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INNOVATIVE NUTRITIONAL STRATEGIES TO COUNTERACT CANCER CACHEXIA

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1- INTRODUCTION

1.1 Definition, classification and diagnosis of cancer cachexia

The incidence of cancer is increasing worldwide. It is estimated an increase of 21% in new cases by 2040 in the European Union and in the European Free Trade Association countries compared to 2020 [1]. Nowadays, the main risk factors for cancer occurrence are modifiable lifestyle elements such as obesity, tobacco, alcohol consumption, and physical inactivity. Therefore, at least 40% of all cancer cases could be prevented with a healthy lifestyle and effective primary health care [2]. Most of the cancer patients develop cachexia, a complex syndrome that is responsible for ~20% of cancer-related deaths [3].

Cachexia is a prevalent but poorly understood syndrome, which is rarely recognized, diagnosed, or treated until clearly established. It is generally associated with several chronic illnesses, including cancer, heart failure, neurological and chronic kidney disease, infections, and chronic obstructive pulmonary disease [4,5]. The main clinical feature is the progressive and involuntary body weight loss accompanied by fatigue, reduced food intake, inflammation, insulin resistance, and increased muscle protein breakdown [5]. Evans *et al*. (2008) described cachexia as "*a complex metabolic syndrome associated with underlying illness and characterized by loss of muscle with or without loss of fat mass*" [4]. Importantly, muscle mass loss impairs the capacity of the host to respond to stress conditions and chronic illness [6]. Fearon *et al.* (2011) produced an improved definition, stating that cancer cachexia is "*a multifactorial syndrome characterized by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment. Its pathophysiology is characterized by a negative protein and energy balance driven by a variable combination of reduced food intake and abnormal metabolism*" [7].

The incidence of cachexia in patients suffering from cancer is very high, reaching up to 80% in patients with gastric or pancreatic cancer [3] and is partly due to the aggressive nature of the therapies and the nutritional deficiency caused by the malignancy. Interindividual heterogeneity has been noted in patients with similar cancer diagnoses. Indeed, different degrees of cachexia are observed in patients with advanced-stage disease [5,8].

Cancer cachexia (CC) is divided into three stages, pre-cachexia, cachexia, and refractory cachexia (Figure 1). In the early stage, an involuntary body weight loss and initial metabolic changes with anorexia are present. The progression of the syndrome is ongoing with systemic

inflammation and persistent weight loss [7]. When patients reach refractory cachexia, the presence of active catabolism and the lack of response to anticancer therapy leads to a reduced life expectancy, which generally does not exceed 3 months. At this stage, the main interventions focus on palliative care to reduce cachexia symptoms and maintain patient quality of life (QoL) [7].

Because of the lack of accordance among definitions, the diagnosis of CC can be a challenge, especially in the early stages. Moreover, the presence of confounding variables such as the adverse consequences of cancer therapy and sarcopenic obesity make the diagnosis of cachexia difficult [9]. Muscle wasting can occur without fat reduction, hence protein degradation may be unnoticed in patients with obesity [5].

Apart from weight loss and fatigue, patients with cachexia often show altered blood biochemistry mainly triggered by a variety of host- and tumor-derived factors [9]. Overall, it is observed an increase in inflammatory markers such as C-reactive protein (CRP), interleukins (IL-1α, IL-1β, IL-6), and tumor necrosis factor-α (TNF-α) [9]. Moreover, significant anemia, hormonal dysregulation, and hypoalbuminemia have also been associated with cachexia [4,9]. So far, several factors have been proposed as biomarkers for CC [10], among them, transforming Growth Factor (TGF-β) superfamily members, Activin A, myostatin, the growth differentiation factor-15 (GDF-15), leptin, ghrelin, and microRNAs [10–12]. However, due to the number of variables, such as underlying diseases, age, and sex, no biomarker for CC has been clinically proven to date [10,12].

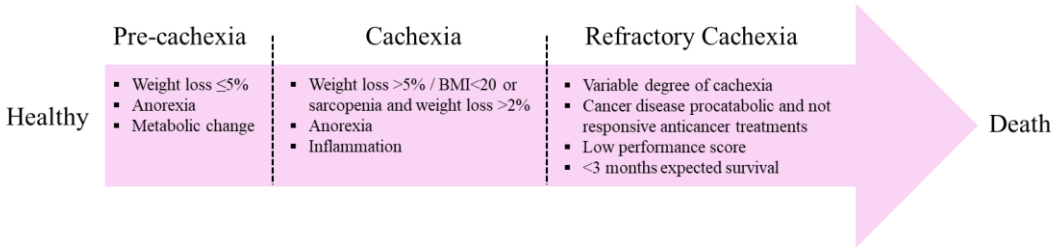


Figure 1. Stages of CC. Adapted from Fearon *et al.* 2011

In 2011, Argilés et al. introduced the cachexia score (CASCO) which was proposed to facilitate cachexia staging and therefore, allows for improved therapeutic efficiency. It consists of a numerical scale that assigns CC to mild (0–25), moderate (26–50), severe (51–75), and terminal (76–100). Thus, the higher the score, the worse the condition. CASCO methodology evaluates, among others, the nutritional status, body weight loss, and QoL. Moreover, the score includes lean body mass (LBM) measurements by bioelectrical impedance analysis (BIA) or dual X-ray absorptiometry (DEXA) [13]. Some other similar methods have been proposed (mini cachexia score, cachexia staging score), however, none of them have been widely used in clinics because

of the huge number of required measurements and questionnaires. In addition, some cachexia scores do not ensure the correct classification of pre-cachexia and cachexia. Accordingly, until now, there is a lack of an accurate diagnostic and classification tool for CC [14].

1.2 Mechanisms of cachexia

1.2.1 Malnutrition

Malnutrition is a key feature of cachexia and differs dramatically from malnutrition due to simple starvation. It mainly results from anorexia and the presence of the tumor, which is draining substrates from the host organism. Additionally, medical and surgical anticancer treatments negatively impact the nutritional status [15]. Cancer hosts may present physical issues that can reduce food intake and nutrient absorption such as diarrhea, vomiting, intestinal obstructions, and malabsorption [16]. Importantly, patients with obstructing head and neck or oesophageal cancers and undergoing radiotherapy are at high risk of malnutrition and body weight loss [15]. Furthermore, intestinal malabsorption, mainly present in patients with gastrointestinal (GI) malignancies, may be an additional challenge by affecting metabolic efficiency [17].

The regulation of appetite is a very complex system and the development of anorexia in cancer patients is attributable to pro-inflammatory cytokines, peptides, hormones, and other humoral mediators. Briefly, cytokines released in the hypothalamus stimulate the production of antagonistic (anorexigenic) molecules by POMC/CART neurons and inhibit the secretion of orexigenic mediators by neuropeptide Y (NPY)/agouti gene-related protein (AGRP) neurons [18]. Together with inflammation, the homeostasis of ghrelin and parathyroid hormone-related protein (PTHrP) have also an impact on the pathogenesis of cancer-induced anorexia [19]. Ghrelin is a stomach-derived hormone involved among others, in the regulation of appetite and glucose metabolism influencing energy metabolism [17]. Altered ghrelin levels have been found in cancer patients, overall increased. Particularly, this elevation has been observed in patients with cachexia and could be due to ghrelin resistance [20,21]. Therefore, anorexia assessment and management are decisive for cancer patient care. Such an assessment is typically reached by questionnaires, like the Functional Assessment of Anorexia Cachexia Therapy (FAACT), which gives an evaluation of the general aspects of QoL considering social and emotional well-being as well as specific anorexia/cachexia-related issues among others. Furthermore, the Mini Nutritional Assessment (MNA) and Patient-Generated Subjective Global Assessment (PG-SGA) have also been used to evaluate the nutritional status of cancer patients, and both incorporate objective and

subjective questionnaires [22,23]. However, cancer-related malnutrition is still largely overlooked and not properly addressed in clinics [15].

1.2.2 Inflammation and cachexia-inducing mediators

Systemic inflammation is mediated by pro-inflammatory cytokines and other factors, produced both by the tumor and the host immune system [5,24]. These factors contribute to protein and fat degradation, altered energy metabolism, and impaired myogenesis [3,25]. Some of the pro-inflammatory cytokines implicated in muscle wasting are IL-1, IL-8, IL-6, TNF-α, TNF receptor-associated factor 6 and tumor necrosis factor-like weak inducer of apoptosis (TWEAK) [24]. Mechanistically, pro-inflammatory cytokines may activate the JAK/STAT3 pathway increasing the transcription of myostatin. Subsequently, myostatin can activate the transcription factors Smad2 and Smad3 leading to reduced protein synthesis by suppressing Protein kinase B (AKT) or increasing protein degradation by elevating Forkhead box O (FoxOs) transcription factors [24]. In addition, pro-inflammatory cytokines can also activate the signaling pathways dependent on NF-κB and p38 mitogen-activated protein kinases, exacerbating protein degradation via the ubiquitin-proteasome system (UPS) and consequently, contributing to muscle wasting [5,24]. Increased levels of TGF-β secreted by many solid tumors have been found in CC. TGFβ-dependent signaling to the skeletal muscle causes an aberrant Ca^{2+} leakage from the sarcoplasmic reticulum, resulting in muscle weakness [5]

Lastly, adipose tissue depletion can also be mediated through pro-inflammatory cytokines by enhancing lipolysis and reducing lipogenesis. Importantly, it has been suggested that fat tissue loss is linked with reduced muscle mass in CC [5]. Indeed, the breakdown of triacylglycerols from adipocytes is thought to precede skeletal muscle protein degradation since it seems to generate signals responsible for the activation of proteolysis. Moreover, pro-inflammatory mediators may promote fat mass depletion in white adipose tissue through the upregulation of the expression of uncoupling protein 1 (UCP1) resulting in lipid mobilization and energy expenditure [3].

1.2.3 Altered energy balance

Overall, cachectic patients present a negative energy balance causing a body store depletion because of the occurrence of multiple factors such as reduced energy intake, increased resting energy expenditure (REE), and the competition between tumor and host for energetic substrates [5,26].

On the one hand, futile cycles that appear in CC represent a source of energetic inefficiency (Figure 2). Tumors generally demand high quantities of glucose compared with normal cells. The increase in anaerobic metabolism, even in normoxia conditions, known as the Warburg effect, negatively influences energy balance leading to wasting. Cancer patients show increased Cori cycle activity in the liver where lactate is recycled to glucose via gluconeogenesis. Converting lactate to glucose requires ATP, and transporting the glucose back to muscles also requires energy. This process is considered energy-inefficient, especially when compared to directly using oxygen-dependent pathways for energy production [27].

Several lines of evidence suggest that inflammatory mediators play a crucial role in the loss of muscle mass and function. In this regard, TNF-α can promote a futile energy-wasting cycle in skeletal muscle inducing proteolysis for amino acid release. This free amino acid source is commonly utilized to provide energy to the tumor and to support tumor protein synthesis. In addition, the metabolism of amino acids requires extra energy for the waste nitrogen elimination, thus, contributing to energy wasting in cachexia [17]. Finally, skeletal muscle can also supply amino acids, particularly glutamine and alanine for glucose formation by gluconeogenesis in the liver [28].

Interestingly, during CC, energy wasting in the liver drives a reduction in the oxidative phosphorylation (OXPHOS) capacity of mitochondria because of changes in cardiolipin content. In addition, reduced consumption of hepatic triglyceride stores has been attributable to hepatosteatosis observed in CC. In summary, CC induces alterations in metabolism, leading to the switch from OXPHOS to glycolysis for energy generation in cancer cells [17].

On the other hand, the elevated REE in cachexia is also due to the UCP upregulation in the skeletal muscle and the adipose tissue resulting in energy dissipation via thermogenesis. This abnormality is favored by inflammation since the UCPs are induced by pro-inflammatory cytokines, such as TNF-α and IL-6 [24,26]. Furthermore, in the context of ATP mismanagement, skeletal muscle mitochondrial ATP generation may be compromised, for instance, by proton leakage from the inner mitochondrial membrane [26].

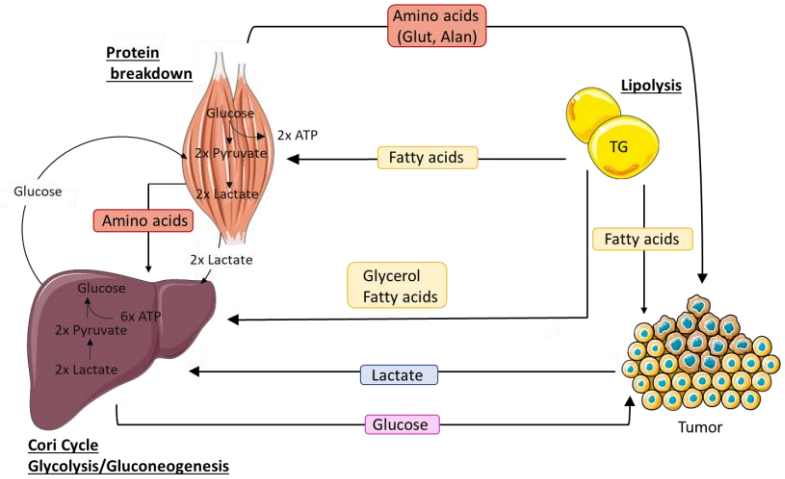


Figure 2. Futile energy-wasting cycles in CC. Adapted from Rohm *et al.* 2019

Mitochondria are the powerhouse of cells, responsible for generating energy in the form of ATP through OXPHOS. Mitochondrial dysfunction, mainly caused by tumor-related factors and systemic inflammation, leads to a disrupted capacity for oxidative metabolism in skeletal muscle (Figure 3). The pathological inflammation mediated by cytokines IL-6, TNF-α, and TGF-β drives mitochondrial abnormalities, including altered mitochondrial mass and morphology [29]. IL-6 can trigger the signal transducer and activator of transcription 3 (STAT3) impinging on the protein balance in skeletal muscle [30]. Furthermore, the NF-κB activation induced by TNF-α is correlated with both decreased muscle oxidative capacity and the regulation of mitochondrial biogenesis. Pro-inflammatory factors reduce the activation of key regulators of muscle mitochondrial biogenesis such as the peroxisome-proliferator gamma-activated receptor (PGC-1α), Nuclear respiratory factor 1 (NRF-1), and Sirtuin 1 (Sirt-1) [29,31]. Lastly, this deficiency in mitochondrial function is related to excessive reactive oxygen species (ROS) generation driving both protein and DNA damage and lastly, apoptosis in the skeletal muscle [29].

Mitochondrial dynamics and disposal play also an important role in maintaining healthy skeletal muscle function. Unbalanced mitochondrial fission and fusion could drive the hyperactivation of protein catabolism through pathways depending on AMP-activated protein kinase (AMPK) and FoxO3 [32]. Mitophagy is the selective degradation of mitochondria by autophagy. It is the main mechanism for the elimination of altered or damaged mitochondria from the cell to prevent further damage and maintain cellular stability. Also, mitophagy was reported to be enhanced in CC, as shown by the upregulation of BCL2 Interacting Protein 3 (Bnip3L) and Parkin1 gene expression in the muscles of cancer patients [33,34].

On the whole, dysfunctional and bioenergetically inefficient mitochondria have been shown in CC resulting in increased ROS levels in tissues, such as the skeletal muscle, and insulin resistance [24,25,35]. Disrupted mitochondrial homeostasis in cancer patients can have a deleterious effect on functional capacity, QoL and survival [29].

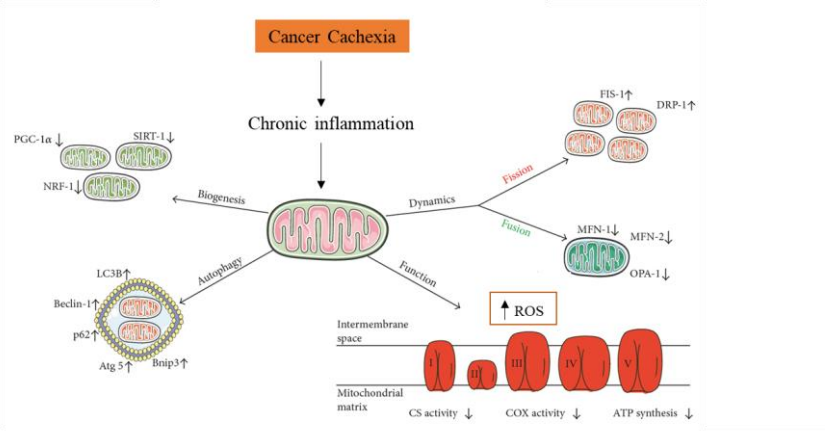


Figure 3. Dysfunctional regulation of mitochondrial dynamics, mitophagy, and biogenesis in cachectic muscle. Adapted from VanderVeen *et al.* 2017

1.2.4 Muscle protein metabolism imbalance

In humans, it is estimated that skeletal muscle contains 50-75% of the body proteins and it comprises approximately 40% of total body weight. Further, represents 30-50% of whole-body protein turnover. The skeletal muscle is a very orderly tissue surrounded by a connective tissue sheath called epimysium. Briefly, bundles of muscle fibers (also called myofibers) are surrounded by another layer of connective tissue known as the perimysium. Each myofiber is likewise bordered by endomysium. Inside the myofibers, there are thousands of filaments, called myofibrils, which contain the functional unit for muscle contraction, the sarcomere (Figure 4) [6].

Skeletal muscle not only contributes to locomotive functions, but it is also a metabolically active tissue that is composed of various types of myofibers [6]. Generally, the adult mammalian skeletal muscle includes type I, IIa, IIb, and IIx myofibers, while type IIb fibers are lacking. Each muscle fiber expresses a specific type of myosin heavy chain isoform (MyHC) which provides differential contractility to the muscle. Type I fibers, also called oxidative slow-twitch fibers, are endowed with high mitochondrial and myoglobin content and are more useful than type II fibers during aerobic (or endurance) exercise when the demand for oxygen increases. On the contrary, type II fibers (fast-twitch) are mainly glycolytic, especially type IIb fibers, being associated with increased intensity and short-duration activity performances [6,36,37].

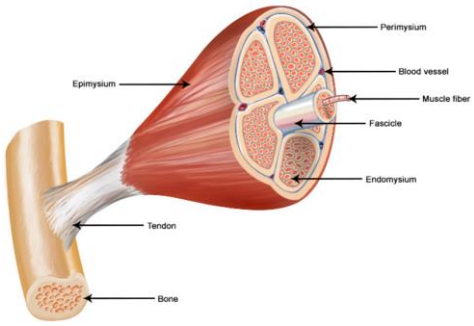


Figure 4. Skeletal muscle structure. From the National Cancer Institute (https://training.seer.cancer.gov/anatomy/muscular/structure.html)

The correct balance between protein anabolism and catabolism (proteostasis) is crucial for the maintenance of skeletal muscle structure and function and both processes depend, among others, on several factors such as nutritional status, hormonal balance, physical activity, and injury or disease [6]. During CC, proteostasis is disrupted, notably towards catabolism, which plays a central role in body and muscle wasting [24]. CC is distinguished by an overactivation of the main catabolic pathways in the skeletal muscle (Figure 5), markedly, those dependent on ubiquitin-proteasome (UPS) and autophagy [38,39].

Under normal physiological conditions, active AKT phosphorylates FoxOs that inhibit the transcription of genes encoding the muscle-specific ubiquitin ligases (MuRF1 and Atrogin-1). This causes a blockage of UPS-mediated protein degradation. In addition, AKT can activate the mammalian target of rapamycin complex 1 (mTORC1), thus promoting protein anabolism. It has been reported that in CC both the pro-inflammatory cytokines and reduced levels of insulin-like growth factor 1 (IGF-1) inhibit the activity of AKT resulting in an altered protein homeostasis which leads to increasing muscle atrophy [5].

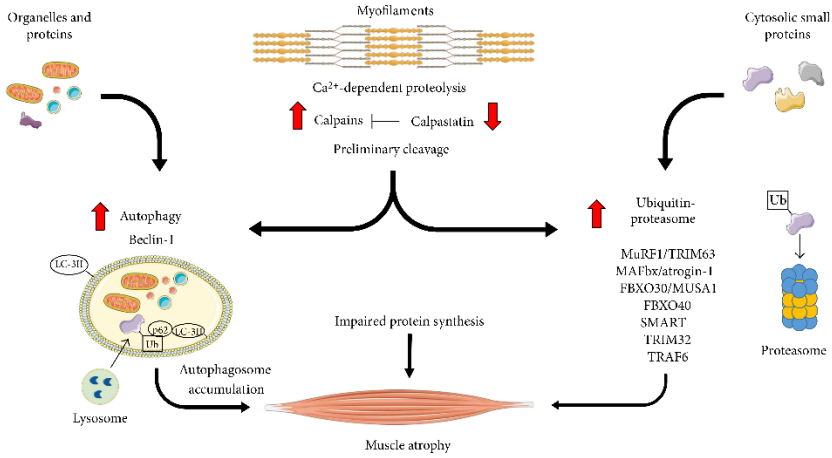


Figure 5. Main catabolic pathways contributing to protein breakdown in CC. From Penna *et al.* 2018

The lysosomal degradative system participates in the degradation of cytoplasmic proteins and organelle recycling. Autophagy is activated under stress conditions and nutrient deprivation to recycle biomolecules for the synthesis of new components, replace damaged cellular constituents, and release stored energy in the cell [39]. Penna *et al.* (2013) reported that autophagic-lysosomal degradation is induced in three different models of cancer-associated muscle atrophy [40]. Nevertheless, not only proteasomes and lysosomes promote the process of protein hypercatabolism during CC, especially in the initial steps for myofilament degradation. Calpains are intracellular calcium-dependent cysteine proteases that are activated by the presence of calcium ions. When calcium levels rise within the cells calpains become activated and can cleave specific target proteins. The system also includes calpastatin which has an inhibitor role [38]. The implication of calpains in CC is multifaceted. They are involved in cleaving key proteins in muscle cells, such as components of the cytoskeleton, leading to muscle protein degradation and atrophy. Moreover, calpains may activate other proteolytic pathways such as the UPS [41].

Interestingly, more atrophy is observed in type II glycolytic fibers than in type I oxidative fibers in conditions such as muscle wasting and aging. The low content of PGC-1α in type II fibers makes them more sensitive to degradation compared to type I fibers [42]. PGC-1α overexpression partly prevents muscle atrophy induced by fasting and disuse atrophy, but also in many systemic diseases by reducing the FoxO-dependent upregulation of ubiquitin ligases MuRF1 and Atrogin-1 [43]. Besides that, it is reported that during aging a change in fiber type from fast to slow appears [44]. Ultimately, type IIx fibers are barely present in active healthy individuals but may become prominent in pathological states or inactive subjects [45].

Last but not least, myogenesis is also compromised in CC. In adults, myogenesis is a physiological process that occurs when new muscle fibers are formed or when damaged muscle tissue is repaired and regenerated. An improper or impaired myogenesis originates the substitution of muscle with connective tissue (fibrosis). In experimental and clinical studies of CC, it has been shown that skeletal muscle increases the expression of Pax7, a marker of satellite cell activation, and reduced levels of myogenin, an indicator of ongoing differentiation, leading to reduced formation of mature myoblasts [46]. Moreover, Hogan *et al.* (2018) described that tumor-derived secreted cytokines impair myogenesis and immune cell homeostasis in the skeletal muscle of tumor-bearing mice [47].

1.2.5 Gut microbiota dysregulations

The human microbiome has been getting an increasing attention in the last few years. Firstly, the association between microbiome in the healthy state and in disease pathogenesis has been widely reported, secondly, technological development has facilitated the characterization of microbial populations. Gut microbiota influences many physiological functions, protects against pathogens, and educates the immune system [48]. Previous studies have demonstrated a link between changes in intestinal microbiota composition and the pathogenesis of CC [49–51] (Figure 6). Particularly, the intensification of systemic inflammation has been associated with increased intestinal permeability and translocation of pro-inflammatory microbial compounds induced by dysbiosis. Mechanistically, dysbiotic states lead to the spread of endotoxins and bacterial products into blood circulation with the subsequent activation of the host immune response [52].

Muscle atrophy may be influenced by the impact of gut microbiota [51,53,54]. In experimental models, some specific bacteria strains prevent skeletal muscle wasting by the activation of the IGF-1/phosphatidylinositol 3-kinase/AKT pathway mediated by the NLRC4 inflammasome. Moreover, other references support the impact of gut microbiota on skeletal muscle metabolism and atrophy affecting the expression of molecules involved in proteolytic systems (ubiquitin ligases and autophagy) and muscle functionality [50].

What is more, the composition of gut microbiota affects nutrient availability. For instance, the microbiota can utilize substrates that are indigestible by the host generating new nutrients. Short-chain fatty acids (SCFAs) are produced from indigestible dietary fibers that may be used as a nutrient source for the intestinal mucosa and also participate in immune responses and tumorigenesis in the gut [48]. Some references have highlighted the positive effect of SCFAs in the modulation of mitochondrial function [55–57]. SCFAs stimulate the muscle oxidative capacity

by boosting mitochondrial biogenesis through the activation of AMPK [58] and, likewise, enhancing OXPHOS and β-oxidation in cell lines [56,59]. Additionally, microbiota-derived substances such as phenolic compounds utilized by differentiated human LHCN-M2 myoblasts line exert a direct effect on muscle glycogen availability and promote glucose uptake [60].

In the context of obesity, the formation of SCFAs by gut bacteria has been associated with promoting body weight gain employing the regulation of food intake, insulin sensitivity, and substrate metabolism. Conversely, decreased levels of SCFAs have been found in both cachectic mice and patients. Additionally, muscle anabolic processes can be influenced by the branched-chain amino acids (BCAA) bioavailability deriving from microbiota metabolism for host utilization [52]. Lastly, a study compared the skeletal muscle of germ-free mice with animals that had gut microbiota. Germ-free animals showed muscle atrophy and disrupted both muscle growth and metabolism, which were restored when animals received microbiota transplantation [61].

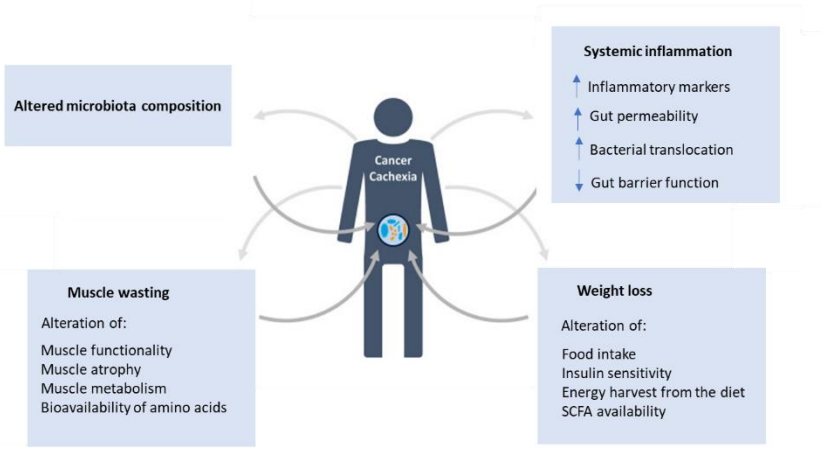


Figure 6. Interactions between the gut microbiota and different aspects of CC
Adapted from Ziemons *et al.* 2021

1.3 Nutritional interventions for cancer cachexia

1.3.1 Conventional nutritional interventions

Nutritional intervention is a fundamental pillar for the treatment of cancer patients, especially when affected by cachexia. Some reports support the beneficial effects of nutritional tools, such as nutritional counselling, specific dietary supplementation, and enteral/parenteral nutrition [62–64]. Many studies provide evidence about the improvement in overall survival (OS), enhanced both body weight loss and muscle wasting and reduced anti-cancer drug toxicity in cancer patients exposed to nutritional interventions [65,66]. The European Society for Clinical

Nutrition and Metabolism (ESPEN) and the American Society of Clinical Oncology (ASCO) respectively, provided nutritional recommendations [15,67] and guidelines for the management of CC in adult patients with advanced cancer [68].

The estimation of energy requirements in patients with cancer can be a challenge. In this context, ESPEN guidelines propose, based on healthy subjects, an energy recommendation of 25-30 kcal/kg/day. This is because no individual estimations of total energy expenditure are usually performed. Moreover, a sufficient macronutrient and micronutrient intake are essential for the prevention of dietary deficiencies and, importantly, for the maintenance or gain of skeletal muscle [69].

Protein intake should be in the range of 1-1,5 g/kg/day and the supplementation with single amino acids or their derivatives is known to exert a stimulus for protein synthesis in muscle tissue. BCAA such as leucine, isoleucine, and valine have been extensively studied for their anabolic effect on the skeletal muscle. Along this line, glutamine, although a non-essential amino acid, has been highly proposed in nutritional supplementation approaches because of its potential to prevent muscle wasting in cancer patients. In addition, β-hydroxy-β-methyl butyrate (HMB), a metabolite of leucine, has been studied for its role in reducing protein degradation rates [69,70]. Cancer patients who received supplementation with arginine, glutamine, and HMB showed improved hand-grip strength. However, no significant maintenance of LBM was observed [71], consequently, the HMB administration in cancer patients still requires additional clinical investigations [72].

L-carnitine is an amino acid derivative that is responsible for the transport of long-chain fatty acids from the cytosol into the mitochondrial matrix for β-oxidation. For this reason, it is mainly stored in tissues that use fatty acids as fuel, such as skeletal and cardiac muscle [73]. On this subject, a double-blind trial investigated the effectiveness of L-carnitine supplementation on cancer patients suffering from advanced pancreatic cancer. Even though no significant differences were demonstrated, the results were encouraging, showing improvement in body weight and body composition [74]. Creatine can be endogenously synthesized and is mainly stored and used in the skeletal muscle because of its role in providing a rapid energy source for muscle contraction. Oral supplementation with creatine has also been shown to enhance both muscle strength and LBM. Moreover, creatine presents antioxidant properties that may avoid the increase of circulating pro-inflammatory cytokines [73].

As outlined, inflammation is one of the main hallmarks of CC. In this regard, the use of eicosapentaenoic acid (EPA), generally known for its anti-inflammatory effect, has been broadly studied in clinics. The literature provides evidence supporting that EPA can reduce the level of pro-inflammatory cytokines (IL-6) and decline the activity of PIF. Nevertheless, until now, EPA oral administration has not shown clear evidence against CC. Several studies described that EPA supplementation increases body weight or LBM in cachectic patients. On the contrary, other reports do not show significant improvement in body weight, nutritional status, and systemic inflammation [70].

The supplementation with micronutrients has also been considered an important component of nutritional support for the prevention of malnutrition in cancer patients. Vitamin C supplementation improved the QoL, fatigue, and appetite loss in terminal cancer patients [75], and vitamin D administration was demonstrated to improve muscle weakness [76]. However, the medical recommendation of micronutrients in CC still needs additional studies [77].

1.3.2 New approaches on the gut microbiota

As stated above, a few studies put in evidence an interaction between the gut microbiota and CC. In this context, Bindels *et al*. (2016) observed that tumor hosts receiving a synbiotic containing both inulin-type fructans and live *Lactobacillus reuteri* were not only able to restore the healthy bacteria composition but also showed a reduced tumor growth accompanied by reduced muscle wasting and prolonged survival [78]. In a prospective clinical trial, patients suffering from head and neck cancer and undergoing cachexia received nutritional intervention consisting of high-calorie high-protein supplements enriched with omega-3 fatty acids, micronutrients, and probiotics for three months. The study demonstrated a significant improvement in body weight maintenance and increased serum albumin and prealbumin levels in patients with a body mass index lower than 19. However, these effects were no longer detectable after the fourth week of treatment [79].

More recently, Clercq *et al.* (2021) reported a clinical trial performed on gastroesophageal cancer patients with cachexia, who were assigned to fecal microbiota transplantation (FMT) either autologous or allogenic, from overweight/obese donors. The donors were considered as “healthy obese” since they did not present with metabolic syndrome. The rationale was that FMT from obese donors could improve appetite, metabolism, and body composition in those patients. Although the disease control rate (DCR) and median survival were improved in patients receiving allogenic FMT, the treatment did not show any effect regarding satiety and cachexia-related parameters [80].

Nowadays, there is an increasing interest in going further to clearly understand the relationship between gut microbiota and cancer [81]. Nonetheless, human studies are still in the initial stages. Indeed, so far, the current European clinical guidelines only mention the use of probiotics for the treatment of radiation-induced diarrhea [15].

1.3.3 Ketogenic diets and supplementation with ketone bodies

Ketogenic diets (KDs) are very low carbohydrate, high-fat, and moderate protein diets. Generally, KDs consist in a ratio of macronutrients of 3-4:1 (3-4g of fat to 1g of carbohydrates and proteins combined) resulting in 80-90% of total energy coming from fats, 6-8% from proteins, and 2-4% from carbohydrates [82,83]. KDs lead to "physiological ketosis", a metabolic condition characterized by an elevation in the blood ketone bodies (KBs) along with a decrease in blood glucose levels which mimics the metabolic effects of fasting. Thus, during nutritional ketosis blood KB concentrations are above 0.5 mmol/L, differing from uncontrolled pathological diabetic ketoacidosis in which the ketonemia can exceed 20 mmol/L [84,85].

During periods in which glucose reserves are low (low carbohydrate diet, fasting, post-exercise), KBs become an alternative source of energy for extrahepatic tissues such as the heart, skeletal muscle, and brain [86] forcing cells to rely on oxidative metabolism [83]. KBs can be converted to an oxidizable state without the need for ATP. They are essentially a more efficient energy substrate than glucose or fatty acids, thus allowing increased muscular work for a given energy cost [87]. Ketogenesis occurs in the liver, particularly in the mitochondrial matrix. Fatty acids are metabolized into mitochondria through β-oxidation resulting in the overproduction of acetyl-CoA. The excess of acetyl-CoA leads to a series of condensation reactions driving the production of acetoacetyl CoA. Once formed, this molecule reacts with another molecule of acetyl CoA forming HMG-CoA in a rate-limiting and irreversible reaction mediated by HMG-CoA synthase 2. Then, it is separated into acetoacetate (AcAc), which is further reduced to β-hydroxybutyrate (β-HB) by β-HB dehydrogenase (β-HBD) (Figure 7) [88]. Although AcAc is the main ketone body produced in the liver, the primary circulating ketone is β-HB [86]. Also, acetone is produced by the spontaneous decarboxylation of acetoacetate and is usually removed via respiration. Subsequently, KBs are exported into the bloodstream where they are eventually imported into other tissues with high metabolic requirements. Once reached the destination, β-HB is oxidized to AcAc by β-HBD. Then, AcAc is hydrolyzed to acetoacetyl CoA succinate through the enzyme succinyl CoA:3-oxoacid CoA transferase (OXCT), and acetoacetyl-CoA thiolase also known as acetyl-CoA acetyltransferase (ACAT) catalyzes the further cleavage of the acetoacetyl

CoA to produce acetyl CoA. Finally, succinate and acetyl CoA serve as substrates for the electron transfer chain complex II and the tricarboxylic acid (TCA) cycle, respectively [88].

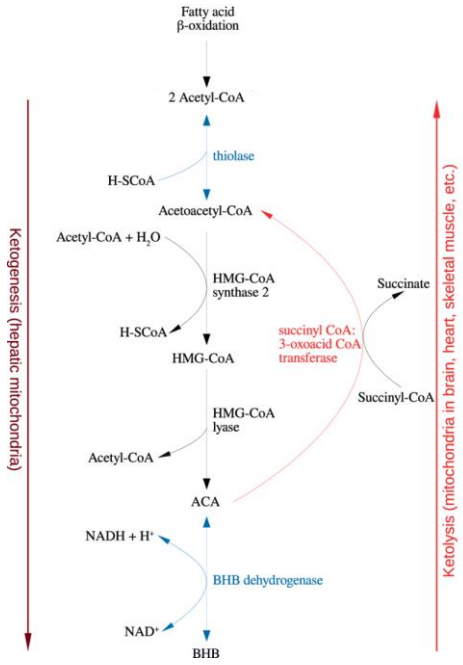


Figure 7. Summary of ketogenesis and ketolysis pathways.
Adapted from Morris *et al.* 2020

Exogenous ketone supplementation (KS) has emerged as an alternative to restrictive KDs. There is abundant evidence suggesting that oral KB administration has the potential to induce acute nutritional ketosis avoiding carbohydrate restriction [87–92]. Salts and esters of β-HB and AcAc, in addition to medium-chain triglycerides (MCT), are considered safe and are commercially available [87].

Nutritional ketosis on muscle metabolism

Historically, KDs were developed in the 1920s as a treatment for refractory epilepsy, but it has also been utilized in different pathological states such as neurological diseases, diabetes, obesity, and cancer [85]. Indeed, clinical evidence supports the use of KDs as a therapeutic approach to counteract the metabolic syndrome thanks to its potential to improve body weight and body composition together with insulin resistance and dyslipidemia [82].

Even though KDs have been advised for the treatment of obesity, KDs may reduce fat mass while preserving lean mass. Multiple lines of evidence support that low-carbohydrate diets and KDs can enhance the expression, content, or activity of proteins involved in OXPHOS, TCA, fatty

acid oxidation, and ketolysis in the brain, skeletal muscle, and adipose tissue [93]. In preclinical studies, KD-fed mice showed improved endurance capacity by up-regulation of both fatty acid and ketone oxidation in skeletal muscle. In addition, the KD relieved the damage induced by IL-6 production in the skeletal muscle in response to exhaustive exercise [94]. The reduction of IL-6-dependent stress prevents the build-up of creatine kinase and lactate dehydrogenase in the muscles during exercise [95]. Furthermore, a ketogenic amino acid-rich diet reverted the high-fat diet-induced shift in muscle fiber type from type I and type IIa to type IIb in the *gastrocnemius* of diabetic mice [96].

In healthy subjects, a KD combined with an exercise training program for 12 weeks resulted in improved fasting insulin and decreased body weight relative to fat mass when compared with the trained subjects who followed the habitual diet. The KD enhances mitochondrial activity and efficiency and induces a shift in macronutrient metabolism toward greater fat oxidation compared to carbohydrates [97], however, these effects were partially related to ketone oxidation in the skeletal muscle.

Nonetheless, there are contradictory data regarding the effects of long-term KDs on muscle mass, exercise performance, and protection against metabolic syndrome. Some references have pointed out that the influence of KDs has little to no effect on skeletal muscle when combined with resistance training. Moreover, KDs can cause dyslipidemia, glucose intolerance, hepatic steatosis, and increased systemic inflammation [86,98]. Animals consuming a KD for seven days showed muscle mass loss similar to the chronic effects of starvation on muscle wasting. The authors reported a decrease in plasma insulin and IGF-1, increased corticosterone levels, and upregulation of genes involved in muscle atrophy and antioxidant defense. Furthermore, some anabolic markers were suppressed in the skeletal muscle [99].

KS has gained interest in recent years because of its ergogenic effects [97,100] in essence, related to glycogen conservation, prevention of proteolysis in skeletal muscle, improvement of endurance performance and recovery after exercise [101,102]. KS alters fuel selection through the reduction of the glycolytic flux and increased KB oxidation in peripheral tissues, particularly in trained individuals [101,103]. Nevertheless, most studies have not been able to support the benefits of KS in physical performance, in part due to the heterogeneity found across studies [100] and because of the high occurrence of GI symptoms (e.g., nausea, abdominal cramps, diarrhea) [102].

In regard to muscle regeneration, mice receiving AcAc injections into the *tibialis anterior* (TA) muscle showed enhanced muscle regeneration after cardiotoxin-induced muscle injury by

stimulating satellite cell activation and proliferation. In addition, AcAc treatment increased the myofiber size in muscular dystrophin-deficient (*mdx)* mice, leading to the preservation of muscle structural integrity [104].

KDs and KS in cancer

KDs have been proposed as adjuvant therapy by targeting the reprogrammed metabolism of cancer cells (Warburg effect). Until now, several studies have demonstrated the beneficial effects of KDs in a wide variety of cancers including breast, endometrial, pancreatic, and bladder cancers among others [82]. KDs might promote a stressful environment for cancer cell growth and proliferation due to the reduction in blood glucose levels accompanied by a reduction of blood insulin and/or IGF-1 exerting the same anti-tumor strategy of fasting [82,105]. The decrease in glucose availability leads to a boosted oxidative stress level in cancer cells caused by the increase in ROS production that may potentiate the antitumoral effect of conventional radio- and chemotherapy [82]. Deficiencies in ketolytic enzymes are important for tumor survival when glucose is insufficient and cells need KBs for energy [106].

Additionally, KDs can regulate gene expression [82,107], in particular, β-HB can inhibit histone deacetylases (HDACs), enzymes that can regulate the transcription of many genes, thus, interfering in multiple stages of cancer. Indeed, increased levels of HDACs are involved in advanced disease and poor outcomes in cancer patients [82]. Concerning the skeletal muscle, hyperacetylation of transcription factors and nuclear cofactors can lead to muscle atrophy, since it results in overactivation of the E3 ubiquitin ligases [107]. Lastly, the endogenous inhibition of HDAC by β-HB, may increase mitochondrial biogenesis by up-regulation of PGC-1α and consequently, potentiate oxidative metabolism [108]. Therefore, epigenetic alterations driven by KDs can interfere both in tumor growth and proliferation and in muscle wasting.

In preclinical studies, a KD sensitizes murine pancreatic cancer tumors to cytotoxic chemotherapy [109] and extends the overall median survival in transgenic mice that develop Pancreatic ductal adenocarcinoma (PDAC), particularly in females [110]. The combined therapy inhibited the Extracellular signal-regulated kinases (ERK) and AKT pathways, commonly activated in PDAC. Interestingly, the use of a KD alone did not impact survival suggesting that dietary interventions alone are not sufficient as anticancer therapy [110].

In a pilot trial, a KD was well tolerated and improved the QoL measured by the EORTC QLQ-C30 questionnaire in patients with advanced metastatic tumors [111]. However, in a systematic review, it was reported that patients with various types of cancers following a restrictive

carb diet, aiming to achieve ketosis, did not show decisive evidence for improved OS or anti-tumor effects [112]. Importantly, it should be noted that in most trials, there was not much adherence to the diet which was primarily attributed to fatigue and GI side effects like diarrhea, constipation, nausea, and vomiting. Moreover, patients found the food unpleasant and had difficulty incorporating the diet into their daily lives [82,111–113] Nonetheless, symptoms can vary and generally decrease after a week of diet adaptation [87]. Noteworthy, in many cases, there is no option to distinguish between the negative effects caused by KDs and anti-cancer therapies [112].

Many patients with cancer experience poor nutritional status and risk of micronutrient deficiency [69]. KDs are deficient in vitamin D and calcium, vitamin C, thiamine, selenium, zinc, and magnesium. Therefore, mineral and vitamin supplements must be prescribed along with KDs [114]. To date, standardized dietary protocols are still lacking [112], hence the use of KDs in the clinic requires close medical supervision and appropriate counseling from dieticians and doctors. KD plans should be personalized and require a thorough evaluation [95].

Lastly, the use of KS as a cancer therapy has also been investigated in preclinical studies [90,115]. The treatment with β-HB has been associated with both inhibition of cancer cell proliferation [116], promotion of tumor growth in hepatocellular carcinoma [117] and stimulation of the dissemination of metastasis in PDAC [118]. In some cases, the tumor metabolic flexibility and the presence of non-cancer cells in the tumor microenvironment (stromal cells and extracellular matrix) could allow ketones as fuel for the tumor mass [118,119].

KDs and KS in cancer cachexia

Preclinical research suggests that KDs and KS may have anti-cachectic properties (Figure 8). A ketogenic formula (KF) enriched with MCT, showed improved body weight loss and muscle wasting in mice with colorectal cancer (CRC) [120]. Moreover, KF-fed animals had considerably lower tumor weights and plasma IL-6 levels in comparison with the tumor-bearing group. It has been observed that β-HB exerts an anti-inflammatory and antioxidant effect [83,121]. In a study published by Shukla *et al.* (2014), KB treatment inhibited the reduction in the size of myotubes exposed to tumor cell-conditioned medium; such a pattern was associated with decreased MuRF1 and Atrogin-1 mRNA levels. Furthermore, mice carrying S2-013 pancreatic cancer cells and fed with a KD for three weeks showed reduced loss of both body weight (without tumor mass) and skeletal muscle compared to controls [115]. A diet supplemented with a ketone ester (R/S 1,3-Butanediol Acetoacetate Diester) prevented skeletal muscle atrophy and inflammation-induced catabolism [89]. Nevertheless, in mice with lung cancer, a KD or ketone ester supplementation did

not improve body weight, muscle mass maintenance and survival [91]. Lastly, even though a KD slowed the tumor progression, the nutritional intervention led to disrupted systemic redox homeostasis and accelerated CC onset in mouse models of IL-6-producing cancers [122].

Finally, in patients with cachexia and malnourished pediatric patients, the KD fosters weight gain and protects against a negative nitrogen balance (an indicator of total protein in the body) [82]. Ketosis prevents protein degradation by decreasing the export of alanine and glutamine for gluconeogenesis in the liver [123]. Furthermore, the mechanism of nitrogen conservation during ketosis was investigated [124]. Sodium β-HB infusions were administered to healthy subjects and subsequently, leucine flux and metabolism were examined. Mild ketosis notably reduced leucine oxidation and therefore, improved the incorporation of leucine into muscle protein, ultimately leading to improved protein synthesis.

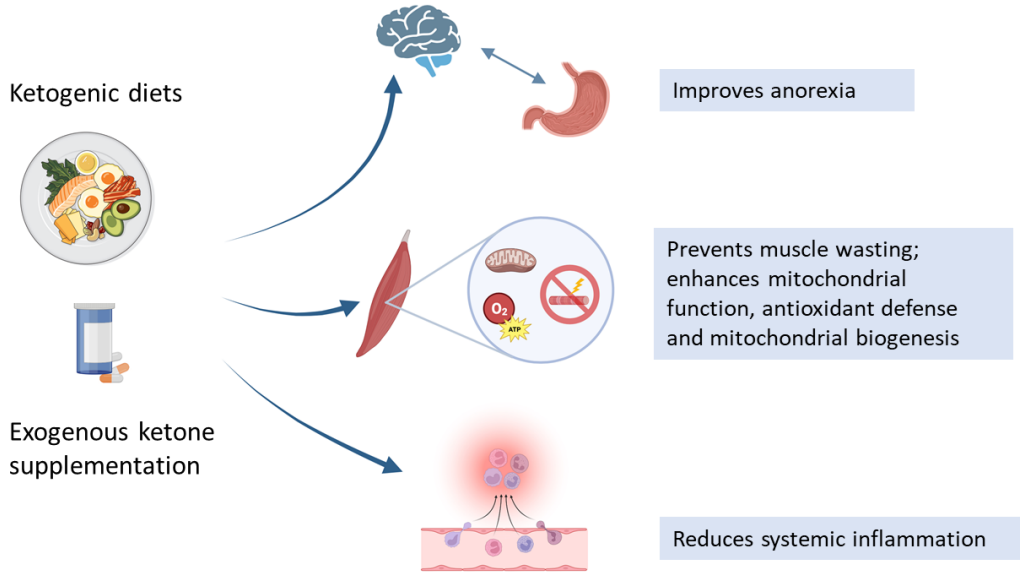


Figure 8. Potential protective effect of nutritional ketosis in CC

An alternative approach to KDs and KS

Although KDs and KS have shown a potential impact against CC, they are not exempt from side effects. As mentioned, a prolonged KD can induce among others, fatigue, dyslipidaemia and a pro-inflammatory state [86,112]. Additionally, patient compliance with the diet proves to be challenging [112,125]. Besides that, KS has also been linked with dizziness and GI distress [102,126].

MCT are composed of medium-chain fatty acids and are potent ketogenic agents [87,127]. The safety of dietary consumption of MCT in humans (up to levels of 1g/kg) has been confirmed in several clinical trials [128]. Furthermore, the Food and Drug Administration (FDA) has also recognized MCT supplementation as a safe nutritional source for exempt infant formulas at levels not exceeding 21g/L [129]. Natural food sources of MCT include coconut oil, palm kernel oil, and milk fat [130]. Its use in the enrichment of KDs has been widely present in several scientific reports [88,131–134]. Fortifying KDs with MCT facilitates patients to achieve ketosis avoiding carbohydrate-restricted diets [135]. Unlike long-chain triglycerides (LCT), MCT are absorbed from the small intestine, without the need to be incorporated into chylomicrons, due to their smaller molecular size (8-12 carbon atoms) and better water solubility, which allows them to reach the liver directly via the portal vein [136].

A parenteral solution enriched with MCT prevented whole-body protein breakdown in tumor-bearing rats with CC [137]. Another study also showed that in a model of lipopolysaccharide (LPS)-induced cachexia, MCT supplementation exerted positive effects on skeletal muscle through the maintenance of the enzymatic activity of proteins participating in oxidative metabolism [136]. Moreover, due to their overall safety and tolerability, MCT have been employed in clinical trials for the treatment of different pathological conditions [138] such as neurodegenerative disorders [132,139], sarcopenia [140,141], GI malabsorption [142] and cancer [135,142], among others. Patients with anorexia nervosa presented increased blood acylated ghrelin levels after a few weeks of treatment with MCT. Consequently, this increase was associated with increased plasma NPY levels [143], suggesting that MCT could be an effective approach to improve appetite in patients with CC.

So far, only one study has been proposed in which patients with refractory cachexia and dysphagia received lipid emulsions of LCT or LCT/MCT (50:50) via parenteral nutrition for 11 days [144]. In this trial, no differences in nutritional markers were demonstrated among patients receiving nutritional supplementation. Thus, no studies have been performed to date to assess the efficacy of supplementation with MCT only against CC.

1.3.4 Calorie restriction and fasting

Calorie restriction (CR) is considered a reduction in calorie intake, typically between 20 and 50% without malnutrition and with an unchanged macronutrient ratio [145]. CR has emerged as a strategy for the improvement of healthspan and lifespan [146]. CR possible application to the prevention and treatment of age-related diseases has been demonstrated in different experimental

models [145,147]. There are multiple types of CR regimens, from time-restricted feeding (TRF), in which people limit the daily period of meal consumption and then fast the rest of the day, to short periods of fasting followed by a normal diet (intermittent fasting, prolonged fasting, and fasting-mimicking diets “FMD” [147,148].

CR has been associated to effective counteraction of obesity and the resulting metabolic syndrome [149,150], but to date, the findings have not been confirmed by other independent studies. In randomized clinical trials, lasting 2 to 12 months, TRF provides modest to moderate weight loss of 3% to 5% compared to no-intervention controls, along with an improvement in some cardiometabolic risk factors [151]. Although the TRF presents a significant reduction in insulin resistance, it had no impact on blood pressure or plasma lipid levels (cholesterol and triglycerides) in obese subjects who followed the intervention for 8 weeks [152]. During the fasting state, there is a metabolic switch from utilizing glucose to the mobilization of fat storage leading to fatty acid and fatty-acid-derived ketone oxidation which preserves both muscle mass and function [153,154], particularly when the dietary strategy is combined with exercise and resistance training [155,156]. However, in humans, a 6h- TRF has shown a lean mass decrease versus both a 4-h TRF and controls [152] showing that soon after mobilizing lipid stores, also the lean compartments are affected.

Interestingly, the decline of age-dependent sarcopenia that occurs during normal aging in mice is prevented by 30% CR. Moreover, some behavioral changes characterized by increased bursts of activity in a short period were observed in CR animals [157]. A systematic review focused on how intermittent fasting affects athletes and sports performance [158] reported that intermittent fasting positively influences body composition and maintenance of lean mass without negatively affecting physical performance. This overall suggests that the improvement in body composition may enhance aerobic capacity.

Beyond the well characterized effects of CR on muscle health, so far, the effects on muscle regeneration are still partly unclear. The age-related decline in the expression of the transcription factor Pax7 in the muscle stem cells (MuSC) can be restored in aged mice by re-feeding after multiple rounds of a calorically restrictive diet [146]. In another study, Benjamin and colleagues (2022) demonstrated that fasting delayed the activation of MuSC as they entered a deep state of quiescence, resulting in greater resilience to nutritional, cytotoxic, and proliferative stress [159]. Mechanistically, increased levels of β-HB driven by fasting, KD or exogenously administration, inhibits HDAC activity within MuSC leading to acetylation and activation of an HDAC1 target protein p53 which is linked to the deep quiescence and enhanced resilience seen during fasting.

Calorie restriction and cancer

The relationship between excessive food intake, insulin resistance and the risk of cancer is well known. According to epidemiological research, there is a substantial causal relationship between increased insulin resistance, type II diabetes, and colon, liver, pancreas, breast, prostate, endometrial, and lung cancer [147]. The increased circulating insulin levels lead to a decrease in insulin-like growth factor binding protein 1 (IGFBP-1) synthesis, resulting in increased serum IGF-1. IGF-1 signaling may promote cellular proliferation and angiogenesis, DNA damage, and reduce apoptosis in pre-cancerous and cancer cells [160,161]. Intermittent fasting and CR are both associated with a lower incidence of cancer [147,162]. Long-term CR can reduce serum IGF-1 levels by 30% to 40% in mice, while in humans, this decrease is observed only when protein intake is reduced [163]. Therefore, these findings suggest that the lowering of IGF-1 level is fundamental for CR-mediated protection from cancer development [164]. Furthermore, upon nutrient deprivation, mTOR signalling is suppressed. Chronic overfeeding can lead to mTOR hyperactivactivation driving metabolic disorders and favouring tumorigenesis, particularly by stimulating tumor growth and metastasis and inhibiting autophagy [165]. Nevertheless, not all types of cancers show a response to CR, indeed, a minor part of tumors are resistant to the effects of CR [164].

Fasting-mimicking diet

The fasting-mimicking diet (FMD) is a plant-based low-calorie, low-protein, low-sugar diet designed to achieve the advantages of fasting while providing micronutrient nourishment and minimizing the stress of fasting. FMD was developed to replace water-only fasting while maintaining and possibly exceeding its effect on key markers of the fasting response, including changes in IGF-1, IGFBP-1, glucose, and ketone bodies (Table 1) [166]. The human FMD consists of a 4 to 5 days regimen: day 1 of the diet provides 1,090 kcal (10% protein, 56% fat, 34% carbohydrate), days 2-5 are identical in the formulation and supplies 725 kcal (9% protein, 44% fat, 47% carbohydrate) and is suggested to be repeated every 3-4 weeks [146]. After this period of restriction, normal eating habits are resumed for the rest of the month, constituting one cycle [167]. This gradual decrease in calories and alteration in macronutrient ratios are key elements of the FMD protocol. FMD is an emerging nutritional plan focused on applying both specific nutrient combinations and complex food compositions used to complement or replace pharmacological or biological therapies [168].

	Fasting (72 hours)		FMD (96 hours)	
	Effect	**Change**	**Effect**	**Change**
Blood glucose	⬇	~45%	⬇	~40%
Ketone bodies	⬆	~10 fold*	⬆	~10 fold
IGF-1	⬇	~40-70%	⬇	~45%
IGFBP-1	⬆	~7-11 fold	⬆	~8 fold

Table 1. Fasting and FMD induce a similar physiological response in mice. (*after 24 hours of fasting) Adapted from Brandshort *et al.* 2015

In mice fed with 4-day FMD cycles twice a month, starting at 16 months of age, a reduction in visceral fat and the incidence of cancer was shown, in addition to rejuvenating the immune system, delaying cognitive decline and prolonging longevity [146]. In healthy adults, three FMD cycles reduced risk factors/biomarkers for cardiovascular disease, diabetes, aging, and cancer with no adverse effects, supporting evidence for the use of FMDs to promote healthspan [146]. Furthermore, a randomized crossover study of 100 healthy participants who received 3 monthly 5-day FMD cycles showed lowered body weight due in part to a reduction in fat mass, a decrease in blood pressure and IGF-1 five days after the third FMD [169].

Fasting, FMD and cancer

Cancer patients are especially vulnerable to malnutrition because both the disease and its treatments threaten their nutritional status. In fact, between 10% and 20% of deaths are attributable to malnutrition rather than malignant disease [68]. The risk of developing CC raises an obvious safety concern for cancer patients participating in fasting interventions [22,162]. Nevertheless, it has been reported that short-term fasting or limited daily calorie consumption for a limited time (4-5 days) can achieve positive outcomes without compromising body weight and preserving lean body mass [170]. Moreover, fasting could aid in resetting the metabolic program during CC, thus attenuating further muscle atrophy [171].

Chemotherapy tolerability

The chemotherapy-induced toxicity profoundly affects the QoL of cancer patients. The most common clinical symptoms typically manifest with fatigue, nausea, vomiting and diarrhea [172,173]. FMD improves cancer treatment efficacy and minimizes chemotherapy side effects [153,170]. Nutrient deprivation can lead to a differential stress resistance (DSR) between normal

and cancer cells. Mechanistically, starvation inhibits growth-promoting pathways to reinvest energy in maintenance and repair pathways in healthy cells. On the contrary, cancer cells fail to adapt to stress conditions since the presence of oncogenic mutations constitutively activates cascades making them more sensitive to chemotherapy, resulting in the proposed differential stress sensitization (DSS; Figure 9) [162,174].

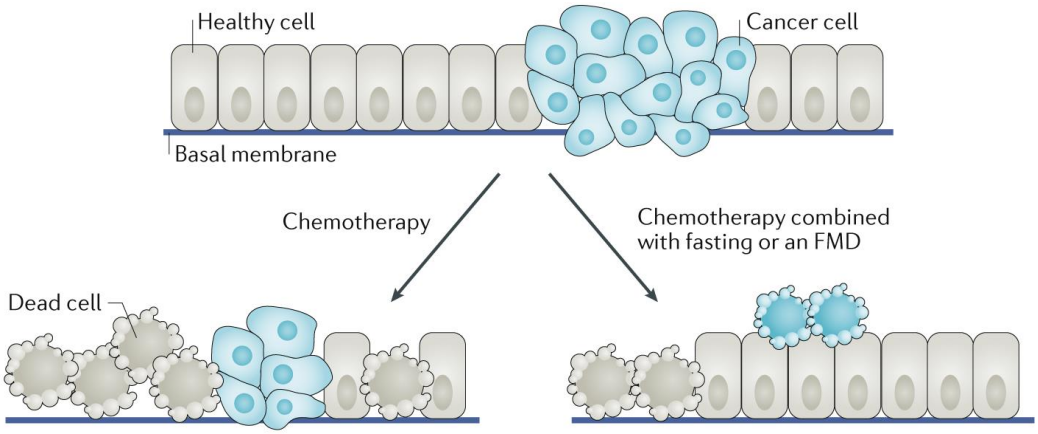


Figure. 9 Differential stress resistance versus differential stress sensitization.
From Nencioni *et al*. 2018

The downregulation of circulating IGF-1 levels is a key mechanism by which fasting increases chemotherapy tolerability. Particularly, the restoration of IGF-1 circulating levels during fasting periods can revert the protective effect of fasting from chemo-toxicity [175]. Transgenic Lid mice (liver IGF-1 deficient) show a prolonged survival rate compared to wild type mice when subjected to different chemotherapy drugs [175]. Therefore, the reduction of IGF-1 leads to a subsequent decrease in growth signalling pathways such as RAS and Akt cascades. This allows normal cells to enter a protective mode, inducing cell cycle arrest and activating repair and protection genes such as FOXO transcription factor, superoxide dismutase (SOD) and DNA repair machinery genes [176]. Additionally, both fasting and FMD can boost autophagy leading to improved cellular health reducing ROS production by removing dysfunctional mitochondria and eliminating toxic aggregates [162].

Tumor growth inhibition

According to the DSS theory, fasting or FMD are able to reduce the growth of different cancer types and importantly, to sensitize cancer cells to chemotherapeutics, radiotherapy and tyrosine kinase inhibitors (TKIs; Figure 10) [145,146,177].

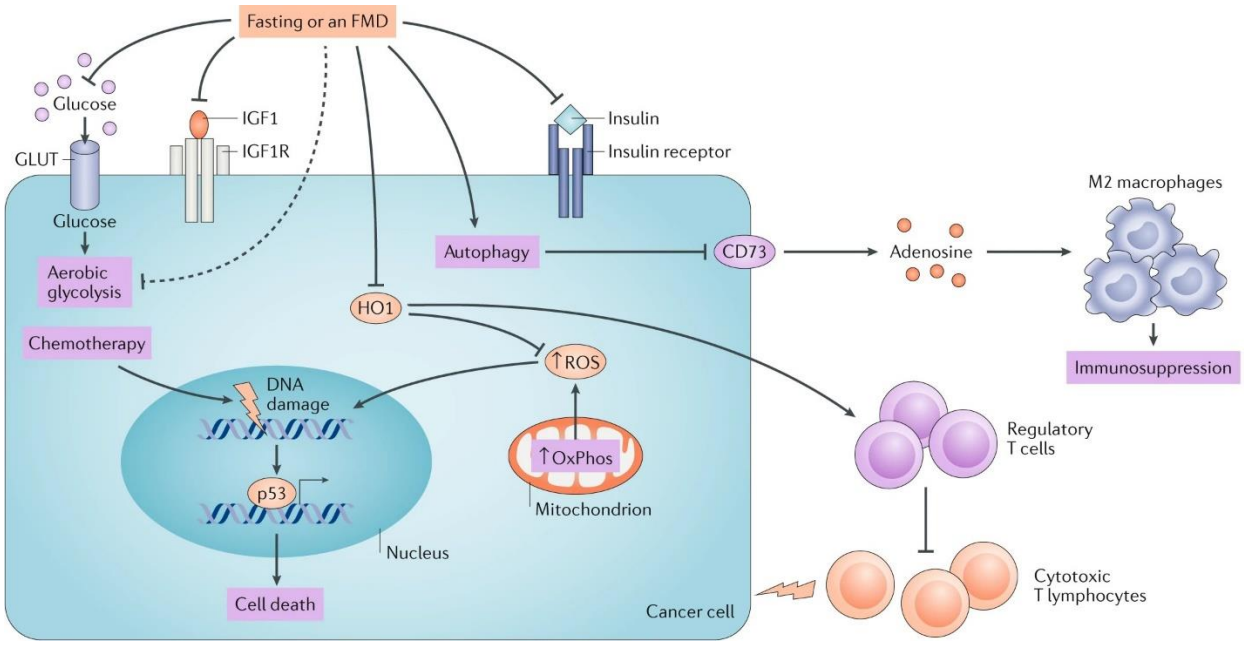


Figure 10. Mechanisms of fasting or FMD-dependent killing of cancer cells in solid tumors. From Nencioni *et al.* 2018

One of the mechanisms that foster cancer cell death is the low availability of tumor growth-promoting nutrients and factors such as glucose, insulin, and IGF-1 that force cells to rely on fatty acid β-oxidation as a source of energy. This switch from aerobic glycolysis to mitochondrial OXPHOS in cancer cells is called the anti-Warburg effect. Consequently, both the increased mitochondrial activity and the decreased cellular redox potential owing to decreased glutathione synthesis from glycolysis and the pentose phosphate pathway, trigger an increased ROS production. Lastly, this rise in oxidative stress conduct to oxidative DNA damage, p53 activation, and ultimately, cell death, especially in response to chemotherapy [178].

In regard to autophagy, fasting may improve the ability of the immune system to identify and destroy nascent tumors in autophagy-competent cancers. In fact, autophagy-deficient cancers present a reduced dendritic cell infiltration as well as an unfavorable cytotoxic T lymphocyte (CTL)/immunosuppressive regulatory T cells (Treg) ratio post-chemotherapy [179]. In a preclinical study, mice bearing colon cancer subjected to fasting every other day for 2 weeks showed that the activation of autophagy via fasting in cancer cells inhibits the expression of the enzyme CD73, which consequently reduces the production of immunosuppressive adenosine by cancer cells [180]. In addition, the CD73 downregulation was shown to prevent macrophage shift to an M2 immunosuppressive phenotype.

Finally, fasting or FMDs can downregulate haem oxygenase 1 (HO1) protein in breast cancer. HO1 protein exerts protection against oxidative damage and apoptosis expression. The

downregulation of HO1 in cancer cells increases CD8+ tumor-infiltrating lymphocyte-dependent cytotoxicity, which may be induced by the downregulation of Treg cells [174]. A study aimed to investigate the potency of different CR approaches against tumor growth and metastatic burden in a 4T1 murine breast cancer model showed downregulation in immunosuppressive markers in response to continuous CR [181]. Tumor-bearing mice subjected to fasting programs demonstrated a significant reduction in the frequency of factor forkhead box protein 3 (FoxP3)+CD4+ and FoxP3+CD8+ Treg cells in both peripheral blood and spleen in comparison to controls. On the contrary, myeloid-derived suppressor cells (MDSCs) frequency, which inhibit cytotoxic T cells and activate Treg cells, decreased in the spleen of CR animals [181]. FoxP3 is a transcription factor considered a master regulator of the development and function of Tregs. Overall, FoxP3 upregulation has been correlated with poor prognosis in several cancer patients since it can exert suppressive activities on effector cells, such as NK cells and macrophages [182]. Also, CD39 is implicated in the suppressive function of Tregs converting ATP into adenosine diphosphate (ADP) and cyclic adenosine monophosphate (cAMP), ultimately leading to the release of an immunosuppressive form of adenosine in the tumor microenvironment (TME). Moreover, it suppresses T-cell proliferation and inflammatory cytokine production [183]. CD39 and FoxP3 are generally expressed on various immune subpopulations, such as Treg cells and CD8+ T cells, but have also been found to be highly expressed in cancer cells [184,185].

Human studies

Chronic dietary interventions are not feasible for most people due to poor compliance with dietary protocols or resistance to drastic lifestyle changes [186]. Indeed, fasting remains a challenging option for cancer patients. A pilot trial with gynecological cancer patients (breast or ovarian cancer) subjected to short-term fasting during chemotherapy indicated that part of the subjects (10%) dropped out from the study due to the fasting regime [187]. Thus, periodic use of FMDs has the potential to exert positive effects and safety for cancer patients [188–192] while minimizing the burden of chronic CR.

A randomized study including 125 patients (phase II DIRECT trial) examined the role of FMD as neoadjuvant chemotherapy in women with HER2-negative stage II/III breast cancer [191,193]. Although only 20% of the patients complied with the dietary intervention during all chemotherapy cycles, the FMD proved to be safe and effective as an adjunct to chemotherapy for breast cancer patients, at least in patients who had a normal BMI (> 18 kg/m^2) at the time of inclusion. The FMD group presented lowered DNA damage in T-lymphocytes after receiving

chemotherapy. However, the FMD did not prevent the chemo-induced toxicity compared to patients who followed a regular diet.

In a feasibility study, 36 patients were subjected to FMD combined with oestrogen therapy to treat breast cancer in combination with moderate daily exercise training that reduced risk factors associated with cancer progression, as well as maintained body weight and muscle mass and function [190]. Vernieri *et al.* (2022) published a prospective, observational study (NCT03340935) in which 101 cancer patients underwent a 5-day FMD combined with standard anticancer therapy. Prominently, patients presented enhanced antitumor immunity at both systemic and tumor levels. The reduced immunosuppressive cell population such as MDSCs and Tregs increased CTL and cytolytic natural killers (CNK) cells as well as driving a shift of CD8+ cells toward an activated/memory phenotype [190].

Effects of fasting on the gut microbiota

Diet is an important factor affecting the composition and metabolism of the gut microbiota. In general, the availability of undigested foods influences the abundance, diversity, function, and balance among microbial species in the intestine [194,195], therefore influencing intestinal homeostasis. Nonetheless, the knowledge about the repercussions of dietary interventions on the animal and human gut microbiota remains limited [196].

Fasting and CR may alter the gut environment by changing the nutrient supply and energy sources, which can directly influence the growth of certain microbial phylum and SCFAs production [196]. Variations in the gut microbiota diversity may occur during fasting. In mice subjected to nutrient deprivation for 24 hours, the abundance of *Akkermansia, Parabacteroides* and *Muribaculaceae* increased, while the populations of *Lactobacillus* and *Bifidobacterium* decreased. Interestingly, this modulation that occurred during fasting was reversible when refeeding ensued [194]. According to Rangan *et al.* (2019), who studied the effect of FMD in a murine model of inflammatory bowel disease, 2 cycles of 4-day FMD reduced markers associated with systemic inflammation and altered gut microbiota composition. The *Lactobacillaceae* levels increased approximately 45-fold after 2 days of re-feeding with the normal diet after the second FMD cycle [197].

On the other hand, obese individuals subjected to intermittent energy restrictions to lose body weight, showed an increased intestinal microbial richness and diversity. The abundance of *Escherichia coli,* commonly associated with obesity and related metabolic disorders [198,199],

was decreased at different time points of the study during the CR period. By contrast, several species with anti-obesity abilities, *Faecalibacterium prausnitzii* [200,201], *Parabacteroides distasonis* [201], and *Bacterokles uniformis* [202] were significantly increased. Nonetheless, these gut microbial variations returned to baseline levels at the end of the low-controlled fasting phase [203]. Finally, although there is evidence to suggest that CR influences the gut microbiota, the effects that such alterations may have on cancer patients still require further research.

2- AIM OF THE STUDY

Cachexia is a common condition in cancer patients, significantly impairing their QoL, affecting the tolerance and efficacy of cancer treatments and shortening OS [7]. Skeletal muscle wasting is one of the main features of CC and is triggered by different mechanisms caused by tumor-related factors and systemic inflammation. One example is mitochondrial dysfunction [46] which leads to impaired oxidative metabolism in the skeletal muscle [29,31] and oxidative muscles have been reported to have greater protection against degradation compared to other muscle types [42,204]. Additionally, both pro-inflammatory cytokines and dysfunctional mitochondria can exacerbate autophagy [46], further promoting muscle atrophy in CC [205,206].

Nutritional support is an essential element in the management of cancer patients as it contributes to the maintenance of body weight, improvement of QoL, and protection against drug toxicity [15,62,69]. Oral nutritional supplementation has been one of the clinically validated strategies to prevent and/or treat malnutrition in cancer patients [67,68,70,207], especially for those undergoing therapy [208].

Nutritional ketosis induced by KD and KS impacts the oxidative metabolism of skeletal muscle. It ameliorates mitochondrial activity and efficiency, by increasing the content and expression of proteins involved in OXPHOS [93,97,101,103] through the use of energy-efficient substrates such as fatty acids and KBs [83,86]. Furthermore, it exerts an anti-inflammatory and antioxidant effect [83,121] which may counteract inflammation-induced catabolism in the skeletal muscle. The use of MCT as a dietary supplement shows similar metabolic effects to those observed by KD and KS [87,92,135].

Based on the above premises, this study aims to investigate the potential effect of MCT supplementation on the prevention of body weight loss and muscle wasting in experimental models of CC through the maintenance of muscle oxidative capacity. More specifically, we have studied the activity and content of mitochondrial proteins responsible for oxidative metabolism in the skeletal muscle. Furthermore, some specific markers of autophagy and mitophagy have also been examined.

Calorie-restricted plant-based diets, known as FMDs, have been shown to induce physiological responses similar to fasting while providing micronutrient-rich foods and minimizing the burden of fasting [146,167]. As adjuvant therapy, the FMD have demonstrated promising findings in modulating tumor growth [209,210] by sensitizing cancer cells to the therapy

[162,174] and improved anticancer immunosurveillance [162,179,180]. Furthermore, FMDs have been demonstrated to be effective in mitigating the adverse effects induced by chemotherapy [153,170]. It is important to note that chemotherapy-induced toxicity contributes to the catabolic condition both in experimental models [206,211] and in cancer patients [5,7]. Apart from that, several studies (especially in trained individuals) have documented that CR can preserve muscle mass and function [158] and even reduce the risk of sarcopenia [153,171]. Nonetheless, there is still a knowledge gap regarding the specific impact of FMD on CC.

Considering all the foregoing arguments, this project is designed to test the efficacy of FMD in ameliorating CC and potentially alleviating chemotherapy-induced toxicity in experimental models of CC. Firstly, by assessing the functional and oxidative capacity of skeletal muscle, and secondly, by studying the host immune response against the tumor. To explore the effects of FMD on a more translational experimental model of CC, a longitudinal study was designed to investigate either tumor incidence or the preventive effect of periodic FMD cycles in the progression of CC. This objective was mainly addressed through preclinical assessments and, since CR modulates the diversity of the gut microbiota [196], the gut microbial population before and after tumor onset was also investigated.

3- MATERIALS AND METHODS

Reagents

All materials were supplied by Sigma-Aldrich/Merck (St. Louis, MO, USA) unless differently specified.

Cancer cell line and tumor cell inoculation

C26 colon-adenocarcinoma cells were obtained from Prof. M.P. Colombo (National Cancer Institute, Milano, Italy). Cells were maintained in high glucose DMEM, supplemented with 10% fetal bovine serum (FBS), 11% sodium pyruvate, 1% L-glutamine and 1% penicillin and streptomycin and maintained at 37°C in a humidified atmosphere of 5% CO2 in air. The medium was refreshed every two days. When cells reached 90% of confluence were trypsinized, centrifuged at 1,000g and resuspended in sterile saline to obtain a concentration of $5x10^5$ cells/mL.

C26 cell viability

C26 cells ($4x10^4$ cells/well) were seeded in triplicate in 6-well plates. Cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS, 1% sodium pyruvate, 1% L-glutamine and 1% penicillin and streptomycin and maintained at 37°C in a humidified atmosphere of 5% CO2 in air. The next day, the medium was refreshed and cells were treated with 10 mmol/L of sodium β-hydroxybutyrate (Na β-HB) for 24h or 48h. Subsequently, cells were fixed for 10 min with 1mL of 4% paraformaldehyde in phosphate-buffered saline (PBS), washed with PBS, and kept at 4°C. Then, cells were stained with crystal violet solution (0.1%) for 30 min at RT, subsequently dissolved in 1mL of acetic acid solution (10% in water). The absorbance of the solution was measured at 590 nm in a BECKMAN COULTER DU® 800 Spectrophotometer.

Animal models and experimental design

All the experimental animal procedures were performed in compliance with the Italian Ministry of Health Guidelines and the Policy on Humane Care and Use of Laboratory Animals (NRC, 2011). The Bioethical Committee of the University of Torino approved the experimental protocol. The animals were maintained on a regular dark-light cycle of 12:12 h with controlled temperature (20-23°C) and humidity (40-60%).

Two models of CC were used in this study. The BALB/c mice, purchased by Charles River (Charles River, Wilmington, MA), were anesthetized with isoflurane (2%) and inoculated subcutaneously with 5×10^4 of C26 cells on the back. The Villin-Cre/ $Msh2^{loxP/loxP}$ (VCM) was

developed by crossing B6. Cg-Tg (Vil1-cre) 997Gum/J (Villin-Cre) and B6. Cg-Msh2tm2.1Rak/J (Msh2loxP) mice, purchased by The Jackson Laboratory (Bar Harbor, CA, USA). The offspring bears a conditional knockout of the Msh2 gene in the enterocytes accelerating the formation of intestinal adenomas/adenocarcinomas [212]. The presence of each transgene construct was assessed by a "quick and dirty" DNA extraction from the distal tail tip (2 mm) of conscious, non-anesthetized animals [213] using Melt Curve Analysis (RT-qPCR) using the following primers: Villin-Cre (forward: 5′-TTCTCCTCTAGGCTCGTCCA-3′ and reverse: 5′-CATGTCCATCAGGTTCTTGC-3′) and Msh2loxP (wild-type: 5′-GATGATGTGTGAAGCCTGCAT-3′, mutant: 5′-CCTCTTGAGGGGAATTGAAGT-3′ and common: 5′-AGGTTAAAAACCAGAGCCTCAACT-3.

Animal experiment 1: MCT administration set-up

For the set-up, healthy 8-week-old wild-type BALB/c male mice received a daily dose of Pure C8 MCT Oil™ (MCT; 5-10g/kg/day) by gavage. Body weight, food intake, and blood ketones were recorded regularly. After two weeks of monitoring, the animals were euthanized.

Animal experiment 2: C26 hosts supplemented with MCT by gavage

In this experiment, 8-weeks-old wild-type BALB/c male mice were randomized according to body weight and divided into four groups, namely: controls, controls supplemented with MCT (5g/kg/day), C26-bearing mice (C26), and C26 supplemented with MCT. Animals were *ad libitum* fed a standard diet and started to receive the supplement the day after tumor transplantation (Figure 13). During the experiment, body weight, food intake and muscle strength were monitored regularly. The sacrifice was performed on day 15 and, animals that did not show body weight loss on day 15 were sacrificed on day 18.

Animal experiment 3: C26 tumor-bearing mice supplemented with an MCT-enriched diet

A new experiment consisting of 6-month-old wild-type BALB/c female mice were randomized and divided into four groups namely: controls, controls supplemented with MCT, C26, and C26 supplemented with MCT. In this case, only females were used to avoid the fighting traits among male cage mates. Animals were *ad libitum* fed a purified diet and started to receive the supplement the day after inoculation of the C26 cells. The animals received 5g/kg/day of MCT during the first week as an adaptation period to the supplementation. Subsequently, 10g/kg/day of MCT were administered until the endpoint (Figure 19). MCT was added to plates containing ground food (0.15-0.3g) and mixed until a homogeneous mixture was obtained. Body weight, food intake and muscle strength were monitored regularly. Blood ketones and glucose levels were

measured at baseline (the day before C26 inoculation) and on day 13. On day 15, animals were euthanized.

Animal experiment 4: Dietary enrichment with MCT in the VCM model

After reaching twelve months of age, male $Msh2^{loxP/loxP}$ mice (used as controls) and VCM mice were randomized and divided into three groups; controls fed with a standard diet (SD; n=3), VCM fed with a standard diet (VCM+SD; n=5) and VCM fed with MCT-enriched diet (VCM+MCT; n=8). The experiment lasted 1 month and the endpoint was set at ~13 months of age. The animals received 5g/kg/day of MCT during the first week as an adaptation period to the MCT. Subsequently, 10g/kg/day of MCT was administered until the endpoint (Figure 26). MCT was added to plates containing ground food (0.15-0.3g) and mixed until a homogeneous mixture was obtained. Body weight, food intake and muscle strength were monitored regularly. Blood ketones and glucose levels were measured at baseline (two days before MCT administration) and on days 9 and 24. On day 28, animals were euthanized.

Animal experiment 5: C26 hosts treated with chemotherapy and fed FMD

To achieve a model system that mimics the human disease more closely than the canonical C26 schedule, 7-month-old wild-type BALB/c female mice implanted with C26 cells and treated with chemotherapy (C26-Folfox) were randomized and divided into two groups namely: C26-folfox+SD or C26-folfox+FMD. Animals received a single dose of Folfox (6mg/kg oxaliplatin, 25mg/kg 5-fluorouracil, and 90mg/kg leucovorin) at days 7, 14 and 23 after C26 inoculation. However, being mice were very frail, the dose of chemotherapy was halved at day 23 and the FMD cycle was delayed for two additional days. C26-folfox+FMD received the FMD for 4 days every week and Folfox on the third day of FMD (Figure 30). Hence, the animals had three cycles of chemotherapy and FMD. On day 33, all the surviving animals were euthanized. During the experiment, body weight, food intake, and muscle strength were recorded regularly.

Animal experiment 6: VCM mice fed FMD

After reaching three months of age, male and female VCM mice were randomized and divided into four groups; VCM+SD males and VCM+SD females, VCM+FMD males and VCM+FMD females. The experimental period was ten months long; the endpoint was set at ~13 months of age. Hence animals had ~20 cycles of FMD. Not all the animals were included simultaneously in the experimental set-up, rather they were included as they reached three months of age from our in-house breeding colony. Body weight and food intake were recorded regularly and stools were collected periodically. Muscle strength, blood glucose and ketones were assessed

when the animals reached twelve months of age (Figure 37). Mice were examined daily for signs of distress and eventually euthanized when they reached the endpoint.

Euthanasia and tissue preparation

Mice were anesthetized, exsanguinated by cardiac puncture and euthanized by cervical dislocation. Blood was collected into 2mL tubes, centrifuged at 1,000g, and the plasma separated and stored at -80°C for further analysis. Tumors, spleen, liver, heart, pancreas, *gastrocnemius* (GSN), *tibialis anterior* (TA), *quadriceps* skeletal muscles, and perigonadal fat were also removed and weighed, quickly frozen in liquid nitrogen and then stored at -80°C for further analysis. Spleens of the C26 hosts treated with Folfox were preserved for splenocyte isolation.

Animal diets

Animal chow

Animals were fed a standard laboratory chow. Nevertheless, a purified diet was used in animal experiment 3 to improve the rigor and reproducibility of the studies involving nutritional approaches. Indeed, by using refined ingredients, the macro- and micronutrient nutritional compositions of the purified diets are well-defined, limiting the variability of both nutrients and contaminants [214]. Table 2 represents the total calorie content and percentage of energy from macronutrients of both the standard and purified diet and MCT.

Table 2. The energy content of diets and MCT.

	Standard diet[1]	Purified diet[2]	FMD[3]	MCT[4]
Kcal/g	2.9	3	2,9	9
% energy carbs	60	58	Ref.	-
% energy proteins	26	33	Ref.	-
% energy fats	14	9	Ref.	98

[1] Special Diets Services; VRFI, [2] SSNIFF; SM Rat/Mouse diet-control (1534),
[3] Ketosource Pure Caprylic acid (C8) MCT Oil™.

FMD protocol

The human FMD is a plant-based diet designed to provide sufficient nourishment during periods of low caloric intake consisting of a 5-day regimen [146]. In our experimental setting, the FMD regimen consisted of 4 days as the animals achieved a ~10-20% body weight loss by the fourth day. On day one, animals received 2,4g of FMD/mouse/day to adapt to a period of low

caloric intake. On days 2-4 animals received 1,4g FMD/mouse/day. Hence mice in the FMD group experienced a ~32% and ~60% calorie reduction on day one and days 2-4, respectively. Before supplying the FMD, the animals were transferred into fresh cages to avoid feeding with residual chow and coprophagy. Mice consumed all the supplied food of the FMD regimen daily and showed no signs of food aversion. After the end of the FMD cycle, we supplied a standard diet *ad libitum* before starting another FMD cycle.

Assessment of blood ketones and glucose levels

The analysis of blood glucose and ketone levels requires a small amount of peripheral blood, which can be obtained by the removal of the distal tail tip (2 mm) of conscious, non-anesthetized animals [213]. Tail tip sampling is a minimally invasive procedure and was carried out by snipping less than 2 mm of tissue from the tail tip with sharp scissors. Blood is obtained by direct flow or by gently massaging the tail, collecting the blood directly on a glucose or ketone test strip (Nova Pro device).

Grip strength analysis

The forelimb strength was analyzed using the grasping test [215]. The device consisted of a rectangular grid connected to an isometric force transducer (dynamometer). The grid was positioned on an incline, and the mice held by the tail were lowered towards the device. The animals were allowed to grasp the grid and were gently pulled backward. Just before the animal loses the grip, the force applied was recorded as the peak tension. Three measurements per mouse were taken.

Succinate dehydrogenase (SDH) staining and enzymatic activity

During necropsy, one TA muscle was frozen in melting isopentane for histology. Transverse sections (10µm) were cut on a cryostat at -22°C and stained for SDH incubating for 5 min at 37°C with 1mg/mL nitro tetrazolium blue chloride (NTB) and 27 mg/mL Na-succinate in PBS. Slides were rinsed twice with PBS, and dehydrated using an ethanol scale and xylene. Then, the slide cover was mounted using mounting resin (Permount) and sections were observed under a light microscope (Leica DM750). Several pictures were taken and the whole TA section was digitally reconstructed.

As for the total SDH activity, ~50mg of frozen GSN muscle were homogenized (10% wt/vol) in ice-cold 150mM NaCl, 10mM KH_2PO_4, 0.1mM EGTA using a bullet blender at a speed of 10 for 1 min. The resulting homogenate was centrifuged 5min at 800g and the supernatant was

collected. Protein homogenates (10µl) were incubated with 200µl reaction buffer containing 10mM sodium-succinate, 50µg/ml 2,6-Dichlorophenolindophenol (DCPIP), 10mM phosphate buffer (pH 7.4), 2mM KCN, 10mM CaCl2, 0.05% bovine serum albumin (BSA). The absorbance at 590 nm was measured after 0, 3, 10, and 20 min with a BECKMAN COULTER DU® 800 Spectrophotometer. Subsequently, total protein content was measured according to Bradford, using BSA as a working standard. Hence, the rate of absorbance decrease between 3 and 20 min was corrected for the protein loading and used to calculate the SDH content.

Western blotting

Approximately 50 mg of GSN muscle were mechanically using bead homogenizer in RIPA buffer (50mM Tris-HCl pH 8.0, 5mM EDTA pH 8.0, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (0.5mM PMSF, 0.5mM DTT, 2µg/ml leupeptin, 2µg/ml aprotinin) and phosphatase inhibitors (P0044), sonicated for 10 seconds at low intensity, centrifuged at 15,000g for 5 min at 4°C and the supernatant was collected. Total protein concentration was quantified according to Bradford (Bio-Rad, Hercules, CA, USA), using BSA as a working standard. Equal amounts of protein (15-30 µg) were heat-denatured (except when assessing OXPHOS expression) in sample-loading buffer (50mM Tris-HCl, pH 6.8, 100mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), resolved by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The filters were blocked with Tris-buffered saline (TBS) containing 0.05% Tween and 5% non-fat dry milk and then incubated overnight with antibodies directed against specific proteins (Table 3). Peroxidase-conjugated IgGs (Bio-Rad, Hercules, CA, USA) were used as secondary antibodies. After incubation with Clarity Western ECL substrate (170-5061, Bio-Rad), bands were developed using the ChemiDoc XRS+ imaging system (Bio-Rad). Densitometric analysis of the obtained images was performed using the Image Lab software (Bio-Rad).

Table 3. List of primary antibodies used for western blot analysis.

Target protein	Company	Code	Source	Band
SDH-A	Santa Cruz Biotechnology	sc-377302	Mouse	~72 kDa
Cyt. C	BD biosciences	556433	Mouse	~15 kDa
COX IV	Abcam	ab14744	Mouse	~17 kDa
TOMM20	Santa Cruz Biotechnology	sc-11415	Rabbit	~20 kDa
PGC-1α	Merck Millipore	AB3242	Rabbit	~110 kDa
PINK1	SAB2500794	Merck	Goat	~55 kDa
P62	P0067	Merck	Rabbit	~62 kDa
OXPHOS	Abcam	ab110413	Mouse	~60-10 kDa
VINCULIN	Santa Cruz Biotechnology	sc73614	Mouse	~120 kDa

RT-qPCR.

The GSN muscle (30-50 mg) was homogenized using a Bullet Blender with 500µl of TRI Reagent for the RNA extraction to isolate high-quality RNA using the standard phenol-chloroform method. The RNA concentration was quantified spectrophotometrically (BECKMAN COULTER DU® 800). Total RNA was retro-transcribed using a cDNA synthesis kit (Bio-Rad or Qiagen, Hilden, Germany) and transcript levels were determined by RT-qPCR using the SsoAdvanced SYBR Green Supermix (Bio-Rad) and the CFX Connect Real-Time PCR Detection System (Bio-Rad) with 10 ng of cDNA per well. Every RT-qPCR was validated by analyzing the respective melting curve and run in parallel to no reverse transcriptase control (NRT) to exclude potential artefacts from genomic DNA contamination. Data were normalized to ACTIN gene expression and displayed as fold change over the control group. Primer sequences are listed in Table 4.

Table 4. Sequence of primers used for the detection of mRNAs (RT-PCR).

Gene (mouse)	Sense	Primer sequence (5'-3')	Product size
Cyt. C	Forward Reverse	GGAGGCAAGCATAAGACTGG TCCATCAGGGTATCCTCTCC	131 bp
PGC-1α	Forward Reverse	GCAACATGCTCAAGCCAAAC TGCAGTTCCAGAGAGTTCCA	132 bp
SDH-A	Forward Reverse	ACACAGACCTGGTGGAGACC GGATGGGCTTGGAGTAATCA	156 bp
ACAT	Forward Reverse	CTGGGCGCAGGTTTACCTAT GGTGTTGCTCCTCTGCTCAT	182 bp
OXCT	Forward Reverse	GGGCCCATACCCACTGAAAGA GACATGTCCCCCTCTAATCATGG	133 bp
MURF1	Forward Reverse	GGGCCATTGACTTTGGGACA TCTCCTTCTTCATTGGTGTTCTTCT	119 bp
ATROGIN-1	Forward Reverse	GCAAACACTGCCACATTCTCTC CTTGAGGGGAAAGTGAGACG	93 bp
LC3B	Forward Reverse	CACTGCTCTGTCTTGTGTA TCGTTGTGCCTTTATTAGTG	171 bp
BNIP3	Forward Reverse	GTCGCCTGGCCTCAGAAC CCCATTGCCATTGCTGAAGT	174 bp
β-ACTIN	Forward Reverse	CTGGCTCCTAGCACCATGAAGAT GGTGGACAGTGAGGCCAGGAT	96 bp

Spleen cell immunophenotype

Splenocyte isolation

Fresh spleens were homogenized using the flat plunger end of a syringe and passed onto a 100µm filter. The resulting cells were counted by flow cytometry (BD Accuri C6), immediately centrifuged for 10 min at 1,000g and resuspended in a freezing medium (DMEM supplemented with 20% FBS, 10% dimethyl sulfoxide 1% of sodium pyruvate, 1% L-glutamine and 1% of penicillin and streptomycin) and transferred to the cryotubes for storage (-80°C).

Flow Cytometry

Myeloid-derived suppressor cells (MDSCs), T regulatory cells (Tregs) and T helper (Th) cells were evaluated by flow cytometry. Splenocytes (Spc) were thawed, centrifuged for 10 min at 1,000g resuspended in Roswell Park Memorial Institute (RPMI) 10% FBS and counted. 1×10^6 Spc were dispensed in FACS tubes and prepared for staining of MDSC and Treg cells; Spc were

washed twice in 2 ml of washing solution (PBS containing 0.1% sodium azide and 2% FBS) for 10 min at 1,000g and the pellets were treated with Fc receptor blocker (CD16/CD32; Biologend, San Diego, CA) for 15 min at 4°C. Spc were then incubated for 30 min at 4°C with the following mAbs from BioLegend: Alexa fluor 647 anti-mouse Ly-6G/Ly-6C (Gr-1) and FITC anti-mouse CD11b for MDSCs evaluation; FITC anti-mouse CD4, PE anti-mouse CD25 and Alexa Fluor 647 for Tregs. After washing, cells pellet for MDSCs evaluation were immediately acquired at the Cytometer, whereas pellets for Treg evaluation were resuspended in 350μL Cyto-Fast Fix/Perm Buffer (BioLegend) and incubated for 20 min at room temperature. After washing with permeabilization buffer (BioLegend), samples were incubated with Fc receptor blocker for 15 min at 4°C and then with Pacific Blue anti-mouse FOXP3 for 30 min at 4°C and washed before analysis.

To detect intracellular cytokine production and consequently Th cells, 10^6 Spc were cultured in 48-well plates and activated with 50ng/ml PMA, 500ng/ml ionomycin and 10ng/ml brefeldin A (Sigma) for 18 h. Cells were then harvested, washed in PBS with 0.1% sodium azide and 2% FBS and labeled on the surface with FITC anti-mouse CD4. Cells were then washed twice, fixed and permeabilized with Cyto-Fast Fix/Perm buffer set and labeled intracellularly with Alexa-Fluor 647 anti-mouse IL-17, Pacific Blue anti-mouse IFNγ and PE anti-mouse IL-22 (all from BioLegend) for 20 min at 4°C. After washing, stained cells were acquired on a BD FACSCelesta™ Cell Analyzer instrument (BD Biosciences) and analyzed using FlowJo v10 Software (BD Biosciences).

Gut microbiome analysis

DNA extraction

DNA extraction from stools was performed using the DNeasy PowerSoil Pro Kit (250; Qiagen Cat No./ID: 47014, Cat No./ID: 47016). The DNA concentration was quantified by Qubit™ 4 fluorometer with Qubit™ dsDNA High Sensitivity (Invitrogen). Shotgun metagenomic sequencing was performed using the Illumina DNA Prep kit (Illumina) as described in (PMID: 30936548, PMID: 31530647). Libraries were sequenced on the NovaSeq 6000 Sequencing System (Illumina).

Shotgun metagenomics

Read quality control, adapter removal, and filtering of phiX and mm11 mouse genome-mapped reads were performed following the pipeline described in https://github.com/SegataLab/preprocessing. Specifically, TrimGalore was applied to remove the

sequencing adapter while Bowtie2 for align the reads against the phiX and mm11 genome (REF). Taxonomic profiling was performed with MetaPhlAn4 (REF) in default settings with mpa_vJan21_CHOCOPhlAnSGB_202103 as the markers database. Diversity metrics were computed using the VEGAN R package v2.6.4 (REF). Differences in microbial relative abundances were tested using the Wilcoxon Rank-Sum test and SIAMCAT v2.4.0 in default settings (REF). A paired analysis was performed to compare data of T1 and T2 samples from the same mice model.

Data representation and statistics

Data are shown using the bar (mean) and dot plots (individual values) unless otherwise specified. Prism (version 6, GraphPad) software was used for data representation and statistical tests. ROUT was used to find outliers (Q = 1%) and remove them from the analysis. The logarithmic and normality tests check whether the data distribution is parametric. The two-tailed statistical tests, Student's "t" test or analysis of variance (ANOVA) for normal distribution and Mann-Whitney test or Kruskal-Wallis test for non-normal distribution were used where appropriate.

4- RESULTS

4.1 MCT supplementation in the C26 model

4.1.1 Sodium β-hydroxybutyrate did not affect C26 viability

Nutritional ketosis can drive a metabolic shift in the skeletal muscle by providing an alternative source of energy (KBs) [83,86]. Consequently, it impacts the oxidative capacity of the skeletal muscle by increasing the content and expression of proteins involved in OXPHOS [93]. Moreover, elevated levels of β-HB have been linked to an anti-inflammatory effect [94,120] and the prevention of body weight and skeletal muscle loss [115]. Thus, in light with these evidences nutritional ketosis may prevent skeletal muscle atrophy.

In order to understand the possible beneficial effect of MCT-induced nutritional ketosis in our CC experimental model, as a first step, C26 colon carcinoma cells were treated with sodium β-hydroxybutyrate (Na β-HB) to exclude a possible effect of the KBs in C26 cancer cells. The plan was to treat C26 cells with a high dose of Na β-HB (10mmol/L) to assess its potential cytotoxic effect. The viability of C26 cells was assessed using the crystal violet staining method in which only viable cells were stained. The results revealed that Na β-HB at a concentration of 10 mmol/L did not interfere with the number of viable C26 cells according to phase contrast microscope observations (Figure 11A) and the stained cells (Figure 11B).

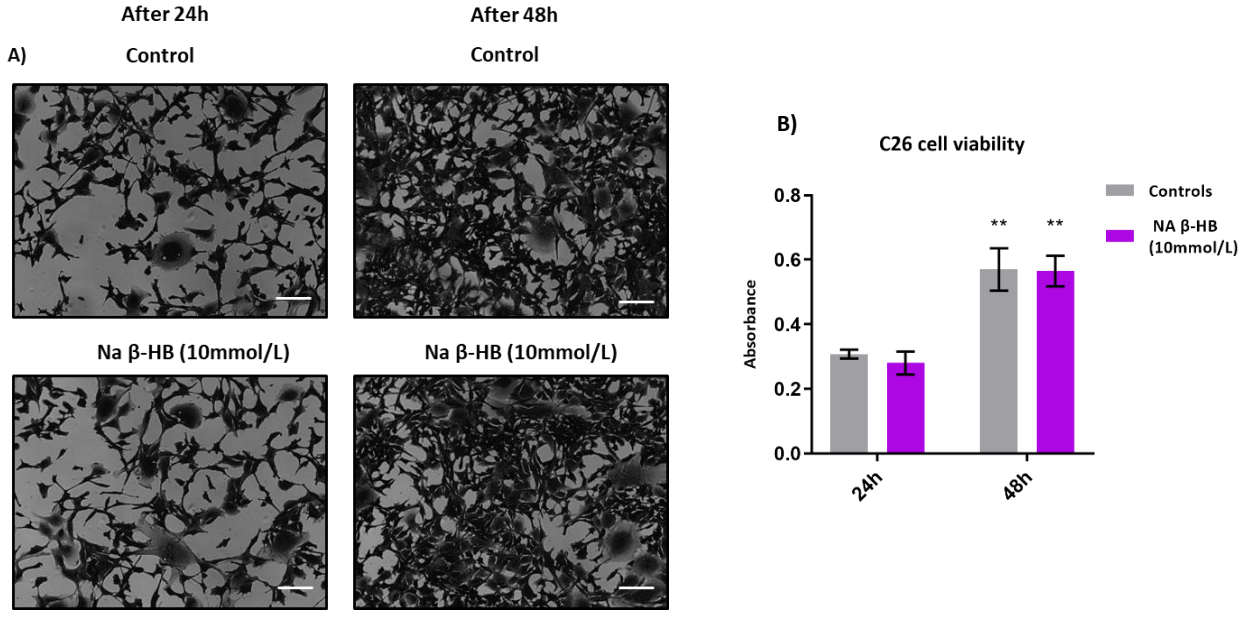


Figure 11. C26 viability 24 and 48h after treatment with Na β-HB (10mmol/L; n=3)
A) Microscopic images (10x) of C26 cells cultured in 12-well plates after 24 or 48 hours of treatment with or without Na β-HB (scale bar: 100 µm). **B)** Histograms representing the absorbances of crystal violet dye

of viable control and treated C26 cells. Absorbance values (mean ± SD) expressed as arbitrary units. Significance of the differences by two-way ANOVA test *p<0.05; **p<0.01 vs controls at 24h.

4.1.2 MCT induced ketosis in the BALB/c mice

As mentioned above, MCT is a potent ketogenic agent [87,127]. Moreover, as demonstrated in a preclinical study [216], MCT supplementation induced ketosis in rats after a daily dose of 5 and 10 g/kg of body weight via intragastric gavage. To confirm the same effect in BALB/c mice, a single dose of 10g/kg via gavage was administered to healthy male mice (n=5).

The animals presented side effects after MCT administration, such as dizziness and disorientation, with full recovery ~1 h after treatment. Consequently, the dose was reduced to 5g/kg in order to avoid the observed side effects. A single dose of 5 g/kg MCT showed elevated ketonemia in animals after 25 minutes of administration (Figure 12) in comparison with baseline levels. The maximal levels of β-HB were observed 35 min after MCT administration (2.18mmol/L±0.217). Subsequently, blood β-HB levels returned to baseline after 4 hours. This result reveals that intragastrical gavage of MCT can induce acute ketosis for at least 1h

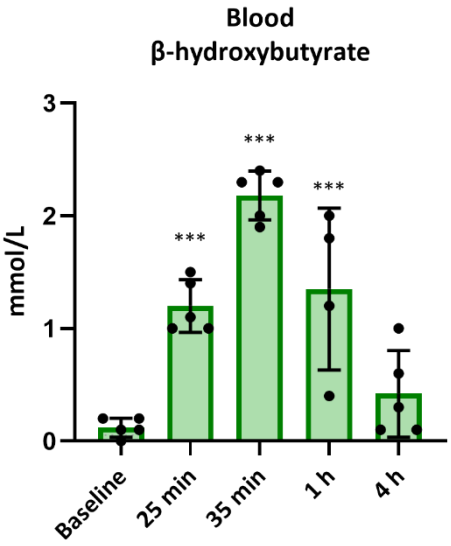


Figure 12. Blood β-HB levels in healthy mice (n=5).
Blood β-HB levels expressed in mmol/L (mean ± SD) at baseline and at different time points after MCT administration. Significance of the differences by one-way ANOVA test *p <0.05; **p<0.01 vs baseline

4.1.3 The supplementation with MCT had no impact on CC in the C26 hosts

To investigate the effect of MCT in mice with CC, an *in vivo* experiment was performed. The experimental schedule included: healthy controls, controls treated with MCT (controls+MCT), C26 tumor-bearing mice (C26) and C26 tumor-bearing mice treated with MCT (C26+MCT). 8 weeks-old male BALB/c mice were subcutaneously inoculated with C26 adenocarcinoma cells and received MCT from the next day after tumor implantation until the end point (day 14; Figure 13). The canonical characteristics of this model are anorexia, progressive body weight loss, muscle wasting, splenomegaly and white adipose tissue depletion [206,217]. Animals that did not show body weight loss on day 15 were sacrificed on day 18.

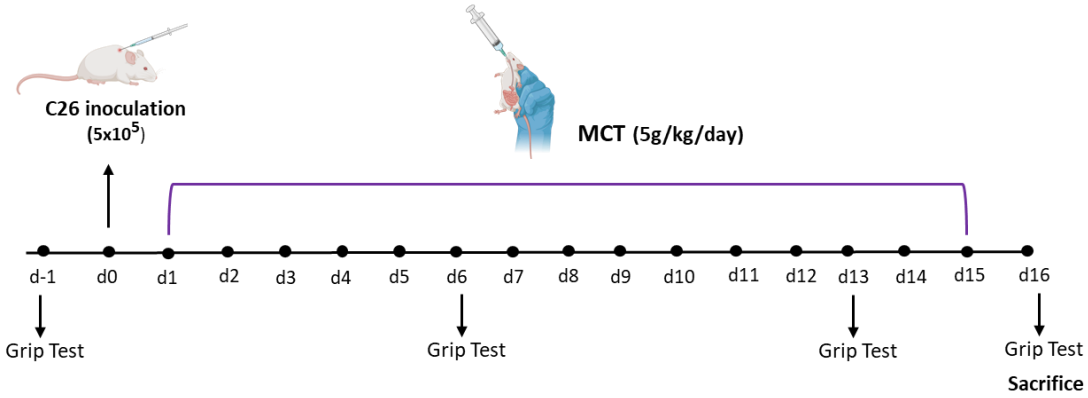


Figure 13. Experimental design. Tumor inoculation, MCT administration, grip test, and sacrifice time points are illustrated in the timeline. The experimental groups consist of healthy controls (n=6), controls+MCT (n=6), C26 (n=10), and C26+MCT (n=10).

Mouse body weight and food intake were recorded every other day and thereafter daily from day 9. As observed, C26 and C26+MCT presented a mild progressive body weight loss starting at day 12 (Figure 14A). However, because of the high variability existing within the groups, only the C26+MCT body weight was statistically decreased at day 15 when compared with controls. Lastly, the final body weight loss between the C26 groups was comparable irrespective of the treatment (C26= -12.27%±8.86; C26+MCT= -14.13%±7.45; Figure 14B).

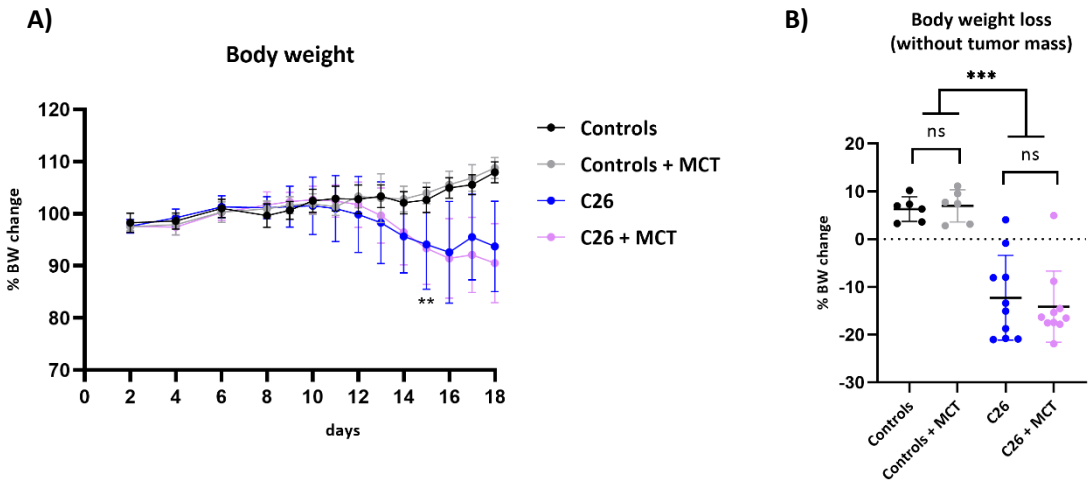
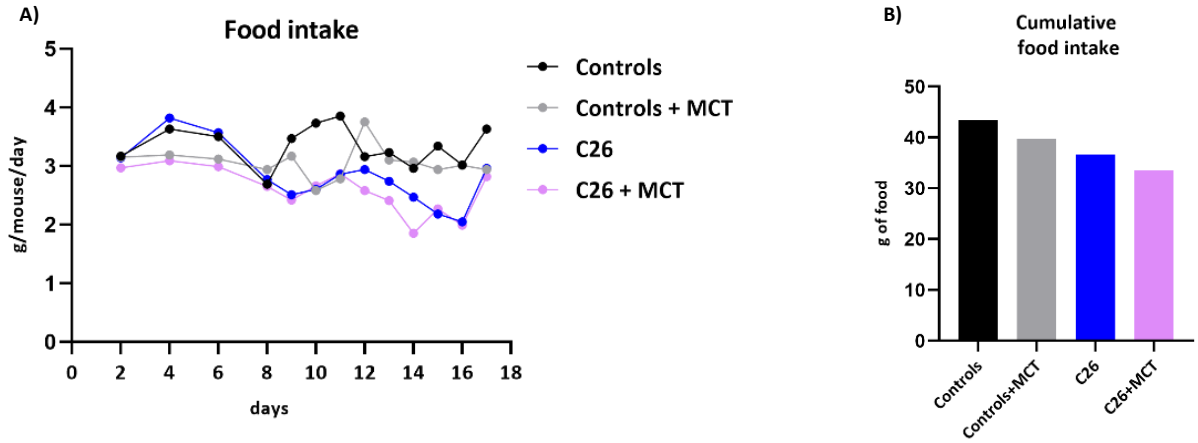


Figure 14. Body weight in controls and C26 hosts.
A) Body weight (mean ± SD) is expressed as a percentage of initial body weight (ibw). **B)** Body weight loss delta (mean ± SD) expressed as a percentage of the delta of the ibw and the final body weight (fbw) excluding tumor mass. Controls; n=6, controls+MCT; n=6, C26; n=10 and C26+MCT; n=10. Significance of the differences by one-way ANOVA test *p<0.05; **p<0.01 vs controls.

The assessment of food and energy intake revealed that mice did not present severe anorexia (Figure 15A, 15C). Nevertheless, the total food intake of the C26 hosts, either treated or untreated, was lower (C26= 38.30g; C26+MCT= 33.56g) compared to controls (controls= 43.36g; Figure 15B). In regard to the total energy intake, the treated animals had an increased energy intake (controls= 125.75kcal; controls+MCT= 132.76kcal; C26= 106.14kcal; C26+MCT= 114.88kcal; Figure 15D) likely due to the extra caloric intake provided by the MCT.



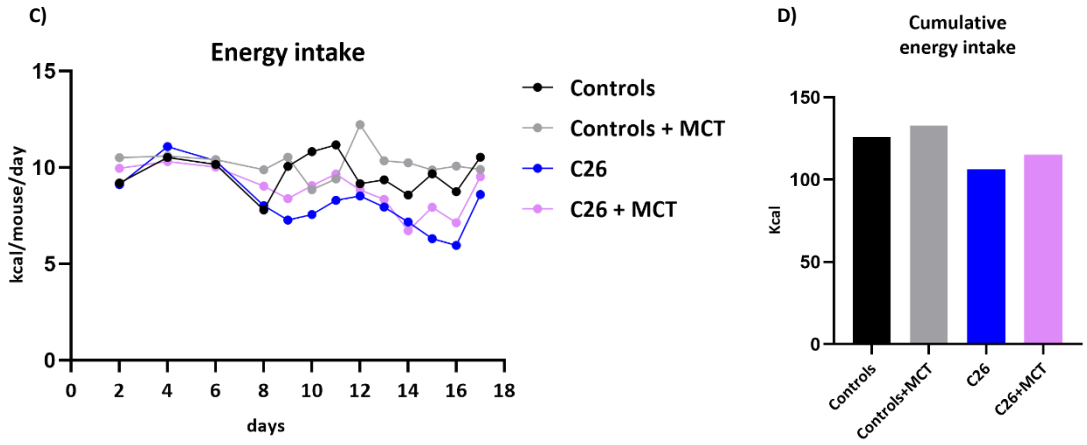


Figure 15. Food and energy intake in controls and C26 hosts.
A) Food intake expressed as average g/mouse/day and **B)** energy intake expressed as average kcal/mouse/day**.** **C)** Cumulative food expressed in grams (g) and **D)** cumulative energy intake expressed in kcal. Controls; n=6, controls+MCT; n=6, C26; n=10 and C26+MCT; n=10.

MCT administration did not prevent cachexia in the C26 hosts. All the C26 tumor-bearing animals showed muscle atrophy (Figure 16A, 16B), a significant reduction in epididymal white adipose tissue (Figure 16E) and splenomegaly (Figure 16F) compared to controls. Furthermore, since CC is also characterized by fatigue and loss of muscle function, the delta muscle strength between baseline and the endpoint (day 16 or 18) was evaluated. The analysis revealed that both C26 and C26+MCT groups did not improve muscle strength compared with the controls. Importantly, only a few tumor-bearing mice showed a slight loss of muscle strength (Figure 16H).

Interestingly, the tumor weight resulted increased in the treated mice (Figure 16I). However, the progression of CC was similar in both groups. The relationship between the tumor burden and cachexia is complex, the progression of CC is not a totally tumor-dependent feature but is partially determined by the host response to tumor growth and dissemination [218]. Overall, the results suggest that despite exerting an effect on tumor growth, MCT did not worsen or improve the cachectic phenotype in the C26 tumor-bearing animals.

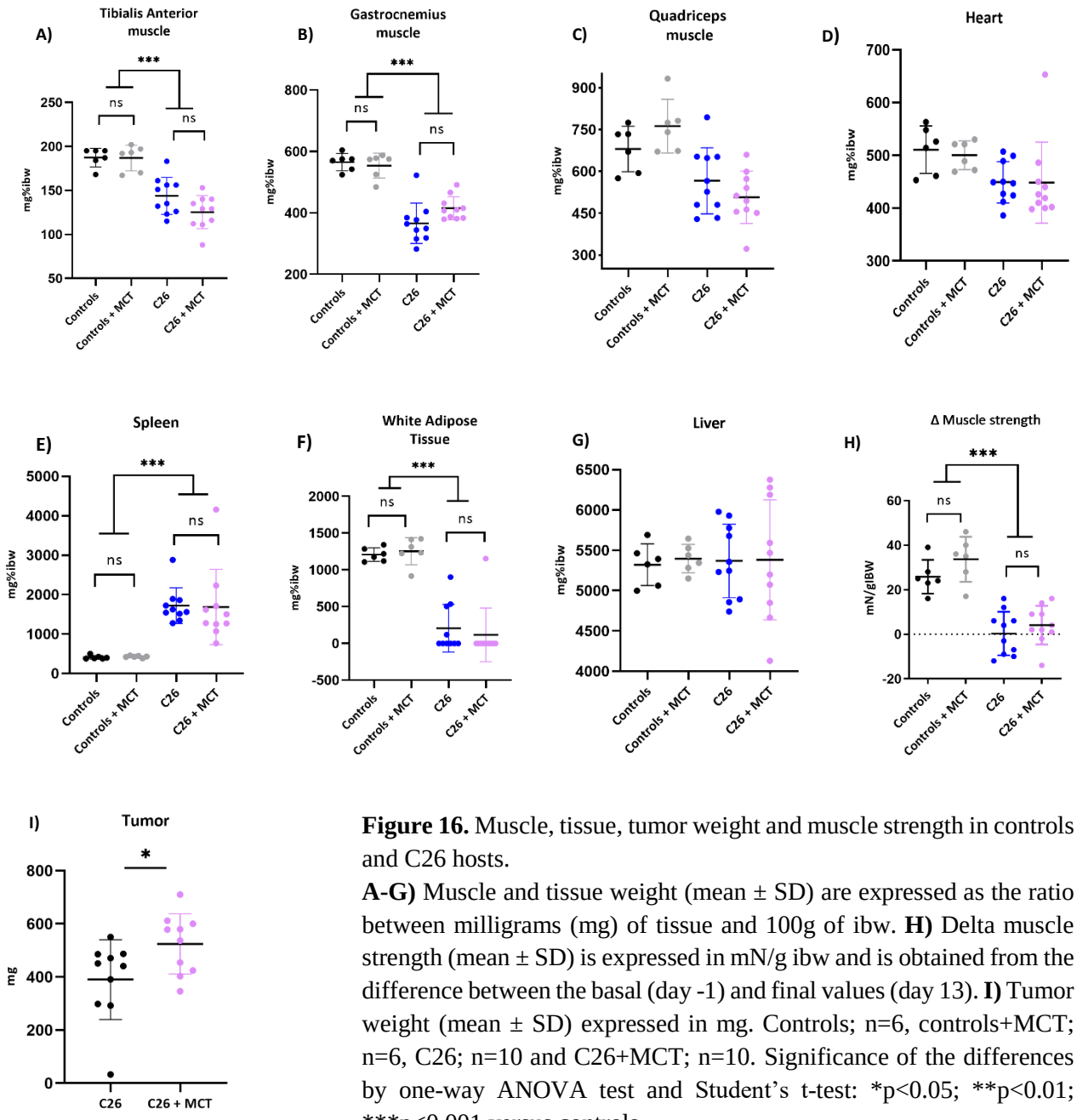


Figure 16. Muscle, tissue, tumor weight and muscle strength in controls and C26 hosts.

A-G) Muscle and tissue weight (mean ± SD) are expressed as the ratio between milligrams (mg) of tissue and 100g of ibw. **H)** Delta muscle strength (mean ± SD) is expressed in mN/g ibw and is obtained from the difference between the basal (day -1) and final values (day 13). **I)** Tumor weight (mean ± SD) expressed in mg. Controls; n=6, controls+MCT; n=6, C26; n=10 and C26+MCT; n=10. Significance of the differences by one-way ANOVA test and Student's t-test: *p<0.05; **p<0.01; ***p<0.001 versus controls.

4.1.4 Effects of the treatment on markers of mitochondrial function and autophagy

Nutritional ketosis, driven by KD and KS, has been linked to increased skeletal muscle mitochondrial activity due to the shift in macronutrient metabolism towards greater fat and KB oxidation compared to carbohydrates [93,97,101,103]. On this basis, although no differences were observed in tissue weight after MCT administration, muscle mitochondrial capacity was evaluated by assessing the activity and expression of proteins involved in oxidative metabolism. In addition, some markers of mitochondrial mass and autophagy in the skeletal muscle were assessed.

Succinate dehydrogenase (SDH), also known as complex II (CII) in the mitochondrial respiratory chain, is responsible for the oxidation of succinate to fumarate. SDH staining on histological sections was performed to evaluate the relative activity of SDH in the skeletal muscle. This analysis can be used to demonstrate changes in overall oxidative capacity by assessing the ratio of oxidative/glycolytic myofibers. A healthy TA muscle contains approximately a 50:50 proportion of oxidative (type I, blue staining) vs glycolytic (type II, unstained) myofibers. However, the results showed that there was no change in metabolic fiber type (ratio of type I/type II fibers) among the experimental groups (Figure 17A, 17B).

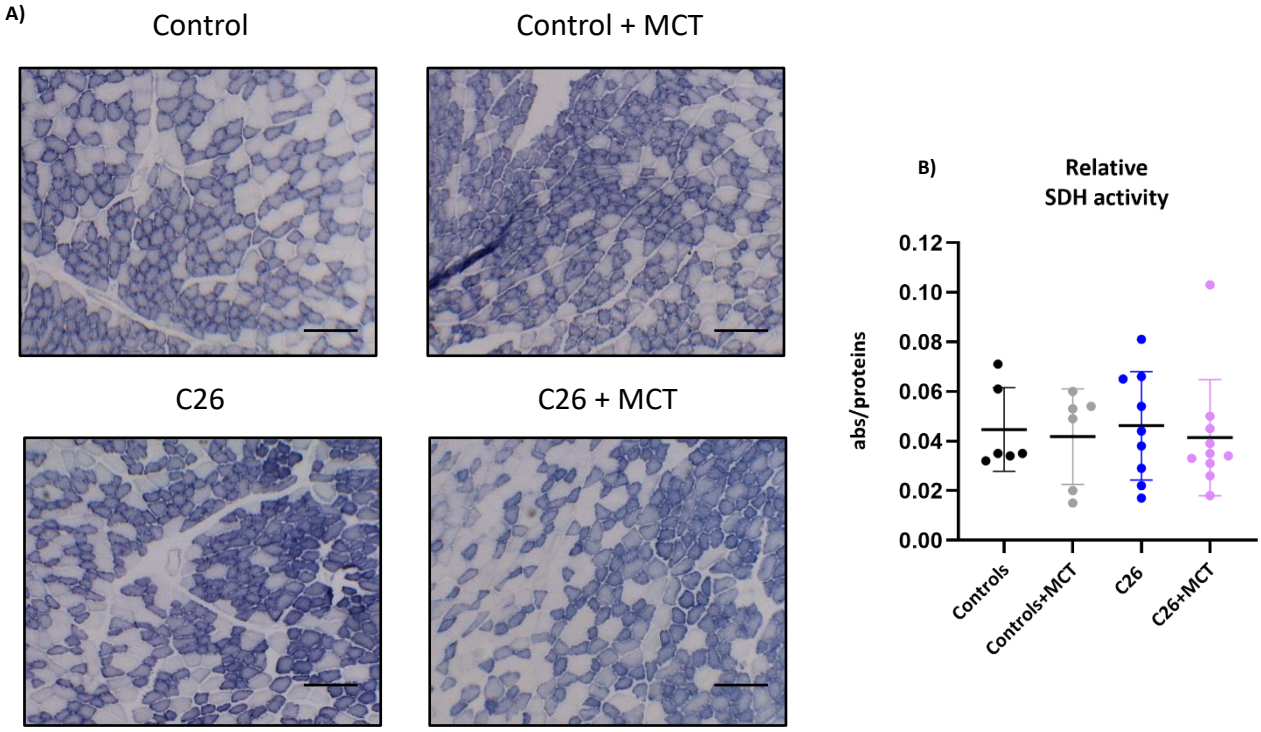
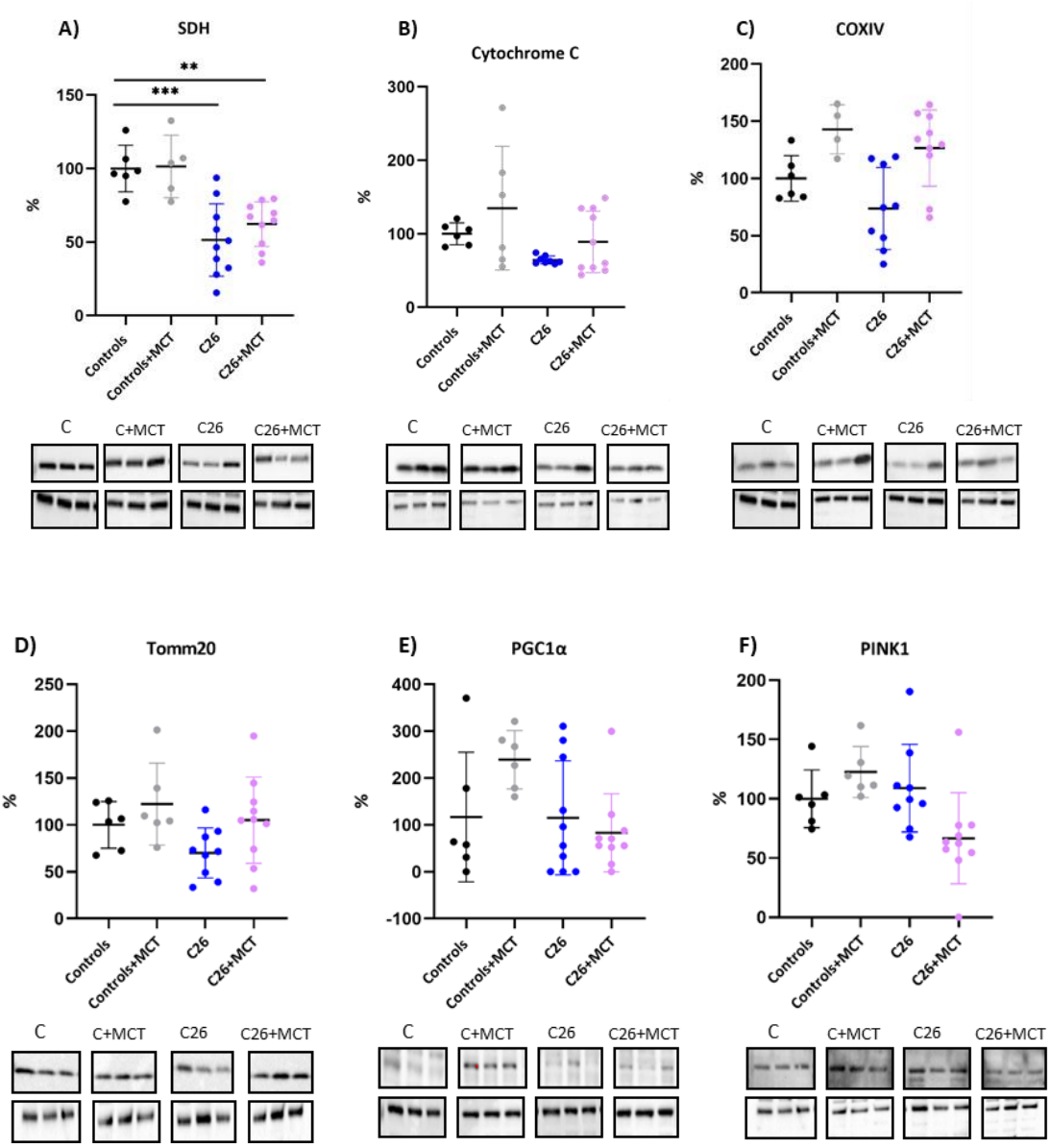


Figure 17. SDH relative activity in the skeletal muscle.
A) Representative images of SDH staining of the TA muscle (x10; scale bar: 100µm). **B)** SDH activity in the *gastrocnemius* muscle. Data (mean ± SD) are expressed as the ratio between absorbance and total protein content after 20 min incubation. Controls; n=6, controls+MCT; n=6, C26; n=9 and C26+MCT; n=10. Significance of the differences by one-way ANOVA test.

As for protein expression, only the SDH protein showed a reduced expression in both C26 groups (Figure 18A). However, no beneficial effect was associated with the MCT supplementation. On the other hand, no significative differences were detected for the rest of the markers commonly associated with mitochondrial mass (Figure 18B-D), and biogenesis (Figure 18E) [31] in the *gastrocnemius* muscle among the groups. Nevertheless, all the MCT-treated groups showed a

tendency to increase the protein expression of cytochrome C, COXIV and Tomm20 in comparison with the untreated mice.

The expression of the marker of mitophagy PTEN-induced kinase 1 (PINK1) did not exhibit alterations under the influence of the tumor or MCT treatment (Figure 18F). Finally, the expression of the ubiquitin-binding protein P62, a marker of autophagy in the skeletal muscle, was more expressed in C26 than in C26+MCT animals compared to controls (Figure 18G).



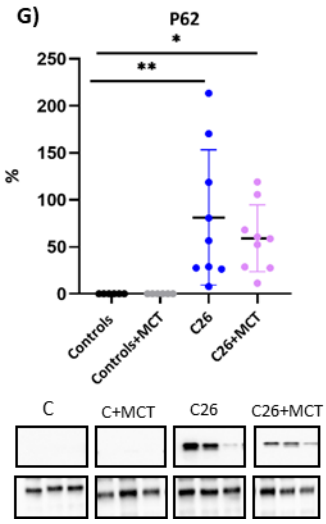


Figure 18. Densitometric analysis and representative blots of proteins involved in oxidative metabolism and autophagy in the *gastrocnemius* muscle of controls; n=6, controls+MCT; n=6, C26; n=10 and C26+MCT; n=10.
Protein levels of **A)** SDH (SDHB), **B)** COX-IV (MTCO1), **C)** Cytochrome C, **D)** PGC1α, **E)** Tomm20 **F)** Pink1 **G)** P62. Data (mean ± SD) are expressed as percentages of controls. Significance of the differences by one-way ANOVA test: *p<0.05; **p<0.01; ***p<0.001 versus controls.

4.1.5 Dietary enrichment with MCT increased ketonemia

Although no protective effect of MCT supplementation was observed in C26 tumour-bearing mice through intragastrical administration, a possible positive impact via other method of supplementation cannot be discarded. Administration by gavage produces an acute ketosis of short duration, less than 4 hours (Figure 12), therefore, the action of MCT may not be sufficient to produce a metabolic shift in the skeletal muscle. On this basis, a new experiment was designed in which animals were supplemented with MCT by enriching the standard diet. Thus, the animals could consume the supplement voluntarily, and also skip the added stress of the gavage. In addition, the MCT dose could be increased as the adverse effects observed after a rapid intake of MCT would be blunted. MCT supplementation was gradual, starting with a low dose (5g/kg/day) administered for 7 days, as an adaptation period. Subsequently, the MCT dose was raised (10g/kg/day) and maintained until the end of the experiment. Blood glucose and ketone levels were measured at baseline and after supplementation on day 13 to monitor nutritional ketosis.

As in the previous experiment, mice were subcutaneously inoculated with C26 adenocarcinoma cells and exposed to MCT administration from the day after tumor implantation until the endpoint (Figure 19). To achieve a translational perspective, 5 months-old female BALB/c mice were used. One animal from the C26 group died the day before the endpoint and therefore its tissues could not be collected.

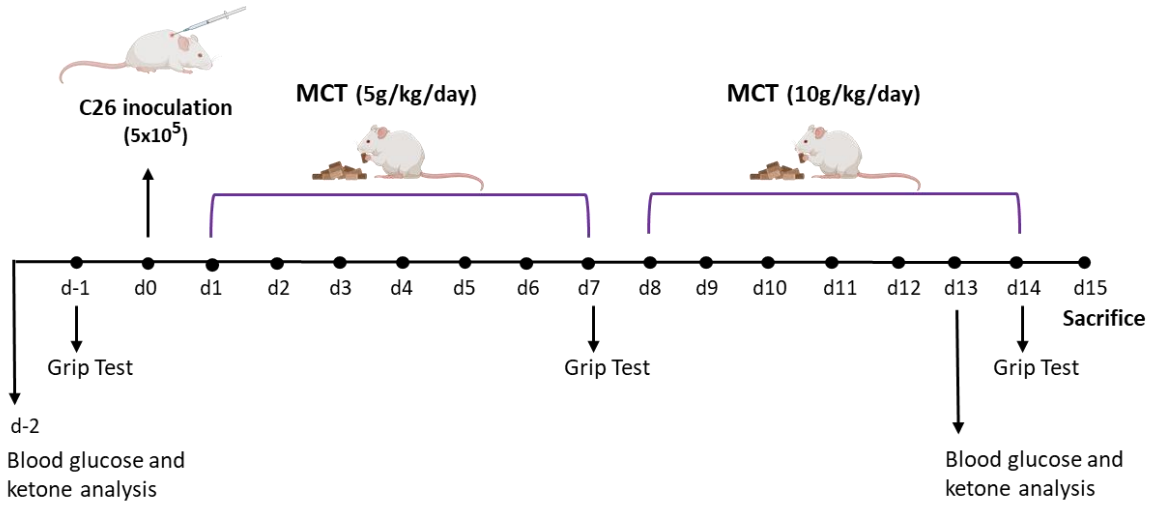


Figure 19. Experimental design. Tumor inoculation, MCT administration, grip test, blood glucose and ketone analysis and sacrifice time points are illustrated in the timeline. The experimental groups consist of healthy controls; n=5, controls+MCT; n=5, C26; n=6, and C26+MCT; n=6.

Defining the exact supplement intake of each mouse is a challenge when animals receive the nutritional supplement *ad libitum*. Hence, we decided to assess the blood ketone levels after one hour of supplement delivery. Consequently, the analysis showed a high dispersion among samples (Figure 20A). Nonetheless, the assessment served to demonstrate that the enrichment of the diet with MCT induced an increase in ketonemia. On day 13, controls+MCT= 1.54mmol/L±0.52 and C26+MCT= 1.15mmol/L±1.5 relative to baseline levels.

Ingestion of ketone drinks significantly decreases overall mean plasma glucose after 1 h. [219]. During exogenous ketosis, carbohydrate stores are abundant, but ketones appear to reduce blood glucose by limiting hepatic gluconeogenesis and increasing peripheral glucose uptake [220]. Thus, in parallel with β-HB analysis, blood glucose levels were also assessed. As shown in Figure 20B, both controls+MCT and C26+MCT reduced blood glucose compared to baseline (on day 13). In the case of the tumor-bearing mice, this decrease could be explained by the anorexia suffered by the animals on day 12 (food intake of C26= 1.08g/animal/day and C26= 1.47g/animal/day).

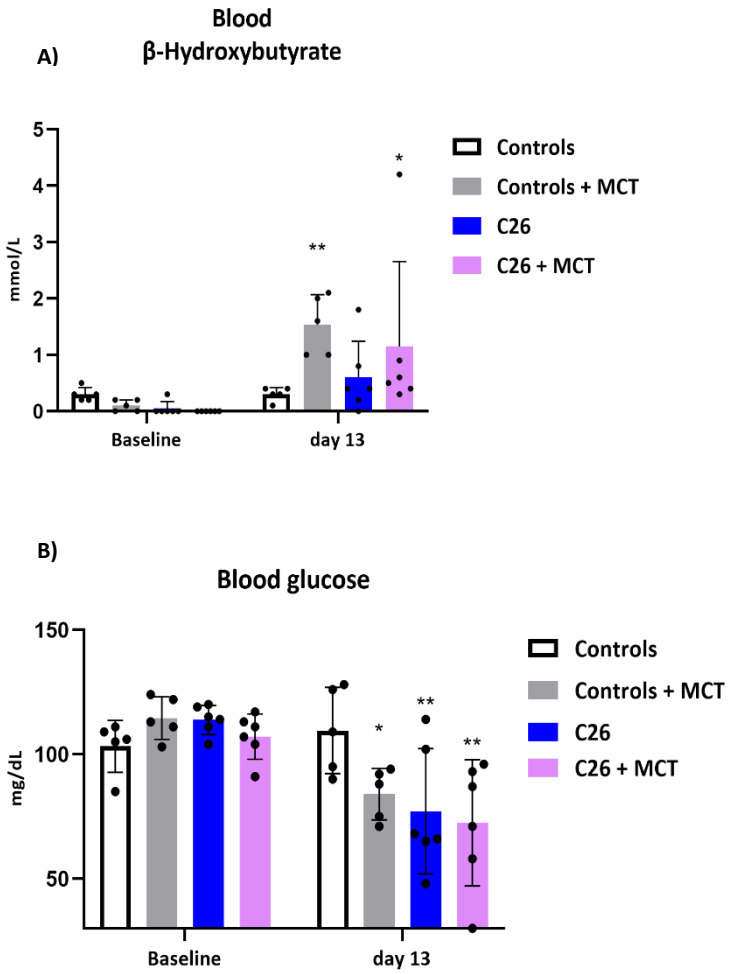


Figure 20. Blood β-HB and glucose levels in controls and C26 hosts. **A)** Blood β-HB (means ± SD) levels expressed in mmol/L and **B)** glucose in mg/dL (means ± SD) at baseline and day 13 after one hour from MCT administration. Controls; n=5, controls+MCT; n=5, C26; n=6 and C26+MCT; n=6. Significance of the differences by two-way ANOVA test *p<0.05; **p<0.01 versus baseline.

4.1.6 MCT enriched diet did not prevent CC

The loss of body weight in the C26 groups is observed from day 10, being statistically significant from day 12 onwards (Figure 21A). At the end of the experiment, the final body weight loss was similar between the two groups (C26= -22.76%±5.63; C26+MCT= -22.55%±9.94; Figure 21B).

The C26 hosts reduced their food intake compared to controls (Figure 22A). The cumulative food intake (from day 2 to 14) was as follows: controls= 21.71g; controls+MCT= 20.62; C26= 17.85g and C26+MCT= 17.83g (Figure 22B). However, the C26 mice supplemented with MCT, showed a higher energy intake compared to the tumor hosts that did not receive it (Figure 22C) reaching values similar to those of controls. The cumulative energy intake was: controls=62.95Kcal, controls+MCT= 66.45Kcal; C26= 51.76Kcal and 66.14Kcal for the C26+MCT group (Figure 22D). Remarkably, this result demonstrated that increasing energy intake is not sufficient to fully reverse the body weight loss observed in C26 hosts.

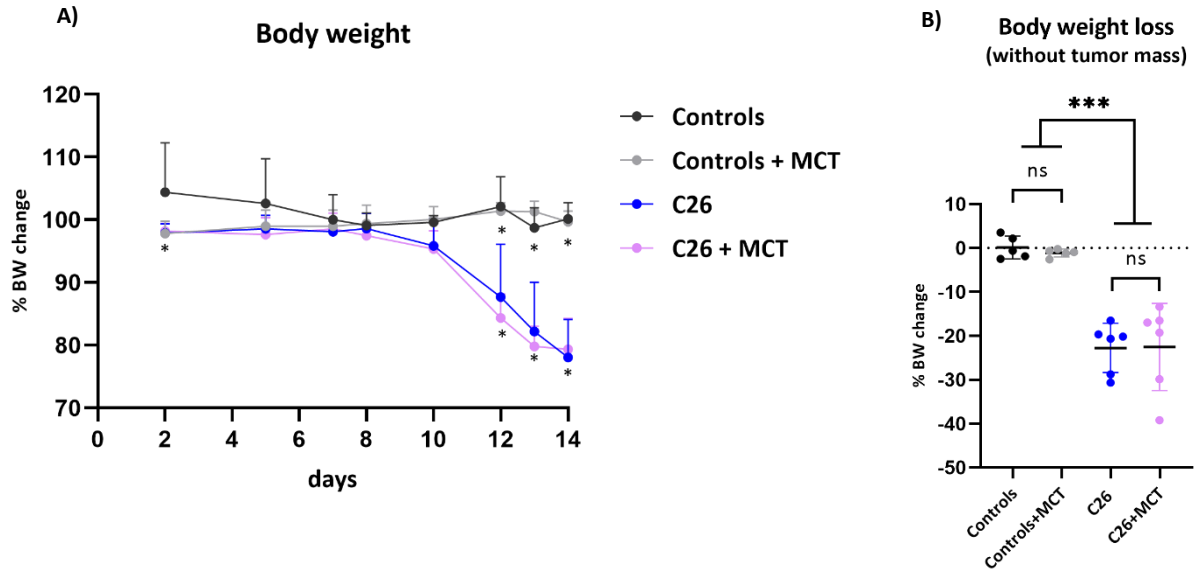


Figure 21. Body weight change and final body weight loss in controls and C26 hosts.
A) Body weight (mean ± SD) is expressed as a percentage of ibw. **B)** Body weight loss delta (mean ± SD) expressed as a percentage of the delta of the ibw and fbw excluding tumor mass of controls; n=5, controls+MCT; n=5, C26; n=6, and C26+MCT; n=6. Significance of the differences by one-way ANOVA test *p<0.05; **p<0.01; ***p<0.001 versus controls.

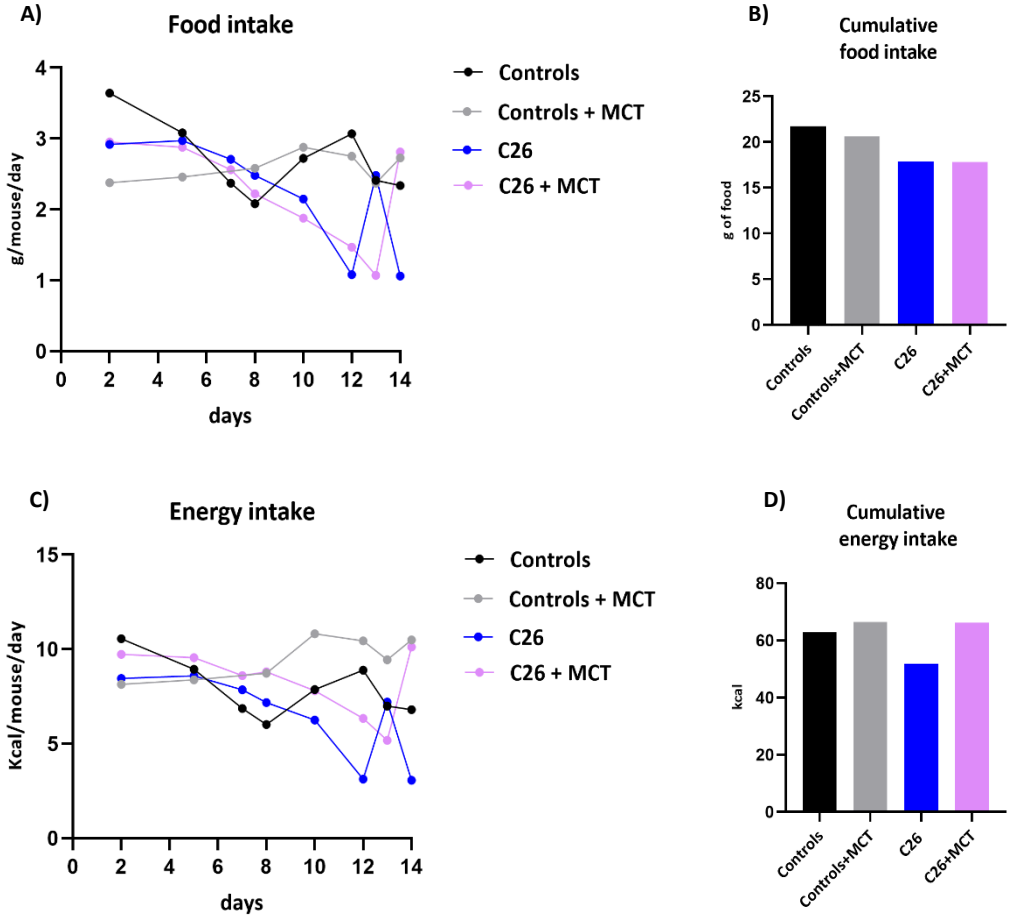
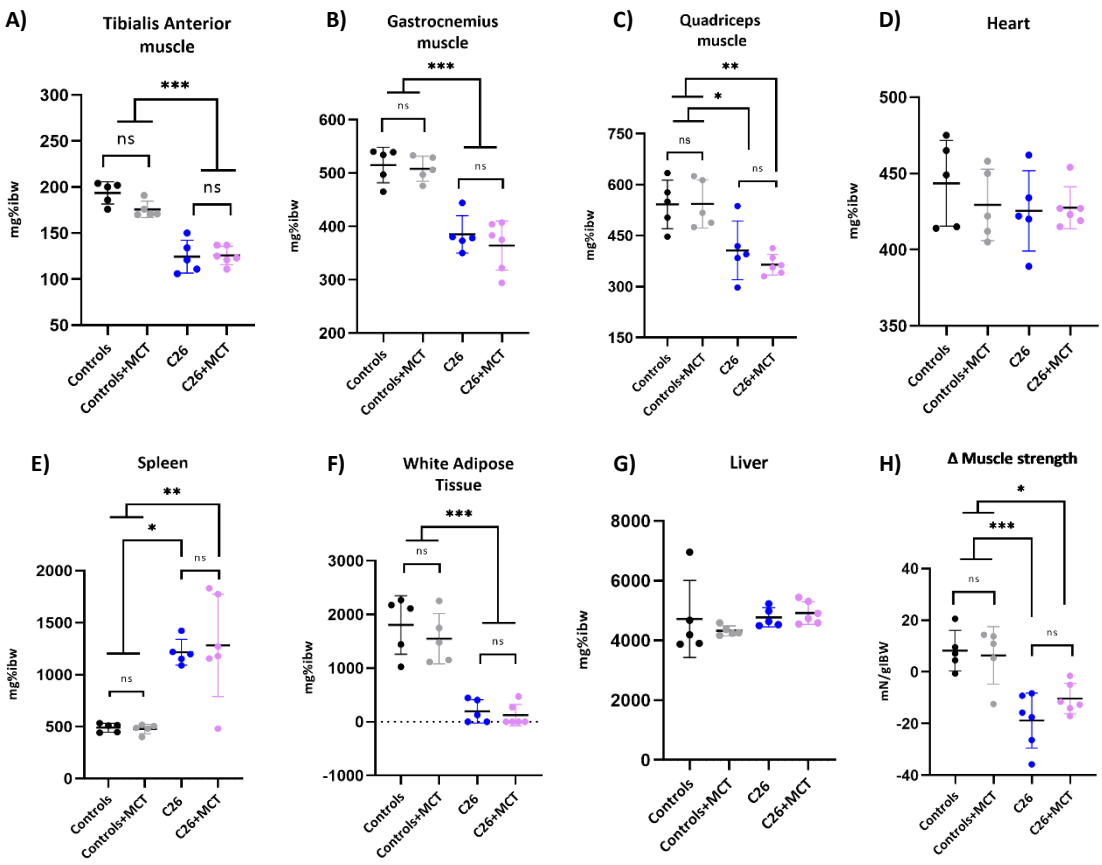


Figure 22. Food and energy intake in controls and C26 hosts.
A) Food intake expressed as average g/mouse/day and **B)** energy intake expressed as average kcal/mouse/day**.** **C)** Cumulative food expressed in g and **D)** cumulative energy intake expressed in kcal. Controls; n=5, controls+MCT; n=5, C26; n=6 and C26+MCT; n=6.

All the tumor-bearing animals showed muscle wasting (Figure 23A-C), along with enlargement of the spleen (Figure 23E) and gonadal white adipose tissue depletion (Figure 23F) irrespective of MCT treatment. As for delta muscle strength, the C26 hosts showed a greater loss of muscle strength compared to controls than C26+MCT mice (Figure 23H). This marked decrease in the C26-bearing mice could be due to the severe muscle weakness shown by the mouse that did not reach the endpoint. Finally, in contrast to the previous experiment, the tumor weights were comparable (Figure 23I).



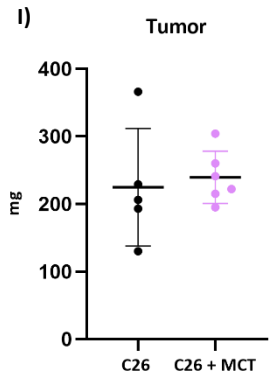


Figure 23. Muscle, tissue, tumor weight and muscle strength in controls and C26 hosts.
A-G) Muscle and tissue weight (mean ± SD) are expressed as the ratio between mg of tissue and 100g of ibw. **H)** Delta muscle strength (mean ± SD) is expressed in mN/g ibw and is obtained from the difference between the basal (day -1) and final values (day 14). **I)** Tumor weight (mean ± SD) is expressed in mg. Controls; n=5, controls+MCT; n=5, C26; n=5 and C26+MCT; n=6. Significance of the differences by one-way ANOVA test and Student's t-test: *p<0.05; **p<0.01; ***p<0.001; ***p<0.001 versus controls.

4.1.7 SDH activity is not enhanced by the treatment

SDH staining was performed on TA muscle slices, to get an overview of the metabolic changes that may be occurring in the skeletal muscle following MCT supplementation. However, no changes in the ratio of oxidative to glycolytic fibres were identified among the groups (Figure 24). It should be noted that the assessment of the SDH enzymatic activity by fibre staining is an approximate method for determining the metabolic profile of muscle fibers.

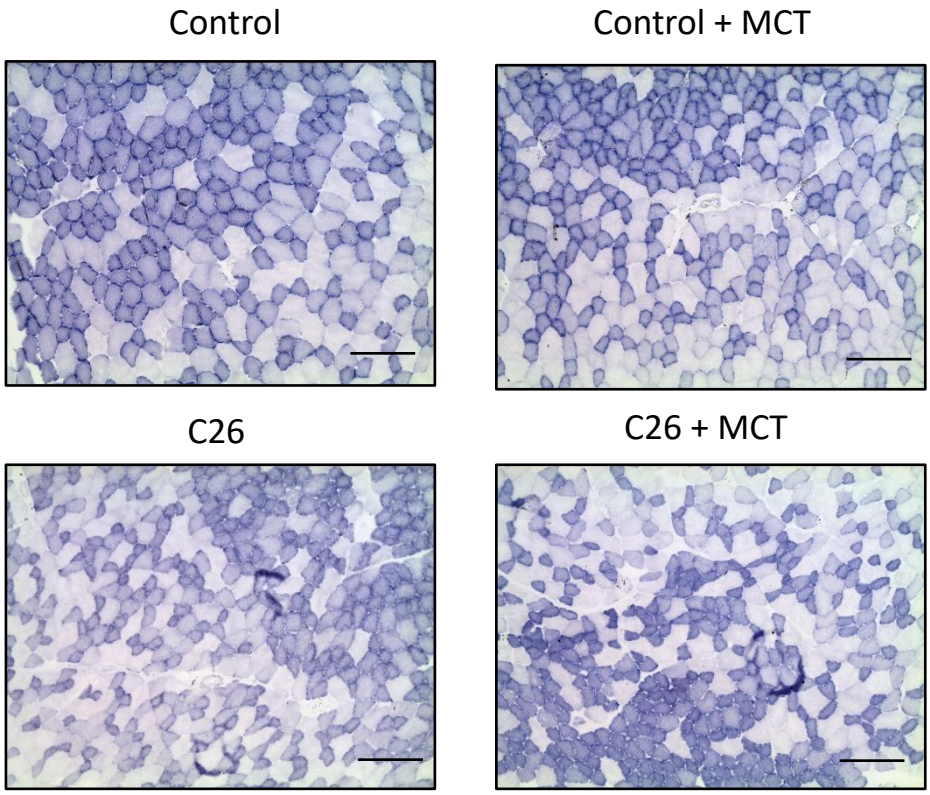
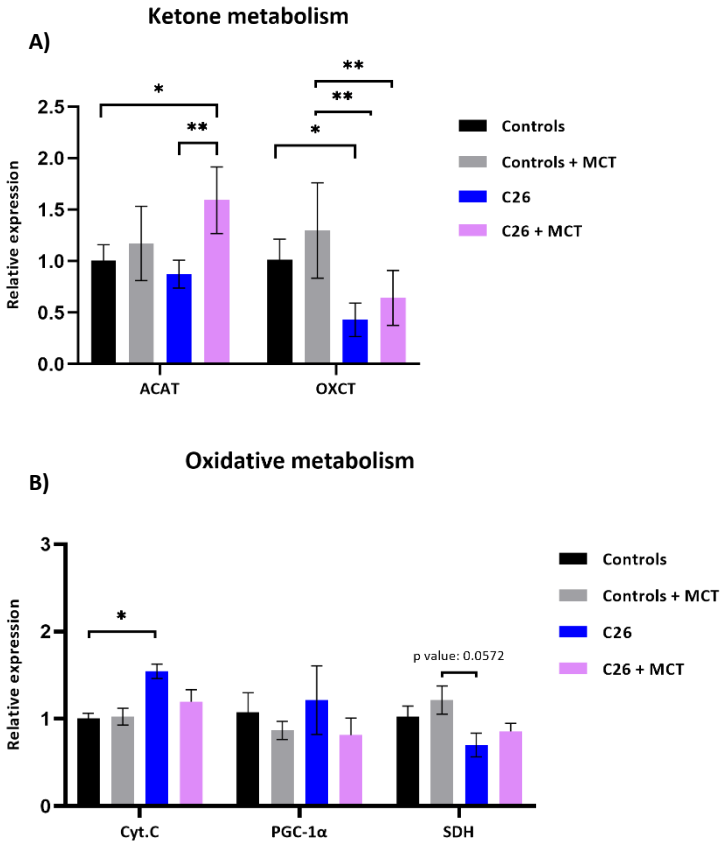


Figure 24. Representative images of SDH staining of the TA muscle (x10; scale bar: 100μm)

4.1.8 The dietary enrichment with MCT modulated gene expression

Although no positive effect of MCT on SDH enzymatic activity was found, the possibility that supplementation could induce changes at the molecular level cannot be discarded. Accordingly, gene expression of key markers of ketone body metabolism, oxidative capacity and proteolysis in the skeletal muscle was investigated (Figure 25).

The gene expression of enzymes involved in ketone body metabolism in the skeletal muscle was influenced by CC and MCT supplementation (Figure 25A). Acetyl-CoA acetyltransferase (ACAT) resulted upregulated in C26+MCT compared with the groups that did not receive the supplement. The ACAT enzyme catalyzes the cleavage of the acetoacetyl CoA to produce acetyl CoA. On the contrary, succinyl CoA:3-oxoacid CoA transferase (OXCT) was significantly downregulated in the C26 hosts compared with untreated controls. This enzyme hydrolyzes the ketone body acetoacetate to acetoacetyl CoA succinate. Concerning proteins related to oxidative metabolism, interestingly, cytochrome C was upregulated in the C26 group in comparison to the controls (Figure 25B). Cytochrome C catalyzes the last step of the mitochondrial electron transfer chain and is considered one of the main sites of regulation of OXPHOS.



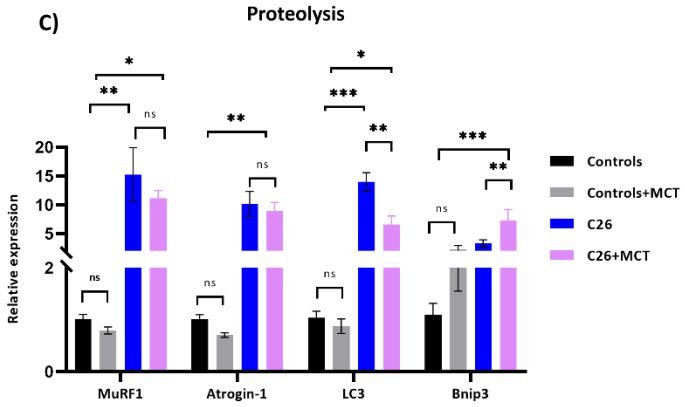


Figure 25. Gene relative expression in the *gastrocnemius* of controls and C26 hosts.
A) Genes involved in ketone metabolism**, B)** oxidative metabolism, and **C)** proteolysis, Data (mean ± SD) is normalized to housekeeping gene expression and displayed as fold change over the control group. Controls; n=5, controls+MCT; n=5, C26; n=5, and C26+MCT; n=6. Significance of the differences by one-way ANOVA test: *p<0.05; **p<0.01; ***p<0.001.

The systemic inflammation that occurs during CC promotes muscle wasting. In particular, the pro-inflammatory cytokines mediate both the activation of proteolysis, and the impaired myogenesis [3,24]. In addition, the inflammatory state is involved in the development of mitochondrial dysfunction leading to an altered capacity for oxidative metabolism in the skeletal muscle, further enhancing muscle atrophy [29]. Besides that, autophagy has important effects on the induction of the inflammatory response [221,222].

Since β-HB have been studied for its anti-inflammatory and antioxidant effect [83,121], the gene expression of some markers involved in proteolysis in the skeletal muscle were studied. Tumor hosts showed an elevated expression of genes belonging to the E3 ubiquitin ligases complex (Murf1, Atrogin-1), and autophagy-related genes (LC3). MCT supplementation led to a reduction of LC3 levels in the C26 hosts. Conversely, BNIP3 expression was found induced in the C26+MCT group (Figure 25C).

4.2 Dietary enrichment with MCT in the VCM model

The C26 tumor-bearing mice rapidly develop a severe CC characterized among others, by a dramatic body weight loss, anorexia and muscle atrophy. Therefore, in overt cachexia models, dietary interventions are challenging as they often require more chronic interventions for both preventive and therapeutic approaches.

The Villin-Cre/Msh2$^{loxP/loxP}$ (VCM) mice slowly and spontaneously develop tumors, usually from 11 months of age, due to the conditional knock-out of the mismatch repair gene Msh2 in the enterocytes of the intestinal mucosa [212]. Consistent with previous findings [223], the VCM model exhibited notable alterations such as significant splenomegaly, anemia, moderate body weight loss, and muscle wasting resulting from tumor development. Thus, it can be considered a new less aggressive model for the study of CC (see Methods).

So far, in a preliminary study, the effect of MCT supplementation in the VCM model has been explored. Male Msh2$^{loxP/loxP}$ mice lacking Cre recombinase were used as controls, while Msh2$^{loxP/loxP}$ in combination with Cre recombinase transgenes (VCM) were used as tumor-bearing animals. Twelve-month-old male mice were randomized as; controls fed with a standard diet (SD; n=3), VCM+SD (n=5) and VCM supplemented with MCT (VCM+MCT; n=8). Nonetheless, not all the VCM mice developed tumors, only 4 animals out of 5 had tumors in the VCM+SD and 4 out of 8 in the VCM+MCT. After four weeks of MCT supplementation, the animals were euthanized (Figure 26).

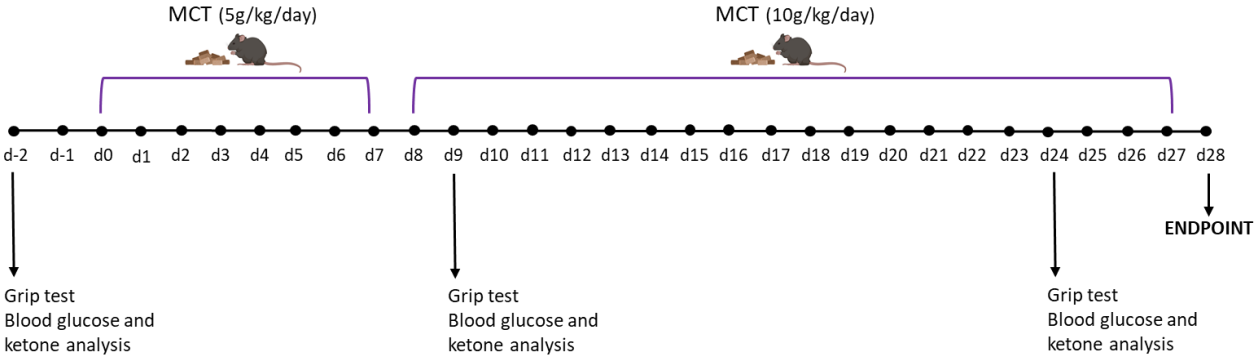


Figure 26. Schematic representation of the experimental design.
The cartoon depicts the experimental plan, including the supplementation with MCT (starting when animals reached 12 months old) grip test, blood glucose and ketones analysis, and sacrifice time points. The experimental groups consisted of three groups, controls (Msh2$^{loxP/loxP}$) mice fed with standard diet (n=3), VCM mice fed with standard diet (n=5) and VCM+MCT mice (n=8).

4.2.1 MCT enriched diet increased ketonemia in VCM mice

The β-HB concentration of all VCM+MCT animals (regardless of tumor presence) increased resulting in "mild ketosis" (β-HB<1.0 mmol/L; Figure 27A). In contrast, blood glucose concentration remained equal to basal levels in both groups (Figure 27B). Blood from the control group was only measured on day 24, the values were: β-HB= 0.07mmol/L±0.12 and glucose=139mg/dL±8.14.

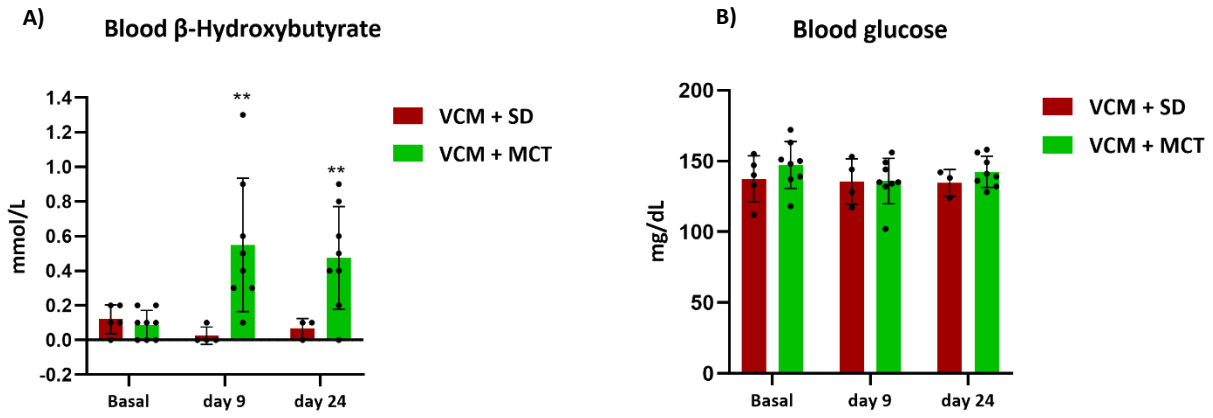


Figure 27. Blood β-HB and glucose levels in controls and VCM mice.
A) Blood β-HB levels expressed in mmol/L and **B)** glucose in mg/dL (means ± SD) at baseline and days 9 and 24 after one hour from MCT administration. Controls; n=3, VCM+SD; n=3, and VCM+MCT; n=4. Significance of the differences by two-way ANOVA test *p<0.05; **p<0.01 versus baseline.

4.2.2 MCT did not impact body weight, muscle strength and food intake

The body weight kinetics were very similar between the groups (Figure 28A). However, on day 15, VCM+SD had a significant body weight loss compared to the control group. This difference is due to the presence of one animal in the SD group that suffered a severe loss of body weight (-39.95%; Figure 28B). Lastly, muscle strength seemed to be lower in the tumor-bearing groups of animals (Figure 28C) in relation to controls. Importantly, the small sample size of this prelaminar study together with the high heterogeneity among the animals would explain the high dispersion observed in this analysis.

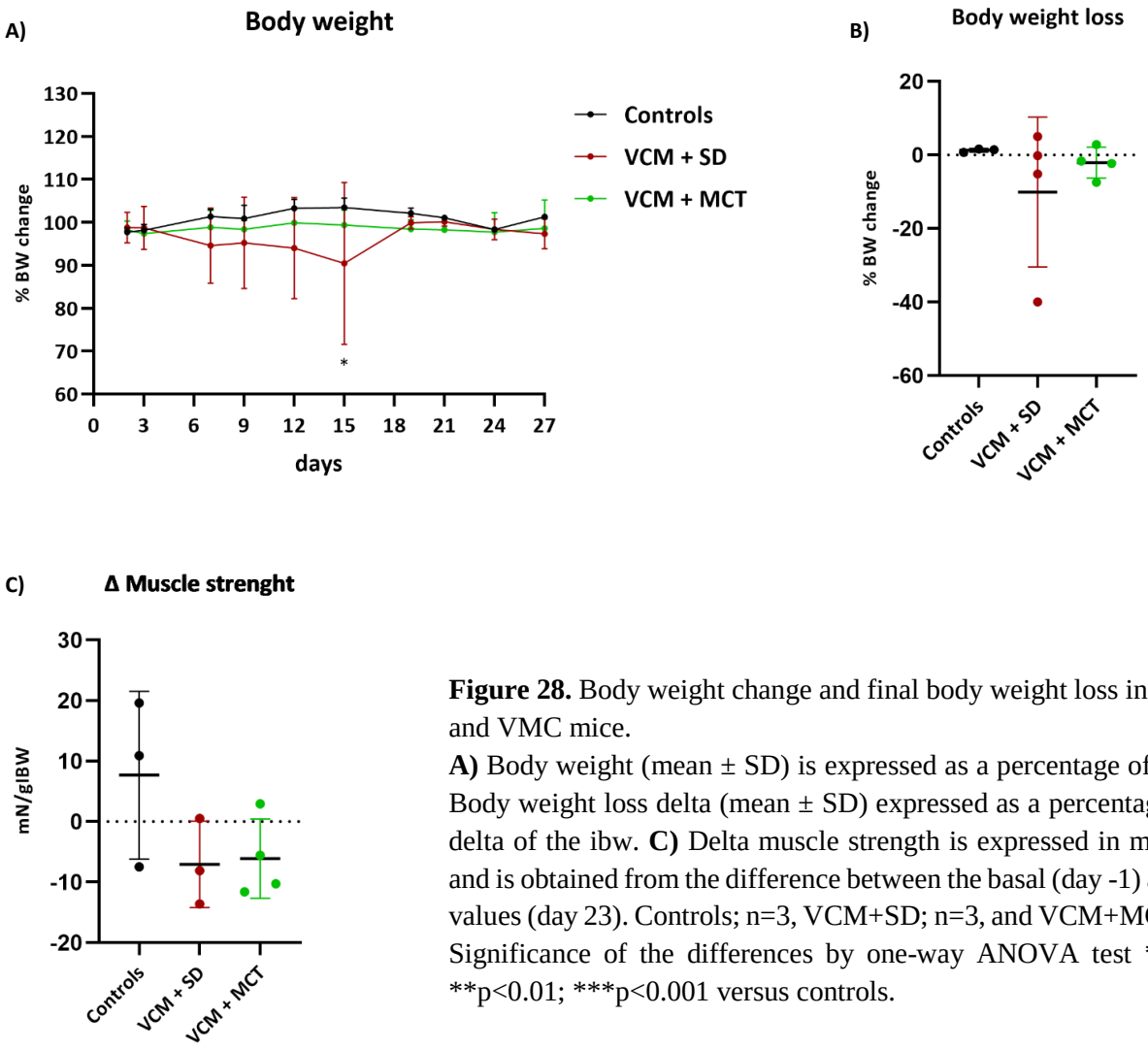


Figure 28. Body weight change and final body weight loss in controls and VMC mice.
A) Body weight (mean ± SD) is expressed as a percentage of ibw. **B)** Body weight loss delta (mean ± SD) expressed as a percentage of the delta of the ibw. **C)** Delta muscle strength is expressed in mN/g ibw and is obtained from the difference between the basal (day -1) and final values (day 23). Controls; n=3, VCM+SD; n=3, and VCM+MCT; n=4. Significance of the differences by one-way ANOVA test *p<0.05; **p<0.01; ***p<0.001 versus controls.

VCM animals also did not show anorexia (Figure 29A, 29B). Despite the fact that the animals supplemented with MCT obtained a high calorie content from the diet (Figure 29C, 29D), this did not interfere with the total cumulative food intake.

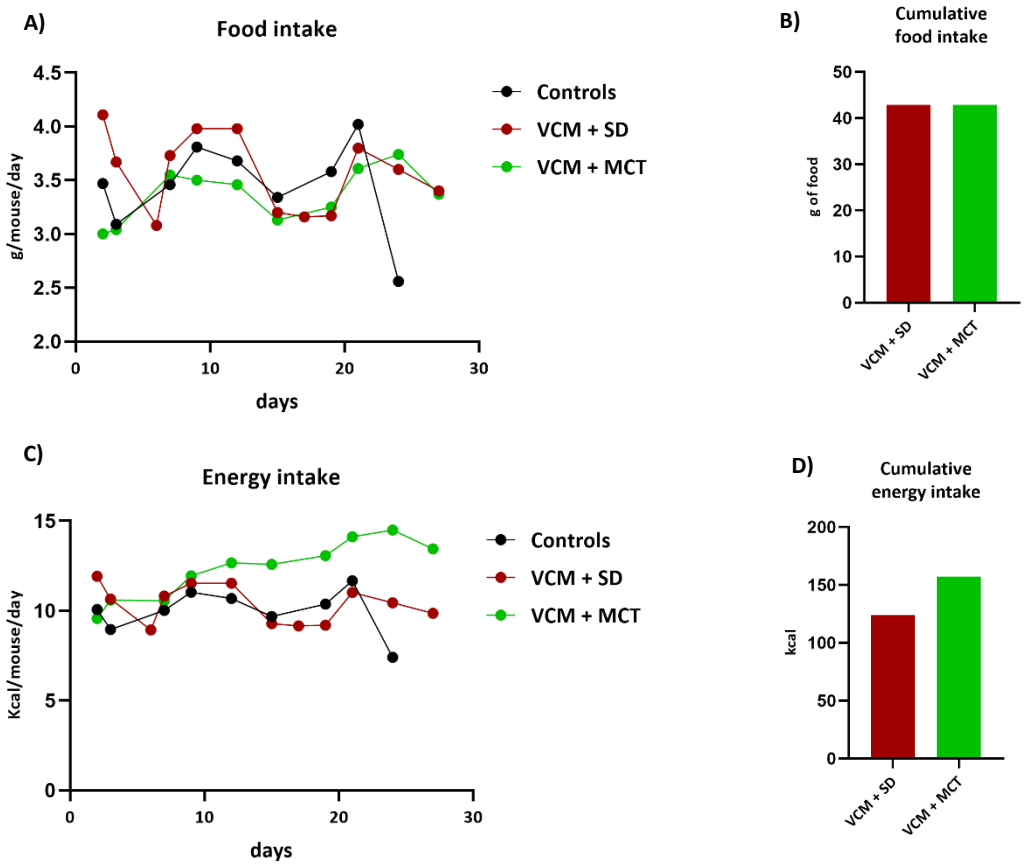


Figure 29. Food and energy intake in controls and VCM mice.
A) Food intake expressed as average g/mouse/day and **B)** energy intake expressed as average kcal/mouse/day**.** **C)** Cumulative food expressed in g and **D)** cumulative energy intake expressed in kcal. Controls; n=3, VCM+SD; n=4, VCM+MCT; n=4.

Tissues were collected and weighed the day of sacrifice. Muscle and organ weights for all the experimental groups are presented in Table 5. Overall, the groups do not show statistical differences, likely depending on the limited sample size. Markedly, the spleen and the liver weight seemed to be increased in both VCM groups. The tumor weight also appeared to be larger in untreated than in treated VCM mice.

Table 5. Tissue weight.

TISSUES	Controls (n=3)	VCM+SD (n=4)	VCM+MCT (n=4)
Tibialis Anterior	153.3±8.62	131.25±26.05	138.75±25.70
Gastrocnemius	483.66±21.55	390.75±83.24	428±71.88
Quadriceps	447±47.57	345±69.68	415±92.13
Spleen	365.33±53.50	1626.75±1531.89	1026±561.17
Liver	4370.33±199.78	5676.75±1702	5397.5±1281.19
Tumor	-	121.5±89.64	78.1±61.10
Heart	357.33±32.47	411±131.65	358±60.27
White adipose tissue	2561.33±586.57	1132.14±1389.70	2861±791.24

Muscle, tissue and tumor weight in controls and VCM mice.
Muscle and tissue weight (mean ± SD) are expressed as the ratio between mg of tissue and 100g of ibw. Tumor weight (mean ± SD) is expressed in mg.

4.3 Effect of FMD on C26 tumor-bearing mice receiving chemotherapy

4.3.1 FMD had no impact on cachexia in the C26-folfox model

Considering the previous evidence that a fasting-mimicking diet (FMD) exerts a protective effect against chemotherapy-induced toxicity (DSR) and makes cancer cells more sensitive to anticancer therapies (DSS) due to a deficient adaptation to stress situations [162,174], this study aimed to investigate the potential synergistic effect of 4-day FMD cycles in combination with chemotherapy (Folfox) in a mouse model of CC.

Female Balb/C mice were subcutaneously inoculated with C26 adenocarcinoma cells, leading to the development of an ectopic tumor of intestinal origin. The experimental timeline, including FMD and chemotherapy protocol, is illustrated in Figure 30. To assess the progression of cachexia induced by the C26 tumor and chemotherapy, we compared our findings with previous reports that included healthy controls [206,223].

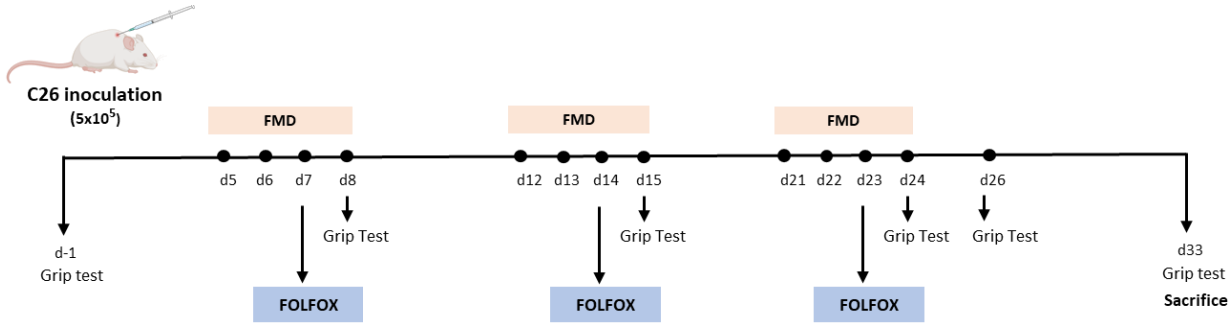


Figure 30. Schematic representation of the experimental design. Tumor implantation, Folfox chemotherapy, FMD protocol, grip test, and sacrifice time points are illustrated in the timeline. The experimental groups consist of C26-folfox animals fed with a standard diet (C26-folofx+SD; n=9) and C26-folfox fed with FMD (C26-folfox+FMD; n=9).

Mouse body weight and food intake were recorded daily from the first FMD cycle. The kinetics of body weight change between C26-folfox fed a FMD (n=9) compared to C26-folfox fed a standard diet (SD; n=9) show a reduced body weight loss recovery for the FMD group (Figure 31A). More specifically, after the first round of chemotherapy and the FMD cycle, the C26-folfox+FMD group seemed to recover better the body weight, by contrast, no recovery was observed after the second round of chemotherapy. Subsequently, C26-folfox+FMD suffered severe body weight loss after the third round of chemotherapy and FMD cycle but at this time, only 6 out of 9 animals from the FMD group and 8 out of the 9 animals from the SD group, respectively, survived until the endpoint. However, it is important to note that no significant differences are reported in the body weight loss of C26-folfox+FMD at the refeeding period (the last day before the next FMD cycle; days

11, 20 and 31) compared with C26-folfox+SD, nor at the endpoint (C26-folofx+SD= -23.12%±3.34, C26-folofx+SD= -25.41%±11 of IBW; Figure 31B).

Consistently, no difference in animal survival was observed in the C26-folfox+FMD group (Figure 31C) compared to the C26-folfox+SD group at the endpoint (day 33), although the study was not designed to determine the impact on animal survival.

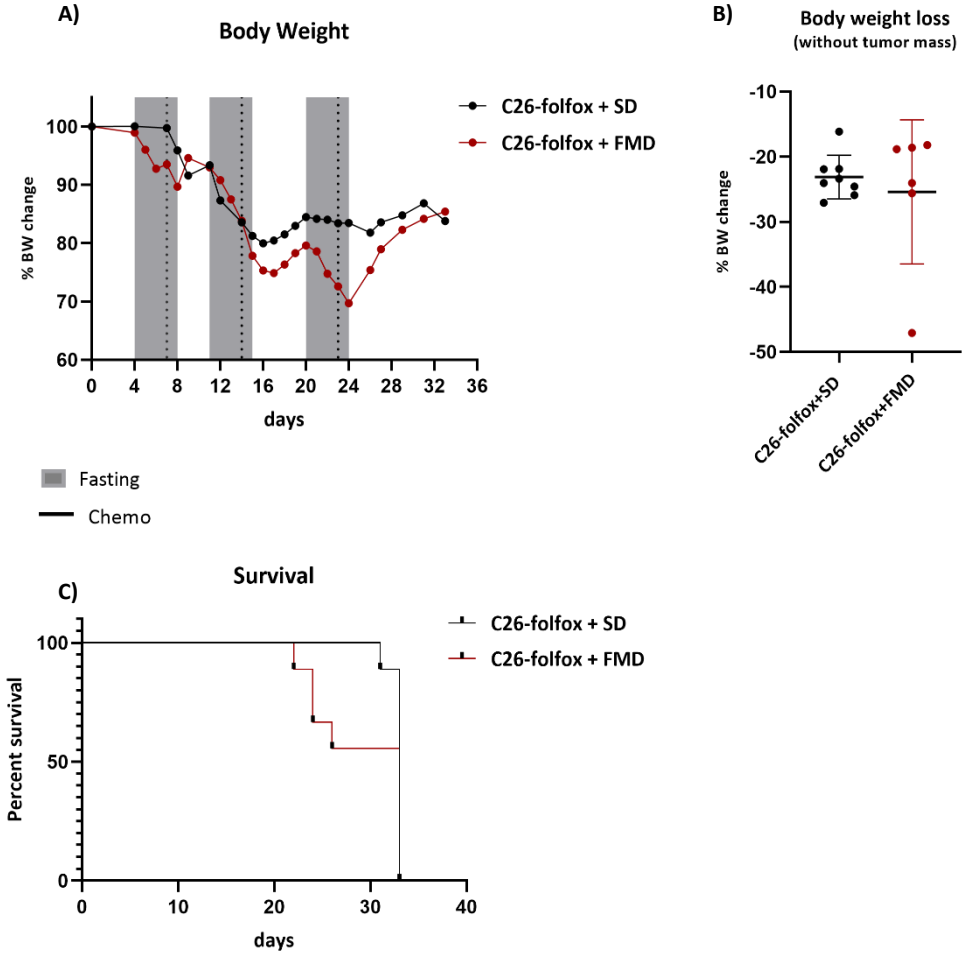


Figure 31. Body weight and overall survival in C26 hosts.
A) Body weight (mean ± SD) is expressed as a percentage of ibw. The grey parts and the dotted lines mark the FMD periods and the administration of chemotherapy in the C26-folfox+FMD group. **B)** Body weight loss delta (mean ± SD) expressed as a percentage of the delta of the ibw and the fbw excluding tumor mass. **C)** Overall survival curve comparing C26-folfox+SD and C26-folfox+FMD groups expressed by Kaplan-Meier curve. Body weight (C26-folfox+SD; n=9, C26-folfox+FMD; n=9) body weight loss (C26-folfox+SD; n=8, C26-folfox+ FMD; n=6).

Muscle strength loss is a common feature of the C26 model [217,223]. In addition, anticancer treatments may exacerbate CC causing muscle atrophy and metabolic perturbations [211,224] along with anorexia and impaired QoL [225]. To investigate whether FMD could exert prevention against

the side effects of chemotherapy, muscle strength was assessed the day next to the Folfox treatment by the grasping test. No differences were observed in the muscle strength of the C26-folfox+SD vs C26-folfox+FMD groups (Figure 32A). Moreover, a similar observation can be made on the delta muscle strength (Figure 32B) which expresses the muscle strength loss, showing no differences among the groups. The C26-folfox+FMD group performed better after the first round of chemotherapy but progressively lost muscle strength in the subsequent tests, consistently with the body weight kinetic.

The overall food and energy intake (Figure 33A, 33C) was slightly decreased by the FMD cycles as presented in the cumulative food intake (Figure 33B; C26-folfox+SD= 57.0g; C26-folfox+FMD= 48.15g) and cumulative energy intake (Figure 33D; C26-folfox+SD= 154.42 Kcal; C26-folfox+FMD= 132.14 Kcal).

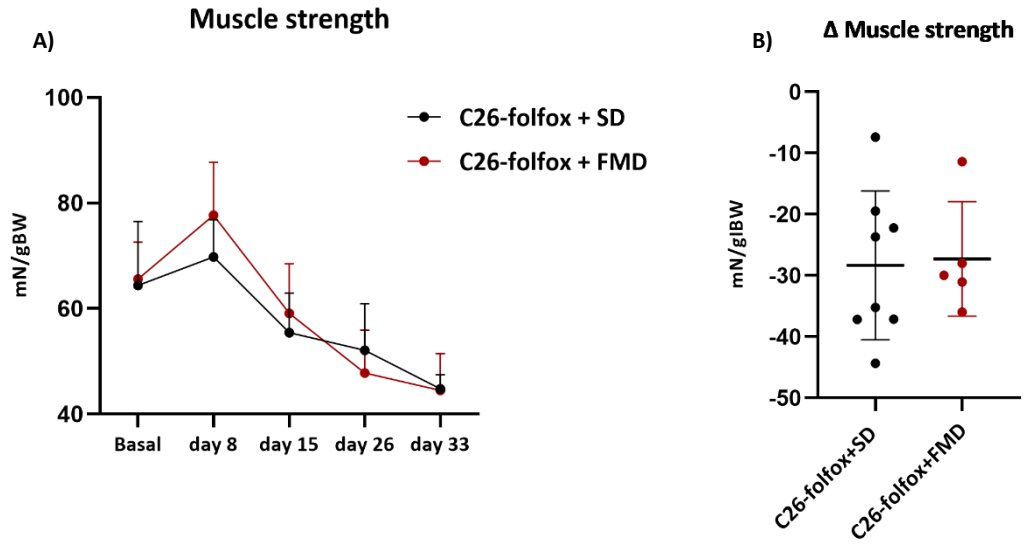


Figure 32. Muscle strength in C26 hosts.
A) Grip strength data (means ± SD) are expressed as the ratio of unit force (mN) and body weight (g) at baseline and the day after every Folfox administration **B**) Delta muscle strength (mean ± SD) is expressed in mN/g ibw and is obtained from the difference between the basal (day -1) and final values (day 33). C26-folfox+SD; n=8 and C26-folfox+FMD; n=5.

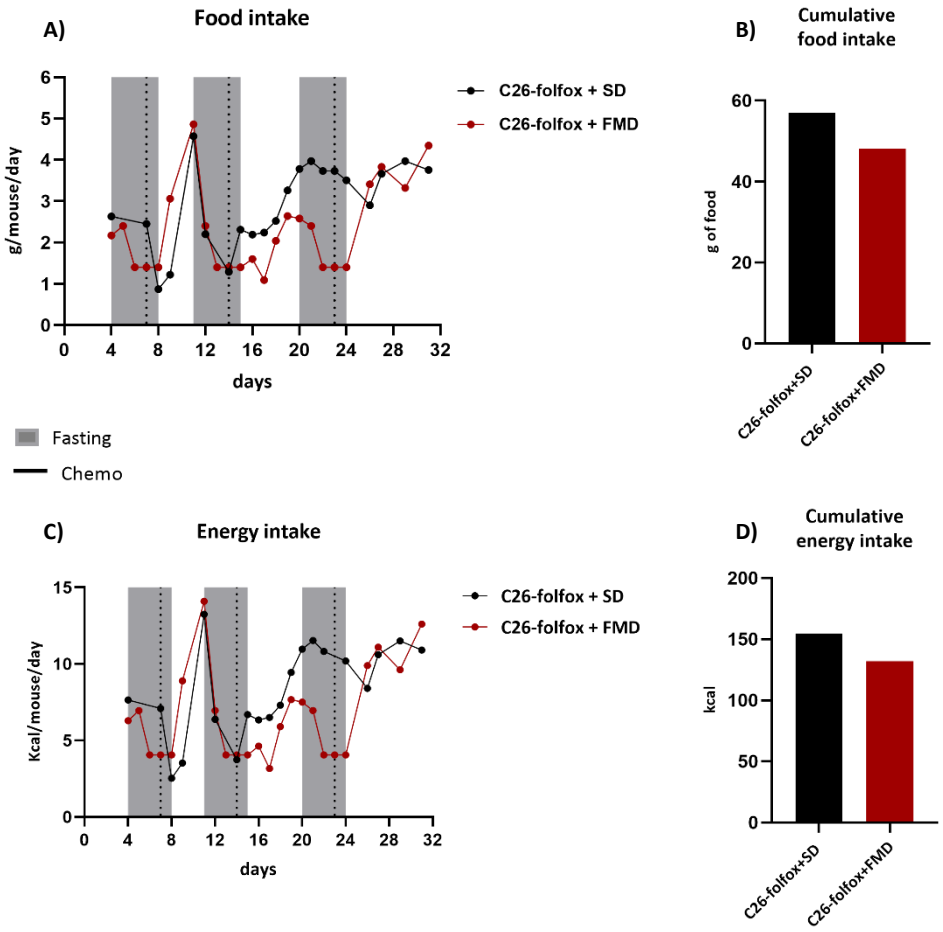


Figure 33. Food and energy intake in C26 hosts.
A) Food intake of 20 days expressed as the average of g/mouse/day. **B)** Energy intake of 20 days expressed as the average of kcal/mouse/day **C)** Cumulative food intake of 20 days expressed in g and **D)** Cumulative energy expressed in Kcal. C26-folfox+SD; n=9 and C26-folfox+FMD; n=9.

FMD did not significantly affect muscle and organ weights (Figure 34). However, in the case of muscle weights, despite a huge variability not allowing to reach statistical significance, FMD seemed to have a positive effect on the weights of the gastrocnemius (Figure 34B) and quadriceps (Figure 34C). The spleen weight (Figure 34F) appeared lower in the C26-folfox+FMD group but again without reaching any statistical significance. On the whole, FMD cycles did not affect the muscle and organ weights of the C26-folfox+SD and C26-folfox+FMD groups. Tumor weight (Figure 34G) shows a tendency to increase in the C26-folfox+FMD group (C26-folfox+SD= 1495.12±306.5mg; C26-folfox+FMD= 1763.6±465.8mg).

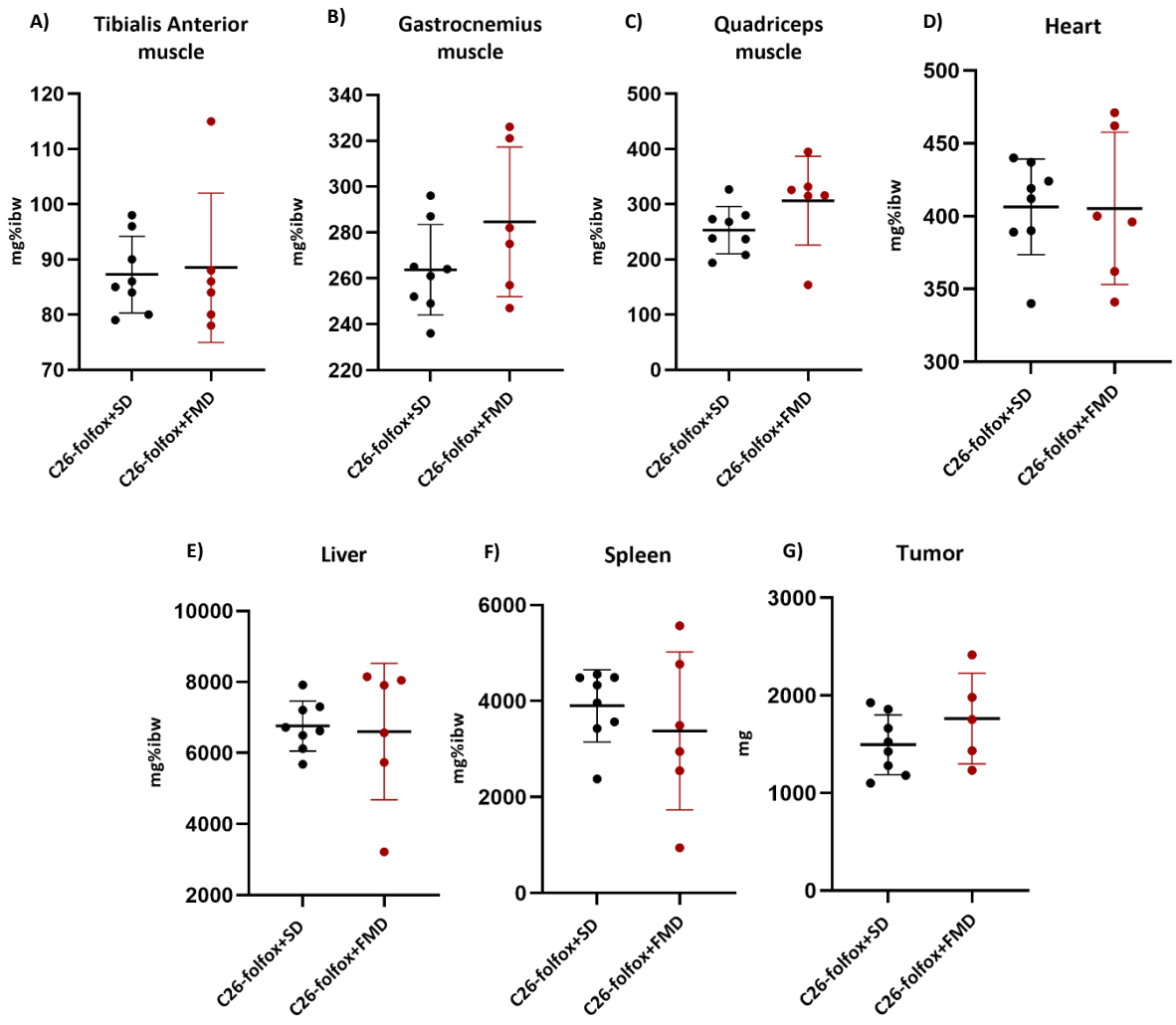


Figure 34. Muscle, tissue and tumor weight in C26 hosts.
A-F) Muscle and tissue weight (mean ± SD) are expressed as the ratio between mg of tissue and 100g of ibw.
G) Tumor weight (mean ± SD) expressed in mg. C26-folfox+SD; n=8 and C26-folfox+FMD; n=6.

4.3.2 Potential impact of FMD on mitochondrial function

Although we observed that FMD did not overtly counteract CC, we decided to evaluate whether FMD could have an impact at the molecular level on muscle metabolism. It has been demonstrated that the metabolic switch occurring during fasting may enhance the maintenance of muscle mass and function [153,154]. Accordingly, some of the proteins involved in oxidative metabolism (OXPHOS) in the skeletal muscle were analyzed (Figure 35). The OXPHOS system consists of five multimeric complexes integrated into the mitochondrial inner membrane, and they work in concert to drive the aerobic synthesis of ATP [226].

Overall, when comparing the FMD and SD groups, the FMD group had a trend to increase in the OXPHOS protein expression levels, indeed, this increase was significant in the complex V (CV; Figure 35E). The CV also known as mitochondrial ATP synthase, plays a role in ATP production

through the energy provided by the proton electrochemical gradient [227]. These findings suggest a potential influence of FMD on the efficiency of mitochondrial function, improving energy production. Next, to assess whether FMD impacted the mitochondrial compartment, succinate dehydrogenase (SDH) enzyme activity was evaluated in the gastrocnemius muscle (Figure 35F). Hence, The C26-folfox+FMD group did not show any increase in mitochondrial activity when compared with mice on SD regimen.

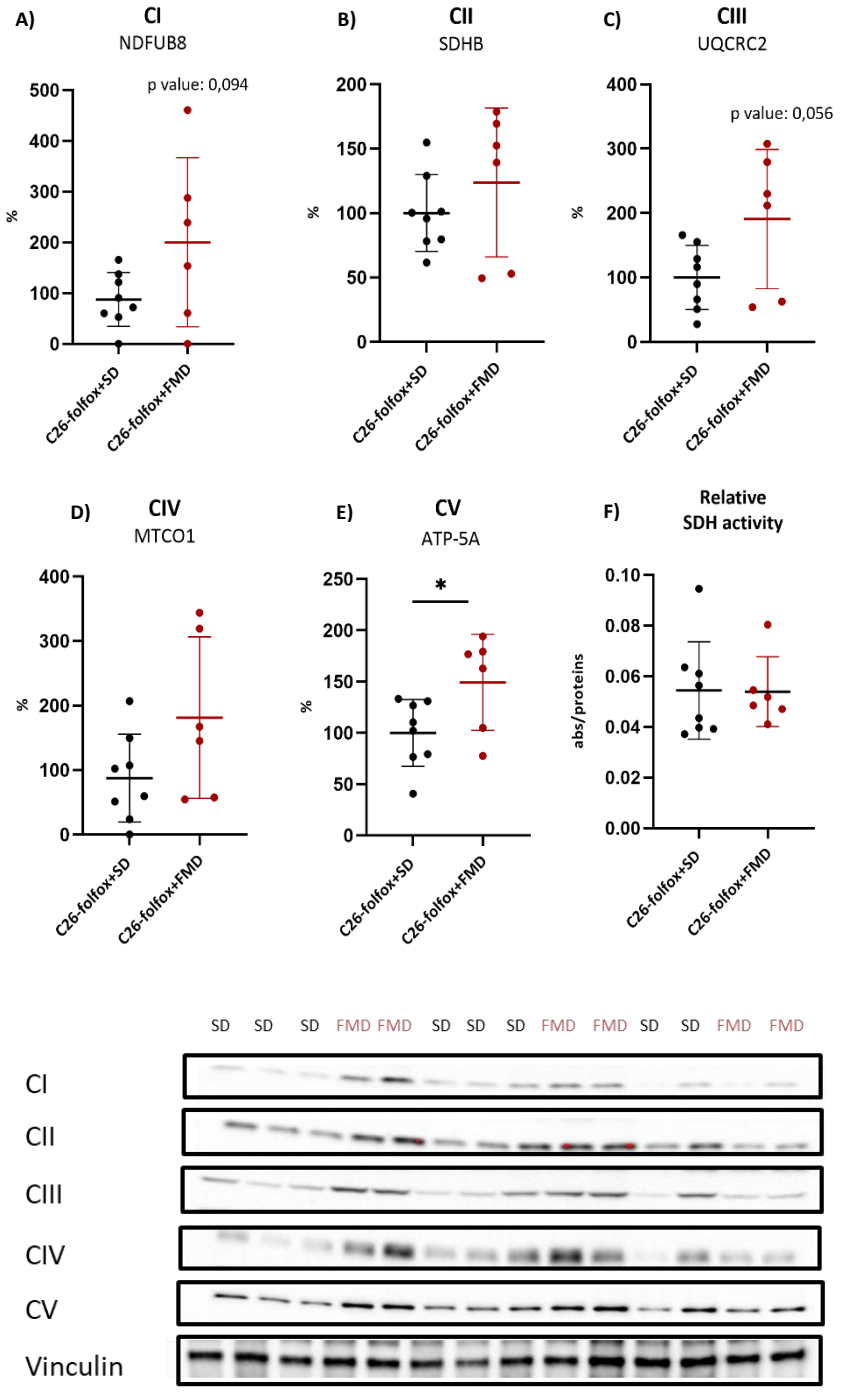


Figure 35. Densitometric analysis and representative blots of OXPHOS respiratory complexes and SDH total enzyme activity in the gastrocnemius muscle of C26 hosts.

Protein levels of **A)** CI (NDFUB8) **B)** CII (SDHB) **C)** CIII (UQCRC2) **D)** CIV (MTCO1) **E)** CV (ATP-5A). **F)** SDH activity data (mean ± SD) is expressed as the ratio between absorbance and total protein content after thirty minutes of incubation. Protein data (mean ± SD) are expressed as a percentage of controls (C26-folfox+SD). C26-folfox+SD; n=8 and C26-folfox+FMD; n=6.Significance of the differences by Student's t-test: *$p<0.05$.

4.3.3 Immunomodulatory effects of FMD in the spleen

Until now, several reports have highlighted that FMD can induce immunological changes, in particular promoting antitumor immunity by activating autophagy, leading to increased infiltration of immune cells into the tumor [180,228]. To characterize the profile of immune cells in the spleen, the expression of specific proteins within the white blood cell population was detected by flow cytometry. FoxP3 and CD39 upregulation represent an important pathway of immune suppression in the TME [182,183]. Overall, CD39 expression is very low in conventional CD4+ T lymphocytes in both tumor-draining lymph nodes and spleen compared to the tumor, in experimental models [229] and in peripheral blood in cancer patients [230]. Our analysis shows that the percentage of MDSCs (Figure 36A) in the spleen significantly decreased (C26-folfox+SD= 50.32%±5.53; C26-folfox+FMD= 34.9%±3.97) in the FMD group, by contrast, Tregs (Figure 36B) did not significantly lower, although they showed a comparable trend. Interestingly, the C26-folfox+FMD group showed a decreased expression of FoxP3 (Figure 36C) and an increased expression of CD39 on CD4+ CD25+ Treg cells (Figure 36E).

T cells that secrete cytokines generate acute inflammation that results in the expansion of CTLs, which play a role in the inhibition of tumor growth [231]. In this analysis, no differences were observed in the percentage of CD4+ T helper 17 (Th17), Th22 and Th1cells between the groups based on the presence of pro-inflammatory cytokines, IL-17 (Figure 36F), IL-22 (Figure 36G) and interferon-γ (IFN-γ; Figure 36H) respectively. In light of these results, FMD partially reduced the immunosuppressive phenotype in the spleen by reducing the frequency of MDSCs and preventing the development and function of Treg cells.

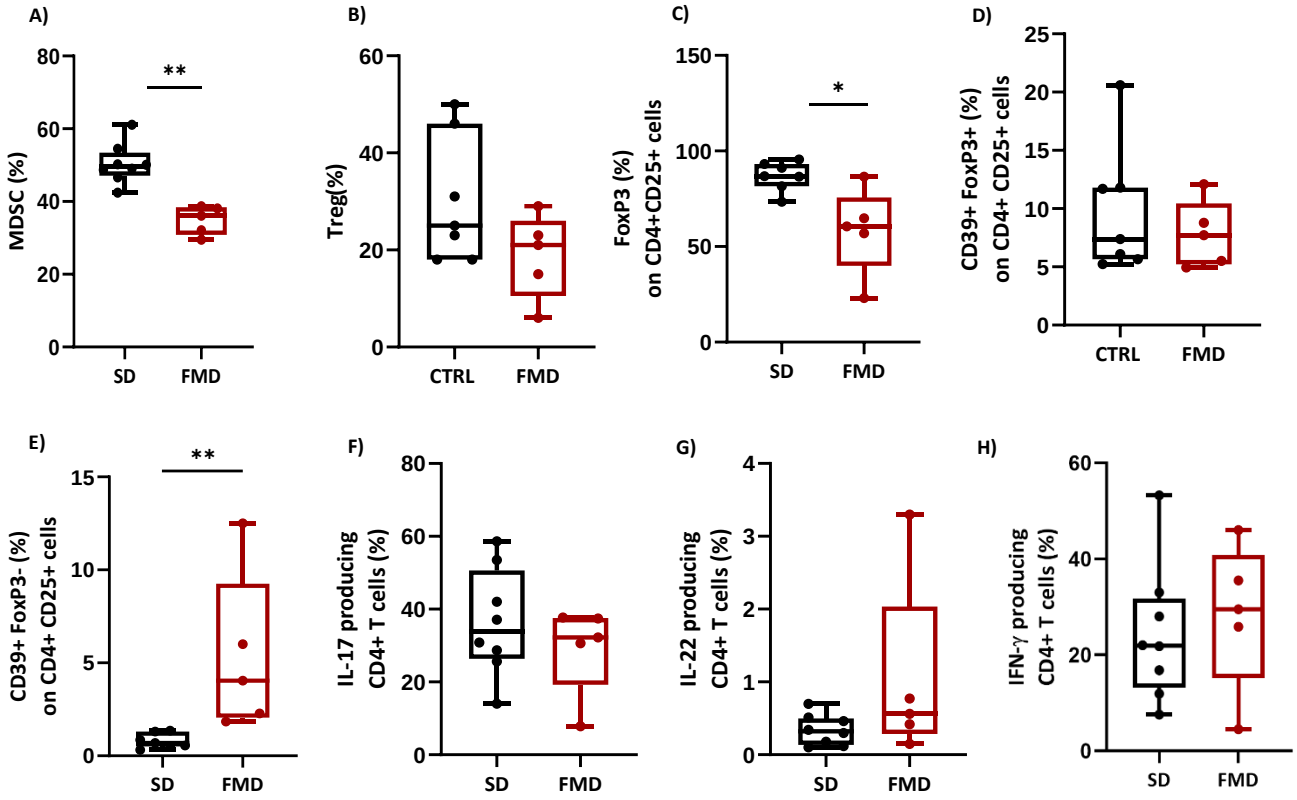


Figure 36. Graphs representing the immune cell characterization in the spleen of C26 hosts.
A) MDSCs and **B)** Treg cells percentage expressed as % of cells in the spleen. **C)** FoxP3 expression in Treg cells expressed as % of FoxP3+ on CD4+ CD25+ cells. **D)** CD39 and FoxP3 expression in Treg cells expressed as % of CD39+ FoxP3+ on CD4+ CD25+ cells. **E)** CD39 expression in Treg cells expressed as % of CD39+ FoxP3- on CD4+ CD25+ cells. **F)** Th17, **G)** Th22 and **H)** Th1 cell (identified as IL-17-producing CD4+ T cells, IL-22-producing CD4+ T cells and IFN-γ producing CD4+ T cells, respectively) percentage among the CD4+ T cells fraction. The data are shown as mean ± SD. C26-folfox+SD; n=8 and C26-folfox+FMD; n=5. Significance of the differences by Student's t-test: *p< 0.05; **p < 0.01 versus controls.

4.4 Effect of FMD in the VCM model

This longitudinal study was designed to investigate the preventive effect of an FMD in the progression of CC. The experiment lasted ten months, as described in the experimental protocol (Figure 37). Villin-Cre/Msh2$^{loxP/loxP}$ (VCM) mice slowly and spontaneously develop neoplasms due to the conditional knock-out of the mismatch repair gene Msh2 in the enterocytes of the intestinal mucosa [212,232]. Consistent with our previous findings [223], the VCM model exhibited notable alterations such as significant splenomegaly, anemia, moderate body weight loss, and muscle wasting resulting from tumor development. Thus, it can be considered a new model for the study of CC.

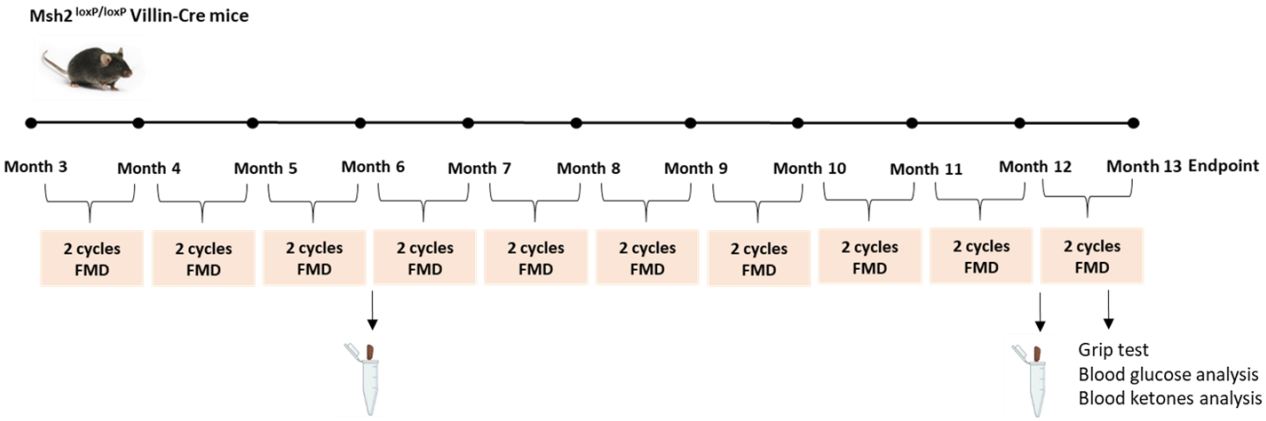


Figure 37. Schematic representation of the experimental design.
The cartoon depicts the experimental plan, including the FMD protocol (twice every month until the endpoint), grip test, blood ketone and glucose analysis, fecal collection, and sacrifice time points. The experimental groups consisted of four groups, VCM mice fed with standard diet males; n=12 and females; n=12 and VCM mice fed with the FMD diet males; n=14 and females; n=12.

In this study, not all the VCM mice developed tumors. Hence, while observing the number of animals that developed tumors starting from the VCM+SD group, either males or females (n= 12), only eight males and six females developed macroscopic tumors. Moreover, in the groups subjected to FMD (comprising 14 males and 12 females), a total of twelve males and seven females exhibited the presence of tumors. However, it is noteworthy that these numbers lacked statistical significance, making it challenging to discern the precise impact of FMD on tumor incidence. Overall, these findings suggest that FMD did not prevent tumor development in the VCM mice. Concerning the body weight change of VCM+SD and VCM+FMD in males (Figure 38A), the FMD group presented with a curve that always remained below the SD group, showing that FMD reduces the excessive body weight gain characteristic of male C57BL/6 mice. On the contrary, such an effect was not observed in females.

Furthermore, no beneficial effect was observed in the survival curves for the FMD group in either males (Figure 38A) or females (Figure 38B). These results overall suggest that FMD impacts differentially on the body weights of females and males, requiring further investigation into sexual dimorphism.

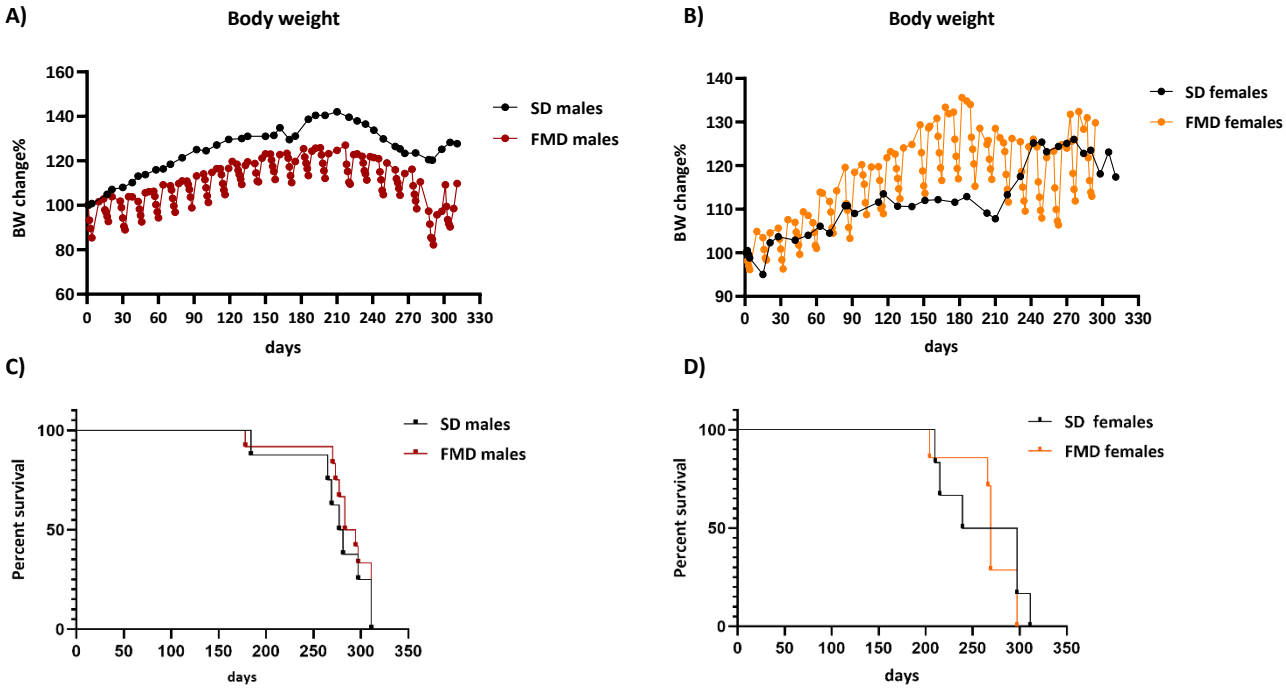


Figure 38. Body weight and overall survival in VCM experimental groups.
Only mice showing macroscopic tumor masses at necropsy are represented. **A)** Body weight change in VCM+SD and VCM+FMD males. **B)** Body weight change in VCM+SD and VCM+FMD females. Body weight (mean ± SD) is expressed as a percentage of the ibw. **C)** Overall survival in VCM+SD and VCM+FMD males. **D)** Overall survival in VCM+SD and VCM+FMD females. Overall survival is expressed by the Kaplan-Meier curve. VCM mice fed with standard diet males; n=8 and females; n=6. VCM mice fed with the FMD diet were males; n=12 and females; n=7.

In regard to energy intake, both males (Figure 39A) and females (Figure 39B) showed similar behaviour during the FMD period. However, only males fed a SD showed a reduction in energy intake from day 240. Likely due to the presence of animals near the endpoint at that time.

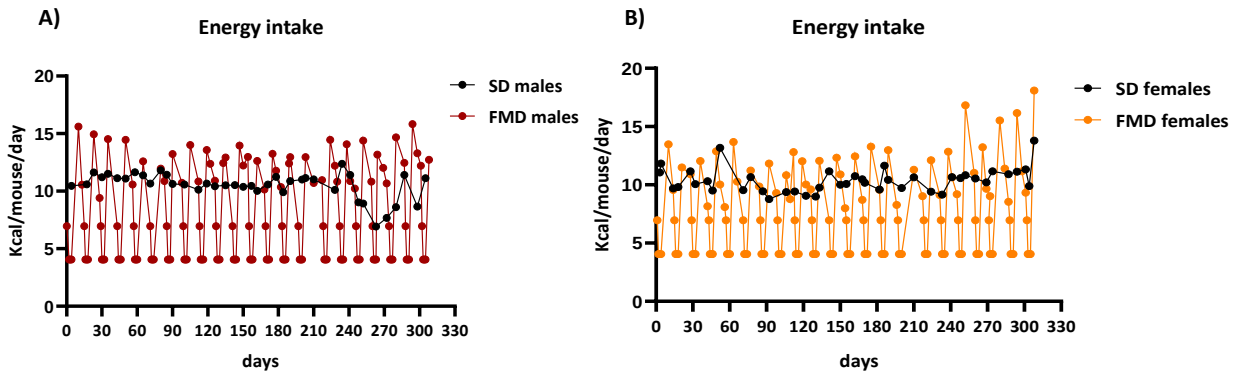


Figure 39. Energy intake in VCM mice.
A) Energy intake in VCM+SD and VCM+FMD males. **B)** Energy intake in VCM+SD and VCM+FMD females. Energy intake is expressed as average kcal/mouse/day. VCM+SD males; n=12 and females; n=12. VCM+FMD diet males; n=14 and females; n=12.

4.4.1 VCM mice presented the features of CC

To check if tumor development could cause cachexia and affect body weight in VCM mice, the body weight loss was compared between tumor-bearing and non-tumor-bearing animals (Figure 40). As shown in Figures 40A and 40B, tumor-bearing VCM mice showed equal body weight loss in males and females. In addition, the FMD did not influence this loss.

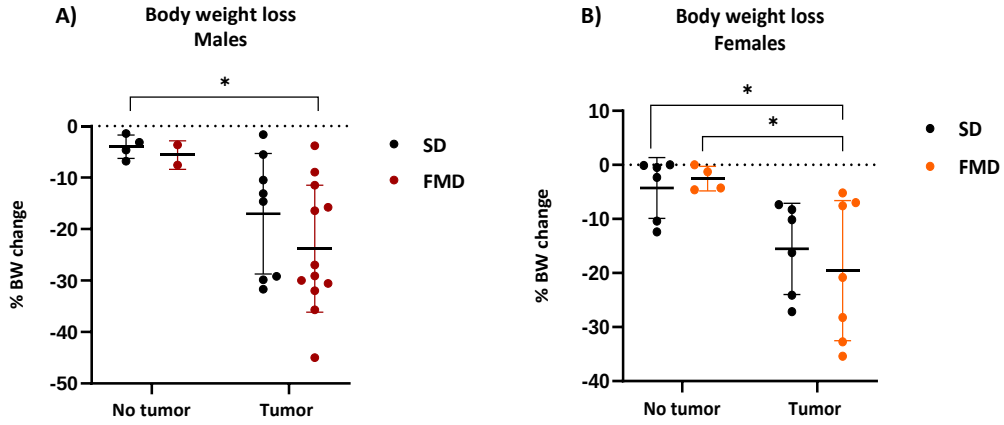


Figure 40. Body weight loss in VCM mice.
Body weight loss delta (mean ± SD) expressed as a percentage of the delta of the ibw and fbw. VCM+SD males; n=12 and females; n=12. VCM+FMD males; n=14 and females; n=12. **A)** Body weight loss (means ± SD) in VCM+SD and VCM+FMD males. **B)** Body weight loss (means ± SD) in VCM+SD and VCM+FMD females. Significance of the differences by two-way ANOVA test *p<0.05.

4.4.2 FMD impacted on systemic metabolism of VCM mice

FMD is a dietary approach based on CR and was shown to stimulate molecular hallmarks of fasting, like low blood glucose levels and increased ketone bodies in circulation [146,168]. Aiming to shed light on the metabolic shift caused by FMD, blood glucose and β-HB levels were assessed when the mice reached twelve months of age (Figure 41) either the last day of the FMD week or one week after the refeeding. During the FMD cycle, both males (Figure 41A) and females (Figure 41B) presented a significant decrease in blood glucose levels that was restored during the refeeding.

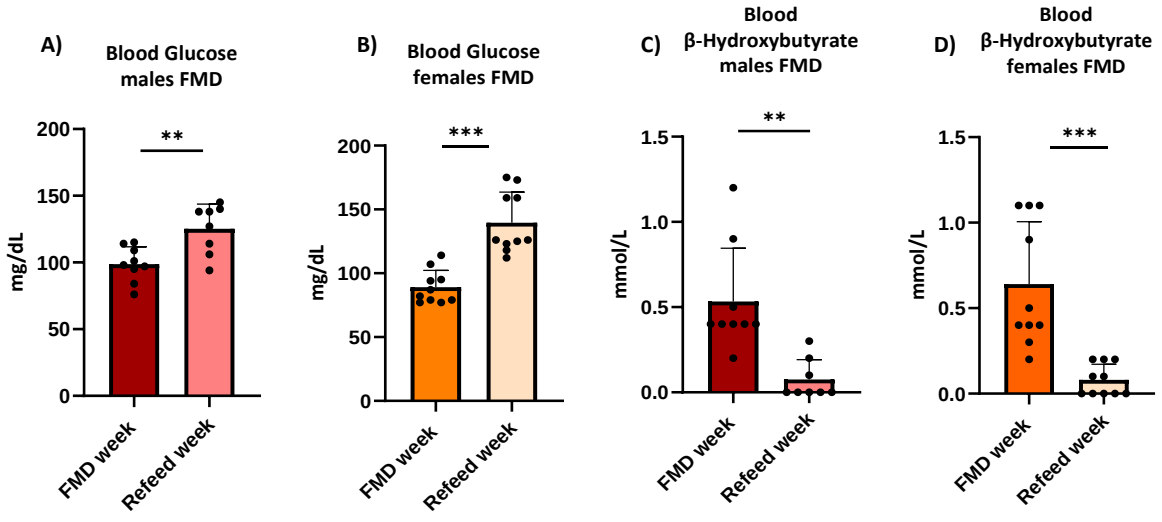


Figure 41. Blood glucose and β-HB levels in VCM.
A) Blood glucose (means ± SD) levels expressed in mg/dL on the last day of the FMD week and one week after refeeding. **B)** Blood β-HB (means ± SD) levels represented in mmol\L on the last day of the FMD week and one week after refeeding. VCM+FMD males; n=9 and females; n=10. Significance of the differences by Student's t-test: *p < 0.05; **p < 0.01; ***p<0.001.

In physiological conditions, the concentration of β-HB is very low (≤ 0.3 mmol/l). In contrast, during nutritional ketosis, the concentration of β-HB increases by ≥ 0.5 mmol\L [84,85]. Differences in β-HB levels were significant in both sexes although there was high variability between samples. Circulating levels of β-HB augmented during the FMD week reaching "mild" ketosis in males (0,53±0.31 mmol\L; Figure 41C) and females (0.64±0.36 mmol\L; Figure 41D). Subsequently, after refeeding, the blood glucose and β-HB levels of the FMD group were comparable to the VCM+SD group in both males and females. Overall, these results suggest that FMD impacted the metabolic state and stimulated a fasting environment in VCM mice.

4.4.3 FMD did not influence tissue weight

The muscle and organ weights for all the experimental groups are presented in Table 6. Overall, the groups show no statistical differences, likely depending on the limited survival at the selected endpoint, however, some interesting observations can be derived from the data. Regarding muscle weight (tibialis anterior, gastrocnemius and quadriceps), VCM+FMD females showed a trend of less tissue wasting compared to VCM+SD females. Consistently, WAT mass seemed to be higher in the VCM+FMD females compared to the SD group. The weight of tumors of the VCM+SD females looked higher compared to the tumors of the VCM+FMD females, although a very high variability in spontaneously occurring tumors was observed. Furthermore, when comparing mice based on sex regardless of the nutritional approach, spleen weight was significantly greater in females than that

observed in males (p-value=0.019). In conclusion, in the chronic setting, FMD had no impact on muscle weights, indicating that it did not affect CC.

Table 6. Tissue weight.

TISSUES	VCM+SD Males (n=3)	VCM+FMD Males (n=7)	VCM+SD Females (n=5)	VCM+FMD Females (n=6)
Tibialis Anterior	140.7 ± 27.47	140.1 ± 32.08	114.2 ± 32.47	136.83 ± 17.37
Gastrocnemius	407 ± 79.16	418.6 ± 43.53	383.6 ± 109.85	478.66 ± 80.2
Quadriceps	425.7 ± 49.65	420.81 ± 14.52	332.4 ± 121.68	434.25 ± 102.61
Spleen	1602.7 ± 727.88	1835 ± 648.33	2942.2 ± 703.36	2634.33 ± 1377.05
Liver	6600 ± 1585.27	5964.3 ± 1041.87	6465.8 ± 1698.78	6916.66 ± 1517.30
Tumor	270.67 ± 177.46	271.63 ± 245.42	396.6 ± 145.47	246.83 ± 133.51
Heart	551.6 ± 49.66	656.3 ± 229.4	648.6 ± 703.36	524 ± 158.38
White adipose tissue	891.7 ± 1544.41	1028 ± 1505.46	431 ± 600.85	1300 ± 1083
Pancreas	442.66 ± 35.38	521.66 ± 188.40	624.6 ± 134	591.2 ± 260.26

Muscle, tissue and tumor weight in VCM mice.
Muscle and tissue weight (mean ± SD) are expressed as the ratio between mg of tissue and 100g of ibw. Tumor weight (mean ± SD) is expressed in mg.

4.4.4 Gut microbiota regulations by FMD

Since possible beneficial effects of dietary restrictions [196], including FMD [197] on the gut microbiota have been previously reported, fecal samples were collected (Figure 37) and metagenomic sequencing analysis were performed to investigate changes in microbial population abundance and diversity. Fecal samples were collected when the animals reached six months (before the frankly appearance of the tumor; T1) and twelve months of age (the time in which tumors are likely present; T2) in males and females of the VCM+SD group, one week after refeeding for the VCM+FMD group.

Microbiome diversity is generally categorized into alpha and beta diversity. Alpha diversity measures the variability of species within a sample while beta diversity accounts for the differences in composition between samples. According to the results, no significant changes in alpha diversity were observed in SD or FMD at T1 or T2. Furthermore, FMD does not influence microbial richness and alpha diversity compared to SD (Figure 42).

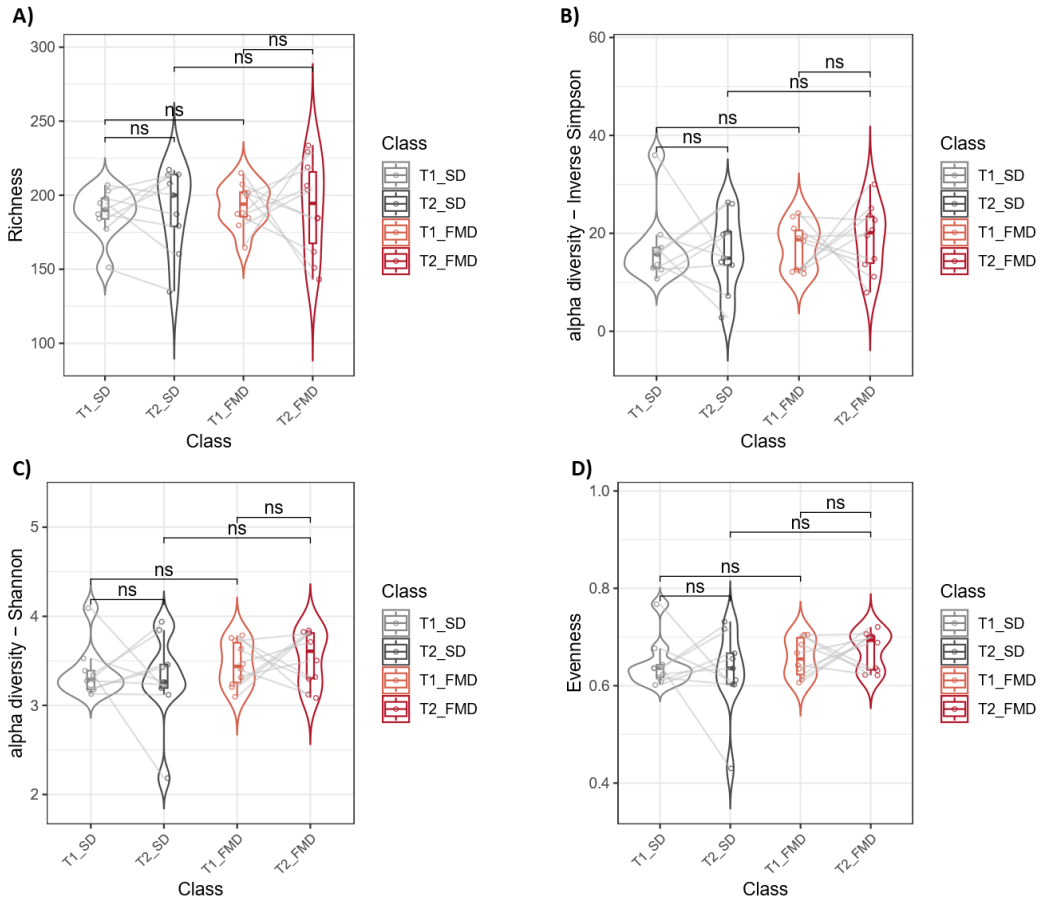


Figure 42. Violin plots reporting the microbial richness and alpha diversity at T1 and T2. **A-C)** Violin plots reporting the alpha diversity computed as richness (**A**), Inverse Simpson index (**B**), and Shannon index (**C**). **D)** Violin plots of Evenness that measure how evenly the microbial abundance is distributed in a sample without considering the number of species. VCM+SD males and females; n=9 and VCM+FMD males and females; n=10. Statistical analysis was performed using the Vegan R package, p-value by Wilcoxon Rank-Sum test.

On the other hand, at T2, the SD group showed more beta diversity compared with T1_SD and the FMD in all time points. This result suggests that the time variable was significantly (PERMANOVA p-value= 0.001) associated with the beta diversity suggesting a possible connection between tumor progression and microbial diversity in SD-fed mice (Figure 43).

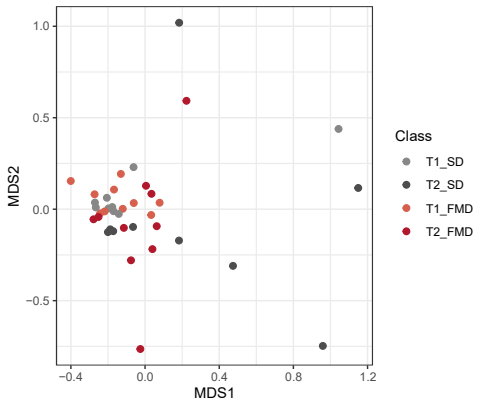


Figure 43. PCA plots (MDS1 vs MDS2) representing sample variability in SD and FMD at T1 and T2. Statistical analysis was performed by computing the Bray-Curtis dissimilarity index with the Vegan R package, and p-value by the Wilcoxon Rank-Sum test.

Regarding the Phyla relative abundance, the Firmicutes/Bacteroidetes ratio is increased in the SD group, especially at T2 (Figure 44A). On the other hand, at T2, the SD group presented a significant increase ($p<0.05$) in Proteobacteria abundance compared with both SD and FMD at T1 (Figure 44B). This relative abundance increases notably in samples 569 (female that did not reach the endpoint and was sacrificed at month 11), 492, and 476 (males that reached the endpoint), and 466 (female upon FMD).

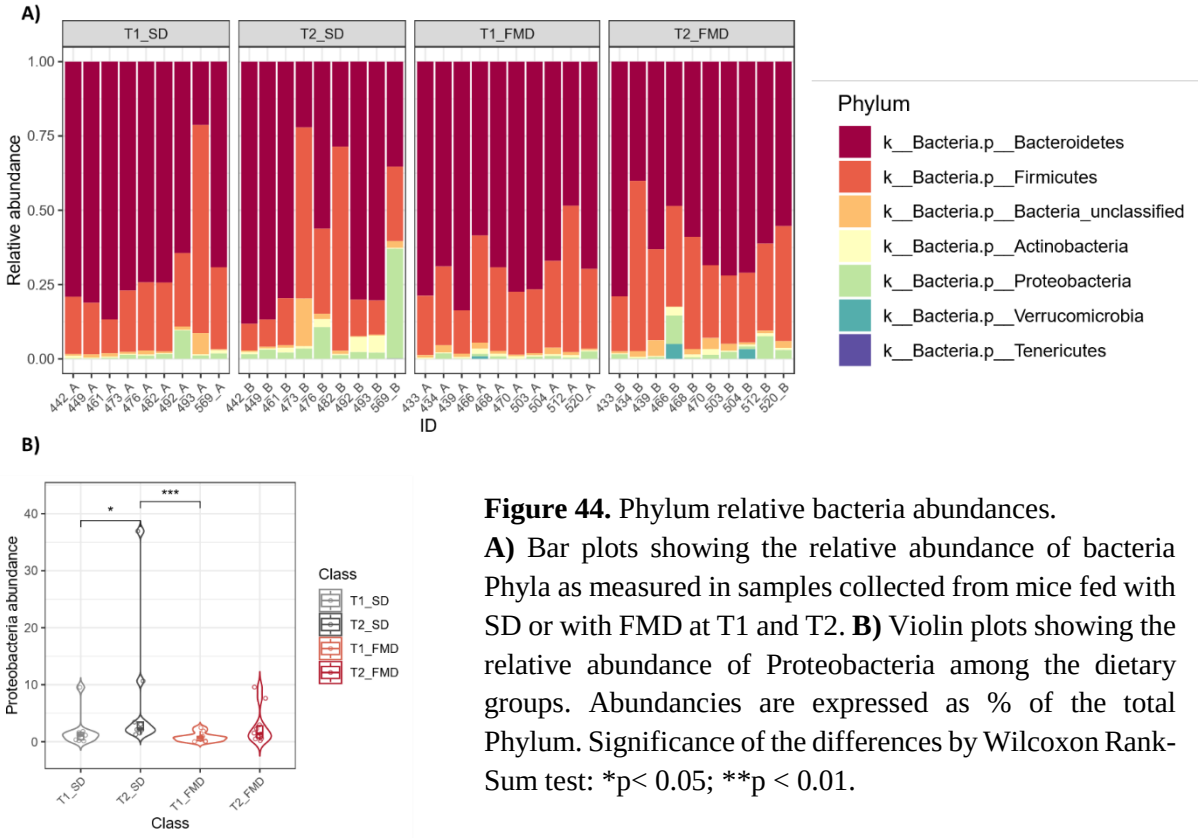


Figure 44. Phylum relative bacteria abundances.
A) Bar plots showing the relative abundance of bacteria Phyla as measured in samples collected from mice fed with SD or with FMD at T1 and T2. **B)** Violin plots showing the relative abundance of Proteobacteria among the dietary groups. Abundancies are expressed as % of the total Phylum. Significance of the differences by Wilcoxon Rank-Sum test: *p< 0.05; **p < 0.01.

A general overview of the differential microbe abundance comparing time points within the same group and differences between the SD and FMD is represented in Figure 45A. When comparing the bacterial abundance in SD over time, it is observed that some species such as *Escherichia coli* and *Parabacteroides goldsteinii* abundance are higher while others such as *Muribaculaceae bacterium* and *Lactobacillus johnsonii* are lower at T2 in comparison with T1. Similarly, in the FMD group, some species increase when animals approach the endpoint. Moreover, a more detailed analysis of the modulated species found in the different samples clustered based on the experimental group and sex is represented in Figure 45B. Finally, significant differences in the abundance of several species are observed between SD and FMD at T2, concluding that both time and diet can modulate intestinal microbial abundance in the VCM mice.

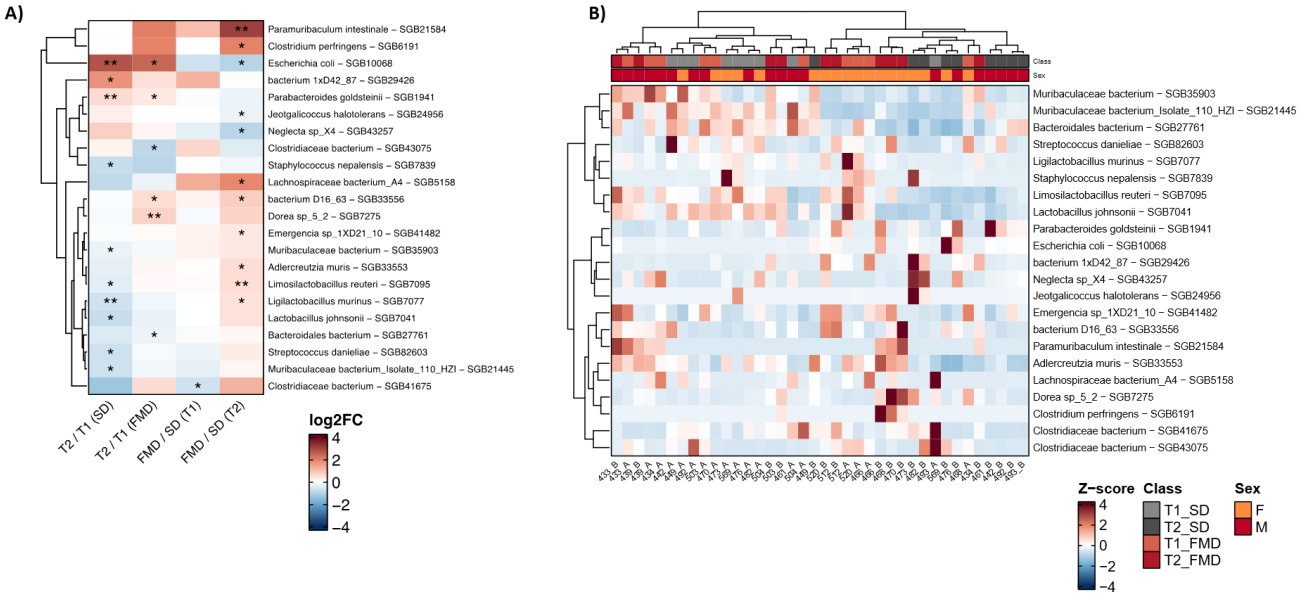


Figure 45. A) Heat map reporting the differential abundance analysis of bacterial species. The color-code represents the log2FC of microbial abundances as computed in the comparisons reported in the columns. **B)** Heat map with Z-score-normalized levels of the differentially abundant bacterial species. Heat map columns are annotated with the study groups and sex information. VCM+ SD; n=9 and VCM+FMD; n=10. Significance of the differences by SIAMCAT test: *p<0.05; **p < 0.01.

Subsequently, an analysis of the differential relative abundance of specific bacteria between the experimental groups has been performed (Figure 46). Interestingly, the *Escherichia coli* (Figure 46A) abundance increased at T2 in both SD and FMD, however, this increase is significantly smaller when animals are subjected to FMD cycles. Similarly, *Neglecta sp* increase in SD fed mice at T2 is prevented by FMD regimen (Figure 46B). On the other hand, *Paramuribaculum intestinale* abundance (Figure 46C) increased in VCM+FMD at T2 compared with the VCM+SD. Furthermore, the FMD prevented the general decrease in the abundance of *Muribaculaceae bacterium* (Figure 46D)

and *Lactobacillus johnsonii* (Figure 46E) observed at month 12. Lastly, the abundance of *Clostridiaceae bacteria* (Figure 46F) was lower in the initial stages (T1) in the VCM+FMD compared to the control group (SD).

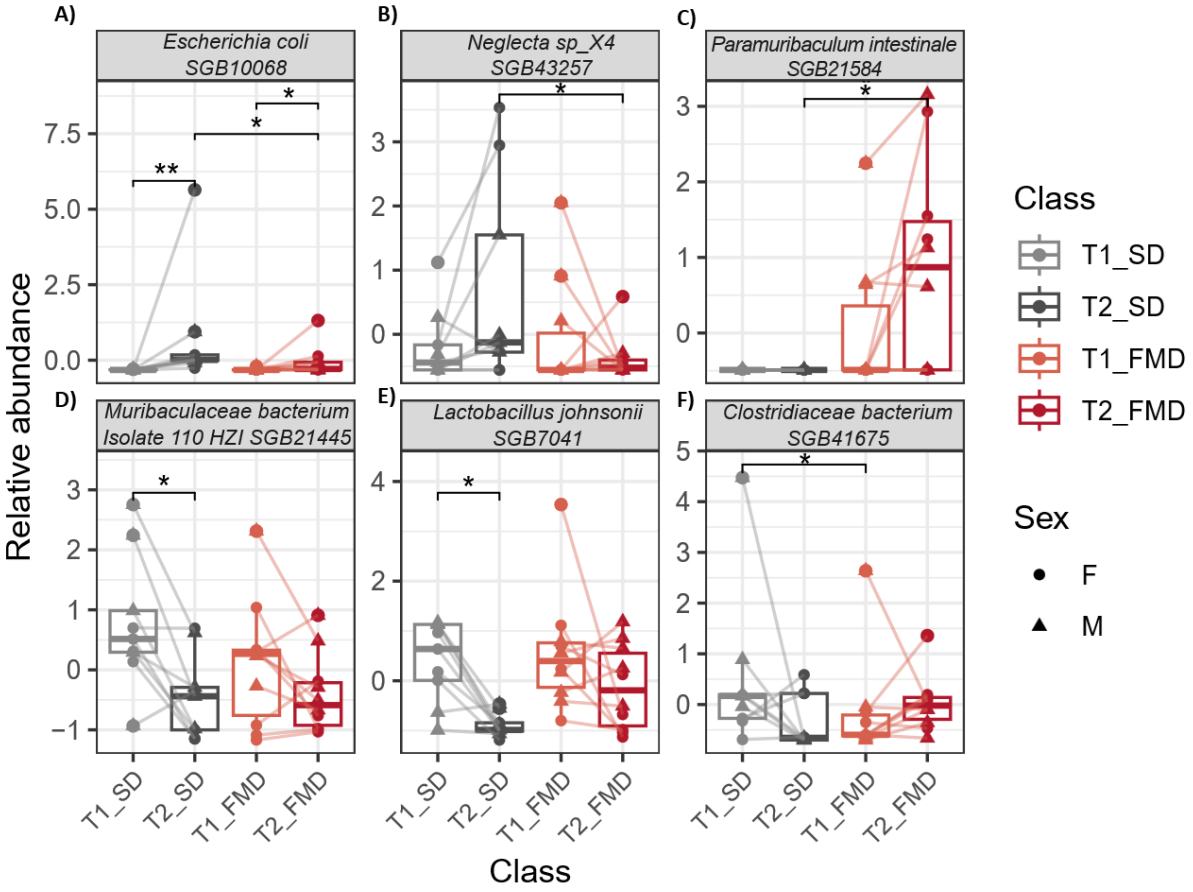


Figure 46. (A-F) Box plots reporting the relative abundances of six bacterial species in the VCM+FMD and VCM+SD. Significance of the differences by Wilcoxon Rank-Sum test: *p< 0.05; **p < 0.01.

In order to gain better insight into the possible influence of animal sex on the relative abundance of bacteria, the abundances of *Escherichia coli* and Proteobacteria (Figure 47) stratified by sex have been evaluated. As observed, *Escherichia coli* (Figure 47A) was more elevated at T2 in both SD and FMD female groups. By contrast, this increment was only present in male VCM+SD (Figure 47A). Concerning Proteobacteria (Figure 47B), both females and males showed comparable differences, exhibiting higher abundance in animals fed with the SD at month 12 compared to the FMD group.

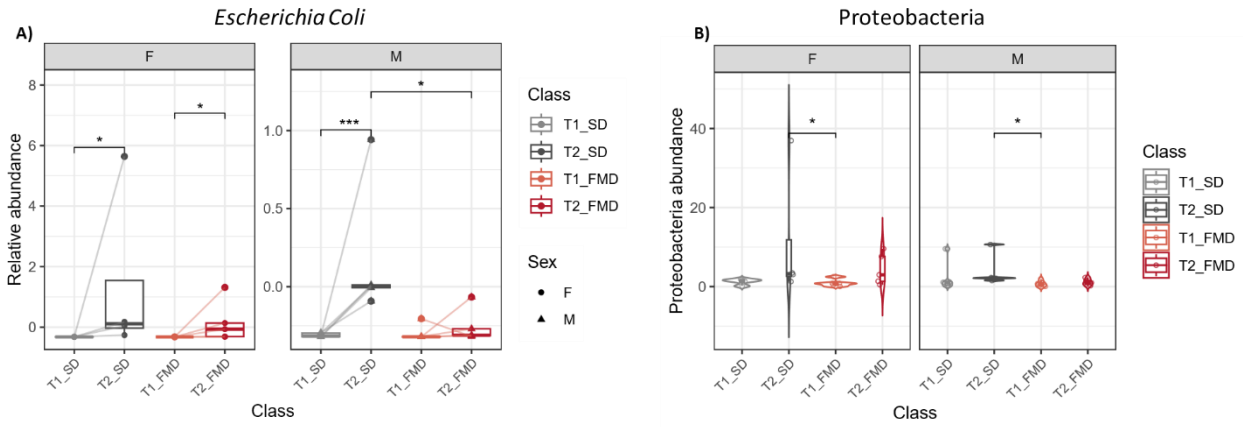


Figure 47. (A-B) Box plots reporting the relative abundances of **(A)** *Escherichia coli* and **(B)** Proteobacteria stratified by sex in the VCM+FMD and VCM+SD. Significance of the differences by Wilcoxon Rank-Sum test: *p< 0.05; **p < 0.01.

5- DISCUSSION

Cachexia is a debilitating disorder with a high prevalence in cancer patients. It is associated with decreased physical and social functioning, affecting QoL, effectiveness of treatments and increased mortality [5,7]. To date, there is no definitive treatment for this condition and research into possible therapies is still ongoing. Despite numerous clinical trials focusing on different types of interventions to treat cachexia the results so far have not provided strong evidence of efficacy [5,233,234]. Some of the current limitations hindering CC research include the use of highly variable definitions, heterogeneous endpoints and the use of experimental models that do not totally reflect the clinical complexity of cancer patients [5,67]. In addition, it is necessary to distinguish whether treatments directly target cachexia or primarily impinge on tumor burden, which would indirectly result in an improvement of cachexia symptoms.

Appropriate nutritional intervention is essential to prevent the risk of malnutrition, especially in patients suffering from CC [15,62,65]. Canonical nutritional counselling is recommended as a key part of multimodal therapy and focuses on adequate calorie-protein intake and, when needed, amino acid supplementation aimed at modulating protein turnover by suppressing protein degradation and stimulating protein synthesis. In addition, nutrients with anti-inflammatory and antioxidant properties, as well as micronutrient supplementation, have also been included in the nutritional guidelines [15,68,69]. Nevertheless, until now, there is no standardized dietary prescription for cancer patients [235].

MCT supplementation against CC

KD and KS have been investigated in several studies as adjuvant cancer therapy and to revert cachexia [89,109–111,115,120]. Both dietary strategies lead to a controlled state of ketosis [85,87,92] which has a beneficial effect on oxidative metabolism and functionality of the skeletal muscle, preventing muscle wasting [89,120]. MCT have been reported to produce metabolic effects similar to those of KD and KS. [87,135]. Furthermore, its origin from natural food sources (e.g. coconut oil) makes it a safe and well-tolerated food [128].

In the present study, we found that MCT supplementation was effective in increasing ketonemia in BALB/c and VCM animals. In particular, we saw that blood β-HB levels returned to baseline after 4h of MCT administration by gavage, an observation which is not in line with previous data reported by Kesl *et al*, (2016) [92], showing an increase in ketonemia up to 8h. Similar increase in β-HB blood levels could be observed when MCT is provided by means of food enrichment, even

if this administration route does not allow to exactly quantify the amount of supplement assumed by each animal. Hyperketonemia in this latter experimental setting is associated with hypoglycaemia. Multiple lines of evidence are consistent regarding the glucose-lowering effect produced by exogenous ketones [219,236,237], which is considered as a marker of the metabolic shift from glucose utilization to fatty acids and fatty acid-derived ketones. Along this line, the data reported in the present study confirm that MCT supplementation can produce a metabolic shift, in both healthy and tumor-bearing mice.

Daily administration of MCT, irrespective of dosage and route of administration, showed no preventive effect against CC. Despite the fact that the C26 tumor-bearing mice showed reduced food intake, the supplemented animals had a higher energy intake than untreated mice due to the energy content of the MCT. Even so, it was not enough to reverse the unintentional body weight loss shown in the tumor-bearing mice, demonstrating that CC cannot be completely reversed by increasing energy intake alone [7]. In apparent contrast to our study, tumor-bearing rats were shown to improve the nitrogen balance when given a parenteral formula containig MCT [137]. Such a supplement, however, was also enriched with amino acids and dextrose, which could have generated a synergistic effect and do not allow to extrapolate the results to MCT only.

While unsuccessful against CC, MCT supplementation could have exerted molecular changes not marked enough to be reflected at the tissue level. Mitochondrial perturbations are generally present in the skeletal muscle of tumor hosts [29,31]. Indeed, a decrease in the activity and content of proteins involved in oxidative metabolism has been observed in the skeletal muscle of cachectic animals [31,211,238], contributing to skeletal muscle wasting [205]. While MCT administration has been proven to boost oxidative metabolism in cachectic mice [136], our findings did not reveal any modulation of oxidative capacity in C26 tumor-bearing mice receiving the supplement.

MCT supplementation counteracted the overexpression of markers of autophagy in the skeletal muscle of C26 hosts suggesting a protective effect against the hyperactivation of the autophagic lysosomal proteolysis that characterizes CC [33,40]. Consistently, oral administration of β-HB was shown to inhibit the upregulation of autophagy-related markers in mice with disuse-induced muscle atrophy [239]. On the contrary, some other authors associated the use of KD and KS with the activation of the autophagic flux [96,240,241]. Concerning mitophagy, we also found a gene induction of BNIP3 in C26 hosts treated with MCT. The ability of KS to enhance energy metabolism efficiency could explain the BNIP3 upregulation, aiming to remove dysfunctional mitochondria during conditions of metabolic stress [204]. However, in the CC scenario, overactivation of

mitophagy leads to a detrimental rather than beneficial effect since it contributes to muscle wasting [34,205].

So far, the activity of ketolytic enzymes in cancer cells [118] and in trained skeletal muscles [97,242] has been extensively investigated, while their function in the skeletal muscle in CC has not yet been fully explored. The ACAT thiolase is not only involved in the KB metabolism, but it also plays a fundamental role by catalyzing the last step of mitochondrial β-oxidation [88]. By contrast, the OXCT enzyme participates in a key step in the metabolism of KBs [101]. The ACAT gene induction observed in the MCT supplemented C26 host may be related to an increase in fatty acid oxidation in the skeletal muscle, probably as a result of excessive lipolysis that occurs during CC [17]. On the other hand, the downregulation of OXCT mRNA levels observed in tumor hosts was partially mitigated when animals received the supplement, which may be associated to a restoration of KB metabolic capacity in the skeletal muscle.

Lastly, according to the literature [116,118] β-HB may have a suppressive or promoting role in tumor growth depending on the type of tumor. Interestingly, in our study, although *in vitro* treatment with a high dose of Na β-HB (10 mmol/L) did not interfere with C26 cell viability, the tumor weight was higher in animals that received MCT by gavage than in non-supplemented mice. However, the increased tumor mass did not influence the aggressiveness of CC in the treated mice, suggesting that the severity of the syndrome is not always associated with the size of the tumor [5,243]. The other way around, this observation could suggest a protective role of MCT administration, since a tumor larger than normal is not associated with worse cachexia. In this regard, beyond cancer cells, the tumor includes a heterogeneous set of immune and stromal cells together with secreted factors and extracellular matrix, which impinge on tumor growth and progression, but can also play a role in mediating effects on distant tissues, contributing to cachexia [244]. Therefore, to clarify which of the two hypothesis reported above is more likely, the histological analysis of the tumor would be necessary to obtain a complete picture of the tumor microenvironment.

Overall, MCT supplementation did not prevent CC in the C26 host. However, it partially enhanced the expression of markers involved in KB metabolism and prevented the overexpression of markers of autophagy in the skeletal muscle. This suggests that nutritional supplementation may modulate some molecular pathways involved in the maintenance of skeletal muscle mass, but this single approach was not sufficient to counteract the syndrome, at least in such a severe model of CC. Therefore, a multimodal therapy combining personalized nutritional support with physical activity and pharmacological treatment should be necessary in the management of CC.

Effects of Fasting-mimicking diet in CC

Calorie-restricted diets, such as the FMD, have received much attention for their potential to improve healthspan and extend lifespan [146]. In addition, short-term fasting or limited daily intake reduces multiple risk factors for age-related diseases without compromising body weight and lean body mass [166,170]. Most studies investigating FMD have focused on its potential to improve the efficacy of antineoplastic treatments by modulating systemic metabolism and enhancing antitumor responses [174,188,190]. Nevertheless, there is a relative lack of research focusing specifically on their impact on CC. Current ESPEN scientific guidelines do not recommend fasting in cancer patients due to the known risks of malnutrition [245]. Hence, it is crucial to further explore the effects of FMD on CC to better understand its potential as a therapeutic approach in this specific context.

Our findings indicated that FMD did not significantly influence overall CC parameters and tumor size in the C26 tumor-bearing mice undergoing Folfox chemotherapy. Furthermore, caution should be exercised when applying FMD in cases of overt cachexia, as was observed in this particular model. The study underlines the need for close monitoring and limited use of FMD due to the premature deaths observed in animals, highlighting the potential risks associated with FMD in fragile host conditions. Of note, FMDs have been used in clinical trials as adjuvant therapy in cancer patients with no underlying diseases and with a BMI above 18kg/m^2 [190,191].

Despite not having seen any preventive effect on the cachectic phenotype in the C26 hosts, we observed a minimal increase in the protein levels of the OXPHOS system concerning mitochondrial function in the FMD group. Furthermore, there was no loss of muscle mass despite reduced energy intake in FMD-fed animals, which would suggest that FMD may have contributed to a more efficient use of energy. The metabolic switch that occurs during fasting through the induction of fatty acid-derived ketone metabolism may improve the maintenance of muscle mass and function [153,154]. Various fasting programs have been associated with increased abundance and activity of electron transport chain proteins in rodent models [246,247]. However, elevated levels of free fatty acids caused by fasting can lead to intramuscular lipid accumulation resulting in reduced mitochondrial capacity [248].

On the whole, this finding suggests that FMD may have a slight impact on mitochondrial function. However, it is important to note that the observed benefit was not substantial enough to justify the use of FMD in the presence of cachexia. Therefore, further molecular analyses are required to elucidate the potential effects of FMD on mitochondrial function.

Furthermore, FMD had a modest positive effect on the spleen immunosuppressive cell populations, but it was not sufficient to influence tumor growth. Similar to our findings, CR and FMD were shown to reduce the percentage of splenic MDSCs and FoxP3+CD4+ Tregs respectively in a tumor-bearing mouse model [181]. On the contrary, an increase in the CD39+ marker was also observed in the Tregs of FMD mice which would be related to the high circulating levels found in cancer patients [249]. However, despite the increase in CD39, cytokine production was not affected. Lastly, it should be noted that analysis of the TME would be necessary to obtain a more comprehensive picture of the effect of FMD on the immune response against cancer.

In the study involving the chronic model of spontaneous intestinal cancer (VCM), we evaluated the impact of FMD on CC and its role in tumor incidence. It is important to note that not all mice developed macroscopic tumors, and the sample size was not sufficient to reach the statistical significance needed to draw conclusive evidence on the effect of FMD on tumor incidence in VCM mice. However, a larger cohort of animals could provide more substantial data to determine any potential effect. VCM mice developing tumors showed a significant loss of body weight in both males and females as observed in previous research conducted in our laboratory [223]. Markedly, VCM was found to be a less severe model of CC compared to C26-bearing mice, which would allow further exploration of nutritional interventions and additional parameters of cachexia. Concerning the FMD, it did not show a significant impact on CC. As shown in the literature [146,166], implementation of FMD resulted in metabolic changes in the animals, with a marked decrease in blood glucose levels and an increase in β-HB. During periodic fasting or FMD, a decrease in insulin levels prompts the release of free fatty acids from the adipose tissue [166], which are utilized by lean tissues like the skeletal muscle as energy sources [250].

Gut microbiota undergoes significant changes in the course of cancer and intestinal inflammatory diseases [197,251]. More specifically, increased Proteobacteria and Firmicutes/Bacteroides ratio together with a decrease in *Lactobacillus* and *Muribaculaceae* has been observed in CRC [252,253]. FMD has demonstrated the ability to reduce intestinal inflammation and promote beneficial gut microbiota [197]. A long-term intervention with FMD cycles prevented the microbiota shift observed during CRC progression, however, the composition of the prebiotic-rich FMD diet may have influenced the growth of beneficial probiotic strains. *Lactobacillus* and *Bifidobacterium* strains have been implicated, among others, in the suppression of IL-6 secretion and NF-kB nuclear translocation along with the production of SCFAs, exerting a protective effect on the intestinal barrier against inflammatory pathologies [254].

The study on VCM mice has provided interesting data regarding the sex-based differences in body weight. Several biological factors may explain these dimorphic effects, such as variations in hormone levels, differences in the distribution and function of adipose tissue between males and females and genetic factors [255]. Furthermore, in terms of sex-specific differences in energy expenditure, females have higher thermogenic activity than males, while resting energy expenditure tends to be higher in males than in females [256]. Importantly, the higher spleen weight showed in females than in males underlines the importance of further investigating the impact of FMD on the immune cell population in this model. Therefore, when conducting nutritional studies, it is essential to carefully consider and address sex-based differences, as they can be confounding factors that interfere with the generalisability of results.

On the whole, FMD did not significantly affect CC parameters in any of the experimental models. However, it did not aggravate CC in C26 tumor-bearing mice despite reduced energy intake and it improved mitochondrial function in the skeletal muscle, suggesting that changes at molecular levels may be present. Nevertheless, increased body weight loss in FMD mice demonstrated that its application may be risky for hosts with high susceptibility to malnutrition, highlighting the need for specialized surveillance and controlled use of FMD. Lastly, the body weight of VCM animals appeared differently according to sex, highlighting the importance of considering sexual dimorphism. Further investigations are needed to better understand the effects of FMD on CC and its implications in the skeletal muscle and the TME.

6- CONCLUSION

The results of the present study provide new insights into innovative nutritional therapies that support the importance of nutritional interventions as part of the multidisciplinary strategy in the treatment and prevention of CC.

The study demonstrated that MCT supplementation proved to be an effective method to induce nutritional ketosis by different routes of administration. However, it did not prevent CC in C26 tumor-bearing mice. MCT was able to positively impact some markers associated with skeletal muscle metabolism of KBs and skeletal muscle wasting in the cachectic mice. Nonetheless, further molecular analyses should be assessed to confirm the involvement of MCT in CC.

In addition, preliminary data obtained from VCM mice would suggest this model for nutritional approaches due to its potential as a more translational and less aggressive model of CC.

FMD did not show significant effects on CC in the mice bearing the C26 colon carcinoma, however, due to the premature deaths and the difficulty in recovery lost body weight after FMD and chemotherapy treatment suggests caution in using FMD during overt cachexia. Additionally, FMD exhibited a modest impact on mitochondrial function, necessitating further molecular analyses. Furthermore, although the spleen immune profile was favoured by the FMD further analysis of the tumor would be necessary to evaluate its possible implications for cancer immunosurveillance.

In the VCM model, significant body weight loss indicative of CC progression was observed, but FMD did not impact cachexia or tumor incidence. Nevertheless, FMD prevented the occurrence of a gut microbial composition associated with CRC progression. Notably, a sex-based difference in body weight response to FMD was observed, highlighting the importance of exploring sexual dimorphism further.

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