50μM). EPA was either added concurrent with TNF-α as a co-treatment or as pre-treatment (2 hour) after which EPA was withdrawn and replaced by TNF-α alone in Dulbecco's modified eagle medium (DMEM). The inhibitory effect of TNF-α on myotube differentiation and modified pattern of myosin heavy chain expression (MyHC) was prevented by both pre- and co-treatment with EPA. TNF-α treatment significantly reduced myotube size, myoblast fusion index and induced cellular necrosis compared to respective untreated controls. These deleterious effects of TNF-α were inhibited by EPA treatment.[52] In addition, activation of apoptosis by caspase-8 induced by TNF-α was completely blocked by EPA co-treatment.[52] Pax7 and MyoD were not measured in the study, limiting the understanding of how EPA affects the

myogenic program. Other studies have reported TNF-α to enhance proliferation and aggregation of myoblast cells while inhibiting chief regulatory molecules of myogenic differentiation, MyoD and myogenin in C2C12 myoblasts (Figure 2).[53,54] A subsequent study evaluated the effects of EPA treatment on TNF-α-induced NF-κB activation in the C2C12 myoblast line (Table 1).[55] They set out to determine whether EPA anti-inflammatory activity was dependent on altered expression and activation of peroxisome proliferator-activated receptors (PPAR).[55] Impairment of skeletal muscle cell differentiation induced by TNF-α was associated with increased NF-kB transcriptional activity as well as inhibition of PPAR-γ expression. On the other hand, EPA co-treatment or pre-treatment was associated with inhibition of NF-kB and up regulation of PPAR-γ expression. IL-6 expression was also significantly inhibited when EPA was administered either as a co-treatment or pre-treatment with TNF-α. The inhibitory effect on NF-kB and IL-6 was specific to EPA and DHA as disrupted morphology was observed in control cell lines with omega-6 and omega-9 fatty acids. Experimental and human studies have suggested that fish oil derived omega-3 fatty acids exhibit an anti-inflammatory effect by inhibiting activation of NK-κB either by decreasing phosphorylation of its inhibitory subunit, IκB, or by binding to PPAR-γ, which in turn interacts with NF-κB to prevent translocation to the nucleus (Figure 2).[17] A recent study explored the long term (5 months) effect of omega-3 fatty acid supplementation on muscle regeneration and inflammation in the mdx mouse model of Duchenne muscular dystrophy (DMD; Table 1).[56] Mdx mice receiving omega-3 (60mg/kg) capsule exhibited increase in muscle regeneration and higher expression of MyoD as compared to control group. Long term omega-3 supplementation resulted in lower expression of inflammatory markers, NF-κB and TNF-α. Measures of Pax7, MyoD and myogenin protein expression in this model would determine which phase of myogenic program of satellite cells is most affected by omega-3 fatty acids.

There are three members of peroxisome proliferator-activated receptor (PPAR) γ coactivator 1 family, namely PGC-1α, PGC-1β and PGC-1 related coactivator (PRC). PGC-1α is a transcriptional coactivator that is a central inducer of mitochondrial biogenesis in cells.[57] Results from several studies suggest that PGC-1α targets the NF-κB pathway and favors an anti-inflammatory environment in skeletal muscle (Figure 2).[57] Muscle loss in pathological conditions such as diabetes, cancer, obesity, uremia and denervation induced atrophy, has been associated with low levels of PGC-1α and/or PGC-1β and high levels of TNF-α and IL-1β.[58-62] High PGC-1α levels could prevent the progression of muscle wasting by suppressing atrogenes *in vitro* and *in vivo*.[60,61,63] A recent study revealed a positive relationship between PGC-1α expression and myogenic differentiation. Transcriptional factors critical for differentiation of skeletal muscle cells, MyoD and myogenin, were enhanced by PGC-1α in C2C12 cells.[64] DHA and EPA treatment for 24 and 48 hours was reported to induce PGC-1α expression in human rhabdomyosarcoma cells.[65] In another study, DHA treatment maintained myotube morphology, diameter and intramyocellular lipid content similar to control levels and retained PGC1α near control levels.[66] Also, DHA treatment caused myotube hypertrophy and showed a protective effect against palmitate-induced muscle atrophy.[66] High concentration of palmitate has been associated with insulin resistance and contributes to muscle atrophy.[67] Peng et al reported that C2C12 myoblast proliferation was significantly reduced by EPA and DHA in a concentration dependent manner whereas ALA did not show any inhibitory effect (Table 1).[68]

b) Omega-3 fatty acids affect glucocorticoid induced muscle degradation

Glucocorticoids are released as a component of the stress response and evoke catabolism by increasing protein degradation and decreasing protein synthesis.[69] Synthetic glucocorticoids, such as dexamethasone, are used to model muscle atrophy. The effects of dietary EPA and DHA on dexamethasone-induced muscle atrophy was evaluated by measuring expression of genes involved in

protein synthesis, degradation, myogenic proliferation and differentiation (MyoD, myogenin, atrogin-1 and MuRF-1) concurrent with histological analysis in rat muscles (Table 1).[70] Four experimental groups consisted of control, dexamethasone alone, omega-3 supplement alone and dexamethasone group with an omega-3 supplement (EPA 180mg and DHA 120mg capsules). The group supplemented with omega-3 showed higher levels of MyoD compared to controls that were not sustained when dexamethasone was added. In contrast, myogenin levels were lower in both omega-3 supplemented and dexamethasone plus omega-3 supplemented groups compared to controls. The dexamethasone group with omega-3 supplementation showed higher activation of atrogenes such as Atrogin-1 and MuRF-1 indicating increased protein degradation and decreased protein synthesis. Ex vivo fatty acid analysis of muscle showed incorporation of omega-3 fatty acids however, DHA and EPA content of muscle were not reported for any experimental group. Diets provided in this study were not matched for calories and no data regarding food intake was presented, limiting the ability to interpret the results from this study.[70] In a model of arthritis-induced muscle atrophy, dietary EPA decreased proteolysis as observed by lower gene expression of TNF-α and atrogin-1 (Table 1).[71] Arthritis increased gene and protein expression of MyoD and myogenin and EPA administration did not modify this effect. No change in expression of these proteins was seen in gastrocnemius of pair-fed and control rats (Figure 2).

c) Omega-3 fatty acids induces myogenesis in cancer induced muscle atrophy

Muscle wasting is an important component of pathophysiology of cancer. A recent study evaluated the effect of EPA and endurance exercise training on muscle depletion in lung carcinoma-bearing mice (Table 1).[72] Control (n=15) and tumor bearing (n=24) mice were randomized into three groups (n=5 for controls and n=8 for tumor bearing) untreated, treated with EPA or treated with EPA and subjected to treadmill running. All groups had free access to food and water during the

experimental period. EPA-treated groups received daily administration of 0.5 g/kg EPA in corn oil starting from the fourth day of tumor growth and untreated groups received corn oil alone. Tumor-bearing mice were killed 14 days after tumor injection. EPA did not prevent muscle wasting when administered alone but was able to improve both muscle mass and strength when coupled with exercise. Untreated tumor bearing mice showed higher Pax7 protein expression whereas endurance exercise combined with EPA had lower levels of Pax7. A single measure of Pax7 does not enable interpretation with regard to alterations in myogenic proliferation or differentiation potential as Pax7 is expressed by satellite cells at different stages within the myogenic program. Measuring other myogenic transcriptional regulatory factors, MyoD and myogenin, would determine the step of satellite cell derived myogenesis affected by treatment. Food intake was partially corrected in the group receiving EPA plus exercise and no pair-fed group was available for comparison. No effect of EPA alone was observed, potentially because the dose of EPA (0.5g/kg) administered might not be sufficient to see desired effects. When a 1g/kg dose was applied, a five fold increase in ratio of protein synthesis to protein degradation was reported in gastrocnemius muscle of cachectic mice bearing the MAC16 tumor.[73]

d) Effect of omega-3 fatty acids on proteoglycans needed for myogenesis

A spectrum of polyunsaturated fatty acids on proliferation and differentiation, as well as on expression of syndecan-4 and glypican-1 in avian myogenic satellite cells has been recently studied (Table 1).[74] Satellite cells were isolated from the pectoralis major and biceps femoris muscles of 13 week old turkeys and 5 week old chickens and cultivated with linoleic acid, α-linolenic acid, arachidonic acid, DHA or EPA. Decreases in proliferation and differentiation were reported in turkey cells treated with EPA and DHA. Evaluation of cell morphology suggested a toxic effect of EPA and DHA on turkey satellite cells. However, proliferation of chicken satellite cells was not depressed by

EPA and DHA and microscopic examination revealed that chicken cells receiving these treatments maintained normal morphology. The difference in response of satellite cells from turkeys and chickens to EPA and DHA may be attributed to differences in muscle membrane lipid composition in turkeys and chickens.[74] After treating with spectrum of fatty acids, glypican-1 and syndecan-4 gene expression was measured in proliferating and differentiating satellite cells from turkey and chicken muscles. Expression of glypican-1 and syndecan-4 was increased during proliferation in all treatments compared to control serum in turkey and chicken satellite cells. Both glypican-1 and syndecan-4 differentially regulate expression of myogenic regulatory transcriptional factors during proliferation and differentiation of satellite cells. Knockdown of glypican-1 in turkey satellite cells has been associated with decreased gene expression of MyoD and myogenin, whereas knockdown of syndecan-4 has been associated primarily with increased MyoD and MRF4 expression during proliferation and differentiation (Figure 2).[75] Single fatty acids as opposed to physiological fatty acid mixtures are likely to evoke different metabolic effects.[76]

e) Incorporation of omega-3 fatty acids into membrane lipids can affect muscle atrophy in dystrophic muscle

In dystrophic hamsters, the effect of flaxseed-enriched diet on myocyte membrane composition, conformation and intracellular signaling has been investigated (Table 1).[77] The hamster model of dystrophy (UM-X7.1, Syrian hamster) used for this study is a model for human limb-girdle muscular dystrophy. The experiment tested four groups in total, the first and second group (n=50 each) were fed either chow diet or flaxseed enriched diet from weaning to death. To determine if ALA reverses well established muscular dystrophy, a third group of dystrophic hamsters (n=30) were fed standard chow from weaning to 100 days (until development of dystrophy) followed by a flaxseed rich diet for next 50 days before death. Healthy animals were fed only chow and used as a control. The

histological analysis of muscles from dystrophic hamsters fed flaxseed enriched diet showed preservation of the typical skeletal muscle morphology compared to those fed the control diet. Significant reduction of Pax7 expression was evident whereas myogenin was highly expressed in ALA fed versus control, suggesting satellite cell differentiation. However, no effect was seen in those fed chow diet for 100 days followed by ALA for 50 days, suggesting an inability of ALA to reverse established injury. Biochemical analysis revealed that ALA and EPA levels in muscles from ALA fed hamsters were almost 2.5 fold higher than controls. Cytoplasmic accumulation of membrane proteins, caveolin-3 and β-catenin, involved in cell adhesion, membrane repair and plasma membrane integrity, were observed in the muscle from chow fed dystrophic hamster whereas normal morphological sarcolemmal pattern of these proteins was maintained in the muscle dystrophic hamsters fed ALA. Diets (chow and ALA rich) used in study were not isocaloric and their macronutrient composition was not reported. Also, the amounts of omega-6 and omega-3 in diet were not reported. The study may have produced different results if a higher amount of ALA was provided in the treatment arm. Providing longer chain fatty acids (i.e., DHA, EPA, and DPA) derived from ALA may have produced different effects.[78] An animal study showed that it is the provision of DHA not the ratio of n-3/n-6 that is critical for skeletal muscle membrane change.[79] Also, supplementation with EPA but not DHA partially improves skeletal muscle oxidative capacity.[80]

Limitations of the studies reviewed

Findings based on cell lines using omega-3 fatty acid treatment or supplementation may not necessarily correspond with physiologic responses *in vivo*. Although C2C12 cells express proteins necessary for myogenic differentiation and display the morphology of individual fiber units, there are striking differences between these cells and human adult muscle, particularly in their degree of maturation and mode of glucose transport.[81,82] Nevertheless, this *in vitro* model is widely used to study

the role of ALA, EPA and DHA in activation, proliferation and differentiation of satellite cells. In reviewed studies using C2C12 cell lines, the effect of omega-3 fatty acids on myogenic regulatory factors (i.e., Pax7, MyoD, myf5 and myogenin) has not been reported. These transcriptional factors are expressed in distinct expression patterns in fusing cells and influence skeletal muscle development.[83] Also, studies demonstrate an inconsistent effect of ALA, EPA and DHA on myoblast proliferation and myotube morphology.[52,66,68] Bovine serum, commonly used in cell culture experiment, is an ill-defined mixture of components in culture media of varied fatty acid composition from batch to batch.[84,85] The fatty acid composition of medium can significantly influence the fatty acid composition of cell lines and potentially alter actions of satellite cells.[86] Chemically designed, serum-free media can be used to ensure consistency of experimental conditions and results.

Animal models are an invaluable component of biomedical research and many factors, like genetic background and environmental factors that can introduce variability, need to be considered.[87] However, most studies overlook the factors of dietary design, diet composition and intake.[9,88,89] Vellaman (2010) reported an association between feed restriction and MyoD/myogenin expression of broiler pectoralis muscle, and decrease in satellite cell proliferation and differentiation have been observed in methionine and cysteine restricted cell culture medium.[90,91] Of the studies reviewed, two studies used "standard chow diet", and another used standard commercial diet (Nuvilab CR1) each of which has undefined macronutrient composition.[70,71,77] The treatment groups were not exposed to isocaloric and isonitrogenous diets, which introduces variation in results as measures of muscle anabolism are highly responsive to anabolic effect of protein and insulin response. Individual omega-3 fatty acids have different therapeutically potential, so it is important to know fat composition of diets used in study as dietary fat alters fatty acid composition of membrane which in turn can effect membrane and cell function.[14-16,92,93] Of the studies reviewed, only two studies determined fatty acid

composition of muscle to confirm incorporation of omega-3 fatty acids in cell membranes after treatment or supplementation, however, in one study DHA and EPA fatty acid levels were not presented.[70,77] In addition to fat composition of diet, dosage of EPA and DHA should also be considered to determine if they are dietary achievable or therapeutic. Most studies that showed a systemic effect of EPA and DHA used intakes of >0.03g/kg.[94] All animal studies reviewed employed higher dosages of EPA and DHA (0.06-1g/kg) but wide variation in dosages makes it difficult to compare between studies. Studies should employ pair-fed controls to determine the extent to which the effect of a treatment on muscle wasting occurred independently of changes of energy intake and differentiate reduced feeding from other causes of muscle loss. Use of undefined or ill-matched dietary designs limits comparison between studies of results within the same study, translation across different study designs, as well as application to clinical studies.

While our understanding of murine satellite cell biology is rapidly expanding, little is known about alterations in activation, proliferation and differentiation of satellite cells in relation to various nutrient interventions. There are few studies in C2C12 cells and five animal studies evaluating effect of omega-3 fatty acids on myogenic program. In contrast, there is no study exploring effects of omega-3 fatty acids on human satellite cells. This is primarily because of difficulty in obtaining human muscle biopsy, particularly from vulnerable populations like older adults and those with existing musculoskeletal disorders. Additionally, there are challenges to isolate satellite cells, like scarcity, location under basal lamina of muscle fibers and separation from other cells within skeletal muscle. However, studies can be designed using commercially available human cell lines. There is need to conduct experiments using human satellite cells to explore effects of omega-3 as it is difficult to determine if phenotype and functions of human and murine satellite cells are equivalent.

Future perspectives

Omega-3 fatty acids show an inconsistent effect on satellite cell proliferation, differentiation and muscle regeneration in experimental systems. Future studies should attend to dietary design (i.e., isocaloric, isonitrogenous and similar fatty acid composition) and food intake in animal studies need to be recorded to control dietary intake as a variable in these studies. There are several inherent limitations of the experimental studies exploring this question to date and few studies have shown mechanisms by which omega 3 fatty acids induce their anabolic effect. There is a need to clarify the nature and mechanisms by which omega-3 fatty acid effect myogenic potential of satellite cells. In C2C12 cell line studies, the effect of omega-3 fatty acids on myogenic regulatory factors (e.g., Pax7, MyoD, myogenin and myf5) need to be explored and at least three need to be measured to determine which part of myogenic program is being affected. Also, it is important to consider differences between human satellite cells and experimental models. Effect of omega-3 fatty acid on human satellite cells has not been studied directly because of practical and methodological issues. However, to use omega 3 fatty acids as potential therapeutic agent to prevent or treat muscle loss in chronic disease population it is important to design clinical studies and investigate effects of these essential fatty acids on myogenic program of human satellite cells.

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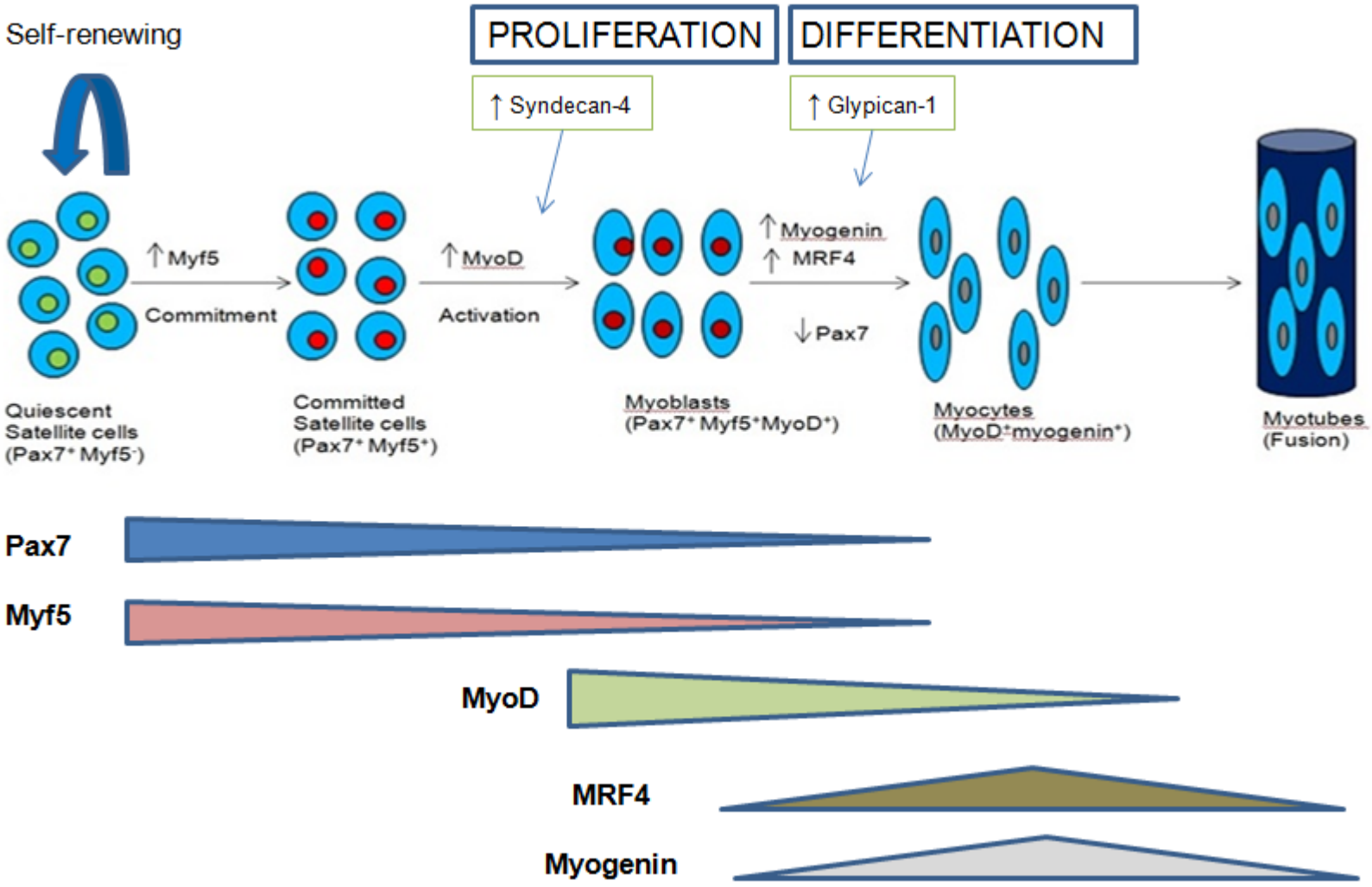
Reference	Objective	Experimental Model	Medium or diet used	Parameters measured	Muscle fatty acid comp.	Treatment (dose)	Outcomes
CELL CULTURE STUDIES							
Magee et al,[52] 2004	To evaluate the responses of differentiation to TNF-α and EPA treatments.	C2C12 cell line	DMEM with fetal bovine serum	Expression of MyHC, myotube size and myoblast fusion index. Caspase-8 activity	-	EPA (50μM)	EPA blocked TNF-α induced reduction of MyHC expression and caspase-8 mediated apoptosis. Increased myotube size and myoblast fusion index
Peng et al,[68] 2012	To determine the effects of omega-3 fatty acids on the proliferation.	C2C12 cell line	DMEM with fetal bovine serum	Cell morphology, Cyclin-E, cyclin-D1 and CDK2 (progression of cell cycle)	-	ALA, DHA and EPA (50μM and 100μM)	DHA and EPA ↓ C2C12 myoblasts proliferation significantly, and the effect was concentration dependent whereas ALA did not show any inhibitory effect.
Bryner et al,[66] 2012	To determine if DHA treatment is protective against palmitate-associated muscle cell atrophy and reducing intramyocellular lipid content.	C2C12 cell line	DMEM with fetal bovine serum	Myotube morphology, PGC1α	-	DHA (100μM)	DHA maintained myotube morphology, diameter and intramyocellular lipid content. ↑ AMPK levels. Maintained PGC1α and ↑ in oxidative metabolism.
McFarland et al,[74] 2011	To examine the proliferation and differentiation responses of cultured satellite cells when administered different types of fatty acids in the media.	Turkey and broiler chicken (isolated satellite cells)	DMEM with chicken serum or horse serum	Syndecan-4, glypican-1, morphology, differentiation, proliferation	No	LA, ALA, EPA, DHA and AA (5μM)	↓ Proliferation and differentiation in turkey cells and no modifcation in chicken cells, ↑ in syndecan-4 expression during proliferation and differentiation and ↑ glypican-1 expression during satellite cell differentiation
Castillero et al,[71] 2009	To examine whether EPA administration is able to prevent an arthritis-induced decrease in body weight and muscle wasting	Arthritic rats	Standard chow	MuRF-1, atrogin-1, MyoD, myogenin and myostatin	No	EPA (1g/kg)	EPA ↓ gene expression of TNF-α, atrogin-1 and MuRF-1. No effect on MyoD and myogenin
ANIMAL STUDIES							
Fiaccavento et al,[77] 2010	To test the hypothesis that n-3 PUFAs could alleviate the dystrophic skeletal muscle damage differently modulating the myocyte membrane composition and conformation and, hence, the intracellular signaling.	Hamster with muscular dystrophy	Chow pellet/ALA enriched-flaxseeds	Pax7 and myogenin expression of satellite cells, molar percentage of EPA, DHA, AA and ALA. Membrane proteins (caveolin-3 and β-catenin)	EPA, DHA	-	↓Satellite cells expressing Pax7, ↑ in myogenin expression, ↑ molar percentage of ALA and EPA. Normal sarcolemmal pattern of caveolin-3 and β-catenin.
Penna et al,[72] 2011	To verify the ability of EPA to prevent muscle depletion in lung carcinoma-bearing mice and to test the ability of endurance exercise training to increase the EPA effect.	Lung carcinoma-bearing mice	Not given	Pax7, atrogin-1	No	EPA (0.5g/kg) and exercise	EPA alone did not prevent the muscle loss induced by tumor growth while the combination with exercise induced a partial rescue of muscle strength and mass. Association of EPA and exercise ↓ PAX-7 accumulation
Fappi et al,[70] 2014	To evaluated whether n-3 supplementation could mitigate the development of dexamethasone-induced muscle atrophy	Rats (dexamethasone induced muscle atrophy)	a standard commercial diet (Nuvilab CR1)	MuRF-1, atrogin-1, MyoD and myogenin	AA, ALA	EPA and DHA (0.1g/kg)	EPA and DHA did not prevent the decreased expression of MyoD and myogenin. Increased expression of Atrogin-1 and MuRF-1
Apolinario et al,[56] 2015	To verify the long term effect of n-3 on muscle regeneration and inflammation.	Mdx mice	Not given	MyoD, NF-κB, TNF-α	No	EPA (0.04g/kg) and DHA (0.02g/kg)	Increase in MyoD, ↓NF-κB and ↓ TNF-α protein expression

Figure legends

Figure 1 - Model for changes in expression of myogenic regulatory factors through the myogenic program. In quiescence, satellite cells reside between basal lamina and sarcolemma as satellite stem cells ($Pax7^{+}/Myf5^{-}$) and/or as committed satellite cells ($Pax7^{+}/Myf5^{+}$). On activation, committed satellite cells upregulate myoblast determination protein (MyoD). Satellite cell progeny then follow one of two fates. They enter the cell cycle to proliferate as myoblasts and differentiate by down regulating Pax7 and up regulating myogenin and MRF4. Alternatively, downregulate MyoD and self renew to give rise to $Pax7^{+}$ satellite cells. Syndecan-4 and glypican-1 are regulators of expression of myogenic regulatory factors during satellite cell proliferation and differentiation.

FIGURE-1


Self-renewing
PROLIFERATION
DIFFERENTIATION
↑ Syndecan-4
↑ Glypican-1
↑ Myf5
Commitment
↑ MyoD
Activation
↑ Myogenin
↑ MRF4
↓ Pax7
Quiescent Satellite cells (Pax7⁺ Myf5⁻)
Committed Satellite cells (Pax7⁺ Myf5⁺)
Myoblasts (Pax7⁺ Myf5⁺MyoD⁺)
Myocytes (MyoD⁺myogenin⁺)
Myotubes (Fusion)
Pax7
Myf5
MyoD
MRF4
Myogenin