BiCyCLE

Body Composition manipulation in CoLorectal cancEr

A series of studies to identify the aetiology of aberrant body composition status in cancer and possible methods of body composition manipulation

Edward Tobias Pring BSc(Hons), MB ChB, MRCS, FRCS(Gen Surg)

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&

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Declaration of Originality

The work presented within this thesis was undertaken during a Clinical Research Fellowship at St Mark's Hospital between October 2017 and April 2021.

I, Edward Tobias Pring, declare and confirm that the content of this thesis is my own original work and that this work has been undertaken within the time stated above as part of my studies for a Doctorate in Philosophy. Where assistance was provided, contributors are credited clearly within the text of this Thesis.

I have not submitted any part of this thesis in support of any other qualification at this or any other institute of higher education.

Copyright Declaration

Thesis Abstract

Host body composition (BC) is associated with outcomes in colorectal cancer (CRC). Muscle and fat appear to be crucial in both disease causation and response.

The aim of this thesis is to identify and understand the aetiology of deleterious changes in BC; explore the underlying immune dysfunction of the disease and ascertain whether we can arrest the process of muscle dysfunction in CRC.

In Chapters 2-5, using our population data, we identify relationships between tumour, environment and host. Visceral obesity (VO) is independently associated with prognostically favourable tumour characteristics whilst myosteatosis is independently associated with an aggressive tumour phenotype and is a strong a predictor of future distant recurrence. The environment and genetic background are implicated; deprivation appears a determinant of sarcopenia and BMI whilst ethnicity determines BC status and inflammatory state.

In Chapters 7-9 we identified that dendritic cell (DC) lipid status is dependent on anatomical location and is associated with BC status. Increased intracellular lipid within circulating DC is significantly associated with myosteatosis. Whilst, in response to microbial stimulation, oxidised fat content is significantly increased in DC in healthy controls compared to the CRC population. Fat scavenger receptor CD36 expression is significantly increased in the CRC DC population suggesting DC in CRC fail to respond appropriately to commensal antigens.

Finally, to ascertain if we could modulate this response through intervention, we undertook a review of the literature which informed the construction of a randomised trial to identify whether using neuromuscular muscular electrical stimulation (NMES) pre- and

post-operatively in rectal cancer patients could alter BC, reduce inflammation and improve outcome.

Host BC and the immune response to CRC is influenced by both the tumour and host's environment. Cellular targets exist which may moderate the immune response. Manipulating host BC by interventions such as NMES may prompt an anti-inflammatory response.

Table Of Contents

List of Tables

List of Figures

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Chapter 1

1 Introduction

1.1 Colorectal cancer

1.1.1 Anatomy and definition

The colon and rectum collectively form the large bowel, an approximately 1.5m long receptacle bounded proximally by the ileocecal valve and distally by the dentate line[1]. Embryologically the colon arises from both the mid gut (caecum to two third of the length of the transverse colon) and hind gut (the final third of the transverse colon to the rectum). The rectum arises from the hind gut, distal to the dentate line the anal canal arises from the proctodeum. The embryological origin determines blood and lymphatic supply to the colon with the superior mesenteric artery and associated vessels supplying the colon arising from the mid gut and the inferior mesenteric artery supplying the structures deriving from the hind gut in conjunction with the rectal branches of the internal iliac artery. The architecture of the wall of the colon broadly consists of four distinct layers, the mucosa (epithelium, lamina propria, and muscular mucosae), submucosa, muscularis mucosa (inner circular muscle layer, intermuscular space and outer longitudinal muscle layer) and serosa. The rectum consists of the same layers but lacks a serosa deep to the peritoneal reflection within the pelvis.

The colon and rectum possess a mesentery, containing adipose tissue, blood vessels and lymphatics. Due to its anatomical location, interposed between the gut and the rest of the body, the mesentery is perfectly placed to sample environmental cues and mediate

both local and systemic responses. Lymph nodes, contained within the mesentery sample bacterial components sampled from the adjacent colon regulating the migration of B cells, T cells, natural killer cells, and dendritic cells to and from the nearby intestinal mucosa[2].

Colorectal cancer (CRC) describes a number of heterogenous diseases of the colon and rectum sharing the common characteristic of uncontrolled and unregulated cell growth that in turn can invade neighbouring tissues and metastasise to distant sites[3]. CRC can occur at any point along the course of the colon and rectum and can be either solitary or synchronous.

1.1.2 Epidemiology of Colorectal cancer

Based on the GLOBOCAN estimates of 2018, 18.1 million new cases of cancer occur globally every year with an associated 9.6 million cancer deaths. Globally the annual incidence of new cases of CRC is 1,096,601 (6.1% of all cancers) and is the third most common cancer, making up 10.2% of all cancers diagnosed in men and women. However, CRC disproportionally makes up 9.2% of all cancer deaths making it the second most common cause of cancer mortality worldwide[4]. There is a geographical variation in incidence worldwide. Globally in 2012 there was an almost three-fold higher age standardised incident rate of colorectal cancer in more-developed countries (29.2 per 100,000) compared to less-developed countries (11.7 per 100,000). Australia and New Zealand exhibit the highest international age standardised incident rate of 44.8 (male) and 32.2 (female) per 100,000, whilst Western Africa has the lowest incidence 4.5 (male) and 3.8 (female) per 100,000[5,6]. This suggests both environmental and genetic factors between different populations that predispose to cancer.

1.2.2 Epidemiology within the United Kingdom

With approximately 41,700 cases per year colorectal cancer is the fourth most common cancer in the UK accounting for 12% of all new cancer cases in 2015. In men 32% of tumours occur in the rectum, followed by 23% in the sigmoid colon and 19% in the right colon and caecum with tumours throughout the rest of the colon accounting for the remaining percentage, the pattern is similar in female patients[7]. Over half of all bowel cancer cases in England were diagnosed in the late stages of the disease in 2014[8]. This impacts on survival as with an increase in stage of cancer of the colon and there is a decrease in five year survival[9]. Potential targets to improve outcome, survival and quality of life remain key in this disease. One such potentially modifiable factor associated with worse outcome is body composition.

1.1.3 Pathogenesis of Colorectal cancer

The majority of colorectal cancers are sporadic in nature with approximately 75% of individuals having no prior family history of the disease[6]. It is hypothesised that CRC develops from normal bowel mucosa via an intermediate adenomatous pre-malignant polyp phase. This pathway on the development of cancer is driven by genetic mutations. This model of colorectal tumorigenesis was confirmed by Vogelstein and colleagues in 1988, who characterised a number of key molecular events that occur in the transition from adenomatous polyp to adenocarcinoma[10].

1.1.3.1 The Genetic Basis of Colorectal Carcinogenesis

Polyp development is driven by alterations in the genes regulating cell growth and differentiation. Epigenetic mutations also lead to aberrant methylation of tumour suppressor genes that results in inactivation of these genes and thus neoplasia. Three distinct pathways of genomic instability are recognised in development of colorectal cancer: Chromosomal Instability (the adenoma carcinoma sequence), Microsatellite Instability, and the CpG Island Methylator Phenotype (CIMP) pathway[11]. Sporadic CRC exhibit many of the same major genetic abnormalities as the inherited colorectal cancer syndromes[12].

1.1.4 Aetiology of colorectal cancer

1.1.4.1 General Factors of Age, Gender and Ethnicity

Multiple biological and environmental factors influence the development of colorectal cancer, it's subsequent presentation and the prognosis and outcome.

Sporadic colorectal cancer is a disease of advancing age with a peak incidence of colorectal cancer in the UK between the age of 85 and 89 years with an incidence rate 503 and 352 cases per 100,000 in males and females respectively[13]. However, over recent years there has been an increase in the incidence of colorectal cancer in the younger population (<50 years of age) that has not been fully explained by environmental factors alone[14].

Males have a higher overall incidence of colorectal cancer compared to females with 56% of all bowel cancer patients occurring in men[13]. There are many factors determining this sexual dimorphism with tumour microenvironment, oestrogen expression, fat

distribution, the Wnt/β-catenin signalling pathway, ion channels, and X-linked genes all appearing to play a role[15,16]. There are also behavioural factors exhibited by men that too may predispose them to colorectal cancer such as a diet higher in red and processed meat and lifestyle factors such as increased alcohol intake[16,17]. Gender also appears to be a related to the site of CRC that in turn may impact on presentation, prognosis and survival. In the UK the proportions of CRC cases in the rectum and sigmoid colon are higher in males (31.5% and 23.1%, respectively) than females (23.1% and 20.4%, respectively). Conversely, the proportion of cases in the caecum and ascending colon are higher in females (17.2% and 9.8%, respectively) than males (12.2% and 7.3%, respectively)[16,18].

Race also appears to have an impact on the incidence of colorectal cancer; in the USA it has been observed that the incidence is highest in African Americans even when socioeconomic factors including cancer screening rates have been adjusted for[19]. This is somewhat at odds with the global pattern of incidence of colorectal cancer discussed earlier however this may be explained by other cause mortality and access to healthcare in low-income nations. Very little is known as to the molecular mechanisms behind this disparity but It has been postulated that this higher incidence of CRC in African Americans is a result of both genetic higher incidence of common genetic risk factors and exogenous factors such as vitamin D and lifestyle may play a part[19]. The concept of ethnicity on outcome and risk in terms of the systemic inflammatory response and body composition is discussed in more depth in Chapter 5.

1.1.4.2 Genetic Predisposition and Family History

As mentioned earlier many of the genetic changes seen in sporadic colorectal cancer are shared with the genetic inherited disorders. An understanding of these genetic disorders and their gene profile has contributed significantly to our understanding of the aetiology of colorectal cancer. As such it is important to understand the genetic basis behind these inherited syndromes to understand the genetic impact on sporadic disease.

1.1.4.2.1 Familial Adenomatous Polyposis

Familial adenomatous polyposis (FAP) is a rare genetic disorder where there is congenital absence of the adenomatous polyposis coli (APC) gene. The APC gene encodes a multifunctional protein responsible for the destruction of β-catenin. APC primarily functions as a tumour suppressor gene regulating intracellular levels of β-catenin. β-catenin is a protein instrumental in the Wnt/β-catenin pathway a major pathway controlling cell proliferation, cell polarity, developmental cell-fate determination, and tissue homeostasis[20]. Loss or truncation of the APC gene results in either complete loss of the protein or ability of its protein to engage with specific binding sites on β-catenin, this leads to unregulated activation of the Wnt/ β-catenin pathway and loss of cell cycle regulation. Other functions potentially related to the role of APC in carcinogenesis include cell adhesion and migration, signal transduction, microtubule assembly and chromosome segregation. In colorectal cancer the APC gene is inactivated early in the Vogelstein pathway. The syndrome of FAP is systemic and all cells reliant on the APC gene are at risk of phenotypic abnormalities. In the colon and rectum every stem cell is affected and has therefore taken its first step along the pathway to carcinogenesis. The syndrome manifests itself as development of hundreds to thousands of colonic polyps

by adolescence, these inevitably progress to cancer before the age of 50 unless prophylactic surgery is undertaken. The genetic abnormality itself is usually inherited from a parent however up to 20% of cases occur spontaneously[21].

1.1.4.2.2 MutYH associated polyposis

MutYH associated polyposis (MAP) is a disease similar to FAP however there are fewer polyps and the age of onset is later. This is an autosomal recessive syndrome and as such requires a mutation in both MutYH genes for the disease to manifest itself[21].

1.1.4.2.3 Lynch Syndrome

Lynch syndrome is perhaps one of the most common genetically inherited colorectal cancer syndromes with an incidence of about 1 in 300 making it responsible for up to 5% of colorectal cancers[21]. Lynch syndrome results from a mutation within one of four so called mismatch repair (MMR) genes (MSH2, MSH6, MLH 1 and PMS2). These genes encode for proteins that are responsible for identifying and repairing errors that arise because of DNA polymerase slippage during DNA replication. These errors commonly occur in the short repeat units of microsatellites (repetitive non coding units of DNA) and gains or losses of repeated sequences within these segments is known as microsatellite instability[22]. Loss of or errors in MMR genes can result in an increased incidence of several primary cancer types including bowel, endometrial, ovarian and pancreatic cancer. However, unlike with FAP there is not the certainty of developing cancer and risk of carcinogenesis is determined by which MMR gene is mutated, patient gender and by exposure to modifiable factors such as smoking and obesity[21]. Awareness of this

pathway and process behind carcinogenesis is important as approximately 15% of sporadic cancers also possess a mutation in MMR genes making sporadic cancers that are MMR deficient more common than colorectal cancer secondary to classic Lynch syndrome and this in turn impacts on treatment and prognosis.

1.1.4.2.4 Family History

Outside of the recognised genetic syndromes is the role of family history in the aetiology of colorectal cancer. Family history of disease is a recognised risk factor however, it is important to recognise that this may not be entirely down to acquired genetic susceptibility but also shared environmental and socioeconomic risk factors. However, heritable factors appear to account for 35% of colorectal cancers and approximately up to 29% of the UK population have a family history (first or second degree relative) of colorectal cancer[23]. These figures will include the genetic syndromes described above however these make up a small proportion (5-10%) of the total numbers. This makes assessment of family history important in terms of appropriate screening, genetic counselling and risk assessment in the clinic.

1.1.4.3 Lifestyle, the Environment and Colorectal Carcinogenesis

Lifestyle and environmental factors are of great importance in the aetiology of colorectal cancer.

1.1.4.3.1 Diet

Dietary factors through the direct intake of carcinogens or procarcinogens can predispose an individual to colorectal cancer. Alternatively, diet can have a harmful impact directly on host body composition namely excessive adiposity that can in turn influence the host inflammatory milieu potentially entering a pro-carcinogenic state. Obesity will be discussed in depth later in this chapter and its relationship with the inflammatory response and CRC throughout this thesis.

Diet is now recognised as an important determinant in developing colorectal cancer. A recent Umbrella Review (meta-analysis of meta-analyses) analysing 45 published meta-analyses identified both protective and detrimental dietary factors, this paper provides the strongest evidence to date regarding the relationship between dietary exposure and CRC risk[24]. High fibre intake has been confirmed as a protective factor in reducing risk of colorectal cancer although the authors were unable to identify specific sources from the published data. High dietary calcium and notably yoghurt intake were also associated with a decreased incidence of colorectal cancer[24]. It is postulated that dietary calcium binds to unconjugated bile acids and free fatty acids within the gut reducing their toxic effects[25]. The relationship between specifically yoghurt intake and reduced incidence of colorectal cancer is perhaps more complicated. Once again, the calcium content is thought to play an important role however, there is also thought to be a role for the lactic-acid producing bacteria within yoghurt such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus* that are thought, by out competing other microbes, to reduce the concentrations of carcinogens produced by other gut flora such as faecal activated bacterial enzymes and nitro-reductase[24]. The role of these bacteria also brings into play the role of diet in influencing the gut microbiome and in turn the

effect of the gut microbiome on the development of colorectal cancer. These concepts are revisited in Chapter 9. This Umbrella Review also confirmed that there was convincing evidence that diets high in alcohol and red meat were associated with increased risk of colorectal cancer[24]. Interestingly this review did not find convincing evidence to support limiting consumption of processed meat for CRC prevention despite the emphasis on this as a factor in the existing scientific dogma[24].

1.1.4.3.2 Smoking

Another major lifestyle factor implicated in a multitude of cancers is cigarette smoking. There are established links between smoking and colorectal cancer, a large prospective study of 184,187 individuals in the US identified long term smoking as an independent risk factor with a significantly higher risk of developing colorectal cancer in current and ex-smokers compared to non-smokers. This study also identified that CRC risk significantly decreased in the ex-smokers and this was dependent upon both the length of time they had stopped smoking and earlier age of smoking cessation[26]. Despite studies such as this it remains inherently difficult to distinguish individual risk factors from the multitude of risk factors that form part of an individual's environment with many of these high-risk lifestyle factors being associated with socioeconomic deprivation.

1.1.4.3.3 Socioeconomic Economics and The Environment

Socioeconomic deprivation is associated with decrease survival from colorectal cancer. There is more uncertainty regarding the true influence of deprivation on the incidence of colorectal cancer particularly due to the difficulty to discern individual lifestyle factors

associated with deprivation. As such the associations relating to survival have been more heavily investigated and can be attributed to decreased screening uptake, later presentation, later referral from primary care and differences in individual patient characteristics that may affect treatment and overall (non-cancer specific) survival[27,28]. The potential aetiology behind an association between deprivation and increased colorectal cancer incidence is more opaque. In one study undertaken in the West of Scotland, deprivation was found to be associated with increased incidence rates in males but not females with the data demonstrating a widening of the gap over time perhaps in part due a reduction in modifiable risk factors in those more affluent combined with the potential increased engagement with the Scottish Bowel Cancer Screening Program by the more affluent widening this gap in the future[29]. This highlights the importance of lifestyle which is so inherently linked to socioeconomic status. There is also an apparent disparity between sexes that in turn could be down to either different lifestyle factors such as labour or different host phenotypic responses to shared lifestyle factors such as fat distribution in men. The role of deprivation and the environment in relation to colorectal cancer and body composition is explored in Chapter 4 of this thesis.

1.1.4.4 Local and Systemic inflammation in Colorectal Carcinogenesis

Local (mucosal/intestinal) and systemic inflammation are both known to bring about carcinogenesis, but the aetiology and biochemical pathways share common themes and yet are different in both their presentation and manifestation.

1.1.4.3.4 Local inflammation and Inflammatory Bowel Disease

Local inflammation is best characterised in patients with inflammatory bowel disease (IBD). IBD has long been recognised as a significant risk factor for developing colorectal cancer. The risk of developing cancer in either ulcerative colitis (UC) or Crohn's Disease (CD) is dependent of disease activity with those suffering florid disease being at greater risk. One study on a UK cohort of patients identified the absolute cumulative frequency of risk for developing CRC in extensive colitis was 8% at 22 years from onset of symptoms in CD and 7% at 20 years from symptom onset in UC. The same study demonstrated an eighteen fold increase in the risk of developing colorectal cancer in extensive Crohn's colitis and a nineteen fold increase in risk in extensive UC compared to the general population, matched for age, sex, and years at risk[30]. From these data UC appears to come with a slightly higher risk and a later meta-analysis estimated an overall prevalence of CRC in any UC patient of 3.7%, based on 116 studies[31]. The pathway to CRC in IBD is complex but it is recognised that as with sporadic CRC the three main genetic pathways (Chromosomal Instability, Microsatellite Instability, and the CIMP pathway) play a pivotal role with a similar array of mutations and resultant loss of gene expression.

The order in which these mutations occur is often different compared to the sporadic cancers for example, DNA methylation and microsatellite instability are frequently found at an early stage in CRC in UC patients but less frequently in the early stages of sporadic CRC[32]. There are often multifocal cellular changes throughout the affected colon and rectum indicative of a field change effect rather than one or two individual foci of disease in sporadic CRC[33]. Chronic local intestinal inflammation is the key driver in this sequence of events and is thought in part to explain the reason for the different sequence of

mutations. The presence of reactive oxygen and nitrogen species, brought about by cellular inflammation, inflicts oxidative stress directly causing DNA damage. Interestingly many of these genetic changes are evident in UC prior to any histological evidence of dysplasia[33].

1.1.4.3.5 Systemic inflammation

The role of systemic inflammation in carcinogenesis is more complex. A normal acute systemic inflammatory response is a vital mechanism allowing a response to pathogens. However, when the resolution of this systemic inflammatory state is impeded or indeed promoted by either host factors or environmental factors a state of chronic sterile systemic inflammation exists. This state is often characterised by activation of immune components that are often distinct from those seen in the acute phase response. The effect of this systemic inflammatory response can be two-fold, firstly it may dampen down normal immune function making the host susceptible to either communicable or neoplastic disease. Secondly, this state of chronic inflammation can cause a breakdown in immune tolerance by tissues resulting in development of non-communicable disease in the host such as type 2 diabetes, chronic kidney disease, cardiovascular disease and cancer[34].

In acute inflammation the inflammatory pathway is usually activated by pathogen-associated molecular patterns (PAMPs) or by directly by noxious stimuli during cellular stress activating damage-associated molecular patterns (DAMPs). The chronic systemic inflammatory response is often triggered solely by DAMPs however, cytokines that induce resolution of inflammation such as lipoxins, resolvins, maresins and protectins are

not activated. This low grade chronic inflammatory state brings about tissue and DNA damage through subsequent oxidative stress[34].

This systemic inflammatory state can be activated and maintained by multiple mechanisms, for example, obesity (characterised by a raised body mass Index (BMI)) induces a chronic systemic inflammatory response[35] and the association between obesity and colorectal cancer is well established following the seminal Umbrella Review by Kyrgiou and colleagues[36]. Systemic inflammation can also help explain some of the more esoteric associations between environmental exposure and colorectal cancer including artificial light at night (blue light spectrum exposure) and increased colorectal cancer risk[37]. In this instance the disruption of the circadian rhythm brought about through night shift work, artificial light and sleep deprivation induces such a sterile chronic inflammatory immune response[34]. This example rather elegantly demonstrates how influential and important the systemic inflammatory response is in carcinogenesis and how sensitive the host is to the environmental processes.

1.1.4.4 The Microbiome

The role of the gut microbiome has been found to be of increasing relevance in our understanding of colorectal carcinogenesis, cancer progression and treatment response[38]. The resident bacteria of the gut not only interact directly with the host intestinal cells but also intimately react with the host immune system at the mucosal interface. Constant sampling of both bacterial antigens and their metabolites provides information to host immune system that can invoke immune response and immunotolerance. This process of interaction and cross talk is a vital part of human gut physiology, and we rely on it for both energy harvest and immune maturation. Studies

have demonstrated that the profile of gut microbiota of CRC patients differs from those of healthy individuals. CRC patients exhibit a lower abundance of potentially protective bacteria such as *Roseburia Spp.* and increased abundance of species such as *Bacteroides, Escherichia, Fusobacterium* and *Porphyromonas* which are known to promote carcinogenesis[38]. This shift in the microbiome of the diseased population is known as dysbiosis. The process of carcinogenesis again is complicated but once again involves the pathways described earlier in this chapter with chronic inflammation and host toxic metabolites such as bile salts playing a key role as mutagens. However, in addition to these, bacterial metabolites also appear to have an important role inducing DNA damage directly and by inducing an immune response and host cellular damage that in turn heightens the immune inflammatory response[38].

1.1.4.5 Increasing Incidence of Colorectal Cancer in the Young

One phenomenon, briefly discussed earlier in this chapter was the increasing incidence of colorectal cancer in the young. As mentioned before the aetiology behind this is not fully understood. Despite this there is little controversy in the fact that there is a shift in disease patterns towards a younger population[14]. Lifestyle is thought to play a major part in the pathogenic process and it was notes that the increase in specifically colon cancer in young adults in China correlated with an increased adoption of a so call Western lifestyle, particularly dietary habits[39]. This rising trend in colorectal cancer cases in young adults is likely to continue to increase but highlights the importance of exogenous and environmental factors in carcinogenesis many of which are modifiable.

1.1.4.6 Protective Factors

We have discussed the aetiology behind colorectal cancer in depth but it is also important to take into account the many factors, particularly relating to diet and lifestyle, that are modifiable and can reduce risk. Diet has been discussed in depth earlier and it was noted that there is robust evidence in the literature that dietary fibre, calcium and yoghurt are all protective agents[24].

In a large study using UK biobank data increased physical activity has been shown to reduce the risk of colon cancer whilst individuals who have a more sedentary lifestyle were shown to be at increased risk of colon cancer. There was no relationship found in this study between physical activity and rectal cancer risk. The study authors postulated that a major factor behind the decreased risk was adiposity and therefore ran their multivariate models with and without adjusting for waist circumference. They noted that adjusting for waist circumference resulted in a modest attenuation of risk estimates in the order of 1-8%. This supported the role of obesity and body composition in the development of colorectal cancer[40]. These findings, notably in colon cancer are echoed elsewhere in the literature suggesting a convincing role for physical activity in colon cancer prevention[41,42].

1.1.5 Tumour staging and prognosis

Colorectal cancer prognosis is based on the grade and stage of the disease.

1.1.5.1 Tumour Grade

Grading describes the degree of differentiation with prognosis and ultimate grade defined by the majority of cells within the tumour that possess phenotype most removed from the individual parent cell. Tumour grade is categorised from grade one to three. Low grade tumours, containing cells resembling the physiological parent are graded as one whilst at the other end of the spectrum cells, classified as grade three, are poorly differentiated and have greater mitotic potential and thus risk of further mutation and spread.

1.1.5.2 Cancer Stage

Two separate systems are primarily used to stage colorectal cancer, Dukes' staging and the TNM staging method. Dukes' staging was initially described for rectal cancer by the St Mark's pathologist Cuthbert Dukes in 1932, this broadly characterised the stage of the tumour into 3 discrete stages based on the histological and survival characteristics A - Tumour limited to mucosa; B - Tumour limited to the bowel wall and C - regional lymph node metastasis[43]. This has been modified further over time with various iterations most notably by Astler and Coller in 1954 who sub-classified the level of extension through the bowel wall and level of nodal disease[44] and by the addition of Stage D by Turnbull and colleagues in 1967 to describe the presence of distant metastases[45]. Dukes' staging is advantageous in that it allows a more simplistic interpretation of disease however it use has decreased in favour of the Union for International Cancer Control (UICC) TNM classification of malignant tumours that allows a more accurate appraisal of disease in keeping with advancing cancer management and treatment. The UICC TNM

classification is currently on its eighth edition published in 2016[46]. The TNM System for Colon and Rectal Cancer is demonstrated below, Table 1.1.

T-stage	
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria
T1	Invading submucosa
T2	Invading muscularis propria
T3	Invading through the muscularis propria into peri-colorectal tissues
T4a	Penetrating to the surface of the visceral peritoneum
T4b	Directly invading or adherent to other organs or structures
N-stage	
N0	No regional lymph node metastasis
N1	Metastasis in 1-3 regional lymph nodes
N1a	One lymph node
N1b	2 to 3 lymph nodes
N1c	Tumour deposits in subserosa, mesentery, or non-peritonealised pericolic or perirectal tissues without regional nodal metastasis
N2	Metastasis in 4 or more regional lymph nodes
N2a	4 to 6 regional lymph nodes
N2b	7 or more regional lymph nodes
M-stage	
M0	No distant metastasis
M1	Distant metastasis
M1a	Metastasis confined to one organ (liver, lung, ovary, non-regional lymph node(s)) without peritoneal metastases

M1b	Metastasis in more than one organ
M1c	Metastasis to the peritoneum with or without other organ involvement

Table 1.1 TNM Stage (Adapted from UICC TNM Staging 8th Edition)[47]

Based on the histopathological and radiological TNM staging described above it is then possible to translate this into a more simplified stratification of staging from 0-4 that allows subsequent prognostication based on population data.

1.1.5.3 Stage of Colorectal Cancer and Outcome

Cancer Research UK publishes contemporaneous data of UK cancer survival. 5-year survival from cancers diagnosed between 2013 and 2017 in the UK based on stage at presentation shown below in Figure 1.1. This figure demonstrates that as the Stage of the cancer advances the five-year net-survival decreases. This allows us to impart prognostically useful and meaningful information to our patients and helps dictate treatment and management.

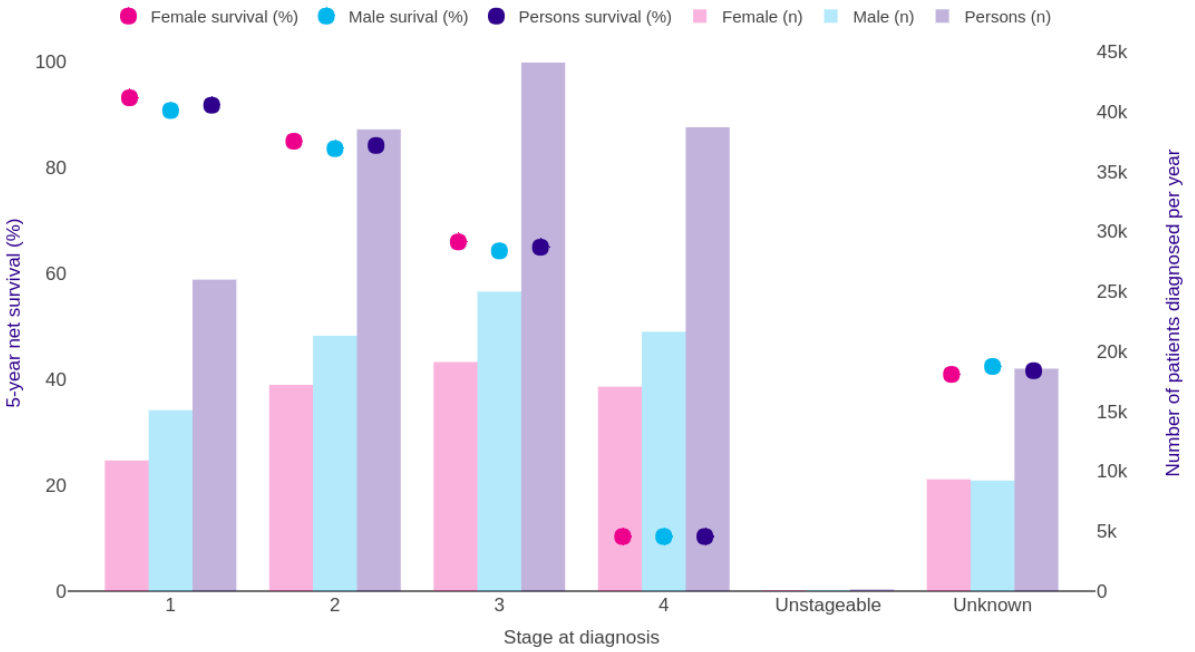


Figure 1.1 UK bowel cancer five-year net survival by stage, with incidence by stage (all data: adults diagnosed 2013-2017, followed up to 2018 (Reproduced with permission from Cancer Research UK - cancerresearchuk.org)[48]

1.1.6 Investigation and Treatment of colorectal cancer

1.1.6.1 Patient Presentation

Perhaps the greatest factors that influences survival in colorectal cancer is stage at presentation, survival has been shown to decrease as stage increases. In 2018 16.9% of patients presenting with colorectal cancer presented with stage 1; 22.7% with stage 2; 27.1% with stage 3 and 22.5% with Stage 4 disease[49]. In the UK the majority (33.3%) of patients present via the two-week wait referral pathway with 21.8% presenting via a direct GP referral and 16.2% presenting via the emergency department. Only 9.8% of colorectal cancer are identified through the national screening program[49].

1.1.6.2 Colorectal Cancer Investigation

In the UK investigation and routine work up for colon and rectal cancer has become relatively standardised. Patients usually undergo a thorough history and examination including a digital rectal examination followed by haematological investigations. A colonoscopy where biopsies will be taken for histological diagnosis. Lesions greater than 15cm for the anal verge are considered colonic and those below 15cm considered rectal. Rectal cancers are further sub stratified into high, middle and low rectal cancers based on the distance of the distal tumour from the anal margin[50]. Computed tomographic (CT) imaging is performed to stage the disease within the thorax, abdomen and pelvis. Local staging of rectal cancer is performed with magnetic resonance imaging MRI. MRI is also used to evaluate the liver should there be concerns regarding liver metastasis. Fluorodeoxyglucose (FDG) Positron emission tomography (PET) is performed on occasions if there is uncertainty regarding the presence or extent of metastases seen on other imaging modalities. Endoanal ultrasound may be used to determine the depth of local invasion of rectal cancers prior to deciding treatment. All patients should be discussed in a multidisciplinary meeting prior to commencing treatment.

1.1.6.3 Radical Treatment of Primary Colorectal Cancer

The National Institute for Health and Care Excellence (NICE) has published guidelines on the management of colorectal cancer[51]. Surgical resection of the affected colonic segment along with its vascular pedicle and associated lymph node package within its mesentery remains the mainstay of radical treatment. Surgery can be performed safely via an open, laparoscopic or robotic approach[52–54]. Total mesorectal excision has become the standard of radical surgical treatment for rectal cancers[55], complete

mesocolic excision is also practiced and has been shown to produce oncologically superior specimens but is technically more challenging and has a potential increase in morbidity in relation to its potential benefits in outcome[56]. Neoadjuvant and adjuvant chemo-radiotherapy may be considered for locally advanced or metastatic disease. Early cancers (carcinoma in situ or cT1-T2, cN0, M0 disease) can be managed with local / endoscopic mucosal/submucosal excision, however this does not allow for histological analysis of the regional lymph nodes. More recently there has been increasing interest in radical radiotherapy for rectal cancers with trials such as STAR-TREC exploring the feasibility of this approach[57].

1.1.6.4 Palliative Treatment

Where radical treatment is not possible, declined by the patient or the extent of disease spread is such that providing a radical option is not possible then treatment may be described as palliative. There is still a role for surgery in the palliative treatment of colorectal cancer. This is performed for symptom control including the management of anaemia by resecting a bleeding tumour or relieving or preventing obstruction through diverting, bypassing or defunctioning. Endoscopic and endoluminal treatments such as stents are also employed but usually as a bridge to surgery. Chemo and radiotherapy remain the mainstay of palliative treatment in combination with holistic palliative nursing and medical care and psychological support.

1.1.6.5 Locally Advanced and Recurrent Colorectal Cancer and Pelvic Exenteration

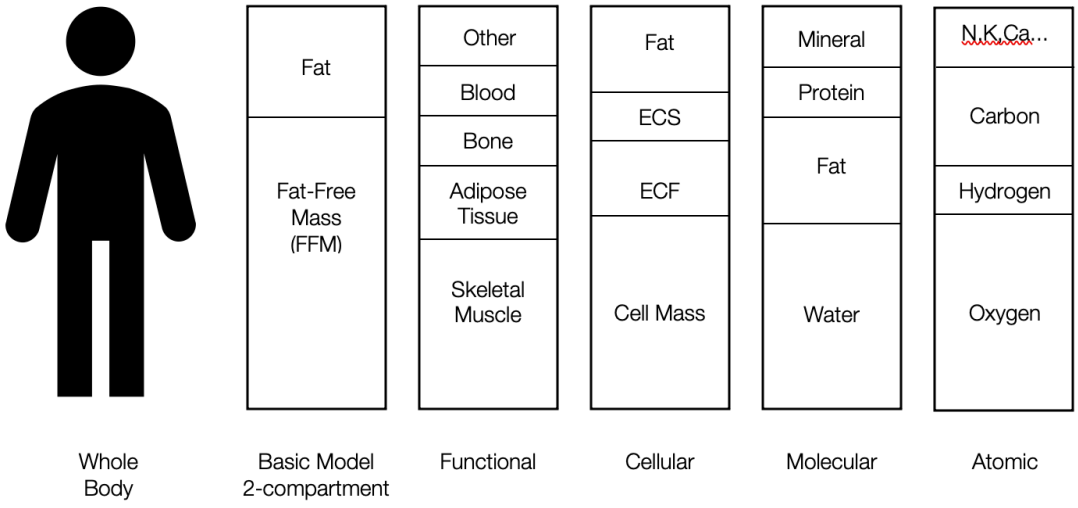
More recently, advances in surgical technique, radiological imaging, oncological and perioperative care have allowed radical surgical resection locally advanced cancers that up to now would have been managed palliatively. Techniques such as high subcortical sacrectomy (HISS) and extended lateral pelvic sidewall excision (ELSIE) have been shown to be technically feasible surgical treatments. Pelvic exenteration surgery, removal of all pelvic viscera involved in the tumour mass, has been shown to improve survival outcomes in this poor prognostic group[58,59,60]. International data published by the PelvEx Collaborative have shown a three year survival of 56.4% following an R0 resection in patients undergoing pelvic exenteration for locally advanced rectal cancer, three year survival decreases in line with increasing resection status, 29.6% with an R1 resection and 8.9% with an R2 resection[61]. These invasive techniques however come at a cost to the patient with increased post-operative morbidity, prolonged hospital stays and costs and post-operative disability[62,63].

1.2 Body composition

1.2.1 Defining Body Composition

The term body composition (BC) describes, quantifies and qualifies the different compartments within the human body. In its simplest form a basic 2-compartment model can be used to define human body composition into either fat or fat free mass (FFM). From this point there are multiple methods of defining individual compartments be it biochemically, physiologically, functionally or anatomically (figure 1.2). In terms of cancer

biology each individual model can be studied in isolation however, because they are inherently linked, they should not be considered as discrete entities.



[ECS: extra-cellular solids, ECF: extracellular fluid]

Figure 1.2 Multicompartmental Models of Body Composition (Adapted from Ellis et al 2000)[64]

1.2.2 Assessment of Body Composition

Assessment of body composition is often performed radiologically and as such we tend to discuss body composition in terms of functional/anatomical compartments. However, there is increasing emphasis on both the molecular and atomic components of body composition as the changes seen radiologically arise from this cellular and biochemical level. Fat and skeletal muscle are the most researched and perhaps most clinically relevant components of body composition in oncology. Less emphasis is placed upon the bone, blood and other elements of fat free mass such as solid organs. Changes in

fat and muscle are well recognised clinically in terms of aging, disease and the host response to disease.

1.2.2.1 Methods of Assessment

There are numerous methods available to assess body composition (Table 1.2.). Each method has both benefits and drawbacks. Assessment should be accurate, time efficient and reproducible, affordable and safe. However, it is important to understand the principals and practice behind the various methods to make an informed decision on the most appropriate method for an individual study.

Setting	Laboratory	Clinical	Bedside
Approach	Gold Standard 4 Compartment Model	Radiological Assessment	Clinical Assessment
Method	Deuterium dilution Underwater-weighing +/- Plethysmography DEXA	Computerised Tomography (CT) MRI DEXA	Anthropometrics Bio-impedance analysis

Table 1.2 Methods of assessing body composition[65]

1.2.2.2 The Four Compartment Model

The gold standard four compartmental model provides accurate data but is highly impractical and of little use beyond a laboratory setting. Briefly it consists of a combination of four components, underwater weighing (UWW), air-displacement plethysmography, deuterium dilution, and DEXA scanning. Underwater weighing involves the complete submersion of the subject in water, measurement of the volume of water displaced, measurement of the subject's underwater weight in combination with their

weight on dry land. This assessment of body composition allows the division of weight into fractions (f) of fat and FFM and using constants for the density (D) of fat and FFM, allows the whole-body density to be calculated (D_b) using the equation:

$1/D_b = f_{fat}/D_{fat} + f_{FFM}/D_{FFM}$.

There are limitations to this method including the variability in residual lung volume and the variation in the density of fat free mass which is why the further components of the four compartments are required. The principals of air-displacement plethysmography are similar however this is reliant upon air displacement rather that water and as such shares many of the limitations of UWW. Deuterium (D_2O) dilution involves the ingestion or injection of D_2O isotope. Background levels of D_2O are checked by analysis of a pre-administration fluid sample (blood/urine/saliva) then levels are checked again at a set "equilibration" time point following administration (2-3 hours). The level of dilution of D_2O in the final sample allows an estimation of compartments based on an understanding of distribution within the total body water of the intra and extracellular fluid[64]. The final component of the four-compartment model is Dual-Energy X-ray Absorptiometry (DEXA), assessment of body composition is estimated based on the attenuation of X-rays as they pass through a subject. The principal is such that attenuation of X-rays that pass through an individual will vary depending on the volume and density of the subject, i.e., the denser the medium the more X-rays will be absorbed. Ultimately, the three final measurements of derived body composition values are bone mineral content (BMC), lean tissue mass (LTM), and fat mass (FM). Assessment via DEXA is unable to differentiate variation in tissue overlying bone, which needs to be subtracted from the final measurements

however, DEXA has been used independently in body composition research in view of its reproducibility, speed of performance and low radiation dose.

Ultimately, by combining the metrics (total body density, total body water and bone) derived from each of these investigations allows an accurate assessment of body composition (% Fat) using the following equation that forms the four-compartment model:

$$100 \times (2.747/D_b - 0.714f_{TBW} + 1.146f_{bone} - 2.0503)^{64}$$

This 4-compartment method is accurate and allows assessment of the whole body and its distinct components. However, this method is rarely used as it is complex, impractical and expensive and as such emphasis has been on newer and evolving methods of body composition assessment.

1.2.2.3 Bioimpedance Analysis

Bioimpedance analysis is a method often used both in and outside the clinic setting. Bioimpedance relies upon the conduction and resistance of an electrical current. Intra and extracellular fluid contains electrolytes which allow these tissues with a high fluid content to conduct electricity more rapidly and efficiently compared to tissues high in fat which act as an insulator with a higher resultant impedance of current. This method of assessment has evolved considerably since its inception in the 1980's. Current machines are able to derive multiple metrics based on bioimpedance that ultimately allows the calculation of total body water, ECF, ICF, lean body mass and fat mass. Various physiologically important metrics can then be calculated based on bioimpedance

including clinically relevant markers such as phase angle. A decreased phase angle has been shown to relate to both low muscle volume and more importantly clinical outcomes for example in critically ill patients decreased phase angle, on admission to the intensive care unit, is an independent predictor of 90-day mortality[66,67]. Overall bioimpedance analysis is relatively cheap, safe and easy to do however it shows high variability and accurate measurement is operator dependent. It also requires assessment with identical and reproducible subject parameters for example, a starved patient, who has been immobile for a set period performed at a set time of day.

1.2.2.4 Anthropometrics

Anthropometrics is an umbrella term for a series of measurements performed by a clinician and include measures such as height, weight, skin fold thickness, waist circumference and limb circumference. These metrics are frequently used in clinical practice as attaining them is both cheap and safe to do however, they do not accurately consider the individual body compartments and are generally used to derive a measure of percentage fat rather than a reflection of fat free mass. There is also a discrepancy between individual operators which affects reproducibility.

The most frequently measurement derived from anthropometrics is Body Mass Index (BMI) calculated using the subject's weight in kilogram divided by their height in squared meters (kg/m^2). Established cut-offs are then used to determine whether a subject is underweight, of healthy "normal" weight, overweight or obese. These cut off vary dependent on sources but are generally considered to be the following[68]:

- BMI <18.5=underweight
- BMI 18.5–24.9=normal weight
- BMI 25–29.9=overweight
- BMI >30=obese.

BMI provides a quick assessment of body composition but relies on the assumption that body mass, adjusted for height squared, is closely associated with adiposity and subsequent associated morbidity and mortality. This is not always the case for example a lean body builder may have a high mass for their stature due to muscle mass essentially meaning they are overweight but not "overfat"[69]. As such BMI can misrepresent reality in when used isolation.

1.2.2.5 Radiological Analysis

In clinical practice and oncological body composition research radiological methods have now become the mainstay of assessment of body composition. Magnetic Resonance Imaging (MRI) and computed tomography (CT) are the assessment methods of choice although DEXA was previously an important modality and has been discussed in depth earlier. MRI is currently used comparatively infrequently however, as the speed of MRI scanning increases and available software available to assess MRI become more readily available, we may see this become used more frequently. It Is advantageous over CT by virtue of the fact that it does impart a radiation dose to the subject and image quality may also allow a more tissue specific assessment.

CT is used in the standard diagnostic work up for many diseases and it is used to stage nearly all colorectal cancer patients. In CRC patients it is performed at set time points

and used in both the pre and post treatment and post-operative setting. The current approach in analysing CT BC is to take a single slice axial image at the level of the third lumbar vertebrate (L3).

1.2.2.5.1 Developing CT Body Composition Analysis

It has been established that single abdominal skeletal muscle or adipose tissue slice areas correlate strongly with corresponding total body skeletal muscle and adipose tissue volumes. The skeletal muscle area 5 cm above the L4-L5 level has the highest correlation with total body skeletal muscle volume and the adipose tissue area 5 cm below L4-L5 the highest correlation with total body adipose tissue volume[70]. Other factors such as age, ethnicity, body mass index and waist circumference as well as technical characteristics such as imaging position only slightly influenced these findings[70]. L3 therefore has been selected as the most appropriate area as it captures the most meaningful representation of skeletal muscle, a recognised and accepted limitation of this is the loss in accuracy and transferability in the terms of adipose tissue values.

Validation of skeletal muscle imaging was conducted in 1998 on cadavers where limbs of cadavers were imaged with CT and MRI and these imaged sections compared to their corresponding axial cadaveric section. The findings of this study demonstrated that MRI and CT estimates of adipose tissue-free skeletal muscle are in good agreement with those obtained from cadaveric sections[71]. Since these studies there has been increasing data in the published literature using CT body composition analysis and this is widely used in studies evaluating patients with cancer. Prado and colleagues were early exponents of this method of assessment in the cancer population with publication of their seminal paper in Lancet oncology[72]. This work provided the parameters from which

the cut off values were derived for previous work from our department, many of the published studies by other groups use.

1.2.2.5.2 CT extraction

CT data is extracted from the picture archiving and viewing system (PACS) in Digital Imaging and Communications in Medicine (DICOM) format. DICOM or .DCM is a standard format to view, store, and retrieve radiological images. Accuracy of the image data is maintained within DICOM by set protocols and as such allows for sharing and accurate and complete reproduction of the image. DICOM contains all data pertaining to the specific radiological image and in doing so differs from formats such as Portable Graphics Format (.PNG) and Joint Photographic Expert Group (.JPEG).

1.2.2.5.3 DICOM Analysis

Once extracted the DICOM data can analysed by software that measures total fat, subcutaneous fat, visceral fat and skeletal muscle cross-sectional area (cm^2) and density (HU). An investigator can then segment these images based on the difference in HU using set cut off values and anatomical distribution to define the different compartments of muscle and adipose tissue. This is a time consuming and laborious process and as such led to the development of automated software. This software, known as Automatic Body composition Analyzer using Computed tomography image Segmentation (ABACS), employs a finite element method deformable model that incorporates priori shape information via a statistical deformation model within the template-based segmentation framework[73]. This essentially allows rapid automatic segmentation of individual CT L3

DICOM images thus negating the need of an individual investigator to manually segment. This method has been validated against manually segmented images demonstrating that it provides an accurate readout of body composition variables[74,75].

Segmentation provides metrics of skeletal muscle area (SMA), visceral fat area (VFA) and subcutaneous fat area (SFA) with values provided as centimetre square (cm^2). In addition to these areas, the software also supply's the mean HU of the muscle within the entire L3 slice which is described as mean muscle attenuation (MA).

Further standardised indices (total fat index (TFI), subcutaneous fat index (SFI), visceral fat index (VFI) and lumbar skeletal muscle index (LSMI or SMI)) can be derived using the patient height giving values in cm^2/m^2. Sex-specific cut-offs can then be applied to the SMI to categorise patients by presence of low muscle volume for example SMI <52.4 cm^2/m^2 for males and <38.5 cm^2/m^2 for females which were values defined from work by Prado and colleagues[72]. The same can be done with visceral obesity thresholds where visceral obesity corresponds to a visceral fat area (VFA). Doyle and colleagues have previously identified that VFA greater than 160 cm^2 in males and 80 cm^2 for females are clinically significant cut-offs for the metabolic syndrome of visceral obesity[76].

1.2.2.5.4 Aberrant Body Composition Phenotypes on CT

To define aberrant body composition, it is important to have a definition of normality and thresholds for disease associated with certain BC phenotypes. As discussed before BMI provides a range of cut-off that determine whether an individual is underweight, normal weight, overweight or obese[68]. Clinically relevant cut-offs for CTBC analysis have also been defined based on population data as described above in the instance of Prado and Doyle[77,78]. Deviation of body composition outside these cut-off values can thusly be used

to determine abnormal body composition. In terms of cancer there are several abnormal body composition parameters of interest.

1.2.3 Abnormal Body Composition States

1.2.3.1 Obesity

The incidence of obesity as defined as a BMI greater than 30 is increasing, globally there are now more people who are overweight than underweight[68]. According to the World Health Organisation, in 2016, more than 1.9 billion adults, 18 years and older, were overweight (BMI>25) and of these over 650 million were obese (BMI >30)[79]. Globally more people now die from disease associated with being overweight or obese compared to being underweight[79]. The development of obesity can be multifactorial however the majority is caused by an energy imbalance where more calories are consumed than expended. Deposition of adipose tissue is also important with different patterns of fat deposition resulting in varying levels of disease risk. Upper body and visceral fat distribution is more strongly associated with metabolic complications of obesity as opposed to a gluteal femoral distribution. Visceral (fat surrounding the organs), omental and mesenteric fat, collectively termed visceral adiposity, appear to play a role in delivering both excess free fatty acids (FFA) and Interleukin-6 (IL-6) to the liver[80]. This may in part explain the low grade chronic systemic inflammatory response seen in the obese and the inability of these individual to regulate their circulating FFA may influence appropriate metabolic and endocrine responses. One of the major determinants of fat distribution is gender. Women tend to have more body fat than men but this tends to be

in a gluteal femoral distribution giving the body a characteristic “pear-shape” as opposed to the classic centripetal truncal obesity seen in men. The consequences of increased adiposity appear to be more apparent in men than women. These differences in the fat phenotype between genders are probably a result of a complex combination of genetic, epigenetic, and hormonal factors[81]. It is not fully understood why intra-abdominal fat, visceral obesity (encompassing mesenteric and omental fat) (VO) and subcutaneous fat obesity play different metabolic roles but it would appear the function of these tissues is profoundly different.

1.2.3.2 Cachexia

Perhaps the most recognised body composition state associated with disease is that of global weight loss and wasting - the syndrome of cachexia. The syndrome of cachexia is characterized by involuntary weight loss, greater than 5%, or weight loss greater than 2% in individuals already showing depletion according to current bodyweight and height (body-mass index [BMI] <20 kg/m2) or skeletal muscle mass [82]. It includes disease-related anorexia in addition to hypermetabolism, hypercatabolism and hypoanabolism from the underlying systemic effects of the pre-existing disease [83]. These factors are inextricably linked to the systemic inflammatory response associated with cancer and are summarised in Figure 1.3.[65]

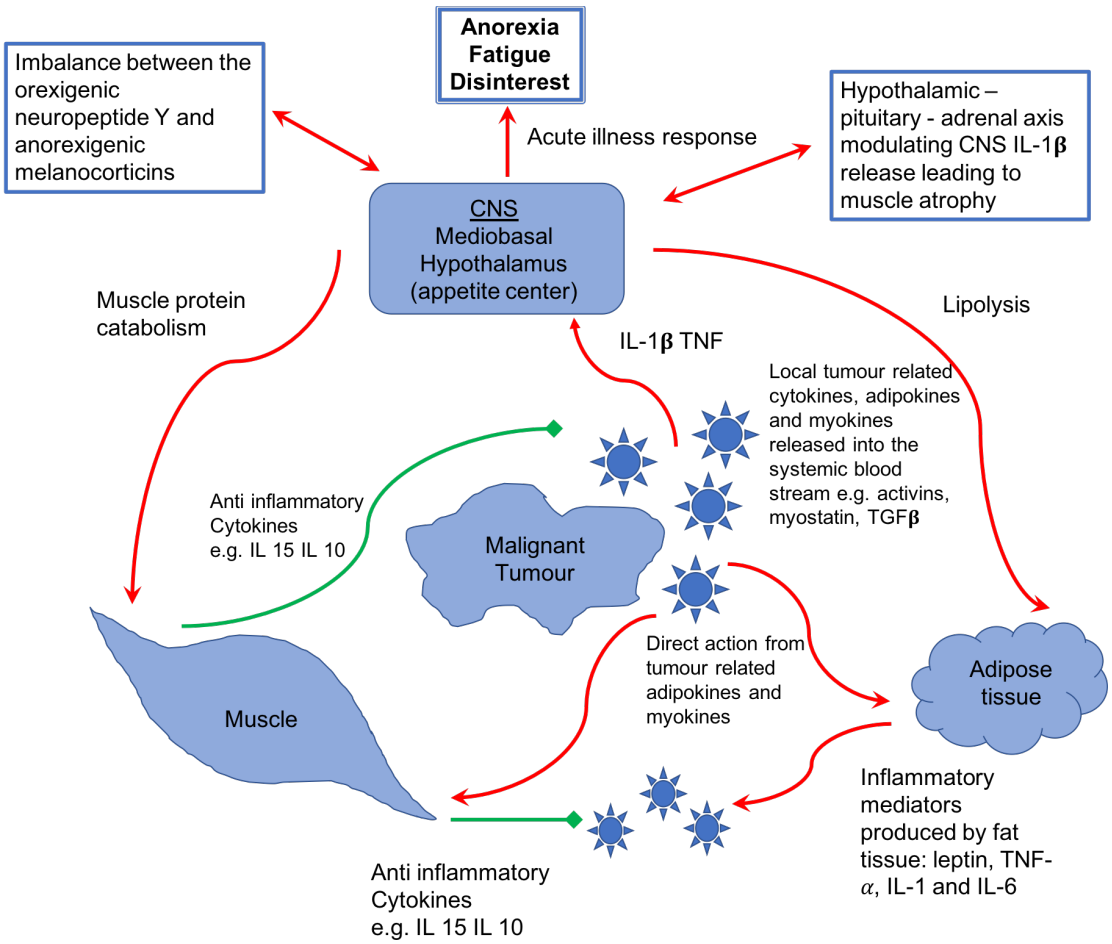


Figure 1.3 A schematic of the various pathways driving anorexia and cachexia in cancer[65]

(Used with permission from Elsevier, Cancer Treatment Reviews)

The presence of cachexia is associated with diminished quality-of-life, poor response to chemotherapy, poor surgical outcomes and poor clinical outcomes in cancer patients [84]. The syndrome has a variable incidence according to the tumour type with certain primary tumours driving a greater degree of cachexia (Table 1.3) [85] [86]. Over eight million deaths a year (half of all cancer deaths worldwide) are ascribed to cancers most frequently associated with cachexia[87] with nearly one third of cancer deaths related to muscle catabolism and the consequent weakened physiology [85].

Primary Cancer	Number of Patients	Percentage with >5% weight loss in preceding 6 months
Gastric	138	67
Pancreatic*	111	54
Non-Small Cell Lung	590	36
Small Cell Lung	436	34
Colon	307	28
Prostate	78	28
Sarcoma	189	18
Breast	289	14

*Weight loss in preceding 2 months

Table 1.3 Frequency of weight loss in cancer patients (ECOG) with solid tumours [86,65]

Cachexia describes this general loss in fat and muscle however there is not always a uniform loss of each tissue, identification of cachexia can also be challenging as premorbid body composition status (obesity) may disguise significant underlying skeletal muscle loss - sarcopenia.

1.2.3.3 Sarcopenia

Sarcopenia is a term derived from the ancient Greek meaning “poverty of flesh”. The term was originally coined in 1989 by Irwin Rosenberg in relation to the progressive muscle loss seen in aging[88,89]. It is defined as a “progressive and generalised skeletal muscle disorder that is associated with increased likelihood of adverse outcomes including falls, fractures, physical disability and mortality”[90]. This definition is important as it considers not only the physical loss of lean body mass in the form of skeletal muscle but also the associated loss of function. The significance of sarcopenia as a disease state is recognised as such by the assignment of an ICD-10 classification in 2012[91]. The term myopenia has also been used to describe muscle loss and has been suggested to be a

better term in that it recognises the specific loss of muscle secondary to disease[92]. However, this term appears to be declining in its use, especially after the Society on Cachexia and Wasting Disease (SCWD) published a position paper on sarcopenia in 2019 highlighting the recent consensus definitions of primary sarcopenia as weight loss secondary to aging and secondary sarcopenia as the process of muscle loss secondary to underlying disease[93].

Sarcopenia is a multifactorial process summarised in Figure 1.4.

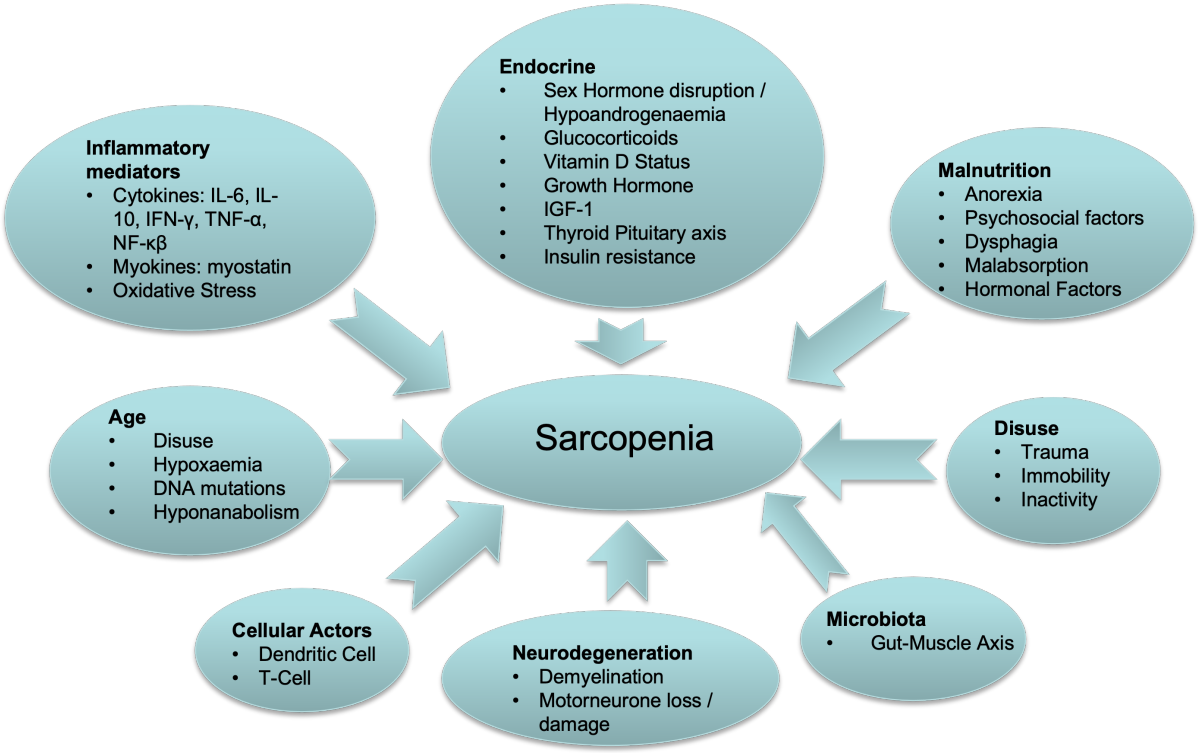


Figure 1.4 Causes of Sarcopenia[94](used with the permission of Elsevier, Cancer Treatment Reviews)

The presence of sarcopenia is a poorly prognostic in a number of disease states, in a meta-analysis of 56 published articles it was found to be associated with a significantly higher risk of mortality independent of the population studied or definition used[95]. The

relationship between colorectal cancer and sarcopenia will be covered in more detail later in this chapter and the remaining thesis however it is important to note that the presence of sarcopenia is a poor prognostic feature in multiple cancer types[96–99].

1.2.3.4 Myosteatosis

Lipid exists in skeletal muscle in two major forms one intracellular; fat infiltration within myocytes (IMCL) and one extracellular; visible fat deposits within the fascia surrounding muscle (lipid infiltration)[100]. CT body composition analysis provides data on mean muscle attenuation (a surrogate of muscle density). Fatty infiltration of skeletal muscle reduces radiographic and actual muscle density and thus reduced CT attenuation; it is specifically extracellular lipid infiltration that we appreciate on CT. In order to quantify IMCL, proton magnetic resonance spectroscopy or muscle biopsy must be employed[100]. Increased ectopic fat deposition, known as myosteatosis, is associated with a systemic pathological and inflammatory state and is seen in ageing, malignancy, diabetes, obesity, and muscle disuse[101,102]. The process that drives myosteatosis is not fully understood but it is not purely explained by the loss of myocytes and the replacement of this void by lipid or adipose tissue. Modulation in neuromuscular activation, decreased muscle blood flow, and the local secretion of proinflammatory cytokines and adipokines are all thought to contribute to the genesis of myosteatosis[100].

As with sarcopenia and visceral obesity, clinically significant cut-off values have been identified for myosteatosis in cancer by Martin and colleagues based on survival analysis in lung and colorectal cancer[103].

1.2.3.5 Sarcopenic Obesity

It is possible to combine the CT derived metrics directly with anthropometrics to identify individuals with particularly pernicious body composition phenotypes. Sarcopenic obesity describes such a state. Patients who are sarcopenic, based on the predefined cut off values described earlier and who are obese in terms of BMI (BMI >30) can be categorised as having sarcopenic obesity. This body composition state has been associated with worse survival and disease specific outcomes and as such are considered a poor prognostic group[72,104].

1.2.4 The Obesity Paradox

The association between obesity and developing colorectal cancer is well established[36]. However, it appears that obesity and outcome also share an unusual relationship with the association between obesity and survival in colorectal cancer forming a non-linear J or U-shaped curve. Essentially the evidence suggests a slight survival advantage in those individuals who are either overweight or have class one obesity[105,106]. This survival advantage conferred by obesity is known as the obesity paradox[107]. There have been a number of hypotheses suggested to explain the survival benefit of obesity from study method, statistical error, selection bias and reverse causality[108] through to physiological explanations such as the greater reserves held in muscle and fat by those who have a BMI>30[109]. Ultimately in cancer, following the evolution of CT BC analysis, it appears that the presence of sarcopenia may be the key, where low muscle volume is the major determinant of outcome. Sarcopenia is associated with the net fall in BMI and as such this may explain this paradox[107]. This explanation highlights the importance of moving

away from BMI as a metric in isolation as it confounds the more complex background physiological picture.

1.3 Body Composition and Colorectal cancer

Throughout this introduction we have discussed body composition in general but have made few references to its specific role in colorectal cancer. Much of the early work into the role of body composition, particularly cachexia and sarcopenia, in colorectal cancer was undertaken by Fearon and colleagues[,93]. Fearon was instrumental in defining cachexia and exploring the relationship between BC status and outcome[65,66,75].

This early work by Fearon led to a number of the earlier studies undertaken at St Mark's Hospital that further explored the relationship between body composition and colorectal cancer and it was from this work that the concept of this thesis arose.

Much of the early work focused on BC status and outcome and a series of important relationships were identified. From here researchers aimed to explore the aetiology behind these changes particularly the relationship between body composition status and the systemic inflammatory and cell mediated immune response in colorectal cancer.

1.3.1 Prognosis in patients with sarcopenia and sarcopenic obesity

Deleterious features of body composition are now accepted to be associated with poorer survival outcomes in patients with colorectal cancer. One of the starkest findings of the early work undertaken at St Mark's by Malietzis and colleagues was the significant association between sarcopenic obesity and 30-day mortality. Of the ten patients who

died within thirty days of major colorectal surgery for the treatment of cancer seven of them had sarcopenic obesity. This data corroborated the significant associations seen between 30 day morbidity and sarcopenic obesity found in the same cohort[111]. On multivariable analysis in this cohort the presence of sarcopenia was an independent predictor of decreased overall and disease free survival[111]. Similar findings from this early work at St Mark's have been replicated by other units in the UK and internationally both in primary and metastatic colorectal cancer. One study by Hopkins and colleagues in Canada specifically looked at stage I to III colorectal cancer. They too identified that sarcopenia is an independent predictor of worse survival in this population. Data from studies from multiple institutions have been amalgamated and undergone meta-analysis, one such meta-analysis from 2021, analysing 44 individual studies, confirmed that colorectal cancer patients with sarcopenia had significantly shorter overall survival (HR=1.83; 95% CI=1.57–2.14), disease-free survival (HR=1.55; 95% CI=1.29–1.88), and cancer-specific survival (HR=1.77; 95% CI 1.40–2.23)[112]. Other studies have identified that not only does sarcopenia impact on survival but also post-operative complications, one study from the Netherlands by van Vugt and colleagues demonstrated that postoperative complication rate was significantly higher in patients with low versus high skeletal muscle and density 20.9% versus 13.6%, $p = 0.006$[113]. The same group also went on if further work to highlight the economic impact of sarcopenia in a cohort of 452 patients undergoing surgery for colorectal cancer, colorectal liver metastases, and hepatopancreatobiliary primary cancers. They found that sarcopenia was independently associated with an increased hospital cost of €4200 per patient[114].

The effect of sarcopenia on the colorectal cancer population should not be underestimated, in the meta-analysis discussed above exploring the effect of sarcopenia

on outcomes in colorectal cancer the authors reported a prevalence of 37% (7009 out of 18,891 colorectal cancer patients)[112]. This highlights the number of patients at increased risk of complications and decreased survival from surgery based solely on their pre-operative body composition status.

1.3.2 Myosteatosis and Cancer

Sarcopenia and cachexia share many parallels and often exhibit clinically visible and measurable signs of deconditioning through either anthropometrics or functional tests. Myosteatosis is more occult in its manifestation and relies on imaging or histological analysis to identify its presence. Myosteatosis is however being viewed with increasing importance in colorectal cancer, numerous studies have linked the presence of myosteatosis with poor outcomes and survival[111,115–117]. In the previously published cohort of colorectal cancer patients at St Mark's Hospital the prevalence of myosteatosis was found to be 77.6% and the presence of myosteatosis was significantly and independently associated with longer hospital stay[111]. There was no association found in this cohort between myosteatosis and outcomes however subsequent larger cohorts links have been established between myosteatosis and survival outcomes. In the Alberta Cohort Hopkins and colleagues observed myosteatosis was independently predictive of worse overall survival and that the presence of both myosteatosis and sarcopenia predicted the worst overall, recurrence free and cancer specific survival of all BC phenotypes[118]. Ultimately a meta-analysis performed by Aleixo and colleagues of eleven studies confirmed that myosteatosis conferred a 73% greater mortality risk in colorectal cancer[119]. Within the large Californian Kaiser Permanente (KP) Cohort associations have also been identified between myosteatosis and major post-operative complications

(Clavien-Dindo three or above[120]) and readmission to hospital following colorectal cancer surgery[121].

1.3.3 BMI, Visceral Obesity and Colorectal Cancer

We have already discussed in depth the causal associations between obesity (BMI) and body composition. There are also numerous significant associations between obesity status and outcomes in colorectal cancer. As discussed earlier central obesity is seen at more pathological state as opposed to a gluteal femoral distribution. CT analysis has allowed the specific influence of this pathological phenomenon of central visceral obesity (VO) to be explored in colorectal cancer. Despite this the associations between obesity and outcomes remain complex and at time contradictory in keeping with the obesity paradox. Associations between BMI obesity, the presence of the metabolic syndrome and worse overall and cancer-related survival outcomes in early stage (stage I-III) colorectal cancer were established by within the KP Cohort of patients in 2016[122]. The same group went on to publish a further study demonstrating that visceral and subcutaneous obesity was prognostic of all-cause mortality in stage I-III CRC. However, the relationship between adiposity and mortality in this cohort was non-linear in both instances with VO producing an reverse L shaped pattern and subcutaneous obesity a J shaped pattern[123]. On further interrogation of the data the authors also identified sex specific differences within the data adding another layer of complexity to the overall picture and suggesting that the sex related dysregulated deposition of excess adiposity is prognostic of mortality[123]. Conversely, within the published St Mark's Cohort it was found that visceral obesity tended to confer a survival advantage following colorectal cancer surgery although statistical significance was not reached[111]. Clearly the

relationship between visceral adiposity remains controversial not just within colorectal cancer but also between primary cancer subtypes, overall, based on the current published evidence, the effect of visceral adiposity on colorectal cancer survival outcomes is seen a negative one whilst in renal cell carcinoma visceral adiposity appears to confer a survival advantage[124]. One possible explanation of this phenomenon is that although obesity increases the risk of developing a renal cell carcinoma, the biology of the tumour arising in an obese individual that is less aggressive in its behaviour.

1.3.4 Body Composition and the Systemic Inflammatory Response

The associations between body composition status and colorectal cancer outcomes are evident however, the aetiology behind these outcomes is somewhat unclear. The temporal relationship between the development of certain BC phenotypes and CRC outcomes are also yet to be fully elucidated. One factor that appears to be important is that of the response of the host immune system and resultant systemic inflammatory state. There are clear independent associations between the host immune response and body composition in colorectal cancer. Within the St Mark's Cohort, sarcopenia and myosteatosis were related a profound host systemic inflammatory response. Patients with CRC who had a clinically significant neutrophil to lymphocyte ratio (NLR) of greater than three being were significantly more likely to exhibit sarcopenia and myosteatosis[125,101]. Furthermore, Cespedes and colleagues, studying the KP cohort, identified that an increasing NLR was associated with sarcopenia in a quasi "dose-response" manner with a greater NLR (NLR>5) being associated with more profound sarcopenia. They found an NLR greater than three and the presence of sarcopenia predicted overall and cancer related death with patients with this combination of traits

having double the risk of death compared to the rest of the cohort[126]. Further evidence to support the relationship between systemic inflammation and sarcopenia and myosteatosis was confirmed by the Glasgow cohort. They identified an association between a hyperinflammatory state characterised by a raised modified Glasgow prognostic score (mGPS), a clinically significant metric derived from the serum albumin and CRP, and the presence of myosteatosis and sarcopenia in colorectal cancer. Their findings also reiterated the associations between these BC phenotypes and poor survival outcomes[127]. These inflammatory changes do not appear to be transitory and in patients with sarcopenia a systemic inflammatory response has been observed to be largely maintained following surgical resection over a twelve month period[128].

Clearly there are relationships between body composition and systemic inflammation, but it is unclear whether the tumour itself is driving this inflammatory state which in turn causes deleterious changes in BC or whether the BC of an individual is modulating the host immune response and inflammatory state, in turn driving the behaviour of the tumour. This then begs the question whether, through modifying the body composition status of a patient, we can manipulate their immune and inflammatory response to mitigate the effects of BC on CRC outcome.

1.4 The Host Immune response

The host immune response is governed by the host immune system. The purpose of the immune system is to defend the host from pathogens and pathological processes through the response to, and recognition of, foreign or abnormal antigens or noxious stimuli. The immune system comprises of an innate and adaptive component. The innate immune response is non-specific and acts as a first line in responding to an insult, should

the innate immune system fail to fully suppress such an immunogenic insult then the adaptive immune system will activate initiating a targeted and specific immune response. Both branches of the immune response have a cellular and humoral aspect to them.

1.4.1 The Immune System in Colorectal cancer

As genetic mutations occur within a cancer the mutated cell, its clones and progeny begin to differ at a molecular level from the host parent cell. This alteration in cell phenotype makes it vulnerable to the host immune response. One of the hallmarks of a successful cancer is its ability to evade the immune system. Despite this the immune response to cancer is well recognised. The most profound aspects of this response occur at the interface between healthy tissue and the tumour but can occur throughout the tumour. The presence of tumour-infiltrating cells, vasculature, extracellular matrix (ECM), and other matrix-associated molecules form a unique inflammatory system known as the tumour micro-environment (TEM)[129]. The interaction between the tumour and the immune infiltrates is complex and in part is key to the process of carcinogenesis. Chronic local inflammation is an initiator and promotor of carcinogenesis and has been seen to be instrumental in the progression from healthy mucosa to carcinoma as described earlier in this chapter. However, as a solid malignancy becomes established the host inflammatory cells drawn to it inadvertently promote carcinogenesis[129].

1.4.2 Tumour associated neutrophils and systemic inflammatory response

One of the most prevalent inflammatory cells within the TEM is the tumour associated neutrophil (TAN). The association between a systemic neutrophilia and poor outcome is

well recognised as described above. An abundance of TAN is also associated with a poor cancer outcome however, TAN appear to have pro and anti-tumoral effects[130]. The association between poor outcome and the presence of inflammatory tumours may in part help explain why we see both systemic inflammation and BC changes consistent with a chronic inflammatory state in patients who have a poor outcome for CRC.

TAN are known to exist in two states either N1 (proinflammatory or anti tumoral subsets) or N2 (anti-inflammatory or tumour promoting subsets). These two populations are distinguished from one another through the expression of adhesion molecules, cytokines and inflammatory mediators, chemokines, and chemokine receptors[131]. N1 cells when in an activated state express a series of chemokine receptors including CCR5, CCR7, CXCR3, and CXCR4. They also produce the proinflammatory chemokines and cytokines such as CCL2, CXCL8, CCL3, and interleukin-6 (IL-6)[131]. The association between circulating levels of IL-6 and deleterious changes in BC are well recognised[132]. The expression of CCR7 within the stroma of the TEM has been associated with poor prognostic features in CRC such as increased age, lymphovascular invasion, higher tumour stage and lymph node metastases. On multivariate analysis CCR7 expression at the tumour margin was associated with significantly decreased and overall survival. Most importantly in the context of this thesis, CCR7 expression in the TEM has been associated with myosteatosis[133]. These data suggest that the immune response within the TEM is associated with both altered host BC but also tumour biology. Signalling via CCR7 appears not only to be important within the tumour but systemically. Mean muscle attenuation on CT at the L3 level was found to negatively correlate with expression of CCR7 by dendritic cells (DC) hence, increased CCR7 expression by circulating DC was associated with myosteatosis.

1.4.3 Dendritic cells

Dendritic cells (DC) were first reported in the scientific literature by Steinman and Cohn in 1973[134] in the USA, at the same time Balfour's UK group also identified these veiled cells which were seen as a key actors operating at the interface between the innate and adaptive immune system. DC consist of a heterogenous group of cells that interact with antigens, process them, then ultimately present them to and thus activate T and B cells inducing the antigen specific immune response[135136]. Conversely DC are vital players in instructing the cellular immune system to exercise a tolerogenic immune response[137]. Humans DC are either migratory or resident, these roles are defined once the immature bone marrow derived dendritic cells, carried within the blood, pass either into mucosal and non-mucosal tissues via resting or inflamed post capillary venules or directly into the spleen or lymph nodes. The DC migrate within the tissue moving from interface tissues into lymphatics, then via lymph into secondary lymphoid organs of the immune system such as Peyer's patches, lymph nodes and spleen where antigen presentation occurs[136137].

1.4.3.1 Dendritic Cells in Cancer

DC play an important role in tumours by exerting both tumourigenic and anti-tumourigenic functions. Colorectal tumour antigens have been shown to induce DC recruitment, maturation and cytokine release to generate effective T cell immune response. It has been seen that DC are able to migrate directly to the site of the tumour and that tumour-infiltrating DC (TIDC) are associated with the delayed tumour

progression and lymph node metastasis in a number of solid tumours[138]. Ultimately, interactions between DC and tumours are complex and not fully understood[139]. However, by their very nature dendritic cells are likely to be at the forefront of abnormal tumour antigen analysis and subsequent presentation and instruction to T cells, whose role in combating tumours is well recognised. Potential dysfunction in this circuitry as a result of influences on the DC by abnormal body composition of the host may lead to tumour evasion of the immune system and poorer outcome.

1.4.3.2 Dendritic Cells and Body Composition in Colorectal Cancer

As highly potent antigen presenting cells, DC are responsible for determining downstream effector responses by other immune cells such as cytotoxic T-cells. As these cells sit at the pinnacle of the hierarchy of the immune system, they are instrumental in not only the host response to a tumour but also the host response to self i.e., the host tissues of muscle and fat. Work previously undertaken at St Mark's on circulating DC established a positive correlation between CD40 (a DC maturation marker) expression and lumbar skeletal muscle area, hence DC maturation was associated with a healthier muscle status. A further positive correlation was identified between expression of CD36 (a fat scavenger receptor) and increasing mean muscle attenuation indicative of myosteatosis[139]. CCR7 was discussed above in the context of TAN however, CCR7 expression by DC (which is associated with lymph node homing) and CD83 (early maturation) shared a negative correlation with mean muscle attenuation.

1.4.3.3 Dendritic Cells and Lipid

The correlation between CD36 and muscle attenuation could suggest that fat could be depleted or metabolised by circulating myeloid cells in these patients suggesting that body composition may impact directly upon the immune system and modulate it in such a way that it impacts on the immunogenic response to cancer[139]. This is recognised further by fat infiltration within dendritic cells which is associated with DC dysfunction. DC in tumour bearing hosts have been found to accumulate lipid bodies (LB) containing electrophilic oxidatively truncated (ox-tr) lipids[140]. These ox-tr LB covalently have been shown in vivo to bind to chaperone heat shock protein 70. This prevents the translocation of the peptide major histocompatibility complex (MHC) class 1, a protein complex necessary for cross presentation, to the cell surface by driving the accumulation of inactivated MHC inside endosomes and lysosomes[140]. As a result of this process and loss of MHC class 1, DC lose their ability to present antigen and hence are considered dysfunctional. Standard lipid bodies alone do not seem to precipitate such a chain of events[140].

This accumulation of especially triglycerides in patients with active cancer has been demonstrated in analysis of DC in peripheral blood and confirmed in mouse models[141]. Scavenger receptors, such as CD36 mentioned above are thought to be the main route by which DC accrue fat. The function of DC, once they become lipid laden has in turn been shown to be impaired with a reduced ability to process and present antigen[141]. These observations suggest a direct relationship between the metabolic and functional processes within the DC population and changes in BC. In turn, BC may influence

changes in stimulatory, migratory and fatty acid-processing potential of DC in patients with CRC[139].

1.5 Summary of Introduction

Colorectal cancer is a major cause of morbidity and mortality worldwide, its incidence is increasing in the young. Multiple factors influence outcomes in colorectal cancer. Many of these relate to the influence on the genetic and epigenetic factors that are involved in tumorigenesis. The environmental and lifestyle factors that in turn are involved in driving or preventing genetic mutation and dictate in part the order in which these mutations occur.

Surgery with or without chemoradiotherapy is the mainstay of radical treatment. Advances in operative technique and perioperative care have allowed treatment of locally advanced and recurrent rectal cancers but these operative approaches come with the cost of significant morbidity to the patient.

Body composition is intimately related to the incidence and outcomes of colorectal cancer. Risk of developing colorectal cancer is associated with obesity. Sarcopenia, myosteatosis, visceral obesity, anthropometric obesity (BMI) and sarcopenic obesity are associated with adverse outcomes including postoperative morbidity, mortality and survival. Abnormal body composition status is related to the host systemic inflammatory response that in turn is related to the interaction between the host immune system and tumour microenvironment. Changes in maturation and function of host dendritic cells in colorectal cancer patients are associated with various body composition phenotypes. Lipid accumulations, specifically oxidised lipid, appears to be linked to loss or dysfunction

of DC antigen presentation and associations have been made between fat scavenger receptors on Host DC and body composition in colorectal cancer.

1.6 Conclusions

Although there is strong evidence to support the relationships between body composition phenotype and outcome little is known about the aetiology of the various body composition phenotypes. Do they precede the disease or are they brought about by a host immune response to the tumour and within the TEM – what has been conceptually called a “bad tumour”.

There is also increasing interest in the arrest of the inflammatory process and subsequent reversibility of the body composition state in disease. Ultimately this poses the question, can we influence outcomes in colorectal cancer by modulating the immune response through the modification of body composition?

1.7 Thesis Hypothesis

Based upon the uncertainty and ambiguity highlighted within this introduction regarding causality and potential manipulability of body composition the following hypothesis is proposed:

"Body composition is dependent on genetic, environmental and tumoral factors but can be manipulated by modulating the immune response to colorectal cancer and thus outcomes improved"

1.8 Thesis Aims

My intention is to attempt to address the above hypothesis in three ways:

Firstly, by examining population data from a cohort of patients with colorectal cancer. Here I would hope to identify genetic, environmental and tumour factors that influence BC looking for potential modifiable targets. (Chapters 2-5)

Secondly, by examining the relationship between dendritic cell function, body composition and the effect of microbial stimulation upon the DC. Looking for points within the immune pathway where one potentially acts to promote or prevent changes through pathways orchestrated by the dendritic cell. (Chapters 7-9)

Thirdly. by exploring methods by which we can directly influence BC and devise a trial that we could implement an intervention in patients who are most in need on intervention to promote healthy BC status – the advanced rectal cancer population. (Chapter 10 & 11)

As such my aims are:

- To explore the association between the prognostic features of colorectal cancer and their individual associations with body composition state

- To identify whether body composition status can be used to predict whether an individual has a poor prognostic tumour with metastatic potential

- To examine the effect of a subject's environment on their body composition status in colorectal cancer

- To identify whether race and ethnicity impact on body composition phenotype and the systemic inflammatory response in colorectal cancer and explore possible explanations for any discrepancies found

- To identify whether it is possible identify and to extract dendritic cells from muscle tissue in colorectal cancer patients

- To explore the relationship between colorectal cancer, body composition status and dendritic cell homing and function in multiple tissue types and identify whether DC function changes within individual tissue types

- To explore the effect of the microbiome on DC function in an in vitro setting and identify whether this can replicate the changes in DC homing and function seen in patients with colorectal cancer

- To review the literature to find evidence to support methods that have been used to preserve or restore body composition status

- Having identified the most promising methods from the scientific literature I intend to formulate a clinical trial to identify whether we can preserve or salvage muscle

quality and quantity; modulate the immune and inflammatory response and influence outcomes in our advanced rectal cancer population

Chapter 2

2 Tumour grade and stage are associated with specific body composition phenotypes with visceral obesity predisposing the host to a less aggressive tumour in colorectal cancer

2.1 Summary

In Chapter 1 we discussed how survival was intimately related to the stage of colorectal cancer. The associations between body composition and outcome were also discussed as well as the controversies relating to obesity and outcome and the obesity paradox. We also discussed the controversy relating to whether BC determines the severity of disease in colorectal cancer and vice versa. Within this we raised the concept of a bad or inflammatory tumour phenotype which influences body composition status. In this chapter we evaluate the relationship between BC status and cancer stage in order to elucidate further this complex relationship.

2.2 Introduction

We discussed the epidemiology of CRC in Chapter 1, CRC is the fourth most prevalent cancer in the UK with approximately 42,000 cases per year[7]; its incidence is increasing in Europe, especially in the young[14]. Body composition (BC) assessment may become a useful adjunct in determining prognosis and outcome from colorectal cancer[142,143]; anthropometrics such as Body Mass Index [BMI][144] are misleading and a poor proxy for adiposity and do not distinguish muscle from adipose tissue and do not define the distribution of adipose tissue[145]. BC defined by computer tomography (CT) as part of routine oncological staging may provide more accurate assessment[107]. Using staging CT scans, we can identify multiple BC parameters of both muscle and fat.

Sarcopenia and myosteatosis are associated with poorer cancer-specific, disease-free and overall survival[146]. Sarcopenia is associated with increased post-operative complications and length of hospital stay[111,147]. Myosteatosis and sarcopenia, are indicative of an elevated systemic inflammatory response suggesting that an inflammatory process, related to the tumour, may alter BC phenotype[101,126,127].

The aetiologies of sarcopenia and myosteatosis are varied and complex but are potentially reversible[65]. Previous work demonstrates that in some patients, poor prognostic body composition features can be reversed following definitive cancer treatment[148]. As stated in Chapter 1 the recent position statement by the SCWD defines sarcopenia as primary (age related) and secondary (disease related)[93]. Differentiating both conditions that manifest as a low skeletal muscle volume, is challenging as patients lack pre-morbid imaging to determine the pre-existing burden of sarcopenia and there is likely to be a combination of primary and secondary sarcopenia present in individual patients.

Associations are well known between obesity, defined by BMI, and colorectal cancer[36]. It is accepted that patients lose adipose tissue in addition to skeletal muscle as part of the syndrome of cachexia and that cachexia is related to poorer outcomes[83,87,149]. Visceral obesity has been found to be associated with worse patient specific outcomes[147] and suggests that in colorectal cancer a potentially more aggressive tumour behaviour is influenced by a pathway associated with obesity.

Sarcopenic obesity describes a situation in which an individual displays low skeletal muscle volume with a BMI of greater than 30kg/m^2; a combination associated with generally poorer outcomes. Owing to a raised BMI, it is likely that little consideration in this group is given to nutritional assessment and support[72].

The association between preoperative body composition and colorectal cancer outcome following surgery is now well established in multiple populations[111,147,150] with these associations appearing to demonstrate homogeneity amongst different populations[151]. Skeletal muscle is increasingly viewed as an important metabolic organ and appears inextricably linked to the systemic inflammatory response associated with cancer[126,152,153]. The inflammatory response and its relationship to tumour characteristics has raised the concept that certain tumours may elicit a more profound effect on BC and hence result in poorer cancer outcomes. Controversy remains regarding the effect of a tumour upon the host body composition phenotype i.e., whether outcome is poorer due to a pre-existing BC phenotype or whether a BC phenotype is orchestrated by a tumour's characteristics. We aimed to determine whether the tumour itself exhibits characteristics that relate an individual patients BC phenotype.

2.3 Methods

2.3.1 Patient Population

1403 consecutive patients undergoing primary colorectal cancer surgery at a single tertiary referral centre in London between May 2007 and January 2017 were identified on a prospectively collected database. Patients with anthropometric data (height, weight and BMI), digital preoperative CT images and post-operative histology and radiological staging data were included in the analysis. Patients were excluded if they had recurrent disease; our complex and advanced rectal cancer cohort were not included in this study; all other exclusions criteria were as Malietzis et al 2016[111]. All prospectively collected data were revalidated from the hospital electronic records prior to analysis.

Data collected prospectively during the perioperative period (within 30 days of surgery) included age, sex, BMI, the American Society of Anaesthesiologists (ASA) physical status classification system, histological grading, TNM stage [Union for International Cancer Control (UICC) Version 5] and included the presence of vascular invasion on histopathology.

2.3.2 Body Composition Analysis

CT images were retrieved in DICOM (.dcm) format from the hospital Picture Archiving and Communication System (PACS) (Sectra, Linköping, Sweden). Automated CT image analysis was performed using Slice-O-Matic v5.0 software (Tomovision, Montreal, Canada) with ABACS L3 Plug-in (Veronoi Health Analytics, Vancouver, Canada). All analysed images were reviewed manually to ensure appropriate segmentation; images

where automated analysis failed were segmented manually using Slice-O-Matic v5.0 by a consultant radiologist experienced in BC segmentation.

Segmentation, based on set Hounsfield Unit thresholds (-29 to 150 for skeletal muscle, -150 to 50 for visceral adipose tissue, and -190 to -30 for subcutaneous adipose tissues), allowed determinations of total skeletal muscle and total adipose tissue surface areas at the level of the third lumbar vertebra (L3). Figures derived from this analysis were, visceral fat area (VFA), subcutaneous fat area (SFA) and skeletal muscle area (SMA). The latter was converted to an index normalised for stature to attain the lumbar skeletal muscle index (LSMI). Mean muscle attenuation (MA) was derived from all muscle segmented at L3. Reduced LSMI (sarcopenia), low MA (myosteatosis) and visceral obesity were defined using predefined sex-specific skeletal muscle index cut points[76,154].

2.3.3 Ethical Approval

Approval was obtained for use of the prospective database in research by the NHS Health Research Authority, UK with ethical approval from the South East London NHS Research Ethics Committee (reference number: 12/LO/1556).

2.3.4 Statistical Analyses

Statistical analyses were performed using SPSS v25.0 (IBM Corp, Armonk, NY). Age was analysed as a continuous variable whilst ASA grade and tumour characteristics were categorised as binary variables as shown in Table 1. Body composition data were binary. Data were analysed using Chi-squared for univariate analysis and binary logistic regression for multivariate analysis. Multivariate binary regression analysis was performed

on the dataset using a model constructed of clinically and statistically relevant independent variables known to impact on body composition and tumour characteristics; these were based on the univariate analysis, previous work[111] and clinical relevance. CT derived body composition parameters were included within the analysis, these measures utilise anthropometrics including height and weight and BMI, Figure 2.1. Binary variables were constructed for the dependent tumour characteristics as demonstrated in Table 6. The model included ASA grade, age and gender and radiological body composition features of sarcopenia, myosteatosis, visceral obesity and sarcopenic obesity. The same multivariate model was used for each tumour characteristic to identify the effect and interplay of body composition on the dependent binary tumour characteristic variable.

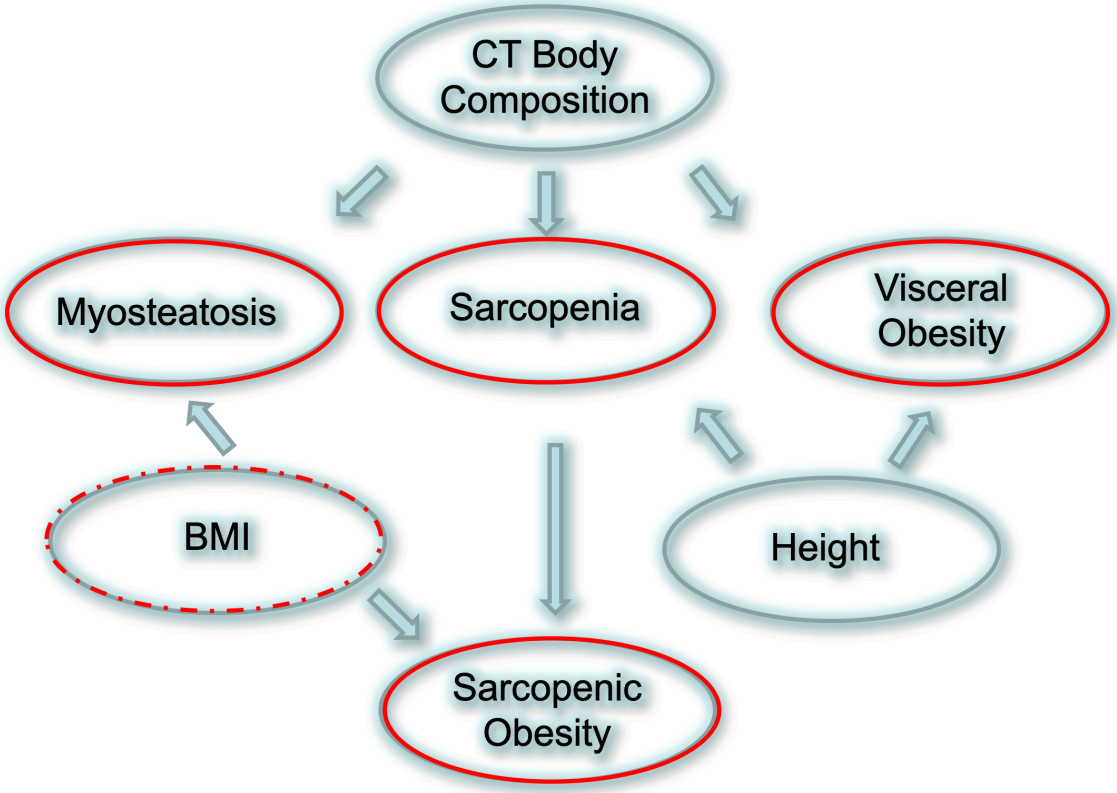


Figure 2.1 The relationship between anthropometric data and CT derived body composition data

2.4 Results

The median age of the cohort was 69 [IQR: 60–76] years. The population demographics are shown in Table 2.1.

		n	%
Gender	Male	450	57%
	Female	345	43%
ASA Grade	ASA 1 & 2	694	87%
	ASA 3 & 4	101	13%
Differentiation (Grade)	Well or moderate	603	90%

	Poor	68	10%
T Stage	T1-2	277	35%
	T3-4	518	65%
Nodal Disease	Negative	484	61%
	Positive	306	39%
Metastases	No Metastases	702	91%
	Metastatic disease	73	9%
Vascular Invasion	Absent	524	67%
	Present	261	33%
UICC Stage	Stage 1	205	27%
	Stage 2	232	31%
	Stage 3	238	32%
	Stage 4	74	10%

Table 2.1 Demographics

2.4.1 Exclusions

The cohort consisted of 1403 consecutive patients who underwent resection of a primary colorectal cancer. Height and weight data were not recorded prospectively at the time of CT in 476 cases, 37 had emergency surgery, 23 had resection for other malignant diseases (such as neuroendocrine tumour [NET]) thus did not have colorectal adenocarcinoma, and in 72 patients the CT analysis was not possible due to poor image quality. Exclusion of these patients produced a sample size of 795. Baseline clinicopathological and demographic characteristics of the cohort are shown in Table 2.1.

2.4.2 Body Composition Parameters

Body composition parameters for sarcopenia and myosteatosis were defined using the cut off values described by Martin et al and were determined using CT data in conjunction to BMI and gender[155]. Visceral obesity was defined using the cut-off values described by

Doyle et al and were gender specific[78]. A diagnosis of sarcopenic obesity was made if an individual both sarcopenic with a BMI of greater than 30kg/m^2.

Men were more likely to be sarcopenic than women p=0.001 (72.9% vs 55.9%), OR=1.95 (95%CI 1.50-2.52). Conversely, women were more myosteatotic P=0.012 (76.2 vs 69.1%), OR=1.44 (95%CI 1.08-1.91). Visceral obesity and sarcopenic obesity were similar between men and women (52.0% vs 57.4% and 21.6% vs 20.3%, respectively) with no significant difference between either group, p=0.51 and 0.15, respectively. Prevalence of individual BC phenotype in relation to stage and grade are shown in Table 2.2-2.5. Results of univariate and multivariate binary regression analyses are summarised in Table 2.6.

Sarcopenia					
		Not Sarcopenic		Sarcopenic	
		n	%	n	%
T Stage	T1-2	112	40%	165	60%
	T3-4	162	31%	356	69%
Nodal Disease	Negative	169	35%	315	65%
	Positive	102	33%	204	67%
Metastases	No Metastases	248	35%	454	65%
	Metastatic disease	21	29%	52	71%
Vascular Invasion	Absent	189	36%	335	64%
	Present	79	30%	182	70%
Differentiation (Grade)	Well/Moderate	200	33%	403	67%
	Poor	24	35%	44	65%

Table 2.2 Prevalence of sarcopenia in relation to pathological stage

Myosteatosis					
		No Myosteatosis		Myosteatotic	
		n	%	n	%
T Stage	T1-2	96	35%	181	65%
	T3-4	125	24%	393	76%

Nodal Disease	Negative	134	28%	350	72%
	Positive	87	28%	219	72%
Metastases	No Metastases	201	29%	501	71%
	Metastatic disease	9	12%	64	88%
Vascular Invasion	Absent	166	32%	358	68%
	Present	51	20%	210	81%
Differentiation (Grade)	Well/Moderate	150	25%	453	75%
	Poor	21	31%	47	69%

Table 2.3 Prevalence of myosteatosis in relation to pathological stage

Visceral Obesity					
		Not Viscerally Obese		Viscerally Obese	
		n	%	n	%
T Stage	T1-2	113	41%	164	59%
	T3-4	250	48%	268	52%
Nodal Disease	Negative	200	41%	284	59%
	Positive	161	53%	145	47%
Metastases	No Metastases	315	45%	387	55%
	Metastatic disease	39	53%	34	47%
Vascular Invasion	Absent	219	42%	305	58%
	Present	138	53%	123	47%
Differentiation (Grade)	Well/Moderate	270	45%	333	55%
	Poor	37	54%	31	46%

Table 2.4 Prevalence of visceral obesity in relation to pathological stage

Sarcopenic Obesity					
		Not Sarcopenic-obese		Sarcopenic-obese	
		n	%	n	%
T Stage	T1-2	229	83%	48	17%
	T3-4	399	77%	119	23%
Nodal Disease	Negative	380	79%	104	22%
	Positive	243	79%	63	21%
Metastases	No Metastases	549	78%	153	22%
	Metastatic disease	59	81%	14	19%
Vascular Invasion	Absent	414	79%	110	21%

	Present	205	79%	56	22%
Differentiation (Grade)	Well/Moderate	469	78%	134	22%
	Poor	49	72%	19	28%

Table 2.5 Prevalence of sarcopenic obesity in relation to pathological stage

		Univariate analysis			Multivariate analysis			
Pathological Feature	BC Status	Chi-Square	p-value	Sig.	Odds Ratio	95% Confidence Interval	p-value	Sig.
T Stage 3 and 4	Age	2.87	0.090		1.01	0.997-1.024	0.142	
	Gender	0.552	0.457		1.06	0.78-1.44	0.711	
	ASA	1.521	0.219		0.979	0.616-1.554	0.927	
	Sarcopenia	5.726	0.017	*	1.154	0.823-1.619	0.406	
	Myosteatosis	10.63	0.001	**	1.387	0.961-2.002	0.081	
	Visceral Obesity	5.32	0.021	*	0.615	0.44-0.861	0.005	**
	Sarcopenic Obesity	3.676	0.055		1.625	1.071-2.465	0.023	*
Nodal Disease Present	Age	5.55	0.018	*	1.001	0.989-1.013	0.889	
	Gender	0.498	0.480		1.071	0.808-1.421	0.632	
	ASA	0.018	0.893		1.054	0.694-1.602	0.804	
	Sarcopenia	0.125	0.724		0.982	0.714-1.35	0.911	
	Myosteatosis	0.863	0.353		0.918	0.652-1.293	0.626	
	Visceral Obesity	13.492	0.0001	***	0.598	0.438-0.816	0.001	***
	Sarcopenic Obesity	0.568	0.451		1.145	0.778-1.685	0.492	
Metastases Present	Age	0.333	0.954		1.001	0.981-1.022	0.891	
	Gender	2.449	0.485		1.191	0.741-1.912	0.47	
	ASA	4.781	0.189		1.64	0.893-3.013	0.111	
	Sarcopenia	0.365	0.546		0.938	0.546-1.612	0.816	
	Myosteatosis	5.531	0.019	*	2.308	1.151-4.629	0.018	*
	Visceral Obesity	1.179	0.278		0.699	0.414-1.178	0.179	
	Sarcopenic Obesity	0.184	0.668		0.856	0.435-1.684	0.652	
	Age	1.770	0.183		1.006	0.994-1.019	0.325	

Vascular Invasion Present	Gender	0.698	0.403		1.148	0.856-1.541	0.356	
	ASA	4.917	0.027	*	1.5	0.987-2.28	0.058	
	Sarcopenia	3.962	0.047	*	1.123	0.803-1.572	0.497	
	Myosteatosis	10.647	0.001	**	1.494	1.032-2.163	0.034	*
	Visceral Obesity	6.692	0.010	**	0.634	0.458-0.877	0.006	**
	Sarcopenic Obesity	0.040	0.842		1.152	0.773-1.717	0.487	
Poorly differentiated tumour	Age	6.335	0.012	*	1.006	0.987-1.026	0.53	
	Gender	6.692	0.010	**	1.844	1.142-2.979	0.012	**
	ASA	1.413	0.235		1.006	0.484-2.091	0.987	
	Sarcopenia	0.329	0.566		0.943	0.558-1.593	0.826	
	Myosteatosis	2.286	0.131		0.483	0.272-0.856	0.013	**
	Visceral Obesity	3.498	0.061		0.491	0.282-0.856	0.012	**
	Sarcopenic Obesity	0.965	0.326		2.01	1.044-3.870	0.037	**

Table 2.6 Results of the univariate and multivariate analyses of body composition status and pathological features

2.4.3 Tumour invasion (T Stage) and CT Body composition

On binary logistic regression analysis, patients with visceral obesity were less likely to have a higher T stage (T3/4) OR 0.62 (95%CI 0.44-0.86, p=0.005). Sarcopenic obesity however was associated with advanced T stage (T3/4) OR 1.625 (95%CI 1.071-.2.465, p=0.023). Sarcopenia, myosteatosis, age, gender and ASA grade were not associated with T-stage.

2.4.4 Nodal metastases (N Stage) and CT Body Composition

Patients with visceral obesity were significantly less likely to have local mesenteric nodal involvement OR 0.60 (95%CI 0.44-0.82, p=0.001). Muscle specific body composition

parameters of sarcopenia OR 0.982 (95% CI 0.714-1.35, p=0.911), myosteatosis OR 0.918 (95%CI 0.652-1.293, p=0.626) and sarcopenic obesity OR 1.145 (95% CI 0.778-1.685, p=0.492) were not related to nodal involvement. As with T stage, age, gender and ASA grade were not associated with nodal involvement.

2.4.5 Vascular invasion (V Status) and CT Body Composition

A strong negative association between vascular invasion and visceral obesity was identified OR 0.63 (95%CI 0.46-0.88, p=0.006). Patients who were myosteatotic displayed a positive association with vascular invasion OR 1.494 (95% CI 1.032-2.163, p=0.034). Sarcopenia and sarcopenic obesity showed no association with vascular invasion OR 1.123 (95% CI 0.803-1.572, p=0.497) and OR 1.152 (95% CI 0.773-1.717, p=0.487). There were no associations between age, gender and vascular invasion, although a higher ASA grade (ASA 3 and 4) demonstrated a trend towards an association with vascular invasion OR 1.50 (95% CI 0.987-2.28, p=0.058).

2.4.6 Distant Metastases and CT Body Composition

Patients who were myosteatotic at the time of surgery were significantly more likely to have metastatic disease OR 2.31 (95%CI 1.15-4.63, p=0.018). There was no association between sarcopenia OR 0.938 (95% CI 0.546-1.612, p=0.816), visceral obesity OR 0.699 (95% CI 0.414-1.178, p=0.179) or sarcopenic obesity OR 0.856 (95% CI 0.435-1.684, p=0.652).

2.4.7 Tumour differentiation and CT Body Composition

Multiple body composition phenotypes were associated with tumour differentiation. Patients with myosteatosis were less likely to have poor tumour differentiation OR 0.48 (95%CI 0.27-0.86, p=0.013). Visceral obesity was also negatively associated with poor differentiation OR 0.49 (95%CI 0.28-0.86, p=0.012). Sarcopenic obesity was associated with poorly differentiated tumours OR 2.01 (95%CI 1.04-3.87, p=0.037) however, sarcopenia alone showed no association with tumour differentiation OR 0.943 (95% CI 0.558-1.593, p=0.826). Female patients also appeared to have an increased probability of a poorly differentiated tumour OR 1.844 (95% CI 1.142-2.979, p=0.012), age and ASA grade appeared to have no bearing on differentiation.

2.5 Discussion

This study has demonstrated that various body composition parameters are strongly associated with specific tumour characteristics and further adds to the literature by demonstrating that tumour characteristics may in part influence and be influenced by differing body composition factors and conversely that BC characteristics may influence outcomes independently of the prognostic potential of the tumour.

Visceral obesity defined using CT analysis was the body composition characteristic positively associated with tumours that are prognostically more favourable including better differentiated, earlier T stage (T1/2), and reduced likelihood of nodal metastases and vascular invasion. Visceral obesity may be a key metabolic factor impacting a tumour's biology predisposing to a more indolent malignancy. Paradoxically, obesity has an established association with the development of colorectal cancer and is seen as an important risk factor[36]. A 2018 review by Xiao and colleagues identified a number of

studies which assessed the effect of visceral adiposity on outcome[124]. Four of the six studies identified a higher mortality related to visceral adiposity in colorectal cancer, however, of the remaining two studies; one found increased survival to be stage dependent and the other found no difference. These studies had mixed methodologies and were inconsistent in their analyses and approach to BC definitions and measurement. The entire colorectal cohort in the review totalled 915 patients in all six trials, our single centre cohort of 795 patients is therefore more statistically robust[124]. A more recent and much larger study by Brown et al suggested the relationship is in fact more complicated; in their cohort of 3262 patients they described that subcutaneous adipose tissue but not visceral adipose tissue was prognostic of colorectal cancer-specific mortality, characterising them essentially as two distinct physiological entities[123]. Despite this, they also described that excess visceral adipose tissue was prognostic of all-cause mortality in a reverse L-shaped pattern which may account for results from the earlier studies.

The effect of visceral adiposity appears dependent on cancer type, in endometrial cancer, poorer outcomes are related to visceral adiposity and this has been hypothesised to be secondary to enrichment of gene sets related to immune activation and inflammation[156]. However, in renal cell carcinoma, its incidence is increased with increasing BMI but the resultant malignancy secondary to the tumourigenic environment born out of obesity are less agressive[124,157,158]. It would appear that colorectal cancer is very similar in this respect and it helps explain the findings of Brown et al[123].

Our findings highlight that the association between visceral obesity and survival is complex and suggest that the increased mortality associated with obesity is not likely to be a result of visceral adiposity.

The incidence of visceral obesity was similar between male and female patients; this does not seem to reflect the pattern of obesity seen in the general population[159]. This infers there is a preponderance towards colorectal cancer in women who are viscerally obese and hence we are seeing a proportional increase here in a subset of the population. This could also be explained potentially by the age of the cohort, as evidence suggest that visceral obesity increases in both men and women with age with one paper suggesting an increase in visceral fat of over 200% and 400% between the third and seventh decade in men and women respectively[160].

Our findings support previous work suggesting sarcopenic obesity is a prognostic marker associated with poorer outcomes in colorectal cancer[151], as sarcopenic obesity was positively associated with advanced T stage. This finding may be explained in part by fat distribution; patients with sarcopenic obesity may carry fat outside the abdominal cavity i.e., subcutaneous fat, this fat may behave in a way which is metabolically more insidious than visceral fat in the presence of a colorectal tumour.

Tumour characteristics were not dependent upon sarcopenia yet its presence is known to be associated with a poorer prognosis in multiple cancer types including colorectal, breast, lung and ovarian[97–99,161]. It would seem reasonable to propose that the sarcopenia and the tumour biology in the surgically treated colorectal cancer patient are independent of one another. Skeletal muscle loss and the mechanism for sarcopenia and its associated poorer prognosis may be brought about through other environmental factors such as malnutrition or inactivity and not related to the tumour itself[87]. This, in turn, adds weight to the argument that sarcopenia is reversible without the need to remove the primary tumour and as such a patient's skeletal muscle status can be addressed whilst waiting to treat the tumour i.e., through prehabilitation. Indeed, it could be strongly

argued that delaying definitive treatment i.e., surgery is merited to potentially allow reversal or improvement of this established poor prognostic feature.

Systemic inflammation is a poor prognostic feature in colorectal cancer[162]. Myosteatosis as a surrogate biomarker of systemic inflammation is also associated with poor prognosis in colorectal cancer[101,127]. It is therefore surprising that poorly differentiated tumours are not associated with myosteatosis as one would expect tumours that are poorly differentiated to have a greater proinflammatory potential as their phenotype differs increasingly from that of the host. It is unexpected that myosteatosis and tumour phenotype do not demonstrate a stronger link, particularly in view of its associations with systemic inflammation and outcome, in general. In turn, this questions what aspect of tumour biology is implicated in the systemic inflammatory response and whether tumour grade is the most important factor in relation to inflammation. Our findings do suggest that patients with myosteatosis are more likely to have metastatic disease and this finding may go some way to explaining at which point myosteatosis occurs in the tumour evolution. It is however unexpected that there appears to be no relationship between local mesenteric nodal invasion and myosteatosis as there is a significant body of evidence linking nodal involvement to the systemic inflammatory response in rectal cancer[163]. However, there is also conflicting evidence for colon cancer [164].

2.6 Limitations

This study was performed retrospectively on a prospectively collected dataset, however the absence in the large dataset of data fields, especially height and weight data will impact on the strength of its findings. The data contained within this data set however are robust in view of the prospective nature of the collection and the final data set

represent one of the largest colorectal surgical data sets with body composition parameters, to date. Survival analyses have not been reported here and have been reported previously from this cohort in other published work[111].

2.7 Conclusions

We have demonstrated that CT defined visceral obesity in colorectal cancer may predispose to tumours of a more favourable grade and stage. Of all the body composition phenotypes, it appears to be most strongly associated with prognostic characteristics of colorectal cancer. We surmise that visceral obesity determines the nature of the tumour and conversely the tumour can then convey features upon an individual's BC phenotype; for example, myosteatosis as a result of tumour metastasis which we will explore in more detail in Chapter 3. Further work is required to assess the links between environmental, socioeconomic status and tumour phenotype as this may confound the findings associated with visceral obesity. These concepts are therefore explored in depth in Chapter 5 and 6.

Chapter 3

3 Myosteatosis presages distant recurrence in patients with colorectal cancer: an argument for radiomic muscle assessment and enhanced surveillance in myosteatotic patients

3.1 Summary

In Chapter 1 associations were described between features of body composition and outcome. There is strong evidence in the scientific literature to suggests that survival outcomes are worse in patients who are either sarcopenic, myosteatotic or have sarcopenic obesity. The influence of visceral obesity on outcomes remains controversial. In Chapter 2 we demonstrated that body composition is related to the stage of the disease with patients who were myosteatotic prior to surgery being more likely to have metastatic disease at presentation. In this chapter we aim to examine this concept in depth and to explore whether we can predict the presence of metastatic disease, in patients with no evidence of metastasis at presentation, based on tumour features and body composition and in doing so propose BC phenotype as a novel radiomic biomarker.

3.2 Introduction

Colorectal cancer (CRC) is the second most common cause of cancer death in the United States[165]. Surgical resection remains the mainstay of treatment with the 5-year relative survival rate far greater in those presenting with localised disease (90%) compared to those with distant disease (14%)[165,166]. However, despite screening, early detection, resection and advances in chemoradiotherapy, recurrence rates have still been reported as being up to 50-60%[167]. Recurrence can be defined as either local, regional or distant and is reliant both on patient, tumour and treatment factors. Identification of prognostic factors for recurrence might improve survival rates in patients with CRC after apparently curative resection, as this would permit earlier detection and hence treatment.

Local recurrence occurs at the tumour site – within the surgical resection bed or at the site of anastomosis, either secondary to seeding of viable tumour cells or regrowth of residual viable tumour. Distant recurrence is purely a result of metastasis. For metastases to occur certain epigenetic and genetic mutations as in EGFR or TGFβRII must occur within the tumour cells[12]. These mutations allow seeding, implantation and propagation to tissue sites distinct from the primary site[168]. It has been postulated that some tumours have an inherent potential to metastasise and do so early. One study by Hu and colleagues provides compelling evidence that metastatic seeding occurs very early in most cases, when the neoplastic mass comprises $<10^6$ cells[169]. Metastatic spread occurs via numerous routes including trans-coelomic, lymphatic, vascular or a combination of these. Colorectal cancer commonly metastasises to liver, lung, peritoneum and bone. Subtle variations in metastasis site exist based on the primary cancer subtype and anatomical location[170].

Survival with recurrence is improving, however, it remains the major cause of mortality in patients with disease and significantly impacts the quality of life and psychosocial wellbeing of patients[171].

Computed Tomography (CT) is perhaps the best technique that can reliably measure muscle and fat distribution in the trunk and can discriminate between the intra-abdominal organ and muscle component of fat-free mass[172]. As such, CTBC analysis has the potential to be a radiomic biomarker, particularly with the evolution in artificial intelligence and deep learning[173]. Sarcopenia is associated with increased post-operative complications and decreased overall survival[174]. Myosteatosis, fatty infiltration of muscle and a marker of poor muscle quality is associated with worse survival independent of sarcopenia and is associated with metastatic disease at the time of presentation (Chapter 2)[117]. Both of these putative radiomic biomarkers are associated with a heightened inflammatory state as demonstrated by a raised neutrophil-to-lymphocyte ratio [NLR], raised modified Glasgow Prognostic Score (mGPS) or a raised platelet to lymphocyte ratio [PLR][126,153,175,176].

Colorectal cancer is associated with raised BMI[36] and both visceral and subcutaneous adipose tissue have been found to be prognostic of mortality in patients with stage I-III colorectal cancer[123]. Radiological features of adipose tissue are often considered in terms of distribution, either visceral or subcutaneous and are related to outcome in colorectal cancer[123]. As discussed in Chapter 1 sarcopenic obesity (SO) describes the poor prognostic combination of sarcopenia and BMI defined obesity, patients with this BC phenotype have a “special risk” for mortality and severe complications in systemic and surgical cancer treatment[104].

Body composition factors have a well-documented association with outcomes and survival. We aimed to assess whether body composition was associated with future distant metastatic disease development and whether a suitable body composition biomarker could be incorporated into risk prediction models of future recurrence or to identify patients for enhanced follow up.

3.3 Method

3.3.1 Dataset

Analysis was performed the prospectively collected database of patients who underwent curative colorectal cancer resections by two colorectal cancer surgeons at St Mark's Hospital from May 2007 to January 2017. All cases meeting the inclusion criteria (Table 3.1) were analysed.

Inclusion	**Exclusion**
Primary colorectal cancer (adenocarcinoma)	Known metastases at presentation
No metastatic disease reported preoperatively	Any locally recurrent disease[§]
Age > 18	Non-adenocarcinomas (e.g. carcinoid)
Curative resection	No recurrence status recorded
Surgical procedure performed by either JTJ/RHK	
Recurrence status recorded	

[§]*Defined as the regrowth of tumour in and around the tumour bed, including the pericolic fat, the adjoining mesentery and lymph nodes (extramural recurrence), or in the suture or staple line of the bowel anastomosis (intramural recurrence)*[177].

Table 3.1 Inclusion and exclusion criteria

Data collected prospectively during the perioperative period included: age, gender, ethnicity, BMI, the American Society of Anaesthesiologists (ASA) physical status classification system grading. Tumour related factors included tumour anatomical

location, TNM stage (Union for International Cancer Control (UICC) 5th edition), presence of vascular invasion and tumour differentiation grade. Other data included anastomotic leak status and post-operative complications using the Clavien-Dindo (CD) classification[120]. All prospectively collected data were revalidated from the hospital electronic records prior to analysis.

3.3.2 Recurrence

Distant recurrence was defined as any macroscopic tumour deposit, distant from the primary site, reported in a patient who had undergone radical (curative) surgery for CRC most often confirmed on CT scanning. Recurrent disease was determined prospectively, missing data was sought from contemporaneous clinic letters, radiological follow up and communications from other healthcare providers. Recurrence data was further validated using registry data from the Public Health England Cancer Registry. The censor date for recurrence was the 13/05/19. To provide clarity within the analysis, locally recurrent disease, define by Abulafi and Williams[177], was excluded from the analysis as the pathways behind the formation of recurrence are different to distant recurrence and as such should be classed as separate phenomena as discussed in the introduction.

3.3.3 Assessment of body composition

Assessment of body composition was performed by the same method described in Chapter 2. Sarcopenia, myosteatosis, sarcopenic and visceral obesity were defined using the same validated predefined cut-off values[76,103].

3.3.4 Statistical analysis

Data were categorised where appropriate (see Table 3.2), age (<50 / =>50), ASA (1,2 / 3,4,5), tumour grade (well, moderate / poor differentiation) T Stage (T1,2 / T3,4) and major morbidity (CD1,2 / CD3,4) were converted into binary variables as described. All other variables were binary by nature or analysed in multiple categories.

Statistical analysis was performed using IBM SPSS Statistics version 25 (*IBM Corp, Armonk, NY)*. Univariate analysis was performed using Chi-square and Kaplan-Meier survival curves to determine the effect of individual BC types on distant recurrence. These analyses were used to construct a multivariate model for Cox regression analysis. The model contained significant factors derived from the univariate analysis and factors of clinical importance such as tumour grade, age, gender and comorbidity (ASA) that did not meet statistical significance in univariate analysis. These factors are known to impact on body composition and as such it was considered important to recognise their impact in the multivariate model.

In addition to the BC parameters the final model incorporated the following patient and tumour factors: age, gender, ASA grade, T-stage, nodal disease, vascular invasion, tumour differentiation (grade) and post-operative anastomotic leak status.

3.3.5 Ethical Approval

Approval was obtained for use of the prospective database in research by the NHS Health Research Authority, UK with ethical approval from the South East London NHS Research Ethics Committee (reference number: 12/LO/1556).

3.4 Results

3.4.1 Demographics

1403 patients underwent surgery between 2007 and January 2017, 57 patients were excluded for recurrent disease at presentation, 47 had local recurrence and we were not able to obtain recurrence data for 243 patients. 1056 patients were included in the analysis, the median age was 68 years [IQR 60-76] and 57% were male. 72% of patients were myosteatotic; 66% sarcopenic; 25% were classified as obese with a BMI> 30; 53% were classified as viscerally obese and 18% had sarcopenic obesity. 157 (15%) patients suffered distant recurrence. Demographics are shown in Table 3.2.

		n	%
Age at diagnosis years	<50	106	10.10%
	50+	950	89.90%
Gender	Male	602	57.00%
	Female	454	43.00%
Ethnicity	White	595	66.70%
	Asian or Asian British	179	20.10%
	Black or Black British	74	8.30%
	Other Ethnic Group	44	4.90%
ASA	ASA 1&2	869	85.40%
	ASA 3&4	148	14.60%
Myosteatosis	Not Myosteatotic	232	28.10%
	Myosteatotic	594	71.90%
Sarcopenia	Not Sarcopenic	276	34.40%
	Sarcopenic	526	65.60%
Visceral Obesity	Not Viscerally Obese	404	46.90%
	Visceral Obesity	457	53.10%
BMI Obesity	BMI<30	613	75.20%
	BMI>30	202	24.80%
Sarcopenic Obesity	S-Ob not present	646	81.90%
	S-Ob Present	143	18.10%
T Stage	T1-2	334	36.20%
	T3-4	589	63.80%
Nodal Disease	Node Negative	644	64.00%
	Node Positive	363	36.00%
Vascular Invasion	Absent	672	67.10%
	Present	330	32.90%
Anastomotic Leak	No Leak	918	91.60%
	Leak	84	8.40%
Major Morbidity	No Major Morbidity (CD 0,1&2)	835	87.20%
	Major Morbidity (CD3,4 & 5)	123	12.80%
Distal Recurrence	No Distant Recurrence	899	85.10%
	Distant Recurrence	157	14.90%

Table 3.2 Patient Demographics

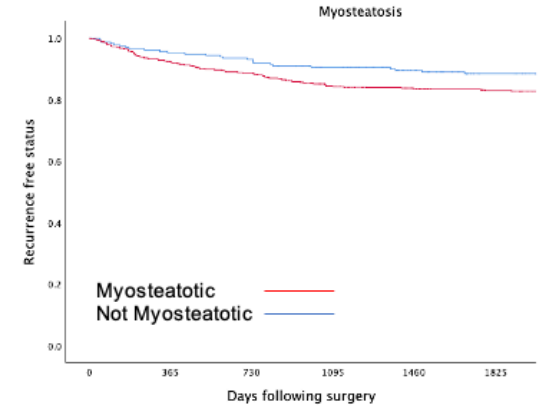
3.4.2 Host Factors and Recurrence

The univariate analyses of host factors and recurrence are shown in Table 3.3 The demographic parameters of age, gender and ethnicity were not related to distant recurrence. High ASA grade, a surrogate measure of pre-operative comorbidity, was significantly associated with distant recurrent disease (p=0.03). Kaplan Meier survival curves for body composition parameters and distant recurrence are shown in Figure 3.1. On univariate analysis preoperative myosteatosis (p=0.04) and visceral obesity (p=0.005) were found to be the only body composition factors associated with distant recurrence. Visceral obesity appeared to confer a protective effect (Figure 3.1d.) whilst the presence of myosteatosis was associated with an increased incidence of distant recurrence (Figure 3.1a.). Neither sarcopenia (p=0.89) nor BMI obesity (p=0.30) showed a significant relationship with recurrence (Figure 3.1b & c.). Sarcopenic obesity was not associated with distant recurrent disease (p=0.81) (Figure 3.1e.). On multivariate Cox regression analysis (Table 3.4) myosteatosis was the only BC phenotype to presage recurrence; HR 2.29 [95%CI 1.087-4.830, p=0.029]. VO ceased to retain its statistical association from the univariate analysis when considered as part of the multivariate model HR 0.764 [95%CI 0.420-1.388, p=0.376].

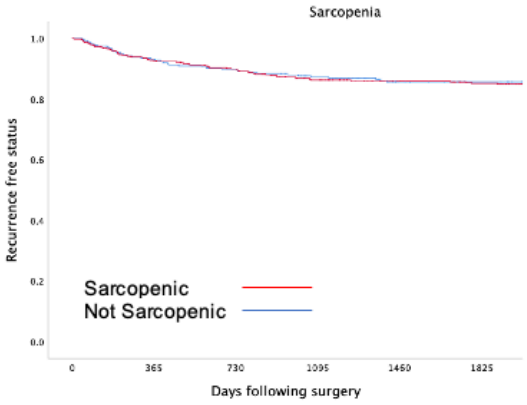
	Chi-Square	**p value**	**Sig.**
Host Factors			
Age	<0.001	0.997	
Gender	1.643	0.2	
Ethnicity	1.771	0.621	
ASA	4.828	**0.028**	*
Myosteatosis	4.159	**0.041**	*
Sarcopenia	0.019	0.891	
BMI > 30	1.092	0.296	
Visceral Obesity	7.925	**0.005**	**
Sarcopenic Obesity	0.059	0.808	
Tumour Factors			

Tumour location	0.462	0.794	
Grade	1.034	0.309	
T Stage	26.444	**<0.001**	***
Nodal Disease	91.485	**<0.001**	***
Vascular	72.693	**<0.001**	***
Technical & Surgical Factors			
Laparoscopic vs. open	1.34	0.247	
Anastomotic Leak	5.186	**0.023**	**
Major morbidity	2.303	0.129	

Table 3.3 Univariate analysis of factors associated with distant disease recurrence



a.



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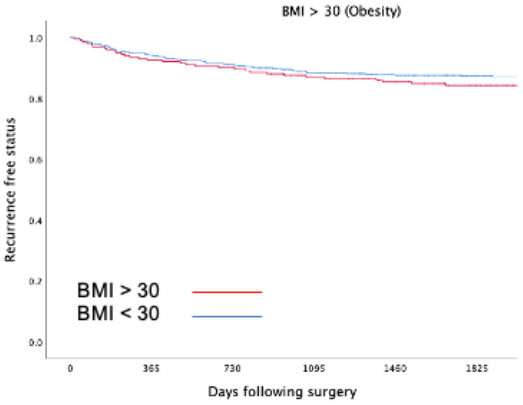

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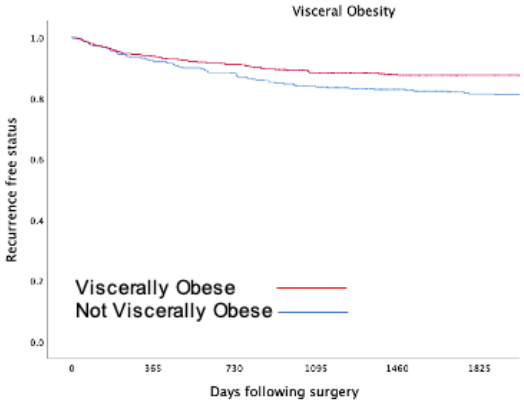

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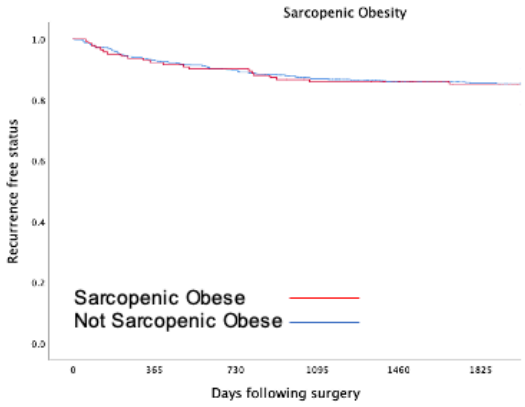


e.

Figure 3.1 (a-e.) Kaplan Meier survival curves body composition and recurrent distal disease

3.4.3 Tumour Factors and Recurrence

Advanced T stage (p=<0.001), nodal positivity (p=<0.001) and vascular invasion (p=<0.001) were all significantly associated with distant recurrent disease on univariate analysis (Table 3.3 and Figure 3.2 b, c & d.). Nodal disease HR 2.468 [95%CI 1.360-4.47, p=0.003] and vascular invasion HR 2.30 [95%CI 1.24-4.25, p=0.008] remained strongly associated with distant metastases on multivariate Cox regression analysis. The significant association between advanced T stage and recurrence was lost on multivariate analysis (Table 3.4).

Variable	**Hazard Ratio**	**95.0% CI Lower**	**95.0% CI Upper**	**p value**	**Sig.**
Advanced Age	0.476	0.187	1.213	0.12	
Female Gender	0.62	0.338	1.138	0.123	
ASA Grade 3&4	1.602	0.841	3.051	0.152	
Myosteatosis	**2.291**	**1.087**	**4.83**	**0.029**	*
Sarcopenia	0.564	0.27	1.178	0.127	
Obesity	1.724	0.79	3.763	0.172	
Visceral Obesity	0.764	0.42	1.388	0.376	
Sarcopenic Obesity	1.154	0.419	3.183	0.781	
Advanced T Stage	1.286	0.598	2.762	0.52	
Nodal Disease	**2.468**	**1.36**	**4.476**	**0.003**	**
Vascular Invasion	**2.298**	**1.242**	**4.251**	**0.008**	**
Poor Differentiation	0.628	0.22	1.791	0.384	
Anastomotic Leak	**2.703**	**1.229**	**5.944**	**0.013**	**

Table 3.4 Cox regression multivariable model of factors associated with recurrent disease

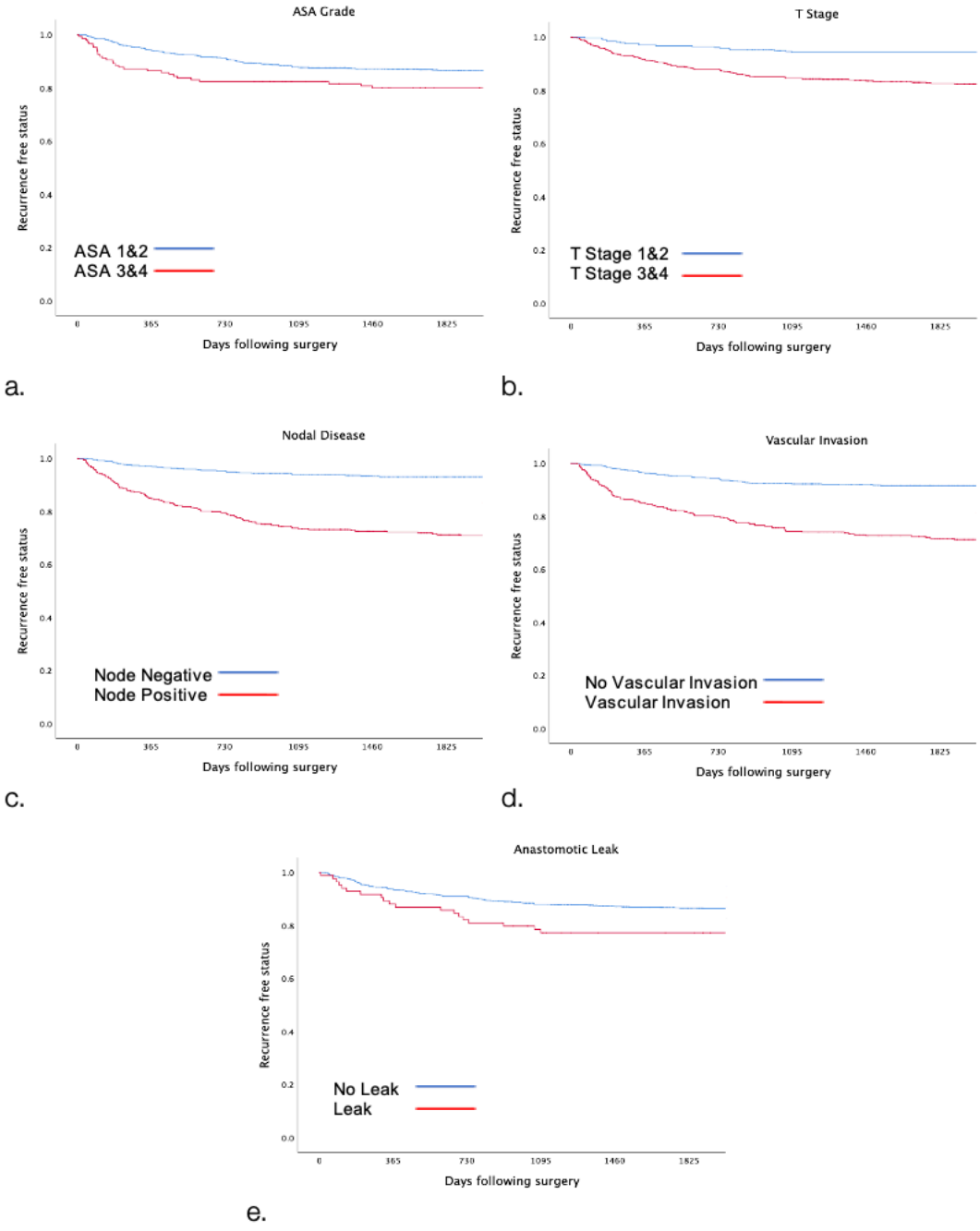


Figure 3.2 (a-e) Kaplan Meier survival curves for other significant variables and distant recurrence

3.4.4 Technical and Postoperative Factors

The approach to surgery (either laparoscopic or open) had no bearing on future distant recurrent disease in our data set (p=0.25). Major post-operative morbidity,

based on Clavien-Dindo classification grade III and above, was not associated with recurrent disease (p=0.13). Anastomotic leak was associated with distant recurrent disease both on univariate analysis (p=0.023) (Figure 3.2e.) and on multivariate analysis HR 2.70 [95%CI 1.23-5.94, p=0.013].

3.5 Discussion

Oncological surveillance and follow up consumes significant resources. Identifying the highest risk individuals for recurrence has the potential to reduce cost and resources. Few clinicians restrict colorectal cancer follow-up visits to clinical examination only and perhaps unnecessarily, order "routine surveillance". Patients prefer specialist investigation as there is often a desire to have a proven negative test result[167].

Distant metastasis is a major determinant of 5-year survival following radical colorectal cancer surgery[178]. Kievit suggested that earlier recognition of cancer recurrences and metastases may have a benefit upon outcome although minimal; a 1% increase in survival[179]. Preventing recurrent disease is therefore relevant and the primary purpose of adjuvant treatment following definitive surgery for bowel cancer. A decision to include adjuvant therapy in a patients' treatment is currently decided based upon both the radiologically identified disease, histopathological findings and physical performance of the patient[180]. All these factors are presented to the patient and a decision is made in combination with the individual's personal wishes. The decisions to treat are based upon a perceived and statistical risk of recurrence. Inevitably there will be some individuals given treatment who benefit and others who will not. Identifying other metrics which may reduce the number needed

to treat (NNT) for potential clinical benefit. This may be achieved through the addition of new variables to existing risk prediction models. Such models will be enhanced with technological advancements such as artificial intelligence. We have seen from our results that myosteatosis, identified on CT using body composition analysis, is one such biomarker. The prognosis from cancer recurrence deteriorates over time from onset, therefore earlier detection and resection might improve outcome[179] and therefore finding new methods of identifying higher risk individuals is needed.

Multivariate analysis identifies myosteatosis to be the only body composition parameter associated with recurrence; as important as tumour differentiation or advanced T stage (T3 and T4) in predicting distant recurrent disease. An advanced T stage, significant on univariate analysis, lost significance in the multivariate model and is likely to be because advanced T stage is related to nodal disease and vascular invasion and such factors influence the development of distant metastases. Adequate resection should ensure a tumour of advanced T stage is pathologically completely removed and thus reduces the specific risk of distant metastases.

Several potential explanations for our findings exist. Myosteatosis is associated with an inflammatory response to cancer, yet another poor prognostic marker. This suggests that certain cancers either have a proinflammatory phenotype or have a phenotype which provokes an excessive immune response[127,152]. From these data it appears that in proinflammatory tumours, recurrence is more likely to be in the form of distant metastasis. These poorly prognostic or “bad tumours”, identifiable by their systemic manifestations, may require more intensive therapy to mitigate future distant disease. A systemic inflammatory response may also suggest a dysfunctional host

immune system, or an immune system that is unable to appropriately identify and destroy circulating and seeding tumour cells. We know Immunological deficits are identifiable in patients with myosteatosis including a significantly increased expression of fat scavenger receptors (a marker of potential dysfunction) on the antigen presenting dendritic cells in myosteatotic patients[139]. This suggests that the immune system within myosteatotic patients may be unable to identify pathological antigens and present them appropriately to the downstream effector cells (T and B Cells) of the immune system. We have therefore aimed to interrogate this further in the work presented in Chapters 7 and 8.

Conversely myosteatosis may be a sign of established metastases; if metastasis is an early process in the pathway of a tumour (when tumour cell number is less than 10^6) as suggested by Hu and colleagues[169] then, by presentation, we would expect a significant proportion of our patients to have undetected metastases. Is the poor prognostic marker, myosteatosis, a manifestation of this and could this in turn explain the minimal benefit of surveillance by Kievit[179] as the die may already have been cast? This in turn may justify the use of more aggressive adjuvant treatment in the myosteatotic patient. It is important to recognise that individuals with deficient muscle volume tolerate chemotherapy doses based standard dosing regimens less well than those without sarcopenia[181] and further work is been undertaken to explore more accurate methods of dose calculation in these patients by colleagues in our group at St Mark's.

On univariate analysis visceral obesity appeared to be protective against metastatic disease however this association was lost on multivariate analysis. The obesity paradox suggests that, although obesity increases the risk of cancer, these patients

fare better from the disease once it establishes[107,145]. The loss of importance of the protective effect of VO on multivariate analysis may be due to thinner patients having more advanced disease - i.e., cancer cachexia and it is the advanced disease that is more important that body composition in determining risk of recurrent disease. The same may be true of ASA grade; the frailer patient with more advanced disease is likely to be assigned a higher ASA grade and as such tumour factors may be more important in determining recurrence than systemic disease (i.e., ASA grade).

Our findings corroborate others in the literature that anastomotic leaks appear an important predictor of future recurrent distant disease[182], again there are strong associations between the immune modulatory effects brought about by the acute sepsis of an anastomotic leak. This fits in with the discussion about immune dysfunction in myosteatotic patients. After surgery there is the potential for cancer cells shed during surgery to implant elsewhere, either locally or at distant sites. In a host, who's immune system is not compromised by overwhelming infection, these circulating cells are more likely to be destroyed and hence we see the risk of recurrence increase in those with anastomotic leaks. Interestingly the same is not true with major morbidity. This may be in part because the Clavien-Dindo method for recording post-operative morbidity does not discriminate well enough between conditions which lead to an immune response – for example a wound infection opened at the bedside is classified as Clavien-Dindo 1 whilst radiological drainage of a deep abscess is classified as Clavien-Dindo 3. Both these conditions can provoke a significant immune response and as such both will both have implications to the host's immune system's response to tumour cells.

Nodal disease and vascular invasion remain important predictors of recurrent disease and their continued strength of association on multivariate analysis adds further validity to our model. The rationale for the impact of these factors on recurrence has been previously described but such tumours are biologically advanced in their nature and are mature in the pathway of genetic mutation that facilitates further distant disease. These factors are already prominent in the adjuvant therapy decision making process.

3.6 Limitations

Both the population demographics and incidence of distant recurrence in our cohort, although lower than the estimates detailed above, are similar to that of the more recent published literature[183] that allow us to draw comparisons with other colorectal cancer patient populations. We accept, however, that recurrence data can never be perfect and despite validation through national registers some patients will be lost to follow up and some recurrences will remain undetected. Also, in view of the size of the cohort, these potentially missed recurrences are unlikely to have a significant effect on our results.

Further work in this area could involve validation of these findings by combining other large international prospectively collected datasets of BC in CRC. Further work should also consider factoring these data into risk prediction algorithms.

3.7 Conclusions

Pro-inflammatory tumours and a systemic inflammatory milieu are associated with myosteatosis. On multivariate analysis preoperative myosteatosis is a significant

predictor of distant recurrence in colorectal cancer. When deciding on adjuvant treatment, myosteatosis, as a radiomic biomarker, should be considered as a risk factor, potentially lowering the threshold to treat. Patients with myosteatosis should also be considered for enhanced surveillance. As the use of artificial intelligence [AI] in medicine increases, radiomic biomarkers such as myosteatosis should be considered in risk prediction models for CRC recurrence.

Chapter 4

4 Sarcopenia in colorectal cancer is dependent on social deprivation in colorectal cancer and BMI alone misrepresents underlying muscle loss in the deprived

4.1 Summary

Chapters 2 and 3 have demonstrated that there is an intimate relationship between myosteatosis, visceral obesity and tumour biology. However, the other major determinant of outcome we considered, sarcopenia, does not seem to be affected by tumour characteristics in our population. We therefore must consider other factors which may be driving poorly prognostic BC phenotypes in our CRC patient population. Socioeconomic deprivation encompasses both variations in lifestyle and the environment between patient populations. We therefore explored the relationship between socioeconomic deprivation, utilising the index of multiple deprivation and its constituent parts to ascertain the effect of environment on BC in our patient population.

4.2 Introduction

Deprivation has long been associated with both incidence and death rate in a number of cancer primary sites[184], including colorectal cancer. These differences in survival cannot be explained by differences in cancer stage alone which suggests an environmental factor associated with deprivation may be influencing outcome[185].

One notable difference described between those who are deprived and those of greater affluence is body mass index (BMI). It has long been recognised that those who are from a more deprived background have a higher BMI, in the UK 35% of men and 37% of women living in the most deprived areas were obese compared with 20% of men and 21% of women in the least deprived areas[186]. This is felt to be down to a number of factors including diet, lifestyle, exercise and access to education[187,188]. There have been no previous studies examining the fuller spectrum of body composition in terms of low muscle quantity (sarcopenia), quality (myosteatosis) and fat distribution in either the healthy or diseased population. As discussed in the preceding chapters there is high quality meta-analytical evidence that these body composition phenotypes profoundly affect outcome and survival in multiple cancer types[119,189–192]. In colorectal cancer, sarcopenia and myosteatosis have been found to be associated with poor overall survival and disease-free survival[103,111,127,150]. Sarcopenic obesity, sarcopenia in the presence of a BMI of greater than 30, has been found to have a particularly poor prognostic outcome[77,151]. It is however, well established that obesity increases your risk of developing a number of cancers including colorectal cancer[36]. The role of visceral obesity and BMI obesity remain contentious and in certain primary cancer types, obesity appears protective such as in renal cell carcinoma, this appears to be the case in our cohort as described in

Chapter 2 and 3 where we saw less aggressive tumour behaviour associated specifically with visceral obesity. In other cancer types it conveys a poorer prognosis as discussed in Chapter 1. This paradox in RCC has been discussed in Chapter 1. One further hypothesis put forward to explain this paradox is the fact that although obesity increases the risk of RCC the associated insulin resistance secondary to obesity may be protective though elevated levels of IGF-1[193]. Less is known about the effect of obesity subtypes in colorectal cancer but it is unclear if a similar biochemical mechanism occurs too. It would therefore be important to examine obesity in relation to deprivation in the colorectal cancer population.

Deprivation is measured in several ways and these vary depending on country and region and approach. Within the UK, deprivation is measured differently among its four constituent countries. In England, the Index of multiple deprivation (IMD) is used. The IMD is the official measure of relative deprivation used in England. This is constructed from data collected in four yearly censuses performed by the UK government[194]. The country is divided into 32,844 small geographical areas based on postal code with each area consisting of approximately 650 households, these are known as Lower-layer Super Output Areas (LSOA)[195]. The Index of Multiple Deprivation ranks each area in England from most deprived to least deprived area. These can then be divided into ranked cohorts e.g., deciles, quintiles etc. The IMD itself is constructed using seven domains, each weighted depending on the significance each one imparts to the final index. The domains and respective weights (given as a percentage) when combined to formulate the final IMD are shown in Table 4.1.

Income Deprivation (22.5%)
Employment Deprivation (22.5%)
Education, Skills and Training Deprivation (13.5%)
Health Deprivation and Disability (13.5%)
Crime (9.3%)
Barriers to Housing and Services (9.3%)
Living Environment Deprivation (9.3%)

Table 4.1 Domains of IMD[195]

In addition to these seven domains there are two further supplementary indices the Income Deprivation Affecting Children Index (IDACI) and the Income Deprivation Affecting Older People Index (IDAOPI). These are created by taking the age specific subsets from the income deprivation domain. We shall not be using the IDACI further within this thesis as it pertains to a paediatric population. The IDAOPI is of more relevance and is discussed further later in this chapter.

In Chapters 2 and 3 we identified that myosteatosis and VO were related to tumour biology. Myosteatosis appeared to be a poor prognostic feature and VO appeared to confer a prognostic benefit. Sarcopenia and BMI appeared to be less dependent on the tumour itself yet remains an important prognostic feature. This poses the question, does the environment play a more significant role in developing sarcopenia in CRC or indeed is sarcopenia a premorbid state in those who are more deprived? This study aims to ascertain whether deprivation, as recorded by IMD, is related to poor prognostic body composition phenotypes in CRC. This may in turn help explain

why patients from deprived groups have a poorer outcome and potentially may demonstrate a target population for enhanced prehabilitation.

4.3 Method

4.3.1 Patient Population

Using data within our cohort from Chapters 2 and 3 we identified 971 consecutive patients undergoing primary colorectal cancer surgery between May 2007 and September 2014. Patients with anthropometric data (height, weight and BMI), digital preoperative CT images, home postal code and P-POSSUM mortality risk estimate were assessed. Patients were excluded with recurrent disease; our complex and advanced rectal cancer cohort were not included in this study; all other exclusions criteria were as Malietzis *et al* 2016[111]. Following exclusions 419 patients were included in the final analysis. All prospectively collected data were revalidated from hospital electronic records prior to analysis.

Data collected prospectively during the perioperative period (within 30 days of surgery) included age, sex, BMI, the American Society of Anaesthesiologists (ASA) physical status classification system, P-POSSUM mortality risk score[196] and post code at the time of surgery. P-POSSUM scores were chosen in this instance as it gave us a more accurate representation of premorbid state for our final analysis. P-POSSUM risk was divided into three categories, those with a 1% (low risk of death), those with a 5% or less risk of death (moderate risk) and those with a greater than 5% (high risk) of death.

4.3.2 Deprivation Data

Post codes were used to obtain the 2015 deprivation data via the UK Ministry for Housing, Communities and Local Government Portal (https://imd-by-postcode.opendatacommunities.org/imd/2015). Data was returned in a Microsoft Excel Spreadsheet Format and contained the information shown in Table 4.2.

Postcode
Postcode Status
LSOA code
LSOA Name
Index of Multiple Deprivation Rank
Index of Multiple Deprivation Decile
Income Rank
Income Decile
Income Score
Employment Rank
Employment Decile
Employment Score
Education and Skills Rank
Education and Skills Decile
Health and Disability Rank
Health and Disability Decile
Crime Rank
Crime Decile
Barriers to Housing and Services Rank
Barriers to Housing and Services Decile
Living Environment Rank
Living Environment Decile
IDACI Rank
IDACI Decile
IDACI Score
IDAOPI Rank
IDAOPI Decile
IDAOPI Score

Table 4.2 Data fields collated from the Ministry for Housing, Communities and Local Government 2015 Census

The decile data was then assigned to each patient for each domain allowing us to assign a deprivation status to each patient. To allow binary regression analysis a decision was made to divide the population in those who fall into the bottom 50%

most deprived (Deciles 1-5) and those in the top 50% least deprived (Deciles 6-10). Figure 4.1 demonstrates the distribution of deprivation in our population and as such a binary split was deemed most appropriate.

4.3.3 CT Body Composition Analysis

CT analysis was performed as in Chapters 2 where there is a detailed description of the method.

4.3.4 Ethical Approval

Approval was obtained for use of the prospective database in research by the NHS Health Research Authority, UK with ethical approval from the South East London NHS Research Ethics Committee (reference number: 12/LO/1556).

4.3.5 Statistical Analyses

Statistical analyses were performed using *SPSS v25.0* (*IBM Corp, Armonk, NY*). Body composition parameters are presented as medians and interquartile ranges (IQR) and are categorized into sex-specific groups. Grouping of the other variables was carried out using standard or previously published thresholds. Data were analysed using binary logistic regression, the model taking into account age, gender, pre-morbid physiology using P-POSSUM mortality risk percentage as a surrogate and body composition features of sarcopenia, myosteatosis, visceral obesity, BMI and sarcopenic obesity. The model was formulated using data from previous univariate and multivariate regression analyses of this cohort[111,151,197].

4.4 Results

4.4.1 Exclusions

Of the 971 patients operated on at our institution between May 2007 and September 2014 P-POSSUM data were absent for 298, 217 patients lacked either CT scans of adequate quality to perform BC analysis or did not have BMI data. We did not hold the UICC stage data for 30 patients and we did not have IMD data for seven patients.

4.4.2 Demographics

419 patients were included in the study, 54% of the population was male, the median age was 68 for men [IQR 67-69] and 70 for women [IQR 68-73]. 74% of the male population was sarcopenic compared to 53% of the female population; 69% of the male population were myosteatotic compared to the female 82%. BMI and VO in the male population was 24% and 53% respectively compared 27% and 62% in the female population and SO was seen in 12% of the Male Population compared to 8% of the female. Demographics are shown in Table 4.3 a & b. Figure 4.1 demonstrates the proportion of the cohort population within the IMD.

Gender						
	Male			Female		
	Median	IQR		Median	IQR	
Age	68	67	69	70	68	73

a.

		Gender			
		Male		Female	
		n	%	n	%
P-POSSUM	<1%	73	30.7	59	32.6
	<5%	135	56.7	99	54.7
	>5%	30	12.6	23	12.7
UICC Stage	Stage 1	66	27.7	52	28.7
	Stage 2	66	27.7	52	28.7
	Stage 3	87	36.6	62	34.3

	Stage 4	19	8.0	15	8.3
Sarcopenia	Not Sarcopenic	62	26.1	85	47.0
	Sarcopenic	176	73.9	96	53.0
Myosteatosis	Not Myosteatotic	75	31.5	33	18.2
	Myosteatotic	163	68.5	148	81.8
Obesity	BMI<30	181	76.4	131	72.8
	BMI>30	56	23.6	49	27.2
Visceral Obesity	Not Viscerally Obese	110	47.4	68	38.4
	Viscerally Obese	122	52.6	109	61.6
Sarcopenic Obesity	S-Ob Not Present	208	87.8	165	91.7
	S-Ob Present	29	12.2	15	8.3

b.

Table 4.3 (a & b). Demographics

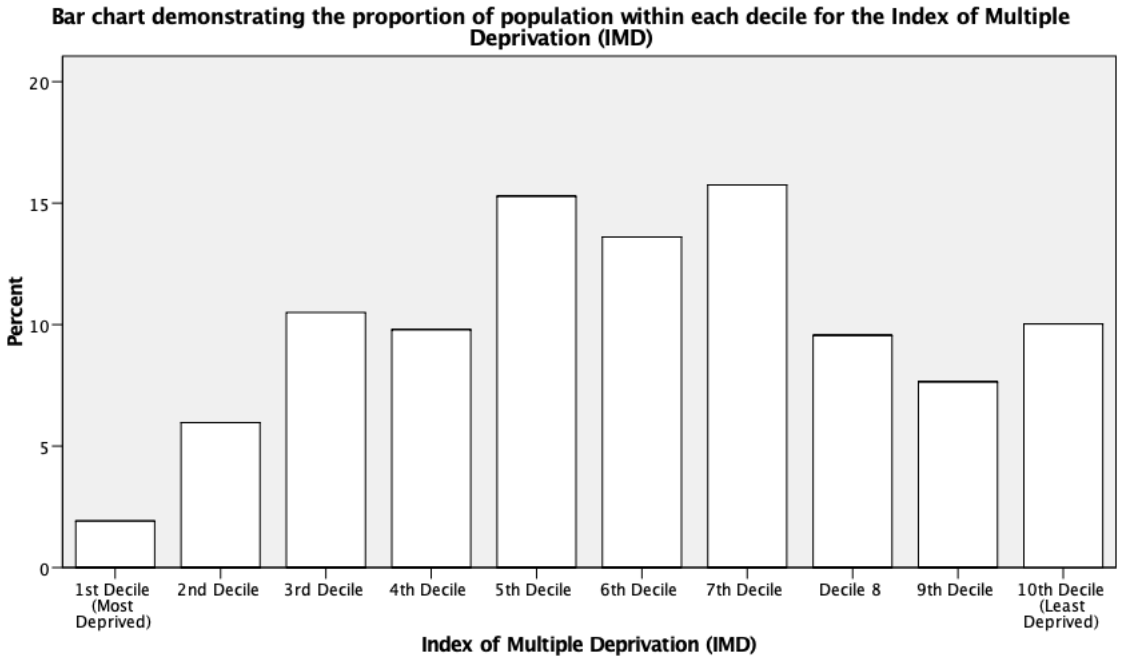


Figure 4.1 Distribution of deprivation domain within cohort

4.4.3 Deprivation and BC phenotype

The results of the univariate analysis are shown in Table 4.4 and the results of the multivariate analysis are shown in Table 4.5.

	Sarcopenia	Myosteatosis	BMI	Visceral Obesity	Sarcopenic Obesity
Age	**0.042**	**0.0001**	0.173	0.085	0.762
Gender	**0.0001**	**0.002**	0.402	0.069	0.199
P-Possum	0.187	**0.036**	0.605	0.795	0.91
UICC	0.281	0.051	**0.039**	0.359	0.738
IMD	0.059	0.068	**0.006**	**0.047**	0.564
Income	**0.04**	0.105	0.121	0.324	0.761
Employment	**0.002**	0.214	**0.001**	0.147	0.697
Education	**0.002**	0.297	**0.0001**	0.234	0.948
Health	**0.001**	0.503	**0.0001**	0.055	0.453
Crime	0.868	0.167	0.632	0.579	0.822
Housing	0.851	0.856	0.91	0.508	0.719
IDAOPI	0.201	0.796	0.416	0.598	0.457

Table 4.4 Univariate analysis (Chi-Square) of body composition, demographic, clinical and deprivation status

Body composition	Deprivation Domain (Bottom vs Top 50%)	OR	95% CI Lower	95% CI Upper	p Value
Sarcopenia	Index of Multiple Deprivation	**1.559**	**1.011**	**2.406**	**0.045**
	Income Deprivation	**1.583**	**1.025**	**2.443**	**0.038**
	Employment Deprivation	**2.004**	**1.287**	**3.122**	**0.002**
	Education, Skills and Training Deprivation	**2.091**	**1.307**	**3.343**	**0.002**
	Health Deprivation and Disability	**2.248**	**1.369**	**3.692**	**0.001**
	IDAOPI	**1.743**	**1.04**	**2.922**	**0.035**
Myosteatosis	Index of Multiple Deprivation	**1.692**	**1.019**	**2.809**	**0.042**
BMI Obesity	Index of Multiple Deprivation	0.596	0.377	0.941	0.026
	Employment Deprivation	**0.505**	**0.318**	**0.8**	**0.004**
	Education, Skills and Training Deprivation	**0.447**	**0.276**	**0.724**	**0.001**
	Health Deprivation and Disability	**0.412**	**0.25**	**0.68**	**0.001**

Table 4.5 Significant results of the multivariate analysis of the relationship between Deprivation status by domain and body composition status

4.4.3.1 Sarcopenia

Sarcopenia is independently associated with deprivation status on multivariate analysis. In our data, 60% of the most deprived were sarcopenic compared to 69% of the least deprived individuals in relation to the combined IMD. However, when factoring these data into our multivariate model we found that patients from the more deprived background, based on the combined IMD, were significantly more likely to be Sarcopenic OR 1.56 (95% CI 1.01-2.40, p=0.045). When IMD is stratified by its

component domains we see that barriers to healthcare OR 2.25 (95% CI 1.37-3.70, p=0.001), barriers to education OR 2.09 (95% CI 1.30-3.34, p=0.002) and employment deprivation OR 2.00 (95% CI 1.29-3.12, p=0.002) present the highest likelihood of being sarcopenic. Income deprivation also increases the likelihood of being sarcopenic but to a lesser extent; OR of 1.58 (95% CI 1.03-2.44, p= 0.038). In patients over 60 years old in relation to the Income Deprivation Affecting Older Persons Index (IDAOPI), a significant proportion of the entire patient cohort [median age 69 years], there is an association with sarcopenia and a higher IDAOPI OR 1.74 (95% CI 1.04-2.92, p=0.035). There were no significant associations between sarcopenia and crime, housing and services and living environment deprivation.

4.4.3.2 Myosteatosis

Once again, in respect of the IMD, we more frequently saw myosteatosis in the more affluent group compared to our more deprived group; 78% least deprived compared to 70% of the most deprived. Despite this, multivariate analysis, finds that the likelihood of myosteatosis is greater in the more deprived population OR 1.69 (95% CI 1.019-2.81, p=0.042). None of the other domains of deprivation confer a significant risk of having myosteatosis.

4.4.3.3 Obesity (BMI)

There was a greater frequency of obese patients in the most deprived population compared to those who were least deprived 32% vs 20%. In multivariate model however it appeared that the chance of being obese was significant less in the most deprived population OR 0.60 (95% CI 0.38-0.94, p=0.026). In particular, employment deprivation OR 0.51 (95% CI 0.32-0.80, p=0.004); Education deprivation OR 0.45 (95% CI 0.28-0.72, p=0.001) and health deprivation OR 0.41 (95% CI 0.25-0.68,

p=0.001) appear to reduce the chance of the patient being obese. Income, crime, housing and services and living environment deprivation appear to be less important factors. There was no association between income deprivation in the over 60's and obesity.

4.4.3.4 Visceral Obesity

We have seen how obesity, classified by BMI is greater in the more affluent population. When we look specifically at visceral obesity (VO), measured at L3 on the abdominal CT scan we find there to be no effect of deprivation upon the likelihood VO. There is a chance that those who are more deprived are in fact less likely to be viscerally obese OR 0.67 (95% CI 0.444-1.01) p=0.056 however statistical significance is not reached for this group. There are no other notable associations in the remaining deprivation domains in relation to VO.

4.4.3.5 Sarcopenic Obesity

There was no association found between sarcopenic obesity and deprivation measures.

4.5 Discussion

Our aim was to explore the relationship between deprivation and the host's body composition. We have identified a series of findings that demonstrate that a group of patients are predisposed to a prognostically poorer body composition phenotype potentially because of their premorbid environmental exposure.

We have identified a breadth of deprivation in our cohort (Figure 1.4) each decile of the deprivation index is represented and offers validity to the associations made in

analysis. We have an ethnically diverse population, with predominantly stage three disease; there is a slight male preponderance that fits with the epidemiology of colorectal cancer. Our data therefore are comparable to other cohorts described in the literature.

Sarcopenia is associated with deprivation as described using the IMD; several factors measured within the IMD seem to be most important – income, employment, health & disability and IDAOPI (in those over the age of 60). Other deprivation factors, housing, living environment and crime are unrelated to body composition status. The aetiology of sarcopenia remains complex, multifactorial and thus controversial with no clear management strategies (Chapter 10)[65] it is well established that sarcopenia relates to outcome in solid tumours as described above but the role of the tumour in sarcopenia is perhaps more contentious. Deprivation itself is known to be associated with poorer outcomes in colorectal cancer with multiple reasons postulated including later presentation and access/uptake of screening[198–200]. In this chapter we are the first to describe an association between deprivation and sarcopenia in colorectal cancer. The hypothesis that a more deprived patient is likely to be more sarcopenic is corroborated by the associations of the individual domains within the IMD that would be predicted to influence an individual's BC phenotype i.e., employment, income, health and education. Conversely indices such as crime have no effect or association, as one would anticipate.

Myosteatosis appears to be related to deprivation when measured using the IMD and we speculate that patients from more deprived backgrounds either have more pro-inflammatory tumours or share the inflammatory features of sarcopenia and thus the sequelae[152]. Ultimately though, it appears that other factors, including individual

tumour characteristics, may exert a greater influence in the aetiology of myosteatosis and its associated poorer outcomes.

Perhaps one of the most unexpected findings in our multivariate analysis was the likelihood of having a BMI of greater than 30 directly attributable to deprivation, taking into account disease, age and comorbidities, is lower in the most deprived population. In the healthy population we would expect a raised BMI to be related to deprivation as this is in keeping with the literature discussed in the introduction to this chapter. It is important to appreciate that our population is not healthy and therefore these findings may be explained by an increased tendency to sarcopenia either through environmental or disease exposure. Deprivation exerts no effect upon visceral obesity that appears to be the similar in both the most deprived and least deprived groups despite the apparent differences in BMI. The patterns in BMI concur with those identified with sarcopenia; essentially those factors of deprivation that are related to sarcopenia also appear to relate to low BMI. This therefore leads us to believe that the impact on BMI is attributable to sarcopenia from deprivation. Interestingly, there was no effect of deprivation in the over 60s age group and obesity in that age group perhaps suggesting obesity to be a more important factor in the younger population.

4.6 Limitations

Older data were used for this study because we held hold robust physiological data for the cohort during this time period. The use of P-POSSUM status in this patient group allowed a better interpretation of their premorbid status, discriminating more subtly in regard to comorbidity than ASA grade alone. It also allowed us to use census data from 2015 that more accurately reflected this population in that it was

contemporaneous whilst the 2019 census data was not. Use of the IMD has its own limitations, it does not consider the individual but the area in which they live based on census information from the collective populace within that area. As such there will be instances where a patient who perhaps would not be considered deprived lives in an area to which that label applies. As such it is suitable for group statistics as used here and not ascribed to an individual based on their postal code alone. However, these study data are suitable to draw conclusions from the population as a whole.

4.7 Conclusion

Deprivation status, as measured by the IMD, is associated with higher rates of host sarcopenia in colorectal cancer, independent of age, cancer stage, concurrent morbidity and gender. This is reflected within the BMI status of these patient groups where we see a predisposition to lower BMI in more deprived patients despite similar levels of visceral obesity between the most and least deprived patients. This suggests that sarcopenia could be a latent pre-morbid condition more frequently identified in this patient group and be a potentially reversable phenomenon with correction of nutrition, use of exercise therapy and other adjuncts as employed in prehabilitation in at-risk groups.

Chapter 5

5 Systemic inflammation and body composition profiles are dependent on ethnicity in colorectal cancer

5.1 Summary

So far in this thesis we have explored the effect of the tumour on body composition and vice versa. We have seen that myosteatosis and VO are related to the tumour biology and explored the concept of a “bad tumour”. We have also seen in Chapter 4 that sarcopenia is the BC phenotype most related to deprivation which we used as a surrogate for lifestyle and the environment. In this chapter we will explore the role of ethnicity and its relation to the systemic inflammatory response. Ethnicity will take into account both genetic and epigenetic factors and we know from previous work within the literature that ethnicity has a bearing on BC phenotype. We aim to explore whether these differences are apparent in the CRC population and does this difference in BC phenotype and ethnicity confer an effect on the systemic inflammatory response.

5.2 Introduction

Muscle and fat in both disease causation and response appear to be crucial elements. Sarcopenia, and myosteatosis, are linked to poorer survival outcomes[111,115]. A raised BMI is associated with an increased incidence of CRC[36] and visceral obesity (VO) and subcutaneous obesity (SO) are prognostic of all-cause mortality in patients with stage I–III colorectal cancer.[123]

The systemic inflammation response (SIR) is both associated with sarcopenia and myosteatosis and with significantly poorer outcomes in colorectal cancer[127,153]. A raised SIR is associated with poorly differentiated and advanced stage colorectal cancer[201,202]. Systemic inflammation can be measured in a number of ways, prognostically significant metrics which are readily available in day to day clinical practice include derivations from the full blood count such as neutrophil to lymphocyte ratio (NLR) [126,152] and platelet to lymphocyte ratio (PLR)[203–205]. Additionally, the modified Glasgow Prognostic Score (mGPS) which utilises the inflammatory protein C-Reactive protein and serum albumin levels has also been shown to be prognostically significant[127,206,207]. Tumours which have an enhanced inflammatory component and that drive a systemic inflammatory milieu particularly within the stroma, are poorly prognostic[208–210]. The process behind the inflammatory tumour is complex and at times paradoxical as an appropriate inflammatory response is vital part of an appropriate immune response to the tumour but is also involved in the pathways of tumorigenesis and tumour evolution[211]. There does seem to be a point where this balance is offset and the inflammatory response to the tumour not only

brings about a limited or ineffective response to the tumour but also impacts on body composition[126]. There is conversely significant evidence that muscle itself is anti-inflammatory and may in fact help reduce the systemic inflammatory response and thus may improve outcome. Inflammatory myokines (cytokines produced by muscle) such as IL6 produced by muscle during exercise, seem to have a dual effect in the inflammatory cascade. IL6 directly increases the circulating IL10 and IL1ra both of which are anti-inflammatory cytokines[212]. Enhanced muscle mass may therefore be an effective anti-inflammatory feature and underlying genetic predisposition to a certain body composition phenotype may have a beneficial effect upon inflammation. Obesity on the other hand is associated with a low grade inflammatory milieu propagated by circulating cytokines which may in part explain why obesity predisposes to a number of cancer types including colorectal cancer[213].

Ethnicity has been found to be associated with certain body composition phenotypes in health; black patients and those of African descent have been found to have greater muscle mass than white men and women[214]. Little is known about the subsequent effect ethnicity has on the inflammatory response nor on the response of the body to disease. There are however known associations between ethnicity and outcome in cancer with black patients having a poorer outcome and these have not been fully explained[215]. Deprivation seems to be a potentially important factor in these associations[216] as this impacts on the access to screening and earlier detection[217].

The relationship between BC and colorectal cancer outcome is well recorded, there is a significant association with a systemic inflammatory milieu, poorer outcome and abnormal body composition. Little is known about the effect ethnicity plays in BC in

cancer and furthermore there is limited literature available on CT assessment of BC in relation to ethnicity. Likewise, the relationship between inflammatory status and ethnicity has not been explored despite the importance of the inflammatory process in cancer biology and tumorigenesis. As technology advances and with the advent of artificial intelligence and deep learning in the field of radiomic biomarkers and risk assessment in cancer[218], an understanding of population based ethnic differences becomes increasingly vital as it will impact on management decisions. We aimed to examine the effect ethnicity has on body composition and inflammation in colorectal cancer with a view to identifying possible targets to assist with prognostication and treatment.

5.3 Methods

5.3.1 Patient Population

Analysis was performed on our prospectively maintained database recording all primary consecutive colorectal cancer resections (1,403 patients) from May 2007 to January 2017 at a single colorectal hospital. Data on ethnicity and body composition were added retrospectively to the dataset using contemporaneous information from the electronic records created during the patients' stay. Ethnicity was recorded from the hospital electronic patient administration (PAS) system. Ethnicity was coded using the UK Office for National Statistics (ONS) Ethnic Category Code 2001. Ethnicity was categorised as White (WBB), Black or Black British (BBB), Asian or Asian British (AAB) or other ethnic group for individuals not fulfilling these criteria. It is important to recognise that in this context Asian and Asian British refer to those

individuals whose heritage is from the Indian Subcontinent. Body composition data from the preoperative CT scans of patients were added to the database from the hospital records. Deprivation status was assigned using the Index of Multiple Deprivation based on patient postcode using data from the English Indices of Deprivation 2015, the make-up of which was described in detail in Chapter 4[219]. Data collected during the perioperative period (within 30 days of surgery) included age, sex, BMI, the American Society of Anaesthesiologists (ASA) physical status classification system grade, and TNM stage [Union for International Cancer Control (UICC) Version 5]. Systemic inflammatory state was classified based on previously identified clinically relevant thresholds. They were as follows: a neutrophil to lymphocyte ratio greater than three[125], a platelet to lymphocyte ratio greater than 150[176] and a modified Glasgow prognostic score mGPS greater than zero[207].

Patients with anthropometric data (height, weight and BMI), digital preoperative CT images and post-operative histology, radiological staging, ethnicity and deprivation data were included in the analysis. Patients were excluded if they had recurrent disease; our complex and advanced rectal cancer cohort were not included in this study. All other exclusions criteria were as Malietzis et al. 2016[101]. All prospectively collected data was revalidated from the hospital electronic records and cancer registry data for Public Health England prior to analysis.

5.3.2 Body composition analysis

CT images were retrieved in DICOM (.dcm) format from the hospital Picture Archiving and Communication System (PACS) (*Sectra, Linköping, Sweden*). Automated CT image analysis was performed using *Slice-O-Matic v5.0* software (*Tomovision, Montreal, Canada*) with ABACS L3 Plug-in (*Veronoi Health Analytics, Vancouver, Canada*). A consultant radiologist [PL], experienced in BC analysis, reviewed all images manually to ensure appropriate segmentation and quality control, for images where automated analysis failed segmentation was performed manually using *Slice-O-Matic v5.0*. Segmentation and cut off values were the same as those used throughout this thesis.

5.3.3 Ethical Approval

Approval was obtained for use of the prospective database in research by the NHS Health Research Authority, UK with ethical approval from the South East London NHS Research Ethics Committee (reference number: 12/LO/1556).

5.3.4 Statistical Analyses

Statistical analyses were performed using SPSS v25.0 (*IBM Corp, Armonk, NY*). Grouping of the variables was carried out using standard or previously published thresholds[111]. Data were analysed using binary multivariate logistic regression, the model took into account age, gender, ASA grade, UICC Stage, IMD (quintile) and ethnicity. The binary dependent variables were the radiological body composition features of sarcopenia, myosteatosis, visceral obesity and sarcopenic obesity as well

as inflammatory status either by NLR >3, PLR >150 and mGPS >0. The reference category for ethnicity was WWB as this was by far the largest population.

5.4 Results

5.4.1 Exclusions

776 patients were included in the final multivariate analysis. Of the 1403 consecutive cases in the cohort, 248 lacked data on ethnicity, 156 patients lacked sufficient data on BC, 129 did not have full staging data available, 77 lacked ASA data, 10 patients did not have IMD data and full demographic data was not available for 7 individuals.

5.4.2 Demographics

56% of the population was male and the median age was 69 [IQR 60-78]. 518 (67%) patients were classified as white, 148 (19%) AAB, 73 (9%) BBB and 37 (5%) were classified as other ethnicity (including, Chinese, Japanese and Middle Eastern heritage) A breakdown of population demographics, disease and body composition characteristics are shown in Table 5.1. A comparison between London's ethnic make-up and our hospital population is shown in Table 5.2.

		n.	Percentage %
Gender	Male	436	56
	Female	340	44
Ethnicity	White	518	67
	Asian or Asian British	148	19
	Black or Black British	73	9
	Other Ethnic Groups	37	5
ASA Grade	ASA 1&2	660	85
	ASA 3&4	116	15
UICC Stage	1	185	24

	2	244	31
	3	261	34
	4	86	11
Sarcopenia	Not Sarcopenic	242	33
	Sarcopenic	491	67
Myosteatosis	Not Myosteatotic	193	26
	Myosteatotic	545	74
Visceral Obesity	Not Viscerally Obese	365	47
	Visceral Obesity	411	53
BMI Obesity§	BMI<30	438	78
	BMI>30	123	22
Sarcopenic Obesity	S-Ob Not Present	570	78
	S-Ob Present	160	21
NLR >3	NLR <3	295	54
	NLR >3	252	46
PLR >150	PLR<150	224	41
	PLR>150	323	59
mGPS > 0	mGPS 0	127	68
	mGPS >0	59	32

Table 5.1 Patient demographics and characteristics

Ethnicity	**% Population London**	**% Population St Marks Cohort**
White	45	67
White Other	15	
Asian	19	19
Black	13	9
Mixed	5	5
Other	3	

Table 5.2 Comparison of 2011 UK Census "Ethnic Diversity in London" to our population[220]

5.4.3 Body Composition Characteristics and Inflammatory Profiles

The most prevalent BC aberrant characteristic was myosteatosis with 785 of the population demonstrating this, 67% of the population were sarcopenic and a greater

proportion were viscerally obese as opposed to BMI obese (53% vs. 21%). The poorly prognostic characteristic of sarcopenic obesity was only present in 22% of the population. NLR and PLR were relatively evenly split in the population with 46% having an NLR >3 and 59% having a PLR >150. We held far fewer data on mGPS compared to the other parameters but of the 186 patients with data available 32% had a mGPS >0 and thus would be classed as suffering an inflammatory state.

5.4.4 Body Composition and Ethnicity

5.4.4.1 Sarcopenia and Ethnicity

Compared to WWB patients BBB were significantly less likely to be sarcopenic OR 0.36 [95%CI 0.206-0.616, p=0.0001] whilst Asian and Asian British AAB were significantly more likely to be sarcopenic OR 2.13 [95%CI 1.34-3.38, p=0.001]. The mixed ethnicity group showed no difference from the WWB OR 1.531 [95%CI 0.67-3.49, p=0.31] Figure 5.1.

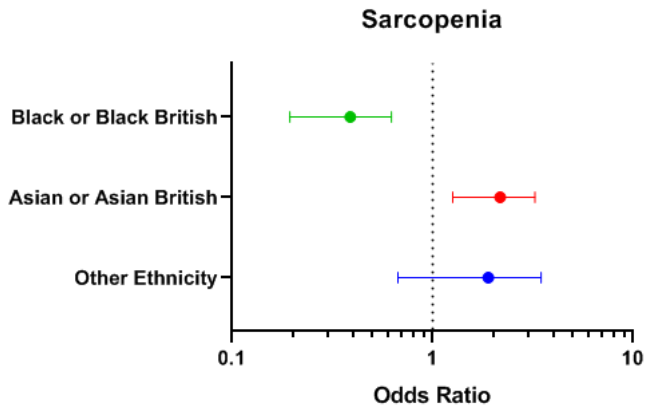


Figure 5.1 Odds ratios of each being sarcopenic defined by ethnic group compared to the WWB population

5.4.4.2 Myosteatosis and Ethnicity

BBB were significantly less likely to be myosteatotic compared to the WWB population OR 0.388 [95%CI 0.21-0.73, p=0.003]. There was no difference between WWB and AAB OR 0.88 [95%CI 0.55-1.40, p=0.59] or the mixed ethnicity group OR 0.973 [95%CI 0.42-2.25, p=0.95] in terms of myosteatosis, Figure 5.2.

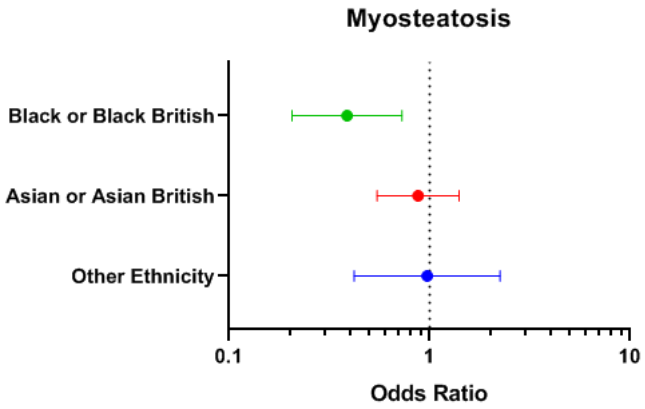
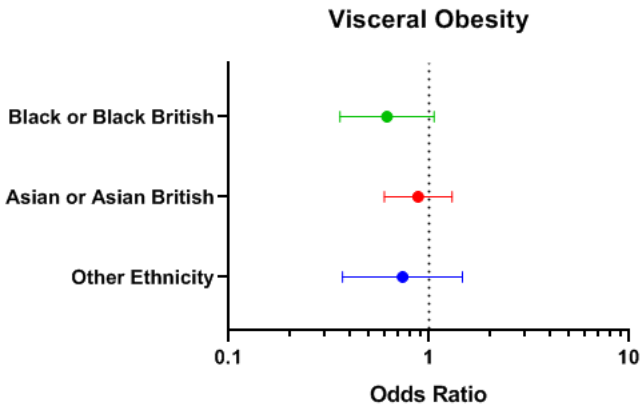


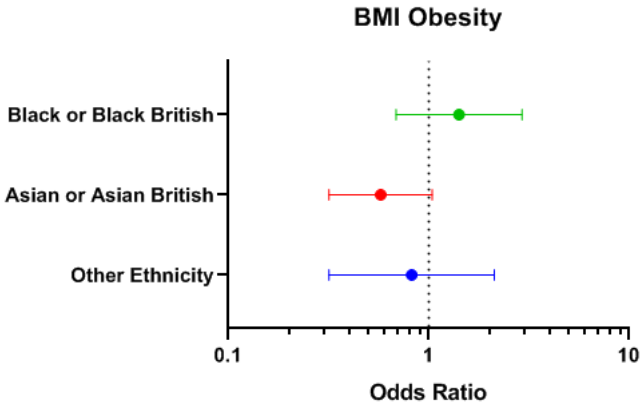
Figure 5.2 Odds ratios of each being myosteatotic defined by ethnic group compared to the WWB population

5.4.4.3 BMI Obesity, Visceral Obesity, Sarcopenic Obesity and Ethnicity

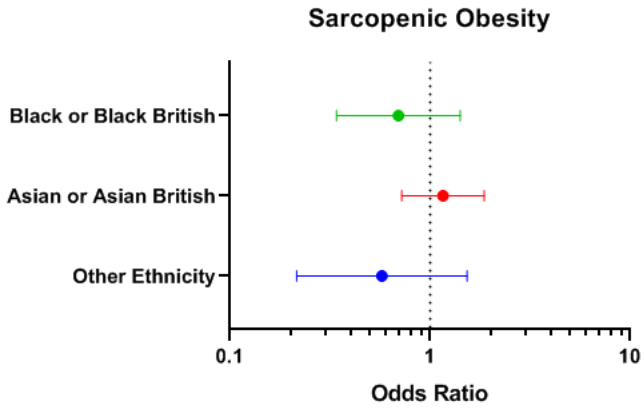
There were no significant differences between ethnic populations in terms of VO, BMI obesity or SO Figures 5.3a-c. There was no significant relationship between ethnicity in terms of BMI obesity which is interesting considering that there are statistical differences in terms of muscle volume between ethnic groups. There was no significant difference in VO between ethnic groups either and therefore changes in BMI may be determined by other factors such as different distributions of fat within populations. That said, the BBB population does appear to tend towards a higher BMI compared to the WWB population which is in keeping with the earlier described results.



5.3a.



5.3b.



5.3c

Figure 5.3 a-c. Odds ratios of each being a certain obesity subtype defined by ethnic group compared to the WWB population

5.4.5 Inflammatory Status and Ethnicity

5.4.5.1 NLR and Ethnicity

NLR appeared to be the Inflammatory metric that discriminated between WWB and other populations. AAB, BBB and mixed ethnicity population were significantly less likely to have an NLR >3 compared to WWB OR 0.37 [95%CI 0.234-0.57, p=0.0001]; OR 0.038 [95%CI 0.20-0.73, p=0.004] and OR 0.406 [95%CI 0.175-0.943, p=0.036] respectively Figure 5.4. This suggests that the WWB population were more likely to have this poor prognostic pro-inflammatory state in disease.

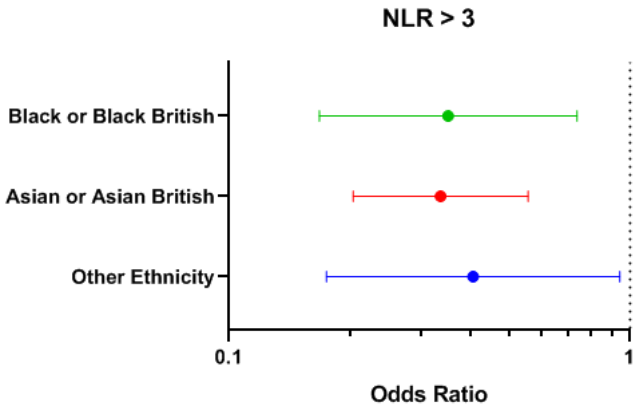


Figure 5.4 Odds ratios of each ethnic group's NLR status compared to the WWB population

5.4.5.2 PLR and Ethnicity

AAB were significantly likely to have a PLR >150 compared to WWB OR 0.642 [95% CI 0.42-0.974, p=0.037]. BBB and the other ethnic group population demonstrated no significant difference in PLR status however both demonstrated a trend towards a lower PLR when compared to the WWB population, in keeping with the NLR data Figure 5.5.

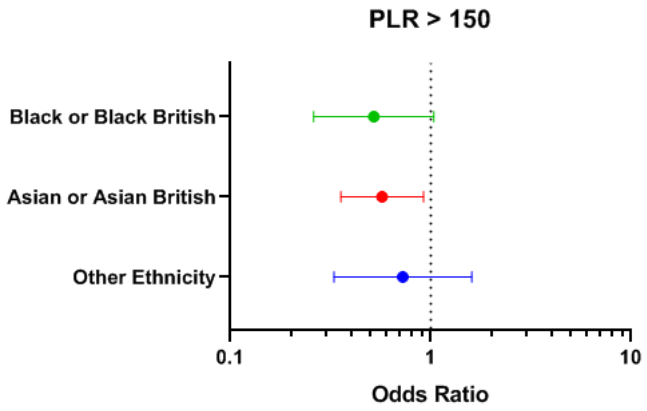


Figure 5.5 Odds ratios of each ethnic group' PLR status compared to the WWB population

5.4.5.3 mGPS and Ethnicity

Sub-group analysis was performed on patients for whom we had both preoperative CRP and serum albumin. 186 were included in this analysis. There was no difference in mGPS between populations however, we are unable to draw conclusions fully from these data, due to the small sample size, Figure 5.6.

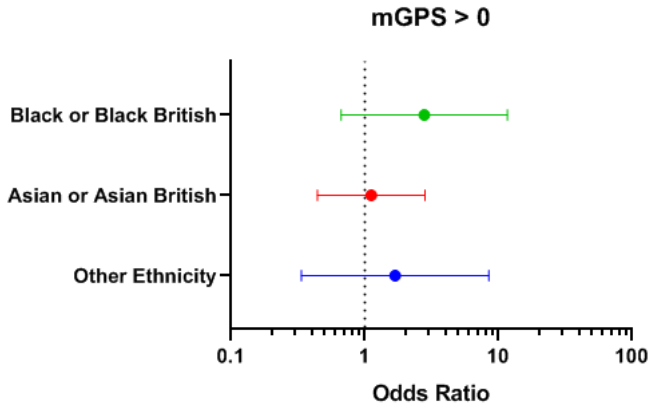


Figure 5.6 Odds ratios of each ethnic group' mGPS status compared to the WWB population

5.5 Discussion

Sarcopenia, myosteatosis and the obesity metrics are important prognostic indicators in colorectal cancer. They are inherently linked to the inflammatory response and underlying tumour biology and immune response to malignancy. Ethnic differences in body composition have been long recognised however there is very limited information in terms of BC analysed by CT and ethnicity and no information regarding the effect of ethnicity on the inflammatory response in colorectal cancer. As one of the most ethnically diverse regions in the UK we were well placed to examine these relationships.

Our local boroughs (regions) of Brent and Harrow are the second and third most ethnically diverse areas in the UK according to the 2011 UK census with a white population of 36.3 and 42.2% respectively[220]. 'Ethnic diversity' in this instance refers to the percentage of the population that is not from the white ethnic group in each local authority area. Essentially despite a lower percentage BBB, our population is comparable to London. We also assert that fewer hospitals will exhibit the level of diversity demonstrated within our hospital due to our location in an ethnically diverse region. This strongly justifies the assessment at our specialist unit as it allows a population size that provides a meaningful output.

Our data show significant differences in body composition and inflammatory status in colorectal cancer patients based upon their ethnicity. Our model assessed disease stage and deprivation suggesting ethnicity alone is an independent predictor of both inflammation and BC in response to cancer. This may suggest that there are inherent premorbid differences between ethnic groups in regard to BC or differing tumour biology and immune response.

Our study found that AAB were more sarcopenic than WWB who in turn were more sarcopenic than BBB. The aetiology behind this phenomenon is likely to be multifactorial with both genetic and environmental factors being key. This is supported by data from previous studies in health that noted differences in muscle mass between white and black individuals[214].

There are very limited data available on the impact of ethnicity on mean muscle attenuation, the surrogate marker of myosteatosis. To our knowledge our study is the first to report significant differences between populations in cancer. We see that the BBB population are significantly less likely to be myosteatotic compared to the WWB population. Despite these favourable BC characteristics described in relation to the BBB population, we know that this does not necessarily convert into more favourable outcomes as numerous studies have demonstrated that patients of Black African descent have worse outcomes[216,221]. This questions the of value of using sarcopenia as a metric in Black patients in the presence of other health inequalities which may be more predominant in determining outcome[215,222]. Important factors such as reduced screening uptake and later presentation may play a more important role in outcome despite the reduced inflammatory state seen in this population and an apparently favourable BC profile[223]. The reduced inflammatory state of these patients is also interesting as it concurs with their BC profile and supports the argument that the presence of high-quality muscle may have a role in promoting a lower inflammatory environment with a significantly lower likelihood of a raised NLR and to a lesser extent, PLR.

WWB were found to have a significantly higher likelihood of NLR to all other ethnicities, which suggests perhaps a genetic predisposition to a neutrophilic

response. A high NLR is a poorly prognostic marker associated with advanced disease such as lymph node metastasis and as such it is interesting that one specific ethnic group displays this inflammatory phenotype[163]. To a lesser extent the same appears to be true with a low PLR and the trend towards significance in all ethnic groups in comparison to WWB. We know that the incidence of inflammatory bowel diseases is higher in northern Europeans and one could question whether this innate tendency to inflammation represents the phenotypic qualities of gut tumours in this population. Another factor that may be responsible, other than genetic and epigenetic susceptibility to inflammatory colorectal tumours, may be the microbiome. Due to potential differences in diet and lifestyle one may also expect a distinct microbiome among different ethnic groups living in a similar geographical area. Dietary intake may too impact on the inflammatory process, diet with high levels of potentially anti-inflammatory agents such a curcumin in turmeric, may result in an inherent anti-inflammatory environment and may account for such differences as demonstrated by the low NLR and PLR of the AAB population[224].

Whilst the AAB population were more sarcopenic they had a lower inflammatory state suggesting a differing aetiology behind their muscle loss. Again, there is likely to be a genetic component to this and one should question whether sarcopenia and inflammatory metrics such as PLR and NLR are as meaningful in this population group.

We found no significant difference between Visceral Obesity and ethnicity however the likelihood of BMI obesity was lower in AAB and a tendency towards sarcopenic obesity compared to other ethnic groups which may be accounted for by significantly

lower muscle volume. This reasserts that BMI as, a metric of morbid status, is not representative of underlying physiological state.

mGPS was not significantly different between ethnic groups perhaps making this a more universal marker of inflammation in colorectal cancer, however our smaller sample size in relation to this does not allow us to draw firm conclusions and further work with a larger sample size should be undertaken to look at this important marker.

When considering inflammation in CRC, ethnicity should be considered, clearly significant differences exist with possible multiple aetiologies – diet, lifestyle, genetic and epigenetic factors tumour factors. We have seen that an ethnic group can be subject to environmental changes such as the Japanese population in Hawaii which saw a decline in gastric cancer incidence compared to the native population in Japan[225,226]. Our local population will be a combination of first and second/third generation ethnic groups and perhaps with new generations born in the UK from varying ethnic backgrounds we may see convergence in some of these characteristics.

Ultimately these findings are important in both clinical practice and research in that outcome may vary with BC in different ethnic groups and as such when approaching treatment and monitoring of sarcopenia or even prehabilitation, ethnicity must be considered. Furthermore, future population analyses involving BC should include ethnicity in their multivariate analyses.

Future work should be performed with larger international population studies, cross continent multi-institutional collaboration to identify the cause of these disparities described is key. Finding underlying aetiological differences may in turn help other populations in treatment of body composition disparities.

Further work should also be undertaken to examine tumour characteristics between ethnic groups as these may have a significant effect on the inflammatory and immune response and be key in determining future treatment and disease management.

Vitamin D levels and other micronutrients play important part in influencing BC in cancer and the effect of these micronutrients should also be examined in different ethnic groups[227]. The role of vitamin D in inflammation and response to CRC has been examined in the past, we know that vitamin D differs in populations and that dark skin types produce less vitamin D especially in countries with limited sun exposure such as the UK[228]. The concern of course being a dampened immune response seen in the BBB and AAB populations may be a result of micronutrient deficiency.

5.6 Limitations

We have already recognised the inherent limitations associated with the smaller sample size of the mGPS analysis. We also have lower proportions of ethnic minorities in this sample compared to WWB but we have one of the most ethnically diverse populations internationally and with London being one of the most diverse cities and us having one of the most diverse populations. International studies will be key in further determining relationships in relation to this.

5.7 Conclusion

Ethnicity is an important determinant of BC and inflammation; the cause is unclear and multifactorial and does not necessarily correspond to outcomes as previously published data suggest that black colorectal cancer patients do worse than their white counterparts. Further work may be able to find factors in different ethnic groups

which preserve BC status and identify ethnic differences in tumour biology. Further international work is required to support the conclusions of this study.

Chapter 6

6 Materials and Methods

6.1 Materials

6.1.1 Human tissue samples

Informed consent was obtained for tissue to be taken from both healthy controls and cancer patients. The protocol, “Microbial Immunity in Inflammatory Bowel Disease and Colorectal Cancer” (MIBDAC) was reviewed and approved by the NHS Health Research Authority and NHS Research Ethics Committee, Harrow (Ref. 17/LO/1636) and LNWUH NHS Trust research and development department (Ref 17/095). Further amendments were obtained to take diseased patient muscle and fat tissue from resected specimens (approved as a non-substantial amendment) and healthy control muscle during anterior cruciate ligament repair surgery (Substantial amendment; Ref. 17/LO/1636/AM04/1). Demographic data, details regarding their hospital stay including complications, tumour characteristics (histopathology), systemic inflammatory status (mGPS, PLR, NLR) and body composition status was obtained from the hospital system as described earlier in this chapter.

6.1.2 Whole Blood Samples

Human venous blood samples were collected from both healthy controls and colorectal cancer patients with active disease prior to surgery. A Vacutainer® system was used to

draw blood into a 10ml vial containing 170 IU of sodium heparin *(BD Biosciences, San Jose, California, USA)*. Patients donated blood between 07:30 and 08:30 on the day of surgery with 30ml samples either been taken by the researcher or anaesthetist. Healthy control bloods were taken between 08:00 and 10:00 with a 50ml sample taken by a research fellow.

6.1.3 Human Colonic Tissue

Healthy colonic mucosal biopsy samples (five from each patient) were obtained from the descending/sigmoid colon during routine or screening colonoscopy where no disease was present. Biopsies were superficial and between 1-2mm in size. Biopsies were transferred from the endoscopy unit in HBSS prior to processing. Colonic biopsies were taken from colorectal cancer patients at the time of surgery. Surgical biopsies consisted of full thickness macroscopically disease-free colon striped of its mesentery. Surgical biopsies were proximally to the tumour and were always taken at least 10cm proximally tumour site. The mucosa was then dissected from the muscularis layers prior to chemical and enzymatic digestion

6.1.4 Human Mesenteric Fat Tissue

Colonic mesentery was taken at the time of colonic resection. The mesentery was taken at the border of proximal resection margin of the specimen with approximately 10g of mesentery being sampled. In the laboratory samples were dissected to remove any peritoneum and ensure there were no lymph nodes present in the specimen. The

samples then went on to be processed, when necessary, samples were stored on ice and processed the following day.

6.1.5 Human Muscle Tissue

Rectus muscle tissue was taken from diseased patients either at the site of their midline laparotomy incision, stoma trephine incision or specimen extraction site. Specimens were either processed immediately or kept on ice overnight. Healthy tissue was taken during primary anterior cruciate ligament repair surgery. Here gracilis muscle is taken during tendon harvest, the stripped muscle was then treated as the muscle from diseased subjects.

6.1.6 Snap-freeze Killed Bacteria

All bacteria were isolated from the caecum of healthy donors by Prof L Hoyles and Colleagues (Imperial College London). Strains were grown anaerobically in Hungate tubes containing Wilkins-Chalgren broth at 37°C for 24hours, following which, cell pellets were killed by snap-freezing with liquid nitrogen and stored at -80°C (Prof Hoyles and Dr A McCartney). Enumeration by flow cytometry following SYBR Green staining was performed I our laboratory by Dr Alistair Noble. The strains of bacteria used are shown in Table 6.1.

Bacterial Species	Phylum	Family
Actinomyces turincensis	Actinobacteria	Actinomycetaceae
Bifidobacterium Longum	Actinobacteria	Bifidobacteriaceae

Bifidobacterium Pseudocatenulatum	Actinobacteria	Bifidobacteriaceae
Clostridium Paraputrificum	Firmicutes	Clostridiaceae
Enterococcus Gallinarum	Firmicutes	Streptococcaceae
Escherichia Coli	Proteobacteria	Enterobacteriaceae
Lactobacillus Plantarum	Firmicutes	Lactobacillaceae
Lactobacillus Rhamnosus	Firmicutes	Lactobacillaceae
Staphylococcus Epidermidis	Firmicutes	Staphylococcaceae
Streptococcus Parasanguinis	Firmicutes	Streptococcaceae

Table 6.1 Bacterial Species, phylum and family

6.1.7 Lipopolysaccharide (LPS)

LPS is a cell wall product common to Gram negative bacterial species, it is immunogenic binding to TLR-4. LPS-RS derived from the photosynthetic bacterium *Rhodobacter sphaeroides* (*Invivogen, Toulouse, France*) was used, this preparation also contains other additional lipoproteins which are known to bind to TLR-2.

6.1.8 Hank's Balanced Salt Solution (HBSS)

HBSS (Sigma-Aldrich, Dorset, UK), a solution containing glucose (1g/L), $NaHCO_3$ (0.35g/L) is a sterile solution utilised in the initial preparation of mucosal biopsies for flow cytometry, its purpose is to maintain pH, osmotic balance and provides cells with water and essential inorganic ions.

6.1.9 Media

6.1.9.1 Roswell Park Memorial Institute Medium 1640 (RPMI)

Dutch modified RPMI 1640 (*Sigma-Aldrich, Dorset, UK*) was initially formulated to support lymphoblastoid cell but since shown to support a number of cell types which are anchorage dependent, RPMI is a sterile liquid containing L-glutamine, $NaHCO_3$ and phenol red dye. It is stored ready for use between 2-8°C. RPMI was used for the preparation of tissue specimens for flow cytometry and as a component of complete medium for cell culture.

6.1.9.2 Complete Medium

Complete medium was made up in 50ml aliquots formulated in the laboratory. 45ml of RPMI was augmented by the addition of 500µl of l-glutamine (2mM), 5ml 10% foetal calf serum (*TCS cell works, Buckingham, UK*), 500µl penicillin/streptomycin (100U/mL) and 50µl gentamicin (50µg/mL) (*Sigma-Aldrich*). This solution was used for overnight culture including bacterial stimulation experiments.

6.1.10 Buffers

6.1.10.1 Dulbecco's Phosphate Buffered Saline (PBS)

PBS (*Life Technologies Ltd., Paisley, UK*) is a buffer designed to maintain the structural and physiological integrity of mammalian cells in vitro. PBS was the primary buffer used to wash and resuspend cells during preparation for flow cytometry, as the main constituent of FACS buffer for flow cytometry and to fix cells with the addition of paraformaldehyde (PFA).

6.1.10.2 Fluorescence Activating Cell Sorting (FACS) Buffer

500ml PBS were augmented by the addition of 10ml FCS (10%); 1ml EDTA (0.5M) preventing cell to cell adhesion and 0.1g sodium azide (NaN_3) acting as a preservative, preventing photobleaching of fluorochromes and prevents antibody shedding. The animal proteins within the FCS of FACS buffer reduce non-specific antibody binding and help prevent cell apoptosis. FACS buffer was the primary buffer used following the viability staining stage.

6.1.13 Paraformaldehyde (PFA)

4% PFA (*Santa Cruz Biotechnology, Inc. Dallas, Tx, USA*) was combined at a 1:3 ratio with PBS to produce a 1% solution of PFA. PFA was used to fix cells prior to performing flow cytometry.

6.1.11 Flow Cytometry Antibodies and Cellular Stains

These are summarised in table 6.2

Ab Specificity/marker of interest	Clone	Fluorochrome / Stain	Isotype	Source	Stock Concen-tration
CD40	LOB7/6	FITC	Mouse IgG2a Anti-Human	AbD Serotec MorphoSys	1.0 mg/ml
Integrin Beta 7	FIB504	BB515	Rat IgG2a, κ Anti-Human B7	BD Biosciences	0.2mg/ml

Total lipid	n/a	BODIPY 493/503	n/a	ThermoFisher	
E06	330003S	TopFluor	Mouse IgM	Avanti Polar Lipids, Inc.	0.25mg /ml
CD45	2D1	FITC	Mouse IgG_1,κ	BD Biosciences	50µg/ml
CLA	HECA-452	PE	Rat IgM,κ	BioLegend®	60µg/ml
CCR7	4B12	PE	Mouse IgA2A	R&D Systems®	20µl/ml
CD36	CRF D-2712	PE	Mouse IgM, κ Anti-Human CD36	BD Pharmingen™ Bioscience	0.2 mg/ml
Beta 7	FIB504	PE	Rat IgG2a,κ Anti-human/ mouse integrin B7	BioLegend®	50µg/ml
CD3	UCHT1	PE-Cy™5	Mouse BALB/c IgG_1, κ Anti human CD3	BD Pharmingen™ BD Bioscience	20µl/ml
CD14	61D3	PE-Cyanine5	Mouse / IgG1,k Anti-Human CD14	invitrogen eBioscience™	5µl/ml
CD16	3G8	Pe-Cy™5	Mouse BALB/c Anti-Human CD16	BD Pharmingen™ BD Bioscience	20µl/ml
CD19	HIB19	Pe-Cy™5	Mouse IgG_1, κ Anti-Human CD19	BD Pharmingen™ BD Bioscience	20µl/ml
CD34	581	Pe-Cy™5	Mouse IgG_1, κ	BD Pharmingen™	20µl/ml

			Anti-Human CD34	BD Bioscience	
CD14	M5E2	PerCP-Cy™5.5	Mouse IgG2a, κ Anti-Human CD14	BD Pharmingen™ BD Bioscience	0.2mg/ml
CD64	Clone 10.1	PerCP-Cy™5.5	Mouse (BALB/c) IgG1, κ Anti-Human CD64	BD Pharmingen™ BD Bioscience	5µl/ml
CD123	6H6	Pe-Cyanine7	Mouse IgG1, κ Anti-Hu CD123	invitrogen eBioscience™	200µg/ml
SIRP α/β CD172a/b	SE5A5	Pe/Cy7	PE/Cy7 anti-human CD172a/b	BioLegend®	200µg/ml
HLA-DR	G46-2.6	APC	Mouse IgG_1, κ Anti-Human	BD Pharmingen™ BD Biosciences	20µl/ml
Live/Dead™ Fixable Near-IR Dead Cell Stain Kit	n/a	See section 6.2.4.1	n/a	Invitrogen Thermo Fisher Scientific	n/a
CD49d	9F10	Brilliant Violet 421™	Mouse IgG1, κ Anti-Human CD49d	BioLegend®	50µg/ml
CD40	5C3	BV421	Mouse IgG_1, κ Anti-Human CD40	BD Horizon™ BD Biosciences	5µl/ml
StreptAvidin	n/a	Brilliant Violet 421™	Anti-Human, Mouse, Rat	BioLegend®	0.1mg/ml

CD103 (integrin αE)	Ber-ACT8	Brilliant Violet 421™	Anti-Human CD103	BioLegend®	50µg/ml
E06	330002S	Biotin	E06 mAb-biotinylated; E06 Mouse monoclonal antibody (IgM)	Avanti Polar Lipids, Inc	0.25mg/ml
CD11c	B-ly6	BV605	Mouse BALB/c IgG_1, κ Anti-Human	BD Horizon™ BD Biosciences	5µl/ml

Table 6.2 Flow Cytometry Antibodies and Cellular Stains

6.1.12 ELISA buffers and reagents

ELISA buffers and reagents (*R&D Systems, Minneapolis, MN, USA*) are shown in Table 6.3

Wash Buffer 0.05% Tween® 20 in PBS pH 7.2-7.4
ELISA reagent Diluent 1%BSA in PBS pH7.2-7.4
Tris Buffered Saline with 1%BSA
Substrate Solution 1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine)
Stop Solution (2 N H_2SO_4)

Table 6.3 ELISA buffers and reagents

6.1.13 ELISA Kits

Human ELISA Kits were all supplied by R&D Systems, IL-6 and IL-10 were analysed using the DuoSet® ELISA system augmented with the above reagents whilst GDF-15 was analysed with the Quantikine® ELISA system. The contents of these kits are summarised in Table 6.4.

Product	Antibodies and Reagents
Human IL-10 DuoSet®	Human IL10 Capture Antibody
	Human IL10 Detection Antibody
	Recombinant Standard
	Steptavidin conjugated to Horseradish Peroxidase (HRP)
Human IL-6 DuoSet®	Human IL6 Capture Antibody
	Human IL6 Detection Antibody
	Recombinant Standard
	Steptavidin-HRP
Quantikine® ELISA Human GDF-15 Immunoassay	GDF-15 Standard
	GDF-15 Conjugate
	Assay Diluent RD1-9
	Calibrator Diluent RD5-20
	Wash Buffer Concentrate
	Substrate Solution (Color Reagent A & Color Reagent B)
	Stop Solution

Table 6.4 ELISA Kits (R&D Systems, Minneapolis, MN, USA)

6.2 Methods

6.2.1 Preparing and procession blood to isolate PBMC

Using a heparinised (170 I.U.) Vacutainer® system (Becton, Dickinson and Co, New Jersey, USA) whole blood was collected from the trial participant (50ml healthy controls, 30ml patient). Blood was diluted using PBS at a ratio of one to one (v/v) in a universal tube. 15ml of Ficoll-Paque Plus (Sigma Aldrich) was added to a Falcon 50ml centrifuge

tube. Each universal of whole blood PBS solution was layered, using a serological pipette, onto the surface of the Ficoll-Paque Plus forming a sharp interface. Without disturbing the two layers the Falcon tubes were transferred to the centrifuge where they were spun at 800g for 30 minutes at room temperature with the brake off.

On completion of the cycle, the tubes were removed from the centrifuge and the layers inspected to ensure the gradients had formed successfully. Red blood cells and granulocytes now sit at the bottom of the tube separated from the PBMC by a Ficoll layer with plasma overlying this. Plasma is extracted at this stage (3ml), without disturbing the PBMC layer, the plasma is frozen at -80°C and stored for future analysis with ELISA. The PBMC layer from each tube is then extracted using a Pasteur pipette and transferred to a clean Falcon 50ml tube. The remaining plasma, Ficoll and red blood cells layers are discarded. The volume of the PBMC solution tube is made up to 50ml with the addition of PBS. The sample is then returned to the centrifuge and spun at 600g at 4°C with the brake applied for five minutes. The supernatant is discarded and the resultant pellet of PBMC resuspended for a second wash in PBS and returned to the centrifuge at 600g 4°C for five minutes, the supernatant is again discarded. The PBMC pellet is transferred for a final wash to a FACS tube and washed as above. The PBMC pellet is resuspended in 1ml of PBS, 5µl of the cell suspension is mixed with 5µl of Gibco® Trypan Blue 0.4% (*ThermoFisher*), 10µl of this mix is taken and placed on a glass haemocytometer, using surface tension this displaces under a coverslip over the grid. Live (unstained cells) are counted in 25 squares. The number (n) is applied to the following calculation to provide the cell number per ml. n x 25 x 2 x 10,000 = Cells in 1ml. The 1ml cell suspension is then either stored on ice at 4°C for use later or washed in PBS as above ready for surface staining (see section 6.2.4 below).

6.2.2 Preparing and processing intestinal tissue

6.2.2.1 Healthy Colonic Samples

Five biopsies taken from the distal colon of healthy donors, obtained at colonoscopy, were received suspended in HBSS, following retrieval they were washed to remove any remaining blood. Cell Isolation and staining was undertaken by Dr L R Durant, within our laboratory. Briefly, the same methods were used as described below in section 6.2.2.2 however small volumes of reagents were used – 12.5ml of HBSS/DTT/EDTA and 12.5ml of Col/Lib in RPMI.

6.2.2.2 Diseased Colonic Samples

Full thickness sections of human colon were obtained at the time of surgery following colonic resection. The samples were taken from tumour free mucosa at least 10cm proximal to the tumour. For transfer and prior to processing the samples were stored on ice. In the laboratory the mucosa was dissected from the muscular layers of the colon, the latter were discarded. The mucosal sample was weighed prior to washing in HBSS. Following three washes the samples were mechanically macerated and washed for a final time in HBSS.

6.2.2.3 Retrieval of Intraepithelial Leucocytes (IEL)

The tissue pieces were suspended in 50ml of HBSS with 500µl Dithiothreitol (DTT) and 1mM EDTA. The samples were incubated on a shaker set at 200rpm for 30 minutes. Following incubation samples were strained through a 100µm Nylon cell strainer – the filtrate containing leucocytes of the intraepithelial layer was retained and stored on ice. The tissue was salvaged from the cell trainer and resuspended in 50ml of HBSS, 500µl DTT and 1mM EDTA prior to incubation on a shaker at 200rpm for a further 30 minutes. The sample was passed through a 100µm Nylon Corning® Cell Strainer (*Corning Incorporated, Durham, NC, USA*) and the filtrate containing the second fraction of IEL retained and stored as above. The tissue was again salvaged in order to obtain the lamina propria leucocytes.

The two IEL filtrate samples were then passed through a 40µm Nylon Corning® Cell Strainer (*Corning Incorporated, Durham, NC, USA*). Both samples were spun in a centrifuge at 600g for 5 minutes. The supernatant was discarded and the pellets resuspended in sterile PBS to wash and spun in a centrifuge at 600g for five minutes. The samples were then ready for preparation for flow cytometry (Section 6.2.4).

6.2.2.4 Retrieval of the Lamina Propria Leucocytes (LPLs)

Following extraction of IEL, the tissue was resuspended in 40ml of RPMI with 5ml Collagenase D (*Roche, Basel, Switzerland*), in 1mg/ml aliquots HBSS and 5ml Liberase TL (*Roche, Basel, Switzerland*), in 0.1mg/ml aliquots HBSS. The sample was then incubated at 37°C on a shaker at 100rpm for one hour. Following incubation, the sample was vortexed to displace cells LPL from the tissue. The suspension was then passed through a 100µm Nylon cell strainer, the solid tissue discarded, then the filtrate passed

through a 40µm Nylon cell strainer. The filtrate was then spun in a centrifuge at 600g for five minutes, the supernatant discarded and the resultant pellet resuspended in PBS. This solution was spun once again as above and the PBS wash discarded. The samples are then ready for preparation for flow cytometry (Section 6.2.4).

6.2.3 Preparing and processing adipose tissue – Cell "Walk-Out" Method

Mesenteric tissue was weighed on a balance then washed in RPMI to remove blood and any surface contaminants. The tissue was mechanically macerated and washed repeatedly in RPMI until there was no blood staining of the solution. The macerated tissue was then placed in a 150 x 15mm polystyrene Petri dish. 50ml of complete medium was added to the Petri dish and the dish agitated to ensure the suspended fat was evenly distributed and incubated at 37°C for 20 hours.

Following incubation, the suspension was passed through a stainless-steel sieve, the adipose tissue retained within in the sieve was washed with 50ml PBS and the wash retained. A further 50ml of PBS was used to wash the petri dish using a Pasteur pipette to suspend cells adherent to the base of the dish. This wash was also retained. All three solutions were passed through a 40µm Nylon cell strainer then spun in a centrifuge for five minutes at 600g, the pellets combined into a single 50ml tube and washed again with 50ml PBS. The samples were then proceeded to processing for flow cytometry (Section 6.2.4).

6.2.4 Preparing and procession muscle tissue

Healthy and Diseased muscle tissue was processed in the same manner. Muscle tissue was weighed, repeatedly washed in RPMI until the solution was no longer blood stained and mechanically macerated. The tissue was then suspended in 40ml HBSS, 5ml (1mg\ml) Collagenase D and 5ml (0.1mg/ml) Liberase TL. The suspension was incubated a shaker at 37°C and 100rpm for 1 hour. Following incubation, the suspension was vortexed, passed through a metal sieve, then 100µm Nylon cell strainer and finally a 40µm Nylon cell strainer. The digested tissue was discarded and the solution spun at 600g in a centrifuge for 5 minutes. The subsequent pellet was resuspended washed in PBS prior to a second spin at 600g for five minutes. The pellet was then resuspended in PBS prior to processing for flow cytometry (Section 6.2.4).

6.2.5 Bacterial stimulation of peripheral blood mononucleocytes

1 x 10^6 PBMC were taken from the PBMC isolates of healthy and diseased donors and reconstituted in complete medium to 1.2 ml. 100µl of solution was pipetted into 12 individual wells of a 96 well plate. 2µl of each heat killed bacterial stock (5 x 10^5) were added to a designated well. The final two remaining wells contained complete medium only, 2µl of Lipopolysaccharide (LPS) (1 µg/ml) solution in PBS (0.2µl in 200µl) was added to one of these wells. The samples were then incubated for 20 hours at 37°C. Following incubation the contents of each well was transferred to 5ml polystyrene FACS tubes and each well washed with 100µl of PBS which was added to the corresponding FACS tube. 1ml of PBS was then added to each FACS tube to wash the cells and cells spun at 600g in a centrifuge for 5 minutes, the wash and spin then repeated; the pellets resuspended were ready for processing for flow cytometry.

6.2.6 Processing for Flow Cytometry

Following extraction cells were resuspended in PBS at a volume to allow 10µl of cell suspension per FACS tube.

6.2.6.1 Viability staining

Live/Dead fixable dead cell stains distinguish dead from alive cells based on loss of membrane integrity and access to available amines in dead cells. Dead cell become stained and thus can be selected out following flow cytometry. Viability staining was performed using LIVE/DEAD™ Fixable Near-IR Dead Cell Stain reconstituted in Dimethyl-sulfoxide (DMSO). Fixable Near-IR Stain is an amine reactive dye that binds covalently to intracellular and extracellular amines of dead cells. Excitation occurs at a maximum of ~633nm whilst emission occurs at ~750 nm. This was prepared by diluting the stock viability stain at a ratio of 1:500 in PBS. 50µl of viability stain was added to each FACS tube and incubated at room temperature in the dark for 15 minutes. The cells were then washed in 1ml FACS buffer and spun at 600g in a centrifuge for 5 minutes. The pellet was then resuspended in FACS buffer with made up to a volume as to be redistributed at 100µl to each FACS tube for the addition of cell surface antibodies. A volume of 15µl/FACS tube of FCS was added to the FACS buffer to reduce non-specific binding.

6.2.6.2 Cell surface antibodies

Fluorochrome-conjugated antibodies were added to each 115µl of cells per FACS tube, each tube received “core antibody cocktail” then each sub-panel received specific

antibodies with fluorochromes corresponding to remaining channels. A "DC lineage cocktail" was used in certain experiments (Table 6.5a) to identify DC by exclusion, this cocktail consisted of a mixture of antibodies recognising other leucocytes (B, T, NK cells, monocytes and stem cells) found in peripheral blood and tissue. Cells which did not express these markers were considered DC in the analysis. These antibody cocktails and sub-panels including volumes are shown in Table 6.5a-k. Fluorescence minus one (FM-1) controls were created for each marker of interest i.e. cells in these control FACS tubes were stained with all markers except the marker of interest (see section 6.2.6.3).

The cells were incubated at 4°C in the dark for 25 minutes. Following incubation 1ml of FACS buffer was added to each tube to wash and FACS tubes were spun at 600g in a centrifuge for 5 minutes, the supernatant discarded. For cells requiring surface marker staining only the cell pellet was resuspended and fixed in 50µl of 1% PFA. Cells requiring intracellular staining were resuspended and fixed in 100µl Leucoperm Reagent A (*AbD Serotec, Watford, UK*).

Antibody cocktails and sub-panels:

Fluorochrome	Marker of interest	Volume/FACS tube
PECy5	CD3	0.675
	CD14	2.00
	CD16	0.075
	CD19	2.00
	CD34	2.00
The lineage cocktail was made up to 10µl/FACS tube by adding 3.25µl of FACS buffer		

Table 6.5a "DC lineage cocktail" for PBMC, muscle and adipose tissue

Fluorochrome	Marker of interest)	Volume/FACS tube (µl)
PECy5	“DC Lineage Cocktail”	10.0
PECy7	CD123	2.00
APC	HLA-DR	2.00
BV605	CD11c	2.00

Table 6.5b “Core antibody cocktail” for all PBMC experiments

Fluorochrome	Marker of interest	Volume/FACS tube (µl)
BB515	Integrin β7	2.00
PE	CLA	2.00
BV421	CD40	2.00

Table 6.5c Panel A PBMC

Fluorochrome	Marker of interest	Volume/FACS tube (µl)
BODIPY 493/503	Intracellular Total lipid	
PE	CD36	2.00
BV421 Streptavidin	Intracellular E06 (Biotinylated)	

Table 6.5d Panel B PBMC

Fluorochrome	Marker of interest	Volume/FACS tube (µl)
PECy5	DC Lineage Cocktail	10.00
PECy7	CD123	2.00
APC	HLA-DR	3.00

BV605	CD11c	2.00
FITC	CD45	5.00

Table 6.5e “Core antibody cocktail” for all muscle and adipose tissue experiments

Fluorochrome	Marker of interest	Volume/FACS tube (µl)
PE	CCR7	2.00
BV421	CD40	2.00

Table 6.5f Panel A muscle and adipose tissue

Fluorochrome	Marker of interest	Volume/FACS tube (µl)
PE	CD36	2.00
BV421 Streptavidin	Intracellular E06 (Biotinylated)	

Table 6.5g Panel B muscle and adipose tissue

Fluorochrome	Marker of interest	Volume/FACS tube (µl)
PE	Integrin Beta7	2.00
BV421	CD49d	2.00

Table 6.5h Panel C muscle and adipose tissue

Fluorochrome	Marker of interest	Volume/FACS tube (µl)
PerCPCy5.5	CD14	2.00
	CD64	2.00
APC	HLA-DR	3.00

PECy7	SIRPα	2.00
BV421	CD103	2.00
BV605	CD11c	2.00

Table 6.5i “Core antibody cocktail” mucosal leucocytes

Fluorochrome	Marker of interest	Volume/FACS tube (µl)
FITC	CD40	2.00
PE	CCR7	2.00

Table 6.5j Panel A IEL and LPL

Fluorochrome	Marker of interest	Volume/FACS tube (µl)
TopFluor	Intracellular E06	
PE	CD36	2.00

Table 6.5k Panel B IEL and LPL

Table 6.5 a-k Antibody cocktails and sub-panels

6.2.6.3 Permeabilisation and intracellular staining

Following cell surface staining the cells were resuspended in 100µl of fixation medium - Leucoperm Reagent A (*AbD Serotec, Watford, UK*) for 15 minutes in the dark at room temperature. 1ml of FACS buffer was added to each tube to wash, centrifuged at 600g for 5 minutes. The supernatant discarded and 100µl of permeabilisation medium - Leucoperm Reagent B (*AbD Serotec, Watford, UK*) added to each FACS tube. E06 Biotinylated and E06 TopFluor were diluted at a ratio of 1:4 in PBS and 1µl added to each FACS tube. BODIPY 493/503 lipid staining dye was prepared by dissolving 10mg

of the lyophilised product in DMSO to a 20mM stock [molecular weight = 262.1085 g/mol]. This was then stored 20µl aliquots at -20°C. 10µl of BODIPY 493/503 stain was taken from these stock aliquots and further diluted for use to a concentration on 1 in 10,000 (2µM) in PBS. Intracellular stains/antibodies (Table 6.6a-c) were added to the now permeabilised cells which were incubated in the dark for 30 minutes at room temperature. A further wash was undertaken with 1 ml FACS buffer. Cells treated with BODIPY or E06 TopFluor were then resuspended and fixed with 50µl of 1% PFA. The samples containing Biotinylated E06 were resuspended in 100µl of Leucoperm Reagent B. 1µl of Streptavidin BV421 was added to each FACS tube. Cells were incubated in the dark for 10 minutes to allow binding of the Streptavidin to the biotinylated portion of the E06 antibody. Following incubation a further wash in 1ml of FACS buffer followed by being centrifuged for 5 minutes at 600g, the supernatant was discarded and pellet resuspended in and fixed in 50µl of 1% PFA.

Intracellular stain and antibody panels:

Fluorochrome/stain	Marker of interest	Volume/FACS tube
BODIPY 493/503	Total lipid	10µl (1 in 10,000 PBS)
PE	CD36	
BV421 - Streptavidin	E06 (Biotinylated)	1µl (1 in 4 PBS)

Table 6.6a Panel B PBMC

Fluorochrome	Marker of interest	Volume/FACS tube

PE	CD36	
BV421 - Streptavidin	E06 (Biotinylated)	1µl (1 in 4 PBS)

Table 6.6b Panel B muscle and adipose tissue

Channel	Marker of interest	Volume/FACS tube
TopFluor	E06	1µl (1 in 4 PBS)
PE	CD36	

Table 6.6c Panel B IEL and LPL

Table 6.6 a-c Intracellular stain and antibody panels

6.2.7 Sample Storage

Following fixation with 1% PFA all samples were stored at 4°C in the dark prior to analysis by flow cytometry.

6.2.8 Flow Cytometry

6.2.8.1 BD FACS Canto II Cell Analyzer

Flow cytometry was performed on the BD FACS Canto II Cell Analyzer (*BD Biosciences, San Jose, CA, USA*). The BD FACS Canto II is an eight-colour flow cytometer configured with three lasers, the technical details relating to the lasers, their associated fluorochromes and their detector bands are shown in Table 6.7. Analysis was performed on BD FACSDiva™ (*BD Biosciences, San Jose, CA, USA*) using a Microsoft® Windows® XP Pro (*Microsoft Corporation, Albuquerque, NM, USA*) operating system. Compensation was carried out using Anti-Mouse Ig, κ/Negative Control Compensation Particles Set (*BD Biosciences*) conjugated to antibodies selected from the experiment

panels in Table 6.5a-k. The ArC™ Amine Reactive Compensation Bead Kit (Thermo Fisher Scientific) was used for compensation of the LIVE/DEAD™ Fixable Near-IR Dead Cell Stain.

LASER	Fluorochromes	Detector Bands
Violet	Pacific Blue™, AmCyan (455, 488 nm), BV605, BV421	450/50; 502 to 525 nm
Blue	FITC, PE, PerCP or PerCP-Cy5.5, PE-Cy7, BB515 (525, 575, 678 or 695, 785 nm)	530/30; 585/42; >670; 780/60 nm
Red	APC, APC-Cy7 (660, 785 nm)	660/20; 780/60 nm

Table 6.7 Technical specification of the BD FACS Canto II Cell Analyzer

6.2.8.2 Principles of Flow Cytometry

Flow cytometry allows the rapid analysis of individual cells or particles suspended in a buffered solution (FACS buffer) as they flow through lasers. Each particle which passes through the laser causes light to scatter and refract additionally fluorochromes bound to the antibodies or within dyes are excited by of a specific wavelength (Table 6.7) and emit a light signal. Light scatter is measured in two directions, forward scatter [FSC] and side scatter. The degree of forward scatter denotes the relative size of the cell or particle whilst the degree of side scatter [SSC] relates to the granularity of the cell. The detection of light emitted following the excitation of fluorochromes corresponds to the presence of that fluorochrome and thus its corresponding conjugate and substrate. The presence of an event of light emission from each cell is detected and recorded in addition the intensity of the emission is also recorded. This allows the operator to identify the frequency of cell which express the marker and also the intensity of light expressed by that fluorochrome. Analysis of the fluorescent intensity of the cell population using descriptive statistics allows the calculation of for example, the median fluorescent intensity [MFI]. Fluorescent intensity increases logarithmically and as use of the median or geometric mean values

are favoured in its analysis. The MFI therefore corresponds to the level of fluorochrome expression within a cell population although it has limitations in its use as it is sensitive to experimental conditions such as voltage and compensation.

6.2.8.3 Analysis of flow cytometry data

Data was exported as .fcs files for analysis in FlowJo v10.7 (*BD Biosciences, San Jose, CA, USA*). Gating is shown in the results section for each experiment. For all samples cells were distinguished from debris and other particles based on size [FSC] and granularity [SSC]. Singlet cell populations were identified from doublets and other aggregates based on cell size [FSC height and FSC area]. Viable cells were identified as those cells lacking staining with the Live/Dead™ Fixable Near-IR Dead Cell Stain. Staining from this point forward depended on the individual experiment. For markers of interest staining was considered positive when the excitation threshold exceeded that of the fluorescence minus one (FM-1) cell population in that channel.

6.2.9 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA were performed to measure circulating amounts of IL6, IL10 and GDF-15 in our experimental cohort. Plasma (3ml) from whole blood was taken as described above and stored at -80°C for protein analysis. Standard were created using serial dilution with Reagent Diluent for IL-6 and IL-10 and Calibrator Diluent RD5-20 for GDF-15 as per the manufacturer's instructions. Plate plans were devised in advance to ensure accuracy and reduce the risk of error.

6.2.9.1 DuoSet® ELISA Kit

IL6 and IL10 ELISA was performed using the DuoSet® ELISA Development System (R&D Systems). The standard protocol was followed for the DuoSet® ELISA Development System as per the manufacturer's instructions (catalogue number DY206-05 (IL-6) and DY217B-05 (IL-10). Briefly, ELISA 96 well plates were prepared the day before and treated with capture antibody, 100µl/well, the plate was sealed and incubated overnight at room temperature. The contents of each well were then discarded and the wells washed three times with wash buffer (400µl/wash) ensuring complete removal of the liquid from the wells as each wash by blotting the well plate against paper towel. The plates were blocked using 300µl of Reagent Diluent to each well and the plate incubated at room temperature for one hour. The wash phase was then repeated as above.

100µl of sample or standard in Reagent Diluent was added to each well, the plates covered and then incubated for 2 hours at room temperature. The wash stage was then repeated. 100µl of Streptavidin-HRP was added to each well, the plate covered and further incubated in the dark for 20 minutes at room temperature. The wash stage was repeated and 100µl of Substrate Solution added to each well and the plate incubated in the dark for 20 minutes at room temperature. 50µl of Stop Solution was then added to each well and the plate agitated to ensure mixing. The plate was then read on an Infinite® F50 microplate reader (*Tecan Trading AG, Switzerland*) at a wavelength of 540nm and data was analysed using Magellan™ software (*Tecan Trading AG, Switzerland*).

6.2.9.2 Quantikine® ELISA

The GDF-15 Quantikine® ELISA came as a pre-prepared kit with coated plates. 100µl of Assay Diluent RD1-9 was added to each well, 50µl of plasma (at a dilution of 1:4 with

Calibrator Diluent) or 50µl standard was added to each well. The sample was incubated on a shaker for 2 hours at 500rpm. Each well was aspirated, the contents discarded and the plates washed with 400µl of wash buffer and blotted dry on paper towel. This was repeated four times. 200µl of GDF-15 Conjugate was added to each well, the plate sealed and incubated on a shaker for one hour at room temperature on a shaker as above. The wash step was repeated and 200µl of Substrate Solution was added, the plate was incubated in the dark at room temperature for 30 minutes.

50µl of Stop Solution was added to each well and the plate was then read on an Infinite® F50 microplate reader (*Tecan Trading AG, Switzerland*) at a wavelength of 540nm the kits) and data was analysed using Magellan™ software (*Tecan Trading AG, Switzerland*).

Chapter 7

7 Dendritic cell lipid content in colorectal cancer varies based on anatomical location and is related to body composition status

7.1 Summary

Chapters two to five have examined population data with the aim of establishing what factors bring about changes in body composition. By identifying both causality but also triggering factors in changes in BC status we may find focussed methods of preventing or reducing the stimulus for a deleterious systemic inflammatory response to the tumour. Chapter two identifies three body composition states which are independently associated with tumour features. Of these three states the presence of visceral obesity is associated with the favourable prognostic features of earlier T-stage, reduced nodal disease burden, absent vascular invasion and better tumour differentiation. We also demonstrated that myosteatosis was associated with poorer differentiation, vascular invasion and metastases and the latter relationship was explored in more detail in chapter three. Sarcopenic obesity, known to be associated with worse outcomes, was itself independently associated with more advanced T-stage and poorer tumour differentiation. These findings highlight how the tumour can influence the host, but perhaps, through the metabolic effects of visceral fat, how the host may affect the tumour's biology. In chapter four we explored how the environment influences body composition status using deprivation indices as a surrogate for environmental and

lifestyle status, here we postulated that sarcopenia could be a premorbid condition which is compounded by disease and treatment and as such may be a reversible condition through the modification of environmental factors. Finally, in Chapter five we demonstrated how the inherited genetics and subsequent ethnic phenotype of the host exerts influence over not only their body composition but also their inflammatory status, implying that the immune response to CRC differs based on predefined and pre-determined factors. These chapters demonstrate the complex interplay between host, the environment, the tumour and ultimately the immune system.

The immune response to cancer is a well-established actor in influencing body composition, surgical and cancer specific outcome. However, it is important to take into consideration the environment and phenotypic features of the host on body composition and cancer outcome. These factors have often been overlooked in previous studies, as by their very nature, animal models do not allow for this complexity. We therefore aimed to explore the effects of the innate and adaptive immune response on the host through an examination of the effect of CRC on key immune pathways and their relationship with body composition features within our patient population.

7.2 Introduction

7.2.1 The role of obesity in systemic inflammation

To fully understand the interplay between body composition and the immune system it is necessary to clarify existing assertions which implicate body composition features in the systemic inflammatory response. The links between obesity and systemic inflammation are well established; studies have identified that the anatomical location of fat influences the level and potency of immune activation[229]. This low-grade immune response has been implicated in the pathogenesis of multiple primary cancer types including colorectal cancer[36]. However, the variation in inflammatory response based on anatomical location may also influence different end organ responses to inflammation. Adipose tissue exerts its systemic inflammatory effects by activating various cell types. One such family are the myeloid cells which include monocytes, dendritic cells and macrophages. Most notably, as part of a presumed stress response macrophages infiltrate adipose tissue, early within the process of adipose tissue hypertrophy, initiating and perpetuating a chronic low grade inflammatory response[230]. The importance of myeloid cells in obesity is characterised by the positive correlation between the number macrophage cells and "obesity" of individual adipocytes. Macrophage number can increase up to four to five fold within the tissue and ultimately constitute 50% of the cell population within the adipose tissue[231]. As well as the direct influence of the cellular immune system on adipose tissue, adipocytes can conversely compound the inflammatory response through alterations in adipokine and cytokine production (Figure 7.1)[232], for example adiponectin, which promotes insulin sensitivity is anti-inflammatory and its' circulating levels decreased in Metabolic Syndrome[233,234]. This is key considering

the links between the Doyles' definition and cut off values of VO in relation to metabolic syndrome described in chapter one[78]. This interplay between the adipocyte, the cellular immune system its downstream effect is likely to be crucial in elucidating our earlier findings of chapter three.

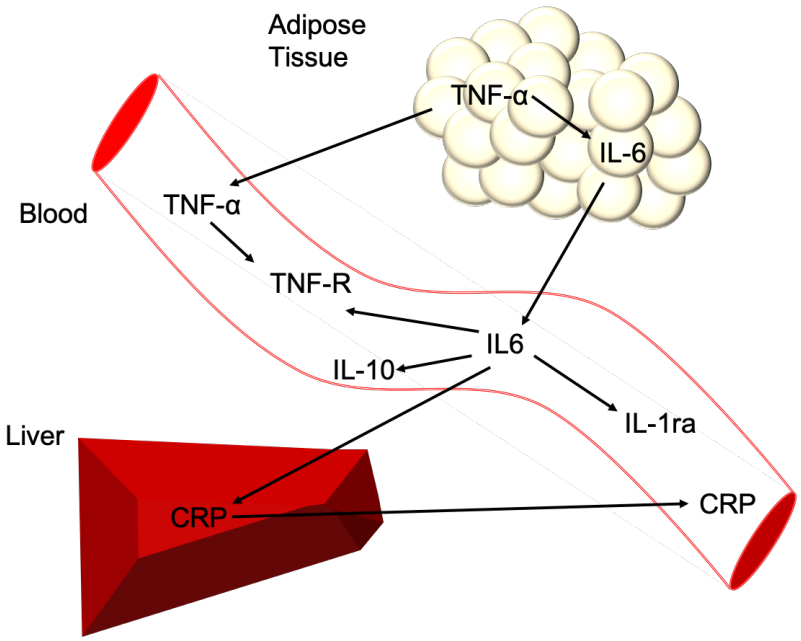


Figure 7.1 Systemic inflammation and adipose tissue

TNF-α is produced in adipose tissue which stimulates *the production of IL-6 in adipose tissue and associated blood mononuclear cells e.g., dendritic cells & macrophages. IL-6 stimulates an increase in synthesis of IL-1 receptor antagonist (IL-1ra), soluble TNF receptor (TNF-R), IL-10, and C-reactive protein (CRP). (Adapted from Petersen et al 2005)[232].*

7.2.2 The role of muscle in systemic inflammation

The systemic inflammatory response is inherently linked to features of muscle tissue. Like adipose tissue the relationship is likely to be complex and multidirectional. The relationship between a heightened systemic inflammatory response and myosteatosis

has been well described in earlier chapters. What is perhaps more complex is the metabolic and pro and anti-inflammatory properties of muscle tissue. Once again IL-6 is implicated; however, unlike adipose tissue, during contractile exercise skeletal muscle upregulates IL-6 mRNA in isolation releasing IL-6 without TNFα[235] and in such is different from the classical picture seen in inflammatory conditions such as sepsis[232] or that of adipose tissue induced inflammation. This muscle derived IL-6 is thought to exert an anti-inflammatory effect by mechanisms including the inhibition of LPS-TNFα production by monocytes[236], inducing hepatic derived acute phase proteins with anti-inflammatory properties and stimulating production of IL-1ra and IL10[237,238]. As such we can assume IL-6 is paradoxically a key component of skeletal muscle's anti-inflammatory cascade which involves a number of mononuclear cell types such as macrophages, T-cells and dendritic cells. Our potential ability to manipulate the anti-inflammatory properties of muscle are explored in more depth in Chapter ten and eleven.

7.2.3 The dendritic cell and colorectal cancer

Dendritic cells are the major antigen presenting cell within the human body, their role and function has been described in Chapter One. Briefly, they are professional antigen presenting cells which sample the host micro-environment providing antigens and co-stimulatory signals to the adaptive immune system. The majority of DC exist in an immature and unaggressive state; they have a high capacity to capture antigen whilst exhibiting limited expression of costimulatory molecules and limited secretion of cytokines[239]. Once stimulated DC down-regulate their antigen capture ability and increase expression major histocompatibility complex II (MHC class II), C-C chemokine receptor 7 (CCR7) and migration markers (see Chapter Nine).

7.2.4 The role of DC in response to colorectal cancer

Dendritic cells are ubiquitous throughout the body, present in all tissue types. In disease DC are found within the tumour microenvironment and it is thought that tumour specific antigen presentation takes place both within the tumour as well as the other more specialised lymphoid organ apparatus. The action of antigen presentation within the tumour therefore intimates that these intratumoural cells potentiate anti-tumour actions of associated intratumoural T-cells[240]. Despite this presence of DC in the tumour microenvironment, in a picture of disease progression, these cells are implicated as part of an immune system which has failed in achieving its end goal. It has been established that in progressive malignancy DC become defective in their ability to differentiate and activate[239] and ultimately successfully present antigen. This dysfunction may manifest itself as increased intracellular lipid which may itself be causative as well as symptomatic of dysfunction.

7.2.5 The effect of intra-cellular lipid in cancer

Early work examining the effect of lipid infiltration on dendritic cell function has been performed in animal models. Initially investigators considered whether DC would be capable of acquiring fat and glycogen stores as an energy source during maturation. In 2005, Maroof and colleagues, undertaking work within our group, identified that DC became “lacy” in appearance on electron microscopy when cultured in vitro with lipopolysaccharide. The cytoplastic inclusion which gave the cells the “holes” of the lace appearance were found to be intracellular lipid. To ascertain whether this phenomenon occurred in vivo DC were isolated from mouse omental lymph nodes and spleens. A proportion of the DCs from each source showed a lacy morphology, more so in DC

isolated from omental nodes in keeping with their perinodal lipid rich environment[241]. Herber and colleagues examined the dendritic cells of tumour bearing mice using the lipophilic fluorescent dye BODIPY 493/503. They explored four tumour models including MC38 colon adenocarcinoma. Notably raised levels of lipid were seen in $CD11c^{+}CD8^{+}$ DCs and $CD11c^{+}CD11b^{+}B220$ – conventional/classic DC (cDC), which includes myeloid DC (mDC) but not in $CD11c^{+}CD11b^{-}B220^{+}$ plasmacytoid DCs (pDC). It was also noted that DC isolated from the tumour microenvironment expressed higher levels of macrophage fat scavenger receptor 1 (Msr1) in comparison to DC isolated from the lymph nodes or spleen[242]. Herber and colleagues later examined possible causation, they identified that in-vitro generated DC cultured with tumour explant supernatants had a greater than threefold increase in intracellular lipid, specifically an increase in all molecular species of triacylglycerol lipid but no increase in cholesteryl esters or phospholipid. DC lipid response to antigen stimulation and further related concepts are explored further in chapter nine. Interestingly Maroof and colleagues identified that increased intracellular lipid and glycogen were acquired in parallel with the developing capacity of DCs to stimulate primary proliferative responses in T cells. Concurrent upregulation of fat scavenger receptors was also seen as DC acquired intracellular lipid and glycogen following exposure to IL-4[241]. Paradoxically, Herber and colleagues suggested that lipid laden DC had a profound defect in their ability to process and present soluble antigens. They propose that lipid ladened DCs are functionally immature cells, effective at picking up soluble proteins but, as described above, limited in their ability to present antigen. They do acknowledge that whether lipid ladened or not, DC present similar levels of MHC class II and other costimulatory molecules equally well[242]. More recently, it has been suggested in work with in vitro healthy human donor DC and mouse tumour models of

cancer that lipid sub-type is important and that oxidised lipids are implicated in DC dysfunction by reducing the expression of peptide–MHC class I complexes on the cell surface, whilst non oxidised lipids did not affect antigen presentation[243].

7.2.6 The association between body composition and fat scavenger receptors in colorectal cancer

Scavenger receptors have a major role in intracellular transport of lipids. CD36 a fat scavenger receptor expressed of various cell types including dendritic cells and macrophages has been associated with pathological processes including fat uptake by macrophages resulting in foam cell formation in atherosclerosis. In atherosclerosis its specific uptake of oxidised low-density lipoprotein has been described as a key factor[244]. The uptake of oxidised lipid by macrophages in atherosclerosis also interferes with macrophage migration resulting in a "macrophage-trapping mechanism" which leads to foam cell deposition[245]. We explore this the concept of potential "DC trapping" in muscle in Chapter eight. This relationships between the CD36 and oxidised lipid is of particular interest in the case of cancer in view of the role of oxidised lipid in DC dysfunction. We know that in myosteatosis there is ectopic fatty deposition within muscle, the mechanism for which is not fully understood. However, earlier work in our laboratory by Malietzis and colleagues identified a positive correlation between circulating DC CD36 expression and increasing mean muscle attenuation (a surrogate marker decreasing fatty infiltration)[139]. This suggests a more complicated narrative defining the function of CD36 in colorectal cancer, its association with a body composition phenotype which is associated with an improved prognosis may suggest a dual role in cancer. We know for example that in DC CD36 mediates uptake of apoptotic cells and provides a mechanism for cross-

presentation of antigens to cytotoxic T cells[246] and such a role may explain this discrepant picture seen in circulating DC which by their nature will be relatively immature. Assessing the relationship between intracellular lipid and CD36 expression, not only in the DC in blood but also in clinically relevant tissues is vital.

7.2.7 Study Hypothesis and Aims

We have seen how different body composition morphologies are associated with different outcomes, we have described how muscle and adipose tissue both play a role in immunomodulation, fat appearing to promote chronic low-grade inflammation and healthy skeletal muscle appearing to be anti-inflammatory. In cancer we have also seen alterations due to lipid metabolism by the DC, which sit at the pinnacle of the hierarchy of the adaptive immune system and how this subsequent lipid accumulation leads to dysfunction. Herber's work has demonstrated alterations in lipid profiles of DC in the tumour microenvironment compared to blood and the spleen. Accumulation of oxidised lipid appears to be the causative agent for antigen presentation in cancer. We know that patients with myosteatosis appear to have deficient CD36 expression and as a fat scavenger receptor it is likely to impact on lipid uptake and metabolism. We therefore aimed to explore whether body composition status was further related to different DC lipid profiles at different anatomical sites and whether abnormal DC dysfunction was associated with these established prognostic markers.

7.2.8 Null Hypothesis

Body composition status and anatomical location have no association with DC function in colorectal cancer

7.2.9 Study aims

To ascertain whether:

- DC can be successfully isolated from multiple tissue sites in humans with colorectal cancer
- The expression of CD36 expression is influenced by body composition at varying anatomical location
- DC fat profile is altered in relation to body composition at different anatomical sites in colorectal cancer
- CD36 expression and oxidised lipid profiles of DC change at different anatomical sites

7.3 Methods

The aim of this study was to characterise the lipid profile of human dendritic cells in colorectal cancer patients and determine any impact on functional behaviour. A detailed description of the methods used are described in Chapter six Material and Methods. An outline of methods is briefly described here.

7.3.1 Tissue Sampling

Colorectal cancer patients, diagnosed on histology and healthy controls were recruited over a fixed period of time in accordance with ethical (Ref. 17/LO/1636), HRA and local research and development (Ref 17/095) approval. Written informed consent was obtained from all participants included in this study. Blood was collected and transported in heparinised (170IU) vacutainers, (30ml CRC patients, 50ml Healthy controls). Blood was obtained from CRC patients between 07:30 and 08:30 on the morning of surgery. Blood from healthy donors collected between 08:00 and 10:00 hours. Full thickness biopsies were taken in the operating theatre from the healthy colon (>10cm from proximal tumour margin) of fresh specimens following colonic resection, the mucosa was dissected free and subsequently processed. Healthy gracilis muscle was harvested during anterior cruciate ligament repair by the orthopaedic surgeon performing the case. Rectus or external oblique muscle biopsies from cancer patients were taken by the colorectal surgeon during surgery. Mesenteric fat was removed from the mesenteric border of the proximal cut edge of colonic resection specimens following colonic resection. All tissue specimens were stored on ice prior to processing.

7.3.2 Tissue processing

7.3.2.1 Isolation of peripheral blood mononuclear cells

Whole blood was diluted 1:1 in PBS, layered over Ficoll-Paque Plus. Samples were centrifuged at 800g for 30 minutes at 18°C and the PBMC extracted at the buffy coat. The cells were washed in PBS and counted prior to fluorescence-activated cell sorting (FACS) processing.

7.3.2.2 Isolation of IEL and LPL leucocytes

Biopsies were washed in HBSS, mechanically macerated and incubated for 30 minutes on a shaker at 37°C in a solution of 50ml HBSS containing 500µl DTT and 1mM EDTA. The supernatants containing the IEL layer were reserved, and the process repeated for a further 30 minutes. The supernatants again were reserved and the biopsies further digested in RPMI containing collagenase D (1mg/ml) and Liberase TL (0.1mg/ml) on a shaker at 37°C for 1 hour. The supernatant containing the LPL were collected and the digested tissue discarded. All supernatants were filtered through a 100µm filter followed by a 40µm filter then washed in PBS, prior to processing for FACS.

7.3.2.3 Isolation of DC from mesenteric fat

DC were obtained through the cell "walk out" method. The peritoneum was dissected from the mesentery and tissue inspected thoroughly to ensure no lymph nodes were present; no lymph nodes were found in any sample. The tissue was mechanically macerated and washed in RPMI until no blood staining was seen. The tissue was macerated then incubated for 20 hours at 37°C in complete medium. The supernatant was collected and the tissue and its container washed thoroughly in PBS to dislodge adherent cells. This PBS wash was added to the supernatant, centrifuged at 600g for 5 minutes washed in PBS again and the cell pellet retained suspended in PBS prior to FACS processing.

7.3.2.4 Isolation of DC from skeletal muscle

The tissue was mechanically macerated and washed in RPMI until no blood staining was seen the biopsies digested in RPMI containing collagenase D (1mg/ml) and Liberase TL (0.1mg/ml) on a shaker at 37°C for 1 hour. The supernatant containing the inflammatory cells from the muscle tissue collected and the digested tissue discarded. All supernatants were filtered through a 100µm filter followed by a 40µm filter then washed in PBS, prior to processing for FACS.

7.3.3 DC Identification

PBMC, IEL, LPL, mesenteric and muscle cells were washed with PBS and labelled with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher Scientific) according to the manufacturer's instructions to distinguish viable from non-viable cells. Cells were washed with FACS Buffer and then labelled with antibodies to identify the dendritic cells as described in chapter 6. Intracellular lipid analysis was performed by fixing using Leucoperm Reagent A and latterly permeabilised using Leucoperm Reagent B. The cells were then stained with BODIPY and either E06 TopFluor or E06 Biotin. Secondary staining with Streptavidin was performed for samples stained with E06 Biotin. Cells were fixed in 1% paraformaldehyde (PFA) prior to flow cytometry.

7.3.4 Flow cytometry

Single-cell suspensions were acquired on the BD FACS Canto II (BD Biosciences). Compensation carried out was prior to each series of experiments on FACS Diva software using Anti-Mouse Ig, κ/Negative Control Compensation Particles Set (BD

Biosciences) conjugated to antibodies used in above labelling experiments. The ArC™ Amine Reactive Compensation Bead Kit (Thermo Fisher Scientific) was used for compensation of the LIVE/DEAD™ Fixable Near-IR Dead Cell Stain according to kit instructions.

7.3.5 Data Analysis

Data analysis was carried out by ETP, except for automated BC analysis which was performed by Dr P Lung.

7.3.6 CT Body composition analysis

Body composition analysis has been described at length earlier in the thesis. DICOM files of the axial plane at level of the third lumbar vertebrate were obtained from the pre-operative CT scans of patients. Auto-segmentation was performed using SliceOmatic v5.0 and the ABACS L3 plug-in using artificial intelligence techniques described in the introduction. Set cut off values were used to determine clinically relevant categorical data, where statistical analysis did not allow for categorical data sets continuous variables were used in the form of LSMI, VFI, fat surface area (cm^2) and mean muscle attenuation.

7.3.7 Flow cytometry data analysis

Data analysis was performed using the FlowJo_v.10 software. Fluorescence minus one (FMO) controls were used to determine gating for cell surface fluorochromes for each tissue in each experiment. Frequency data was obtained for all cell surface markers and viability stains. Median fluorescence intensity was obtained for the intracellular lipid

analysis as these better reflected the marginal shifts seen in these populations on flow cytometry.

7.3.8 Statistical Analysis

Statistical analysis was carried out using GraphPad Prism software version 10. Non-parametric data was analysed using Mann-Whitney *U* tests. Comparisons of non-parametric grouped data was undertaken using Kruskal-Wallis ANOVA. Correlations for non-parametric data were assessed using Spearman's Rank. Descriptive statistics were used to analyse demographic data and performed in SPSS version 25.

7.4 Results

7.4.1 DC and Macrophage Lipid profile in the intraepithelial and lamina propria layer of the colon

The gating strategy for isolating both IEL and LPL is shown in the supplementary data in Appendix 1.

7.4.1.1 Demographics

Sixteen colorectal cancer patients were included in the analysis. 12 patients were male and the median age was 63 [IQR 55.25-72]. Tumour characteristics for the cohort are shown in Table 7.1 and the body composition characteristics are shown in Table 7.2.

		n	*%*
Gender	Male	12	75.0
	Female	4	25.0
T Stage	T1/2	5	31.3
	T3/4	11	68.8
Nodal Disease	Nodal Disease Absent	8	50.0
	Nodal Disease Present	8	50.0
Vascular Invasion	Vascular Invasion Absent	7	43.8
	Vascular Invasion Present	9	56.3
Lymphatic Invasion	Lymphatic Invasion Absent	10	62.5
	Lymphatic Invasion Present	6	37.5
Perineural Invasion	Perineural Invasion Absent	7	43.8
	Perineural Invasion Present	9	56.3
EMVI	EMVI Absent	9	56.3
	EMVI Present	7	43.8
Tumour Differentiation	Well/Moderately Differentiated	13	81.3
	Poorly Differentiated	3	18.8

Table 7.1 Demographic and tumour characteristics of the IEL and LPL DC cohort

		n	*%*
BMI Obesity	Not BMI Obese	8	50.0
	BMI Obese	8	50.0
Sarcopenia	Not Sarcopenic	7	43.8
	Sarcopenic	9	56.3
Myosteatosis	Not Myosteatotic	7	43.8
	Myosteatotic	9	56.3
Visceral Obesity	Not Viscerally Obese	6	37.5
	Viscerally Obese	10	62.5
Sarcopenic Obesity	Not Sarcopenic Obese	12	75.0
	Sarcopenic Obese	4	25.0

Table 7.2 Body composition characteristics of the IEL and LPL DC cohort

7.4.1.2 Fat Scavenger Receptor CD36 expression is significantly associated with obesity in the IEL DC and LPL DC

CD36 expression in the IEL DC was significantly greater in patient who were either BMI (p=0.04) or viscerally obese (p=0.04). No such significant relationships were seen with

the muscle related BC features of sarcopenia, myosteatosis or the combined feature of sarcopenic obesity (Figure 7.2).

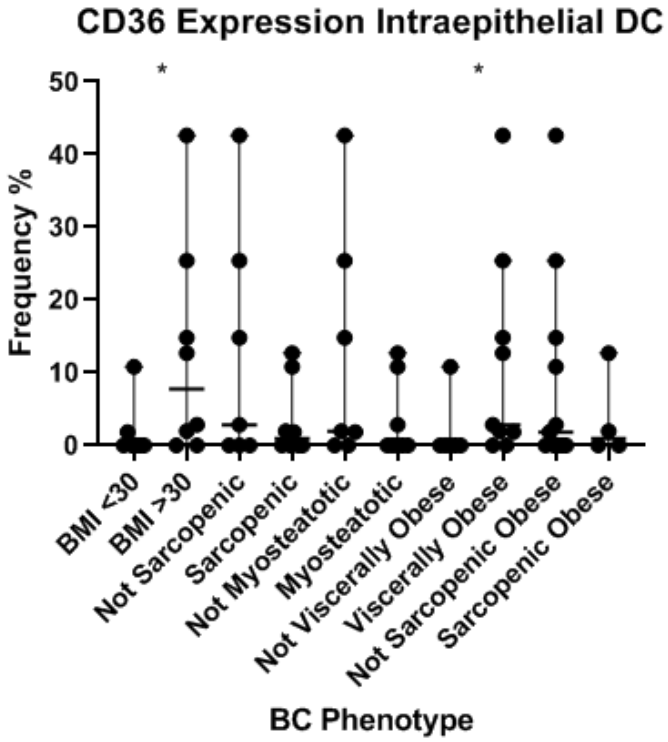


Figure 7.2 CD36 Expression in the IEL DC

No significant difference in frequency of cells expressing CD36 was found in LPL DC of CRC patients with sarcopenia (p=0.94), myosteatosis (p=0.78), BMI obesity (p=0.10) and sarcopenic obesity (p=0.88). The frequency of LPL DC CD36 expression was however significantly greater in patients with visceral obesity (p=0.008) (Figure 7.3).

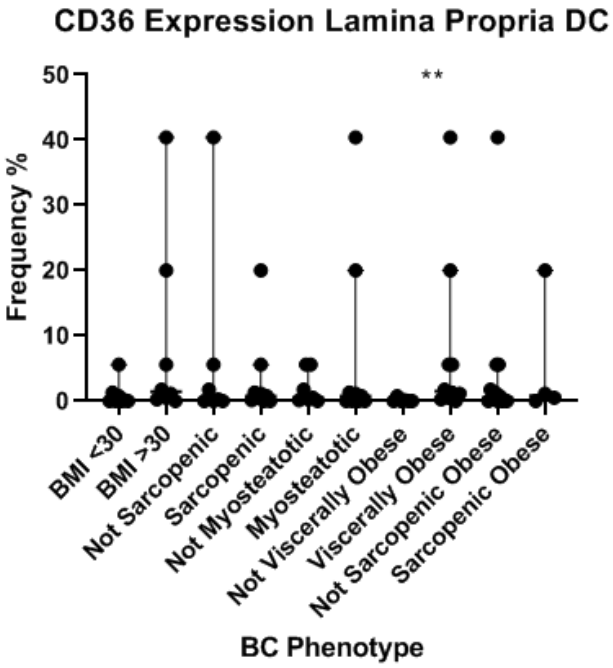


Figure 7.3 CD36 Expression in the LPL DC

CD36 expression was once again significantly raised on the IEL macrophages of patients who were viscerally obese (p=0.048); however, there was no relationship with BMI obesity (p=0.065) (Figure 7.4). Regarding frequency of CD36 expression on macrophages in the LPL there was no significant difference seen between patients with sarcopenia (p=0.55), myosteatosis (p=0.24) and sarcopenic obesity (p=0.88). However, patients with BMI obesity and visceral obesity both appeared to have significantly greater frequency of cells expressing of CD36 (p=0.02 and p=0.003 respectively) (Figure 7.5).

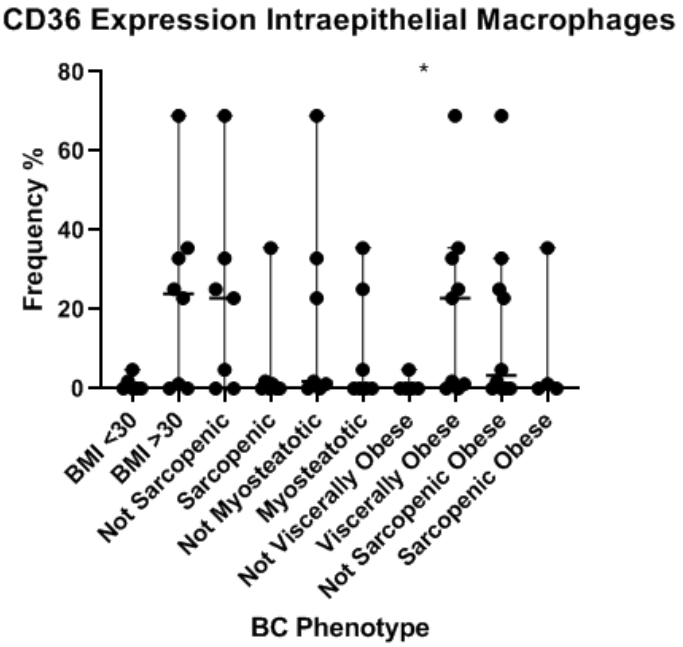


Figure 7.4 CD36 Expression on IEL macrophages

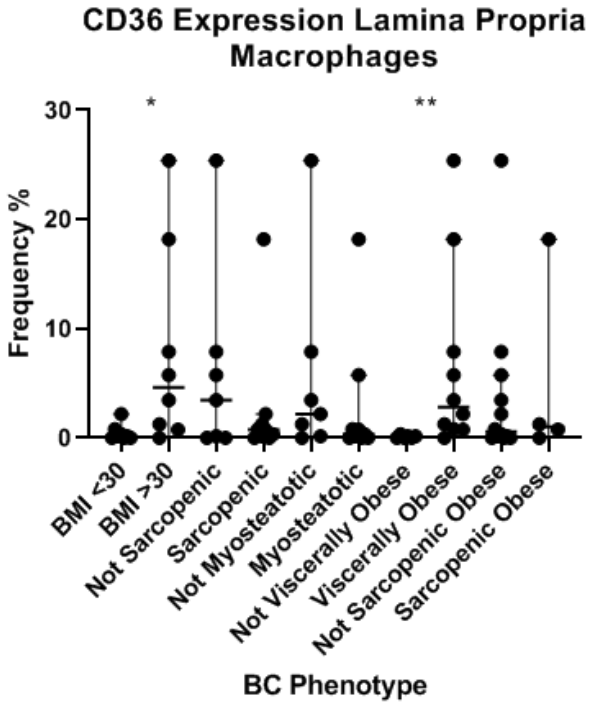


Figure 7.5 CD36 Expression of LPL macrophages

7.4.1.3 Oxidised Lipid within the LPL DC and Macrophages

Intracellular oxidised lipid (ICOL) was measured using E06 TopFluor; intracellular levels were characterised by median fluorescence intensity (MFI). There were no significant relationships between body composition and the intracellular oxidised lipid content of IEL DC (Figure 7.6).

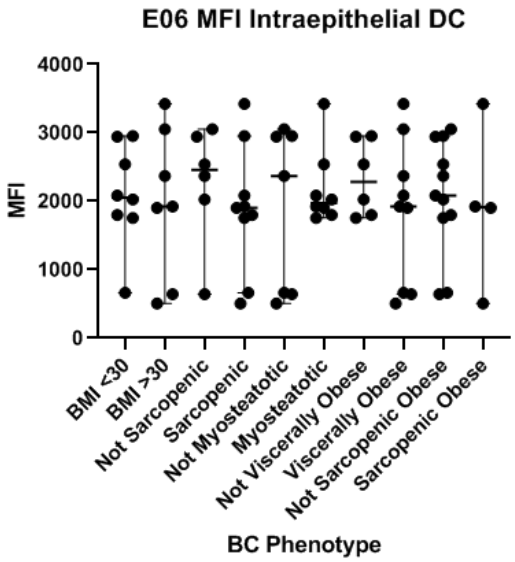


Figure 7.6 Intracellular oxidised lipid in the IEL DC expressed as EO6 MFI

The body composition phenotypes of sarcopenia (p=0.30), myosteatosis (p=0.92), BMI obesity (p=0.19) and sarcopenic obesity (p=0.08) had no association with levels of ICOL in LPL DC. Patients with visceral obesity had significantly lower levels of ICOL compared patients who were not viscerally obese (p=0.022) (Figure 7.7).

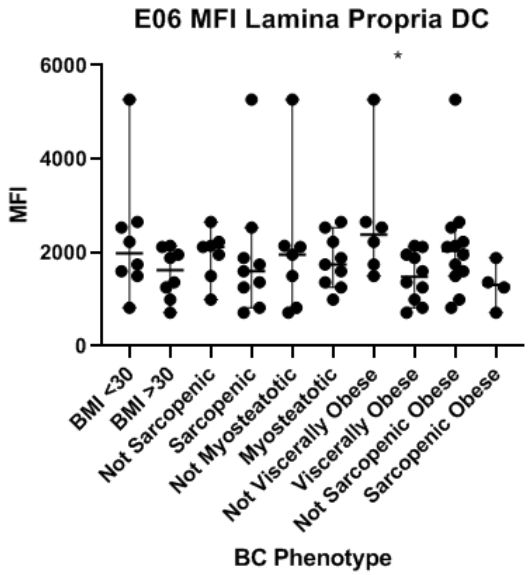
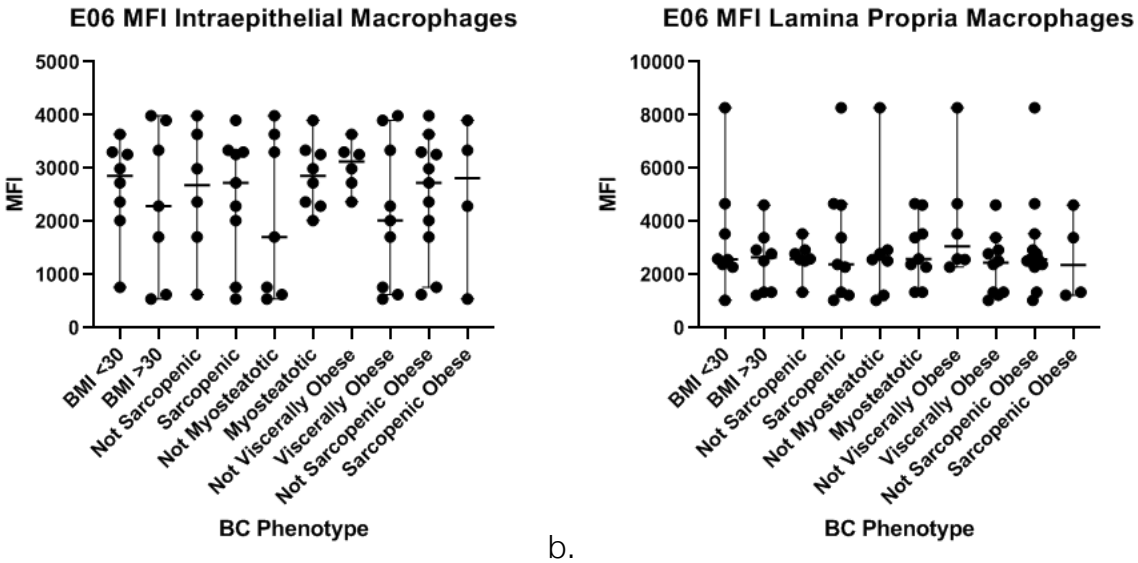


Figure 7.7 Intracellular oxidised lipid in the LPL DC expressed as EO6 MFI

There was no significant relationship between ICOL levels in macrophages and body composition in terms of MFI in either IEL or LPL macrophages (Figures 7.8a & b).



a. b.

Figure 7.8 a & b. Intracellular oxidised lipid in the IEL and LPL macrophages (EO6 MFI)

7.4.2 DC Lipid profile in the Colonic Mesentery

Comparison was made between the mDC of patients with normal BC characteristics and those with abnormal characteristics. pDC numbers obtained were too low to perform any statistical analyses. The gating strategy for the isolation of mesenteric fat is shown as supplementary data in Appendix 1.

7.4.2.1 Demographics

Fifteen colorectal cancer patients were included in the analysis, 11 patients were male and the median age 61 [IQR 55-72]. Tumour characteristics are shown in Table 7.3 and body composition characteristics are shown in Table 7.4.

		n	*%*
Gender	Male	11	73.3
	Female	4	26.7
T-Stage	T1/2	5	33.3
	T3/4	10	66.7
Nodal Disease	Nodal Disease Absent	7	46.7
	Nodal Disease Present	8	53.3
Vascular Invasion	Vascular Invasion Absent	7	46.7
	Vascular Invasion Present	8	53.3
Lymphatic Invasion	Lymphatic Invasion Absent	9	60.0
	Lymphatic Invasion Present	6	40.0
Perineural Invasion	Perineural Invasion Absent	7	46.7
	Perineural Invasion Present	8	53.3
EMVI	EMVI Absent	9	60.0
	EMVI Present	6	40.0
Tumour Differentiation	Well/Moderately Differentiated	12	80.0
	Poorly Differentiated	3	20.0

Table 7.3 Demographic and tumour characteristics of the mesenteric fat cohort

		n	*%*
BMI Obesity	Not BMI Obese	8	53.3
	BMI Obese	7	46.7
Sarcopenia	Not Sarcopenic	7	46.7
	Sarcopenic	8	53.3
Myosteatosis	Not Myosteatotic	7	46.7
	Myosteatotic	8	53.3
	Not Viscerally Obese	6	40.0

Visceral Obesity	Viscerally Obese	9	60.0
Sarcopenic Obesity	Not Sarcopenic Obese	12	80.0
	Sarcopenic Obese	3	20.0

Table 7.4 Body composition characteristics of the mesenteric fat cohort

7.4.2.2 Fat Scavenger Receptor CD36 Expression

The frequency of DC expressing CD36 is significantly diminished in patients with sarcopenia (p=0.013) and sarcopenic obesity (p=0.004); the sample size is low for the sarcopenic obesity population increasing the risk of type 2 error. The associations between visceral obesity and CD36 expression seen in the colonic DC are no longer present with no difference seen in the frequency of cells expressing CD36 between patients with and without visceral obesity (p=0.50) (Figure 7.9).

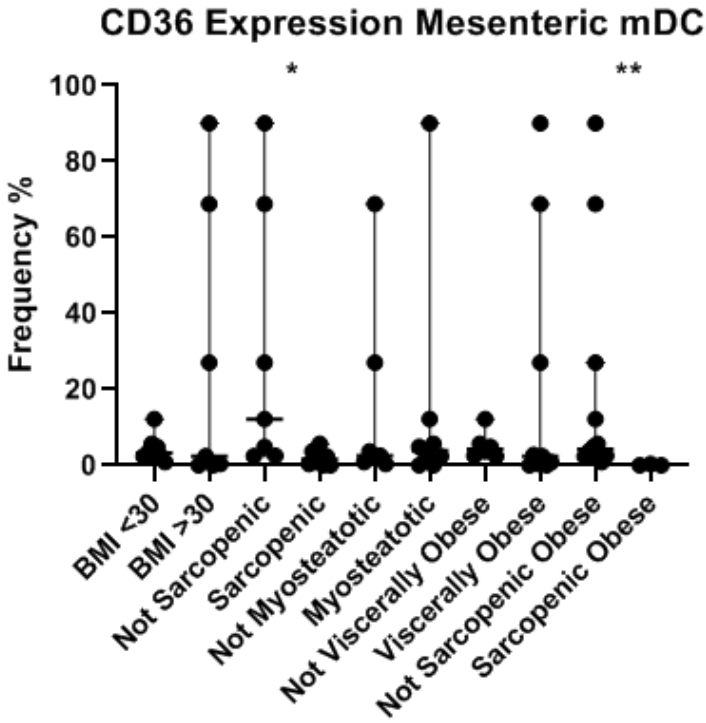


Figure 7.9 CD36 Expression by mesenteric fat mDC

We also performed an analysis of the lineage negative HLA-DR$^-$ CD11c$^+$ cell population. Interestingly we see that these there is a significant difference in the frequency of cells expressing CD36 in patients of different obesity profiles. Here we see increased frequency of cells expressing CD36 in patients with visceral obesity (p=0.04) and BMI obesity (p=0.0059) (Figure 7.10). This interestingly mirrors the expression of CD36 in the myeloid cells in the LPL and perhaps suggests these cells are related in function or behaviour to classic HLA-DR$^+$ mDC.

There was no difference in frequency of cells expressing CD36 between the myosteatotic and non-myosteatotic population.

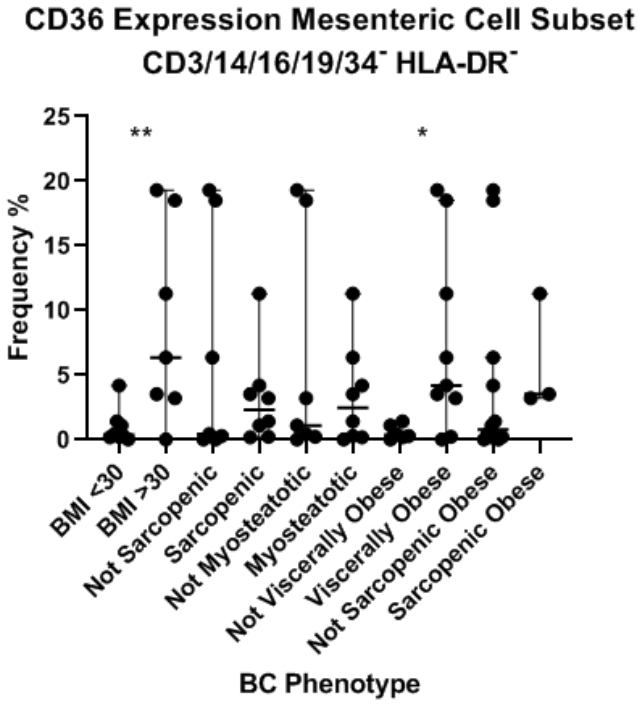


Figure 7.10 CD36 expression by the CD3/14/16/19/34- HLA-DR- CD11c+ cell population

7.4.2.3 ICOL within Mesenteric DC

There was also no significant difference in mesenteric DC ICOL between any of the body composition populations (Figure 7.11), despite noted difference in fat scavenger receptors.

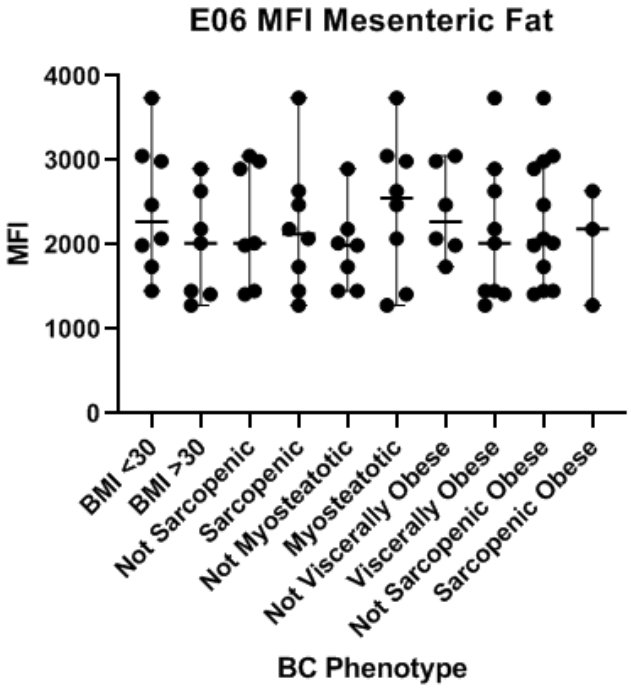


Figure 7.11 Oxidised fat expression (E06 MFI) by mesenteric Fat mDC

7.4.3 Lipid profile of circulating DC

The gating strategy used to isolate DC from peripheral blood mononuclear cells (PBMC) is shown is the supplementary data of Appendix 1.

7.4.3.1 Demographics

Twenty-two patients were included in the analysis, 15 patients were male and the median age was 60.5 [IQR51.5-60.5]. Tumour and body composition characteristics are shown

in Tables 7.5 and 7.6. Seven healthy controls were also included in the analysis, five were female and two were male, median age was 33 [IQR29-35].

		n	*%*
Gender	Male	15	68.2
	Female	7	31.8
T Stage	T1/2	5	22.7
	T3/4/extra luminal disease	17	77.3
Nodal Disease	Nodal Disease Absent	13	59.1
	Nodal Disease Present	7	31.8
	Ungraded[§]	2	9.1
Vascular Invasion	Vascular Invasion Absent	11	50.0
	Vascular Invasion Present	9	40.9
	Ungraded[§]	2	9.1
Lymphatic Invasion	Lymphatic Invasion Absent	14	63.6
	Lymphatic Invasion Present	6	27.3
	Ungraded[§]	2	9.1
Perineural Invasion	Perineural Invasion Absent	10	45.5
	Perineural Invasion Present	10	45.5
	Ungraded[§]	2	9.1
EMVI	EMVI Absent	12	54.5
	EMVI Present	8	36.4
	Ungraded[§]	2	9.1
Tumour Differentiation	Well/Moderately Differentiated	17	77.3
	Poorly Differentiated	5	22.7

[§]Ungraded as resection of locally recurrent rectal cancer

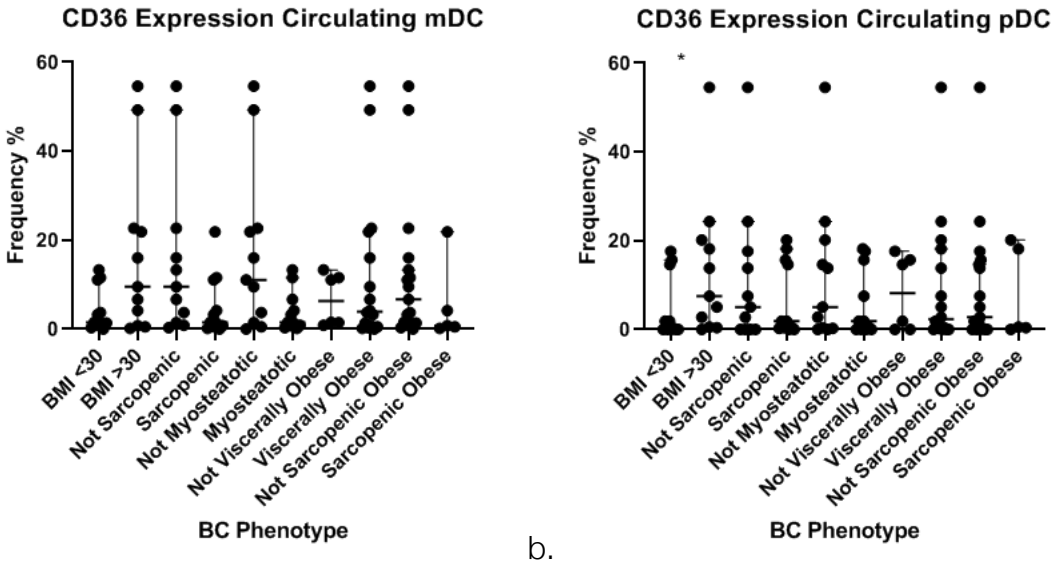
Table 7.5 Demographic and tumour characteristics of the PBMC cohort

		n	*%*
BMI Obesity	Not BMI Obese	11	50.0
	BMI Obese	11	50.0
Sarcopenia	Not Sarcopenic	11	50.0
	Sarcopenic	11	50.0
Myosteatosis	Not Myosteatotic	11	50.0
	Myosteatotic	11	50.0
Visceral Obesity	Not Viscerally Obese	6	27.3
	Viscerally Obese	16	72.7
Sarcopenic Obesity	Not Sarcopenic Obese	17	77.3
	Sarcopenic Obese	5	22.7

Table 7.6 Body composition characteristics of the PBMC cohort

7.4.3.2 Lipid and CD36 profiles between different body composition profiles in disease

There was no association seen when comparing CD36 expression on mDC between BC profiles in disease (Figure 7.12a). CD36 expression did however appear to be significantly greater in the pDC population of those patients with BMI obesity (p=0.05), (Figure 7.12b). There was also no significant difference between ICOL (Figure 7.13a &b) or intracellular total lipid (ICTL) between different patients with different body composition profiles (Figure 7.13c & d). Differences became apparent when we factored in data from a healthy control population.



a. b.

Figure 7.12a & b CD36 expression in circulating mDC and

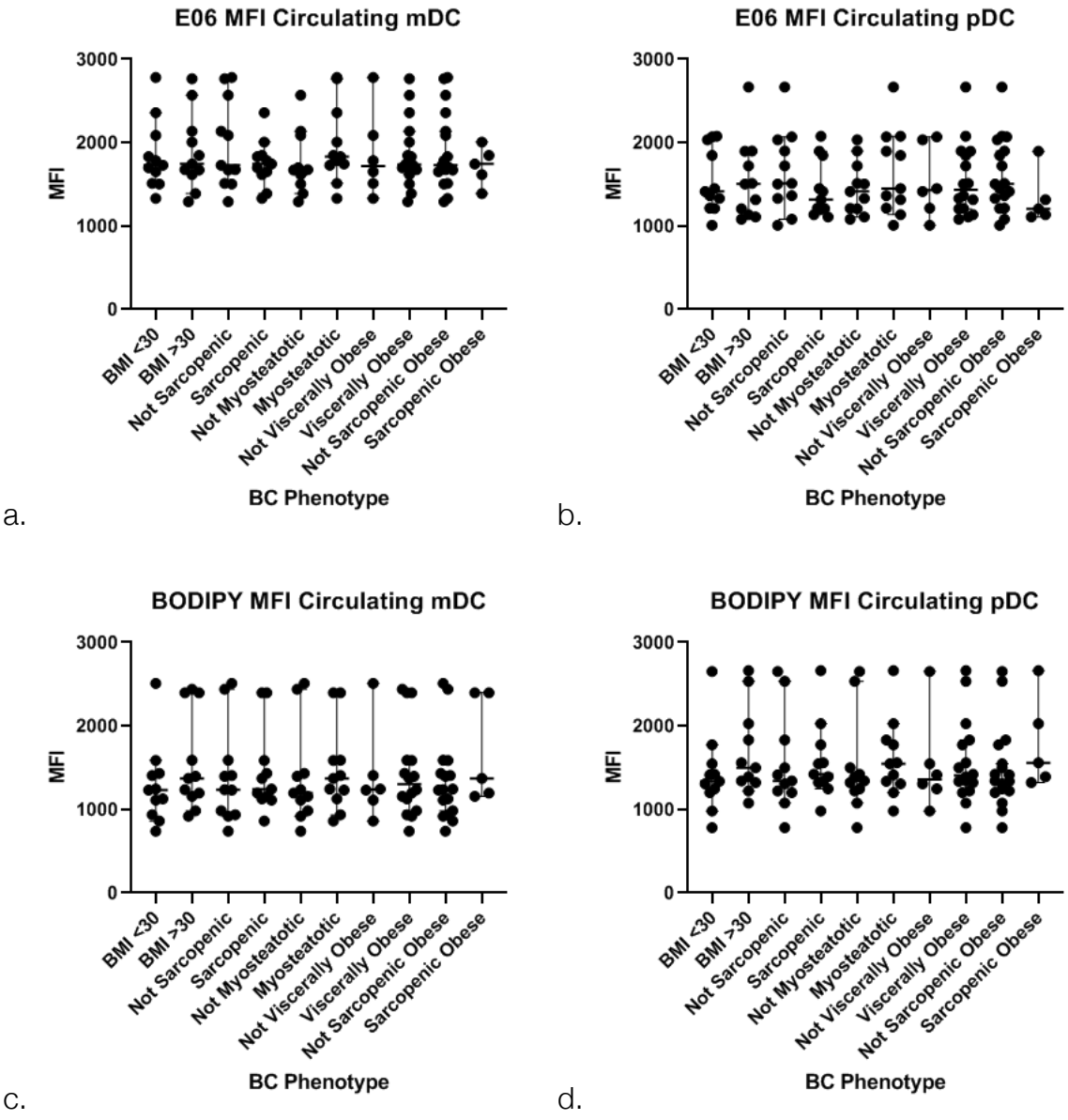


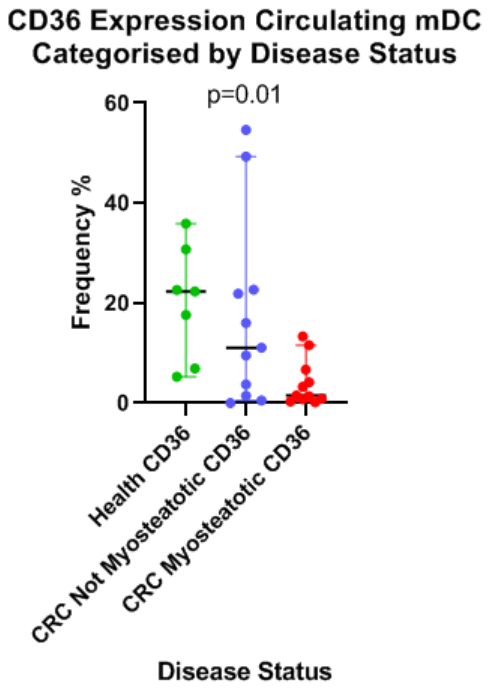
Figure 7.13 a-d expression of intracellular lipid in circulating DC in relation to BC E06 MFI (ICOL) shown in a & b; BODIPY MFI (TICL) shown in c & d.

7.4.3.3 Lipid and CD36 DC profiles in patients with and without myosteatosis

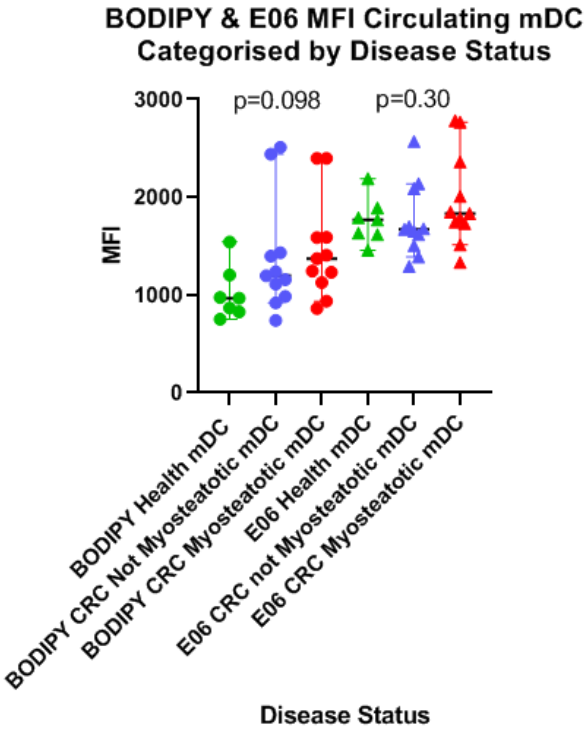
Earlier work by Malietzis in our lab demonstrated a correlation between increasing mean muscle attenuation (i.e. those who are less myosteatotic) and increasing expression of CD36[139]. We therefore examined this specific patient population in greater detail. Kruskal-Wallis test was performed to determine a significant difference between CD36 expression

and ICOL and ICTL in three categories – health, CRC myosteatosis and CRC myosteatosis. There was a significant difference in frequency of CD36 expression between the three groups (p=0.01) (Figure 7.14a). There was no significant difference between ICOL (p=0.30) and ICTL (p=0.098) between groups (Figure 7.14b).

However, when individual comparisons were performed between health and CRC patients with myosteatosis and health and CRC patients without myosteatosis; significant associations became apparent (Table 7.4). When comparing health to the CRC population without myosteatosis there was no difference in CD36 expression (p=0.37), ICOL (p=0.68) or ICTL (p=0.15). However, when we compared healthy controls to the CRC myosteatotic population we saw significantly diminished expression of CD36 (p=0.001) and significantly raised ICTL (0.03) in the myosteatotic CRC group. These findings (summarised in Table 7.7) demonstrate the how changes in DC lipid profile are part of a spectrum which is apparent from health through the disease subtypes.



a.



b.

Figure 7.14 a & b Analysis of CD36, BODIPY and E06 expression by circulating mDC in health (green), CRC patients without myosteatosis (blue) and CRC patients with myosteatosis (red)

Disease Status	p value		
	BODIPY (MFI)	CD36 (%)	E06 (MFI)
Health vs CRC Not Myosteatotic	0.15	0.37	0.68
Health vs CRC Myosteatotic	**0.03***	**0.001***	0.43
CRC Not Myosteatotic vs CRC Myosteatotic	0.50	0.09	0.48

Table 7.7 Comparisons of expression of CD36, E06 and BODIPY in mDC of healthy controls, CRC patients without myosteatosis and CRC patients with myosteatosis

7.4.4 DC Lipid profiles in muscle

Dendritic cells were successfully isolated from the skeletal muscle of CRC patients and from a healthy control. The gating strategy used to isolate the DC on flow cytometry from the CRC patients is shown in the supplementary data within Appendix 1.

7.4.4.1 Demographics

Nineteen muscle biopsies from colorectal cancer patients were analysed, thirteen were male and the median age was 60 years [IQR 52-72]. All these samples were successfully analysed for intracellular oxidised lipid by used of median fluorescence intensity. The earlier experiments did not result in the isolation of enough DC to allow assessment of the frequency of cell surface marker (CD36) expression as such, fewer patients were included in the analysis for CD36 cell surface expression, evolution of the method allowed an increase in the yield of DC isolated and the final 11 consecutive experiments were used in the CD36 analysis. The tumour characteristics of the full muscle cohort and the

subset analysed for CD36 expression are shown below in Tables 7.8 & 7.9. A single healthy participant (male, age 33) was also recruited in order to demonstrate proof of concept of the method and identify whether there were any DC present in healthy muscle; the purpose of this sample was to firstly demonstrate that DC were present in healthy muscle tissue and to provide an avenue for future work. This is discussed in more detail in section 3.4.3 and the flow cytometry data is shown in Figure 7.15.

		n Total cohort	% Total cohort	n CD36 subset	% CD36 subset
Gender	Male	13	68.4	6	54.5
	Female	6	31.6	5	45.5
T Stage	T1/2	4	21.1	2	18.2
	T3/4/extraluminal disease	15	78.9	9	81.8
Nodal Disease	Nodal Disease Absent	10	52.6	6	54.5
	Nodal Disease Present	7	36.8	4	36.4
	Ungraded[§]	2	10.5	1	9.1
Vascular Invasion	Vascular Invasion Absent	9	47.4	6	54.5
	Vascular Invasion Present	8	42.1	4	36.4
	Ungraded[§]	2	10.5	1	9.1
Lymphatic Invasion	Lymphatic Invasion Absent	11	57.9	6	54.5
	Lymphatic Invasion Present	6	31.6	4	36.4
	Ungraded[§]	2	10.5	1	9.1
Perineural Invasion	Perineural Invasion Absent	8	42.1	4	36.4
	Perineural Invasion Present	9	47.4	6	54.5
	Ungraded[§]	2	10.5	1	9.1
EMVI	EMVI Absent	10	52.6	6	54.5
	EMVI Present	7	36.8	4	36.4
	Ungraded[§]	2	10.5	1	9.1
Tumour Differentiation	Well/Moderately Differentiated	15	78.9	7	63.6
	Poorly Differentiated	4	21.1	4	36.4

[§]Ungraded as resection of locally recurrent rectal cancer

Table 7.8 Demographic and tumour characteristics of the full muscle cohort and the subset analysed for CD36 expression

		n Total cohort	% Total cohort	n CD36 subset	% CD36 subset
BMI Obesity	Not BMI Obese	9	47.4	3	27.3
	BMI Obese	10	52.6	8	72.7
Sarcopenia	Not Sarcopenic	9	47.4	7	63.6

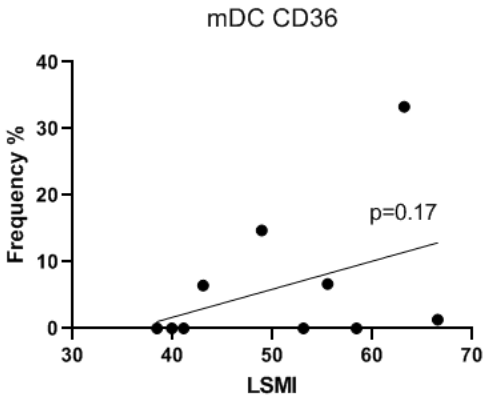
	Sarcopenic	10	52.6	4	36.4
Myosteatosis	Not Myosteatotic	6	31.6	3	27.3
	Myosteatotic	13	68.4	8	72.7
Visceral Obesity	Not Viscerally Obese	10	52.6	8	72.7
	Viscerally Obese	9	47.4	3	27.3
Sarcopenic Obesity	Not Sarcopenic Obese	15	78.9	9	81.8
	Sarcopenic Obese	4	21.1	2	18.2

Table 7.9 Body composition characteristics of the full muscle cohort and the subset analysed for CD36 expression

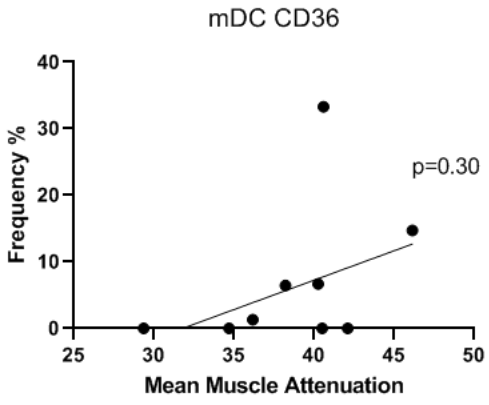
7.4.4.2 DC CD36 expression in muscle tissue

Cell numbers were too low to draw meaningful conclusions on frequency; evolution of the isolation technique in the latter experiments increased cell yield. We therefore attempted to ascertain whether there were any correlations between the expression of CD36 and linear measures of body composition (Figures 7.15 a-e). There were no significant correlations found between CD36 and any of the BC indices. If we look at CD36 expression in terms of MFI we do however see a significant increase in fluorescence intensity from CD36 in the viscerally obese compared to the non-viscerally obese population (p=0.012) and a trend toward a significance increase of CD36 MFI in the BMI obese compared to the BMI non-obese population (p=0.051). No other significant differences were seen in the other population groups.

a.



b.



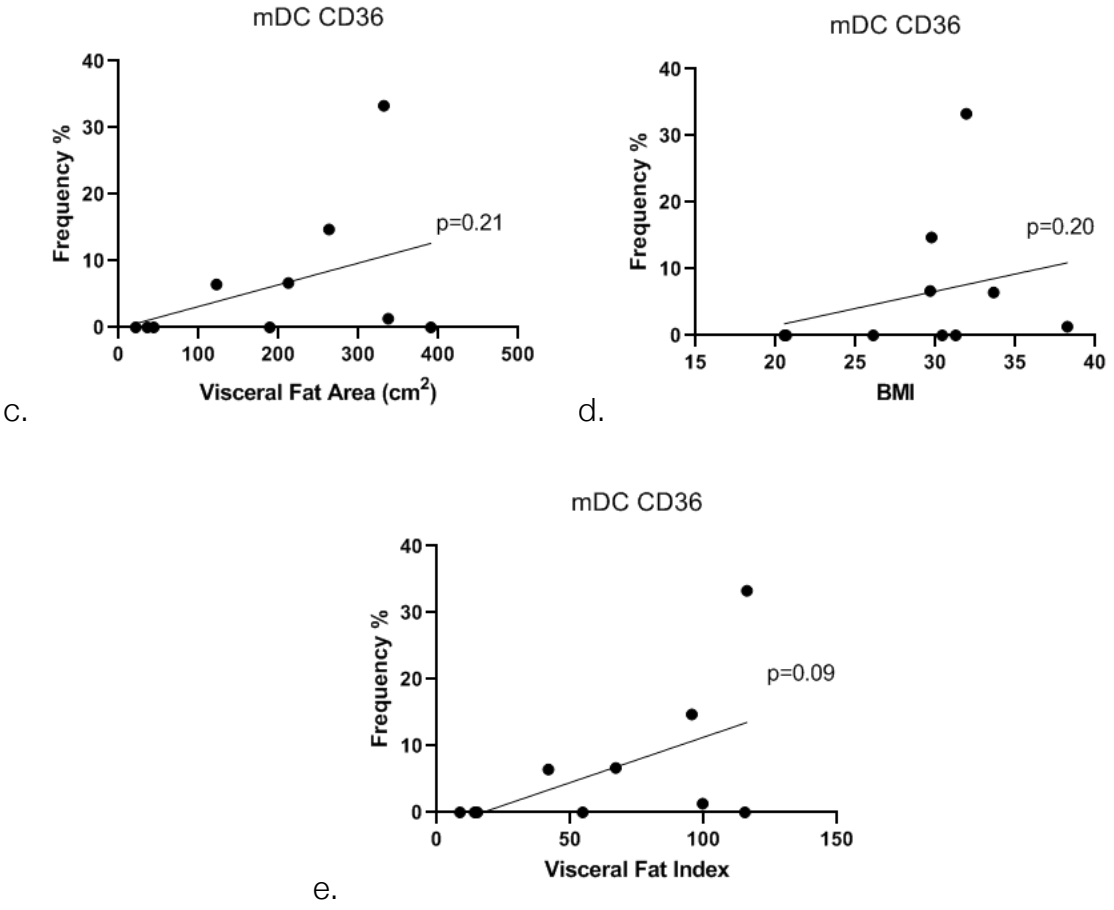


Figure 7.15 a-e Correlation of CD36 to body composition

Comparing CD36 expression and **a.** *Lumbar Skeletal Muscle Index (LSMI);* **b.** *Mean Muscle Attenuation (MMA) (HU);* **c.** *Visceral Fat area (VFA) (cm^2);* **d.** *Body Mass Index;* **e.** *Visceral Fat Index. p values following assessment of correlation by Spearman's Rank are shown.*

7.4.4.3 ICOL in muscle related DC is related to myosteatosis

There did not appear to be a significant difference in the MFI of E06 and hence ICOL in the mDC and pDC of CRC patients with and without sarcopenia (p=0.84), visceral obesity (p=0.83), BMI obesity (p=0.36) and sarcopenic obesity (p=0.36). Interestingly myosteatotic patients appear to have significantly more ICOL within their mDC compared

to non-myosteatotic patients (p=0.035) (Figure 7.16a). This was not true of the pDC population where no significant relationship was seen (p=0.28) (Figure 7.16b).

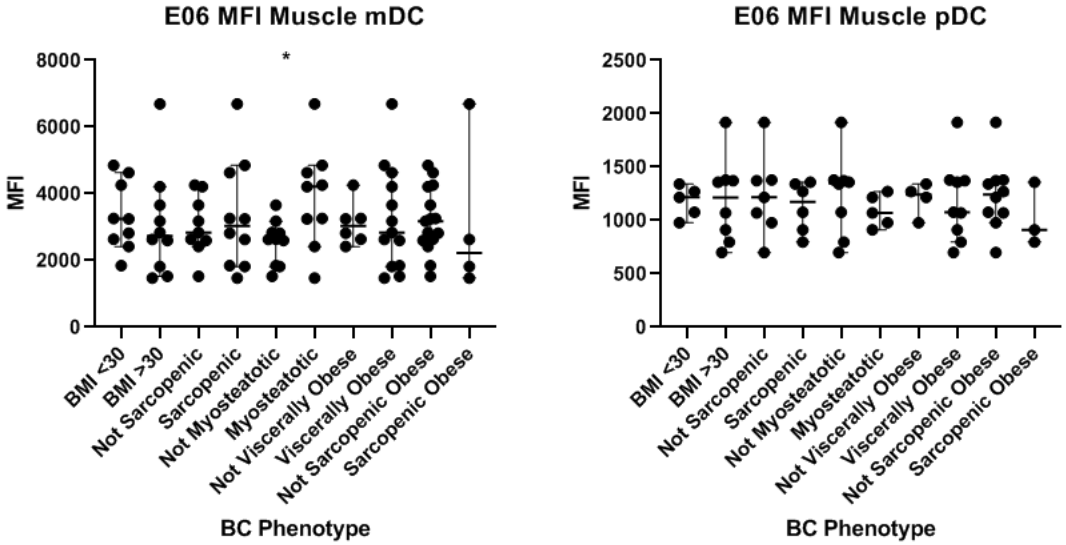


Figure 7.16 a & b E06 MFI expression by muscle mDC and pDC

However, when we examine the lineage negative HLA-DR negative population cells we see further relationships in relation to ICOL and myosteatosis the putative mDC and pDC of this population. Myosteatotic patients demonstrate greater intensity of ICOL compared to non-myosteatotic patients of most notable significance in the CD123 expressing population of putative pDC (p=0.02) (Figure 7.17b). The CD11c expressing population of these cells, the putative mDC, also appear to have greater ICOL content compared to the non myosteatotic population however the threshold of significance is not reached p=0.056) (Figure 7.17a).

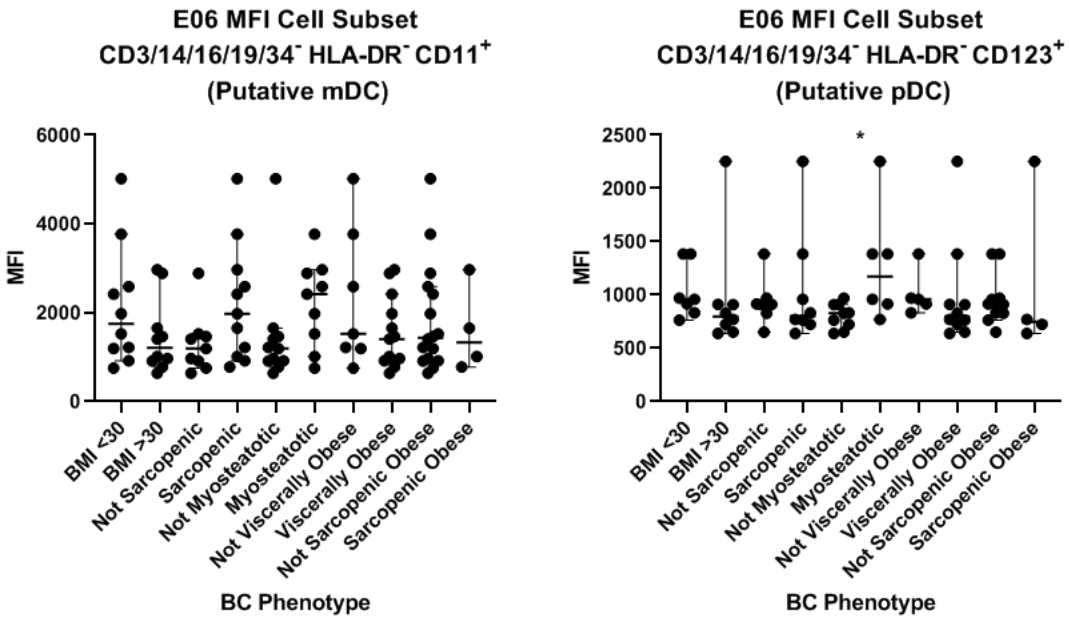


Figure 7.17a & b. E06 MFI expression by CD3/14/16/19/34- HLA-DR- CD11c+ (putative mDC) and CD3/14/16/19/34- HLA-DR- CD123+ (putative pDC) cells

7.4.4.4 Healthy muscle contains DC which express CD36 and contain oxidised lipid

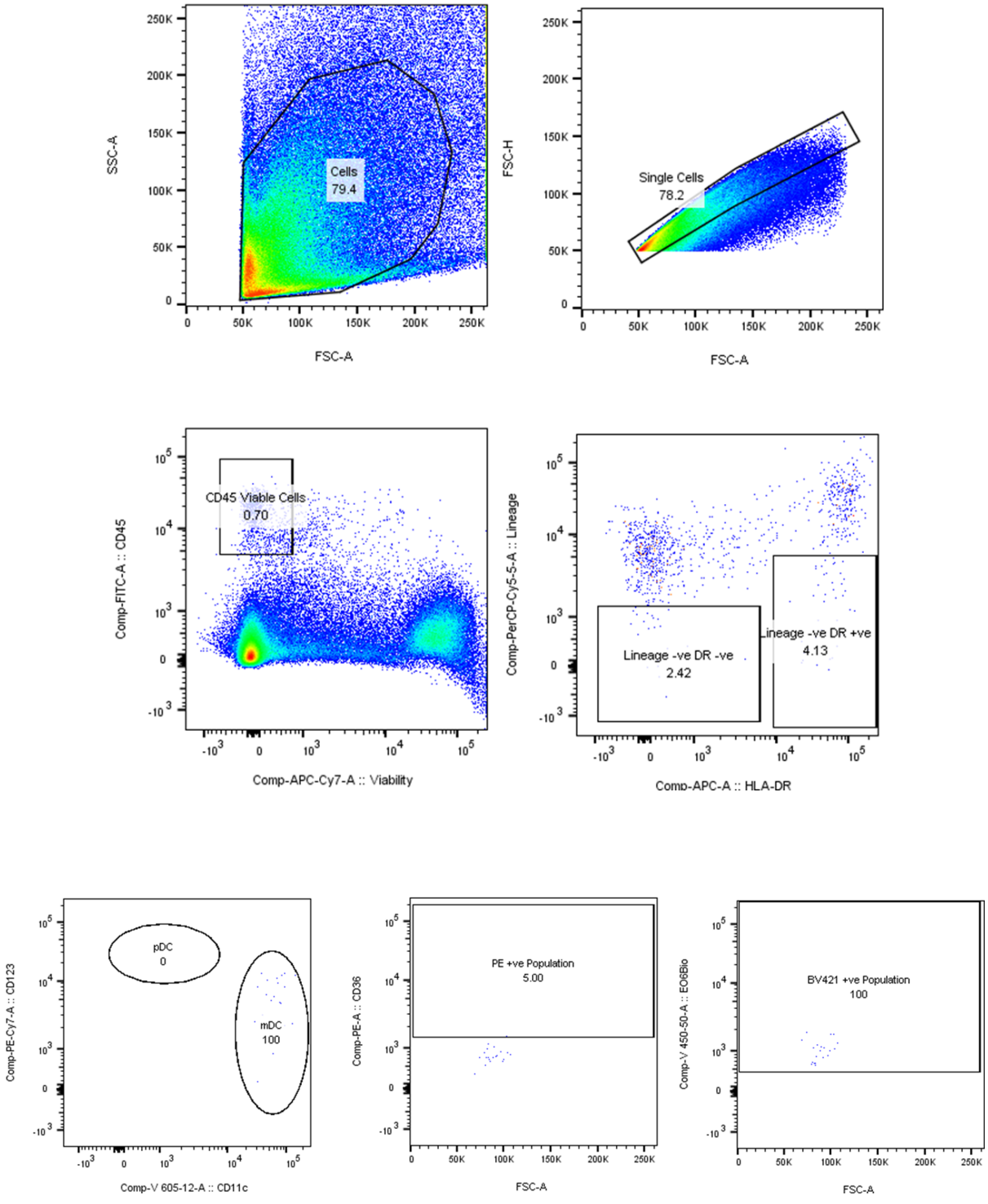


Figure 7.18 Flow cytometry data demonstrating DC isolated from healthy muscle (gracilis) expressing CD36 and containing intracellular oxidised lipid

These data, shown in Figure 7.18, demonstrate proof of concept that it is possible to successfully isolate DC from the skeletal muscle of healthy individuals and it is interesting to note the high level of expression of E06 in the BV421 channel – the entire population of DC appears to contain intracellular oxidised lipid. CD36 expression seems markedly low but this is in keeping with CD36 expression in the diseased population.

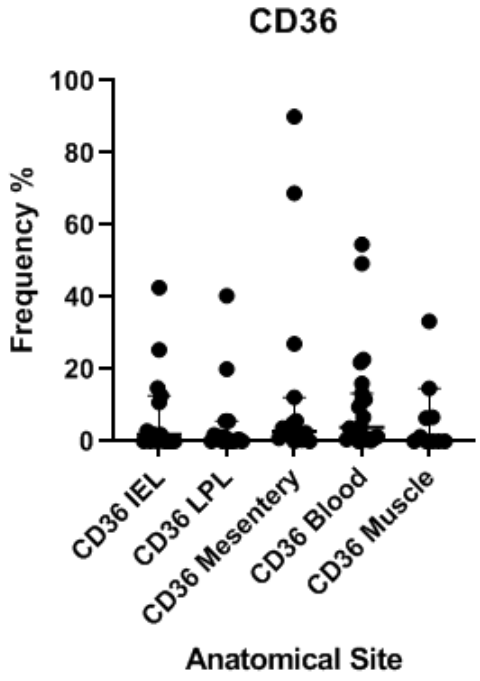
7.4.4.5 DC CD36 and lipid profiles at are significantly influenced by anatomical location

All data for the patients described in each tissue cohort was compared to identify if and how the lipid profile of DC changes at each anatomical location. Analyses were undertaken comparing each tissue site. Figures 7.19a-c demonstrate the expression of each marker for each experiment at each tissue site whilst Tables 6.10 to 6.12 show the statistical relationship between each tissue for each marker of interest.

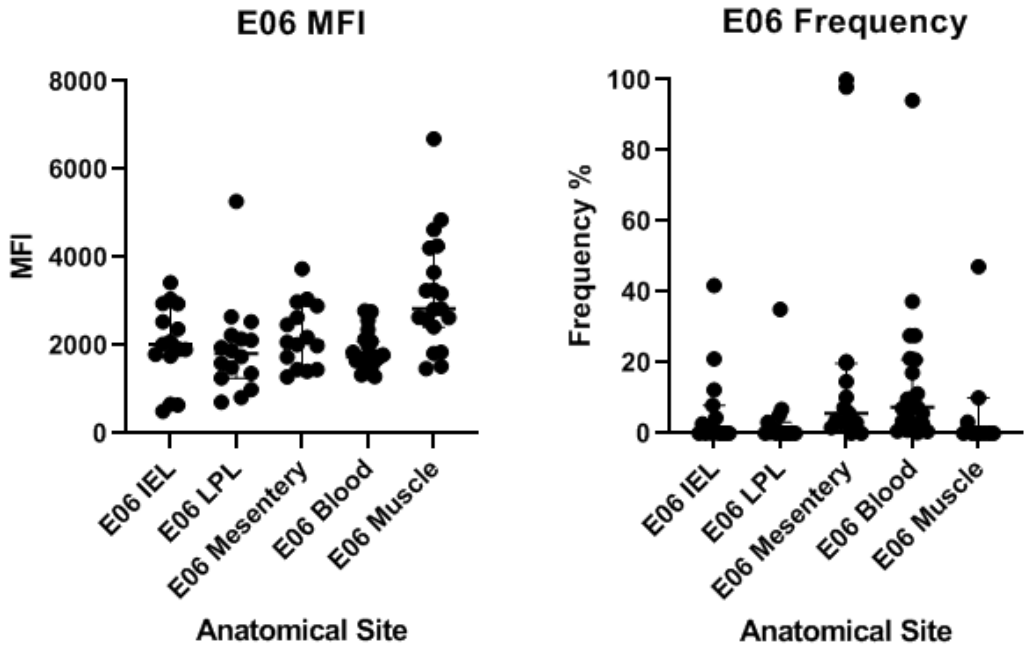
CD36 expression shows variation both within tissues and between tissues, the expression of CD36 appears to be greatest in the mesentery and blood. We find that there in significantly greater CD36 expression in these two tissues when compared to the DC of the LPL. This may relate to the lipid rich milieu of both these tissues or alternatively suggest immature DC with an enhanced antigen presentation capability, in keeping with CD36 alternative function of antigen processing.

Comparison of E06 between tissues is challenging because different FACS antibodies were used in the analysis of the colonic DC compared to the mesenteric, circulating and muscle DC. Although E06 antibody binds specifically to oxidised fat, E06 TopFluor was conjugated with a fluorochrome activated in FITC whilst E06 Biotin was unconjugated and required secondary staining with Streptavidin to be activated in BV421. Therefore, it could be considered inappropriate to compare the MFI of each E06 TopFluor and E06

Biotin. We have included this data for illustrative purposes only but will not draw conclusions from comparisons in MFI between colonic DC and the remaining tissues. That having been said the E06 MFI in muscle was significantly greater than all tissues with what we can regard as a true and valid statistically significant increase compared to blood and mesentery. We included the frequency data too in this instance as this perhaps may allow a more valid comparison across all tissue. These results are interesting in that there appears to be an inverse relationship between frequency and MFI. The frequency of DC expression of E06 was significantly diminished in muscle compared to mesenteric and circulating DC. Whilst the proportion of cells expressing E06 was significantly greater in Blood and Mesentery to IEL and LPL. This increased frequency of cells containing ICOL may be explained by the increased expression of CD36 on mesenteric and circulating DC compared to the colonic (LPL) DC.



a.



b. c.

Figure 7.19 a-c. Comparison DC expression of CD36 (frequency) (a.) and E06 (MFI) (b.) and E06 (frequency) (c.) within different anatomical locations

CD36 %	p value				
	IEL	LPL	Mesentery	Blood	Muscle
IEL		0.91	0.23	0.14	0.81
LPL			**0.06**	**0.019**	0.88
Mesentery				0.89	0.31
Blood					0.11
Muscle					

Table 7.10 Statistical differences in frequency of CD36 expression by DC resident within different tissues

E06 MFI	p value				
	IEL	LPL	Mesentery	Blood	Muscle
IEL		0.42	0.78	0.14	**0.013**
LPL			0.18	0.76	**0.001**
Mesentery				0.15	**0.017**
Blood					**<0.0001**
Muscle					

Table 7.11 Statistical differences in frequency of E06 MFI by DC resident within different tissues. N.B. IEL and LPL DC used E06 TopFluor (FITC) and Mesentery, Blood and Muscle DC were assessed using E06 Biotin wit Streptavidin (BV421)

E06 %	p value				
	IEL	LPL	Mesentery	Blood	Muscle
IEL		0.93	0.057	**0.009**	0.43
LPL			**0.011**	**0.0001**	0.26
Mesentery				0.70	**0.023**
Blood					**0.0037**
Muscle					

Table 7.12 Statistical differences in frequency of cell containing ICOL (E06) by DC resident within different tissues

7.5 Discussion

A series of experiments was undertaken to explore the relationship between body composition and DC lipid profiles in colorectal cancer patients. Earlier work had suggested a correlation with CD36 and increased muscle attenuation on CT[139]. No previous work has been undertaken to explore the lipid profiles of DC in muscle, and the majority of published work in humans focusses on circulating DC; earlier work in mouse models has been performed on different tissues, spleen, omentum and tumour tissue, that has shown anatomical influence[241,242]. Four Tissue types were chosen for analysis. Colonic mucosa was used because it provides the primary site at which the immune system and DC in particular encounter colonic specific antigens of the microbiome, tumour and colonic environment in general, tissue proximal to the tumour used as we hoped to look for non-tumour specific environmental responses. Mesentery was chosen as it contains lymphatics both afferent and efferent, related to cancer and because it is a tissue whose volume and morphology is independently associated with disease progression and outcomes[124,247]. Circulating DC were examined as they are numerous, readily obtainable and much of the earlier work exploring lipid profiles has been done in circulating DC demonstrating relationships to BC[139]. These DC are also at the interface between the tissues allowing us to profile the immature immune profile of the participants. Finally, muscle was used as the end organ of interest. Muscles relationship with the

immune system, the systemic inflammatory response and cancer outcomes has been the theme of this thesis and direct examination of the immune profile in muscle provides an opportunity to explain some of these associations.

7.5.1 Successful isolation of DC from multiple tissue types

Our data demonstrate that through various methods we can successfully extract DC from multiple tissue types. We report the presence of DC within the muscle tissue of both patients with CRC but also a healthy individual. The method of muscle DC isolation proved the most complex as this was a new technique refined in our laboratory hence fewer earlier results as DC isolates were insignificant until the technique evolved sufficiently. Following technique refinements, we were able to isolate sufficient DC for analysis by flow cytometry. Due to the size of colonic mucosal biopsies this study provides the first opportunity to examine the difference in behaviour of IEL DC macrophage and LPL DC and macrophages. There is no previous work on human IEL DC in the literature reflecting the number of IEL DC seen in this study. The mesenteric DC were obtained by the "walk out" method which had previously proved successful for the isolation of DC from the omentum and colon[248,249]. This is the first time this method has been used to obtain DC from the colonic mesentery and has been shown to be successful.

7.5.2 $CD45^+$, $CD3/14/16/19/34^-$, $HLA\text{-}DR^-$ populations

Within our mesenteric fat and muscle tissue there were cell populations similar in size to the population that were $CD45^+$, $CD3/14/16/19/34^-$, $HLA\text{-}DR^+$, the key difference in this

population is the lack of HLA-DR. These cells share many of the characteristics of there CD3/14/16/19/34^{-}, HLA-DR^{+} cousins. These cells exist in two distinct populations of cells expression CD123 and CD11c and they express CD36 and contain intracellular oxidised fat. However, this populations of putative dendritic cells appear to behave differently to the HLA-DR population especially in terms of significant association in relation to body composition status in colorectal cancer. Myosteatotic muscle contains lipid laded DC and also these putative DC with high E06 – are these DC which have become non-functional due to lipid metabolism as a result of chronic inflammatory process instituted by tumour and associated microbiome? One questions whether this population consists of a population of HLA-DR expressing cells who through a response to disease or a result of maturity have lost their antigen presenting ability including expression of HLA-DR and thus their status as a classic DC. This may explain some of the findings relating to intracellular oxidised fat and function loss described in other studies[140,250].

7.5.3 Increased CD36 expression is related to favourable BC phenotypes

We have seen with these data that increased CD36 expression is associated with favourable body composition phenotypes, this concurs with earlier work examining the role of the receptor in circulating DC in relation to BC. This change in CD36 may be a result of its role in antigen presentation[246], DC expressing higher levels of CD36 might be able to successfully present antigen to cytotoxic T-cells and hence share an association with better prognostic phenotypes, this may also explain why Herber and colleagues didn't find a relationship between CD36 and intracellular lipid in mice[242]. The associations with CD36 are related to visceral adiposity, obesity may lead to conditioning of these cell

types, a fatty milieu may potentially be a driver to upregulate CD36 and thus condition the DC further to an antigen presentation role. We also identified that the effect of BC phenotype on significant relationships with CD36 at different sites and when comparing circulating mDC and pDC. It's interesting to note that significant relationships in frequency were greatest in the mesentery and LPL where exogenous lipid exposure is likely to be raised.

7.5.4 Intra cellular lipid profiles change throughout the anatomical locations

Distinct DC lipid profile are associated with anatomical location. Muscle for example appears to contain a low number of cells packed with high levels of oxidised lipid whilst circulating DC appear to have a higher frequency of cells containing lipid but a lo MFI suggesting perhaps a lower total lipid content. This may be an example of changing metabolic functions of DC at different anatomical locations, or those in end tissue organs such as muscle or mesenteric fat may have DC which are trapped and perhaps truly dysfunctional. These DC in muscle are of particular interest both because of their high lipid burden but also the association between ICOL (E06) expression and myosteatosis. Myosteatosis is a state of fatty infiltration, thought to be inflammatory in nature. Could it therefore be postulated that these lipid ladened DC are perhaps trapped through dysfunction similar to the macrophage foam cells of atherosclerosis. Could this be part of the mechanism through which myosteatosis forms?

7.5.5 DC lipid profile are associated with prognostic BC phenotype

Our results regarding DC lipid profiles suggest agreement with earlier assertions regarding intracellular DC oxidised lipid, DC dysfunction and disease. We found patients with poorly prognostic BC phenotypes were more likely to have a significant association with increased intracellular oxidised lipid, DC dysfunction has been thought to be tumour induced However, our data, controlled for the presence of colorectal cancer and suggests a particular subset of tumours drives specific dysfunctional changes in DC and these immunogenic tumours also cause concurrent deleterious inflammatory changes in muscle[140,251]. We also identified that circulating DC of myosteatotic patients have an increased staining for intracellular lipid with a concurrent deficiency in CD36 expression. This may allude to the role of CD36 as a fat scavenger receptor which is down regulated by excess intracellular lipid either as part of a negative feedback loop or as part of the systematic cell failure in antigen presentation.

7.6 Limitations

One of the advantages of the experimental design is that the presence of cancer as a variable allows examination of more nuanced variables such as those of body composition. However, colorectal cancer is a heterogenous disease and as such there remains a significant amount of variability within the population which can lead to skewed findings. Although we controlled for time of day, we were unable to control for seasonal variations in antigen exposure or concurrent disease (however minor or seemingly insignificant). These variables will impact on the behaviour of cells as sensitive as DC and may therefore impact on results. We made the decision not to age match healthy controls in this work, the reason being we wanted to use as controls, individuals who have as

optimum body composition as possible without confounders such as age-related sarcopenia or raised BMI. However, age matched controls are equally advantageous and it would have been of particular interest to have added an aged match population too as a comparator.

We have discussed above how we are unable to draw conclusions regarding the results of the E06 MFI between colonic DC and the other tissues due to the use of a different antibody investigating the same antigen. Due to the restrictions on available reagents and equipment it was not possible to circumvent this problem and as such we have tried to devise other means of interpreting the data in this instance, namely the use of frequency data.

7.7 Further work

We have demonstrated that lipid profiles within DC and macrophages in response to body composition, we found in conjunction to this that these changes were rarely related to concurrent tumour characteristics perhaps suggesting a degree of independence. Further work covered later in this thesis focuses on the causation behind changes in these lipid profile and the effect that intracellular lipid accumulation has on function, cytokine expression, maturation and migration.

Future work, beyond the bounds of this thesis should include further characterisation of DC lipid in healthy human skeletal muscle. Our work has demonstrated the presence of DC within muscle and our refined isolation protocol provides successful yields. This report is the first to our knowledge to successfully isolate DC from the IEL in sufficient quantity to illustrate their profile. We have also demonstrated that these DC are inherently

different from the DC within the LPL. The role, function and significance of these IEL DC should be performed as part of future work.

7.8 Conclusions

Data from this study demonstrated that an increased CD36 expression is associated with favourable BC phenotypes especially visceral obesity, in keeping with Malietzis' earlier findings that CD36 expressed on circulating DC was associated with increased muscle attenuation. Our findings did not demonstrate a significant association between CD36 and myosteatosis in circulating DC but we did not repeat the work on correlation performed previously on this DC subset. Interestingly the instance where CD36 was not associated with a favourable prognostic BC phenotype was in the mesenteric fat itself where increased frequency of cells expressing CD36 was associated with sarcopenia and sarcopenic obesity.

High lipid burden was associated with poor prognostic BC phenotype especially myosteatosis in circulating DC and muscle. In muscle we saw an especially high lipid burden within certain DC in myosteatosis including the HLA-DR$^-$ population which shares many other characteristics with classic DC suggesting these cells may have at one time also had a DC function but perhaps through lipid saturation their HLA-DR expression has been lost.

Ultimately, we have to ask why do we see these lipid profiles; is it a result of the tumour and its tumour antigen driven inflammatory process, or perhaps exogenous antigenic stimuli such as the microbiome dictating down steam DC behaviour? Further work is required looking at maturation, migration and function as well as possible pathways of causation.

Chapter 8

8 Dendritic cell migration and maturation in relation to body composition in colorectal cancer

8.1 Summary

Chapter 7 described in detail the rationale behind our pursuit of the role of the dendritic cell in the complex interplay between body composition and colorectal cancer. The findings demonstrated that the lipid profile of DC is associated with variations in body composition in cancer and that DC lipid profile is associated with anatomical location. We also raised the possibility that appropriate DC migration signalling may be adversely influenced by lipid profile and whether this has a potential role in the dysfunctional immune response to colorectal cancer. In this chapter we explore the effect body composition has on DC cell migration and maturation in colorectal cancer to ascertain whether certain migration and maturation profiles as associated with certain DC profiles.

8.2 Introduction

8.2.1 DC and their role in innate and adaptive immunity

Dendritic cells have a critical role in the initiation of T cell immune responses; as such they are able to determine whether a tolerogenic or immunogenic response to antigen is required[252]. A tolerogenic response is required to self-antigen and those of commensals to prevent the symptoms seen within auto-immune disease, conditions in which this mechanism has dysfunction, or loss of symbiotic organisms such as within the gut microbiome. DC maturation has been increasingly implicated in tolerogenicity and immunogenicity. Historically it was thought that immature DC were responsible for tolerance whilst mature DC induced immune response[253]. However, this concept has been superseded by a more complex belief that DC in different stages of maturation can induce proliferation of $CD4^{+}CD25^{+}$ regulatory T-cells[254] and thus an immune response. This raises the question of whether the potential self-immune response seen in myosteatosis with its raised systemic inflammatory milieu[101] is a result of inappropriate maturation or homing signalling.

8.2.2 Process of DC maturation and role of CD40

CD40, a co-stimulatory molecule and part of TNF receptor superfamily is present on numerous different cell types including DC, B-cells and monocytes. Carcinomas are also known to express CD40. The interaction between CD40 and its ligand CD40L, expressed mainly on T-cells, plays a key in immune regulation between T cells and antigen presenting cells. Cross linking of CD40 to CD40L induces potent proliferation of B-cells[255]. Defects in CD40-CD40L interactions such as those seen in X-linked hyper IgM

syndrome result in immunodeficiency from decreased antibody formation which in turn results in recurrent infections and increased incidence of gastric cancers and lymphomas[256]. In addition to these data, it has been suggested that CD40 has an important role in eliciting an antitumour response by improving the antigen recognition phase of the response and the T-cell cytotoxic effector stage of the response through increasing cytokine secretion and upregulation of co-stimulatory molecules[257].

8.2.3 DC maturation and BC in Cancer

There are established associations between CD40 expression and body composition phenotype in cancer. Expression of CD40 on circulating DC increases in patients with a higher lumbar skeletal muscle index (LSMI), essentially denoting an increase in CD40 expression with increased muscle mass and thus a favourable prognosis[139]. However within the same patient population it was noted that the expression of CD83, another DC co-stimulatory molecule and marker of early DC maturation, correlated with decreasing mean muscle attenuation and thus poor prognosis[139]. DC maturation and BC appear to be closely associated however, this study demonstrates an uncertainty about the role of DC maturation in relation to BC in CRC and highlights an area for further work.

8.2.4 Process of DC migration and homing markers

Maturation is also a key factor in migration, immature DC have a limited migratory capacity. Once stimulated DC change their behaviour, further endocytosis by the cell is diminished and increasing cellular migration is seen[258]. The ability to migrate is key to the existence and function of dendritic cells, they roam great distances following their

inception in the bone marrow. Their role as sentinels within the circulation and multiple tissue types necessitates a complex and intricate method of direction both for themselves but also their downstream effector cells. DC also require the ability to change their location and tissue specificity or that of their effector lymphocytes in response to antigen stimulation as such DC have evolved the ability to direct migration through a diverse range of soluble and membrane-bound proteins[258]. However, more recently it has been recognised that DC are able to migrate in an integrin independent manner especially in a three dimensional tissue environment[259].

The focus of this thesis is primarily the role of the DC in response to CRC and hence gut pathology, and we will concentrate primarily on markers associated with gut homing. Gain or loss of these markers by DC may suggest important functional sequelae that explain why certain tumours have a poorer prognosis as a result of enhanced tumour immune evasion. One such example of a gut homing marker is alpha 4 beta 7 Integrin (α4β7) also known as lymphocyte Peyer patch adhesion molecule; this complex is responsible for T-cell homing into gut associated lymphoid tissue. This is brought about through binding to mucosal addressin cell adhesion molecule (MAdCAM), which is present on the high endothelial venules of mucosal lymphoid organs[260–262]. It is therefore expected that the DC of individuals with a disease should exhibit DC homing towards the disease site. There has been significant focus on the role of DC homing in autoimmune disease especially inflammatory bowel disease. Indeed, previous work in our lab has identified that homing markers expressed by DC in Crohn's disease differed on disease location; for example, patients with large bowel Crohn's disease had increased β7 expression but reduced CCR9 (small bowel homing) expression. Interestingly patients with small bowel disease demonstrated increased CLA expression, skin homing as well

as increased CCR9. Notably, patients with quiescent disease demonstrated an increase in chemokine (C-C motif) receptor (CCR7), lymph node homing[263]. Interestingly, increased expression of CCR7 has also been found to be correlated with low levels of mean muscle attenuation and hence myosteatosis in colorectal cancer[139].

The recognition of this association of lymphocyte migration and disease anatomy has led to the development of specific antibodies which can inhibit this pathway. The biological therapy Vedolizumab blocks α4β7 and is used in the successful treatment of IBD[264]. Like IBD, in cancer we see an uncoordinated dysfunctional immune response and a resultant florid systemic inflammatory response with inappropriate end organ inflammatory change, such as myosteatosis[101]. One may therefore question whether there is an inherent abnormality in the DC – T-cell circuitry in CRC brought about through loss of the ability of DC to orchestrate homing.

8.2.5 Known axes and migration processes

In health and disease, the DC migration relationship appear to involve cross talk between a number of seemingly unrelated organs. These relationship pathways are known as axes. Several immune axes have been identified including the gut-brain axis and gut-skin axis. The concept of a gut-brain immune axis has become well established in recent years. Here, we see immune cells from the intestine, sampling antigen then having a profound effect on neurological immune behaviour. This signalling pathway is thought to arise because of antigen or microbe associated molecular patterns from the gut microbiota interacting with gut lymphocytes. This interaction through a pathway of CD4+ T-cell differentiation is known to induce DC and other myeloid cells to increase cytokine production especially IL-6 and IL-23. This inappropriate activation of the immune system

at one site may explain the development of immune mediated degenerative disease at other sites. For example there is emerging evidence that the T-cell driven inflammation, which mediates dopaminergic neurodegeneration in Parkinson's disease, is triggered in the gut mucosa[265]. It has been postulated that similar mechanisms involving a dysfunctional gut-brain axis are also responsible for multiple sclerosis, depression and myalgic encephalomyelitis[266–268]. The concept of a gut-skin axis also is being viewed with increasing interest and has been use to explain the extraluminal manifestations of IBD such as pyoderma gangrenosum[269] with interest now in ways of manipulating this mechanism[270]. Most recently the concept of a gut-muscle axis has been raised; this concept has been considered in athletes where concern over improper diet and overtraining disrupting intestinal homeostasis has been thought to induce enhanced inflammation. This concept is relatively nascent and is yet to be fully explored but we hypothesise that myosteatosis is a result of a dysfunctional gut muscle axis and that an examination of homing markers may help elucidate this. This also raises the theoretical concept of yet undefined axes which may play an important role in the immune response to colorectal cancer including a potential gut-fat axis.

8.2.6 Null Hypothesis

There is no association between body composition status and DC maturation and homing

8.2.7 Aims

- Characterise the homing and maturation profile of DC in relation to body composition

- Identify whether changes in BC are related to DC homing and maturation changes at different anatomical locations

- Postulate based on findings, whether targeting homing be a suitable method of addressing deleterious effect of body composition – similar to disease management in IBD

- Is there evidence to support the concept of a gut muscle axis

8.3 Methods

8.3.1 Isolation of circulation and tissue leucocytes

Isolation of leucocytes was undertaken utilising the same methods described in detail in the material and methods (chapter 6) and to a lesser extent in chapter 7. No further methods were employed in the isolation of cells for the experiments of this chapter.

8.3.2 Flow cytometry and gating

Detailed description of FACS has been given in chapter 6 with a truncated explanation in chapter 7. The same methods were used for this cohort but no permeabilisation or

intracellular staining was performed on this cohort as analysis was of cell surface markers only.

8.3.3 CT BC analysis

The method of CT body composition analysis has been described in detail in earlier chapters. This method of analysis was performed for this cohort using the cut-offs described in earlier chapters.

8.3.4 Flow cytometry analysis

Data was captured using FACS Diva software and exported in .fcs files. Analysis was undertaken in FlowJo (version 10.7), positive frequency of markers was considered based on gating determined using fluorescence minus one (FMO) samples.

8.3.5 Statistical analysis

Descriptive statistics were analysed in SPSS (version 25). All other analyses were undertaken using GraphPad Prism (version 10); data were non-parametric and therefore Mann Whitney U test and Kruskal Wallis tests were employed in their analyses. Correlations were assessed using Spearman's rank.

8.4 Results

8.4.1 Myeloid cell homing and maturation in the colonic mucosa

Macrophages and DC were successfully isolated from the IEL and LPL layer of the colon. These experiments explored the behaviour of myeloid cells which were not directly associated with the tumour but present in apparently healthy mucosa distant to the tumour site.

8.4.1.1 Demographics

Sixteen colorectal cancer patients were included in the analysis. 12 patients were male and the median age was 63 [IQR 55.25-72]. Tumour characteristics for the cohort are shown in Table 8.1 and the body composition characteristics are shown in Table 8.2.

		n	*%*
Gender	Male	12	75.0
	Female	4	25.0
T Stage	T1/2	5	31.3
	T3/4	11	68.8
Nodal Disease	Nodal Disease Absent	8	50.0
	Nodal Disease Present	8	50.0
Vascular Invasion	Vascular Invasion Absent	7	43.8
	Vascular Invasion Present	9	56.3
Lymphatic Invasion	Lymphatic Invasion Absent	10	62.5
	Lymphatic Invasion Present	6	37.5
Perineural Invasion	Perineural Invasion Absent	7	43.8
	Perineural Invasion Present	9	56.3
EMVI	EMVI Absent	9	56.3
	EMVI Present	7	43.8
Tumour Differentiation	Well/Moderately Differentiated	13	81.3
	Poorly Differentiated	3	18.8

Table 8.1 Demographic and tumour characteristics of the IEL and LPL DC cohort

		n	*%*
BMI Obesity	Not BMI Obese	8	50.0
	BMI Obese	8	50.0
Sarcopenia	Not Sarcopenic	7	43.8
	Sarcopenic	9	56.3

Myosteatosis	Not Myosteatotic	7	43.8
	Myosteatotic	9	56.3
Visceral Obesity	Not Viscerally Obese	6	37.5
	Viscerally Obese	10	62.5
Sarcopenic Obesity	Not Sarcopenic Obese	12	75.0
	Sarcopenic Obese	4	25.0

Table 8.2 Body composition characteristics of the IEL and LPL DC cohort

8.4.1.2 Maturation of myeloid cells from the IEL to LPL

On comparing frequency of DC expressing CD40 in the IEL against those in the LPL there was no significant difference seen (p=0.81) (Pooled anatomical data Figure 8.20). There was no significant difference in maturation between macrophages (CD14/64 expressing myeloid cells) in the IEL compared to the LPL (p=0.86) in our colorectal cancer cohort Figure 8.1).

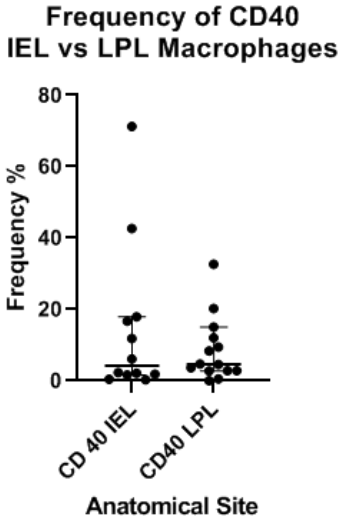


Figure 8.1 CD40 expression of macrophages of the gut

8.4.1.3 Dendritic cell maturation in the LPL but not the IEL is associated to body composition

Within the IEL there was no significant associations found between DC maturation in patients with sarcopenia (p=0.23), myosteatosis (p=0.12), BMI obesity(p=0.23), visceral obesity (p=0.18) and sarcopenic obesity (p=0.75). However, maturation of the LPL DC appears to be associated with obesity. Patients with visceral obesity demonstrated significantly greater frequency of expression of CD40 compared to patients who were not viscerally obese (p=0.03), Figure 8.2. BMI obesity also appeared to have a pro-maturation effect but the threshold for statistical significance was barely reached (p=0.054), Figure 8.3. There was no association found between sarcopenia (p=0.54), myosteatosis (p=0.46) and sarcopenic obesity (p=0.29).

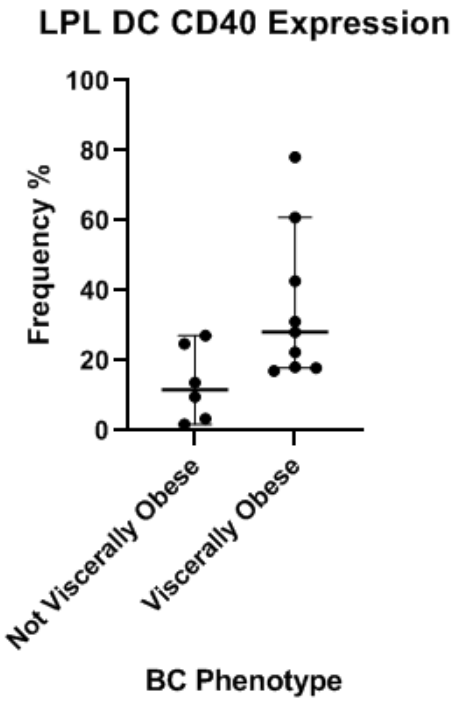


Figure 8.2 CD40 expression by LPL DC in relation to VO

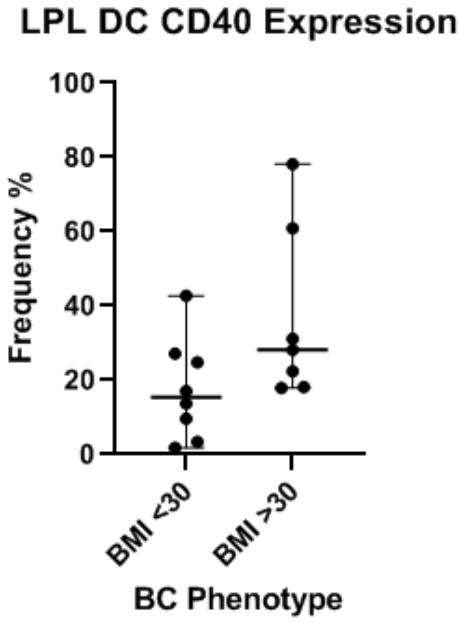


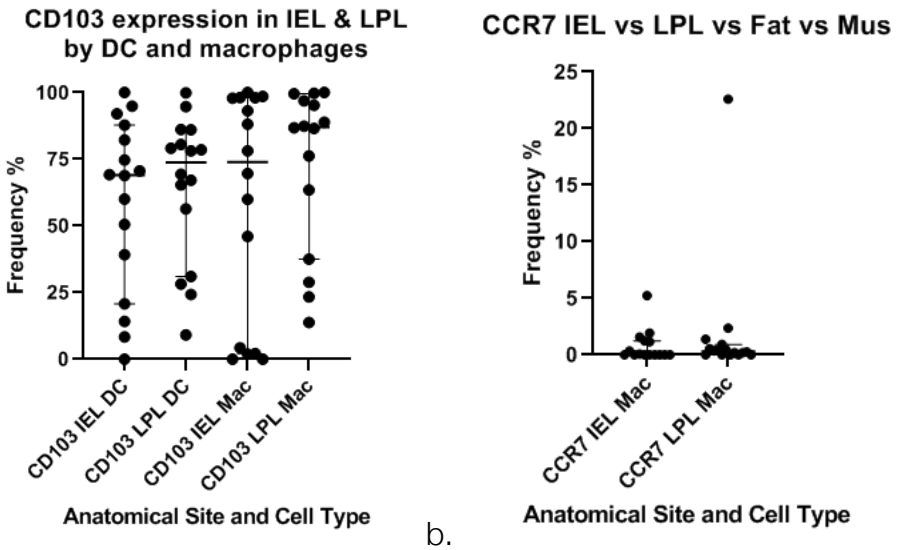
Figure 8.3 CD40 expression in DC in relation to BMI

8.4.1.4 Macrophage maturation is not associated with body composition status in CRC

When comparing patients with the different body composition phenotypes There was no significant difference in the frequency of cells expressing CD40, either in IEL nor in LPL macrophages.

8.4.1.5 CCR7 and CD103 expression is not significantly different in the IEL or LPL

The lymph node homing marker CCR7 and mucosal homing marker CD103 were assessed in the IEL and LPL population. On comparing all samples in the IEL and LPL there was no significant difference in DC CD103 (p=0.67) and CCR7 (p=0.54) macrophage CD103 (p=0.31) and CCR7 (p=0.48) expression (Figure 8.4a & b). Data for IEL and LPL DC CCR7 expression are shown in the pooled data at the end of this results section, Figure 8.21.



a. b.

Figure 8.4 a & b. a). CD103 expression in IEL and LPL by DC and macrophages & b). CCR7 expression by macrophages in the IEL and LPL

8.4.1.6 Lymph node homing signalling of DC is related to body composition status in LPL but not in the IEL

The relationship between DC homing and body composition status in the IEL layer was assessed. There was no significant difference in frequency of expression of either CCR7 or CD103 in the IEL layer DC.

Once again, we see differences in the behaviour of DC in the LPL layer where we find patients who were sarcopenic or myosteatotic had significantly greater expression of the lymph node homing marker CCR7; p=0.02 and p=0.05 respectively, Figure 8.5. It is important to note that the frequency of DC expressing CCR7 was low in all groups with a median cellular expression of 1.4% [IQR 0.91-5.03] in the sarcopenic group compared to a median of 0.2% [IQR 0.08-0.44] in non-sarcopenic group. There was no significant difference in the expression of the CD103 in the sarcopenic (p=0.68), myosteatotic (p=0.60) and sarcopenic obese populations (p=0.17). There was significantly decreased

mucosal homing seen in patients with visceral obesity compared to those without visceral obesity (p=0.03); there was a decrease in expression of CD103 seen in the BMI obese population but this decrease was not significant (p=0.16), Figure 8.6.

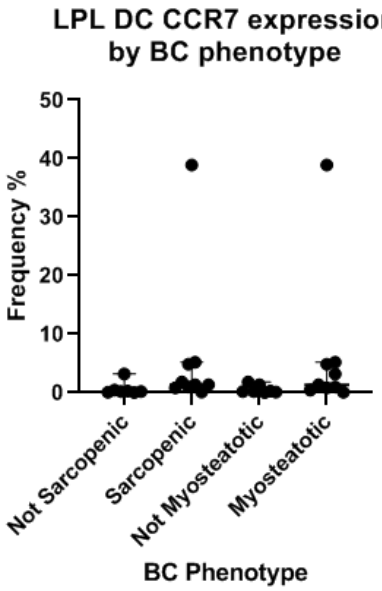


Figure 8.5 LPL DC CCR7 expression by BC phenotype

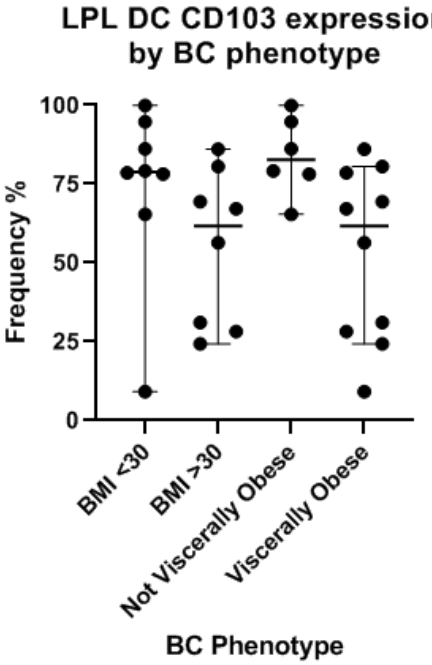


Figure 8.6 LPL DC CD103 expression in relation to BMI and VO status

8.4.1.7 Macrophages of sarcopenic patients significantly increased expression of CCR7

Within the IEL population of macrophages there were no significant differences in expression of either CCR7 or CD103 in patients of differing body composition phenotypes. In the LPL, as with the DC population, sarcopenic patients show a similar lymph node homing response in their macrophages as they do in their DC population. Again, CCR7 expression was significantly diminished (p=0.04) with negligible expression in the non-sarcopenic group the increased frequency of CCR7 expression seen in the sarcopenic group may suggest increased stimulation in this group, Figure 8.7. The other body composition parameters had no such significant differences in their macrophage population regarding CCR7.

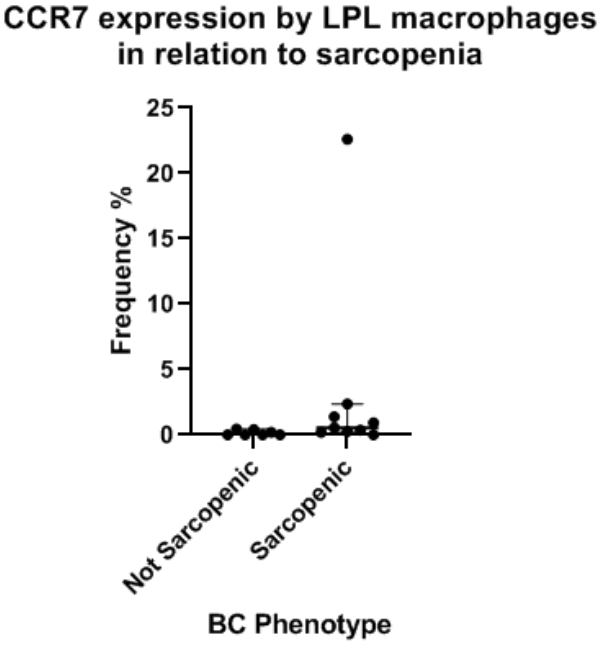


Figure 8.7 CCR7 expression by LPL macrophages in relation to sarcopenia status

Patients with visceral obesity did not share the significant decrease in CD103 expression although there was a trend towards significance seen in this difference (p=0.056).

Similarly, to DC, frequency of CD103 expression by macrophages was not associated with any of the other body composition parameters assessed.

8.4.2 DC homing and maturation in the colonic mesentery

Frequency of expression of CD40, CCR7, CD49d (alpha-4 integrin) and Beta7-integrin were assessed in DC isolated from the colonic mesentery. DC were defined as lineage negative cells expressing CD45, HLA-DR and CD11c. We also analysed the population of cells which were HLA-DR negative but expressed CD45 and CD11c. We considered this population because they shared many characteristics with the DC population and intended to examine them to ascertain whether these cells may be DC which have had HLA-DR (MHC-class II) down regulated and thus lost their antigen presenting capacity on entering the tissue. Interestingly when we examine leucocyte cell number identified within mesenteric fat at flow cytometry, they were significantly greater (p=0.008) in patients with lymphatic invasion (approximate median $0.3x10^6$ leucocytes in lymphatic invasion positive (L1) patients compared to $0.036x10^6$ in lymphatic negative patients (L0)), Figure 8.8.

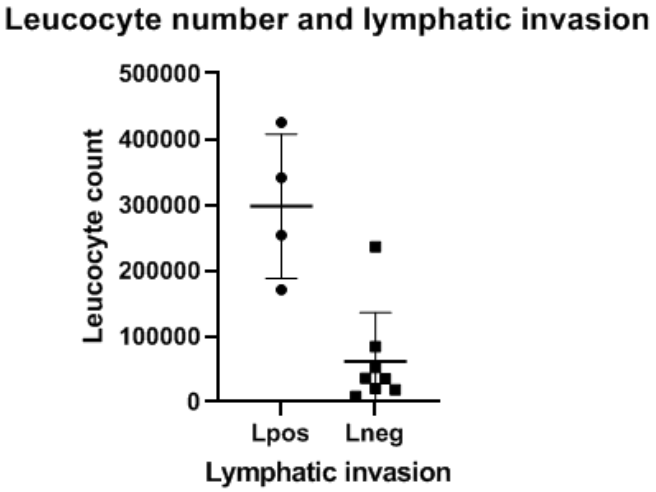


Figure 8.8 Leucocyte number in mesenteric fat is significantly greater in patients with tumour lymphatic invasion

8.4.2.1 Demographics

Fifteen colorectal cancer patients were included in the analysis for CCR7 and CD40, 11 patients in this analysis were male and the median age 61 years [IQR 55-72]. Tumour characteristics for CCR7 and CD40 are shown in Table 8.3 and body composition characteristics are shown in Table 8.4.

Eight colorectal cancer patients were included in the CD49b (Alpha4) and Beta7 analysis. Six were male and the median age 63.5 years [IQR 51.5-71.5]. Tumour characteristics and body composition characteristics for this CD49d and Beta7 sub-set are shown in Table 8.5 and body composition characteristics are shown in Table 8.6.

		n	*%*
Gender	Male	11	73.3
	Female	4	26.7
T-Stage	T1/2	5	33.3
	T3/4	10	66.7
Nodal Disease	Nodal Disease Absent	7	46.7
	Nodal Disease Present	8	53.3
Vascular Invasion	Vascular Invasion Absent	7	46.7
	Vascular Invasion Present	8	53.3
Lymphatic Invasion	Lymphatic Invasion Absent	9	60.0
	Lymphatic Invasion Present	6	40.0
Perineural Invasion	Perineural Invasion Absent	7	46.7
	Perineural Invasion Present	8	53.3
EMVI	EMVI Absent	9	60.0
	EMVI Present	6	40.0
Tumour Differentiation	Well/Moderately Differentiated	12	80.0
	Poorly Differentiated	3	20.0

Table 8.3 Demographic and tumour characteristics of the CCR7 & CD40 mesenteric fat cohort

	n	*%*

BMI Obesity	Not BMI Obese	8	53.3
	BMI Obese	7	46.7
Sarcopenia	Not Sarcopenic	7	46.7
	Sarcopenic	8	53.3
Myosteatosis	Not Myosteatotic	7	46.7
	Myosteatotic	8	53.3
Visceral Obesity	Not Viscerally Obese	6	40.0
	Viscerally Obese	9	60.0
Sarcopenic Obesity	Not Sarcopenic Obese	12	80.0
	Sarcopenic Obese	3	20.0

Table 8.4 Body composition characteristics of the CCR7 & CD40 mesenteric fat cohort

		n	***%***
Gender	Male	6	75.0
	Female	2	25.0
T-Stage	T1/2	2	25.0
	T3/4	6	75.0
Nodal Disease	Nodal Disease Absent	4	50.0
	Nodal Disease Present	4	50.0
Vascular Invasion	Vascular Invasion Absent	5	62.5
	Vascular Invasion Present	3	37.5
Lymphatic Invasion	Lymphatic Invasion Absent	4	50.0
	Lymphatic Invasion Present	4	50.0
Perineural Invasion	Perineural Invasion Absent	3	37.5
	Perineural Invasion Present	5	62.5
EMVI	EMVI Absent	5	62.5
	EMVI Present	3	37.5
Tumour Differentiation	Well/Moderately Differentiated	6	75.0
	Poorly Differentiated	2	25.0

Table 8.5 Demographic and tumour characteristics of the CD49d & Beta7 mesenteric fat cohort

		n	***%***
BMI Obesity	Not BMI Obese	2	25.0
	BMI Obese	6	75.0
Sarcopenia	Not Sarcopenic	5	62.5
	Sarcopenic	3	37.5
Myosteatosis	Not Myosteatotic	4	50.0
	Myosteatotic	4	50.0
Visceral Obesity	Not Viscerally Obese	2	25.0
	Viscerally Obese	6	75.0
	Not Sarcopenic Obese	6	75.0

Sarcopenic Obesity	Sarcopenic Obese	2	25.0

Table 8.6 Body composition characteristics of the CD49d & Beta7 mesenteric fat cohort

8.4.2.2 DC maturation is significantly increased in viscerally obese patients

A significantly greater proportion of DC in patients with visceral obesity expressed CD40 compared to patients without visceral obesity (p=0.05), no significant relationships were identified for BMI obesity (p=0.12) sarcopenia (p=0.46), myosteatosis (p=0.40) or sarcopenic obesity (p0.99), Figure 8.9. Within the lineage negative, HLA-DR negative population of cells, we identified a subset of cells which express CD11c and thus have many of the hallmarks of mDC with the exception of HLA-DR. In this subgroup we saw a significantly increase in the proportion of cells expression CD40 in BMI obese patients (p=0.02) however no statistically significant relationship was seen in terms of visceral obesity in this subgroup (p=0.24), Figure 8.10. There were no further relationships seen in this cell population in relation to body composition status.

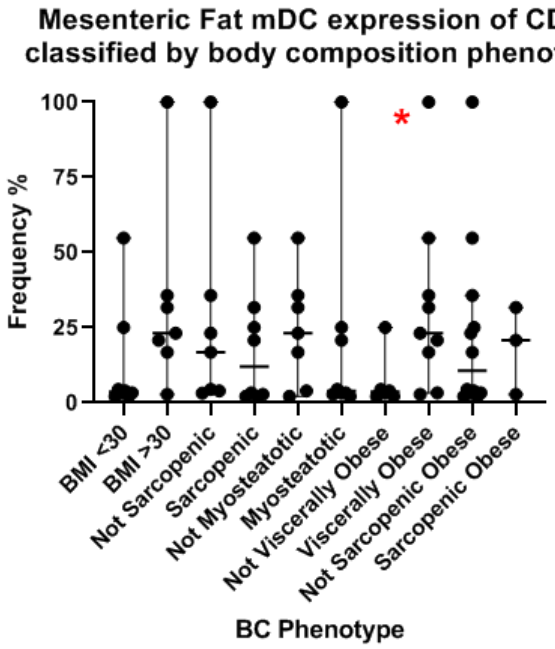


Figure 8.9 CD40 expression by mesenteric fat mDC demonstrating the significant difference in maturation in the viscerally obese population compared to the non-viscerally obese population

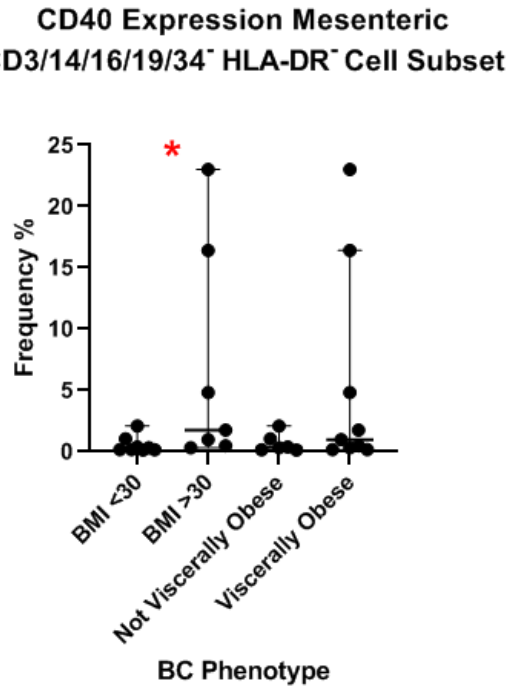
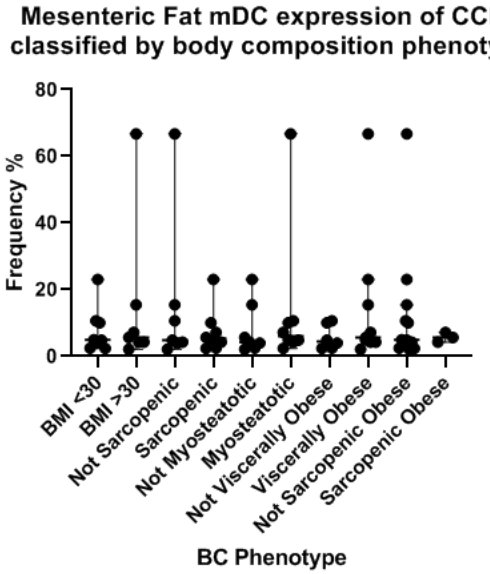


Figure 8.10 CD40 expression by mesenteric fat CD3/14/16/19/34-, HLA-DR-, CD11c+ cells in relation to obesity subtypes

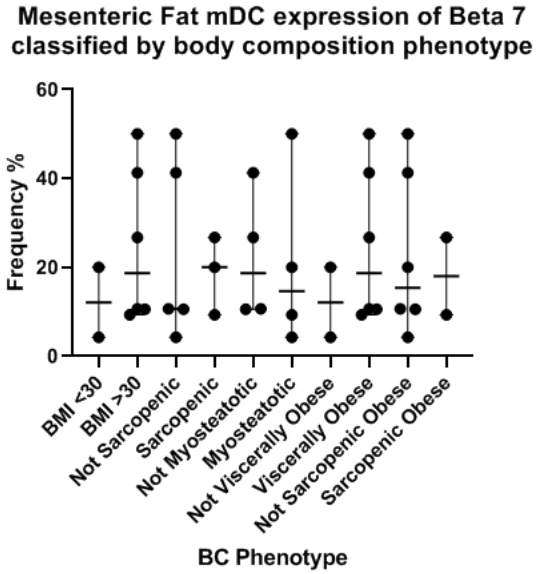
8.4.2.3 Lymph node and gut homing of mesenteric fat DC was not associated with body composition status

Frequency of expression of CCR7, CD49d and Beta7 integrin were assessed in the population of CD11c expression DC derived from mesenteric fat (Figure 8.11a-c). There was no evidence of significant differences in the frequency of cells expressing these markers in the various body composition states. This suggests that the homing behaviour of the DC in mesenteric fat, based on the markers examined, are seemingly not influenced by body composition status. We did however identify that CCR7 expression was significantly greater in the mesenteric DC compared to the LPL DC (p=0.0002) and

intramuscular DC (p=0.02) which is in keeping with the assertion that mesenteric fat has perinodal qualities, Figure 8.21.



a.



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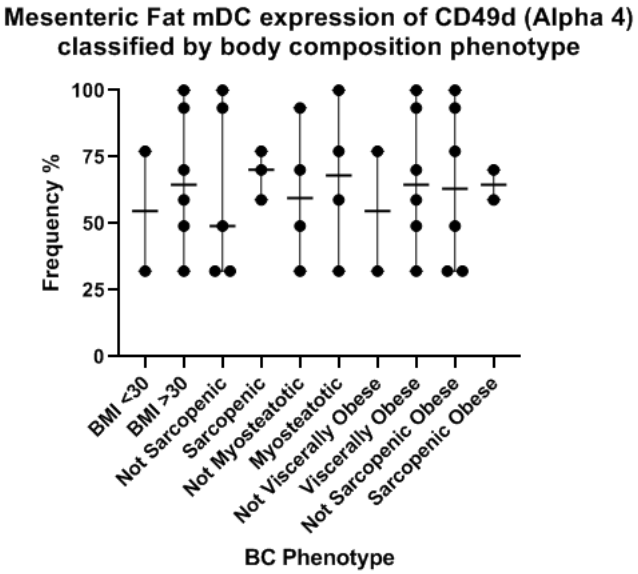


c.

Figure 8.11a-c. Expression of homing makers on mesenteric fat mDC

8.4.3 Maturation and migration of circulating DC

CD40, CLA (skin homing) and Beta 7 (gut homing) expression were assessed in the PBMC DC population. CCR7 was not assessed as previous work has examined the

effect of BC on this marker[139]. DC were defined as lineage negative HLA DR positive cells. They were further sub classified into myeloid DC (mDC) expressing CD11c and plasmacytoid DC (pDC) expressing CD123.

8.4.3.1 Demographics

Twenty-two patients were included in the analysis, 15 patients were male and the median age was 60.5 years [IQR 51.5-60.5]. Tumour and body composition characteristics are shown in Tables 8.7 and 8.8.

		n	*%*
Gender	Male	15	68.2
	Female	7	31.8
T Stage	T1/2	5	22.7
	T3/4/extra luminal disease	17	77.3
Nodal Disease	Nodal Disease Absent	13	59.1
	Nodal Disease Present	7	31.8
	Ungraded[§]	2	9.1
Vascular Invasion	Vascular Invasion Absent	11	50.0
	Vascular Invasion Present	9	40.9
	Ungraded[§]	2	9.1
Lymphatic Invasion	Lymphatic Invasion Absent	14	63.6
	Lymphatic Invasion Present	6	27.3
	Ungraded[§]	2	9.1
Perineural Invasion	Perineural Invasion Absent	10	45.5
	Perineural Invasion Present	10	45.5
	Ungraded[§]	2	9.1
EMVI	EMVI Absent	12	54.5
	EMVI Present	8	36.4
	Ungraded[§]	2	9.1
Tumour Differentiation	Well/Moderately Differentiated	17	77.3
	Poorly Differentiated	5	22.7

[§]Ungraded as resection of locally recurrent rectal cancer

Table 8.7 Demographic and tumour characteristics of the PBMC cohort

		n	*%*
BMI Obesity	Not BMI Obese	11	50.0

	BMI Obese	11	50.0
Sarcopenia	Not Sarcopenic	11	50.0
	Sarcopenic	11	50.0
Myosteatosis	Not Myosteatotic	11	50.0
	Myosteatotic	11	50.0
Visceral Obesity	Not Viscerally Obese	6	27.3
	Viscerally Obese	16	72.7
Sarcopenic Obesity	Not Sarcopenic Obese	17	77.3
	Sarcopenic Obese	5	22.7

Table 8.8 Body composition characteristics of the PBMC cohort

8.4.3.2 mDC in sarcopenic patients have significantly increased expression of CD40

Body composition status is associated with frequency of expression of CD40; patients with sarcopenia demonstrated a significant increase in mDC expression of the maturation marker CD40 (p=0.0004), Figure 8.12. The pDC population did not share this maturation profile with no significant difference between the sarcopenic and non-sarcopenic group (p=0.42). Maturation of mDC and pDC was not however influenced by myosteatosis (pDC p=0.98; mDC p= 0.67), visceral (pDC p=0.99; mDC p=0.99), BMI (pDC p=0.67; mDC p=0.49) or sarcopenic obesity (pDC p=0.9; mDC p=0.08).

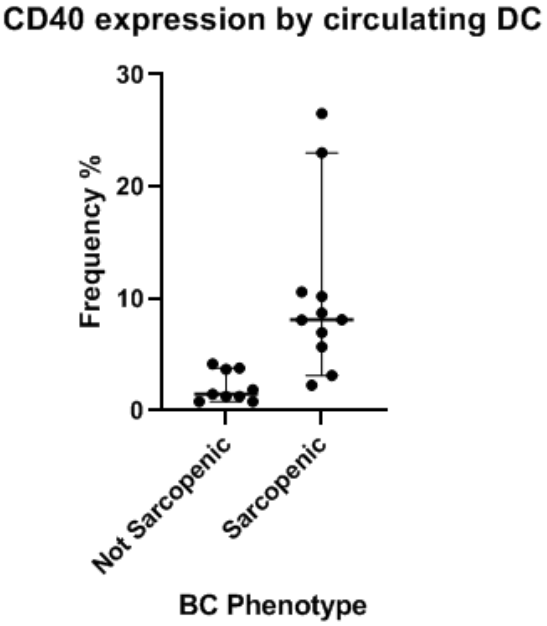


Figure 8.12 Expression of CD40 on circulating DC occurs more frequently in sarcopenic patients

8.4.3.3 The homing profile of circulating DC is associated by host body composition

CLA, a skin homing marker and Beta7, a gut homing marker were assessed. Myosteatotic patients consistently expressed significantly greater gut homing Beta7 receptors on mDC compared to non myosteatotic patients (p=0.03), Figure 8.13, pDC did not share this significant relationship (p=0.10). No other body composition parameters showed a propensity for gut homing of either mDC or pDC. Interestingly, patients within the poorly prognostic sarcopenic obesity sub-group had significantly increased expression the skin homing marker CLA on both mDC (p=0.05) and pDC (p=0.02). The sample size of this population was small and thus there is an increased risk of type 2 error however this requires further investigation. No other body composition phenotypes were associated with specific skin homing.

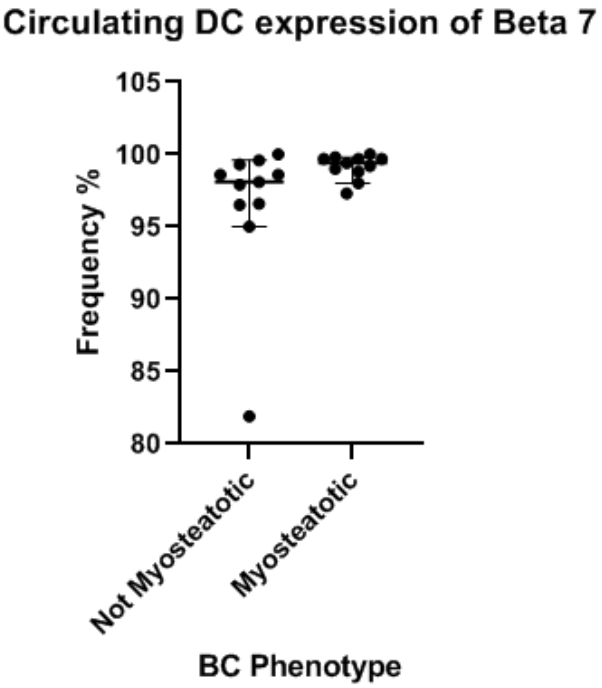


Figure 8.13 Beta7 (gut homing) expression by circulating DC was significantly greater in patients with myosteatosis

8.4.4 Muscle DC profile of maturation and migration

Dendritic cells were successfully isolated from the skeletal muscle tissue of colorectal healthy and colorectal cancer patients. The data from the healthy subject demonstrate the presence of DC in the muscle of healthy individuals and demonstrate that the method of DC isolation is successful. DC from diseased patients underwent analysis. The sample size of these patients precluded analysis based on categorical data as there were too few data points in the comparator groups to draw statistically relevant conclusions. Assertions were therefore made based on correlative statistics, where instead of using categorical cut-offs i.e. sarcopenic or not, we assumed a spectrum of body phenotype was linear and would as such share a linear relationship with DC features. Such assumptions were made by Malietzis et al 2016 allowing them to draw conclusions regarding DC behaviour.

8.4.4.1 Demographics

Eleven colorectal cancer patients were included in the analysis, 6 were male and median age 57 years [IQR 50-70]. Tumour and body composition characteristics are shown in Table 8.9 and Table 8.10 respectively. We also examined gracilis muscle from a single male healthy patient, aged 33, to explore whether there was evidence of maturation and gut homing with a view to providing proof of concept for future studies.

		n	*%*
Gender	Male	6	54.5
	Female	5	45.5
T Stage	T1/2	2	18.2
	T3/4/extraluminal disease	9	81.8
Nodal Disease	Nodal Disease Absent	6	54.5
	Nodal Disease Present	4	36.4
	Ungraded[§]	1	9.1
Vascular Invasion	Vascular Invasion Absent	6	54.5
	Vascular Invasion Present	4	36.4
	Ungraded[§]	1	9.1
Lymphatic Invasion	Lymphatic Invasion Absent	6	54.5
	Lymphatic Invasion Present	4	36.4
	Ungraded[§]	1	9.1
Perineural Invasion	Perineural Invasion Absent	4	36.4
	Perineural Invasion Present	6	54.5
	Ungraded[§]	1	9.1
EMVI	EMVI Absent	6	54.5
	EMVI Present	4	36.4
	Ungraded[§]	1	9.1
Tumour Differentiation	Well/Moderately Differentiated	7	63.6
	Poorly Differentiated	4	36.4

[§]Ungraded as resection of locally recurrent rectal cancer

Table 8.9 Demographic and tumour characteristics of the full muscle cohort

		n	**%**
BMI Obesity	Not BMI Obese	3	27.3
	BMI Obese	8	72.7
Sarcopenia	Not Sarcopenic	7	63.6
	Sarcopenic	4	36.4
Myosteatosis	Not Myosteatotic	3	27.3
	Myosteatotic	8	72.7
Visceral Obesity	Not Viscerally Obese	8	72.7
	Viscerally Obese	3	27.3
Sarcopenic Obesity	Not Sarcopenic Obese	9	81.8
	Sarcopenic Obese	2	18.2

Table 8.10 Body composition characteristics of the full muscle cohort and the subset analysed for CD36 expression

8.4.4.2 Increasing lumbar skeletal muscle index negatively correlates with DC maturation in muscle

Lumbar skeletal muscle index (LSMI) is used in combination with BMI and gender to determine clinically relevant sarcopenic status. It allows correction of axial area for individual height. As LSMI increases sarcopenia decreases. We identified a significant negative correlation between LSMI and frequency of DC expression of CD40 (r= -0.72; p=0.022), Figure 8.14. CD40 expression did not significantly correlate with changes in BMI (r=-0.53; p=0.12), mean muscle attenuation (MA - i.e. a surrogate of fatty infiltration of muscle) (r=0.33; p=0.35), visceral fat surface area at L3 (VFA) (r=-0.41; p=0.24) and visceral fat index (VFA corrected for height) (r=-0.50; p=0.14).

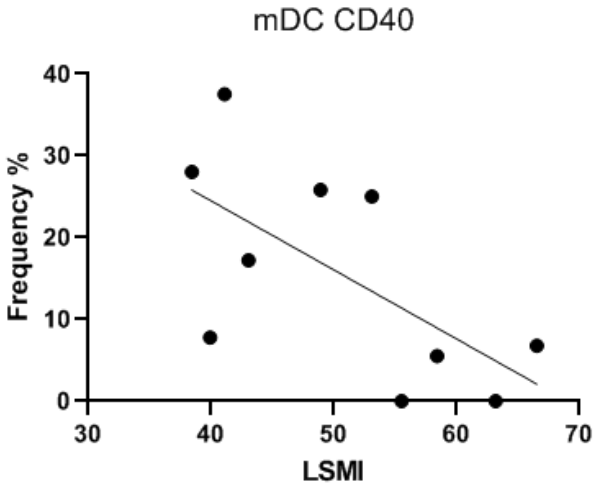


Figure 8.14 Significant negative correlation between CD40 expression and LSMI

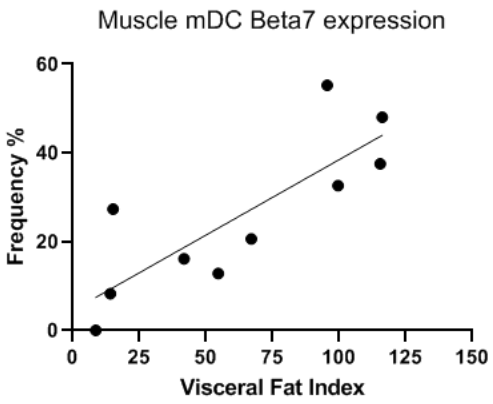
8.4.4.3 CCR7 expression does not correlate with body composition status

None of the body composition parameters described above correlated with CCR7 expression; however, variations were seen in CCR7 expression in different patients

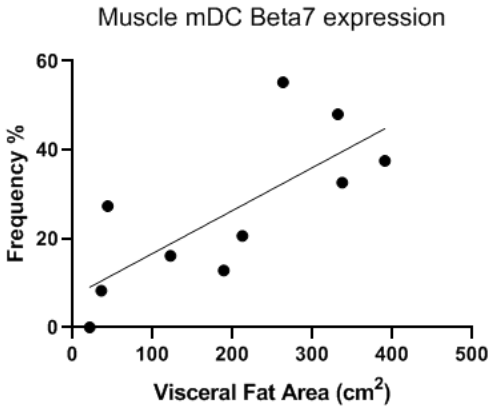
suggesting a role for lymph node homing in muscle which would require further evaluation.

8.4.4.4 Alpha 4 Beta 7 expression and muscle tissue

Alpha 4 and Beta 7 integrin were assessed as component parts using the antibody CD49d for Alpha 4 and Beta 7. There was no relationship between LSMI, MA, body mass index either Alpha 4 or Beta 7 integrin. The obesity parameters however seem to be more influential; there were significant positive correlations with VFI and VFA and Beta7 expression (r=0.84; p=0.004 and r=0.81; p=0.007 respectively), Figure 8.15a & b. Interestingly, there was not a shared correlation with alpha 4 integrin with either VFI or VFA (p=0.35 and p=0.45 respectively), Figure 8.16a & b. This suggests that the Beta7 subunit is potentially binding another integrin partner and initiating an alternative homing signal.

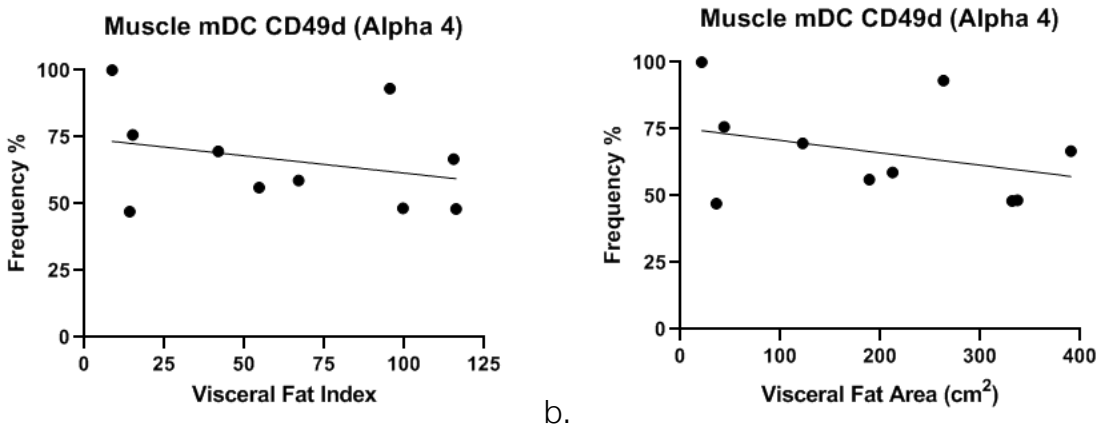


a.



b.

Figure 8.15a & b Beta7 Expression correlates with increasing VFI and VFA



a. b.

Figure 8.16a & b Alpha4 expression does not correlate with increasing VFI and VFA

8.4.4.5 Dendritic cells in in healthy muscle exhibit maturation and gut homing but no lymph node homing

As in Chapter 7 we were able to demonstrate the presence of DC within the skeletal muscle of a healthy male individual. The gating strategy and proportion of lineage negative and HLA DR positive cells is shown in Figure 8.17.

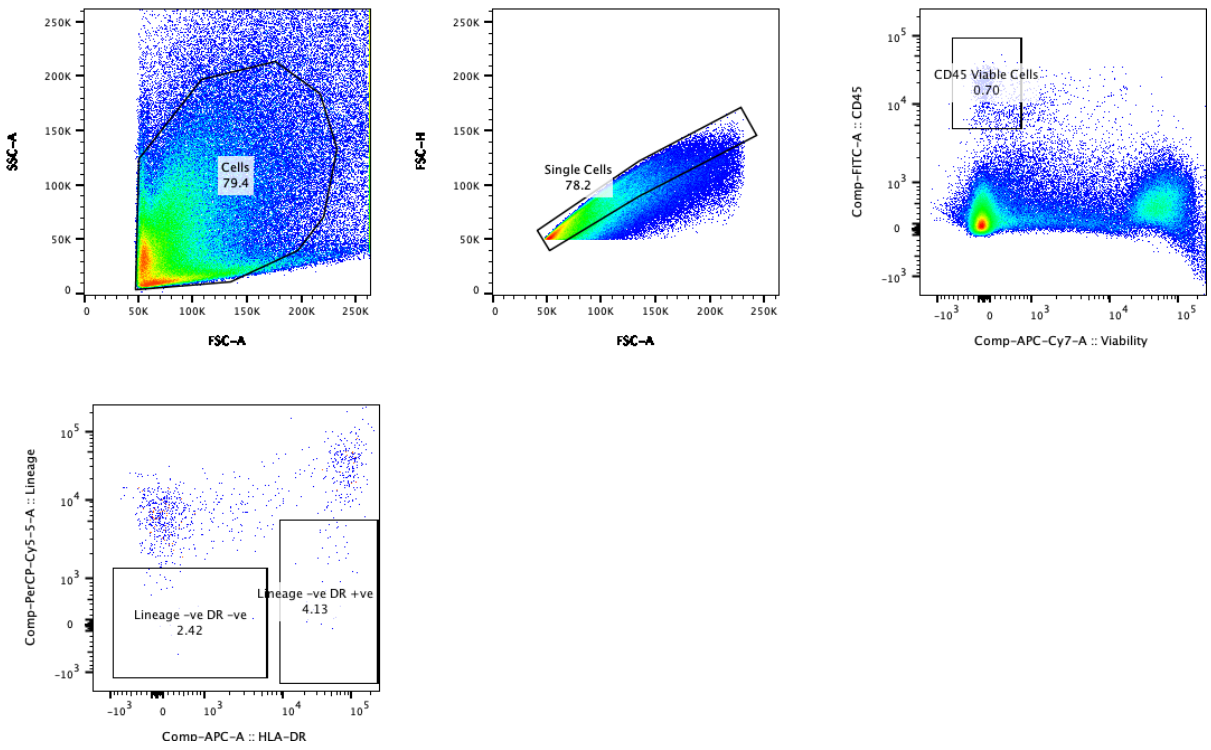


Figure 8.17 Gating strategy for healthy muscle

There were no pDC isolated from our experiment but numerous mDC were identified. 50% of the mDC express CD40, which we would expect form resident tissue DC. 100% of the cells expressed Alpha 4 integrin but only 11% Beta 7 integrin, suggesting another binding partner for alpha 4 and perhaps raising the possibility that alpha 4 integrin, by its ubiquity in muscle, is also involved in homing to healthy muscle. There was no CCR7 expression seen in the mDC of this individual. The raw data is shown in Figure 8.18.

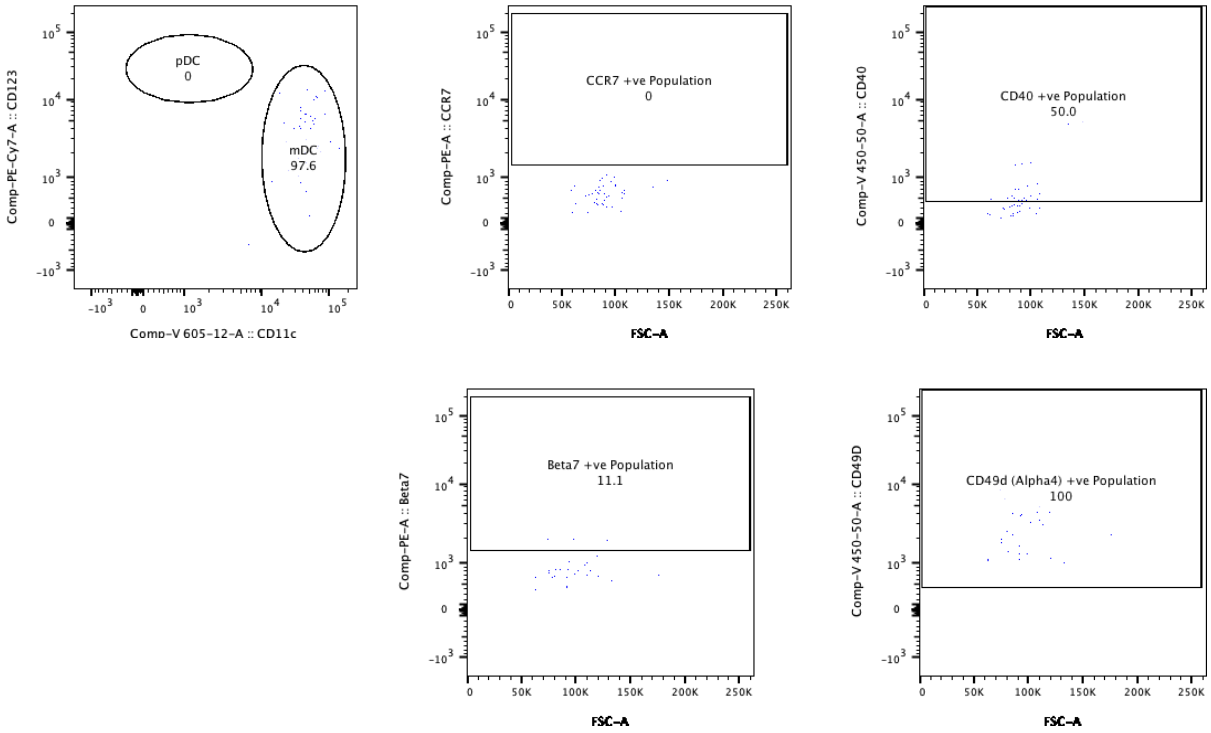


Figure 8.18 mDC of healthy muscle and frequency of maturation and migration markers

Interestingly in the healthy individual there were very few $CD45^+$, $CD11c^+$, $CD3/14/16/19/34^-$, $HLA\text{-}DR^-$ cells, Figure 8.19 perhaps suggesting the presence of this putative DC population, discussed in chapter 7, is a pathological phenomenon seen in CRC.

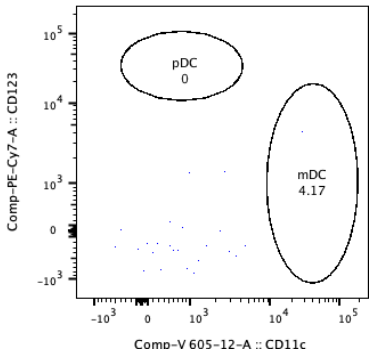


Figure 8.19 CD45+, CD11c+, CD3/14/16/19/34-, HLA-DR- cells in healthy muscle

8.4.5 Direct Tissue Comparison of Serial Markers

From the work above we have established association with changes in body composition and CD40 and CCR7. We examined these functional markers in a number of tissues, an analysis was performed to ascertain how expression of these markers change within the various tissues.

8.4.5.1 CD40 expression by DC is tissue dependent in colorectal cancer

CD40 expression was examined in all four tissues. We compared expression of CD40 in each tissue. Significant differences exist between CD40 expression on circulating DC compared to DC of the LPL and mesentery. CD40 expression is significantly deficient on circulating DC compared to the LPL and mesenteric DC. There was however no significant difference in the CD40 expression between circulating DC and intra-muscular DC Table 8.11 & Figure 8.20.

p-value				
CD40 %	LPL	Mesentery	PBMC	Muscle
LPL		0.35	0.0001	0.19
Mesentery			0.05	0.99
PBMC				0.17

Table 8.11 significant difference in CD40 expression by DC in each tissue

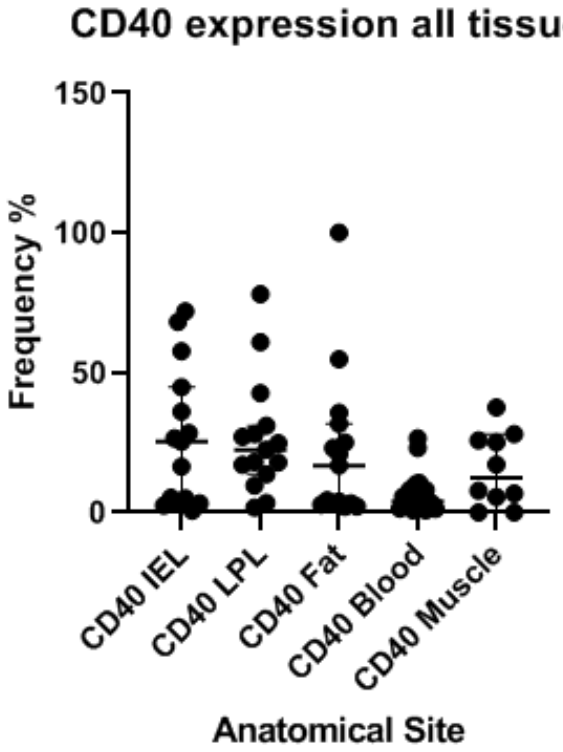


Figure 8.20 CD40 frequency of expression by DC at all tissue sites

8.4.5.2 CCR7 expression is greatest in the mesentery compared to other tissues

CCR7 expression is also tissue specific, here we found that expression of CCR7 was significantly greater in the mesentery compared to either the muscle or LPL DC populations. This would fit with the assertion that DC within the mesentery have

significantly increased lymph node homing properties in the perinodal fat of the mesentery (Table 8.12 and Figure 8.21).

p-value			
CCR7%	LPL	Mesentery	Muscle
LPL		0.0002	0.46
Mesentery			0.02

Table 8.12 Significant relationships between frequency of CCR7 expression between tissues

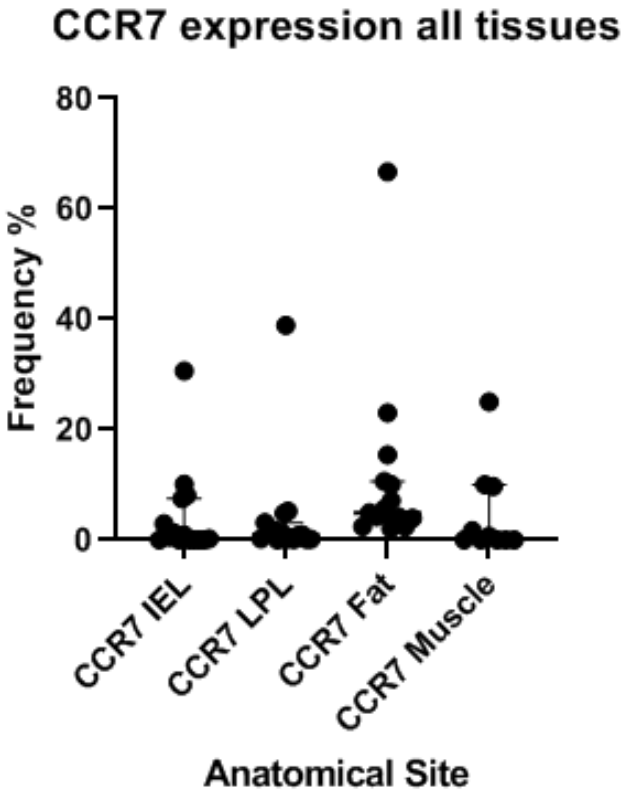


Figure 8.21 CCR7 frequency of expression by DC at all tissue sites

8.5 Discussion

These experiments focussed on the effect of body composition status on maturation and migration of macrophages and DC in the IEL and LPL of the colon and DC of the colonic mesentery, blood and skeletal muscle. Our aim was to examine the effect of CRC on DC maturation and homing and to explore the associations between the DC and the end organs of gut, adipose tissue and muscle in relation to body composition status. We have seen how DC maturation is related to the ability of DC to migrate and present antigen but that the process of maturation and role of DC throughout the process of maturation, is complicated and not fully understood; however, we have seen that maturation of DC, stimulated by antigen presentation, is key in determining the role of DC and establishing subsequent migration. We have established that the expression of integrin is an essential part of the DC – T-cell circuitry with T cells orchestrated by DC signalling and that this circuitry is altered in disease such as IBD[264]. We have seen how, using monoclonal antibodies such as Vedolizumab we can manipulate the course of lymphocytes in their migratory pathway. We have demonstrated that certain immune axes exist between the gut and distant organs and that there is a developing concept of a gut-muscle axis in health. Body composition is associated with DC maturation and has been found to be related to elements of DC homing, namely CCR7 lymph node homing[139].

8.5.1 DC Maturation significantly increased in solid tissues – but not muscle in disease

CD40 expression on DC is influenced by host tissue; unsurprisingly unstimulated circulating DC have significantly deficient expression of CD40 compared to solid tissue DC. This would suggest that those DC which have become resident in the solid tissues

have been stimulated by antigen and are carrying out an endpoint function. Interestingly we do not see a significant difference in CD40 expression between blood and muscle in colorectal cancer, although expression of CD40 in the muscle of diseased patients was diminished on comparison to our healthy muscle donor. Further samples are required to investigate whether muscle in health contains DC with higher CD40 expression and if so, this raises an interesting question as to the role of the mature DC in muscle in disease. Are DC failing to mature or are DC being re-directed to other organs due to alterations in homing. The fact that our healthy donors' CD40 expression was higher than any of the diseased patients CD40 could be a suggestion of such dysfunction. One consideration is whether naïve DC are triggering a cytokine pathway in muscle leading to the muscle damage (myosteatosis) seen in CRC patients.

8.5.2 Lymph node homing is highest in the mesenteric fat and putative DC exist with loss of HLA-DR (MHC-class II)

It comes as no surprise that lymph node homing is highest in the DC of the colonic mesentery. These DC are intimately associated with the lymph node rich environment and although no lymph nodes were present in the fat mesentery samples, there is undoubtably a web of lymphatic vessels which will contribute to this lymph node pool. This concurs with the work of Bedford and colleagues who found that abdominal (omental) fat has perinodal qualities and clearly this suggest that there is a significant level of traffic towards the lymph nodes from the gut where the DC will present their captured antigen to effector cells[248]. This ultimately suggests appropriate DC homing function in these patients. One of the starkest observations was the significant increase in leucocyte number seen in the mesentery of patients with lymphatic invasion. High leucocyte

number was almost diagnostic of lymphatic invasion with only one patient out of 15 with a high leucocyte count but no lymphatic invasion. It would be interesting to re-examine this one patient to see whether, in retrospect, there was lymphatic invasion on their histology.

8.5.3 CCR7 expression is significantly higher in LPL of patients with sarcopenia and myosteatosis

We described how, in earlier experiments, CCR7 expression was higher in the circulating DC of patients with myosteatosis. It is therefore an interesting observation that we see a similar picture in the LPL of these sarcopenic and myosteatotic patients. What is perhaps more interesting is that we do not see this relationship in the muscle or the mesenteric fat. Are we therefore seeing the DC in this poor prognostic group bypassing the mesenteric pathway for some reason, and failing to present tumour antigen to the nodal system perhaps through a homing malfunction, and entering the peripheral circulation straight from the gut?

8.5.4 Body composition influences DC and macrophage behaviour in the LPL but not IEL

Our findings demonstrated there is no gross significant difference in DC maturation or lymph node homing between the IEL and LPL DC. However, the influences of BC become apparent in the LPL but not the IEL which suggests an evolving role of the DC as they migrate to the LPL. This may be explained by the behaviour of DC and the way they initiate the primary immune response through interaction with other naïve DC. Knight

and colleagues proposed that DC from mouse bone marrow or blood did not stimulate primary T cell responses directly. However, these DC donate processed antigen and MHC class 2 to other DC they interacted with. If these recipient DC had not been exposed to the same antigen the recipient DC initiated primary immune responses. However, DC from LN that had passed through the peripheral tissue could stimulate primary responses directly[271].

We noted a significant difference in maturation (CD40 expression) in relation to adiposity, with those patients with VO and BMI obesity displaying a greater tendency to have mature DC in the LPL. VO appears to be associated with better prognostic tumours (chapter 2) and therefore one may assume a more effective DC and subsequent immune response in this patient group. This increase in maturation seen herald an appropriate functional DC response to the tumour or gut microbiome. Earlier published work, described above, that CD40 expression was found to be higher on the circulating DC of patients with a higher LSMI and thus increased DC maturation in the blood is a prognostic of a better BC status and thus outcome. This fits in with our findings of an appropriate early maturation in this prognostically advantageous group. It is also interesting to see where in the path of the DC lifecycle that BC influence is exerted on the DC.

8.5.5 DC resident in the mesentery are more likely to be mature in the presence of visceral obesity

The presence of an increased frequency of mature DC in this VO subgroup is in keeping with the associations found with DC maturation in the LPL which may suggest transference of DC from the LPL through the mesentery or a systemic effect of the mesentery on DC in general.

The mesentery appears to have a profound immune function and in certain autoimmune diseases such as Crohn's disease, resection of the mesentery can provide immunomodulation and disease quiescence demonstrating these diseases of the gut are also diseases of the mesentery[272]. No such models or hypotheses have been developed for the cancer patients, particularly since in an oncological resection the surgeon would remove the mesentery en bloc to ensure appropriate nodal resection. That having been said, these data show the mesentery to be immunologically active and as such, resection of the mesentery may also have a profound effect on the systemic inflammatory milieu and this may be both beneficial and detrimental. Once again, these findings are in keeping with the concept that VO and DC CD40 expression are findings associated with a better prognosis.

Once again within the mesentery we see this population of $CD45^+$, $CD3/14/16/19/34^-$, $HAL\text{-}DR^-$, $CD11c^+$ cells which share many of the characteristics with the classical myeloid DC discussed above. As discussed earlier this is keeping with Bedford's earlier work in the omentum in Crohn's Disease[248]. With this population though we see reinforcement of the significant relationship with CD40 and increased LSMI and VO in the BMI metric, which will consider both of these values, albeit in an arbitrary manner. BMI obesity was found to be associated with increased CD40 expression in these cells.

8.5.6 CD40 expression on circulating DC is significantly lower than solid tissue but expression is associated with sarcopenia

On examination of the DC population within the PBMC we see findings which conflict with earlier work. Indeed, as with Malietzis' muscle volume appears to be a key factor in DC maturation; however, in our population we find increased DC maturation is

associated with sarcopenia. This stark difference in findings is interesting, Malietzis did not look at dichotomous variables but a scale (LSMI), this significant difference may thus have been brought about by a bell-shaped curve distribution or non-linear relationship in CD40 expression where it initially rises with LSMI then falls when LSMI reaches a certain point. This could in turn explain why his different population did not reflect our findings. Further work needs to be done to examine this phenomenon as seasonal variations may indeed influence these results. It is important to say that the circulating DC population expressing CD40 may be of limited importance and impact in view of its size compared to the solid tissue DC. Our data is also supported by the fact that we see a significant negative relationship between CD40 and LSMI in the resident muscle DC. This loss of mature DC in the sarcopenic population may represent less metabolically active and immunogenic muscle tissue in this poor prognostic group, this may be a result of reduced myokine and other muscle related cytokine expression e.g. IL10.

8.5.7 The gut muscle axis

Patients who have increased expression of Beta7 on their circulating DC are more likely to be myosteatotic. This presence of an increased gut homing integrin in a poor prognostic condition may suggest more florid gut disease and greater disruption of the gut mucosal barrier. In health we see high Alpha 4 expression in the muscle DC (although, n=1) and this needs to be explored further and in greater detail. However, in the viscerally obese patients we see a positive correlation between Beta7 expression and the presence of visceral obesity. This suggest that patient who have excess intrabdominal adiposity have a potentially great propensity for gut homing DC in their muscle tissue. There is however no relationship with myosteatosis suggesting a less pernicious explanation for

these gut homing DC in the muscle. These data clearly demonstrate a link between the gut and muscle as evidenced by the expression of gut homing makers on resident DC in the muscle. More work needs to be done to define muscle homing integrins and assess their presence and change in their patterns of expression in multiple tissues in both health and disease.

8.6 Limitations

One of the greatest limitations of this work is the lack of healthy controls with which we can compare our data. Comparing different prognostic groups within a colorectal cancer population is helpful and provides strongly controlled data using the presence of CRC as a constant. However, CRC is a heterogenous disease and these analyses do not account for disease subtype. We also require large numbers of sample patients from the cancer population to be able to draw conclusions and as such when you have a comparatively rare event such as sarcopenic obesity it makes it very difficult to draw conclusions.

As describe in the Chapter 7, our method for DC isolation from muscle evolved over time impacting on the number of successful experiments compared to the other tissue experiments as such the conclusions we can draw from the muscle data are limited too. We have shown healthy muscle data here but this only serves as proof of concept and provides a snapshot illustration of what the DC behaviour may be like in health. Further work must be done on this population to define the role of DC in healthy muscle further. As the only work to be done, to our knowledge, of DC behaviour in the muscle tissue of CRC patients we must make certain assumptions and a larger body of work is needed to draw further conclusions. We have however seen that we can't draw conclusions on

DC behaviour in the circulation and relate it to the function of tissue DC as these populations clearly behave in very different ways.

Finally, although we tried to control for time of day and disease we could not control for seasonal variation and this may also explain conflicting findings in DC behaviour.

8.7 Conclusion

DC homing and maturation changes in different tissues with DC carrying out certain specific roles and actions at each tissue site. The passage of DC is not likely to be linear but in fact a complex web where DC will pass from one tissue to another and back again sharing information and defining the immune response. These data show that body composition, particularly visceral obesity, plays an important role in maturation and homing of DC but our understanding of the processes that drive these responses are limited. We suggest that muscle and fat related cytokine production may be a key factor in this. Overall, the DC appear to be of a prognostically favourable phenotype in the viscerally obese. Further work, covered in Chapter 9, examines the effect of BC on DC function, homing and maturation in more detail in an attempt to elucidate why certain BC phenotypes predispose the DC to particular behavioural functions. Finally, these data support the concept of a gut muscle axis particularly through the expression of gut homing integrin on resident muscle DC and the fact that different prognostic BC state influence their expression.

Chapter 9

9 Pathways to dysfunction of the Dendritic cell in colorectal cancer

9.1 Summary

In Chapters 7 and 8 demonstrated that body composition status was associated with changes in DC character and behaviour. We ascertained that the dendritic cell population in colorectal cancer is both influenced by and exerts influence upon body composition. Phenotypic changes in this seminal cell will have functional sequelae on its downstream effector cells but will also function differently as a result of the disease process. Migration and maturation are altered as is the lipid profile of DC. What remains to be seen is what effect do these changes have on the function of the DC in colorectal cancer and indeed what effect does lipid accumulation have on maturation and migration itself. This in turn would allow us to identify pathological cellular pathways which require further investigation.

In Chapter 2 we demonstrated that patients with VO had prognostically favourable tumour characteristics and in Chapter 8, we identified that in the viscerally obese, mucosal homing in the lamina propria layer of the colon was significantly diminished. Mann and colleagues have previously demonstrated an association between the lymph node homing marker CCR7 and expression of Signal regulatory protein alpha (SIRPα or CD172a) in the colon. They demonstrated that a greater proportion of colonic DC express CCR7 alongside enhanced endocytic capacity for bacterial sampling; this difference was most striking in $CD103^{+}SIRP\alpha^{+}$ DC[273]. What remains to be identified is

how does mucosal homing relate to function in health and disease and could this explain our findings in the context of body composition.

In this chapter we further evaluate the changes in DC behaviour between disease and health in the colon to contextualise the picture of function in terms of body composition. We explore the relationship between DC lipid status and maturation and migration as well as exploring the relationship to cytokine production. Finally, we examine the effect of the microbiome upon DC in disease in health to identify culprits in dysfunction.

9.2 Introduction

9.2.1 SIRPα and its role in the host response to cancer

SIRPα is an inhibitory receptor present on monocytes, tissue macrophages, subsets of DC, granulocytes, bone marrow progenitor cells and some neurons, especially those in synapse rich areas[274]. It consists of three Ig-like domains, a transmembrane region and a cytoplasmic region which has four Tyr residues with immunoreceptor Tyrosine-Based Inhibitory Motifs which are capable of activating in intracellular signalling pathways[275]. SIRPα interacts with the protein CD47, a protein with five transmembrane regions with a single Ig-like domain that interacts with the NH_2-terminal domain of SIRPα. CD47 is ubiquitously expressed on host cells. On binding CD47, SIRPα transmits what is known as a "don't eat me" signal in relation to the cell expressing CD47 which prevents engulfment by phagocytosis. Cancer cells are known to upregulate CD47 as part of their process of immune evasion and in fact CD47 was indeed first identified on ovarian cancer cells[276]. In one study on stage III-IV colorectal cancer CD47 was upregulated in the tumour of 82 out of 95 cases analysed and correlated with the incidence of distant metastases[277]. As such, CD47 has been seen as a potential immunotherapy target where by its inhibition can result in subsequent activation of a myeloid cell mediated immune response[278]. The SIRPα/CD47 interaction more has more recently been recognised as an important component of cellular adhesion and migration especially in inflammatory processes where monocytes are required to infiltrate across the junctions between endothelial cells by diapedesis[279]. Notably, in an experiment on gene targeted mouse models, those mice deficient in the gene encoding CD47 succumbed to E. coli peritonitis following intraperitoneal bacterial inoculation whilst their heterozygous

littermates survived. At four hours following inoculation the influx of polymorphonucleocytes into the peritoneum was diminished in the CD47 knock-out mice. At 24 hours the PMN number had equalised between groups but there were significantly more macrophages on abdominal lavage in the CD47 knock-out group with a 100 fold increase in bacterial burden[280].

These studies demonstrate the importance of SIRPα/CD47 interaction both in relation to the host response to cancer and also the ability to mount a successful inflammatory response.

9.2.2 Cytokine profiles in relation to body composition

IL-6 and IL-10 were discussed in the introduction and their relationship to muscle and adipose tissue briefly in Chapter 7. These two cytokines are intimately related to body composition and numerous relationships have been identified both implicating and influencing them in disease behaviour. Growth Differentiation Factor 15 (GDF-15) (macrophage inhibitory cytokine-1), a divergent member of the transforming growth factor β superfamily, has emerged as a cytokine of interest in more recent years and is being investigated as a marker of sarcopenia in a number of diseases including cancer.

9.2.3 IL-6 in colorectal cancer

IL-6 is implicated in the both the aetiology and evolutionary biology of CRC such as tumour angiogenesis[281]. As discussed in the introduction and earlier chapters IL6 can be produced at multiple sites by multiple cell types including cells of the immune system such as DC and other myeloid cell. Plasma IL-6 levels is significantly elevated in colorectal

cancer patients compared to benign adenoma patients and healthy controls and increased circulating IL-6 is found in more advanced stages of CRC and in patients who have irresectable disease[282].More importantly, perhaps in the context of sarcopenia and myosteatosis, IL-6 is known to be produce in the tumour micro environment from such cell types as colon cancer associated fibroblasts within the stroma[281]. It is therefore, not surprising that high circulating levels of IL-6 are associated with a decreased overall survival in gastrointestinal cancer patients[283]. Interestingly, ethnicity also appears to influence IL-6 production in cancer. We identified in Chapter 5 several associations between inflammatory status and ethnicity, there is evidence that gene-polymorphism in IL6 and subsequent cancer risk. A recent meta-analysis demonstrated in their ethnicity subgroup analysis of the relationship between IL-6 and cancer risk a significant association of increased overall cancer risk with the presence of the rs1800795 polymorphism in African and Asian populations, the rs1800796 polymorphism in the Asian only and the rs1800797 polymorphism in the African population[284]. However the relationship between ethnicity, IL-6 and body composition is yet to be explored.

9.2.4 IL-6 and immune regulation

In mouse models, IL-6 has been found to play a major role in preventing DC maturation, with an increased number of mature DC found in IL-6 knockout mice. The authors of this study also identified that Signal transducer and activator of transcription 3 (STAT3) activation by IL-6 was required for the suppression of LPS-induced DC maturation and additionally that IL-6 regulated the phosphorylation of STAT3 in DC[285]. IL-6 has also been found to have a profound effect on the function of the DC population in colorectal cancer. In an experimental model, $CD11b^{+}CD11c^{+}$ cells obtained from PBMC of healthy donors

were treated with IL-6, it was found that surface expression of HLA-DR and the activation marker CD86 were significantly diminished through a STAT3 dependant process[286].

In the same series of experiments in CRC patients, it was found that gene expressions of a number of pro-inflammatory proteins including IL-6 were higher in tumour infiltrating $CD11b^{+}CD11c^{+}$ cells compared with those within the circulation[286]. Ultimately the authors concluded that the expression of surface HLA-DR and CD86 on $CD11b^{+}CD11c^{+}$ cells in the tumour microenvironment was down-regulated and as such their ability to present antigen to and activate T cells was attenuated compared with circulating $CD11b^{+}CD11c^{+}$ cells. This suggests that immunosuppressive phenotype might be induced in part by IL-6, in the tumour microenvironment of colorectal cancer patients[286].

9.2.5 IL-6 in relation to body composition

As with many of these cytokines the relationship between IL-6 and body composition is complex. We have described in detail the relationship between body composition phenotypes and IL-6 production but less so on the effect IL-6 plays on BC. In a meta-analysis of individuals with and without sarcopenia in all disease types there was no significant difference found between serum IL6 levels in people with sarcopenia versus controls[287]. Studies specifically in cancer are few and the evidence limited, one study colorectal cancer specific study demonstrated IL-6 levels to be lower in patients with sarcopenia compared to those without sarcopenia and found the levels of IL-6 predicted the presence sarcopenia[288]. The relationship between IL-6 and cachexia is more robust and there is more convincing evidence that elevated IL-6 drives cachexia by targeting adipose, skeletal muscle, the gut, and liver tissue[289].

9.2.6 IL-10 in colorectal cancer

Like IL-6 the role of IL-10 in colorectal cancer is complex and it can be both pro and anti-inflammatory in its effect. IL-10 receptor has been found to correlate with both IL-10 and phosphorylated STAT3 in both CRC tumour tissue and the resection margins. A positive correlation has also been found between the expression of IL-10RA on tumour cells and the Ki-67 proliferation index and a negative correlation between IL-10RA and clinical stage in CRC. Interestingly the highest expression of IL-10RA in these patients was found in the surgical margins of patients suggesting an IL-10 mediated immune response to the tumour[290].

9.2.7 IL-10 in relation to body composition

The influence of IL-10 on body composition and the role tissues play in producing IL-10 have been discussed in the introduction and earlier chapters, we further discuss IL-10 in Chapter 10 especially in its relation to exercise physiology and muscle. Essentially, IL-10, released by muscle in response to exercise is thought to exert profound anti-inflammatory effects[212]. Interestingly one study found in patients with cancer cachexia found a tendency towards positive correlation between IL-10 concentrations in the subcutaneous adipose tissue and IL-10 concentrations in the tumour but this was not significant.

9.2.8 GDF-15 and its relationship with colorectal cancer, body composition and the immune system

Serum levels of GDF-15 have been shown to be significantly higher in patients with metastatic colorectal cancer and correlate with the extent of liver involvement[291]. It has also been shown to promote epithelial-mesenchymal transition and metastasis both in vitro and colorectal cancer mouse models, and there is a significant correlation between GDF-15 levels and lymph node metastases and tumour budding in humans[292].

Serum concentration of GDF15 has been found to increase with aging and there is an inverse relationship with serum levels and muscle mass and muscle endurance in individuals with age related sarcopenia[293]. Paradoxically, in cancer patients raised circulating levels of GDF15 have been found to be associated with weight loss, decreased appendicular lean body mass, muscle strength, and poor survival[294]. As with IL-6 and IL-10 the role and effect of GDF-15 remains controversial and conflicting and further research is required in this area to elucidate the pathways and mechanisms involved. As its alternative name suggests, GDF-15 is known to influence the myeloid cell population. In cancer, GDF-15 has been found to be a potent suppressor of dendritic cell maturation that inhibits expression of co- stimulatory and major histocompatibility complex (MHC) class II molecules, reduces IL-12 levels and elevates TGF-β1 secretion[295]. This is remarkably similar to the behaviour of IL-6 described earlier in this this chapter and like IL-6, it too may have a role in tumour immune evasion by downregulating antigen presentation by DC to T-cells and also preventing cytotoxic T lymphocyte activation.

9.2.9 The microbiome immune function and cancer

The relationship between the microbiome in the pathogenesis of colorectal cancer have been discussed in depth in the introduction to this thesis. Needless to say, the relationship between the microbiome and the immune system is complex and indeed complicated further by the presence of colorectal cancer. For the purpose of this thesis we are concentrating on the microbiome of the colon but we do acknowledge there is likely to be substantial microbial stimuli and thus influence, from other anatomical sources with their own established microbiome such as the proximal gut and skin. It is estimated the human colon contains more than 70% of the microbes in the huma body with a broad range of phyla differing in their proportion. The vast majority of these are Firmicutes (64%), followed by Bacteroidetes (23%) then Proteobacteria (8%) with Actinobacteria (3%) making up the smallest proportion of the major phyla of the colon[296]. DC interact with microbes through a multitude of receptors with Toll-like receptors being most critical. The host immune system relies on appropriate immune tolerance to maintain a healthy microbiome with a symbiotic function, however immune surveillance is necessary should certain microbial populations expand inappropriately or seed in an ectopic location. Notably an alteration in the composition of the colonic microbiota can affect microbial-host interactions and immune homeostasis[296]. In CRC it is also important to recognise that the immune system is potentially deficient and therefore the response to the gut microbiota may be impacted. Little is known about the effect of the microbiome on the lipid metabolism of circulating DC.

Body composition is also known to be related to the composition of the gut microbiota for example there are differences in the composition of the gut microbiota between lean and obese individuals, however a clear “obese-type” microbiota profile has yet to be

defined[297]. It is therefore possible that the innate immune system is involved in the regulation of a microbiota profile in keeping with a healthy body composition and indeed that the microbiome of individuals with colorectal cancer can be modified in such a way that we can manipulate their BC status.

9.2.10 Rationale

Earlier chapters have demonstrated the immune profile of homing, maturation and intracellular lipid associated with varying BC features. Earlier work has identified important functional features which may be impacted by colorectal cancer. The SIRPα/CD47 interaction in the gut is crucial and the relationship between CD103 and SIRPα is paramount but it is vital we improve our understanding of this in the context of health and CRC.

There is also a growing body of evidence in relation to cytokine expression and body composition further exploration of the relationship between cytokine expression, DC functional status and body composition may help untangle the nature of the complex interaction between DC phenotype and body composition status in cancer. In addition to this we have seen the importance of cytokine and myokine production and also the paradoxical relationship that these cytokines such as IL-6 bring about.

Finally we have identified that the gut microbiome has profound influences on the pathogenesis of colorectal cancer, that the immune system is dysfunctional in colorectal cancer and that therefore the metabolic and functional response of DC to bacterial stimulation may be affected. It is also recognised that the microbiome of the colon both influences and is influenced by BC status, particularly obesity and as such further exploration of these concepts are required in the context of this thesis.

9.2.11 Null Hypothesis

The function and behaviour of DC is not influenced by body composition in colorectal cancer

9.2.12 Aims

- Explore the significance of CD103 profile on the function of DC in the LPL of the colon by comparing healthy controls with colorectal cancer patients.
- Identify relationships between DC lipid status, homing and maturation.
- Examine the relationship between circulating plasma cytokine concentration and body composition
- Examine the relationship between circulating plasma cytokine concentration and DC maturation, homing status and lipid profile
- Using a model of the gut microbiota examine the effect of bacterial stimulation on the lipid profile of circulating DC in health and disease.

9.3 Method

9.3.1 Isolation of DC from tissue and blood

Healthy colonic biopsies were processed and analysed by Dr L R Durant in our laboratory using the methods and reagents described in Chapter 6. Isolation of DC and analysis of all other tissues was performed as described in Chapters 6, 7 and 8.

9.3.2 Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed on the plasma of fourteen colorectal cancer patients and five healthy controls. IL-6 and IL-10 were performed using the DuoSet® ELISA system (*R&D Systems, Minneapolis, MN, USA)* following the manufacturer's instructions. The GDF-15 ELISA was performed using the pre-prepared Quantikine® ELISA system (*R&D Systems*) according to the manufacturer's instructions. The plate was read on an Infinite® F50 microplate reader (*Tecan Trading AG, Switzerland*) at a wavelength of 540nm and data analysed using Magellan™ software (*Tecan Trading AG, Switzerland*). Further details of these methods are described in more detail in Chapter 6.

9.3.3 Bacterial Stimulation

Bacterial stimulation was performed on the PBMC of seven consecutive colorectal cancer patients and five healthy controls. Our experimental model utilised PBMC stimulated for 20 hours with snap freeze killed bacteria, previously isolated by Prof L Hoyles and her team from the caecum of healthy donors. PBMC were isolated and incubated overnight in complete medium with either LPS or the individual bacterial species listed in Table 6.1. Control stimulation was performed using medium alone. Following stimulation, the PBMC underwent FACS staining with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain reconstituted in Dimethyl-sulfoxide (DMSO) and the PBMC core antibodies described in Table 6.5a & b and CD36 (Chapter 6). Cells were then fixed and permeabilised using Leucoperm Reagents A & B (*AbD Serotec, Watford, UK*) and intracellular staining of lipids performed using BODIPY dye and E06 Biotin with secondary staining using Streptavidin.

9.3.4 Body composition analysis

Automated BC analysis was undertaken using SliceOmatic v5.0 with ABACS plug-in by Dr P Lung using DICOM format images extracted from the preoperative staging CT of patients. Details of this method, including its thresholds and cut-offs have been described extensively in the earlier chapters of this thesis.

9.3.5 Statistical analysis

Statistical analysis was carried out using GraphPad Prism software version 10. Non-parametric data was analysed using Mann-Whitney *U* tests. Comparisons of parametric grouped data for the bacterial data analysis was undertaken using two-way ANOVA with graphical display in heatmaps constructed in GraphPad Prism. Correlations for non-parametric data were assessed using Spearman's Rank. Descriptive statistics were used to analyse demographic data and performed in SPSS version 25.

9.4 Results

9.4.1 SIRPα CD103 in the LPL

The expression of SIRPα by myeloid cells within the colon has major functional implications in cancer through its interaction with CD47 as described above. We have demonstrated that in Chapters 7 and 8 that seemingly "healthy" colonic mucosa, distinct from the immediate tumour microenvironment, is subject to influences related to body composition status. VO and BMI obesity are associated with a pro-maturation status of the colonic LPL DC. Sarcopenia and myosteatosis on the other hand are associated with

increases in CCR7 expression and thus enhanced lymph node homing status. We also identified that in VO CD36 expression was significantly increased and the oxidised lipid profile diminished in these cells. We know from work by Bedford and colleagues that a greater proportion of colonic DC express the lymph-node-homing marker CCR7 alongside enhanced endocytic capacity for bacterial sampling and that this difference was most striking in $CD103^{+}SIRP\alpha^{+}$ DC[273]. Finally we identified that CD103 expression was downregulated in the VO population. We therefore sought to identify whether the profile of SIRPα expression in relation to CD103 (mucosal homing) differs from health to disease and whether SIRPα expression was related to BC status.

9.4.1.1 Demographics

The demographics of the colorectal cancer population are shown in Table 9.1, the median age of the population was 63 [IQR 55.25-72]. 12 age matched healthy controls were included in the study with a male to female ratio of 6:6, median age of the healthy population was 66.

		n	*%*
Gender	Male	12	75.0
	Female	4	25.0
T Stage	T1/2	5	31.3
	T3/4	11	68.8
Nodal Disease	Nodal Disease Absent	8	50.0
	Nodal Disease Present	8	50.0
Vascular Invasion	Vascular Invasion Absent	7	43.8
	Vascular Invasion Present	9	56.3
Lymphatic Invasion	Lymphatic Invasion Absent	10	62.5
	Lymphatic Invasion Present	6	37.5
Perineural Invasion	Perineural Invasion Absent	7	43.8
	Perineural Invasion Present	9	56.3
EMVI	EMVI Absent	9	56.3
	EMVI Present	7	43.8
Tumour Differentiation	Well/Moderately Differentiated	13	81.3

	Poorly Differentiated	3	18.8

Table 9.1 Demographic, tumour and body composition characteristics of the CRC population

9.4.1.2 Profile of mucosal homing and SIRPα DC in health and disease

There are stark significant differences between CD103 and SIRPα expression between health and disease, SIRPα expression is significantly diminished in colorectal cancer patients. The cell population in CRC is divided primarily between those which are $CD103^{+}SIRP\alpha^{-}$ and $CD103^{-}SIRP\alpha^{-}$, the differences in cell populations are shown in Figure 9.1 and the significant differences between populations. These data suggest significant downregulation of SIRPα in disease, what is more, in the mucosa of apparently healthy colon. This demonstrates that in established colorectal cancer immunomodulation or dysregulation appears to occur throughout the whole organ.

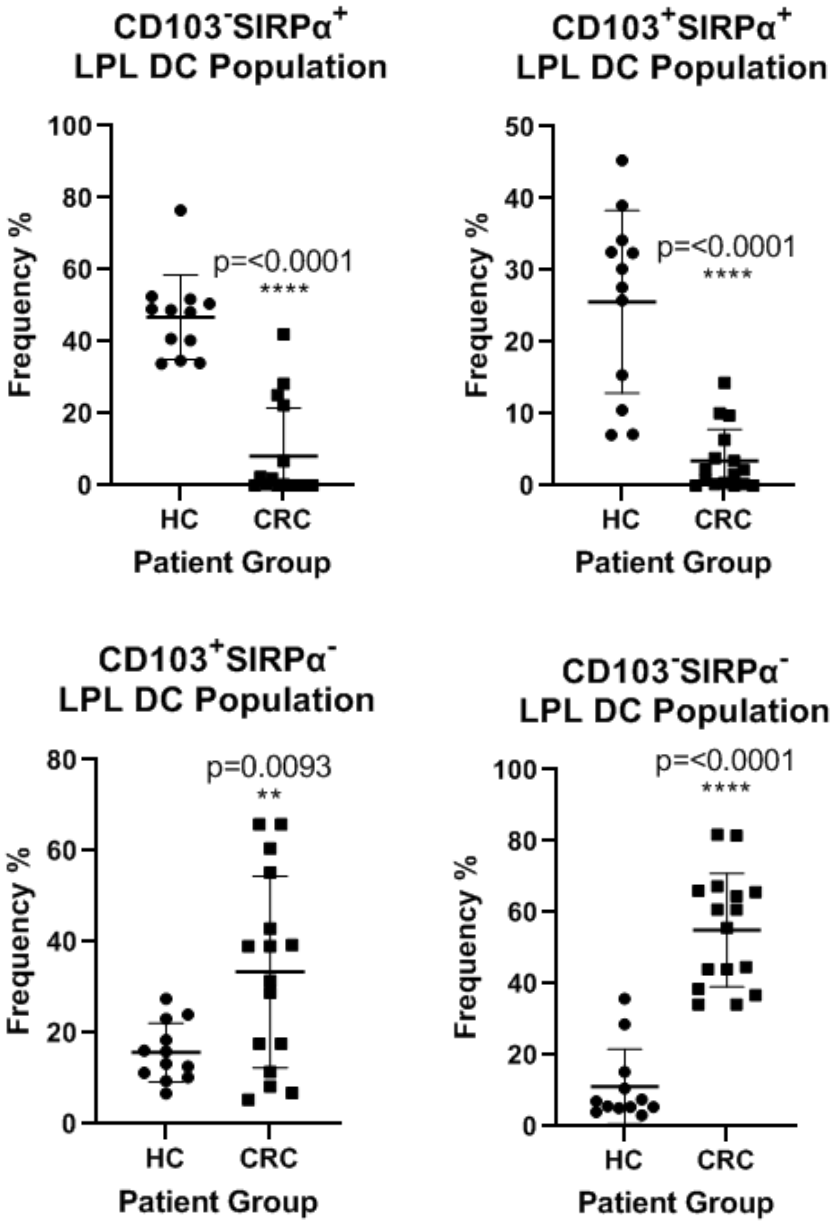


Figure 9.1 Differing populations of CD103 and SIRPα expressing DC in healthy controls and CRC patients with respective p values shown

9.4.1.3 SIRPα expression by LPL DC in relation to body composition status

The above data have demonstrated that proportion of LPL DC expressing SIRPα are significantly diminished in colorectal cancer, when we further analyse the colorectal cancer population by BC status and tumour characteristics, we find no significant

differences between groups. This suggest that the presence of the colorectal cancer itself exerts greater influence than either tumour characteristics or BC characteristics.

9.4.2 The Function of Circulating DC in CRC

In Chapters 7 and 8 we identified significant differences in homing, maturation and lipid status in circulating DC. These abstract findings can be dissected further to help us deduce functional status of the DC. Demographics for the circulating DC were the same as the demographic data previously described.

9.4.2.1 Correlations between MFI and frequency of DC containing lipid

We have seen a complex relationship between E06 (ICOL) and BODIPY (ICTL), we have preferentially used MFI as an indicator of uptake as this should represent the amount of lipid within cells rather than the frequency of cells containing lipid, the rationale behind this, in line with our findings in the analyses is that frequency was less dynamic, in that most cell contained lipid and therefore the frequency never changed but there was a dynamic shift in the intracellular content of lipid as seen by the shifts in MFI. To put this into context we correlated the MFI of E06 and BODIPY with the frequency of cells expressing/containing E06 and BODIPY. E06 MFI and BODIPY MFI correlated (r=0.47, p=0.03) but E06 MFI did not correlate with BODIPY % (r=0.10, p=0.65) or E06% (r=0.09, p=0.70). E06 % did not correlate with BODIPY % (r=0.38, p0.08) or BODIPY MFI (r=-0.09, p=0.70). BODIPY % correlated with BODIPY MFI (r=0.52, p=0.012).

Correlations in the pDC population were limited to E06% and BODIPY % (r=0.50, p=0.02) and BODIPY MFI and BODIPY% (r= 0.63, p=0.0015).

9.4.2.2 Correlations between lipid and functional cell surface marker expression

Table 9.2 summarises the correlations found between mDC lipid status and cell maturation, homing and fat scavenging status. The frequency of cells expressing the CD40 maturation marker positively correlated the frequency of cells containing oxidised lipid suggesting that ICOL may have a role in promoting maturation or indeed the function of mature DC results in a shift in lipid profile to this oxidised state. Interestingly there was a negative correlation between the frequency of cells containing BODIPY and expression of the fat scavenger CD36 receptor. This may be because of down regulation due to lipid saturation or indeed functional loss of CD36, which has an antigen presenting function, as the DC become adipocyte like.

mDC	**CD36 %**		**CD40 %**		**BB515 % (alpha 4)**		**CLA %**	
	r	**p**	**r**	**p**	**r**	**p**	**r**	**p**
E06 MFI	-0.27	0.22	-0.34	0.14	-0.19	0.41	-0.35	0.13
E06 %	-0.26	0.24	0.46	**0.04**	0.18	0.42	0.15	0.52
BODIPY MFI	-0.19	0.40	-0.19	0.43	-0.07	0.75	-0.34	0.14
BODIPY %	-0.49	**0.02**	0.37	0.10	-0.05	0.81	0.19	0.42
CD36 %			-0.35	0.13	0.001	0.99	-0.04	0.88

Table 9.2 Correlation between ICOL and ICTL and cell surface markers in circulating mDC

The pDC population did not demonstrate any significant correlations between lipid profile and frequency of cellular expression of CD36, CLA and alpha 4 integrin.

9.4.2.3 Lipid status and function in muscle mDC

No significant relationships were identified between lipid profile and expression of the functional cell surface markers examined, Table 9.3. We also examined the relationship

between cellular maturation and homing to ascertain whether maturation of tissue DC led to loss of homing function. Interestingly no correlations were found.

mDC	CD36 %		CD40 %		CCR7		CD49d %		Beta 7	
	r	p	r	p	r	p	r	p	r	p
E06 MFI	-0.41	0.25	-0.21	0.55	0.06	0.88	0.49	0.17	0.19	0.61
E06 %	0.23	0.51	0.59	0.07	0.44	0.19	-0.28	0.45	-0.10	0.80
CD36 %			-0.46	0.18	-0.28	0.44	-0.29	0.41	-0.02	0.97
CD40					0.59	0.08	0.12	0.75	0.30	0.40

Table 9.3 Correlation between lipid profile, maturation and homing in muscle mDC

9.4.2.4 Lipid and functional correlations of $CD45^+$, $CD3/14/16/19/34^-$, $HLA\text{-}DR^-$, $CD11c^+$ cells within muscle

In our earlier chapters we have described this population of cells which resemble DC in all but the expression of HLA-DR, we have also seen how HLA-DR can be down regulated in inflammation as described in the introduction. Interrogation of this population of cells reveals a number of interesting correlations which perhaps suggests that this population of cells are the more active and relevant population who have lost HLA-DR and become specialised effectors with an assigned role or conversely pathologically dysfunctional DC with inappropriate homing signals. The relationships between lipids and these markers are shown in Table 9.4.

There were positive correlations in this group of cells with the frequency of cells containing ICOL and lymph node and gut homing integrin (α4β7), Figure 9.2a – c. This is conclusive evidence of a gut-muscle axis and the manifestations of this require further clarification. CD36 expression also correlated with CD40 expression suggestion cell maturation, whilst CD40 in turn correlated with α4 expression but not β7, which leaves the putative binding partner to α4 open to speculation in this cellular subset, these data

are shown in figure 9.3a – c. There was also a positive correlation found between cell maturation and α4 expression which likely corresponds to the same population of cells.

CD3/14/16/19/34$^-$ HLA-DR$^-$ CD11c$^+$	CD36 %		CD40 %		CCR7		CD49d %		Beta 7	
	r	p	r	p	r	p	r	p	r	p
E06 MFI	0.08	0.84	0.37	0.29	0.28	0.44	0.07	0.87	-0.05	0.89
E06 %	0.057	0.09	0.58	0.09	0.67	**0.04**	0.71	**0.03**	0.84	**0.004**
CD36 %			0.65	**0.05**	0.79	**0.01**	0.79	**0.01**	0.33	0.34
CD40					0.63	0.06	0.80	**0.008**	0.26	0.47

Table 9.4 The relationship between intracellular lipid and cell surface markers and "putative DC" with absent HLA-DR

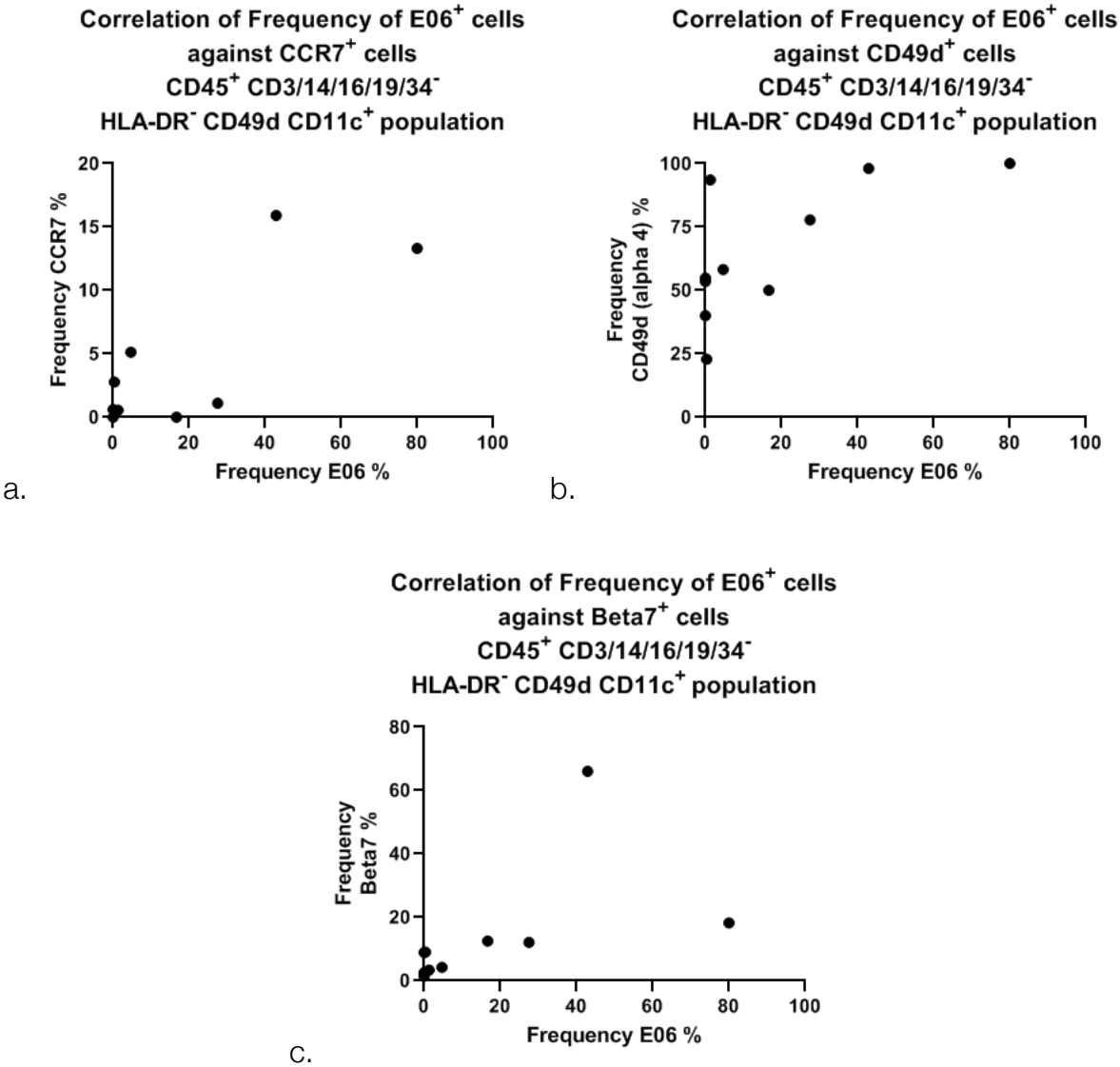


Figure 9.2a-c Correlations of the frequency of cells containing ICOL and cell surface markers, CCR7, Alpha4 integrin an Beta7 integrin

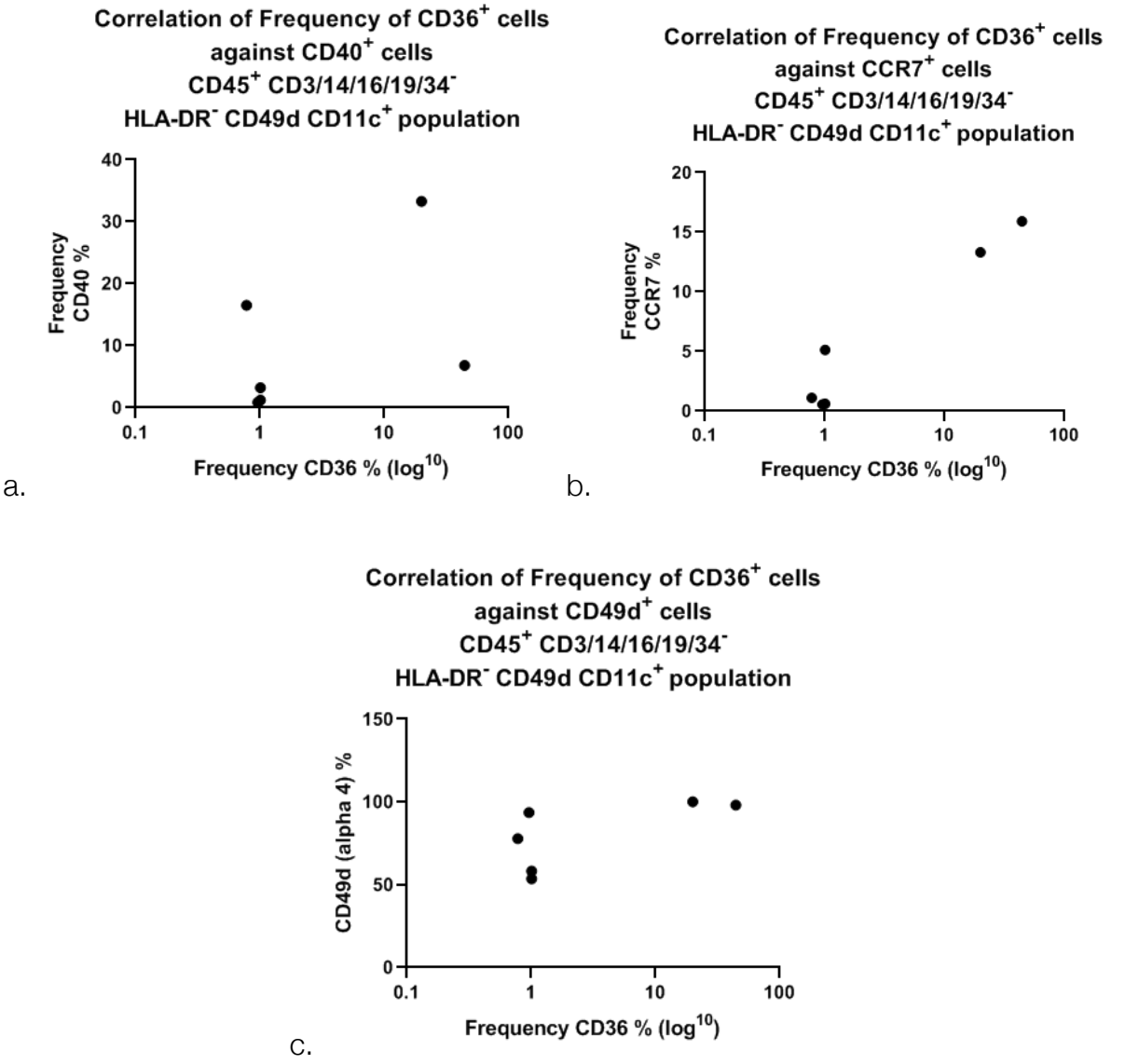


Figure 9.3a-c. Correlations between CD36 and CCR7, Alpha4 and Beta7

N.B. Data for CD36 is shown on a logarithmic scale for clarity and to aid visual interpretation; four data points not plotted on CD36 graphs as values of zero recorded and thus mathematically cannot be displayed on a logarithmic scale

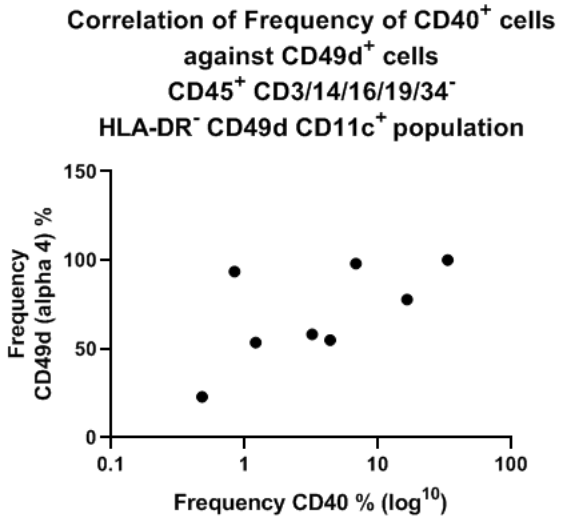


Figure 9.4 Correlation of cell maturation and alpha 4 expression

N.B. Data for CD40 are shown on a logarithmic scale to aid visual interpretation. two data points not plotted on CD40 graph as values of zero recorded and thus mathematically cannot be displayed on a logarithmic scale

9.4.3 Plasma cytokine concentration, body composition and DC function

ELISA were performed on a consecutive subset of CRC patients and healthy controls to ascertain whether there were associations between these inflammatory markers, body composition status and DC function.

9.4.3.1 Demographics

Samples from fourteen colorectal cancer patients underwent ELISA. Median age 56.5 years, [IQR 49.75-67.0]. Further information on demographics, tumour characteristics and body composition status are shown in Table 9.5. Seven healthy controls, two males and five females were included in the analysis median age 33 years [IQR 29-35].

	n	**%**

Gender	Male	9	64.3
	Female	5	35.7
T Stage	T1/2	4	28.6
	T3/4	10	71.4
Nodal Disease	Nodal Disease Absent	8	57.1
	Nodal Disease Present	5	35.7
	Ungraded	1	7.1
Vascular Invasion	Vascular Invasion Absent	8	57.1
	Vascular Invasion Present	5	35.7
	Ungraded	1	7.1
Lymphatic Invasion	Lymphatic Invasion Absent	9	64.3
	Lymphatic Invasion Present	4	28.6
	Ungraded	1	7.1
Perineural Invasion	Perineural Invasion Absent	7	50.0
	Perineural Invasion Present	6	42.9
	Ungraded	1	7.1
EMVI	EMVI Absent	9	64.3
	EMVI Present	4	28.6
	Ungraded	1	7.1
Tumour Differentiation	Well/Moderate	10	71.4
	Poor	4	28.6
BMI Obesity	BMI < 30	6	42.9
	BMI > 30	8	57.1
Sarcopenia	Not Sarcopenic	8	57.1
	Sarcopenic	6	42.9
Myosteatosis	Not Myosteatotic	10	71.4
	Myosteatotic	4	28.6
Visceral Obesity	Not Viscerally Obese	5	35.7
	Viscerally Obese	9	64.3
Sarcopenic Obesity	Not Sarcopenic Obese	11	78.6
	Sarcopenic Obese	3	21.4

Table 9.5 Demographics of CRC patients on which ELISA was performed

9.4.3.2 ELISA for IL-10

IL-10 ELISA was performed on the plasma of 14 colorectal cancer patients and seven healthy controls. IL-10 was detectable in only two cancer patients and two healthy controls. Neither patient shared tumour nor body composition characteristics. No further

analyses were able to be carried out on the IL-10 dataset in view of the lack of circulating IL-10 found in the majority of patients and controls.

9.4.3.3 IL-6 and body composition characteristics

Six out of fourteen (43%) CRC patients were found to have detectable IL-6 within their plasma, three out of seven control patients also had detectable IL-6. There was no significant difference in IL-6 levels between colorectal cancer patients and controls ($p=0.51$), Figure 9.5. In our population, there was no significant association between IL-6 concentration and sarcopenia ($p=0.44$), myosteatosis ($p=0.99$), and visceral obesity ($p=0.48$). Interestingly raised BMI demonstrated a trend towards a significant association with decreased IL-6 concentration, but the significance threshold was not met ($p=0.054$), Figure 9.6. This is interesting, especially since there was no relationship found with sarcopenia and VO in our cohort, this is likely to be due to sample size, the data comparing sarcopenic and non-sarcopenic patients is in Figure 9.7. Here we can see that although significance is not reached there appear to be more patients who are sarcopenic with a higher IL-6 concentration (almost a mirror image of the BMI data, – these sarcopenic patients are likely to have a lower BMI as they have reduced muscle and thus can explain this finding that the those with a BMI > 30 are significantly less likely to have a raised IL-6 concentration. This supports evidence in the published literature that sarcopenia and raised IL-6 are intimately related.

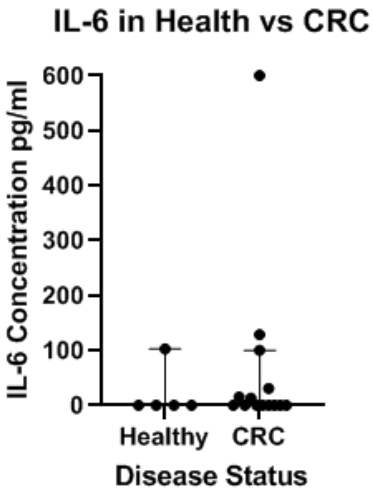


Figure 9.5 comparison of IL-6 concentration between healthy controls and colorectal cancer patients

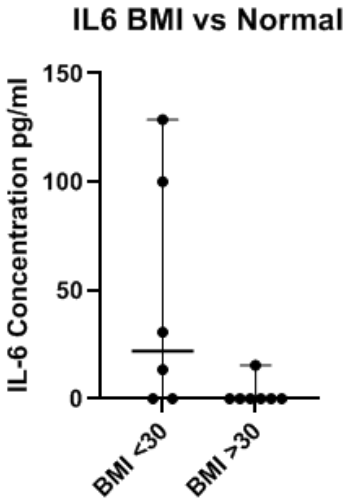


Figure 9.6 IL-6 concentrations in CRC patients with BMI less than and greater than 30

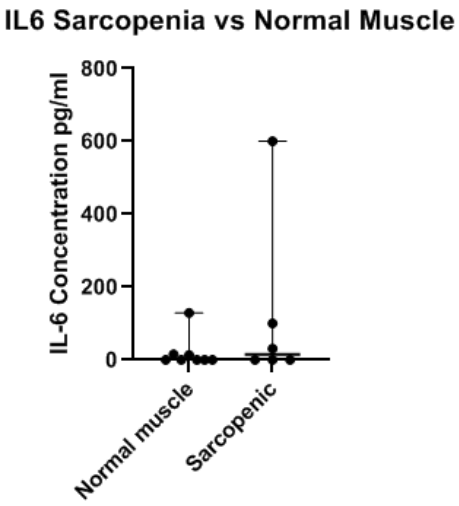


Figure 9.7 IL-6 concentrations in CRC patients with and without sarcopenia

9.4.3.4 Plasma GDF-15 concentration and body composition status

GDF-15 was present in the plasma of all healthy controls and colorectal cancer patients. The concentration of GDF-15 in CRC was significantly greater than in healthy controls (p=0.002), Figure 9.8. There was no statistical difference found between GDF-15 concentration and the various body composition states of colorectal cancer patient however there perhaps was a trend towards significance in the sarcopenic population (p=0.08), with the sarcopenic patients having a lower plasma concentration of GDF-15, Figure 9.9.

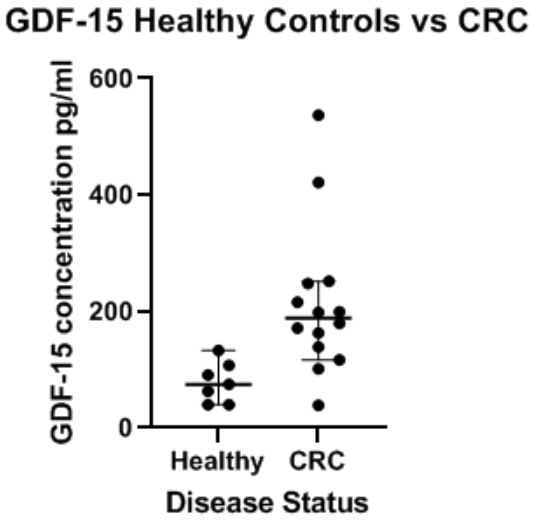


Figure 9.8 Difference between GDF-15 concentration in health and colorectal cancer

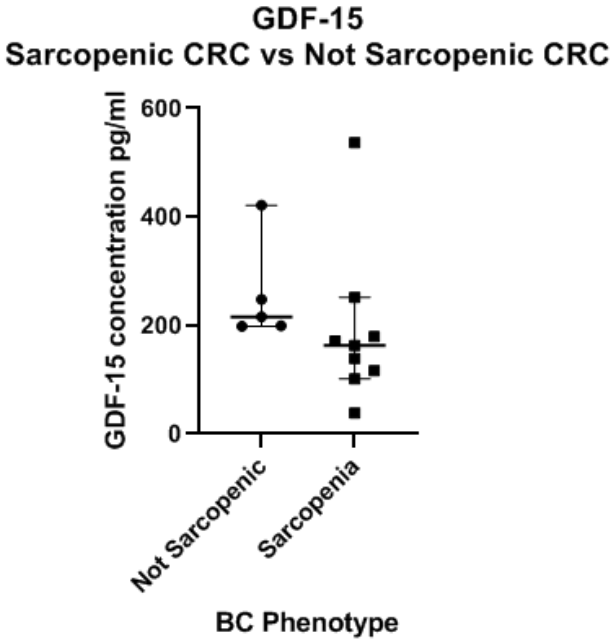


Figure 9.9 Difference between GDF-15 colorectal cancer with and without sarcopenia

9.4.3.5 IL-6, GDF-15 and Dendritic Cell Function

Correlations between plasma IL-6 concentration and the frequency of DC homing markers and median fluorescent intensity of MFI of intracellular total lipid (ICTL) and

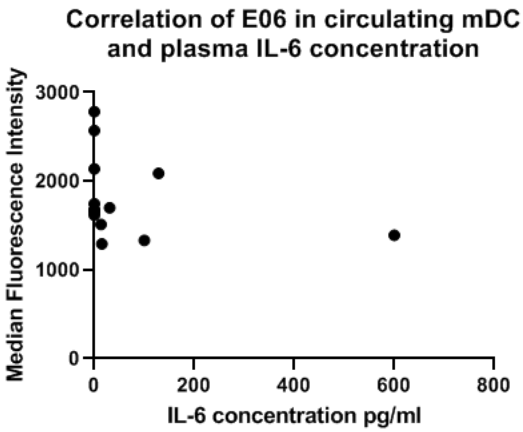
intracellular oxidised lipid (ICOL). We focused on blood, mesenteric fat and muscle. Muscle and fat were examined as we view these as both effector organs and end organs in terms of body composition in that we hypothesis that they exert an effect on the systemic immune system whilst also being under its influence. Blood acts as an interface between these tissues.

9.4.3.6 IL-6 plasma concentration and dendritic cell function

Correlations between plasma IL-6 concentration and the frequency of DC homing markers, median fluorescent intensity of MFI of intracellular total lipid (ICTL) or intracellular oxidised lipid (ICOL) were assessed in circulating and resident tissue DC.

Several notable associations were found between IL-6 plasma concentration and circulating and resident tissue mDC. IL-6 plasma concentration negatively correlated with E06 MFI (ICOL) in circulating mDC (r=-0.467, p=0.047), Figure 9.10a. IL-6 plasma concentration negatively correlated with CD36 expression on muscle mDC (r=-0.698, p=0.048), Figure 9.10b. IL-6 plasma concentration negatively correlated with Beta7 expression on muscle mDC (r=-0.782, p=0.0099), Figure 9.10c.

IL-6 plasma concentration did not correlate with expression of any cell surface markers of interest on the mDC within the mesentery. However, IL-6 negatively correlated with CD36 (r-0.614; p0.033) and CD40 (r-0.614; p=0.033) expression in the mesenteric $CD45^{+}$; $CD3/14/16/19/34^{-}$; $HLA\text{-}DR^{-}$; $CD11c^{+}$ cell population, Figure 9.10d & e.



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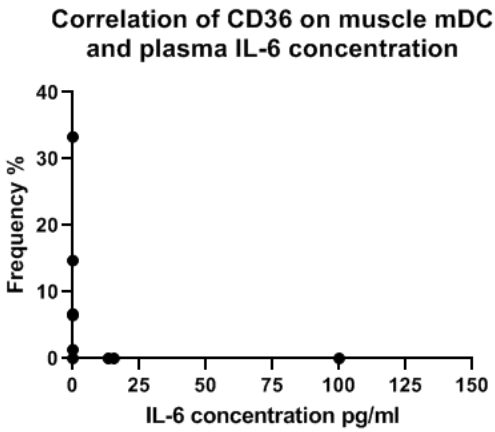

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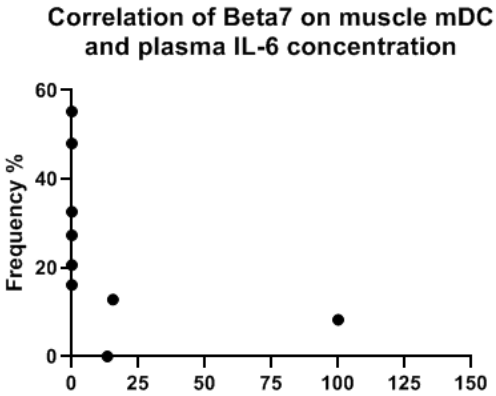

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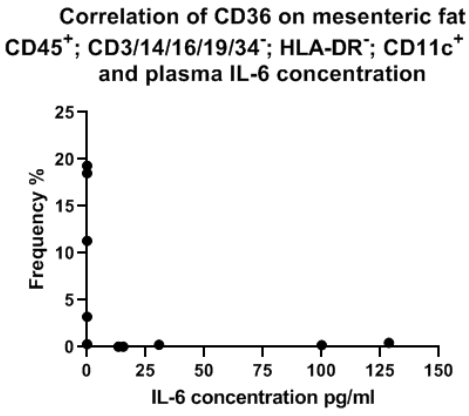

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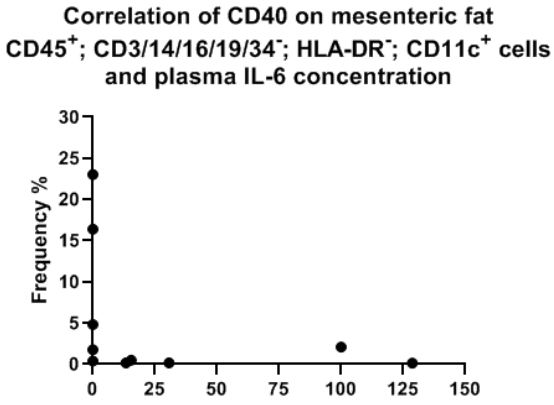


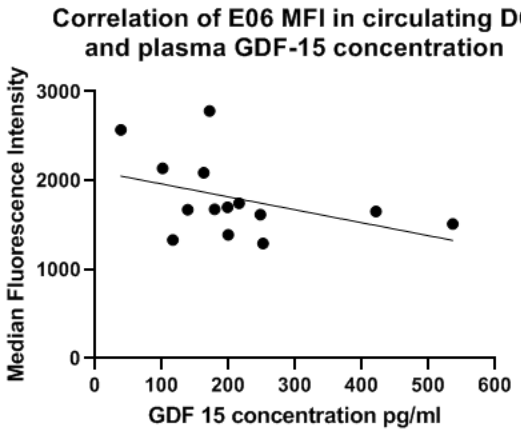
e.

Figure 9.10a-e IL-6 plasma concentrations and correlations markers of interest on circulating and resident tissue DC

9.4.3.7 GDF-15 correlation and dendritic cell function

Correlations between plasma GDF-15 concentration and the frequency of DC homing markers and median fluorescent intensity of MFI of intracellular total lipid (ICTL) and intracellular oxidised lipid (ICOL) were also assessed as above.

GDF-15 plasma concentration negatively correlated with E06 MFI in circulating mDC (r=-0.534, p=0.026), Figure 9.11a. On muscle mDC GDF-15 plasma concentration positively correlated with CD40 expression (r=0.728; p=0.016), Figure 9.11b and positively correlated with CD49d (alpha 4) expression (r=0.667; p=0.029), Figure 9.11c. GDF-15 plasma concentration negatively correlated with Beta7 expression on mesenteric fat mDC (r=-0.714; p=0.044), Figure 9.11d. This negative correlation was also shared with Beta7 expression on the $CD45^{+}$; $CD3/14/16/19/34^{-}$; $HLA\text{-}DR^{-}$; $CD11c^{+}$ cell population (r-0.785; p=0.024), Figure 9.11e. Interestingly, GDF-15 plasma concentration negatively correlated with CCR7 (r-0.649; p=0.025) expression in the $CD45^{+}$; $CD3/14/16/19/34^{-}$; $HLA\text{-}DR^{-}$; $CD11c^{+}$ cell population, Figure 9.11f.



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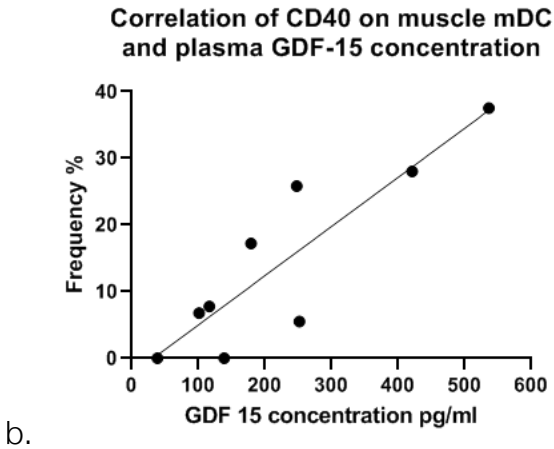

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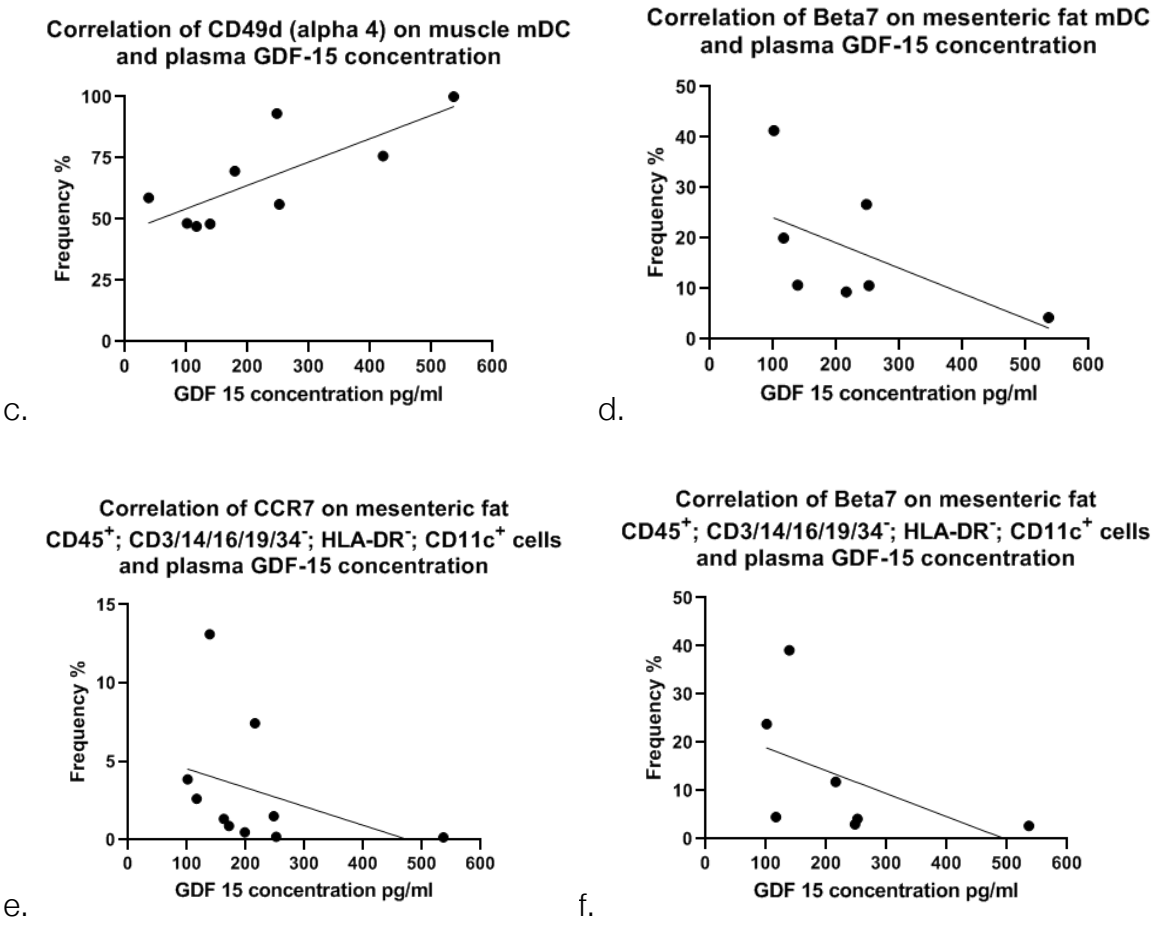


Figure 9.11a-f GDF-15 correlation and dendritic cell function

9.4.3.8 The effect of the microbiome on CD36 expression and intracellular lipid

In our final series of experiments in this thesis we explored the effect of the microbiome on the circulating DC in health and disease. As described above the microbiome is known to influence the host cancer biology and is a major immunogenic reservoir in health and disease. The composition of the microbiome is influenced by multiple factors, such as deprivation[298], ethnicity[299] and can affect the immune milieu and disease pathology[300]. The microbiome is also a modifiable entity which can be influenced by changes in diet and lifestyle[301] and as such may be a target for immunomodulation of DC function and ultimately the deleterious effects on muscle through the gut muscle axis described in Chapter 8.

We developed an in vitro model to study the effects of the microbiome on DC function. Snap frozen bacteria previously isolated from the caecum of healthy adults were used. A broad selection of phyla was taken to examine whether certain strains exerted more of an effect than others. Due to the nature of the model, PBMC were be subjected to not only the cell surface products of the bacteria but their intracellular contents too. In our analyses we also included cell profiles at fresh timepoints, following stimulation in complete medium alone and stimulation with LPS derived from the photosynthetic bacterium *Rhodobacter sphaeroides.* We examined cell surface expression of CD36 and frequency of cells containing intracellular total and oxidised lipid content using MFI. Analyses between health and disease were undertaken using two-way ANOVA and data are displayed on heatmaps with a column for each individual healthy control (on the left side of the heatmap, each prefixed HC) and CRC patients (on the right side of the heatmap each prefixed CRC). Stimulation status in shown on the y-axis of the heatmap. Colour intensity corresponds to expression of the marker of interest shown as a percentage, missing data is shown by a X.

9.4.3.8.1 Demographics of bacterial stimulation cohort

Five CRC patients were included in the stimulation experiment, the median age was 50 years [IQR 48-64] and the male to female ratio was 1:4. The remaining tumour characteristic and body composition data is shown in Table 9.6. 7 Healthy controls were included in the analysis, two males and five female median age 33 years [IQR 29-35].

		n	***%***
Gender	Male	1	20.0
	Female	4	80.0
T Stage	T1/2	0	0.0
	T3/4	5	100.0

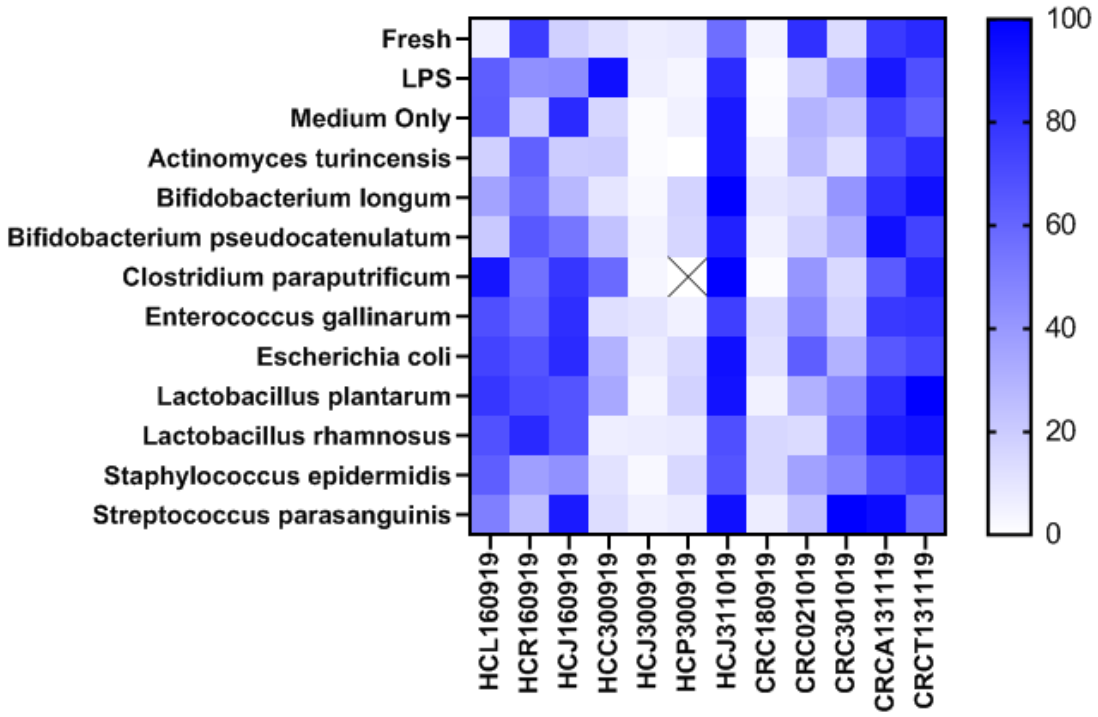
Nodal Disease	Nodal Disease Absent	2	40.0
	Nodal Disease Present	3	60.0
Vascular Invasion	Vascular Invasion Absent	2	40.0
	Vascular Invasion Present	3	60.0
Lymphatic Invasion	Lymphatic Invasion Absent	3	60.0
	Lymphatic Invasion Present	2	40.0
Perineural Invasion	Perineural Invasion Absent	2	40.0
	Perineural Invasion Present	3	60.0
EMVI	EMVI Absent	2	40.0
	EMVI Present	3	60.0
Tumour Differentiation	Well or Moderate	3	60.0
	Poor	2	40.0
BMI Obesity	Not BMI Obese	3	60.0
	BMI Obese	2	40.0
Sarcopenia	Not Sarcopenic	3	60.0
	Sarcopenic	2	40.0
Myosteatosis	Not Myosteatotic	3	60.0
	Myosteatotic	2	40.0
Visceral Obesity	Not Viscerally Obese	3	60.0
	Viscerally Obese	2	40.0
Sarcopenic Obesity	Not Sarcopenic Obese	5	100.0
	Sarcopenic Obese	0	0.0

Table 9.6 Demographic, tumour and body composition characteristic data for CRC patients included in the stimulation experiment cohort

9.4.3.8.2 Bacterial Stimulation and BODIPY

There was no difference in the frequency of cells containing lipid (BODIPY) between either CRC patients or healthy controls in mDC or pDC (p=0.41 and p=0.24) (Figure 9.12a and 9.13a). However the lipid content (MFI) of the mDC of colorectal cancer patients appeared to be significantly greater than that of the healthy controls (p=0.0025), Figure 9.12b, this was not replicated in the pDC population where no significant difference was observed (p=0.08), Figure 9.13b. There was no significant difference between the frequency of cells containing total lipid (mDC p=0.98; pDC p=0.50) or indeed the MFI of BODIPY between stimulated with each, bacteria, LPS, medium alone or fresh time points (mDC p=0.21; pDC p=0.12). The heatmaps do demonstrate a diverse lipid profile

between subjects, particularly in the mDC, but this does not appear to be related to health and disease.



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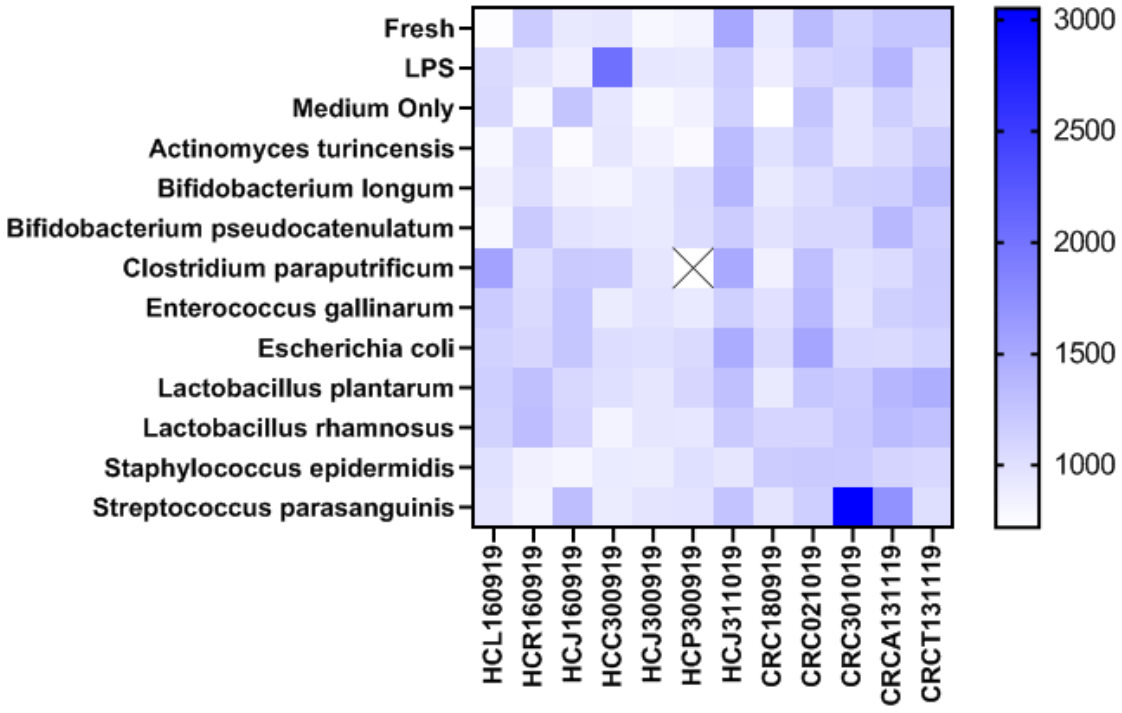
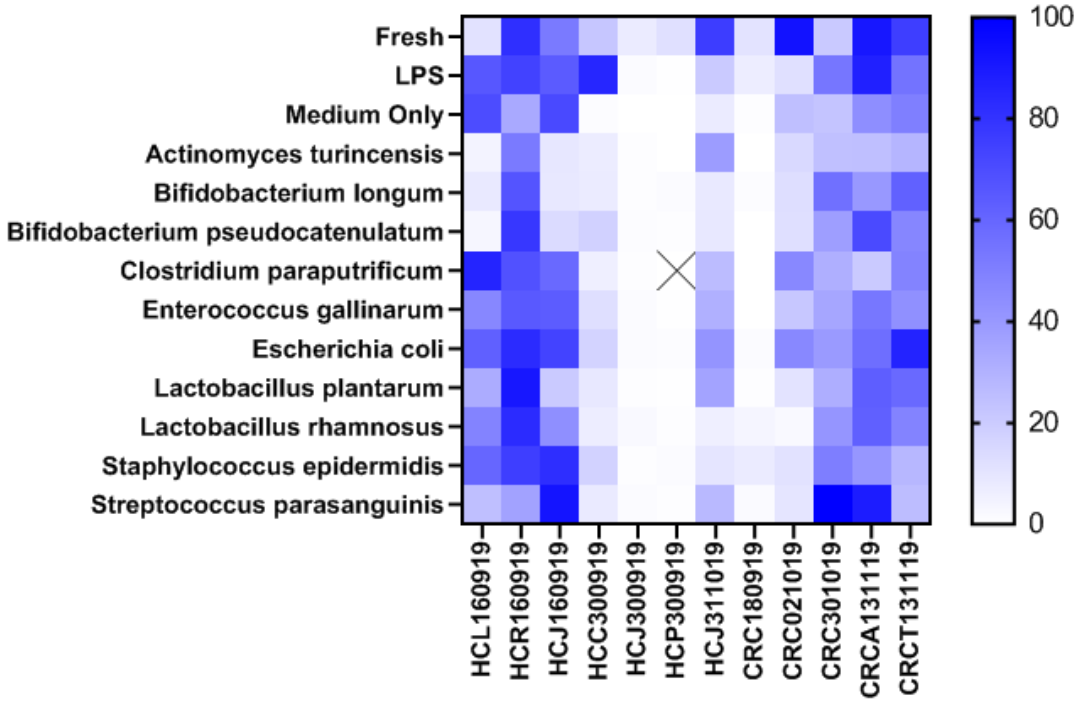


b.

Figure 9.12a & b; a. Heatmap demonstrating the frequency of mDC containing BODIPY (ICTL) in healthy participants and colorectal cancer patients. b. Heatmap demonstrating the MFI of BODIPY (ICTL) in mDC of healthy participants and colorectal cancer patients



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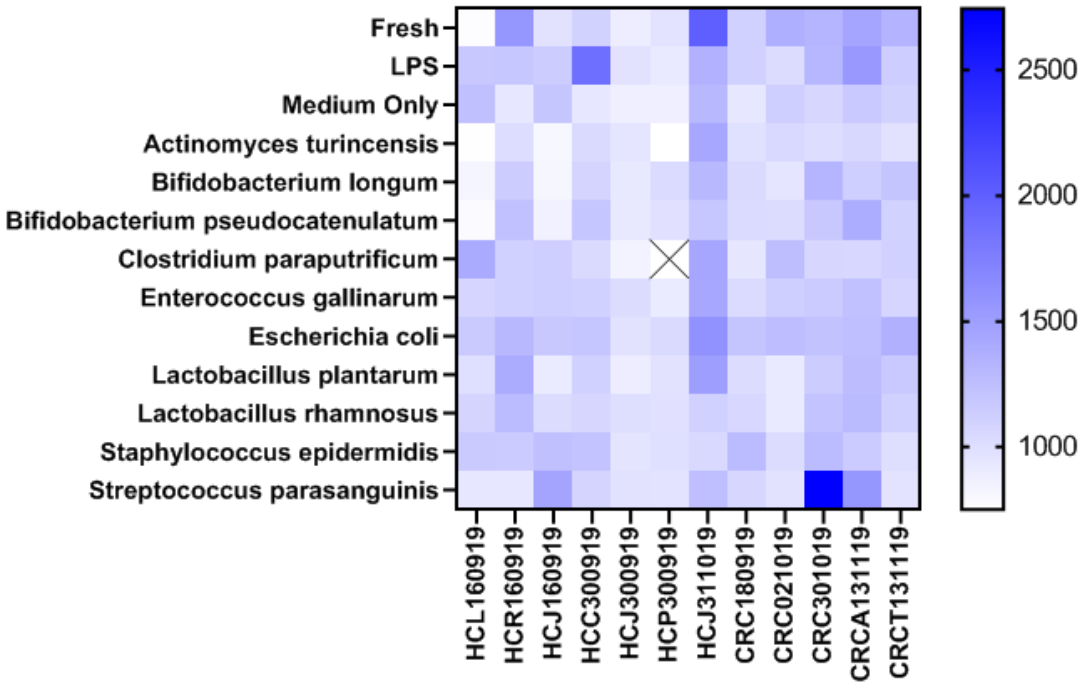


b.

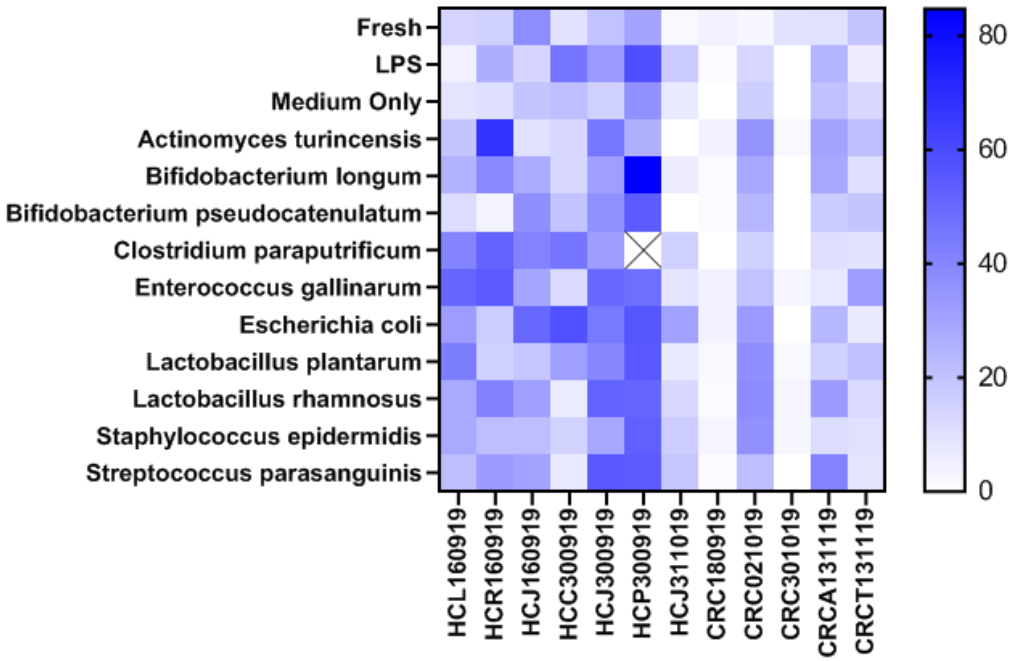
Figure 9.13 a & b; a. Heatmap demonstrating the frequency of pDC containing BODIPY (ICTL) in healthy participants and colorectal cancer patients. b. Heatmap demonstrating the MFI of BODIPY (ICTL) in pDC of healthy participants and colorectal cancer patients

9.4.3.8.3 Bacterial Stimulation and E06

The frequency of mDC containing oxidised lipid was significantly greater in the healthy control population compared to the CRC patients (p=<0.0001) Figure 9.14a. The level of intracellular lipid also appeared to be significantly greater in the healthy population with a significantly greater MFI compared to the CRC group (p=0.0025), Figure 9.14b. There was no significant difference in the frequency of mDC containing oxidised lipid between the stimulation agents or fresh timepoints (p=0.64), however there was a significant difference in the E06 MFI p=0.013 between stimulation groups, with levels of oxidised lipid increasing in response to any form of stimulation compared to relatively low intensity at the fresh timepoint, Figure 9.14b, this suggests that oxidised lipid increases in response to stimulation in both health and disease but the effect in health is significantly greater. The fact that we see a greater frequency of cells at the fresh time point in health compared to CRC, Figure 9.14a, also suggests that the mDC is disease are dysfunctional and potentially have a decreased ability to respond appropriately to stimulation.

The behaviour of the pDC is somewhat different, regarding frequency of cells containing oxidised lipid, we do not find a significant difference between health and disease (p=0.46), Figure 9.15a. However, we see a significant difference to the frequency of cells containing ICOL in response to stimulation by different agents (p=<0.0001) with LPS and E. coli appearing to be the most immunogenic, followed to some extent, by s. epidermidis. These stimulatory effects appear to be similar in both health and CRC,

Figure 9.15a. The MFI of E06, and thus content of ICOL, in the pDC population is significantly greater in health compared to disease (p=0.0019), Figure 9.15b and with more clarity, the amalgamated cohorts, Figure 9.15c. Once again, as with the pDC frequency data we see a significant difference in ICOL in response to stimulation agent (p=<0.0001). Stimulation with LPS and E. coli result in the greatest increase in ICOL and once again this response is seen in both health and disease. Lastly, it is important to note that with ICOL we do not see as overt a difference between individuals as we saw with ICTL (BODIPY) perhaps suggesting disease status is a more important factor that the individual with regards the generation of ICOL.



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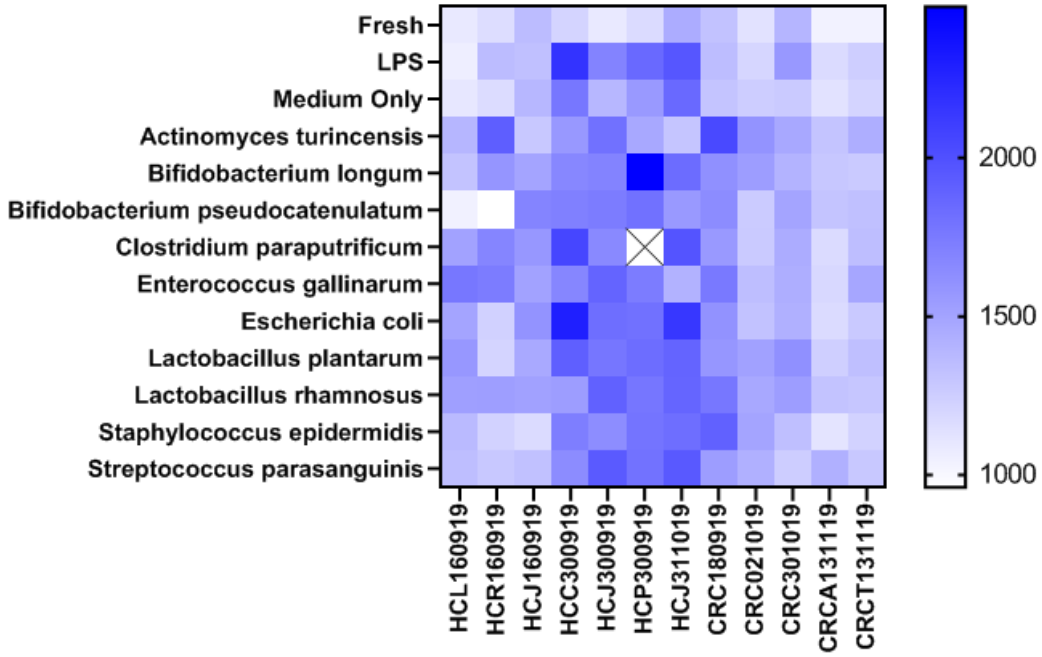
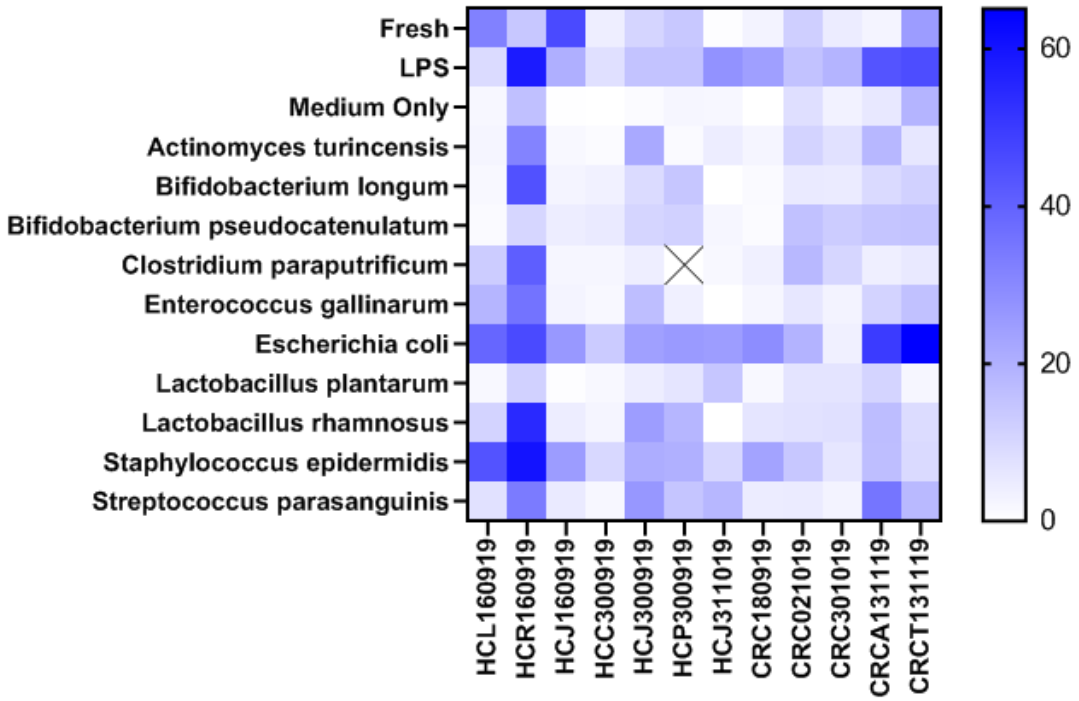


b.

Figure 9.14 a & b; a. Heatmap demonstrating the frequency of mDC containing E06 (ICOL) in healthy participants and colorectal cancer patients. b. Heatmap demonstrating the MFI of E06 (ICOL) in mDC of healthy participants and colorectal cancer patients



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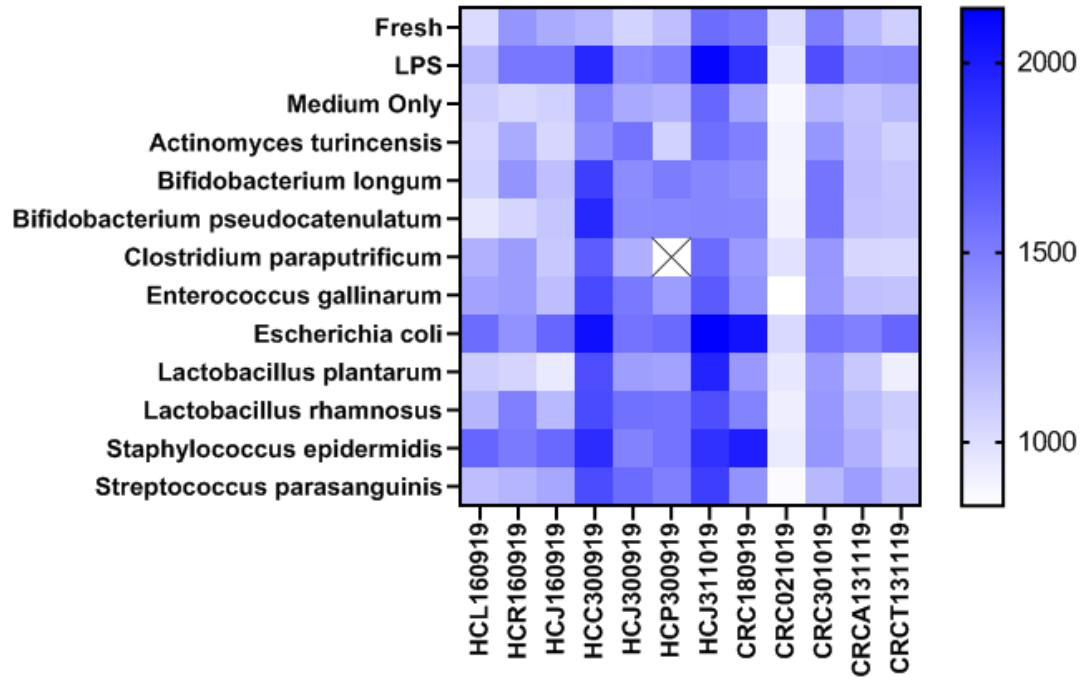

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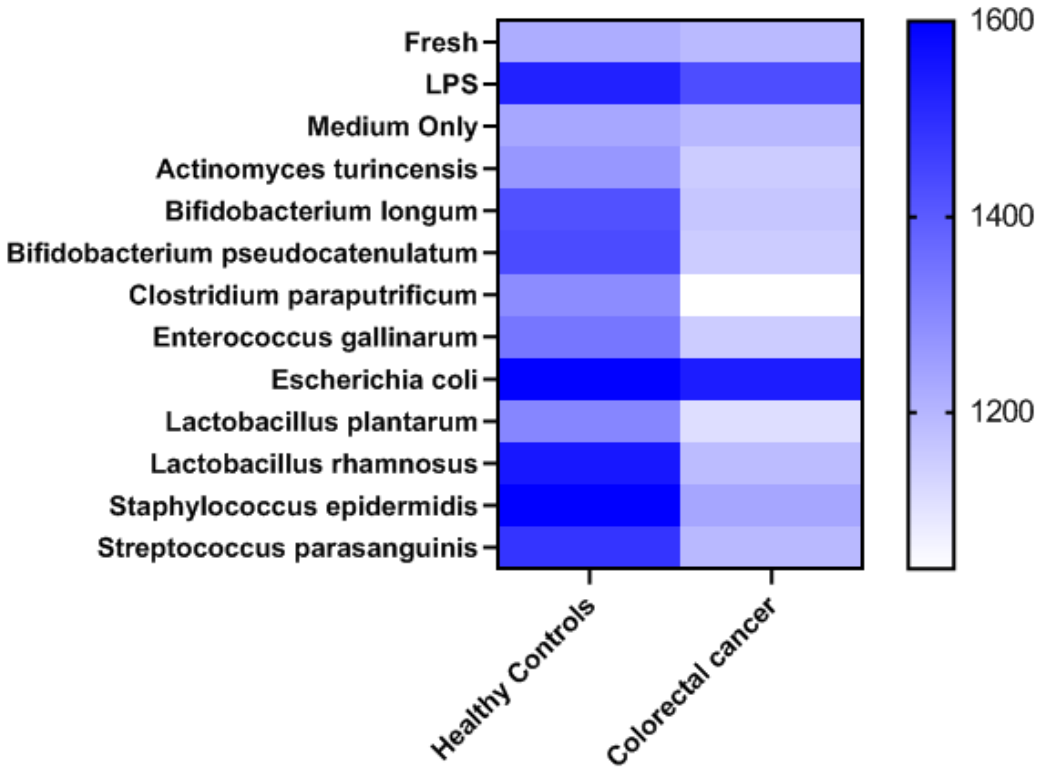


c.

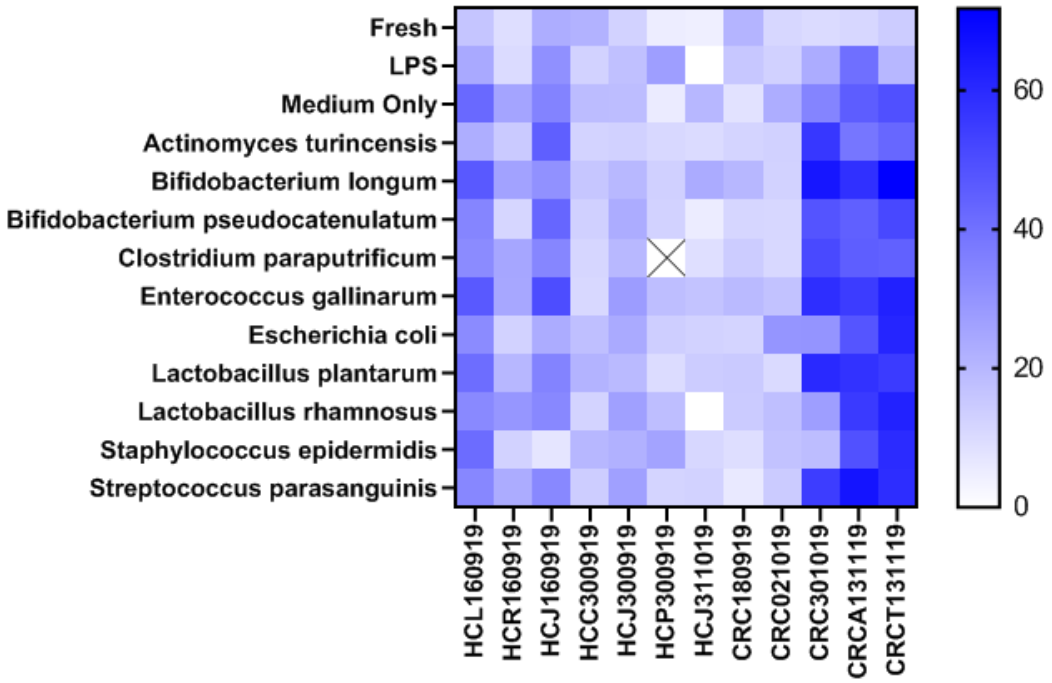
Figure 9.15 a-c; a. Heatmap demonstrating the frequency of pDC containing E06 (ICOL) in healthy participants and colorectal cancer patients b. Heatmap demonstrating the MFI of E06 (ICOL) in pDC of healthy participants and colorectal cancer patients. c. combined

data for MFI of E06 in health compared to disease demonstrating the stark deficiency in E06 in the CRC cohort.

9.4.3.8.4 Bacterial Stimulation and CD36

Finally we looked at the fat scavenger receptor CD36, which as described in earlier chapters, has antigen presenting properties too. In Chapter 7 we identified that CD36 expression is significantly influenced by obesity in both circulating DC and DC resident in muscle, mesenteric adipose tissue and the colonic LPL. Stimulation of mDC revealed an interesting pattern. We see that the mDC of colorectal cancer patients significantly up regulated in colorectal cancer ($p=<0.0001$) and from the heatmap this appears to be enhanced by simulation in a subset of CRC patients, Figure 9.16a. However looking at the entire cohort of health and disease there was no significant difference between CD36 expression based on stimulation status. This suggests there is a relevant subset of patients for whom CD36 expression is more important and more work is required to investigate the characteristics of this subgroup.

Interestingly the pDC population appear to behave very differently, the healthy control population have significantly greater expression of CD36 compared to the deficient expression of CD36 in the diseased population ($p=0.019$), Figure 9.16b. there was no significant difference in CD36 expression as a result of stimulation, however the heatmap suggests that once again there is a subgroup of individuals who upregulate CD36 on their pDC in response to stimulation, whilst the majority do not. Once again defining features of individuals who up regulate this fat scavenger receptor is of importance and should eb the subject of future work.



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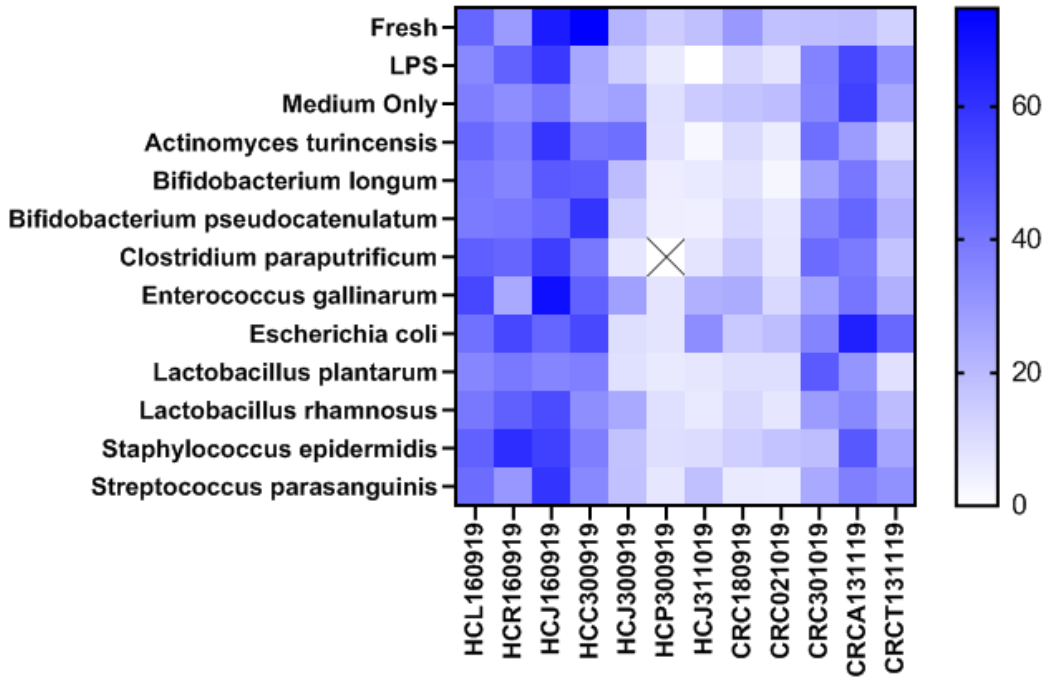

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Figure 9.16 a & b; a. Heatmap demonstrating the frequency of mDC expressing CD36 in healthy participants and colorectal cancer patients. b. Heatmap demonstrating the frequency of pDC expressing CD36 in healthy participants and colorectal cancer patients

9.5 Discussion

In this chapter we have explored have attempted to further define and explain the meaning of the experimental findings from chapters 7 and 8 whilst introducing further concepts and questions raised in the chapters exploring the underlying associations between BD and host characteristics such as deprivation. To this end we have created models where we compare health to disease, to illustrate the broader picture of changes in an attempt to understand what the functional and immunological deficits identified in the earlier chapters actually mean.

9.5.1 Patients with CRC are deficient in SIRPα in apparently healthy mucosa

The data above demonstrate clear differences between health and disease on a "whole organ scale". The data show that the immunological sequelae of CRC are not restricted to the tumour microenvironment but extend into the neighbouring tissue and beyond. This broad sweeping down-regulation of SIRPα both in DC with and without mucosal homing capability suggests a possible explanation for cancer evasion. We know that colorectal cancer can upregulate CD47, the ligand of SIRPα, and this forms part of the immune evasion strategy in disease[277]. This over-expression of CD47may be responsible for the impressive downregulation of SIRPα seen in the CRC population, either through saturation of its binding partner and subsequent loss of the ligand complex by either ectodomain shedding or endocytosis of the ligand complex [302,303]. The SIRPα CD47 complex has been considered a potential target for immunotherapy and this significant shift in expression could be a further mechanism through which pharmacological immunomodulation may act[278].

We noted in Chapter 8 that CD103 was diminished in the VO patients and that this group appeared to have a more favourable prognosis (Chapter 2). The question remains is whether these patients do not have mucosal homing because their tumours are more "benign" and therefore mucosal homing is less of a priority for the DC seeking foreign or abnormal host antigen or reduced mucosal homing is a protective feature in itself. Further investigation of the relationship between CD103/SIRPα an obesity should be undertaken as increasing co-expression of these cell surface receptors may be of some benefit in the colorectal cancer patient. However, it is important to note that due to the globally low level of SIRPα in the CRC cohort there was no significant association between SIRPα expression and BC status.

9.5.2 Lipid status is associated with maturation homing

The earlier chapters discussed how DC lipid status was associated with BC phenotype, in this chapter we explored the effect of lipid status on co-expression of cell surface markers. We identified that cell maturation (CD40 expression) positively correlated with the number of cells containing ICOL (E06 positive staining). We saw in the stimulation experiments that the frequency of mDC with E06 staining increased on stimulation more so in health tan disease. This concurrent expression may therefore be a sign of normal or healthy immune function and the accumulation of oxidised lipid either through uptake or metabolic activity may be part of the DC maturation process following antigen presentation.

Lipid accumulation (total lipid) appears to lead to negatively correlate with CD36 expression. This may be for several reasons, CD36 as a fat scavenger receptor will increase intracellular lipid through uptake, it therefore would be logical for a lipid saturated

cell to down regulate CD36 to reduce uptake. However, CD36 also has antigen presentation capabilities and we know that lipid accumulation is associated with myosteatosis and thus poor prognosis (Chapter 7). Therefore are these cells with high lipid content by down regulating their CD36 also concurrently losing their ability to present antigen successfully.

When we examine putative DC (CD3/14/16/19/34$^-$ HLA-DR$^-$ CD11c$^+$ cells) in mesentery further questions regarding the role of CD36 are raised. CD36 correlates with maturation in this HLA-DR negative group and one wonders whether as these cells mature losing their class II status, do other mechanisms of antigen presentation become more important perhaps, in preparation for trafficking to the lymph node (which could explain their CCR7 expression correlating with CD36 expression). Interestingly the level of oxidised fat correlates with CCR7 expression but not significantly with CD40 or CD36 suggesting a complex underlying picture which requires further investigation. Perhaps the most striking association in this cell group is with the gut homing alpha integrin (CD49d). Positive correlations were seen between this marker, oxidised lipid, cd36 and CD40 – Does this suggest that these gut homing cells are the successful functional DC which have matured, possess antigen presenting capability and are metabolically active and thus have a greater frequency of cells containing E06? Expression of the binding partner of Alpha 4, Beta 7, also positively correlates with the frequency of cells containing ICOL, suggesting there is a propensity for these cells with ICOL to home to the gut from the mesentery, however, the purpose of this migration is yet to be identified.

It is important to note that these data support the argument that these CD3/14/16/19/34$^-$ HLA-DR$^-$ CD11c$^+$ cells are DC which have lost class II as part of their functional journey and this cell population seems to be of greater importance than perhaps the more

immature HLA-DR expressing DC which are yet to present antigen and be assigned a role.

9.5.3 Cytokine expression, body composition and DC function

IL-6 and GDF-15 are of recognised importance in terms of colorectal cancer and body composition status. Our data did not necessarily support the evidence in the literature linking IL-6 with BC phenotype but there were trends and patterns to this effect although significance was not reached. A larger sample size may elucidate underlying statistical relationships which were not identified in these experiments. Elevated plasma GDF-15 however was not only significantly associated with CRC but also with normal muscle volume (i.e. not being sarcopenic). It is interesting that this poor prognostic group has lower levels of a cytokine associated with their underlying disease but this may relate to the role of muscle in generating GDF-15, there is indeed evidence to suggest that GDF-15 is in fact an exerkine (a cytokine produced in response to exercise) by skeletal muscle, which in turn induces lipolysis[304]. This may also explain why it is increased in CRC as perhaps the metabolic needs of the tumour to grow require an increase in lipolysis and thus an increase in gene expression for GDF-15 is favourable and advantageous.

A number of correlations between IL-6 and DC status were identified as part of this work, IL-6 levels seemed to negatively correlate with cell surface marker expression namely CD36, CD40 and Beta 7. There was also a slight negative correlation with E06 content in DC. This work supports the evidence that IL-6 is a cytokine which has, in cancer, primarily pro-inflammatory and poor prognostic connotations. This loss in metabolic DC activity, antigen presentation capability, maturation and homing ability fit in with this profile of IL-6 and may in part also explain the prognostic implications.

GDF-15 was perhaps more paradoxical in its picture, like IL-6 it negatively correlated with ICOL levels in DC, however, GDF-15 strongly correlated with DC maturation. This may be explained by the presence of cancer with a greater metabolic demand leading to greater antigen exposure to DC and thus greater rates of DC maturation or conversely, muscle mass may be exerting a direct effect on DC maturation, in that GDF-15 is associated with greater muscle mass and thus is skeletal muscle in some way, through various metabolic and cytokine pathways helping to promote DC maturation?

9.5.4 ICOL is a product of stimulation in a healthy DC

The final aspect of the puzzle encountered throughout this thesis is in part answered by our experiments on DC stimulation. We see, categorically, that ICOL is significantly increased in health rather than diseased DC in response to stimulation. ICOL until now has been associated with DC dysfunction in cancer but many of these experiments have been in vitro or in animal models[242,243]. Also, these studies primarily looked at the loss of antigen presentation but did not necessarily investigate whether this is in fact an appropriate loss and whether other methods of antigen presentation such as CD36 expression took over form classic HLA-DR class II presentation. Oxidised lipid accumulation appears to be a healthy response to stimulation, which is in fact diminished in colorectal cancer. The increase of CD36 in the mDC of the colorectal cancer population in response to stimulation compared to healthy controls perhaps suggests a metabolic demand in these mDC in CRC patients who are attempting to increase their energy stores in response to metabolic dysfunction. Interestingly the opposite relationship with CD36 occurs in the pDC. We see diminished CD36 expression on the pDC of CRC patients compared to healthy individuals. This firstly confirms that these two

cell types have vastly different functions especially in the context of a mature immune system and that both pDC and mDC are of importance in response to stimulation and exposure to foreign antigen.

9.6 Limitations

There were slight differences in the harvesting and management of the colonic tissue between health and diseased subjects. In healthy individuals, mucosal biopsies were taken during colonoscopy whilst the surgical biopsies comprised of full thickness tissue from which the mucosa was stripped in the laboratory. The advantage of the latter method allowed a yield of cells great enough for analysis of DC in the IEL layer, a method which could not be carried out in health as removal of this volume of tissue would be unjustifiable. This is unlikely to have impacted on the behaviour of the DC and as such we feel our results are justifiable however, further work would be required to validate this method to confirm it is comparable i.e. the experiment should be repeated with endoscopic biopsies from colorectal cancer patients.

The experiments involving IL-10 did not yield enough samples with detectable IL-10, this may be a true reflection of the cytokine picture or a technical problem with the assay. Further analysis of serum samples with a different ELISA kit may yield different results or confirm our findings and should be considered.

The stimulation experimental model compares health against disease, the next stage in the evolution of the model would be to explore the relationships directly too body composition in both health and disease. This would require a healthy cohort with CT data and an expansion of the diseased cohort to allow us to draw significant conclusions. The data, in its current form, shows unexplained variations in lipid profile and CD36

expression and we know from work presented in the earlier chapters that body composition fundamentally effects the behaviour of the DC or vice versa. Therefore the anomalies may be explained by more nuanced examination within both cohorts.

9.7 Conclusions

Body composition appears to influence DC lipid status, maturation and homing. DC function is significantly impaired in colorectal cancer in the gut, through down regulation of SIRPα. DC in the circulation, muscle and mesentery also share functional relationships with cytokine production and are intrinsically dysfunctional in the presence of certain BC phenotypes. Muscle may play a role in immunomodulation as seen through the expression of GDF-15 and DC maturation. Oxidised lipid considered a marker of DC dysfunction and previously associated with loss in DC antigen presentation capability appears to be an essential part of DC function in health which is in fact lost in cancer but interestingly present increased levels in the resident DC of muscle in myosteatotic patients.

Chapter 10

10 A Narrative Review of Management Strategies and the Direction of Future Research for Optimising Body Composition in Cancer

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Pring ET, Malietzis G, Kennedy RH, Athanasiou T, Jenkins JT. Cancer cachexia and myopenia – Update on management strategies and the direction of future research for optimising body composition in cancer – A narrative review. Cancer Treat Rev. 2018;70.

10.1 Summary

The earlier chapters from this thesis have concentrated on the interaction between the tumour, the environment, host ethnicity and the body composition of the host. We explored in depth the role of the immune system, orchestrated by the dendritic cell, from which we identified associations between the host immune response and the changes in body composition phenotype which would help us to explain some of the associations in our colorectal cancer population. The purpose of this thesis was to identify targets along the pathway of the deterioration in body composition where we could act to reverse or arrest some of these changes. This is a topic which has previously been studied in some depth and therefore, with the aim of finding an efficacious treatment to manipulate body composition in our colorectal cancer population, we undertook a review of the literature. Due to the disparate nature of the topic a systematic review was not technically

feasible as such a narrative review was performed. The findings of this review culminated the clinical trial described in Chapter 11.

10.2 Introduction

The role of cancer and its associations with changes in body composition along with the pathways to developing sarcopenia and cachexia have been discussed in depth in Chapter 1. In Chapter 2 we demonstrated the direct and independent associations between the tumour and body composition. Whilst in Chapter 3 we showed that patient with myosteatosis were more likely to develop metastases, and therefore have either a more biologically aggressive tumour or unrecognised advanced disease at presentation. Sarcopenia, an important predictor of outcome seemed to share associations with social deprivation and may possibly be a premorbid condition which suggests modification may be possible.

Many of the studies focussing on modifying body composition have looked at either cachexia or sarcopenia, this is because these two states were readily quantifiable and have long been recognised as poor prognostic features. Over eight million deaths a year (half of all cancer deaths worldwide) are ascribed to cancers most frequently associated with cachexia[87] with nearly one third of cancer deaths related to muscle catabolism and the consequent weakened physiology [85].

Multiple methods have been trialled in an attempt to treat cachexia and sarcopenia. The goal has been to improve either surgical or general cancer outcomes via the preservation of muscle mass directly by nutrition, physical therapy or muscle stimulation or combat the underlying inflammatory, metabolic and endocrine processes. More recently there has been greater emphasis on a multimodal approach. Figure 10.1 shows which interventions can tackle which pathway in the development of sarcopenia or cachexia.

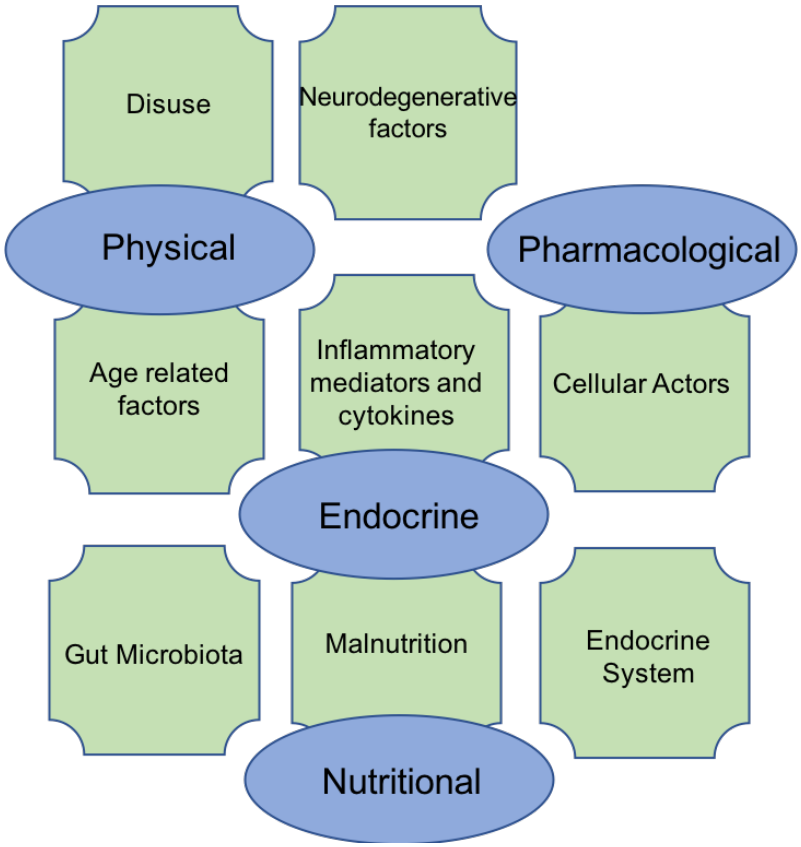

Figure 10.1 Factors which drive sarcopenia (green) and the class of interventions which may be used to treat them (blue)[65]

New treatments and strategies for managing sarcopenia are emerging; identifying sarcopenia and deleterious changes in body composition are becoming more practical now that imaging with modalities such as CT are commonplace; coupled with increasing evidence for the independent prognostic importance of sarcopenia in relation to short and long-term outcomes.

Ultimately, we can conclude that the aetiology behind these changes in body composition are complex and multifactorial. They are known to impact on survival outcome and therefore restoration of muscle mass may impart some protective effect to the host. Hence, we assert that altering the process of cachexia with body composition modification will improve prognosis and treatment outcome.

This narrative review aims to extensively explore the current published literature on the management of cancer cachexia and cancer related sarcopenia. In doing so, we have identified both high quality single studies and where possible synthesised literature based on multiple studies assessing treatment interventions. We aim to examine the advances in the treatment of sarcopenia and cachexia syndrome describing their role in improving prognosis and survival. By interrogating the current available evidence in the management of cachexia and sarcopenia and appraising it in terms of its hierarchy and strength, we can inform future research strategies including the development of a clinical trial within our colorectal cancer cohort

10.3 Methods

PubMed, Embase and the Cochrane databases were searched for studies published until April 2017. Key words included sarcopenia, myopenia, cachexia, depletion and malignancy. This search elicited 733 articles from Medline and Embase and a further 75 from the Cochrane database. One reviewer (ETP) identified suitable articles based on their design, content and validity, the inclusion and exclusion criteria are given in Table 10.1 and a flow diagram demonstrating study selection in Figure 10.2. The preference was for meta-analyses, systematic reviews and randomised clinical trials (RCTs). A manual search of the references of selected articles, reviews, meta-analyses, and practice guidelines was used to identify any further studies that eluded the initial searches. Selected articles were agreed mutually by the authors. Studies were characterised according to the treatment methods employed – endocrine, pharmacological, nutrition and exercise, physical therapy and direct muscular

stimulation.

Inclusion	Exclusion
Published and peer reviewed Randomised Controlled Trials, Systematic Reviews, Meta-analyses	Non synthesised observational and case-controlled studies
Presence of cancer and Body composition changes – muscle or fat with appropriate outcome monitoring	Body composition changes other than those in fat or muscle e.g., Biochemical, fluid based, bone profile changes.
Publications from any time point included within the EMBASE, MEDLINE or Cochrane databases	Meta-analyses and systematic reviews which have not been published in the peer reviewed literature
Demonstrate potential improvement in cachexia or sarcopenia	Papers not in the English language Individual trials**
	Body composition changes other than those in fat or muscle e.g., Biochemical, fluid based, bone profile changes.

*** No studies were identified during the review which met the inclusion criteria but were not published in the English Language*

Table 10.1 Inclusion and exclusion criteria for studies

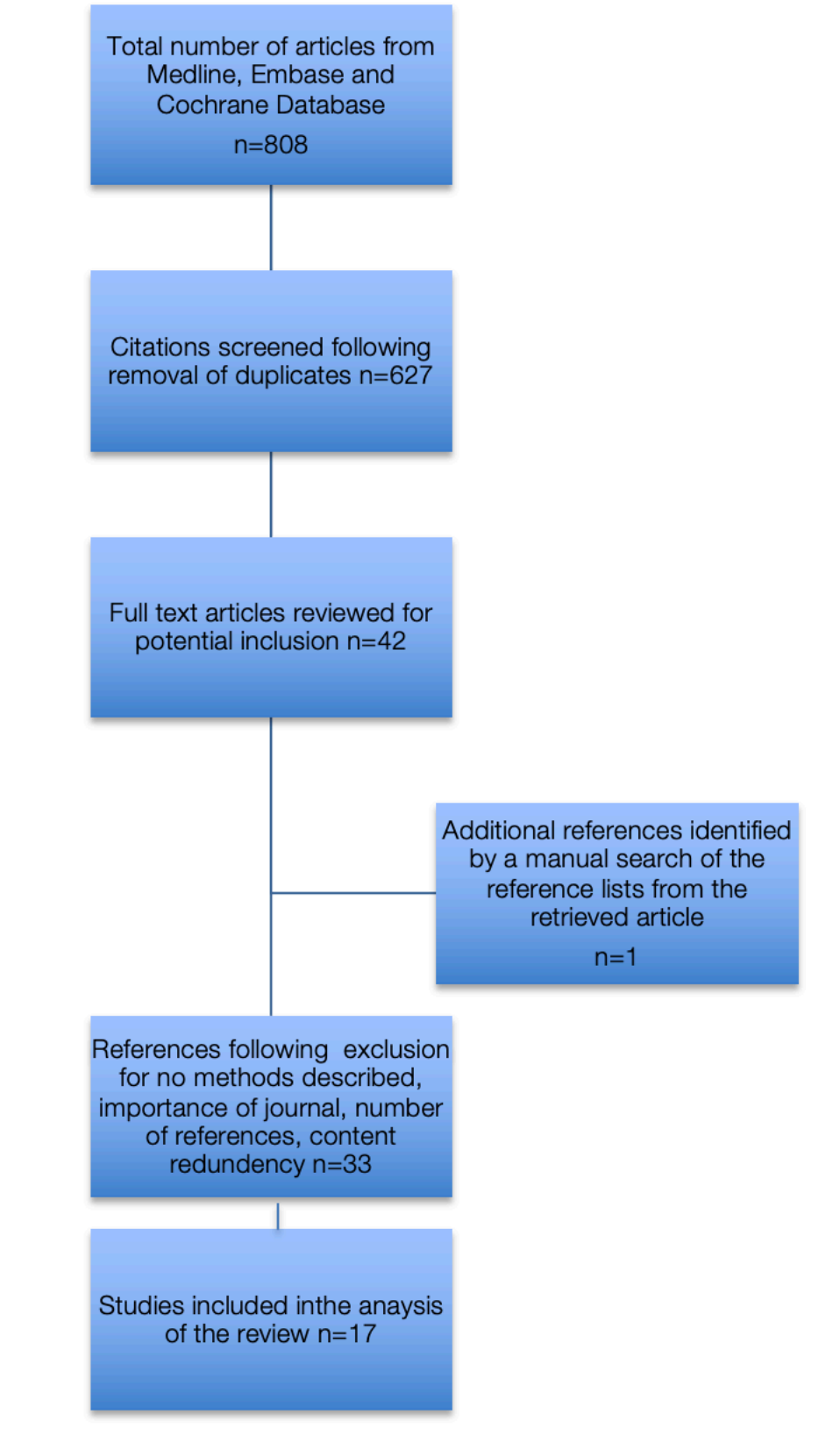

Figure 10.2 Flow diagram of study inclusions and exclusions

10.4 Review findings

To better understand the rationale behind the treatments considered it is important to understand the pathophysiological and biochemical process responsible for sarcopenia. As discussed earlier in this chapter the biochemical process is multifactorial, complex, and our understanding is evolving. Figure 10.1, in the introduction, shows the proposed mechanism of action of the various agents described in the review. The treatments described below are believed to potentiate their actions by disrupting these processes. We examined and categorised treatments by modality, separating them into endocrine, pharmacological, mechanical and nutritional therapies.

10.4.1 Endocrine therapies

Hormonal expression and regulation are an important component for the cachexia process and has been proven to be an appropriate avenue of exploration of treatment and management options, either by the administration of exogenous hormones or manipulation and up regulation of hormone systems within the body. A summary of the studies relating to endocrine therapy is in Table 10.2.

Study Author & Journal	Study Date	Therapeutic Agent	Study type	Number of trials included	Number of patients	Outcomes	Proposed mechanism of action
Ruiz Garcia et al. Cochrane Database	2017	MEGACE	Meta-analysis	35 (total) 23 (cancer patients alone)	4234 (total) 3428 (Cancer alone)	Weight Gain: 1.96 kg (95% CI 1.11-2.81kg) Quality of life gain: Standardized mean difference 0.32 (95% CI -0.02-0.65)	Appetite gain via cytokine associated inhibition of TNF alpha
Greig et al Support Care Cancer	2013	MEGACE & Folmoterol	Phase I/II clinical trial	1	13	Mean Quadriceps Volume (control vs. treatment): Left 0.99 vs 1.05L p=0.012 Right 1.02 vs 1.06L p=0.004 Lack of appetite symptom score 76.2 vs. 23.8 p=0.005	As above with alpha agonist inhibition of the ATP-dependent ubiquitin-proteasome pathway and inhibition of caspase-mediated apoptosis
Dobs et al. Lancet oncology	2013	Enobosarm	Phase II clinical trial	1	100 Placebo n=34 1mg Enobosarm n=32 3mg Enobosarm n=34	Change in Lean body mass (median kg): Placebo: 0.02 (-5.8 to 6.7) p=0.88 1mg: 1.5 (-2.1 to 12.6) p=0.0012 3mg: 1.0 (-4.8 to 11.5)	Androgen receptor modulation altering interaction with coactivator/ corepressors
Temel et al. Lancet Oncology	2016	Anamorelin	ROMANA 1 Phase III clinical trial	1	484 total 161 Placebo 323 anamorelin	Lean body mass increase at 12 weeks: -0.47kg (95% CI-10 to 0.21) 0.99kg (95%CI 0.61-1.36) p=<0.001	Agonist of the ghrelin receptor leading to transient increases in growth hormone and insulin-like growth factor
Temel at al. Lancet Oncology	2016	Anamorelin	ROMANA 2 Phase III clinical trial	1	495 total 165 placebo 330 anamorelin	Lean body mass increase at 12 weeks: -0.98 (95% CI – 1.49) 0.99kg (95%CI 0.61-1.36) p=<0.001	As above
Lundholm et al Clinical Cancer Research	2007	Insulin	RCT single center	1	138 total 69 Insulin 69 Control	Body fat in Trunk and Leg between groups over time significantly increased (p<0.03) in Insulin group vs. control group. No significant difference in lean body mass	Counteracts insulin resistance which occurs early in the cachexia pathway

Table 10.2 Summary of endocrine studies and their relevant outcomes

The anabolic effect of steroids is well recognised although different steroid families will elicit different effects and end results. Corticosteroids are appetite stimulants and improve both appetite and quality of life when compared to placebo [305,306].

Progestins similarly increase weight, primarily in the form of fat and water but they increase the risk of thromboembolism, although derivatives of progestins have been used in palliative care for a number of years. Megestrol acetate (MEGACE) is an orally active synthetic derivative of progesterone. It has been used to improve appetite and weight in cancer associated anorexia [305]. MEGACE interacts with the physiological inflammatory

pathways, decreasing serum levels IL-1, IL-6 (a putative mediator of muscle wasting) and TNF alpha in cancer patients [307]. A meta-analysis of 35 trials (23 cancer-specific) encompassing 3963 patients (3428 with cancer) with a specific focus on effectiveness, published by Cochrane, has demonstrated a benefit of MEGACE compared to placebo in improving appetite and increasing weight in cancer [308]. However, in this analysis the quality of studies forming the analysis were very low in the majority. Whilst the evidence for MEGACE in cancer patients indicates that it is safe, the quality current evidence is low, meaning it's use, as a single agent in the broad population of patients with cancer cachexia cannot be recommended.

The long acting beta 2 agonist Formoterol exerts a powerful selective protective action on heart and skeletal muscle by antagonising the protein degradation associated with cancer cachexia [305,309]. A phase I/II pilot study examining MEGACE in combination with Formoterol was found to increase quadriceps muscle mass as measured on MRI, hand grip strength and appetite without improvement in quality of life, body weight or muscle function [310]. Although the sample size is small (13 patients) and the findings are difficult to extrapolate to clinical practice, it highlights a potential benefit to adopting a polypharmacy approach.

Of the non-steroidal selective androgen receptor modulators (SARM), Enobosarm [GTx-024] has been shown to be useful in the prevention of muscle wasting of cachexia and can increase muscle mass by direct stimulation of muscle androgen receptors or indirectly through non-muscle androgen receptor pathways mediated by muscle fibroblasts [311–313]. Enobosarm in comparison to placebo has been shown in a phase II clinical trial, [funded by the drug manufacturer GTx], to increase both lean body mass on DEXA imaging and physical function in cancer patients with sarcopenia[314]. This

randomised, double-blind placebo-controlled study was undertaken at sites in US and Argentina; its methodology was well constructed and process appeared sound despite relatively small patient numbers. One Hundred patients with various cancers were enrolled in the efficacy trial and 156 patients to examine drug safety. It is postulated that using Enobosarm or related SARM agents may potentiate the positive effects of anabolism via the steroid pathways without the potential risks and side effects of androgen treatment.

In consequence, two phase III trials, POWER 1 (platinum and taxane) and POWER 2 (platinum and non-taxane), are now underway. These trials aim to examine changes in muscle mass and physical strength in stage III and IV non-small cell lung cancer using Enobosarm whilst simultaneously initiating chemotherapy treatment. These identically designed randomised, double-blind, placebo-controlled, multicentre, and multinational trials are yet to conclude however, preliminary data suggest an increase in muscle mass in the treatment group and in POWER 1 an improvement in stair climbing power [315,316].

Ghrelin, the gut hormone implicated in food intake regulation, stomach acid secretion and gut motility, also stimulates the release of growth hormone from the pituitary gland [305,317]. Cancer patients are believed to develop ghrelin resistance, producing increased endogenous levels of circulating ghrelin, potentially to combat anorexia as a counter regulatory mechanism. Anamorelin hydrochloride, an orally available mimetic of the secretagogue ghrelin, is a possible therapeutic agent, capable of increasing lean body mass. The ROMANA 1 and ROMANA 2 randomised double-blind phase III trials examined body weight, lean body mass, fat mass, hand grip strength and appetite-centred quality of life following administration of anamorelin or placebo in stage III/IV non-small cell lung cancer. Both demonstrated increased body weight, lean body mass, fat

mass, and appetite-centred quality of life without a significant increase in hand grip strength. Anamorelin was well tolerated by patients [318]. The data from these trials were robust and there is convincing evidence in this patient group that there is a significant gain in lean body mass but as yet, no evidence directly relating to outcomes. The lack of functional improvement is disappointing and the lack of concordance between lean body mass and handgrip strength is unexpected. Several explanations are postulated by the authors to explain this apparent discrepancy between values derived for lean body mass and function. They suggest that values derived for a gain in lean body mass may be confounded by an expansion of the extracellular water space although reported rates of oedema were low and such accumulation of extracellular fluid is not a known action of ghrelin. They also suggest, in view of the underlying advanced pathology that pleural effusions and ascites may add to lean body weight but again refute this argument by demonstrating that nearly half of the lean body mass gain was appendicular in distribution, i.e. on the limbs, and therefore would not include weight gain in these body cavities [318]. We question therefore, whether grip strength may not be an appropriate or even functionally relevant end point for the study in view of the comorbid trial population and in future studies, radiological assessment of muscle volume and quality by computer tomography may provide a less ambiguous result, in addition to a combination of varied functional metrics designed for a geriatric or frail population such as “muscle power test sit to stand” and the Berg Balance scale [319,320]. Pooled overall survival was included as a secondary endpoint within the study. Median survival over one year, showed no difference between study groups (8·90 months [95% CI 8·3–9·8] for anamorelin vs. 9·17 months [7·9–11·0] for placebo); hazard ratio 1·06, 95% CI 0·89–1·26; p=0·47).

Having shown anatomical improvement further studies need to look at these broad outcome measures and from a surgical point of view, the impact on postoperative outcomes and disease-free survival. Some positive work has come out of The ROMANA 3 trial has recently completed and has examined a further twelve weeks of treatment, demonstrating that anamorelin continued to be well tolerated. Over the entire 0-24 week treatment period, body weight and symptom burden were improved with anamorelin [321].

Insulin administration in cancer patients can decrease whole body protein breakdown and increase muscle protein synthesis [322,323]. A RCT to examine the effects of insulin on a group of 138 patients with heterogeneous diseases and cancer cachexia was conducted by Lundholm et al [324]. The control group (n=69) were treated with best palliative care including nutritional support; the treatment group (n=69) received long-acting insulin in addition to best palliative care and nutritional support for a median of 150 days (range 7-548 days). The treatment group demonstrated an increase in whole body mass but not lean body mass, improved metabolic efficiency on exertion and an increased median survival by 50% in the treatment group (0.11IU/kg insulin daily) compared to the control group [324]. Despite disease heterogeneity, patient characteristics including weight, height and age were similar between treatment and control groups and supports the proposition that insulin has a role in managing these patients.

10.4.2 Pharmacological treatments

Omega-3-Fatty acids (N-3-FA) including Eicosapentaenoic acid (EPA) and Docosahexaenoic acid have cardioprotective properties but may reduce both cachexia

associated tissue wasting and tumour growth [325,326]. Several meta-analyses have investigated the potential benefits of these relatively harmless and easily tolerated agents; the results of these analyses have been mixed.

A Cochrane meta-analysis of five trials in 2007 found the data were insufficient to demonstrate that oral EPA is no better than placebo in the treatment of cancer cachexia, although there were no safety implications from EPA use [327]. Hence the use of EPA in clinical practice despite its low risk was not justifiable. Further corroboration was obtained from a systematic review of seven RCTs in 2008 by Mazzotta et al who found no benefit in relation to weight, lean muscle mass, symptoms, survival and quality of life using EPA and Docosahexaenoic acid (DHA) [328]. A systematic review published in 2007 assessed effectiveness and safety of N-3-FA in symptom relief of cancer cachexia syndrome and examined seventeen studies (including four of the five studies of the previous review); several biochemical, clinical and quality of life parameters improved but methodological flaws compromised the quality of their recommendations [329].

Non-Steroidal Anti-Inflammatory Drugs (NSAIDS) may combat cachexia owing to their direct interaction with the systemic inflammatory response [SIR]. Two systematic reviews have examined their effectiveness cachexia management but study heterogeneity with different cancer types, clinical parameters, definition of effects and weakness of the individual studies meant the reviews were unable to recommend the widespread use of NSAIDS [330,331]. Despite these recommendations, there was evidence of an observed benefit and statistical significance was achieved but the studies were small, few in number and of limited methodological quality. As such, further larger randomised controlled trials are required to address this issue.

A double-blind placebo controlled phase III RCT by the Cannabis in Cachexia Study Group found no benefit in cancer related anorexia-cachexia [332]. Overall mixed results have been reported and when weighed against the potential complications associated with its psychotropic side effects [333] there is little evidence to support its routine use in cachexia or sarcopenia.

Recently biological therapies have sparked interest due to their disruptive interaction with inflammatory pathways. Data from a phase II clinical trial designed to determine whether selumetinib, a tumour suppressive agent with anti-inflammatory properties, especially to IL6, was found to be safe and efficacious in the management of cholangiocarcinoma compared to standard therapies [334]. Selumetinib induced rapid and significant skeletal muscle gain at an average of 3.9kg in patients, without concurrent gains in adipose tissue (n=20). Significant muscle gain, was not observed in the standard therapy comparator group (n=30) [335]. Selumetinib treated patients' muscle cross-sectional area increased by +13.8 (11.9) cm^2/100 days compared with a loss of - 7.3 (14.3) cm^2/100 days for non-selumetinib-treated patients ($P<0.001$). This translated to approximately + 2.3 vs. -1.2 kg of skeletal muscle on a whole-body basis, respectively. Tissue gains noted for selumetinib-treated patients were restricted to skeletal muscle. Adipose tissue was lost in both groups [335]. These actions were determined in part to be secondary to IL-6 suppression, as inhibition of MEK1/MEK2 and associated kinases alone would lead to muscle catabolism. Due to the lack of a placebo-controlled design within this study the data, although promising, have limitations. However, these data not only demonstrate an interesting and novel approach to tackling sarcopenia and cachexia but also highlights the importance of immunological research in this area. Nevertheless, these agents are

expensive and their long-term side effects have not been evaluated. Table 10.3 summarises the pharmacological therapies described above.

Study Author & Journal	Study Date	Therapeutic Agent	Study type	Number of trials included	Total number of patients in trials	Outcomes / Conclusions		Proposed mechanism of action
Dewey et al. Cochrane Database	2007	Oral EPA	Meta-analysis	5	587	Insufficient data to establish whether EPA better than placebo		Inhibits IL6 and decreases tumour associated proteolysis inducing factor thus preventing lipolysis and muscle protein degredation
Mazzotta et al. J Pain and Symptom Management	2008	Oral EPA & DHA	Systematic review	7	1319	Unable to support independent use of EPA and DHA but may be benefit in multimodal usage		As above
Colomer et al. Br J Nutr	2007	N-3-FA	Systematic review	17	1081	Supplementation seems to be associated with improved clinical, biochemical and QoL – Recommends prospective trials		As Above
Reid J et al. Palliat Med	2013	NSAIDS	Systematic review	4	241	Insufficient data to make appropriate recommendations recommend further trials and earlier intervention and multimodal therapy	COX-2 inhibition with resultant prostaglandin and cytokine reduction	
Solheim TS et al. Acta Oncol	2013	NSAIDS	Systematic review	13 (6 comparative)	681 (in comparative group)	There is evidence that NSAIDs can improve weight in cancer patients however this evidence is insufficient to make a recommendation for treating cachexia	As above	
Strasser F et al. J Clin Oncol	2006	Cannabis extract and Δ-9-Tetrahydrocannabinol	Phase III RCT	1	243 randomized 164 completed treatment	No improvement in appetite and QoL compared to placebo	Increased appetite via cannabinoid receptor related processes	
Prado et al 2012 British Journal of Cancer	2011	Selumetinib	Phase II Clincal trial (sub study)	1	50	84.2% of patients gained skeletal muscle after commencing treatment – Need for randomized trials	MEK 1/2 inhibition, & inhibits secretion of IL-6, IL-1β, TNFα	

Table 10.3 A summary of the studies utilising pharmacological agents

10.4.3 Physical Therapy and Neuro-Muscular Electrical Stimulation (NMES)

Exercise employing repetitive muscle use leads to an increase in muscle mass. Exercise also imparts an anti-inflammatory effect by attenuating the cellular response to inflammatory stimuli and pro-inflammatory cytokines [336,337]. By virtue of these two observations, prescribed exercise may be a powerful tool in the treatment, management and potentially prevention of cancer cachexia and sarcopenia. Exercise itself is immunogenic, increasing cytokines, promoting the acute inflammatory response and

paradoxically those that limit this response [338]. Studies into exercise resistance training in breast cancer patients receiving adjuvant therapy have documented a significant increase in lean body mass [339,340]. In prostate cancer patients where resistance exercise training is employed similar results have been reported with retained muscle mass and strength, decreased fatigue and improved quality of life in the treatment group [341–343]. Evidence suggests that half of patients with cancer or having received curative treatment are willing and able to complete such exercise programs [344]. Exercise therapy is promising but it is unlikely to be a panacea as compliance can be highly variable [16-97%] [345]; it is labour intensive and is dependent on patient comorbidity.

For patients who are unable to exercise or for whom compliance is an issue, there may be the possibility of mimicking the effects of exercise by direct neuromuscular electrical stimulation (NMES). The use of exogenous electrical stimulation of muscle has the potential to directly increase muscle mass by mimicking the exercise process. A battery powered stimulatory unit is secured by self-adhesive electrodes to the patient's skin superficial to the muscle body and once activated produces smooth regular contractions. A typical program consists of 30 to 60 minutes of stimulation, generally of the quadriceps with or without additional lower limb muscles, for example calves, hamstrings, or glutei, three to five times each week, for four to eight weeks [346]. NMES can be used to produce a muscle contraction equivalent to 20% to 40% of a maximum voluntary contraction [347] thus meeting the criteria of the American College of Sports medicine definition of planned exercise [348].

A Cochrane review of NMES in a number of diseases which cause cachexia such as COPD, CCF, HIV/AIDS and cancer suggested NMES may be an effective treatment for

muscle weakness in adults with advanced progressive disease, and could be considered as an exercise treatment for use within rehabilitation programs [346]. Two of the studies, a randomised phase II trial and its pilot study, included in this review looked specifically at cancer cachexia. Both studies were conducted in patients with non-small cell lung cancer receiving palliative chemotherapy. The pilot study demonstrated positive results [349] however, the phase II study of 49 patients in which 30 were randomised to NMES found that there were no significant differences in quadriceps muscle strength, thigh lean mass or physical activity level between groups [350]. A summary of exercise and nutrition studies is in Table 10.4.

10.4.4 Nutritional therapy

In patients with the syndrome of cancer cachexia there is a nutritional deficit of 250-400 kcal/day compared to 200 kcals in a patient with advanced cancer alone. Patients with cancer cachexia would need to increase their calorific intake by 300-400kcal/day and protein intake would have to increase by 50% from 0.7-1g/kg/day to have an effect upon anabolic resistance [351]. A meta-analysis conducted in 2012 assessing thirteen studies with 1414 patients determined that with a mean increased calorific intake of 432kcal/day across the studies that oral nutritional interventions have no effect on survival, with the effects on body weight and energy intake being inconsistent. It was determined that there may be statistically significant improvements in quality of life however, as the improvements were only slight, it may not be clinically relevant [352]. Despite the lack of significant evidence there are multiple guidelines from numerous societies based on

consensus statements advocating nutritional management in the cancer patient; such as the ESPEN guidelines [353].

Study Author & Journal	Study Date	Therapy	Study type	Number of trials included	Number of patients	Outcomes	Propsed mechanism of action
Courneya KS et al J Clin Oncol	2007	Aerobic or Resistance Exercise (Breast Cancer)	Multicentre RCT	1	242	Showed an improvement in body composition – Aerobic training prevented fat gain resistance training added lean body mass. Neither prevented total weight gain	Anabolism of exercise. Anti-inflammatory effects of exercise possibly involving IL10 and IL15 Positive effects upon insulin resistance
Segal RJ et al J Clin Oncol	2008	Aerobic or Resistance Exercise (prostate cancer)	RCT	1	121	Neither intervention prevented weight gain but resistance training helped avoid a gain in body fat.	As Above
Jones S et al Cochrane Database	2016	NMES	Systematic review	18 total 2 cancer specific	65 (cancer specific)	Potentially effective for patients with progressive diseases such as cancer. Though the quality of evidence is low.	Anabolism of exercise and the anti-inflammatory cytokines produced by muscle – IL10, IL15
Baldwin C et al Cochrane Database	2017	Oral Nutritional interventions (Calorific intake)	Meta-analysis and systematic review	13 (8 studies provided data on weight)	1414	Oral nutritional intervention an inconsistent effect on body weight. Further studies required	Increased caloric intake and addressing malnutrition and caloric imbalance

Table 10.4 A summary of the described studies relating to exercise and nutrition

10.4.5 Current advances in management

We have demonstrated that there have been numerous approaches to managing cancer cachexia and sarcopenia, however, to date, success has been limited and this in part may be due to single treatment options. We have seen dual therapy used in the case of Formoterol and MEGACE although the sample size was small in this trial hence the individual results should be interpreted with caution but the rationale of dual treatment in this scenario is pertinent. Research into this complex and multidisciplinary field continues to expand and now several high-quality trials are in progress to examine multimodal

approaches to managing cancer cachexia. Two full trials registered with the US national library of medicine (www.clinicaltrials.gov) are examining multimodal therapies in managing cachexia. The MENAC trial, a multinational trial run by the Norwegian University of Science and Technology, is examining change in body weight, muscle mass and physical activity following exercise in combination with Ibuprofen in advanced cancer patients with cholangiocarcinoma, lung and pancreatic primaries [354]. The second registered study based at the Markey Cancer Center, Kentucky examines the effects of vitamin D in combination with exercise and protein supplementation exploring mitochondrial function and anabolic resistance as potential targets of action of vitamin D on muscle metabolism, size and strength [355]. Studies such as this may help identify at risk groups, the vitamin D depleted, allowing focussed or bespoke care in the future based on individual parameters. A third trial, currently running as a feasibility study at The University of Edinburgh, is investigating the combination of exercise and nutritional supplementation on cancer cachexia, the outcome focus from this study, other than feasibility of the regime in the specific population; is on function, quality of life and nutritional status [356].

Taking treatment beyond interventions into the multidisciplinary arena is also vitally important and progress evident. Multimodal cancer cachexia clinics have become established in several units, for example the McGill Cancer Nutrition Rehabilitation Program clinic in Montréal. Here nutritional, psychosocial and physical support can be provided in a multidisciplinary environment [357]. A retrospective analysis of Quality of Life (QoL) in 374 patients with cancer cachexia was assessed. Baseline QoL scores were severely impaired but clinically important improvements were observed over three visits to the clinic. Patients who gained weight and increased their 6 min walk test (6MWT) had

the greatest improvements in QoL. This suggests that a multimodal approach to management of cancer cachexia and sarcopenia results in clinically important improvements in QoL [358].

10.5 Discussion

Successful treatment of cachexia and sarcopenia should be safe, well tolerated and clinically relevant and cost effective. Sarcopenia is seen in early-stage cancers but remains poorly recognised, often only becoming apparent only in the late stage of disease. As clinicians our goal is to identify the syndrome early and attempt to halt its progression. A number of endocrine and pharmacological agents have been identified in clinical trials to attenuate muscle loss and include anamorelin and Enobosarm as in the ROMANA and POWER Trials, respectively. The evidence on biological therapies remains limited yet encouraging. These agents have potentially significant side effects and are expensive. However, if their use in the treatment of cancer increases then we may find that the associated side effects are positive and beneficial to patient body composition. Nutritional support and supplementation, although lacking strong evidence a direct attenuation of sarcopenia and cachexia, remain important due to the calorific and protein deficit that accompanies cancer. Physical exercise or using NMES have shown promise, are safe, yet they are time consuming and compliance may ultimately restrict their utility. Nevertheless, it is unlikely that significant harm in promoting exercise in combination with nutritional support will be incurred due to the established benefits on general health and wellbeing.

Cachexia is a well-recognised phenomenon in clinical practice, however, at this time, the assessment sarcopenia alone has only recently become popularised. Yet assessment of

sarcopenia and its subsequent recognition and management is potentially more important to patient outcomes [111], treatment costs [114] and may have a major impact on drug and chemotherapy dosing and therapy regimes [72,151,359,360]. Routine assessment of sarcopenia is anticipated to integrate into regular clinical practice and may ultimately replace old metrics such as BMI. Severity classification of cancer cachexia requires further development in relation to the predictive value of the system for outcomes such as treatment toxicity, quality of life, hospitalisation, and survival [82].

10.6 Study Limitations

Due to the diverse and varied nature of studies and trials within this review it was not possible to perform a true systematic review and therefore a narrative review was favoured as it was felt it would capture the diversity of approaches. As such not all studies were included and there was no opportunity to amalgamate data in a meaningful manner and undertake a meta-analysis. However to minimise bias and ensure as much data was captured as possible, we aimed to follow the methods of systematic review where possible and be transparent about the data used and discarded.

10.7 Conclusion

This review demonstrates the potential for advancements in the management of sarcopenia and cachexia. It highlights the need for a multimodal and multidisciplinary approach to managing cancer cachexia and sarcopenia. It also demonstrates the current deficit in the research literature of such multimodal therapeutic approaches to managing cancer cachexia. As part of a holistic approach to care we must utilise methods of body composition modification to expand our armoury to address the systemic burden of cancer. Well-constructed clinical trials addressing cachexia and sarcopenia in cancer

must identify what can be employed to meaningfully modify body composition and in doing so, improve outcomes for our patients.

Chapter 11

11 BiCyCLE NMES - Neuromuscular electrical stimulation in the perioperative treatment of sarcopenia and myosteatosis in advanced rectal cancer patients: design and methodology of a phase II pilot study

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Pring ET, Gould LE, Malietzis G, Lung P, Bharal M, Fadodun T, et al. BiCyCLE NMES—neuromuscular electrical stimulation in the perioperative treatment of sarcopenia and myosteatosis in advanced rectal cancer patients: design and methodology of a phase II randomised controlled trial. Trials. 2021;22(1):1–12.

11.1 Summary

The work in this thesis has explored the aetiology behind changes in body composition in colorectal cancer. Our final aim was to find a method by which one could preserve muscle mass and maintain muscle quality and by doing so improve outcomes in the colorectal cancer surgical patient. One such method could be muscle preservation in the post-operative period using neuro-muscular electrical stimulation (NMES), described in Chapter 10. NMES has shown initial promise in the palliative lung cancer population however in later trials questioned its efficacy. However, in a critically ill population there is more convincing evidence.

Advanced rectal cancer patients are rendered critically unwell in the post-operative period and by the nature of their advanced disease may have more profound BC changes and poorer outcomes, as discussed in Chapter 1. This group of patients would be a subset of patients who could benefit from such an intervention. In this chapter we describe the trial protocol for use of NMES in these patients.

11.2 Introduction

Radical multi-visceral resection of pelvic tumours, known as pelvic exenteration, is being utilised to successfully treat a number of intra-abdominal malignancies[60]. Pelvic exenteration for locally recurrent (LRRC) or primary advanced rectal cancer has a high morbidity and mortality. The *PelvEx Collaborative* analysed data from 1184 patients who underwent surgery for LRRC, and found that 2% of patients died within 30 days of surgery and 32% of patients experienced a major complication[361]. Despite this high morbidity and mortality, these complex procedures are increasingly practiced in specialist centres. Following surgery, these patients enter a catabolic crisis where incapacitation and high protein and fat metabolism lead to a marked loss in skeletal muscle[362–364]. Sarcopenia and myosteatosis are independently associated with poorer post-operative outcomes following surgery for colorectal cancer[111,126] (Chapter 1). The aetiology behind sarcopenia and myosteatosis is complex and multifactorial and includes inflammatory changes, hormonal changes, loss of function, fatigue and energy balance (Chapters 2-5; 7 & 8)[94]. However, strategies to preserve skeletal muscle mass, quality and function may improve these outcomes (Chapter 10).

A meta-analysis of resistance exercise training in patients with non-metastatic cancer showed significantly increased skeletal muscle mass [365]. Exercise can also impart an anti-inflammatory effect by attenuating the cellular response to inflammatory stimuli and pro-inflammatory cytokines such as IL-6, TNFα and TGFβ[336,337]. However, exercise programmes following a rectal cancer diagnosis and exenterative surgery are not always possible or practical due to patient anxieties and the need to expedite treatment, and the pain or disability associated with the extensiveness of the surgery

itself. Furthermore, restriction upon rehabilitative resources, especially physiotherapy, often leaves patients immobile for long periods with resultant muscle atrophy. An alternative approach to traditional physiotherapy could be functional electrical stimulation (FES) via neuromuscular electrical stimulation (NMES). This is currently used in clinical practice for a number of diseases, indeed, at the National Clinical FES Centre at Salisbury, UK over 2500 patients are currently undergoing FES[366]. NMES of the lower-limb muscles requires less motivation than traditional exercise and can be undertaken whilst the patient is seated or lying down[350]. NMES can be used to produce a muscle contraction equivalent to 20% to 40% of a maximum voluntary contraction [347] thus meeting the criteria of the American College of Sports medicine definition of planned exercise [348].

A study of anterior cruciate ligament [of the knee] (ACL) reconstruction patients by Hasegawa and colleague demonstrated NMES, implemented during the early rehabilitation stage, was effective in maintaining and increasing muscle thickness and strength in the operated limb [367]. There is also evidence from meta-analyses that NMES increases muscle strength and shows potential benefit for joint range of motion, muscle atrophy, outcomes of ventilation and activity limitations in critically ill patients[368]. A Cochrane review of NMES in a number of diseases that cause cachexia (muscle and fat loss secondary to disease) such as COPD (Chronic obstructive pulmonary disease), CCF (Congestive cardiac failure), HIV/AIDS and cancer, suggested that NMES may be an effective treatment for muscle weakness in adults with advanced progressive disease, and could be considered as a treatment within rehabilitation programs [346]. Two studies, a phase 2 randomised trial and its pilot study,

investigated NMES in cancer cachexia. Both studies were conducted in patients with non-small cell lung cancer receiving palliative chemotherapy [349,350]. The pilot study [349] demonstrated positive results however, in the phase 2 study of 49 patients, in which 30 were randomised to NMES, there were no significant differences in quadriceps muscle strength, thigh lean mass or physical activity level between groups [350]. The study team did however recommend further NMES studies in patients with cancer in other settings. Notably these two studies by Maddocks' and colleagues focussed on palliative lung cancer patients, a surgical complex rectal cancer cohort is fundamentally different both by virtue of the impact of the insult of surgery on muscle but also the radical or curative nature of the treatment. The differences between these studies by Maddocks et al and our study are summarised in Table 11.1.

BiCyCLE NMES Study Population	Maddock's Study Population
Post-operative "tumour free"	Active cancer
Confined to bed rest	Active and mobile population
Intensive inpatient support	Outpatient community care
Aiming for recovery up to or beyond baseline	Palliative and functionally declining population

Table 11.1 Differences between BiCyCLE NMES and earlier studies by Maddocks' et al

Previous studies have examined NMES in the palliative setting, with inconclusive conclusions regarding efficacy[349,350]. No work has yet been done on clinical outcomes of NMES use in colorectal cancer nor any trial in the post-operative setting for cancer surgery. A phase II trial is required to determine whether there is evidence of a potential benefit prior to justifying a phase III study. The previous studies performed

in cancer patients have not examined the relationship to the systemic inflammatory response, nor has there been an assessment of immediate post-operative outcomes. Our study aims to provide evidence of early indicative evidence of efficacy in relation to key health outcomes, including skeletal muscle mass and quality (myosteatosis), markers of systemic inflammation and post-operative recovery outcomes in rectal cancer patients undergoing radical pelvic surgery.

11.3 Study design, methods and analysis

The study is being undertaken at St Mark's Hospital, the sponsor in London North West University Healthcare (LNWH) NHS Trust. The study sponsor will have various roles and responsibilities including auditing, indemnity and monitoring. Ethical agreements and amendments were processed and communicated through the NHS Health Regulation Authority, Queens Square Research and Ethics committee and LNWH Research and Development Department

.

Patients will be blinded as to which trial arm they enter and a sham protocol will be used by the control arm. Body composition analysis of the images will be done by automated software used by an assessor blinded to the intervention to remove operator or interpretation bias.

Our aim is to compare the effect on muscle of therapeutic NMES and current best practice against placebo NMES and current best practice alone in patients undergoing advanced radical surgery for complex rectal cancer.

11.3.1 Outcomes

11.3.2 Primary outcome

The difference in mean muscle attenuation (MA), of all skeletal muscle groups captured on the axial CT image at the level of the third lumbar vertebrate (L3), measured in Hounsfield units and hence the degree of myosteatosis between the pre-operative and six-month post-operative CT scan in the NMES treatment group and the placebo NMES group.

11.3.3 Secondary outcomes

Our main secondary outcomes include change in total skeletal muscle cross-sectional area, at the L3 level between treatment and non-treatment groups as well as between time points for individual patients. Difference in quality of life between groups using the validated questionnaires ED-5Q-5L & EORTC QLQ – CR29. Post-operative complications and length of hospital stay between both arms and comparison of the systemic inflammatory response between each group. A comprehensive list of secondary outcomes is shown in Table 11.2.

Domain	Specific measurement	Metric	Method of aggregation	Time point
Lumbar Skeletal Muscle Index (LSMI)	The difference in Lumbar skeletal Muscle Index (LSMI=height / area of skeletal muscle in cm2 at L3) derived from the third lumbar vertebral axial level	Change in LSMI at each time point	CT Scan; SliceOmatic software version 5.0 with ABACS L3 Plug-in automation tool	Pre-surgery 3 to 6 months post-surgery
Visceral Adipose Tissue (VAT) Surface Area	Visceral Adipose Tissue area (cm2) derived from the third lumbar vertebral axial level	Change in VAT at each time point	CT Scan; SliceOmatic software version 5.0 with ABACS L3 Plug-in automation tool	Pre-surgery 3 to 6 months post-surgery
Systemic Inflammation	C-Reactive Protein and serum albumin	Modified Glasgow Prognostic Score	Ordinal Values mGPS=0 mGPS=1 mGPS=2	Preoperatively and 6 months post-surgery
Cellular Immune Response	Neutrophil count Lymphocyte count	Neutrophil to Lymphocyte Ratio (neutrophil/lymphocyte count	Clinically relevant categorical cut off values NLR<3 NLR>3	Pre-operatively and 6 months post-surgery

Post-operative Complications	Any post-operative complication	Clavien-Dindo Classification	Clavien-Dindo Score 1-5	Between 0- and 90-days post-surgery
Length of Hospital Stay	Inpatient stay post-surgery	Days	Median Length of Stay	From surgery to Hospital Discharge
Disease Free Survival	Days to reported first recurrence / death / 5 Years post-surgery	Days	Kaplan-Meier Survival analysis	From date of surgery to 5 years post-surgery
Overall Survival	Days to death / 5 years post-surgery	Days	Kaplan-Meier Survival analysis	From date of surgery to 5 years post-surgery
Quality of Life (General)	EuroQol 5-level EQ-5D-5L Mobility, self-care, usual activities, pain/discomfort and anxiety/depression	Visual analogue scale Score 1-100 & Score in each of the 5 domains	Change in scores between events	Pre-surgery, 6 & 12 months post-surgery
Quality of Life (Colorectal specific)	EORTC QLQ - CR29 Function and Symptoms	4 multi-item scales and 19 single items assessing common symptoms and problems in colorectal cancer	Change in score between events	Pre-surgery, 6 & 12 months post-surgery
Function	Berg Balance Scale (BBS)	Berg balance score (0-56)	Change in BBS score	Pre-surgery and 6 months post-surgery
	30 second sit to stand	Number of full sit-to -stand actions completed in 30 seconds	Change in number of successful actions	
	6-minute walk test	Distance walked in 6 minutes	Change in metres	
Thigh circumference	Thigh circumference, 5cm above the superior pole of the patella	Circumference in cm	Change in Thigh Circumference between groups	
Dose response to NMES	Pre- and post-operative mean muscle attenuation (L3) (MMA) & hours of device usage	Change in MMA/time	Linear regression	3-6 months post-surgery
Patient satisfaction	Free text responses and satisfaction scores derived from each domain (See patient satisfaction survey supplementary material).	Qualitative and visual analogue scale 1-10	Qualitative responses Median score between groups on each domain assessed	8 weeks post-surgery
Bio-impedance analysis	Phase angle = (Xc/R)*180°/π	Cellular resistance (R) and Cellular Reactance (Xc)	Change in phase angle at each time point	Baseline, day two post operatively, day twenty-eight post-operatively (if in hospital) day of discharge, first post-operative follow up appointment

Table 11.2 Secondary Outcomes

11.3.4 Sample size estimation

We powered this pilot study based upon the primary outcome. Using data from the Alberta Cancer Registry Martin and colleagues described a standard deviation of MA at 8.6 Hounsfield units (HU) for males and 10.2 HU for females[154]. We therefore assumed an overall mean SD of 9.4 HU for both male and female patients. A difference in MA between groups of 8HU was considered to be of clinical importance, and the calculation was based on showing a difference of this size between groups.

The proposed analysis will adjust the differences at 6 months for the MA values at baseline. To allow for this approach, this adjusted is included in the sample size calculation. The size of the association between baseline and outcome MA values is relatively unknown. A fairly weak correlation of about 0.3 between the time points was assumed.

The calculations were performed using a 5% significance level and 90% power. Based on the information above, it was calculated that to show a difference in MA of 8 units between groups would require a sample size of 27 per group (54 patients in total).

To allow for an estimated dropout rate of 5%, 58 patients will be recruited into the study. The dropout rate of 5% is an estimate based on the fact that the treatment period is short and supervised for the most part in hospital. For the primary outcome to be measured we require the pre- and post-operative CT scans and therefore anticipate a very low dropout rate due to the fact these are routine scans and the time period is relatively short. The dropout rate for the secondary outcomes may be higher as the time from surgery progresses however the trial is powered to the primary outcome and as such, we are not taking into account the potential drop-out from the trial outside this time period encompassing the primary outcome metrics.

11.4 Trial protocol

11.4.1 Recruitment and eligibility screening

The inclusion and exclusion criteria are shown in Table 11.3. Following diagnosis of locally advanced rectal cancer patients are discussed in a multidisciplinary team (MDT) meeting. Some of these patients may be felt to be suitable for radical surgery – i.e., surgery performed with the intention of a cure. If patients are deemed fit for and consent to surgery then this is performed by one of three specialist surgeons in St Mark's Hospital, London, UK.

Inclusion criteria	Exclusion criteria
○ **Adults aged 18 and above** ○ **Male or female** ○ **Primary or recurrent locally advanced rectal cancer amenable to elective radical exenterative surgery** ○ **ASA grade I-III** ○ **Able and willing to consent** ○ **Participation in other concurrent trials is acceptable – following discussion with trial team of both studies**	○ Lack of patient consent ○ Widespread metastases not amenable to curative resection ○ Contraindication to NMES ○ Pre-existing neuromuscular degenerative disease ○ Participation in other trials where agreement on participation not made in advance by trial teams ○ Patients with solitary colon cancer above the level of the peritoneal reflexion which does not require complex pelvic surgery

Table 11.3 Inclusion and Exclusion Criteria

Patients who meet the inclusion criteria will be identified by the clinical team in the colorectal outpatient clinic or MDT and will then be approached by the study team with written information on the trial and given the option to enrol in the study. Consent

to take part in the trial will be obtained at the next outpatient clinic appointment, which will occur in the weeks preceding surgery. Patients may be included in concomitant studies provided agreement is obtained from each trial team.

11.4.2 Randomisation

Randomisation, performed after the assessment of baseline outcomes, will take place by computer generated randomisation software (https://www.sealedenvelope.com) on a one-to-one basis. Recruitment will be performed by the study team; randomisation will be performed by the study principal investigator to ensure allocation concealment. Patients who are randomised to the either arm will be blinded as to intervention and will be taught by the research team to use the stimulator this will be at their clinic appointment following consent.

The NMES intervention lasts a total of ten weeks with follow up over 5 years. The trial algorithm is shown in Figure 11.1 and schedule of enrolment, interventions, and assessments

in Figure 11.2.

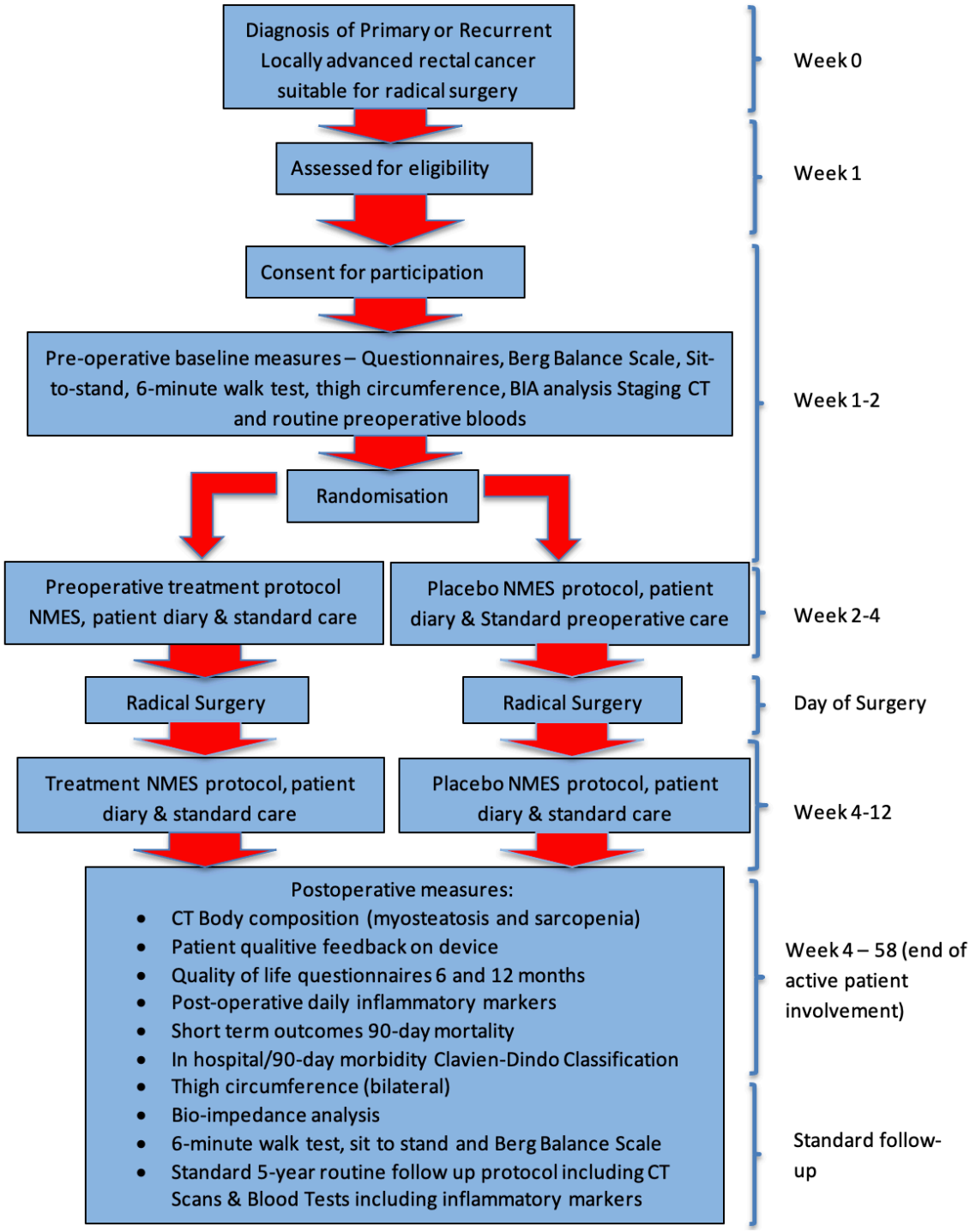

Figure 11.1 Study Algorithm

Procedures	Visits								
	Routine Staging CT either at St Mark's or patients home hospital	First outpatient clinic at St Mark's	Second outpatient appointment or Pre-Assessment	Surgical Admission	4-8 weeks post-surgery	3-6 months	12 months	Routine 6 monthly follow up appointments for 5 years post-surgery	Routine 12 monthly follow up appointments for 5 years post-surgery

Staging CT	x					x	x		x
Eligibility		x							
Medical history and demographics		x	x						
Consent			x						
Device Training			x	x					
Device Use			x	x	x				
Blood Tests			x	x	x	x	x	x	x
Patient device satisfaction questionnaire					x				
Patient QoL Questionnaire						x	x		
Bio-impedance analysis (BIA)			x	x	x	x			

Figure 11.2 Schedule of enrolment, interventions, and assessments

11.4.3 Blinding

Patients and the assessor of the primary outcome will be blinded. Patients will be blinded as to which arm they are in, the devices appear identical except for a small, coloured plastic tab indicating whether they are treatment or placebo devices. The assessor of the primary outcome, a consultant radiologist, will be blinded as to which trial arm the participant is in; assessment of the primary outcome is also automated and therefore will not allow bias. The clinical team caring for the participant will not be aware of which trial arm the patient is in. It is not possible for the individuals providing the NMES therapy to be blinded as they will need to be aware of which arm the patient is in in order to provide effective advice.

11.4.4 Data collection

Surveillance CT scans performed as part of sequential screening (i.e., not emergency or non-routine imaging) are performed as standard in this patient group and will

undergo analysis measuring mean muscle attenuation (myosteatosis) and muscle area (sarcopenia) at the level of the third lumbar vertebrate. Routine bloods including CEA (Carcinoembryonic antigen) and inflammatory markers will be measured at each elective routine clinic visit and these data recorded, these samples will be collected, analysed and disposed of in accordance with the LNWH NHS Trust policy. Quality of life will be assessed at 6 and 12 months using validated quality of life questionnaires (ED-5Q-5L & EORTC QLQ – CR29). The Berg Balance scale, 30 second sit-to-stand test and 6-minute walk test[369–372] will be used to assess functional outcome these tests will be performed at the patients three months post operatively clinic appointment. Pre and post-operatively we will measure bilateral thigh circumference at 15cm above the superior pole of the patella (which has been shown in earlier studies to correlate with muscle volume on MRI) [373]. Bio-impedance analysis (BIA) will be undertaken at baseline, day two post operatively, day twenty-eight post operatively (if in hospital) day of discharge and first post-operative follow up appointment. We will record data from the device satisfaction questionnaires from both groups. Standard outcome data and covariates to be collected are shown in Table 11.4.

Data collection will be undertaken using a case report form (CRF) designed by the investigators, the data will be transferred from the CRF to a Microsoft Excel Database *(Microsoft Corporation, Redmond, Washington, USA)*, stored on the LNWH NHS Trust secure network. All collected data will be reviewed by the principal investigator prior to analysis and specific searches on written notes or electronic record systems will be made to address any missing data points. The data collected as part of this

trial will be subject to review by the independent Trial Data Monitoring committee on request as per the trial Data Monitoring Charter.

Demographics o Patient age o Patent birth year o Patient gender o Patient ethnicity coding o Weight o Height o Body Mass Index	Outcome measures o 90 day post-operative complications (Clavien-Dindo classification [120]) o Disease Outcomes including 30-day survival o Death / recurrence / disease free survival o Patient compliance diary data o Device usage
Disease o ASA grade (American College of Anaesthesiology) physical status o Past medical history (Other or previous illnesses and surgery)	Body composition parameters o CT Body Composition at the level of the L3 vertebra o Bio-impedance analysis o Anthropometric measurements
Cancer Specific Characteristics o Type of cancer (primary/recurrent cancer, location and subtype on histology) o Grade of Cancer o Stage of Cancer	Inflammatory markers o Serum C-reactive protein, o white cell count o serum albumin o platelet count
Treatment factors o Details of Surgery o Chemotherapy o Radiotherapy	Patient specific and functional outcomes o Quality of Life - ED-5Q-5L - EORTC QLQ – CR29 o Patient satisfaction

Table 11.4 Outcomes and Covariates

11.4.5 Data Monitoring and Compliance

A sponsor approved independent data monitoring committee (IDMC) has been appointed to the trial as part of good trial governance to ensure safety, scientific validity and integrity of the trial. The data monitoring committee will have access to

raw data and will review any significant adverse events or safety concerns within the trial. The IMDC will make recommendations and report directly to the sponsor representative and chief investigator. The trial Data Monitoring Charter is included for review within the supplementary material.

11.5 Study Intervention

11.5.1 Stimulation of Muscle

We will endeavour to stimulate two major muscle groups during the study, the quadricep muscles and paraspinal muscles. The muscles of the quadriceps, particularly vastus lateralis and vastus medialis will be stimulated in both legs, this will be performed with a view to preserving muscle mass and encouraging earlier ambulation and better function.

We will also stimulate the erector spinae muscles and the muscles of the lower back. The reason for this is twofold, firstly, it is felt that some of the earliest muscles to atrophy following surgery or during bed rest are the core muscles of the back especially as these patients will not be sitting up or utilising these important supportive muscles in the first stages of their recovery. Patients are nursed on their side during the first 14 days following major pelvic surgery, which means they tend not to use their core muscles to flex or extend their back or support their weight leading to loss. This lateral position however would afford easy access to place the electrodes. Secondly, we are focussing on the muscle groups at the third lumbar

vertebrate – the level this stimulation would take place, using the device in this location would give us the best chance of demonstrating the benefits of the device with regards muscle preservation. This site is well away from the operative site and tumour bed in these individuals and therefore there would be no risk of stimulating the tumour bed whilst using the device in this position.

Neuro-muscular stimulation will be delivered by a *MicroStim Exercise Stimulator* MS2v2 (*Odstock Medical Limited (OML), Wiltshire, UK*) using two self-adhesive electrodes placed on the anterior thigh over the body of the vastus medialis and lateralis and the muscles of the lower back. At their second clinic appointment at St Mark's patients will be trained by the research fellow or other competent research team member (physiotherapist or specialist nurse) to use the NMES. A study specific instruction leaflet will be given to this group along with the standard instruction manual by OML.

The program will commence pre-operatively and consist of daily stimulation to one thigh at a time followed by the lower back each for 15 minutes, increasing to 60 minutes within one week as tolerated. One treatment session for both thighs would last between 60 to 90 minutes in total per day – this can be taken in up to three discrete sessions. Treatment will last for two weeks pre-operatively and eight weeks postoperatively.

NMES would be used preoperatively to familiarise patients with and increase confidence in using the device prior to surgery and to aid prehabilitation.

11.5.2 Intervention training

Training in using the MicroStim 2v2 has been undertaken by the trial principal investigator at the device manufacturer, OML. Patient training will be conducted by the study PI or a trained member of their study team. Patients using the device will be observed and educated on correct usage by the study team. They will be asked to keep a usage diary and the device will record usage statistics via an inbuilt recorder. These data will feed into the analysis to provide a dose response model within the final analysis.

11.5.3 The Therapeutic NMES arm

Patients will be blinded as to which arm of the trial they are in. Therapeutic NMES will be delivered by a *MicroStim Exercise Stimulator* MS2v2 using two self-adhesive electrodes placed on the anterior thigh over the body of the vastus medialis and vastus lateralis muscles and the lower back.

Pulse waveform (symmetrical biphasic squared), frequency (40 Hz), and width (350 microseconds) would be used for the duration of treatment with the NMES. The amplitude (device output 0-120 mA, tested across 1000**Ω**) will be set to elicit a visible and comfortable muscle contraction; patients will be encouraged to subsequently increase the amplitude as tolerated. A “compliance diary” will be kept by the patients during their treatment period detailing their time spent using the device and the settings at which they are using it.

This program is adapted from one found to be of benefit in a pilot study of patients with non-small cell lung cancer which itself was based on an NMES exercise program developed for patients with COPD. The stimulation parameters were selected to favour gains in function and strength over endurance (frequency), minimise skin irritation (pulse width), and allow for sufficient recovery of the muscles between contractions (duty cycle)[349,350].

11.5.4 The placebo NMES arm

A modified model of *MicroStim Stimulator* MS2v2 (*Odstock Medical Limited, Wiltshire, UK*) will be provided to the placebo group who will apply two self-adhesive electrodes placed on the anterior thigh over the body of the vastus medialis and vastus lateralis muscles and the lower back as in the treatment group. This placebo device will be programmed to provide sub-therapeutic electrical stimulation. The device manufacturers have tailored a program to come on and off at specified timings with ramps of specified duration. The placebo devices output is restricted to around 18V and this gives little or no muscle recruitment. Patients will however perceive a sensation of electrical stimulation.

11.5.5 Both Groups

Patients in both arms will receive standard care including enhanced nutritional support (parenteral nutrition for a minimum of 5 days or until taking sufficient calories enterally) and physiotherapy in line with current guidelines and local hospital practices. Routine daily blood tests for inflammatory markers will be taken until discharge.

11.6 Assessment of outcomes

Patients from both the treatment and control arms will receive standard five year follow up. Histopathological data will be recorded following processing of the resected specimens by the pathologist. Quality of life data, patient satisfaction, bio-impedance analysis, CT Body composition and functional measurements will be taken as detailed below.

Early-stage analysis to identify changes within the pre- and post-operative CT scans and analysis of NMES satisfaction and the initial inflammatory data, functional and quality of life data will take place at six months following the recruitment of the final patient. We will then continue long term follow up for the standard 5 year follow up period or until patient death. Final analysis will take place at 5 years following recruitment of the final patient.

11.6.1 Body composition assessment

11.6.1.1 CT body composition parameters

CT image analysis using *SliceOmatic* version 5.0 software (*TomoVision, Montreal, Quebec, Canada*) will be performed. Total skeletal muscle and visceral adipose tissue surface area (cm^2) will be evaluated on a single image at the third lumbar vertebra (L3) using HU thresholds of –29 to 150 for skeletal muscle, –50 to 150 for visceral adipose tissue (VAT) and –190 to –30 for subcutaneous adipose tissues. CT body composition analysis of all the included images will undergo automated segmentation

using the ABACS L3 automated plug-in software[374] (*Voronoi Health Analytics, BC, Canada*), which complements SliceOmatic. The automated process will be directed by a radiologist who will be blinded to the treatment group of individual patients. The automated segmentation process provided by the ABACS L3 plug-in also removes the possibility of operator bias in the analysis of the images. The sum of skeletal cross-sectional muscle areas will be normalised for stature (m^2) and reported as lumbar skeletal muscle index (LSMI) (cm^2/m^2). Outcome variables will be continuous, categorical variables will be defined from these data using the cut-off values described earlier[72,76,154].

11.6.1.2 Anthropometrics and Bio-impedance analysis

Bio-impedance analysis (BIA) will be undertaken using a SECA mBCA 525 analyser (*SECA, Hamburg Germany*). This will be performed at baseline, day two post-surgery, either hospital discharge or at 28 days post-surgery (whichever is first) and at 6 months. Patients will undergo analysis in a fasted state. Posterior upper arm skin fold thickness and waist circumference will be performed at baseline and 6 months. We will measure thigh circumference at 15cm above the superior pole of the patella (which has been shown in earlier studies to correlate with muscle volume on MRI)[373]. Phase angle from BIA and patient BMI will be utilised as categorical variables with other outcome variables being continuous.

11.6.2 Functional Assessment

It is important that we measure not only the anatomical effects of NMES i.e., increased muscle mass on CT and anthropometric changes but we identify whether

these patients demonstrate both a functional and physiological improvement. To that end we will assess functionality preoperatively at diagnosis and post operatively at 3 months using the validated instruments of the 6-minute walk test[372], 30 second sit-to-stand test[371] and Berg Balance scale (BBS).[369,370] These assessments will be undertaken in the complex cancer clinic by either a specialist physiotherapist or research fellow. These data will be treated as continuous outcome variables except for BBS which will be categorical.

11.6.3 Quality of Life

We will examine quality of life and patient experience of using the device. Quality of life will be measured using the validated questionnaires described above. On completing the intervention participants will complete a questionnaire on compliance, comfort and usability of the device in the postoperative setting. Qualitative data and free text comments from this will also be collected.

11.6.4 Systemic inflammatory response

To monitor the inflammatory response, we will use commonly utilised postoperative inflammatory markers, namely c-reactive protein (CRP) and values derived from the full blood count and biochemistry including neutrophil-to-lymphocyte ratio (NLR) and modified Glasgow Prognostic Score (mGPS). These inflammatory markers are well-established metrics linked to both sarcopenia, myosteatosis and prognosis in colorectal cancer[125,152,153]. We have chosen these markers for a number of reasons; they are routinely taken, cost effective and allow for comparison with substantial historical data. We may also require results from other trusts, due to the national

spread of our patient population, and we cannot support them in obtaining non-routine tests as part of this study.

11.7 Planned statistical analyses

This study will be performed in line with the CONSORT criteria (http://www.consort-statement.org/consort-2010). Initially outliers, patterns of attrition and missing data will be identified using a combination of graphical displays and descriptive statistics allowing decisions on the assumption of normality. The primary analysis will be of observed data only, with patients with missing data omitted from the analysis. If the primary outcome has >10% missing data points, a sensitivity analysis will be performed using multiple imputation to estimate the missing values.

Data will be analysed by a statistician blinded to the intervention. Analyses of primary and secondary endpoints will be based on the full analysis set defined according to the intention to treat principle. Safety analysis will be performed for the on all enrolled individuals with disclosure of any significant adverse events. The full analysis set consists of all participants consented and randomised with valid baseline assessments. Participants will be analysed according to the study arm they were assigned at randomisation.

The primary outcome is myosteatosis at 3-6 months post-surgery, derived from the mean muscle attenuation on CT body composition analysis. This will be analysed using analysis of covariance (ANCOVA), with muscle attenuation values at baseline used as a covariate in the analysis.

The secondary outcome measures measured on a continuous scale, and with a baseline measurement will be analysed using equivalent methods as the primary outcome. For continuous outcomes with no baseline measurement, group comparisons will be made using either the unpaired t-test or Mann-Whitney test, depending on data distribution. The Chi-square test, or Fisher's exact test, will be used to compare categorical outcomes between the study groups.

Significance will be assumed when $p<0.05$.

11.8 Adverse event reporting

Adverse event reporting in this trial is carried out in accordance with the NHS Health Regulation Authority (HRA). All serious adverse events (SAE), whether or not related to participation in the trial will be reported immediately to the trial sponsor, these will be reviewed by the trial sponsor and should further investigation be required the trial sponsor may pause the trial to carry out investigation. Should a SAE occur as a direct result of the trial device or as a direct result of participation in the trial the trial will be paused and a non-CTIMP safety report form will be submitted to the relevant research and ethics committee with 15 days of the Chief Investigator becoming aware of the event. In this instance the SAE report will be unblinded as required by the HRA. Patient reported adverse events deemed expected or non-serious will be reported in the final publications arising from this trial, we expect many of these minor events to be reported in the patient satisfaction questionnaire.

11.9 Discussion

Patients who undergo major pelvic surgery have limited mobility due to postoperative pain and disability. These patients are therefore at much greater risk of suffering from muscle wasting than patients undergoing more routine colorectal surgery. This is a result of greater loss of function, greater immobility and potentially a more profound immunogenic inflammatory response.

Currently these patients receive postoperative physiotherapy, due to limited time, postoperative pain, patient choice and resource availability it is unlikely that the patients are exercised to their full potential. A prescribed program with a NMES device would allow patients to choose when they undertake muscle stimulation exercise for example once they had received adequate analgesia or at a time convenient to them. This would hopefully improve compliance and bring about a hypertrophic response in the muscle.

We know that in muscle disuse in healthy individuals NMES may provide an effective treatment to preserve muscle volume[367]. Maddocks' work in cancer patients[349,350] however did not demonstrate a significant increase in muscle volume and therefore one may question the rationale behind use in this patient group (these differences are summarised in Table 11.1). The cancer population in these studies is different from our own in several respects beyond the diagnosis alone and as such we may find NMES to be a more suitable intervention in our patient group. Maddocks' work was performed in a palliative population with active cancer whilst postoperatively our patients will be theoretically cancer free with perhaps a few exceptions in patients who have solitary metastases (which, by the criteria of inclusion, are amenable to

curative treatment). In view of their palliative status, Maddocks' population would be expected to decline in health over time whilst our population would be expected to make a recovery up to or even beyond their preoperative state and therefore NMES may increase the rate of or facilitate this recovery. Our population is confined to bed rest for over a week's duration following surgery and therefore activity provided by NMES may help arrest the muscle loss associated with disuse as in Hasegawa's population whose anterior cruciate ligament repair cohort was subject to muscle loss through disuse rather than disease[367]. Finally, our patients will receive intensive inpatient support by the ward physiotherapists and the research team, they will receive positive reinforcement of their use of the device and will be asked to complete an exercise diary which the physiotherapy team, will review with them at each point they receive formal physiotherapy sessions. This level of direct input and positive reinforcement is notably more than in the previous NMES studies of Maddocks' and therefore we would hope compliance and correct usage would be increased.

The inflammatory effects of exercise are known to be paradoxical in that exercise drives both a pro and anti-inflammatory response[212,375]. We propose that the metabolic result of exercise in cancer patients will drive a beneficial anti-inflammatory response. This immunomodulation may in part help support the body's immune system in the early stages of post-surgical recovery and as such may potentially support the cellular immune system in being able to identify and destroy malignant cells shed at the time of surgery.

In our patient group NMES will potentially allow a higher degree of exercise than the patients would otherwise be able to undertake due to their incapacity. Our hope is that this promotes muscle preservation, allowing earlier mobilisation and a more expedient return to “normal” exercise and function, further reinforcing the preservation of muscle mass. Increased muscle mass and quality are associated with improved long term outcome such as disease free survival[111] we intend to follow our cohort for 5 years to see if NMES provides evidence to. support improvement in these oncological outcomes through muscle preservation.

11.10 Conclusion

Exercise in healthy individuals leads to increased muscle mass, exercise can bring about an anti-inflammatory effect due to muscle physiology thus obfuscating a key pathway driving secondary sarcopenia. Preservation of muscle mass through early post-operative intervention with NMES would allow a more rapid return to normal exercise and normal function leading to greater muscle preservation and subsequently improved outcomes.

11.11 Trial Status

BiCyCLE NMES has completed recruitment of patients, recruitment in March 2021. Protocol version 6 dated 05/06/20 is currently approved by the research ethics committee and the HRA.

Chapter 12

12 General Conclusions

12.1 Thesis Summary

We hypothesised that “Body composition is dependent on genetic, environmental and tumoral factors but can be manipulated by modulating the immune response to colorectal cancer and thus outcomes improved”. During this thesis we have explored the influence of tumour, environmental and immune factors on body composition with an aim to either proving or disproving this hypothesis. We identified what approaches have been used to manipulate body composition to date and the evidence of their efficacy before devising a trial to ascertain whether we can arrest or reverse the post-surgical impact upon body composition and through doing so whether we can in turn modify the immune response and improve outcomes.

Our current knowledge and understanding of the associations between body composition and colorectal cancer outcome were discussed in the first part of this thesis. We expanded the understanding of this in Chapter 2 where, we demonstrated that various body composition phenotypes were associated with the grade and stage of colorectal cancer. Visceral obesity appearing to be associated with a prognostically favourable suite of tumour characteristics. Chapter 3 explored the role of the tumour on body composition further in respect to distant metastatic disease. Myosteatosis was as potent a predictor of future distant recurrence than anastomotic leak, vascular invasion and lymph node disease at the time of surgery.

Few relationships were identified between sarcopenia and disease characteristics and so we explored environmental and genetic aspects which may affect muscle mass and volume and the systemic inflammatory response. Our findings in Chapter 4 demonstrated striking links between deprivation and body composition, sarcopenia was significantly associated with socioeconomic deprivation and as a pre-existing poor prognostic feature in colorectal cancer may help explain outcomes in this group. This evidence that environmental and lifestyle exposures are key in determining BC suggests that we in turn may be able to identify at risk individuals and use diet and lifestyle modifications to manage sarcopenia in certain individuals. Chapter 5 explored ethnicity and its relationship with body composition in colorectal cancer, we found that ethnicity not only predisposed to various body composition phenotypes in CRC but also the associated systemic inflammatory picture.

Having identified a relationship between body composition, tumour characteristics and environmental factors such as deprivation we went on to examine the links between the tumour, the environment and body composition through a series of studies exploring the relationship between body composition and the immune system. The second part of the thesis focused on the relationship between behaviour and function of dendritic cells and body composition. Chapter 7 demonstrated associations between DC lipid profile and body composition including that DC containing significantly higher levels of intracellular oxidised lipid were resident in the muscle of myosteatotic patients. Chapter 8 explored the concept of the gut muscle-axis identifying evidence of gut homing by the DC resident in muscle tissue which have greater Beta 7 expression in the viscerally obese population. We also identified an increased frequency of Beta 7 expression by circulating mDC in

myosteatotic patients. Chapter 9 aimed to explain these functional relationships in more depth including through a stimulation model which demonstrated a predilection for the mDC of healthy individuals to have higher intracellular levels of oxidised lipid compared to CRC patients following stimulation by colonic bacteria, a possible method of immunomodulation. This counters the existing narrative that high intracellular oxidised lipid content is a dysfunctional phenomenon. We also explored the relationships between cytokines and DC behaviour finding a number of significant correlations between GDF-15, IL-6 and DC behaviour. The role of GDF-15 was of particular interest where we found serum GDF-15 levels were associated both with the presence of CRC but also normal muscle volume suggesting the role of GDF-15 as an exerkine (exercise induced myokine) both related to muscle volume and disease state.

The final stage of this thesis was to identify possible ways in which BC could be manipulated either pharmacologically, mechanically or endocrinologically, Chapter 10. From our earlier work exploring cytokine relationships and immune function we chose to further explore muscle manipulation by exercise, known to modulate the cytokine response, using NMES, we devised a randomised control trial as a feasibility study to investigate whether prescribed muscle stimulation following exenterative surgery was possible and could maintain muscle mass and dampen the immune reaction to surgery whilst promoting an oncologically positive response, the methodology of this trial is described in Chapter 11.

12.2 General Discussion and Future Research

The associations between body composition and outcomes in colorectal cancer have become more established and accepted over the last 10 years, especially during the last four years of the construction of this thesis[113,117,247]. Work in this unit between 2013 and 2016, by Malietzis and colleagues was instrumental in establishing and conceptually cementing many of these links, especially the relationship between the systemic inflammatory response and body composition in colorectal cancer[111,152]. This thesis has built on much of this earlier work expanding on nascent concepts and developing novel avenues of study as the field of body composition science advanced.

The overarching goal of this thesis was to determine whether body composition in colorectal cancer was modifiable. To this end we established what factors influence body composition; the environment, host genetic characteristics (ethnicity), disease biology and the associated host immune response to the tumour. The latter was further explored as a possible target for manipulation. To do this it was necessary to dissect the minutiae of the behaviour of the dendritic cell in certain conditions and anatomical locations. These data helped evolve the concept of the gut-muscle axis[376], demonstrate how the DC behaviour and function changed in different anatomical locations of interest and in part provided an explanation for prognostic differences between BC states. Finally we aimed to translate our assertions from the earlier part of the thesis into clinical practice through the BiCyCLE NMES trial[377]. We hypothesised that by improving muscle mass and quality perioperatively, one could modulate the immune system through myokines such as IL6 and IL10, reducing the inflammatory burden of the innate and adaptive immune system thus promoting a measured and appropriate immune response to the tumour.

12.3 Body composition features and disease

12.3.1 Visceral Obesity

In Chapter 2 we established an independent association between visceral obesity and favourable tumour characteristics of earlier T-Stage (T1/2) and a more differentiated tumour, VO patients were also significantly less likely to have nodal disease and vascular invasion. This can be explained in one of two ways either, these patients have retained their visceral fat because of their less aggressive tumour or the presence of VO although potentially driving carcinogenesis through chronic inflammation (Chapters 1 and 7) it predisposes to a less aggressive tumour phenotype as postulated in RCC[124]. The fact that patients with sarcopenic obesity, a metric considering BMI, did not share these associations may support the concept tumour behaviour and biology is a more important factor some patients. Further evidence supporting VO as a protective characteristic was identified in Chapters 7 and supported by work in Chapter 9. Here we demonstrated that the dendritic cells of patients with visceral obesity had potentially favourable characteristics compared to other BC phenotypes. Patients with VO has significant greater expression of CD36 by DC and macrophages of the colonic IEL and LPL. CD36 has a joint role of being both a fat scavenger but also antigen presenting complex[246]. In the colon, at the anatomical "coal face" for the immune system, antigen presenting cells in VO individuals have a greater functional potential for antigen presentation and perhaps a greater metabolic capacity, through lipid uptake, compared to CRC patients with other BC states. Other associations between VO and CD36 were seen elsewhere, namely in the mesentery where putative DC, described in chapters 7 and 8, lacking class II, had significantly elevated CD36. We also identified that VO was associated with the presence of more mature DC in the mesentery whilst analysis of skeletal muscle demonstrated a

significant positive correlation between an increase in both visceral fat index and visceral fat area and expression of Beta 7 integrin, a gut homing marker. One interesting finding worth revisiting, in view of its paradoxical insinuation, was that E06 MFI, intracellular oxidised lipid (ICOL), was significantly diminished in the colonic LPL DC of VO individuals, this would fit with earlier published work suggesting that a low intracellular oxidised fat level is beneficial as antigen presentation is disrupted by high levels of ICOL[140,243]. However, in Chapter 9 we demonstrated that healthy mDC have a significantly higher levels of E06 following stimulation compared to mDC from CRC patients. This would therefore imply dysfunction of the LPL DC of the VO patients and not corroborate our overall findings. Two explanations could be posed, one being that the level of ICOL in DC is dynamic and in the experimental models we use we see a snapshot of what is happening which may not represent the true evolution of DC in vivo. The second relates to the activity of the pDC, we see in blood that they mirror the effects of mDC following stimulation with increased ICOL seen in the pDC of CRC patients following stimulation. When we isolated the LPL DC, we did not differentiate them based on expression of CD123 (Chapter 6), this means we may have a mixed population of mDC and pDC in the isolates. Ultimately this relationship with ICOL in the LPL DC is unexplained and requires further experiments to explore this relationship further.

12.3.2 Myosteatosis

Myosteatosis has been recognised a poor prognostic indicator throughout this thesis, in our colorectal cancer cohort, in Chapter 2 we demonstrated myosteatosis to be a powerful independent predictor of the presence of metastases and vascular invasion. Chapter 3 explored the relationship between the tumour and body composition further

in regard to distant metastases. Myosteatosis was as strong a prognostic indicator of future distant metastasis as concurrent nodal disease, vascular invasion and anastomotic leak. The parallel with anastomotic leak is interesting as the only other predictor found not directly related to the tumour. Anastomotic leak may predispose to distant disease recurrence as there is a systemic immune response to the insult of a leak which may lead to immune dysfunction and a failure by the innate immune system to identify and destroy circulating tumour cells[182,378]. A similar process of immune system dysfunction may also occur in the myosteatotic patient population. Chapter 7 found other associations with myosteatosis which would support such a pattern of immune dysfunction, we demonstrated that the mDC of myosteatotic patient had significantly greater intracellular total lipid, a phenomenon described before in DC and suggestive of dysfunction as they become adipocyte-like. The relationship between intracellular lipid and DC stimulation was examined in greater depth in Chapter 9. Here we identified that the presence of colorectal cancer determined the metabolic response of the DC. Bacterial taxa had little effect on the metabolic response but the presence of cancer appeared to attenuate the stimulatory response. This increased lipid level seen in the DC of the myosteatotic patients in Chapter 7 may demonstrate that these cells have been unable to appropriately respond to an immune stimulus, scavenge lipid but are unable to utilise it as an energy source. Interestingly, as described in Chapter 8, the circulating DC of myosteatotic patients also had a significantly greater expression of Beta 7 integrin compared to non myosteatotic patients but this relationship was seemingly lost in the muscle with no correlation between muscle attenuation and either Alpha 4 or Beta 7. This differs to VO where we saw a significant positive correlation between gut homing and Beta 7 expression on the resident muscle DC but no relationship to gut homing in the circulating

DC. This highlights a clear immunological difference between these two prognostically different groups in regards the potential gut-muscle axis.

12.3.3 Sarcopenia

Chapters 2 and 3 failed to find significant associations between sarcopenia and tumour characteristics and we therefore went on to explore genetic and environmental influences on the host. Here associations became more apparent with certain ethnic phenotypes being associated with body composition and inflammatory profiles (Chapter 5). Deprivation was also associated with sarcopenia (Chapter 4). These results demonstrate that sarcopenia is, although poorly prognostic, is a premorbid state. The presence of sarcopenia may change as the cancer burden increases (although this was not apparent in our population – Chapter 2) but one would expect sarcopenia in advanced disease as part of the syndrome cancer cachexia. Interestingly sarcopenia and sarcopenic obesity were also associated with the fat scavenger profile of DC in mesenteric fat, Chapter 7. This unusual and unexpected observation suggests that the immune metabolic function and behaviour of DC within the fat of these patients is abnormal. This may mitigate or demonstrated mitigation of the beneficial effects VO as described above. Sarcopenia was also associated with DC maturation, patients with sarcopenia were significantly more likely to express CD40 on their circulating mDC. This may be a result of environmental and lifestyle factors (Chapter 4) which are driving the chronic inflammatory state, seen in sarcopenia (Chapter 1), and have resulted to stimulation and DC maturation described in Chapter 8. Corroborating this, within the end organ (muscle) CD40 expression by DC significantly decreased as LSMI increased showing that these immune profile in the blood is intimately related to the diseased end organ – the muscle. This then poses the question

of whether one can modulate the immune response of certain at risks individuals by altering antigen exposure. Conversely one may be able to explain these findings by the suggestion that those who are sarcopenic lack the ability to regulate their immune system and this we see unregulated maturation of DC in this population. Therefore, by restoring muscle mass, through early intervention in the at-risk population e.g., those of socioeconomically deprived background, could we in turn modulate their immune system and arrest this DC activation.

12.4 Translating body composition science into clinical practice – further work

Throughout this thesis we have seen the profound effect that the quantity and quality of muscle tissue have on both the immune system and outcomes in colorectal cancer. Muscle is a rapidly modifiable tissue but is lost rapidly through multiple processes following surgery. Preservation of muscle tissue may help modulate the immune system and promote destruction of circulating tumour cells or seeded metastases following surgery. We have aimed to explore this concept in the BiCyCLE NMES trial in our first step towards translating our findings into clinical practice. However, this thesis has highlighted several other areas where one could investigate the benefits of assessing and restoring body composition in colorectal cancer.

12.4.1 Delaying definitive treatment to restore body composition

Sarcopenia is related to deprivation status and ethnicity (Chapters 4 and 5); it is also related to poor outcomes. This raises the question of whether one can delay surgery to pre-habilitate patients for their surgery, improving muscle mass and thus providing the

immune benefits of muscle. A number of studies such as PREPARE ABC[379] are underway exploring the effect of prehabilitation and post-operative rehabilitation on improving outcome in CRC. Should prehabilitation demonstrate a significant positive effect on both oncological and surgical outcomes then there may be an argument for delaying surgery in certain patient groups to allow time to build up muscle mass. It is likely to be of increased benefit if targeted at the most in need group such as the economically deprived.

Conversely, we have seen how visceral obesity appears to confer a beneficial oncological profile. Obesity, especially visceral obesity, presents a series of technical problems for the surgeon which has been shown result in poorer post-operative outcomes[380,381]. Therefore, by performing sub-optimal surgery, due to the technical difficulties resulting from VO, we may be conferring a worse outcome on what should be a group of individuals with a good prognosis. As such there may be a strong argument for delaying surgery in the VO patient as their tumour is statistically likely to be less aggressive (Chapter 2) thus improving their body habitus may improve outcomes further. Our group is exploring these concepts further and work should be undertaken to explore the safety and feasibility of delaying surgery to reduce VO in this patient population.

12.4.2 Chemotherapy dosing

Patients with more muscle loss are at increased risk of side-effects and complications from chemotherapy. These complications occur, in part, due to patients being administered an inappropriate chemotherapy dose[360]. Currently, there are two approaches to calculate chemotherapy dose, firstly, flat dosing - giving a fixed dose without considering the size of the patient (height and weight); secondly, dosing based

on body surface area, a crude calculation based on weight and height. Neither of these methods consider different volumes of muscle and adipose tissue in a patient. Adipose tissue and muscle process chemotherapy drugs differently, patients who have lower muscle metabolise drugs slower. These patients are therefore at risk of overdosing, whilst patients with high muscle volumes are at risk of underdosing. Patients who are overdosed are at increased risk of side effects which can be debilitating, impacting on quality of life, and are more likely to have their chemotherapy stopped[382]. This reduces the effectiveness of chemotherapy and increases the chance of the cancer returning. Patients with a high volume of muscle are at risk of underdosing and therefore are not receiving an adequate level of chemotherapy to treat their cancer. To address this problem further work is being undertaken by multiple groups to explore the use of alternate dosing strategies in colorectal cancer such as the LEANOX trial[383]. Trials such as this may shift the paradigm of treatment based on CT BC analysis and are the focus of colleagues in our group based on work from this thesis[384].

12.4.3 Prognostication

In Chapter 3 we demonstrated how myosteatosis was a potent predictor of future metastatic disease. The reason for this was not fully elucidated. However, being able to predict which individuals would be at greater risk of recurrence would be of huge benefit to surgeons, oncologists and patients. Multiple prediction tools exist and help determine who would benefit from chemotherapy and the chance of associated risk to the patient such as PREDICT in breast cancer[385]. Myosteatosis is a clinically relevant and easily obtainable metric which could be used as part of such a predictive tool. Further research

needs to be undertaken to explore whether this is both feasible and reliable. Data on myosteatosis should be taken for large population groups to help facilitate this.

12.4.4 Modulation of the microbiome and gut muscle axis

In Chapter 9 we demonstrated, through an in vitro model, how the microbiome can influence the behaviour and response of DC. We saw that CRC patients and the healthy population responded differently to stimulation which suggested that there was immune dysfunction to the microbiome in CRC characterised by the lipid profile of DC. The microbiome therefore is a potential target which we could exploit or modify to influence the immune system in CRC patients.

Further trials are needed to explore the effect of the microbiome on BC and the role of the gut-muscle axis in sarcopenia, myosteatosis and obesity. This may be of increased relevance once again in the deprived population, whom we have shown to be at increased risk of sarcopenia based on their lifestyle and environment.

12.4.5 Assessing fitness for surgery

Another potential use of BC assessment in clinical practice is in the assessment of fitness for surgery. Current methods such as cardiopulmonary exercise testing (CPET) are employed to provide a physiological assessment of fitness. CPET is time consuming, expensive and often excludes certain individuals from participating either through disease or disability which may have no bearing on their physiological fitness. Sarcopenia, myosteatosis and VO also to impact on outcome from surgery and may relate to physiological status too. To date no one has studied whether CPET and CT BC

parameters are related and as such our group is undertaking a multicentre study to explore the relationship between the findings at CPET and CT BC profile. Should the results demonstrate concordance between the two, CTBC could provide an easy method of preoperative physiological assessment which could be performed 24 hours a day and provide useful and informative data for patients and clinicians prior to surgical intervention.

12.5 Thesis Conclusion

Work in this thesis has demonstrated that body composition is a clinically, diagnostically and prognostically relevant marker. Myosteatosis is associated with a poor prognosis but could be a useful tool for identifying patients in need of enhanced surveillance or for a decreased threshold for the administration of adjuvant therapy. Muscle is an immunomodulatory tissue and could be targeted successfully in the peri-operative period to assist the innate and adaptive immune system in the initial post-operative days to ensure an appropriate host responds to residual circulating tumour cells. Sarcopenia appears to be a premorbid characteristic influenced by ethnicity and the environment, this means it is potentially modifiable if diagnosed on initial assessment and may benefit form targeted nutrition, microbiotic and exercise support. Obesity is controversial, a raised BMI predisposes to colorectal cancer but patients with VO are in a prognostically favourable group and have an immunological profile which appears comparatively less dysfunctional compared to other BC subgroups. The results of the BiCyCLE-NMES Trial may help answer these questions and will form the basis of future research within the group. Further research, examining the immunological pathways in VO may allow us to

use biological agents to influence the immune systems in such a way that it improves outcome.

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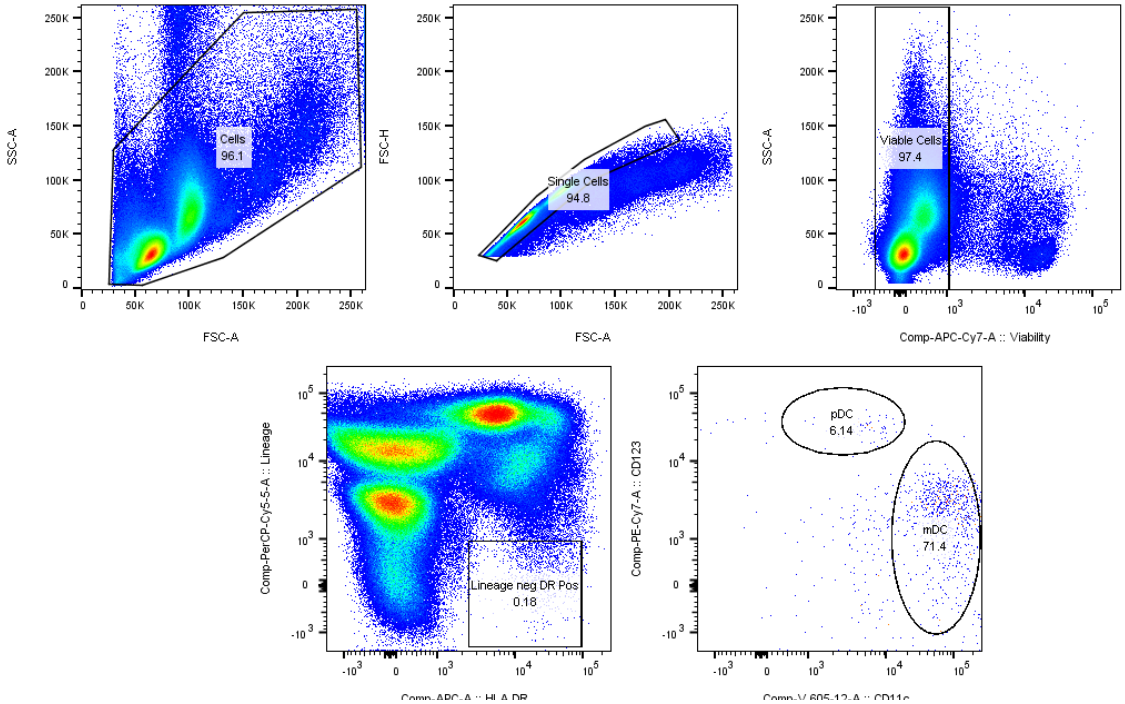
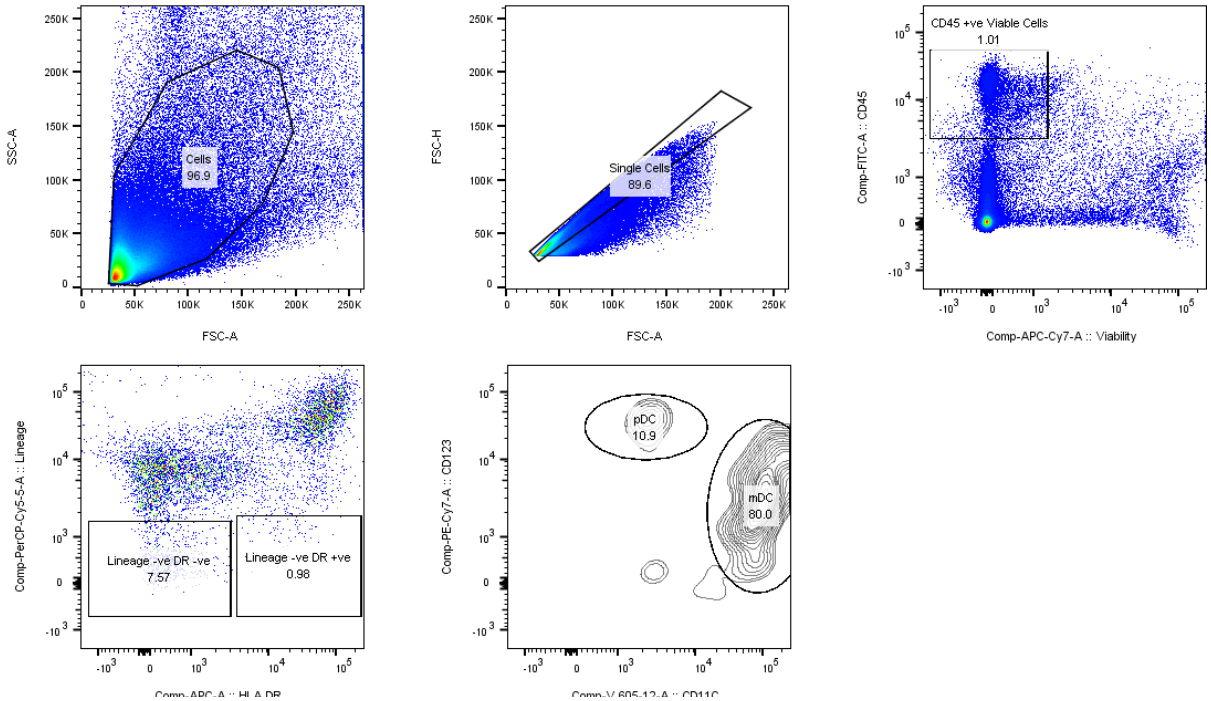
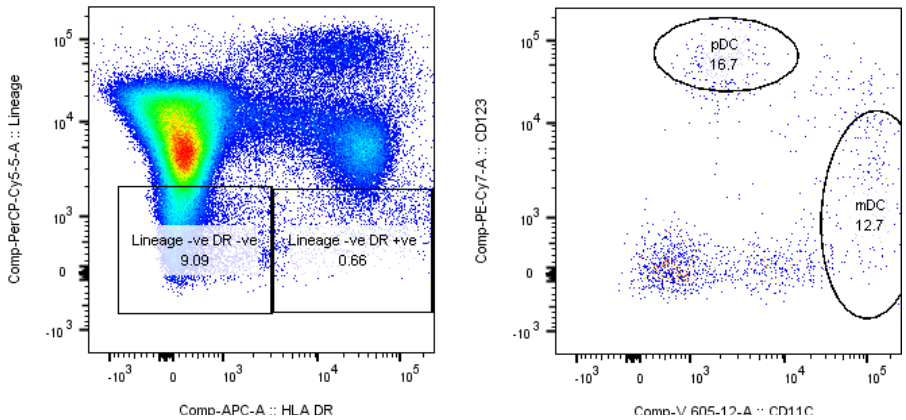
Appendix 1 Gating strategies for Flow cytometry by tissue type



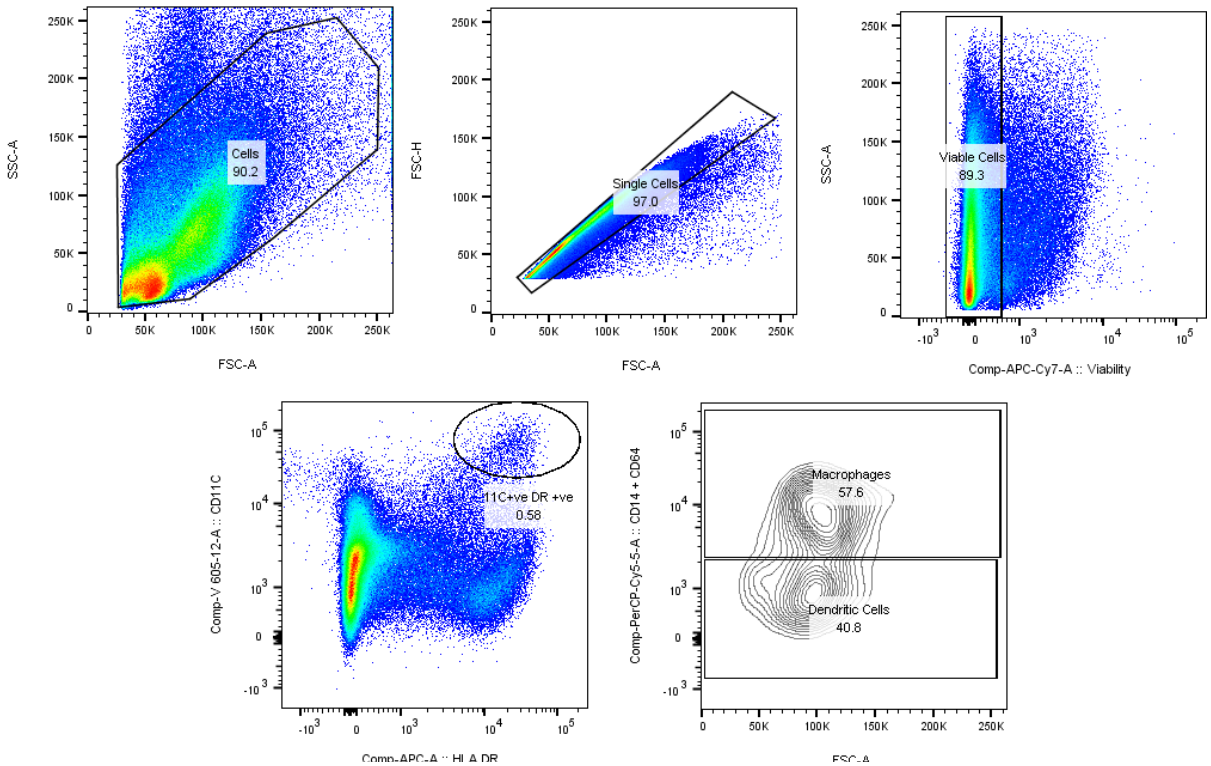
Supplementary Figure 1 PBMC Gating Strategy



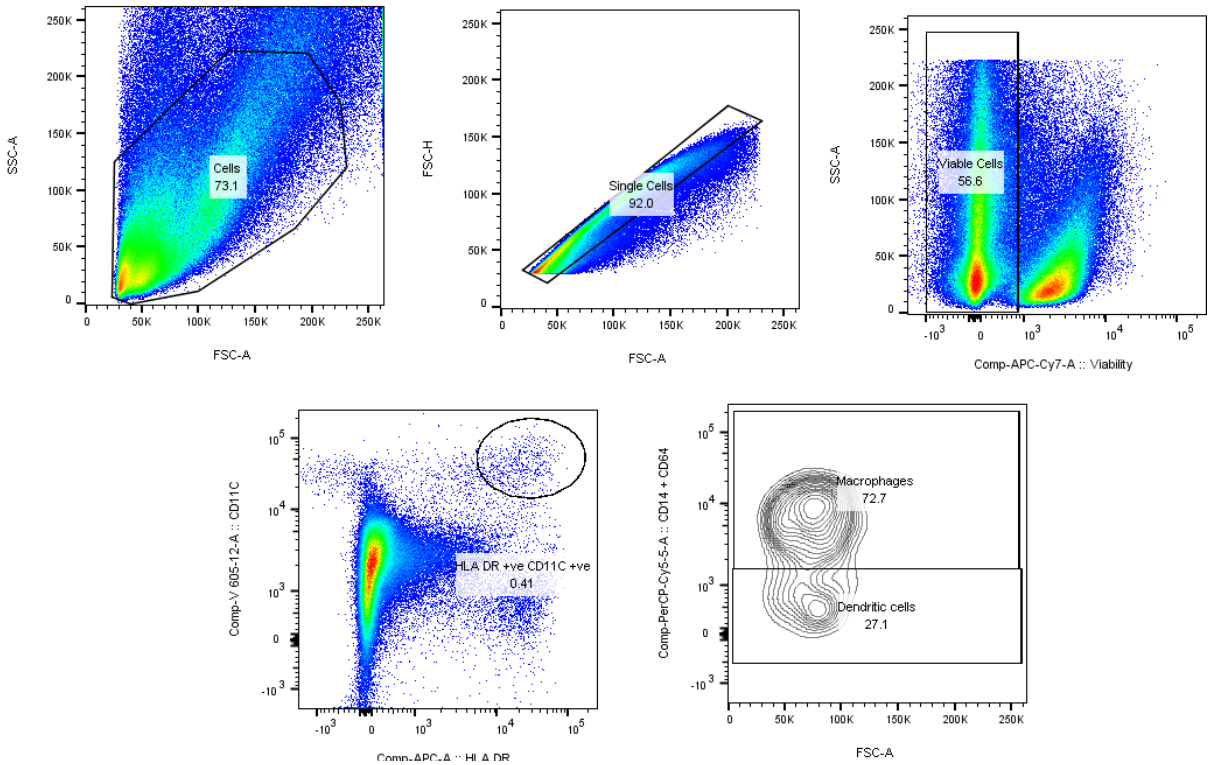
Supplementary Figure 2 Muscle Gating Strategy



Supplementary Figure 3 Fat Gating Strategy



Supplementary Figure 4 Lamina Propria Layer Gating Strategy



Supplementary Figure 5 Intraepithelial Layer Gating Strategy

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