Title

Effects of a Ketogenic Diet in Pancreatic Cancer and Associated Cachexia in a KPC Mice Model

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Publication Date

2022

Peer reviewed|Thesis/dissertation

Effects of a Ketogenic Diet in Pancreatic Cancer and Associated Cachexia in a KPC Mice Model

By

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Nutritional Biology

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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2022

ACKNOWLEDGEMENTS

To my mom Dr. Cristina Penso, my dad Dr. Carlos Cortez, and my sister Cintia Cortez, thank you for being my roots, my wings, and my safety net. You have always encouraged me to follow my dreams, no matter how long they take or how far away they me. I could not have done this without your support.

To my mentor Dr. Gerardo Mackenzie, thank you for guiding me and letting me lead. You always balanced high expectations with kindness, and I could not have asked for a better mentor.

To Dr. Cecilia Rodriguez-Lanzi, thank you for embracing this project as your own. I could not have accomplished all this work without you.

To my collaborators, especially Brian Hong, and all the interns that worked with me, thank you for your efforts and your expertise. You made this very complex project possible to complete.

To all my Davis friends, thank you for being my support system and my Bubbletown family.

To my friends in Mexico, thank you for being my connection to home and my anxiolytics.

To my dissertation committee members, Dr. Patricia Oteiza and Dr. Jon Ramsey, thank you for your investment and insightful comments.

To all the mice that unknowingly sacrificed their lives, thank you for giving your life a scientific purpose.

To myself, thank you for being resilient.

ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is a leading cause of cancer-related deaths, with chemotherapy as the standard of care for all stages of disease. Gemcitabine (GEM)-based chemotherapy regimens persist as front-line treatment options, but without substantial improvements in survival. Cancer-associated cachexia (CAC), a multifactorial disorder characterized by the involuntary and ongoing wasting of skeletal muscle with or without loss of adipose tissue, is a critical contributor to PDAC mortality. Thus, there is an urgent need for better therapeutics for PDAC and CAC; and the exploration of dietary interventions is a critical component. A ketogenic diet (KD), characterized by a very low carbohydrate and high fat composition, has shown anti-tumor and anti-cachectic potential. Still, there is limited understanding on the effect of KDs in PDAC and CAC progression, even less in combination with chemotherapeutics, such as GEM. Therefore, the overall aim of this thesis work was to evaluate the effect and mechanisms of a KD alone or in combination with GEM on morbidity and mortality in a clinically relevant genetically engineered *LSL-Kras*$^{G12D/+}$*; LSL-Trp53*$^{R172H/+}$*; Pdx1-Cre* (KPC) mouse model of PDAC.

Chapter 1 provides a thorough review of the current literature regarding the use of KDs in pancreatic cancer and CAC, with a focus on cellular mechanisms and clinical perspectives. A summary of the evidence reported is that a KD can reduce tumor growth and act as an adjuvant therapy in various cancers, including pancreatic cancer. In addition, the chapter also discusses the limited research published on KDs effect on CAC mitigation. The main cellular mechanisms that may explain KD's potential anti-tumor and anti-cachexia effects are described, focusing primarily on reprogramming of cell metabolism, epigenome, and the gut microbiome.

Chapter 2 describes the effects of a KD with or without GEM in the pancreas/tumor of KPC mice, with the main objective of determining whether a KD plus GEM increases survival of KPC mice following PDAC detection. Following tumor size determination, male and female KPC mice

were fed a control diet (CD; %kcal: 70% carb, 14% protein, 16% fat), a KD (%kcal: 14% protein, 1% carb, 85% fat), a CD + GEM (CG), or a KD + GEM (KG) group. GEM was administered to the CG and KG groups at 100 mg/kg by intraperitoneal injections twice per week for 3.5 weeks (7 total injections). Throughout the survival study, mice were monitored daily until an endpoint criterion was reached and they were euthanized. We observed that a KD plus GEM extended overall median survival in KPC mice when compared to a CD. Mechanistically, KG treatment significantly reduced AKT, ERK, IGFR and AMPK phosphorylation in pancreatic tumors compared to CG-treated mice. Furthermore, following KG treatment, palmitic acid, myristoleic acid, palmitoleic acid, asclepic acid and linoleic acid were reduced in pancreatic tumors. Moreover, data from the relative abundance of fecal bacteria using 16S rRNA sequencing analysis of the microbiota showed that KG treatment leads to increased relative abundance of *Faecalibaculum* and the reduction of *Lactobacillus* at one-month of treatment. In summary, results from chapter 2 of this thesis highlight that a KD has a synergistic effect with GEM that benefits survival in KPC mice and that such response is multifactorial.

The aim of chapter 3 was to evaluate whether feeding a strict KD alone or in combination with GEM mitigates CAC in the autochthonous KPC mouse model, and to elucidate the potential mechanisms involved. In the survival study, KD alone or combined with GEM resulted in the mitigation in the decline of muscle strength over time, as determined with a forelimb grip strength dynamometer. When analyzed by sex, in females, both KD and KG had significantly higher grip strength force than CD-fed groups. In addition, female KPC mice fed a KD had higher gastrocnemius weights compared CD fed mice. Caloric intake was recorded to evaluate anorexia, which was diminished in the KG groups when compared to CG and lessened in KG-treated female mice.

A cohort of mice was allocated to either the CG or the KG groups after tumor detection and euthanized at two months post-interventions to collect the gastrocnemius muscle, which was

used to evaluate potential mechanisms involved in muscle strength preservation. Mechanistically, findings from this chapter signal towards sex-specific differential effects of KG treatment, including the inhibition of autophagy in KPC female mice, increased total acetyl-lysine levels and reduced phosphorylation levels of eIF2α in the KG-fed female KPC mice when compared to CG-treated mice. Findings from chapter 3 contribute to the beneficial potential of a KD in combination with GEM for the preservation of skeletal muscle mass in KPC mice, deserving further evaluation.

Finally, Chapter 4 delves into the safety evaluation of a KD in combination with GEM. In particular, we examined the hepatic safety profile of a KD in combination with GEM in KPC mice. Feeding a strict KD in combination with GEM failed to significantly affect mouse body weight, liver weight, liver aminotransferases, liver markers of inflammation and oxidative stress, or liver enzymes involved in ketone bodies and glucose metabolism. In addition, KG did not increase markers of liver-lipid accumulation nor serum cholesterol and triglyceride levels. In summary, results of chapter 4 indicate that a KD in combination with GEM appears safe in KPC mice with no apparent hepatotoxicity. These safety data support the evaluation of a KD as an adjuvant dietary treatment for pancreatic cancer.

Overall, a KD in combination with GEM has the potential to be beneficial as a treatment strategy for PDAC in KPC mice. Our findings indicate a beneficial effect of a KD in combination with gemcitabine in survival and in the preservation of skeletal muscle function in KPC mice with PDAC and CAC. The mechanisms of the favorable effect appears to be multifaceted, including decreased autophagy and increased cellular response and acetylation. Furthermore, we demonstrate that a KD in combination with GEM used for the treatment of PDAC and CAC in KPC mice, appears safe with no deleterious effects on liver physiopathology or function. Additional research is warranted to further investigate how such diet-treatment combination can be optimized for clinical advantage in PDAC-patients.

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Chapter 1. Ketogenic diets in pancreatic cancer and associated cachexia: Cellular mechanisms and clinical perspectives

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive and extremely therapy-resistant cancer. It is estimated that up to 80% of PDAC patients present with cachexia, a multifactorial disorder characterized by the involuntary and ongoing wasting of skeletal muscle that affects therapeutic response and survival. During the last decade, there has been an increased interest in exploring dietary interventions to complement the treatment of PDAC and associated cachexia. Ketogenic diets (KDs) have gained attention for their anti-tumor potential. Characterized by a very low carbohydrate, moderate protein and high fat composition, this diet mimics the metabolic changes that occur in fasting. Numerous studies report that a KD reduces tumor growth and can act as an adjuvant therapy in various cancers, including pancreatic cancer. However, research on the effect and mechanisms of action of KDs on PDAC-associated cachexia is limited. In this narrative review, we summarize the evidence of the impact of KDs in PDAC treatment and cachexia mitigation. Furthermore, we discuss key cellular mechanisms that explain KD's potential anti-tumor and anti-cachexia effects, focusing primarily on reprogramming of cell metabolism, epigenome, and the gut microbiome. Finally, we provide a perspective on future research needed to advance KDs into clinical use.

Keywords: Ketogenic diet; pancreatic cancer, cancer cachexia, pancreatic ductal adenocarcinoma, microbiome, ketone bodies, cell metabolism.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive and deadly disease with a five-year survival of ~10% [1]. Surgery, which offers the only realistic hope, is a viable option in a limited number of patients, whereas current chemotherapy and radiation therapy regimens offer minimal benefit [2]. Following diagnosis, PDAC patients experience progressive weight loss and nutritional deterioration, leading to a rapid decline in their quality of life. Approximately 80% of PDAC patients suffer from cachexia, a complex metabolic disorder characterized by loss of skeletal muscle mass (SKM). Unfortunately, there are no effective strategies to mitigate PDAC-induced cachexia. For this reason, new treatment strategies for PDAC and PDAC-associated cachexia are needed, and the exploration of dietary interventions is a critical component.

Ketogenic diets (KD) have been gaining attention for their anti-tumor, an-ti-inflammatory potential [3]. Characterized by a very low carbohydrate, moderate protein d high fat composition, this diet mimics changes in metabolism that are similar to fasting. Numerous animal studies have tested the effects of a KD on tumor growth and survival, including pancreatic cancer (PC), with the majority showing promising results [4]. Moreover, the evidence for a beneficial effect of a KD in clinical trials shows promise and merits further research [5, 6]. Unfortunately, evidence on the effect and mechanisms of action of KDs on PDAC-associated cachexia is sparse. Therefore, in this review article, we discuss the potential beneficial role of a KD as a complimentary dietary therapy for PDAC treatment and associated cachexia, and summarize the cellular mechanisms modulated by a KD.

Pancreatic cancer: Biology and current treatments

Pancreatic cancer is a highly lethal disease, ranked the seventh leading cause of cancer mortality in the world [7]. In the United States, it is the third most common cause of cancer-related deaths, and it is estimated to become the second by 2030 [8]. PDAC is the most common and aggressive type of PC, with the lowest survival rate among all solid tumors [9]. Risk factors

associated with the development and progression of PDAC are age, smoking, alcohol abuse, long-standing chronic pancreatitis, obesity and type 2 diabetes mellitus (T2DM) [10].

The majority of PDACs are believed to arise from pancreatic intraepithelial neoplasias (PanINs), which are microscopic lesions (<5 mm) composed of flat or papillary neoplastic epithelium [11]. PanINs progression involves gradual acquisition of mutations in oncogenes and tumor suppressor genes [12]. The genes most commonly affected during PanINs progression and PDAC are *KRAS* (~90%), *TP53* (~74%), *CDKN2A* (~35%) and *SMAD4* (~31%) [11, 13]. Besides multiple genetic/epigenetic alterations, PDAC harbors a complex and dense tumor microenvironment (TME) which acts as a physical barrier to drug perfusion, and could be responsible for drug resistance [14].

Pancreatic tumors have a remarkable resistance to conventional treatment options [15]. The main PDAC treatment is surgical resection followed by chemotherapy, radiotherapy, and targeted therapy [14]. Although surgery is the only potentially curative treatment, tumor resection is possible in less than 20% of patients [16]. For a long time, gemcitabine (GEM) monotherapy was the primary treatment for unresectable PDAC, but the combination of 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX), or the addition of Nab-Paclitaxel to GEM, are now the standard-of-care [17]. Although these combination chemotherapies are increasingly effective for PDAC, they are associated with significant toxicities and, unfortunately, tumor response rates remain low, and many patients ultimately relapse and require second line therapy [18-20].

PDAC-associated cachexia

A major contributor to PDAC mortality is cancer-associated cachexia, a complex multifactorial disorder characterized by the involuntary and ongoing wasting of SKM with or without loss of adipose tissue [21, 22]. Up to 85% of PDAC patients suffer from cachexia and around 30% of deaths are directly associated to it [23]. The diagnostic criterion for cachexia is

weight loss greater than 5% within 6 months, weight loss greater than 2% in individuals with body-mass index (BMI) less than 20 kg/m^2, or sarcopenia with more than 2% weight loss [22].

The key role of the pancreas in nutrient digestion and glucose homoeostasis, plus its interaction with other digestive organs, likely adds to the incidence and intricacy of PDAC-associated cachexia [24]. Significant preoperative weight loss is frequent even in early stage diagnosis [25]. Tumors compete with other organs and tissues for energy fuels and biosynthetic substrates, which promotes an elevated resting energy expenditure and a negative energy balance [26]. Cachexia causes a shift in body fuel utilization where proteolysis and lipolysis are increased,but lipogenesis and SKM protein synthesis are decreased [27]. PDAC cachectic patients have an increased risk for malnutrition during chemotherapy, which in term affects their response to treatment [28, 29]. Because chemotherapeutic dose is determined by body weight or surface area, patients with a lean body mass (LBM) equivalent to underweight are more susceptible to suffer toxicities [30]. Cachexia is associated with chemotherapy toxicity, functional impairment, surgery complications, and mortality [31, 32]. For these reasons, the preservation of SKM is extremely important [33]. Despite increased understanding of the mechanisms of cachexia, there is still no standard of care, no licensed drug treatment, and no evidence-based guidelines for its management [34].

The pathophysiology of cachexia is complex, yet likely characterized by inflammation and increased catabolism in combination with decreased muscle anabolism [34]. In addition, pancreatic tumors probably have specific mechanisms that exacerbate cachexia [25, 35]. These major mechanisms implicated in the development of PDAC-associated cachexia are highlighted in Figure 1.

Multi-organ systemic inflammation occurs in both the tumor and the cachectic muscle, with signals/factors from one site affecting other sites, and with multiple deregulated signaling pathways involved [36, 37]. Pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-8, interferon-γ (IFN-γ), monocyte chemoattractant protein-1 (MCP-1)

and other catabolic factors (activin and myostatin) are released into circulation by the tumor [38]. These signals, can in turn, activate metabolic pathways that increase proteolysis and/or decrease protein synthesis, which culminate in SKM wasting [23]. TNF-α is a major mediator of muscle catabolism associated with poor nutritional status in PDAC patients [23, 39]. IL-6, overexpressed in PDAC, correlates with cachexia, chemotherapy response, and survival [23, 36, 39]. Both TNF-α and IL-6 can activate the transcription factor nuclear factor kappa-light-chain-enhancer of B cells (NF-κB), known to inhibit differentiation of SKM [23, 40]. Activation of the ATP-dependent ubiquitin-proteasome proteolytic pathway (UPP) causes the breakdown of myofibrillar proteins and plays a prominent role in SKM degradation in cancer cachexia. NF-κB is a major regulator of cachexia, in part, by regulating two E3 ligases of UPP: (1) muscle atrophy F box protein (MAFbx/atrogin-1) and (2) muscle RING finger-containing protein 1 (MuRF1). Of note, upregulation of MAFbx is a marker of acute muscle atrophy present in the majority of cancer cachexia cases, while MuRF1 mediates the ubiquitination of the sarcomere's thick filament [41].

The increased levels of cytokines can also contribute to muscle wasting and cachexia through additional mechanisms, such as the activation of janus tyrosine *kinase (JAK)/* signal transducer and activator of transcription (STAT) signaling [41].Prolonged activation of the IL-6/JAK/STAT axis is an established mechanism in tumorigenesis and in muscle wasting during cancer cachexia [42]. Activation of the JAK/STAT pathway correlates to a poor PDAC outcome, while increased levels of phosphorylated STAT3 are essential for muscle wasting [43].

Transforming growth factor-β (TGF-β) family members, such as myostatin and activin A, promote muscle loss through the myostatin/activin receptor type IIB (ActRIIB). Myostatin is a key negative regulator of muscle growth, whereas activin A is involved in cell growth and differentiation [23, 35]. TGF-β proteins facilitate Forkhead box O (FoxO) and SMAD2/3 activation, which in term increase the transcription of atrophy-related genes associated with the UPP, including MAFbx/atrogin-1 and MuRF1 [41, 44, 45]. Interestingly, SMAD-2/3 transcription factors can also activate FoxO3 [46]. Inhibition of myostatin/activin/SMAD2/3 signaling ameliorates

muscle atrophy in cancer [44]. Overall, FoxO, SMAD2/3, NF-κB and STAT3 play a crucial role in cachexia-related muscle atrophy by triggering the activity of MuRF-1 and MAFbx [23, 44, 47].

The insulin/IGF-1-dependent phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of the rapamycin (mTOR) system is a major anabolic pathway involved in muscle development and regeneration. Insulin-like growth factor binding proteins (IGFBPs) help regulate several cellular processes through the IGF-1/PI3K/AKT, NF-κB, TGF-β, and JAK-STAT pathways [35]. PDAC cachexia is characterized by a lower levels of circulating anabolic factors like IGF-1 [35]. The decline in IGF-1 levels suppresses AKT activity, which is a signal that stimulates muscle growth [26]. AKT also activates mTOR complex 1 (mTORC1), consequently activating ribosomal protein S6 kinase-β1 (S6K1) and eukaryotic translation initiation factor binding protein 1 (4E-BP1), which play a dominant role in myogenesis [39]. Moreover, activated AKT inhibits FoxO3a transport, thus blocking the up-regulated expression of MAFbx and MuRF-1 [39].

Another mechanism that contributes to cachexia is related to alterations of the zinc homeostasis and the upregulation of the zinc transporters ZIP4 and ZIP14. Zinc deficiency is a common occurrence in several cancers, including PC [48]. Tumor cells exhibit dysregulated zinc uptake and efflux due to alterations in the activity of many zinc transporters [49]. For instance, the zinc transporter ZIP4 has been shown to induce tumor growth and cachexia in mice bearing orthotopic pancreatic tumors [50]. Yang *et al.* observed that in PC cells, ZIP4 stimulates the tumor release of surface heat shock proteins HSP70 and HSP90 via extracellular vesicle (EV), which can then activate mitogen-activated protein kinase 14 (p38 MAPK)-mediated muscle catabolism and promote cachexia, and that knocking down ZIP4 in PC cells mitigated muscle wasting and cachexia, by attenuating HSP70/HSP90 release from tumor cells [50]. Another zinc transporter implicated in cachexia is ZIP14, with increased expression upregulated in muscles of mice and patients with PDAC-associated cachexia. Shakri *et al.* observed that ZIP14 alters zinc homeostasis and that high levels of zinc in mature muscle fibers induce myosin heavy chain loss, therefore causing muscle breakdown. Moreover, in muscle progenitor cells, elevated zinc levels

prevent muscle formation by inhibiting muscle cell differentiation. ZIP14 is up-regulated by proinflammatory conditions and can be induced by TNF-α and TGF-β [51, 52]. Importantly, ZIP14 is not expressed is healthy muscle cells, suggesting that anti-ZIP14 antibodies might help prevent PDAC-associated cachexia [53].

Ketogenic diet in cancer

Ketogenic diets (KDs) are low-carbohydrate, moderate protein, high-fat diets characterized by the intentional restriction of dietary carbohydrate intake or nutritional ketosis, which generates ketone bodies (KBs) and induces a metabolic shift [54, 55]. Given that there is no exact definition for KD macronutrient composition, its variability could serve different clinical purposes [56]. Evidence suggests that KDs produce anticonvulsive, antioxidant and anti-inflammatory effects [57]. Clinically, KDs are a treatment for epilepsy and are therapeutic in other neurological conditions such like Alzheimer, traumatic brain injury, and stroke [58].

During the last decade, there has been increased interest in evaluating the impact of KDs in cancer prevention and treatment. Based on the premise that in contrast to healthy brain tissue, brain tumors are incapable of using KBs as an energy source, KDs have been suggested as metabolic therapy for malignant gliomas and have shown to reduce neuroblastoma (NB) tumor growth and prolong survival [59-61]. Moreover, KDs have also shown beneficial effects in other cancer types [59, 60]. A 2017 systematic review reported that KD had an inhibitory effect on tumor growth and enhanced survival time in a variety of animal cancer models [62]. For example, when gastric adenocarcinoma xenografts were fed either a KD or a standard diet *ad libitum,* survival in the KD group was significantly prolonged [63]. In mice bearing subcutaneously implanted colon tumors, a KD also had a tumor-suppressive effect [64]. In an orthotopic hepatocellular carcinoma study, tumor size, growth rate, and weight were lower in KD fed mice when compared to those fed a chow diet [65]. In pancreatic cancer xenografts, Shukla *et al.* showed that mice fed

a KD exhibited less tumor burden along with reduced proliferation of tumor cells when compared to a chow diet [66].

In addition, when combined with existing drugs, KDs may provide a promising approach to increase the therapeutic effects of existing cancer therapies at lower levels of overall toxicity [67]. Therefore, KDs are gaining attention as potential adjuvant therapy for cancer, with numerous preclinical studies showing promising results in multiple cancer types [56, 68, 69]. For example, in a mouse glioma model, KD alone had no effect on median survival, but increased that of bevacizumab-treated mice [70]. In mice with two different syngeneic glioblastoma tumors grown orthotopically, efficacy against tumor growth and pro-survival was greater when the glutamine antagonist 6-diazo-5-oxo-L-norleucine was administered together with a calorically restricted KD [71]. In NB xenografts, a KD supplemented with medium-chain triglycerides (MCTs) enhanced the anti-angiogenic efficacy of cyclophosphamide [72]. In breast cancer in vitro and in vivo models, a KD in combination with melatonin showed a synergistic effect against cisplatin- and vincristine-resistance [73]. In a mouse model of anaplastic thyroid carcinoma, a KD inhibited tumor growth when in combination with the antioxidant N-acetylcysteine [74]. Furthermore, Hopkins *et al.* documented that feeding a KD enhanced the efficacy of PI3K inhibitors in multiple tumor models, including PC. The effect was due in part to the ability of a KD to reduce insulin feedback, which represents a resistance mechanism during PI3K inhibitor treatments [75].

KDs can also potentiate the effect of radiation. In lung cancer xenografts, KD inhibited tumor growth and extended survival by enhancing radiation response [76]. Ketocal® (KC), a commercially available ketogenic formula, significantly increased survival in mice with malignant glioma when given with radiation, plus 9 out of 11 study animals were apparently cured of their implanted tumor [68]. In a PC xenograft model, KD improved radiation sensitivity and significantly increased survival [77]. Moreover, a KD seems to also improve immunotherapy. A recent study showed that a KD enhances immunotherapy in multiple types of tumor (lung, melanoma) [78]. The proposed mechanism appears to be directly related to the ability of a KD to increase 3-

hydroxy-butyrate levels which boosted the expression of CTLA-4 on $CD8^+$ T cells. Additional research is needed to validate these studies in immunotherapy-resistant tumor models, such as PDAC.

Although the preclinical evidence is abundant, only few studies have assessed the beneficial effects of KDs in humans with cancer [54]. In a study by Hagihara *et al*, a KD plus chemotherapy had a synergistic effect in the treatment of cancer and influenced long-term survival of advanced cancer patients [79]. Among women with ovarian or endometrial cancer, a KD might have improved physical function [54]. In post-pancreatectomy cancer patients, meal compliance, energy intake rate and meal satisfaction scores were higher in KD compared to general diet, with no differences in adverse events, nutritional status, and serum lipids [18]. Besides, KD appears to modulate PC-related metabolites and benefit pancreatobiliary cancer patients post-surgery [80]. In advanced cancer patients, a KD was shown to enhance the efficacy of chemotherapy treatment and improve quality of life [81]. Also encouraging are the numerous clinical trials evaluating the efficacy of KD alone or in combination with chemotherapy or radiotherapy (i.e: NCT03955068, NCT01716468 NCT03535701, NCT03962647, NCT03285152), including a clinical trial that is evaluating the effect of a ketogenic drink for pancreaticobiliary cancer patients (NCT03510429). A critical point to consider when analyzing the effects of KD in cancer treatment, is that the macronutrient composition of the KDs varied between studies and many trials lacked standardized protocols, so comparison among studies is difficult [59, 82, 83].

Ketogenic diet in cachexia

Cancer cachexia was believed to be resistant to nutritional interventions, yet evidence indicates that nutrition can impact it in PDAC patients [84]. Because muscle protein balance is affected by nutritional factors, some studies evaluated the impact of KDs on SKM during cancer progression [85]. The study by Shukla *et al.* showed that a KD can reverse PC-induced cachexia through the inhibition of systemic inflammation and muscle wasting [66]. The metabolic alterations

induced by KDs, in particular the production of KBs, are associated to reduced degradation of SKM's proteins, plus decreased secretion of pro-inflammatory cytokines and metabolites involved in pathogenesis of cancer cachexia [35, 81].

The favorable changes in body composition from KD appear to be influenced by the degree of nutritional ketosis achieved [86]. KDs may exert a protective effect against muscle mass loss, potentially through maintenance rather than a net hypertrophic effect [85]. In aged mice, Wallace *et al*. observed that a KD resulted in preservation of SKM and increased markers of mitochondrial biogenesis, oxidative metabolism, and oxidative stress response, while decreasing protein synthesis and proteasome activity [87]. The above evidence indicates that KDs have the ability to preserve SKM and assist in mitigating cancer-associated cachexia. However, there are some conflicting reports that feeding a KD leads to muscle atrophy in mice [88]. The differences observed could be due to the composition of the KD, the length of dietary treatment, or the fact that mice in Nakao *et al.'s* study lost weight, while in Wallace *et al*.'s study maintained their body weight throughout the KD intervention [87, 88].

Cellular Mechanisms of KDs in PDAC and Cachexia

Multiple cellular mechanisms might explain the beneficial effects of a KD in PDAC and cachexia. These include anti-inflammatory, anti-angiogenesis, cell metabolism effect, epigenetic as well as the modulation of the microbiome. Given their key role in PDAC growth and cachexia, in the sections below, we describe in more detail the effect of KDs on cell metabolism, epigenome, and the gut microbiome as major cellular mechanisms of the beneficial role of KDs in PDAC (Figure 2).

Ketogenic diet and cell metabolism

Metabolic reprogramming is a hallmark of cancer cells with many pathways that regulate cell metabolism being altered. For example, the IGF1/PI3K/Akt/mTOR system is often hyperactive in cancer cells due to chronic hyperglycemia and hyperinsulinemia, plus mutations in genes that

code for pathway proteins [89]. In PC, PI3K/AKT/mTOR and Ras/Raf/Mitogen-activated protein kinase/ERK kinase (MEK)/extracellular-signal-regulated kinase (ERK) pathways are upregulated and favor cancer cells proliferation and growth [56, 90]. AMP-activated protein kinase (AMPK) regulates glycolisis and is involved in tumorigenesis and PC progression [91]. In addition, activity of lactate dehydrogenase (LDH), responsible for conversion of pyruvate to lactate, is also increased in tumor cells [92]. LDH is a target gene of c-Myc, an important regulator of glucose metabolism, cell growth and proliferation, and is frequently amplified in PDAC [93-95]. In PDAC, high serum LDH levels are a poor prognostic indicator [93]. Pyruvate kinase (PK) regulates the final rate-limiting step of glycolysis. PKM2 has a critical role in reprogramming cancer metabolism, is upregulated in various tumors, and contributes to tumor growth and angiogenesis by regulating hypoxia-inducible factor 1 (HIF-1α) [96]. Furthermore, genes involved in glycolysis and glycolytic transport to the mitochondria are dysregulated in tumor cells and cause mitochondrial alterations [68].

Several mechanisms support the rationale of using a KD to modulate cancer metabolism [97, 98]. KDs mimic many of the metabolic and anti-inflammatory properties of calorie restriction (CR), including reduced blood glucose, insulin, and IGF-1, as well as the oxidation of fatty acids and generation of ketones [99]. Decreased blood insulin/IGF-1 levels by KD lead to an inhibition of the PI3K/Akt/mTOR and Ras/Raf/MEK/ERK cascades [66, 99].Hyperglycemia inhibits AMPK, which in term activates mTOR, but ketosis activates AMPK and inhibits mTOR [100-102]. Importantly, KBs can be used by healthy cells as energy sources, but many tumors are largely unable to metabolize them [103]. Therefore, KD tumor suppressive effects are mainly based on the Warburg effect [56]. Since cancer cells depend heavily on glycolysis, by reducing glucose, insulin, IGF-1 levels, plus lactate production, KDs potentially induce selective starvation in cancer cells [93, 104] . Shukla *et al.* showed that treatment with KBs reduced expression of GLUT1, LDH and c-Myc in PC cells, which might contribute to the inhibitor effects of KBs in PDAC growth [66].

In addition, fatty acid oxidation occurs primarily in the mitochondria and is dependent upon efficient and well-integrated mitochondrial electron transport chain activity [77]. Ketosis decreases ROS production in healthy tissues [97, 105]. Therefore, KDs may disrupt cancer cell metabolism and limit the energy source of the tumors, while still providing fuel for the host [106]. Results from the studies by Chang *et al*. showed that malignant gliomas have differential expression of ketolytic and glycolytic enzymes, and suggest that expression profiles could potentially be useful as biomarkers for KD therapy response [107]. Consistently, Zhang *et al.* demonstrated that xenograft tumors expressing low levels of BDH1 and OXCT1 are more responsive to KD therapy. In their studies, KD inhibited growth of Panc-1 and significantly prolonged the mean survival of mice with Panc-1 xenograft tumors, but in mice with HeLa xenografts KD increased tumor growth and significantly lowered survival, which suggests pancreatic cancer cells are more responsive to KD therapy, likely because they have less ability to metabolize KBs [108]. Besides ketolytic enzyme expression, the timing of KD administration may play a role in the effects of KDs against tumor growth. In a meta-analysis by Klement *et al*., it was described that mice that were in ketosis at the time of tumor cell injection seemed more protected than those receiving KD after tumor transplantation [109].

A summary of the Warburg effect and metabolic reprogramming in PDAC cells in the presence or absence of glucose is shown in Figure 3.

Although malignant cells lack key mitochondrial enzymes required to metabolize KBs, muscle cells maintain this ability [110, 111]. CR facilitates mitochondrial fat oxidation in SKM through upregulated AMPK signaling, working in opposition to IGF-1-mediated activation of mTOR [112]. KDs may contribute to SKM maintenance or growth through upregulation of mTOR signaling [113] [86]. Huang *et al.* showed that a normal-protein KD activated mTOR-related proteins that drive protein synthesis over autophagy [86]. In addition, Roberts *et al*. showed that, in aged mice, a KD preserved muscle mass and motor function by increasing protein acetylation levels and modulating mTORC1 signaling in a tissue-dependent manner [114]. In addition, a KD

can modulate amino acid metabolism. Douris *et al.* documented that consuming a KD for 80 weeks decreases amino acid catabolism in mice, with animals maintaining an improved glucose homeostasis, and showing a reduction in the hepatic expression of genes responsible for amino acid catabolism including branch-chain amino acids catabolism [115].

The previously discussed metabolic alterations in cancer cells contribute to the secretion of cytokines and metabolites involved in cancer-induced cachexia, and the catabolism of the SKM can then provide metabolites and energy sources for tumor growth. Conversely, the metabolic alterations induced by the KD lead to decreased secretion of pro-inflammatory cytokines and metabolites associated with cachexia [35]. In mice with PC xenografts, significantly lower glucose concentration and tumor weight, plus significantly higher βHB, muscle weight, and carcass weight was observed in a group fed a KD when compared to a group fed standard chow. Therefore, KD may be associated with diminishing tumor growth and inhibiting cancer-induced cachexia [66]. The anti-cachectic effects of the KD are not surprising considering that during prolonged fasting or starvation there is a metabolic shift to fat metabolism and ketosis in order to spare protein [81].

KBs reduce inflammation and oxidative stress, so they may have anti-catabolic effects during inflammation-related muscle atrophy [116]. Previous studies have suggested that βHB has potent anti-catabolic actions in muscle during acute inflammation [117]. Nakamura *et al.* demonstrated that the elevation of plasma IL-6 concentration was inhibited when given Ketonformula, which, together with increased βHB levels, suppressed systemic inflammatory responses and colon tumor progression, in term improving body, and muscle weights [69]. Besides, KDs may strengthen bioenergetic signaling that upregulates mitochondrial fat oxidation and endogenous antioxidant defense and downregulates inflammation [112]. In sedentary rats, a KD improved oxidative capacity and aerobic energy metabolism in muscle, without compromising muscle performance [118].

Ketogenic Diet and the Epigenome

Epigenetic alterations are emerging mechanisms of cancer progression, including PDAC [119, 120]. The epigenetic modifications of the genome involve changes to chromatin structure, DNA methylation, and histone modification, amongst the main ones. Interestingly, stage specific DNA methylation, and histone modifications have been detected in early tumorigenesis, PDAC progression and metastasis [120, 121]. KD could inhibit tumor growth and/or improve the effectiveness of cancer therapies through epigenetic modifications, such as DNA methylation, that affect the regulation of genes involved in tumor survival and proliferation [56, 67].

Besides epigenetic changes at the tumor sites, the regulation of gene expression in cachectic SKM can also be controlled by epigenetic mechanisms, through acetylation and deacetylation of histones, which are modified in a post-translational manner through histone acetyltransferases (HATs) and histone deacetylases (HDACs) [122]. HDACs are enzymes that remove acetyl groups and condense chromatin. Sirtuins (SIRTs) are also capable of histone deacetylation [123]. Histone acetylation plays numerous roles in muscle control and different classes of histone deactylase have different effects on SKM [85]. Histone acetylation via Classes I and II HDAC inhibition is a possible anti-catabolic mechanism of ketones [116]. Interestingly, the balance between HATs and HDACs is perturbed in muscle wasting [124]. Hyperacetylation of transcription factors and nuclear cofactors regulating gene transcription in muscle wasting may influence muscle mass. In addition, hyperacetylation may render proteins susceptible to degradation by different mechanisms, including intrinsic ubiquitin ligase activity exerted by HATs and by dissociation of proteins from cellular chaperones. In recent studies, inhibition of p300/HAT expression and activity and stimulation of SIRT1-dependent HDAC activity reduced glucocorticoid-induced catabolic response in SKM, providing further evidence that hyperacetylation plays a key role in muscle wasting. Although epigenetic markers are inherited, there is evidence that some can be modified by environmental variables, including diet [123, 125].

HDAC inhibitors are capable of reducing cancer cell proliferation and enhancing programmed cell death, and βHB and ACA have demonstrated HDAC inhibitor effects [67].

At the molecular level, βHB and ACA affect epigenetic marks by inhibiting HDAC1, and this modulates protein expression at the post-translational level, affecting DNA methylation and acetylating histone and non-histone proteins [126-128]. Of note, evidence also suggests that βHB can have a direct epigenetic effect via a novel histone modification known as β-hydroxybutyrlation of H3K9, which results in improved gene regulation [129]. KBs affect epigenetic marks by inhibiting HDAC1, modifying proteins at the post-translational level by butyrylation, affecting DNA methylation and acetylating histone and non-histone proteins. Epigenetic changes stimulated by KBs potentially modulate the expression of proteins involved in carcinogenic pathways [56].

The epigenetic effect of a KD might be due to the production of βHB. Shimazu et al. have shown that in the brain, βHB is more than an energy molecule; it plays important roles in cell signaling. The signaling functions of βHB are linked to the epigenetic regulation, since it is an endogenous class 1 HDAC inhibitor [127]. Another postulated epigenetic mechanism could be associated to the ability of a KD to increase adenosine levels. A study in epileptic rats fed a KD observed reduced gene expression due to ameliorated DNA methylation and increased adenosine levels , which blocks DNA methylation [130]. Roberts *et al*. showed that a KD extends median survival in mice, in part, by increasing protein (and histone) acetylation [131]. In addition, KD has been linked to increased global histone acetylation, with a specific increase in the expression of protective genes, such as Foxo3a which plays an important role in cachexia. Finally, the epigenetic regulations by KDs have been postulated to affect patient treatment response [119, 132].

Furthermore, recent studies have indicated the importance of the TME on tumor growth, as well as in PDAC treatment response [133]. KD could also modulate the activity of the pancreatic TME by affecting their epigenetic state. Wallace *et al*. reported increased markers of neuromuscular junction turnover, mitochondrial biogenesis, oxidative metabolism, and oxidative

stress response, and decreased ER stress, protein synthesis, and proteasome activity as mechanisms through which a KD results in the preservation of SKM and function in mice [134].

Ketogenic diet and the gut microbiome

The role of the microbiota in mediating the anti-tumor effects of KDs still needs to be fully investigated. Such a role appears possible given findings that indicate an important contribution of the gut microbiome to PDAC growth and treatment [135]. Emerging evidence also indicates that the gut microbiota is an important mediator of PDAC progression [136]. Individuals with PDAC appear to have microbial alterations with increased *Bacteriodetes,* but decreased *Firmicutes* and *Proteobacteria* compared to healthy controls [137]. Furthermore, ablation of the gut microbiota with wide spectrum antibiotics reduced the PC burden in a murine xenograft model [138] . Several strategies are being explored to modulate the gut microbiome in PDAC patients in order to shift the TME from an immunosuppressive to an immune-active state [135]. Riquelme *et al.* demonstrated that human fecal microbial transplantation positively affects PDAC tumors by modulating the gut microbiota and the immune system [139].

Several studies have explored the effect of a KD on the gut microbiome. Interestingly, a KD reversed the dysbiosis associated with certain neurological disorders, including autism, multiple sclerosis, and refractory epilepsy [140-142]. Moreover, individuals consuming a KD for 8 weeks significantly shifted their gut microbiota community, diversity, and function in a manner distinctive from high-fat diets. Moreover, KD decreased the gut and adipose tissue levels of pro-inflammatory Th17 cells [143]. In infants with refractory epilepsy, an increased proportion of circulating Th17 cells was partially reversed following KD consumption [144]. These data provides a premise for future investigations into the potential role of immune responses as a mechanism underlying the efficacy of a KD. Along these lines, the beneficial effects of a KD alone or in combination with chemotherapy/immunotherapy in PDAC and its impact on the microbiota is yet to be explored.

Besides its role regulating tumor growth, the gut microbiota may play an important function in the context of cancer cachexia. Cancer-associated gut microbiota dysfunction can alter mitochondrial energy metabolism in SKM, contributing to the negative energy balance in cachectic patients. The amino acid bioavailability and metabolites generated by the gut microbiota can influence energy expenditure in the muscle cells [41]. In addition, the gut microbiota may influence the efficacy of chemotherapeutic agents utilized in PDAC. For example, some bacteria species have the ability to metabolize GEM and decrease its activity [145]. Hence, modulation of the gut microbiota could potentially sensitize the tumor to chemotherapy, and has potential as a therapeutic target in the modulation of disease progression [136]. In cancer cachexia, gut barrier function and microbiota composition appeared to be altered independently of chemotherapy [146]. Still, the mediators of PDAC-induced cachexia, PDAC treatment and their interplay with microbiota remain elusive, and additional research is warranted to evaluate if select microbiome changes induced by a KD could be beneficial in PDAC.

Another important aspect to consider in future studies is the determination of the microbiome profile in each PDAC patient. This information will guide the implementation of specific diets, such as KDs, based on the species comprising their microbiome. One caveat is the need to monitor the gut microbiome regularly after the start of the diet to assess whether it was successful in modulating the gut microbiome profile. If the dietary intervention does not exert a strong and continuous effect, fecal microbiota transplants might be needed to fully change the microbiome at the start of the intervention, which could then be further sustained and enhanced through a KD. Nevertheless, additional research is needed in this area to determine the impact of KDs on the gut microbiome.

Clinical perspectives and future directions

Although KDs are an established therapy for epilepsy in humans, the evidence for a clinical beneficial effect of KDs for cancer patients is less consistent, but nonetheless promising

[111, 147]. KDs seem to be most beneficial when used as an adjuvant therapy with other treatment strategies. Even though many studies included only a small number of participating patients, they have provided promising indications that a KD is safe, feasible and improves outcomes in patients with several types of advanced cancer [5, 6].

Regarding its effect mitigating cancer-cachexia, the majority of the studies indicate that a KD preserves muscle mass, suggesting that it might prove to be instrumental in limiting cachexia development. Unfortunately, the current evidence in clinically-relevant PDAC models and/or human trials is limited, and further work is required to elucidate applicability of a KD in PDAC-cachectic patients. Given that the evidence suggests that the effect of the diet increases with time, investigations at an earlier stage in disease progression are warranted to evaluate long-term effect of KDs in mitigating PDAC cachexia.

Even though we have made significant progress in the understanding of the cellular mechanisms of a KD in cancer and cachexia, some important questions remain unanswered. There is no standardized KD, so variability in compositions (fat, protein and carbohydrates levels) may lead to different effects. Hence, a standardized treatment protocol that includes the length and regimen for a KD remains to be established [111].

A major limitation of any dietary intervention is its compliance for a long period. Indeed, strict diets are difficult to maintain throughout cancer treatment. Thus, it is important to consider whether an intermittent KD schedules might have a beneficial effect similar to those observed using a strcit KD. This possibility has been reeently investigated and the authors observed that intermittent administration of KBs induced T-cell dependent tumor growht inhibition [78]. Another possibility that requires further evaluation, is whether dietary supplements, such as a ketone esters (KE), that increase blood ketone levels provide the physiological benefits of ketosis, without extreme dietary restrictions. For example in animals and humans, feeding a KE-diet [30% kcal from (*R*)-3-hydroxybutyl (*R*)-3-hydroxybutyrate] have shown to increase circulating βHB concentrations and lowered plasma cholesterol, triglyceride, and glucose levels, compared with

pair-fed, isocaloric diets in which the KE was replaced by fat or carbohydrate [148]. Importantly, the authors showed that feeding a KE-diet enhances motor and physical performance [148]. Of note, KEs are proven to be safe in rats [149], and humans [150]. Thus, although studies in cancer models are lacking, this evidence supports the possibility that a KE-diet might be beneficial for cancer-associated cachexia.

An additional concern of the applicability of a KD in PDAC patients is that anorexia, in addition to the side effects of chemotherapy (nausea, vomit, diarrhea, dysgeusia) contribute to a high risk of malnutrition in PC patients [49, 151, 152]. Patients following a KD have reported constipation, diarrhea and fatigue, and other less common effects such as increased level of low-density lipoprotein cholesterol and some shakiness, but no significant adverse effects [153]. Moreover, the anatomical location of the pancreatic tumor (i.e. head of the pancreas) might affect the digestion of lipids due to an obstruction of the secretion of digestive enzymes into the duodenum. This may represent a major problem for proper lipid digestion and absorption, in particular when consuming high amounts of fat. Indeed, pancreatic exocrine insufficiency (PEI) often occurs with pancreatic tumors [151], contributes to cachexia and correlates with poor survival in advanced PDAC patients [154]. Due to the high prevalence of PEI in PDAC, patients usually receive pancreatic enzyme replacement therapy (PERT), the standard therapy for PEI, which is associated with weight maintenance and increased survival in PC patients [151, 155, 156]. Still, PERT prescription needs to be individualized and residual pancreatic function, clinical data, nutritional parameters, and dietary fat intake considered [156, 157]. Thus, these commons clinical practices (such as PERT) should be considered when implementing KD interventions in PDAC patients.

As the field of precision nutrition/medicine gains traction, it is becoming more evident that a “systems biology” approach is necessary. Given the heterogeneity of PDAC tumors and their complex TME, it appears critical to genotype each tumor to determine its metabolic profile. As

KDs are gaining in popularity, genetic variants for the prediction of a KD response must be considered [158].

In summary, the evidence to date strongly suggest that KD strategies are beneficial in PDAC and PDAC-associated cachexia, particularly when used adjuvant to treatment. We believe that in order to advance in the implementation of KD strategies, research should focus on understanding the long-term effects of KDs in humans bearing PDAC. Moreover, besides physiological factors such as age and gender, future studies need to consider additional factors such as lifestyle, dietary intake, body composition, gut microbiome composition, genotype, epigenetics, and the interplay of all factors, in order to maximize a patient's likelihood of successful response to KDs in combination with standard of care.

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FIGURES

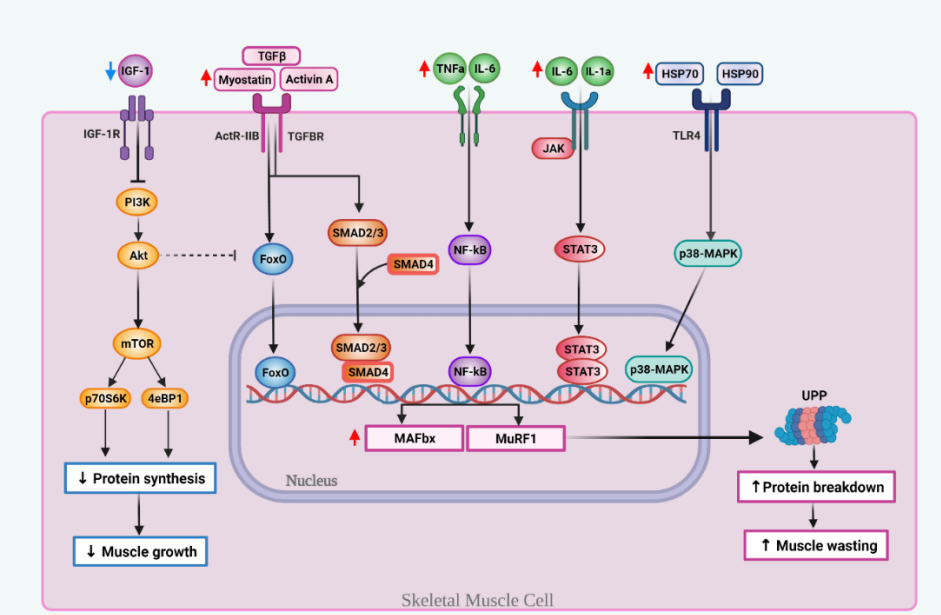

Figure 1. Key molecular mechanisms involved in muscle wasting during PDAC-associated cachexia. Cachexia is associated with decreased levels of the insulin-like growth factor 1 (IGF-1), which inhibits protein synthesis, in part, by suppressing the PI3K-Akt-mTOR pathway. Transforming growth factor-β (TGF-β) family members, like myostatin and activin A, bind to the ActRIIB receptor complex or TGFβ receptor and activate the forkhead (FOXO) family transcription factor or Smad2/3. Smad2/3 makes a complex with Smad4.. Pro-inflammatory cytokines like tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and IL-1a can activate the nuclear factor kappa-light-chain enhancer of B cells (NF-κB) and/or the janus tyrosine kinase/ signal transducer and activation of transcription (JAK/STAT). The tumor releases surface heat shock proteins Hsp70 and Hsp90 that activate Toll-like receptor (TLR4) and upregulates p38-MAPK. The translocation to the nucleus of FoxO, SMAD2/3-4 complex, NF-κB, STAT3 or p38-MAPK induces the subsequent transcription of muscle atrophy F-box protein (MAFBX) and muscle RING finger-containing protein 1 (MURF1), two genes that activate the ubiquitin-proteasome pathway (UPP) and induce proteolysis, which in term increases and induces muscle wasting.

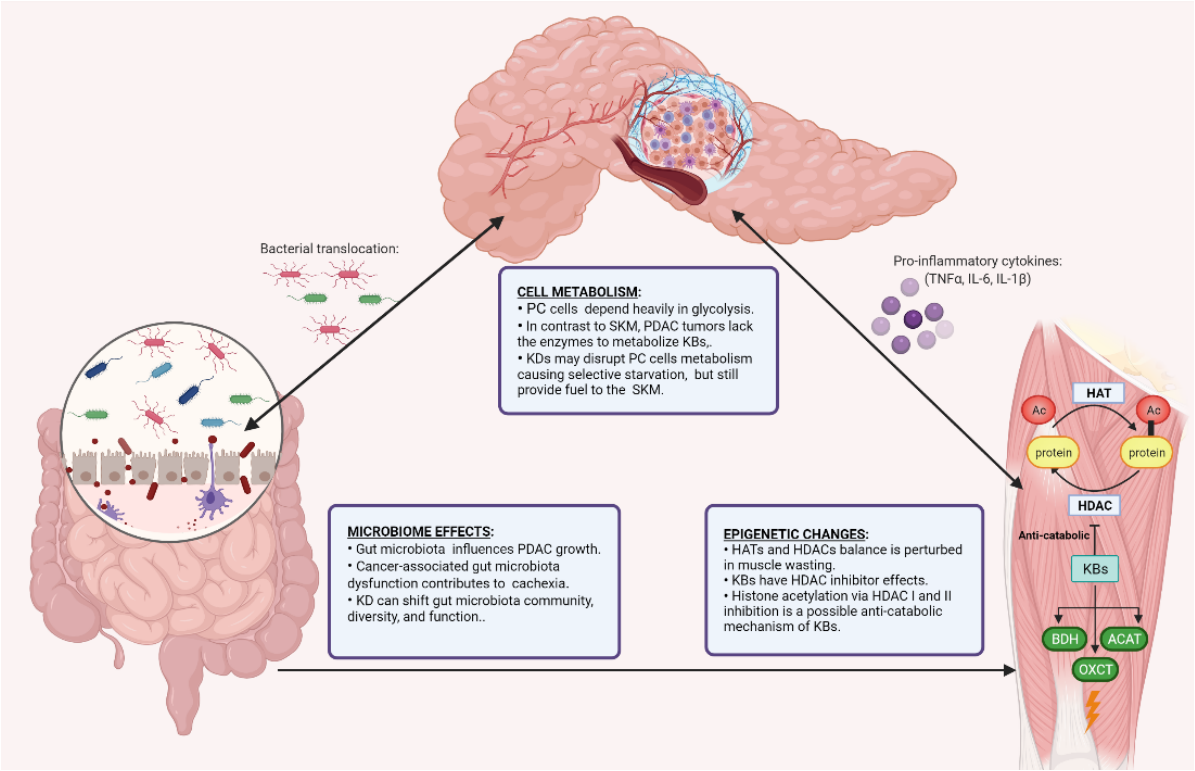

Figure 2. **Major proposed mechanisms of the beneficial role of KDs in PDAC.** Cell metabolism, epigenome and the gut microbiome have a complex interplay in pancreatic ductal adenocarcinoma (PDAC) and associated cachexia. PDAC cells depend on glycolysis and lack enzymes to that allow them to use KBs as a fuel. On the other hand, skeletal muscle (SKM) has mitochondrial enzymes D-beta-hydroxybutyrate dehydrogenase (BDH1), succinyl CoA: 3-oxoacid CoA transferase 1 (OXCT1) and acetyl-CoA acetyltransferase (ACAT1), which allow KBs utilization. The regulation of gene expression in cachectic SKM can be controlled by epigenetic mechanisms through acetylation and deacetylation of histones. The balance between histone acetyltransferases (HATs) and histone deacetylases (HDACs) is perturbed in muscle wasting. A KD could modulate the gene regulation of pancreatic tumors and SKM by affecting their epigenetic state. HDAC inhibition by KBs could have anti-catabolic effects. Finally, the gut microbiome is altered in PDAC and cachexia, influencing PDAC progression and potentially cachexia. The KD can shift the microbiota profile and have a role regulating tumor growth and cancer cachexia.

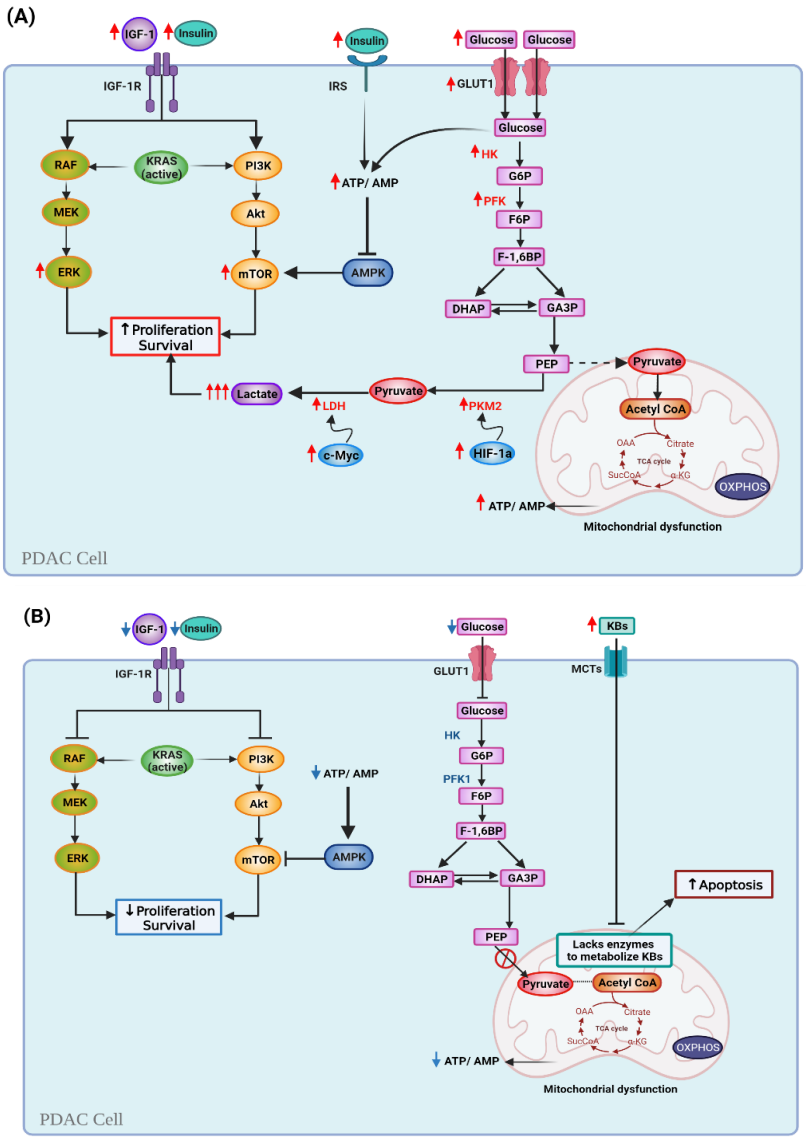

Figure 3. Pancreatic cancer cell metabolism during a normal carbohydrate rich diet versus a ketogenic diet. (A) Cancer cells undergo various metabolic modifications to satisfy their energy needs. High levels of insulin and insulin-like growth factor-I levels (IGF) induce upregulation of insulin/IGF-1-dependent phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of the rapamycin (mTOR) system and the Ras/Raf/Mitogen-activated protein kinase/ERK kinase (MEK)/extracellular-signal-regulated kinase (ERK) cascade. Kras mutation affects glucose dependency. The expression of glucose transporter 1 (GLUT1) is stimulated, so glucose uptake and glycolysis increase. Pyruvate kinase isozymes M2 (PKM2) and lactate dehydrogenase (LDH) are over-expressed, so lactate levels increase. Hyperglycemia inhibits AMP-activated protein kinase (AMPK), which in term activates mTOR **(B)** KDs reduce circulating glucose, which halts glycolysis. Decreased blood glucose, insulin, and IGF-I levels inhibit the PI3K/Akt/mTOR pathway, and lactate production, therefore inducing selective starvation in cancer cells, thus targeting proliferation and survival. Ketosis activates AMPK, which inhibits mTOR. Moreover, mitochondrial dysfunction and lack of the mitochondrial enzymes that metabolize ketone bodies (KBs) cause the mitochondria to decrease ATP production. Note: Upward red arrows mean upregulation; downward blue arrows mean downregulation

Chapter 2. A ketogenic diet in combination with gemcitabine increases survival in pancreatic cancer KPC mice

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Running title: Keto diet plus chemotherapy enhances PDAC survival

ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) continues to be a major health problem. A ketogenic diet (KD), characterized by a very low carbohydrate and high fat composition, has gained attention for its anti-tumor potential. We evaluated the effect and mechanisms of feeding a strict KD alone or in combination with gemcitabine in the autochthonous LSL-KrasG12D/+; LSLTrp53 R172H/+; Pdx1-Cre (KPC) mouse model. For this purpose, both male and female pancreatic tumor-bearing KPC mice were allocated to a control diet (CD; %kcal: 70% carb, 14% protein, 16% fat), a KD (%kcal: 14% protein, 1% carb, 85% fat), a CD + gemcitabine (CG), or a KD + gemcitabine (KG) group. Mice fed a KD alone or in combination with gemcitabine showed significantly increased blood β-hydroxybutyrate levels compared to mice fed a CD or CG. KPC mice fed a KG had a significant increase in overall median survival compared to KPC mice fed a CD (increased overall median survival by 42%). Interestingly, when the data was disaggregated by sex, the effect of a KG was significant in female KPC mice (60% increase in median overall survival), but not in male KPC mice (28% increase in median overall survival). Mechanistically, the enhanced survival response to a KD combined with gemcitabine was multifactorial, including inhibition of ERK and AKT pathways, regulation of fatty acid metabolism and the modulation of the gut microbiota. In summary, a KD in combination with gemcitabine appears beneficial as a treatment strategy in PDAC in KPC mice, deserving further clinical evaluation.

Keywords: pancreatic cancer, Ketogenic diet, ERK and AKT signaling pathways, gemcitabine, pancreatic ductal adenocarcinoma, Lipid metabolism, microbiome

INTRODUCTION

Despite extensive efforts to develop new treatment strategies, pancreatic ductal adenocarcinoma (PDAC) continues to be a major health problem, with a five-year survival of approximately 11% (1). While surgery is a viable option in a limited number of patients, the majority of PDAC patients (>80%) are diagnosed with advanced, unresectable or metastatic disease (2). For these patients, the standard treatments include combination of gemcitabine plus nanoparticle albumin bound (nab)-paclitaxel (Abraxane®), or the combined therapy of leucovorin modulated 5-Fluorouracil (5-FU), irinotecan, and oxaliplatin (FOLFIRINOX) (2-5). Unfortunately, these chemotherapeutic strategies still provide limited clinical benefit. Hence, there is an urgent need to develop therapies that can improve outcomes in PDAC patients, and the exploration of dietary interventions is a critical component. During the last years, there has been considerable interest in the anti-tumor evaluation of ketogenic diets (KDs) (6). KDs are characterized by a high fat, moderate protein and very low carbohydrate content. These diets mimic changes in metabolism that are similar to fasting by elevating circulating levels of ketone bodies (i.e., acetoacetate, β-hydroxybutyrate, and acetone), which serve as an alternative energy source (7) and as signaling molecules (8). Numerous studies have indicated that a KD inhibits tumor growth and increases survival (9-11), including in PDAC (12-14). Multiple cellular mechanisms might explain the beneficial effects of a KD in tumor growth. These include anti-inflammatory, anti-angiogenesis, cell metabolism and epigenetic effects, as well as modulation of the microbiome (15). Unfortunately, many of these studies were performed using xenograft models of pancreatic cancer, which do not closely recapitulate human PDAC, so their clinical significance are limited (16). Recently, Yang et al., reported that a KD was effective as an chemotherapy adjuvant reducing tumor growth in syngeneic subcutaneous pancreatic tumors and prolonged survival in the clinically relevant LSL-KrasG12D/+, LSL-Trp53R172H/+, Pdx1-Cre (KPC) genetically engineered mouse model of pancreatic cancer (17). However, this study was performed using a small cohort of only male mice. In this study, we evaluated the impact of feeding a strict KD alone

or in combination with gemcitabine in the autochthonous and clinically relevant KPC mouse model of pancreatic cancer (18,19). Furthermore, we examined whether there might be sex-related differences in the response to a KD in PDAC. We observed that a KD in combination with gemcitabine extends survival in KPC mice and that female mice appear to be slightly more responsive to the KD. The mechanisms by which a KD plus gemcitabine increases survival response appear to be multifactorial, including inhibition of ERK and AKT pathways, regulation of fatty acid metabolism, and the modulation of the gut microbiota.

MATERIALS and METHODS

Animal studies: All animal use procedures were approved by the University of California, Davis Animal Care and Use Committee.

Genetically engineered transgenic mice: The genetically engineered LSL-KrasG12D/+; LSL-Trp53R172H/+; Pdx-1-Cre (KPC) mice were bred at the UC Davis Animal Facility in Meyer Hall. KPC mice were generated from three mouse parental strains (LSL-KrasG12D/+; LSL-Trp53R172H/+ and Pdx-1-Cre), obtained from National Cancer Institute (NCI) mouse repository, following established procedures described by Hingorani and colleagues (18). After weaning, mice were individually housed in polycarbonate cages in a room with controlled temperature (22-24°C) and humidity (40-60%), maintained on a 12-hour light-dark cycle, and fed chow diet ad libitum LabDiet 5001 (LabDiet, Saint Louis, MO) until enrolled in the studies.

Survival study: Enrollment of KPC mice was based on tumor size, measured using a high-resolution ultrasound imaging of the pancreas with the Vevo 2100 System with a 35MHz RMV scan-head (Visual Sonics, Inc.), when KPC mice were around 3-4 months old (Fig. S1A). Imaging was obtained and tumor volumes measured following previously published guides (20,21). Once tumor size was assessed (Fig. 1A), male and female KPC mice were assigned randomly to one of four groups: a control diet (CD), ketogenic diet (KD), a control diet plus gemcitabine (CG) or a ketogenic diet plus gemcitabine (KG).

Dietary Interventions: Following tumor size determination, male and female KPC mice (7- 12 mice per sex per group; 16-23 mice/group) were allocated to either a control diet (CD; %kcal: 65% carb, 15% protein, 20% fat), a KD (%kcal: 1% carb, 15% protein, 84% fat), a CD + gemcitabine, or a KD + gemcitabine group. Mice were fed ad libitum, and food was changed and food intake was recorded three times per week. The composition of diets were adapted from the study by Roberts et al (22), and are shown in in Supplemental Table 1. The Envigo (Indianapolis, IN) mineral mix TD94046 was used for the control diets and the TD79055 was used for the ketogenic diets due to their lower carbohydrate contents. For both diets, TD40060 (vitamin mix) was used.

Chemotherapy treatment: Gemcitabine (>99% 2'-Deoxy-2',2'-difluorocytidine; dFdC; Gemzar; LY-188011) from Fisher Scientific (Hampton, NH) was administered to the CG and KG groups at 100 mg/kg by intraperitoneal injections twice per week for 3.5 weeks (7 total injections). Throughout the survival study, mice were observed daily for signs of significant weight loss; hemorrhagic ascites; and for other signs of clinical failure including loss of thermoregulation, inactivity, and presence of malignant ascites. Endpoint criteria included the development of abdominal ascites, weight loss exceeding 20% of the initial weight, or extreme weakness or inactivity. When an animal reached the endpoint criteria, it was euthanized by carbon dioxide 6 asphyxiation, blood was collected and tissues, including pancreatic tumors were dissected, weighted, and then stored in liquid nitrogen, RNA later and 10% buffered formalin.

Blood Glucose and Ketones: Non-fasting glucose levels were measured using a glucometer (Easy Plus II, Home Aid Diagnostics Inc, Deerfield Beach, FL), and β-hydroxybutyrate levels were measured using the Precision Xtra glucose and ketone monitoring system (Abbott) according to the manufacturer's instructions.

Mechanistic study: A cohort of mice was allocated to either the CG or the KG groups after tumor detection and euthanized at two months post-interventions. We chose CD + gemcitabine as our control group to specifically depict the contribution of a KD to the effect. At

the end of the 2 months, pancreas and pancreatic tumors were dissected, weighed, sectioned and then stored in liquid nitrogen, RNA later and 10% buffered formalin.

Metabolic measurements: Blood samples were collected via cardiac puncture and serum was isolated after centrifugation at 3,000 x g for 10 minutes (min) at room temperature. Insulin was assayed using the V-PLEX mouse metabolic kit and mouse leptin kit. Inflammation-related biomarkers were assayed using the V-PLEX Proinflammatory panel I kit (Meso Scale Discovery).

Histology: After necropsy, pancreas specimens were fixed in 10% buffered formalin overnight at 4°C. Tissues were processed and embedded by routine methods. Tissue sections (5 µm) were stained with hematoxylin and eosin or Masson's Trichrome (Chromaview, Thermo Scientific). Tumors were classified by morphologic pattern (glandular, spindled, solid), and each morphologic pattern was scored as a percentage of total tumor surface area. Presence and extent of tumor necrosis, and presence and type of background pancreatic fibrosis (e.g., inter-lobular, intra-lobular) were also scored. All histologic sections were evaluated in a blinded fashion. Immunohistochemical Staining. KPC pancreas was fixed in 10% buffered formalin overnight at 4°C and maintained in 70% ethanol for paraffin embedding. Paraffin sections were 7 deparaffinized, rehydrated, and heated for 12 min at 95°C in 10 mM (pH 6) citrate buffer (M15704, Fisher scientific). Afterwards, sections were incubated with 3% hydrogen peroxide (59105926, Millipore corporation) for 10 min and blocked-in animal-free Blocker (SP-5030, Vector laboratories) for 1 hour (h) at room temperature and then incubated overnight at 4°C with primary antibody against p-ERK1/2 (1:200 dilution, Cell Signaling Technology Cat# 4376, RRID:AB_331772). The following day, paraffin sections were incubated with biotin-conjugated secondary antibody for 1 h at room temperature (856743, Life technologies), horseradish peroxidase streptavidin for 1 h at room temperature (856743, Life technologies), and developed by DAB (SK-4100, Vector laboratories) followed by hematoxylin (MHS16, Sigma) staining. Sections were then dehydrated, mounted in Cytoseal 60 mounting medium (8310-16, Thermo

Scientific) and analyzed using an Olympus BX51 microscope. Scoring: At least 5 fields per sample (at magnification x200) were scored. We calculated the percentage of positive cells (brown staining) by dividing the number of labeled cells by the number of cells in each field and multiplying by 100.

Western Blot Analysis. Pancreas tissue homogenates were prepared as previously described (23) and western blots were performed. Aliquots of total homogenates containing 25–40 μg protein were separated by reducing 8%-12.5% (w/v) polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. Membranes were blocked for 1 h in 5% (w/v) nonfat milk and subsequently incubated with the following antibodies from Cell Signaling Technologies (Danvers, MA): p-ERK (Cat #4376, RRID:AB_331772), ERK (Cat# 9102, RRID:AB_330744), p-Akt (Ser473; Cat #4060, RRID:AB_2315049), AKT (Cat #9272, RRID:AB_329827), p-AMPKα (Thr172: Cat #2535, RRID:AB_331250), AMPKα (Cat #2532,RRID:AB_330331), p-4E-BP1 (Thr37/46; Cat #2855, RRID:AB_560835), 4E-BP1 (Cat #9452, RRID:AB_331692), HKII (Cat #2867, RRID:AB_2232946), PDH (Cat #3205, RRID:AB_2162926), LDH (Cat #2012, RRID:AB_2137173), p-IGFR-R (Cat #3024, 8 RRID:AB_331253), IGFR-R (Cat #9750, RRID:AB_10950969), and PKM2 (Cat #4053, RRID:AB_1904096), using a 1:1000 dilution, overnight at 4 °C. After incubation for 1 h at room temperature in the presence of secondary antibodies (either HRP or biotinylated antibodies, followed by 1 h incubation with streptavidin when biotinylated antibody was used in a 1:5,000 dilution), the conjugates were visualized and quantified by chemiluminescence detection in a ChemidocTM Imaging-System, Bio-Rad Laboratories (RRID:SCR_008426), Inc. β-actin (Cat #A1978) from Millipore-Sigma, Saint Louis, MO, was used as a loading control. The densitometric analysis was performed using the Image J Program (RRID:SCR_003070).

RNA preparation and RNA-seq analysis: Mice for the RNa-seq data were treated for two months with diet and chemotherapy. Tissues were stabilized in RNAlater (Invitrogen). Total RNA was extracted following the manufacturer's instructions using a RNeasy mini kit (74104,

QIAGEN) from frozen pancreas/tumors. RNA quality was confirmed using Nano drop one (Thermo scientific). Library preparation and RNA-sequencing were performed by Novogene Co., LTD (Beijing, China). In brief, mRNA was enriched using oligo(dT) beads, and rRNA was removed using the Ribo-Zero kit. The mRNA was fragmented, and cDNA was synthesized by using mRNA template and random hexamers primer, after which a second-strand synthesis buffer (Illumina), dNTPs, RNase H and DNA polymerase I were added for the second-strand synthesis, followed by adaptor ligation and size selection. The library was sequenced by the Illumina Novaseq platform. Raw data was aligned to mm10 genome using HISAT2, read counts and normalized read count were generated using the feature Counts, and the differentially expressed genes were identified using DESeq2 (RRID:SCR_000154).

Tissue fatty acid analysis: Fatty acid content in KPC pancreatic tumors of CG and KGtreated mice was measured using gas chromatography (GC). Briefly, pancreatic samples were freeze-dried and direct-methylated with sodium methoxide as previously described (24). Cis-10-17:1 methyl ester (Nu-Check Prep Inc., Elysian, MN) was added as an internal standard prior to 9 methylating reagent. Fatty acid methyl esters (FAME) were analyzed by GC using a CP-Sil88 column (100 m, 25 μm ID, 0.2 μm film thickness) in a TRACE 1310 gas chromatograph (Thermo Scientific) equipped with a flame-ionization detector (GC-FID, Thermo Scientific). Each sample was analyzed twice by GC using a 175 °C plateau temperature program (24). The FAME were quantified using chromatographic peak area and internal standard-based calculations.

Microbiota analysis: Fecal samples were collected from KPC mice at baseline and one month after dietary intervention ± gemcitabine treatment (from the KPC survival study groups). All fecal samples were collected directly from the animals on Eppendorf tubes and immediately frozen in liquid nitrogen. Genomic DNA was extracted from all samples using a commercially available kit (Qiagen QIAamp PowerFecal Pro DNA Kit, Cat. 51804) and following manufacturer's instructions. DNA concentrations of each sample were evaluated using Qubit® dsDNA High Sensitivity Assay Kit (Cat. Q32851) with Qubit® 4.0 Fluorometer, following manufacturer’s

instructions. Quality assessment was performed by agarose gel electrophoresis to detect DNA integrity, purity, fragment size and concentration. The 16S rRNA amplicon sequencing of the V3-V4 hypervariable region was performed with an Illumina NovaSeq 6000 PE250. Sequences analysis were performed by Uparse software (Uparse v7.0.1001) (25), using all the effective tags. Sequences with ≥97% similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation. For each representative sequence, Mothur software was performed against the SSUrRNA database of SILVA Database (26). OTUs abundance information were normalized using a standard of sequence number corresponding to the sample with the least sequences.

Data Availability: The accession number for the RNA-seq data reported in this study is NCBI GEO: GSE208398. The accession number for the microbiome data is under Bioproject number: PRJNA858994. 10

Statistical Analysis. The data, obtained from at least three independent experiments, were expressed as the mean ± SEM. Statistical evaluation was performed by t-test or one-factor analysis of variance (ANOVA) followed by the Tukey test adjusted for multiple comparisons. Analyses were performed by GraphPad (Prism version 9.2, RRID:SCR_002798) and R version 4.0.4. Two-sided $P<0.05$ was regarded as statistically significant.

Kaplan–Meier methods and the log-rank tests were used to compare unadjusted survival outcome (time from the start of treatment to death) between treatments in overall and key subgroups. There is no censoring in the survival outcome. To adjust for possibly unbalanced age and sex between treatment groups and explore potential interactions, linear regression models for survival days since treatment to death were performed, which include diet (CD, KD), gemcitabine (no, yes), sex, age at the start of treatment (centered at 90 days), interaction between diet and gemcitabine, 2- and 3-way interactions between sex and treatments (diet and gemcitabine). All interactions were removed from the final model due to non-significance. Model diagnosis was performed to ensure that the assumptions of linear regressions hold.

For the microbiota analysis, alpha- and beta-diversity were assessed by using standard metrics (e.g. Simpson and Shannon H diversity index) and Bray-Curtis Principal Coordinates of Analysis (PCoA) respectively. Statistical significance was determined by Kruskal–Wallis or Permutational Multivariate Analysis of Variance (PERMANOVA). Comparisons at the Phylum and Genus level were made using classical univariate analysis using Kruskal–Wallis combined with a false discovery rate (FDR) approach used to correct for multiple testing. Finally, LEfSe (Linear discriminant analysis Effect Size) was also employed to determine the features most likely to explain differences between classes.

RESULTS

A KD plus chemotherapy extends survival in KPC mice.

We first conducted a survival study in the clinically relevant KPC mouse model to evaluate the effect of feeding a strict KD alone or in combination with gemcitabine as a treatment protocol in male and female KPC mice bearing pancreatic tumors. For this purpose, we enrolled KPC mice with similar tumor sizes, measured by high-resolution ultrasound imaging of the mouse pancreas (Fig. 1A and Fig. S1). Male and female KPC mice were divided into control diet (CD), KD, CD + gemcitabine (CG), or KD + gemcitabine (KG) groups (16-23 mice/group) (Fig.1B). While a KD alone had no significant effect on KPC survival, the combination of a KD with gemcitabine synergistically prolonged survival. The overall median survival times among the 4 groups were 80, 94, 88, and 119 days for CD, KD, CG, and KG groups, respectively. While KD alone or CG treatments were unable to extend KPC mouse survival, KPC mice fed a KG had a significant increase in overall median survival compared to KPC mice fed a CD (increased overall median survival by 42%, Fig. 1C). A 26% increase survival was observed when comparing KG group with CG. Interestingly, when we separated by sex, the effect of a KG was significant in female KPC mice (60% increase in median overall survival ($p=0.028$), but not in male KPC mice (28% increase in median overall survival compared to CD mice ($p=0.089$), (Fig.1 D-E and Fig. S1B). The median

survival times for CD and KG groups were 77 and 123 days in females; and 80 and 103 in males, respectively. Interestingly, the weights of the pancreas/tumors were comparable among the groups, with only a significant decrease of the tumor weight of KG-treated female mice was observed compared to KD alone (Fig. 1F). Of note, treatment with KD, CG or KG was well tolerated with no body weight loss throughout the treatment, as compared to the baseline body weight levels (Fig. 1G).

Histopathologic evaluation of the tumors showed the classic glandular morphology of PDAC, as originally described by Hingorani et al. (18). Spindled and solid patterns were also observed as secondary or primary patterns (also described by Hingorani et al. (18); Fig. 1H). Interestingly, an increased proportion of CG and KG tumors demonstrated classic glandular morphology compared to CD and KD tumors (Fig. 1H), whereas the latter showed an increased proportion of spindled and solid patterns, generally considered indicative of more aggressive behavior (27,28). In addition, the increase in glandular morphology in the KG-treated group was found to be the result of a marked predominance of glandular morphology in the tumors of the female mice. Additionally, some tumors showed some degree of necrosis. Overall, we noted a slight increase in tumor necrosis in females fed the KD compared to CD, with and without gemcitabine (Fig. S2). The residual background pancreas (when present) showed a combination of intra- and interlobular fibrosis. No significant difference in the pattern of fibrosis (intra- versus interlobular) was noted among the four groups (Fig. S3).

To adjust for possibly unbalanced age and sex between intervention groups and further explore whether a KD ± gemcitabine's survival effect is sex-dependent, we conducted linear regression models for survival days since treatment to death, which was adjusted by sex and age at the start of treatment. As shown in Table 1, gemcitabine significantly extended mean survival by 25.8 days ($p=0.002$), KD extended mean survival by 13.8 days with a trend towards significance ($p=0.052$). Compared to CD, KG significantly extended mean survival by 39.6 days ($p<0.001$). Although Kaplan-Meier curves in sex subgroups suggested that the effect of KG is

likely more effective in females than in males, the interactions between sex and treatments in linear regressions were not significant and hence removed from the final model. Thus, the treatment effect was comparable across both sexes, benefiting both females and males.

Mice fed a KD, alone or in combination with gemcitabine, showed significantly increased blood ketones compared to mice fed a CD or CG (Fig. 2A). The β-hydroxybutyrate levels in the KD and KG groups remained elevated throughout the study. The increase in β-hydroxybutyrate 13 levels were observed in both female and male mice fed a KD or KG (Fig. 2A). In contrast, glucose levels were significantly higher in the CD and CG groups when compared to KD only at 1 month (Fig.2B). When disaggregated by sex, such effect was only observed in males (Fig. 2B).

Furthermore, since KDs have been shown to exert anti-inflammatory effects (29), we assessed the levels of several pro-inflammatory cytokines in the serum of male and female KPC mice at endpoint. In males, there was a decrease of TNFα in KD compared to CD and a decrease in IL-6 in the KG-group when compared to KD. In addition, higher levels of IL-1β were observed in KG males compared to CD. In males, higher levels of KC/GRO were observed in the KG group compared to CD and CG groups. On the other hand, in females, no significant changes in serum cytokines were observed among the CD, KD, CG and KG groups (Fig. 2C). Moreover, no significant differences, in both males and females, were observed in IFNɣ, IL-10, or MCP-1 levels among the groups (Fig. S4).

Evaluation of cellular mechanisms of a KD related to tumor growth. To elucidate the cellular mechanisms underlying the beneficial effects of a KD plus gemcitabine on pancreatic tumors, we conducted a study in which male and female KPC mice bearing pancreatic tumors (3 months old) were treated with either CG or KG for 2 months (Fig. 3A). We chose CD plus gemcitabine as our control group to specifically depict the contribution of a KD to the effect. After 2 months of treatment, no differences in the weights of the pancreas/tumors were observed between CG- and KG-treated mice (Fig. 3B).

To investigate whether KD plus gemcitabine induces changes in PDAC tumors in females that would suggest general antitumor activity, we initially performed an RNA-Seq analysis followed by HALLMARK gene set enrichment analysis (GSEA) on female pancreatic tumors obtained from KG or CG mice after 2 months of treatment. KG treatment was broadly associated with increased changes in the expression of genes involved in early and late estrogen response, 14 xenobiotic metabolism, glycolysis and fatty acid metabolism. In contrast, KG treatment was associated with decreased changes in the expression of genes involved in allograft rejection, interferon alpha and gamma response, PI3K-AKT-mTOR as well as unfolded protein response (Fig. 3C).

A KD inhibits the Akt and ERK pathways in KPC mice.

Two pathways commonly activated in PDAC are PI3K-AKT-MTOR and Kras/MAPKs (30,31). GSEA data indicated that PI3K-AKT-MTOR was one of the pathways downregulated in the KG group, compared to CG (Fig. 4A). Thus, we validated these data by assessing the activation status of key proteins in the PI3K/Akt/mTOR, as well as the Raf/MEK/ERK pathways by immunoblot. Although there was no significant difference in AKT, ERK or AMPK phosphorylation in pancreatic tumors between KG and the other groups in the survival study (Fig. S5), KG treatment significantly reduced AKT, ERK, IGFR and AMPK phosphorylation in pancreatic tumors of female, but not male, KPC mice, compared to CG-treated mice, at 2 months of treatment (Fig.4B-C). In contrast, no significant changes were observed in the expression levels of phosphorylated 4EBP-1 between the two groups (Fig.4B-C). To confirm these results, we assessed ERK activation by immunohistochemistry of tumor sections prepared from CG and KGtreated female and male KPC mice. KG reduced p-ERK levels by 79% in females, compared to CG-treated mice (Fig. 4D; $p<0.08$ for females).

Furthermore, because AKT activation can be regulated by insulin, we assessed serum insulin levels. Compared to CG-treated mice, after two months KG treatment reduced insulin levels by 85.5% in female and 78.2% in male KPC mice (Fig. 4E).

A KD alters glucose metabolism in pancreatic tumors.

Among many signatures, GSEA of differentially expressed genes in tumors from KG and CG-treated female mice identified glycolysis signatures as highly affected (Fig. 5A). Thus, we assessed the expression levels of several enzymes linked to glucose metabolism in the pancreas/tumors of KPC mice. In both female and male KPC mice in the survival study, KG reduced hexokinase 2 (HK2) levels when compared to CD (Fig. 5B), but no changes were observed in animals euthanized at two months between KG and CG groups (Fig. 5C). Moreover, no significance differences were observed in the expression levels of LDH, PKM2 and PDH (Fig. S6).

A KD affects the concentrations of fatty acids in pancreatic tumors.

as highly enriched in KG treated mice compared to CG-treated mice (Fig.6A). To understand more comprehensively, which fatty acids are affected in pancreatic tumors, we analyzed the fatty acid composition of tumors isolated from female and male KPC mice treated with a KG or a CG for 2 months. As shown in Fig.6B, there were no significant differences in concentrations of total saturated fatty acids (SFA), total cis-monounsaturated fatty acids (c-MUFA), total n6-polyunsaturated fatty acids (n6-PUFA) and n3- PUFA in the pancreas of KG mice compared to CG mice. This holds true when separated by sex.

Interestingly, when examining changes of individual fatty acids between KG and CG, we observed that KG significantly reduced concentrations of asclepic acid (cis11-18:1), palmitoleic acid (cis9-16:1), and eicosatrienoic acid (20:3n-3), while increased margaric acid (17:0) content, compared to CG (Fig. 6C). Distinctively in female KPC mice, KG significantly reduced the

concentrations of palmitic acid (16:0), myristoleic acid (cis9-14:1), palmitoleic acid (cis9-16:1) and linoleic acid (18:2n-6), and significantly increased the concentrations of margaric acid (17:0) and 16 stearic acid (18:0) when compared to CG-treated females (Fig. 6D). No significant changes in any fatty acid concentrations were observed between KG and CG-treated KPC male mice.

A KD plus gemcitabine alters gut bacterial composition in KPC mice.

Given that diet influences the composition of the gut microbiota, and the gut microbiota can affect PDAC growth and response to treatment (32,33), we next performed 16S rRNA sequencing to evaluate the impact of a KD alone or in combination with GEM (KG) on the gut microbiota. For this purpose, we collected fecal samples at baseline (KPC mice fed chow diet, prior to dietary and/or chemotherapeutic treatments) and after 1 month of treatment with CD, KD, CG or KG and assessed the α-diversity among groups. As expected, at baseline, there were no significant differences on the microbiota composition/diversity among the four groups. As shown by the Shannon and Simpson diversity indices, there were no significant differences on the microbiota diversity in the CD-fed group pre to post dietary intervention, but a significant difference was observed in both gemcitabine-treated groups ($p<0.001$). When comparing mice fed a CD with those in the KG group, a significant difference was observed ($p=0.0003$). Interestingly, a significant difference was observed when comparing animals fed a KD with those in the KG group, as depicted by the Shannon index ($p=0.0163$) (Fig.7A).

We next analyzed the taxonomic components for all groups to confirm the specific changes of the microbial community. At the phylum level, *Firmicutes* and *Bacteroidetes* dominated the gut microbiota, and lower levels of *Proteobacteria* were detected. Compared to CD-fed mice, there was an increase in the relative abundance of *Firmicutes* in both KD-fed groups at 1 month of treatment. At 1 month of treatment, the ratio *Firmicutes/Bacteroidetes* was significantly higher for the KG (ratio = 9) group when compared to all others [CD (ratio =2.9), KD (ratio =5.3), CG (ratio =2.5)] (Fig. 7B).

At the Genus level, all post-treatment groups increased the levels of *Faecalibaculum, Romboutsia* and *Erysipelatoclostridium*, while reduced Lactobacillus levels, compared to baseline levels. *Romboutsia* levels were higher in the CD groups, while the increase in *Erysipelatoclostridium* was more apparent on the KD-fed animals. Interestingly, *Dubosiella* increased only in both GEM-treated groups. Of note, the levels of *Faecalibaculum* were significantly increased in the KG-treated mice when compared to all three other groups (Fig. 7C). Based on the differences in microbial community composition among groups, we next performed a Bray-Curtis principal component of analysis (PCoA) to define the similarity of species diversity among groups on operational taxonomic unit (OTU) level (Fig. S7). Although there was a significant impact of treatment on microbial beta-diversity (PERMANOVA: F-value: 4.6619, $p< 0.321$; not shown).

Finally, given that only KD plus gemcitabine increased overall survival, we aimed to identify some key species of bacteria that were differentially present in the KG group compared to KD or CG groups alone by performing a Linear discriminant analysis Effect Size (LEfSe) analysis. The linear discriminant analysis (LDA) histogram was used to calculate the significant changes in the gut microbiota and interpret the degree of consistent difference of relative abundance between treatment groups. LDA results showed several discriminative features in the KG group ($LDA > 3.6$, $p < 0.05$), compared to either KD or CG groups (Fig. 7D). The major species that were significantly increased in KG versus KD and KG versus CG include: genus_*Faecalibaculum,* class_*Erysipelotrichia,* order_*Erypiselotrichales* and family_ *Eryspelotrichales.* Moreover, major species that were significantly decreased in KG versus KD and KG versus CG were order_*Lactobacillales,* family_*Lactobacillae*, genus_*Lactobacillus* phyllum_*Bacteroidetes,* order_*Bacteroidales*, class_*Bacilli* (Fig. 7D). Of note, increases in order_*Erypiselotrichales* and a decrease in order_*Lactobacillales,* family_*Lactobacillae,* genus_*Lactobacillus* was observed when comparing as the KD versus CD (Fig. S8).

DISCUSSION

Dietary interventions hold promise in cancer treatment, including PDAC. Previous studies in animal models suggested that a KD is an effective adjuvant therapy for pancreatic cancer, yet the significance of the clinical benefit of KDs was limited due to the use of xenograft models, only one sex, or the use of small cohorts (12-14). We observed that in the clinically relevant KPC mouse model, mice fed a strict KD in combination with gemcitabine exhibited a significant increase in overall median survival, compared to KPC mice fed a CD, and this beneficial effect was superior in female mice compared to male mice. Although our linear regression model indicates that the effect of a KD plus gemcitabine is likely not sex-dependent, benefiting both males and females, the survival curves suggest that the effect of a KD plus gemcitabine is somewhat more effective in females. Indeed, when disaggregating the data between females and males, the effect of a KD plus gemcitabine was significant in female KPC mice (60% increase in median overall survival), but not in male mice (28% increase in median overall survival). It is important to note that treatment with a KD alone had no effect on KPC survival, indicating that the dietary changes themselves were insufficient to cause the tumor responses.

Consistent with our findings, other investigators have recently evaluated the use of a KD in preclinical KPC allograft tumor models. For instance, Hopkins et al. observed that a KD rendered PI3K inhibitors, which are normally inactive against PDAC, effective in a KPC cell linebased orthotopic allograft tumors (14). In addition, Yang et al. recently showed that a KD synergized with a clinically relevant chemotherapeutic regimen of gemcitabine, nab-paclitaxel and cisplatin, significantly increasing survival in subcutaneous KPC allograft tumors (17). Overall, 19 these findings, together with our data, strongly indicate that a KD is an effective adjuvant dietary strategy for PDAC, and supports the initiated clinical trials (i.e.NCT04631445), currently underway, to investigate its benefit in humans.

Mechanistically, the survival response to a KD plus gemcitabine appears to be multifactorial, including the inhibition of ERK and AKT pathways, regulation of fatty acid

metabolism and the modulation of the microbiota. Interestingly, we noted some discrepancies between ours and Yang et al.'s RNA-Seq data (17). For example, while allograft rejection, interferon alpha and gamma response gene sets were down regulated in our data, they noted the opposite. These discrepancies might be the result of the differences in tumor types used in the analysis (KPC tumors versus allografts), differences of the tumor microenvironment, or the variances in the duration of KD intervention and other interventions (i.e: gemcitabine). Therefore, and as suggested by our RNA-Seq analysis, at this time, we cannot rule out that other mechanisms, including modulation of xenobiotic metabolizing enzymes or estrogen responses, could also contribute to the effect of a KD in PDAC.

Many features contribute to the reduced effectiveness of gemcitabine, including the dysregulation of signaling pathways related to cell metabolism (34), such as the insulin/IGF-1R, ERK and PI3K/AKT pathways. For example, the PI3K/AKT pathway is aberrantly activated in multiple tumor types, regulating tumorigenesis, cancer metabolism and drug resistance (35,36). On the other hand, the deregulation of the ERK pathway is a signature of many epithelial cancers, including PDAC (37), whereas the upregulation of the insulin/IGF-1R pathway in PDAC occurs in over 70% of patients (38). Interestingly, compensatory upregulation of IGF-1R and ERK signaling limits the efficacy of select inhibitors, such as autophagy inhibitors, and their concurrent inhibition synergistically increases autophagy dependence and chloroquine sensitivity in PDAC (39). Therefore, the fact that a KD inhibits ERK, AKT and IGFR activation might explain, at least in females, the survival benefit of its combination with gemcitabine.

Lipid metabolism is essential for cancer progression (40), with increased levels of specific fatty acids known to regulate pancreatic cancer progression (41). For example, Lien et al. recently showed that the upregulation of stearoyl-CoA desaturase, which synthesizes MUFAs from SFAs, is essential for cancer cells to grow (42). Interesting, they suggest that modifying the composition of the dietary fat could lead to higher tumor inhibitory effect. For instance, altering the KD fat composition, by using palm oil instead of lard as the source of fat, slowed tumor growth, by

increasing tumor saturated fatty acid levels, lowering MUFAs and decreasing tumor stearoyl-CoA desaturase activity. Although we did not observe significant differences in overall saturated fatty acids or MUFAs between KG and CG groups, we observed a reduction in select MUFAs in the KG group compared to the CG group. Since the KD used in our study was mainly prepared with Lard, it would be important to evaluate whether a KD from other fat sources that increase saturated fatty acids might provide an additional beneficial effect. Several studies have also shown a positive association between higher consumption of certain fatty acids and pancreatic cancer risk. For example, high linoleic acid intake was shown to increase the risk of pancreatic cancer when compared with the individuals with the low linoleic acid intake (43). In a prospective nested case-control study, Yang et al. identified a fatty acid pattern using principal component analysis, associated with an increased risk of prostate cancer, which was characterized by higher levels of 14 and 16 carbon SFA and MUFA including myristic acid, palmitic acid, myristoleic acid and palmitoleic acid, along with low levels of α-linolenic acid (44). Interestingly, many of the fatty were reduced in pancreatic tumors following KG treatment, such as palmitic acid, myristoleic acid, palmitoleic acid, asclepic acid and linoleic acid. Additional studies are warranted to validate whether one or more of these fatty acids could explain, in part, the beneficial effect of a KD in PDAC, and whether the modifying the type of fat used in the KD could lead a higher tumor inhibitor effect.

The gut microbiota is an emerging mediator of PDAC progression (45), with many strategies to modulate the gut microbiome in PDAC being actively explored (46). For example, the transplantation of human fecal microbes can affect PDAC tumor response by modulating the gut microbiota and the immune system (47). In addition, two bacterial communities (*Faecalibaculum* and *Lactobacillus*) have been recently documented to play a critical role regulating tumor growth. Zagato et al. identified that *Faecalibaculum rodentium*, belonging to the *Erysipelotrichaceae* family, was strongly under-represented during the early phases of tumorigenesis in the ApcMin/+ mice compared to wild-type mice, and that it was responsible for

inhibiting intestinal tumor cell proliferation (48). Moreover, *Faecalibaculum* can inhibit tumor growth in breast cancer models (49). On the other hand, bacteria belonging to the genus Lactobacillus, which are gut commensals with an ability to produce indoles from tryptophan (50), can drive suppression in the pancreatic tumor microenvironment promoting tumor growth (51). Our findings, showing that KG treatment leads to increased relative abundance of *Faecalibaculum* and the reduction of Lactobacillus, might provide a partial explanation of the beneficial effects of KD in combination with gemcitabine observed in KPC mice. Future investigations will determine if the selective modulation of these bacteria can be used to improve the therapeutic response in PDAC.

In summary, a KD in combination with gemcitabine is beneficial as a treatment strategy for PDAC in KPC mice. The mechanisms by which KD plus gemcitabine increases survival response are multifactorial, including inhibition of ERK and AKT pathways, regulation of fatty acid metabolism and the modulation of the microbiota. These data in an autochthonous and clinically relevant mouse model strongly suggest that a KD should be evaluated concomitant to chemotherapeutic treatment in the clinical setting.

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FIGURES

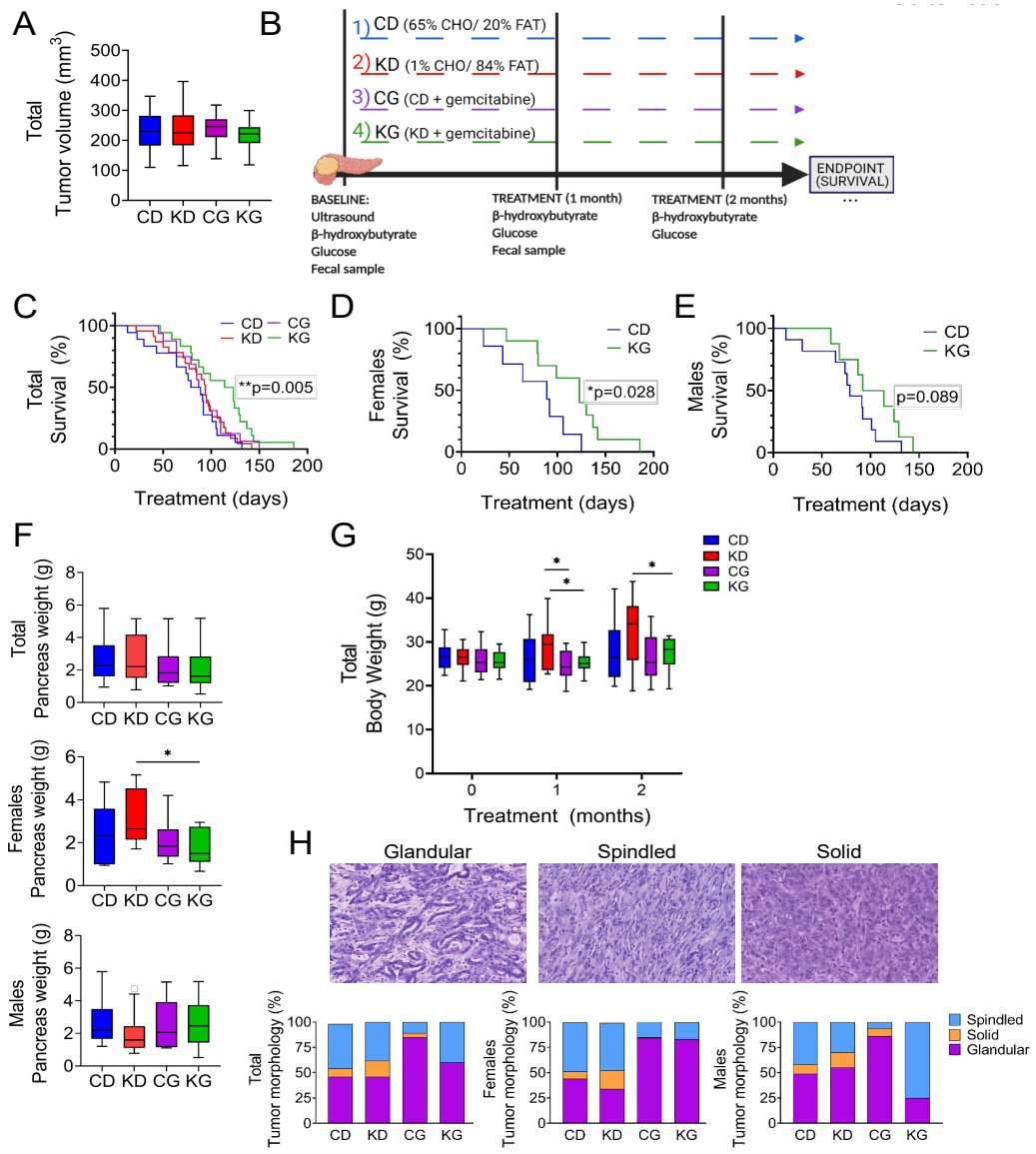

Figure 1. A ketogenic diet plus gemcitabine extends median overall survival in KPC mice. **A:** Mean tumor volume of KPC mice at enrollment. **B:** Schematic outline of the survival study design. **C:** Kaplan-Meier survival curves of male and female KPC mice fed a control diet (CD), ketogenic diet (KD), control diet plus gemcitabine (CG) or ketogenic diet plus gemcitabine (KG). **D-E:** The effect of KG extending median overall survival was more pronounced in female KPC mice than in male KPC mice. Of note: KD and CG groups had no significant effects, compared to CD group, and are not displayed for clarity. **F:** Pancreatic tumor weight at endpoint for total cohort (left), females only (center) and males only (right) are shown. *p < 0.05. **G:** Body weight progression. **H:** Histopathological analysis of pancreatic tumor morphology isolated from female and male KPC mice treated with CD, KD, CG or KD. Tumor morphology was predominantly glandular. However, spindled and solid patterns were also observed, as previously described (18). Representative images of morphologic patterns: Glandular (left), Spindled (center), Solid (right). Hematoxylin and eosin-stained sections. All images digitally scanned at 20X original magnification.

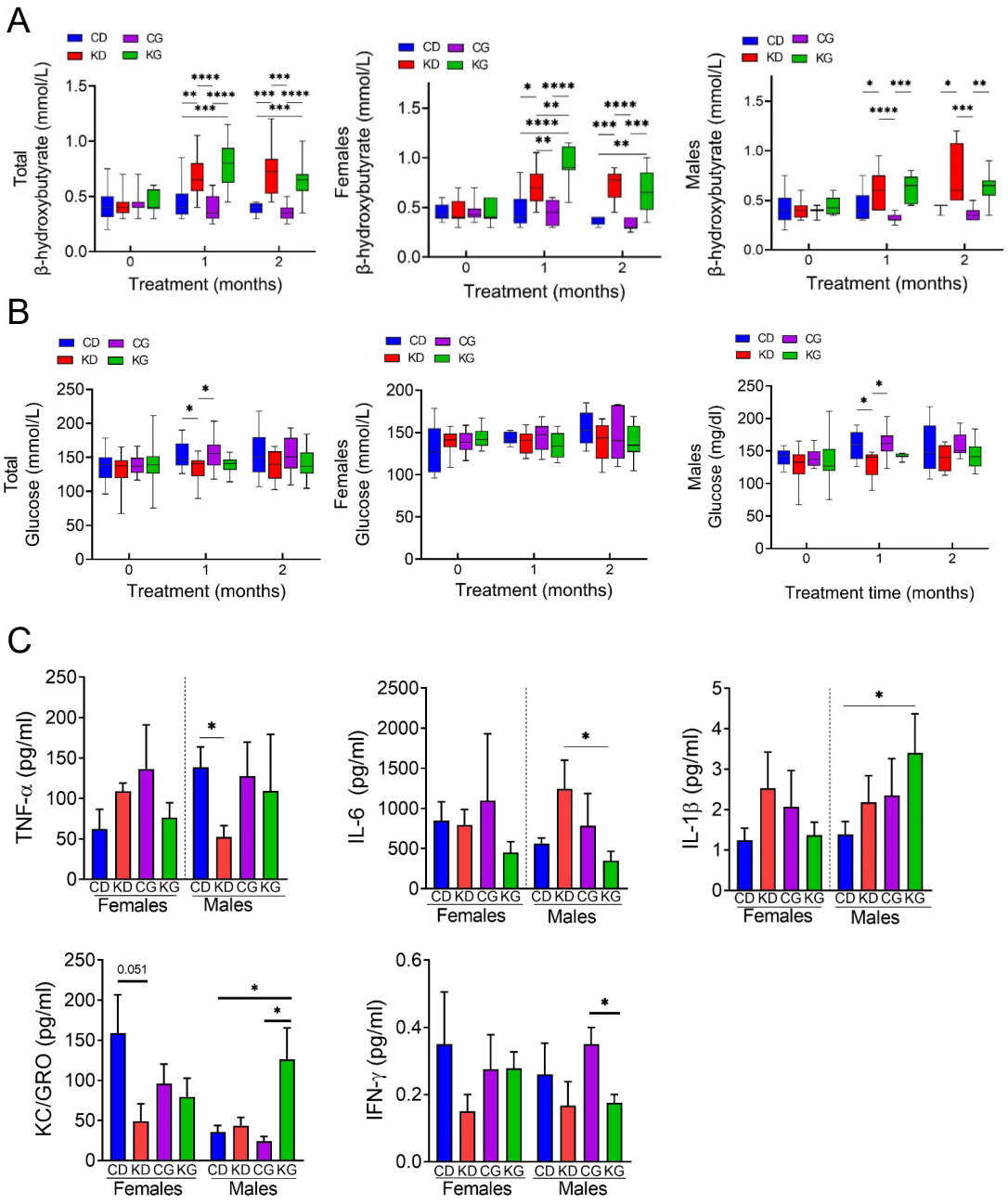

Figure 2. Metabolic changes of KPC mice fed ketogenic diet with or without chemotherapy. A: Blood β-hydroxybutyrate levels at baseline and after each month in each group in total cohort (left), females only (center) and males only (right) are shown; *p<0.05. **B:** Circulating levels of non-fasting glucose in total cohort (left), females only (center) and males only (right) are shown. **C:** Cytokines TNFα, IL-6, IL1β, KC/GRO and IFNγ were measured in serum obtained from KPC mice fed a CD, KD, CG or KG at euthanasia. Results are expressed as mean ± SD; *p<0.05.

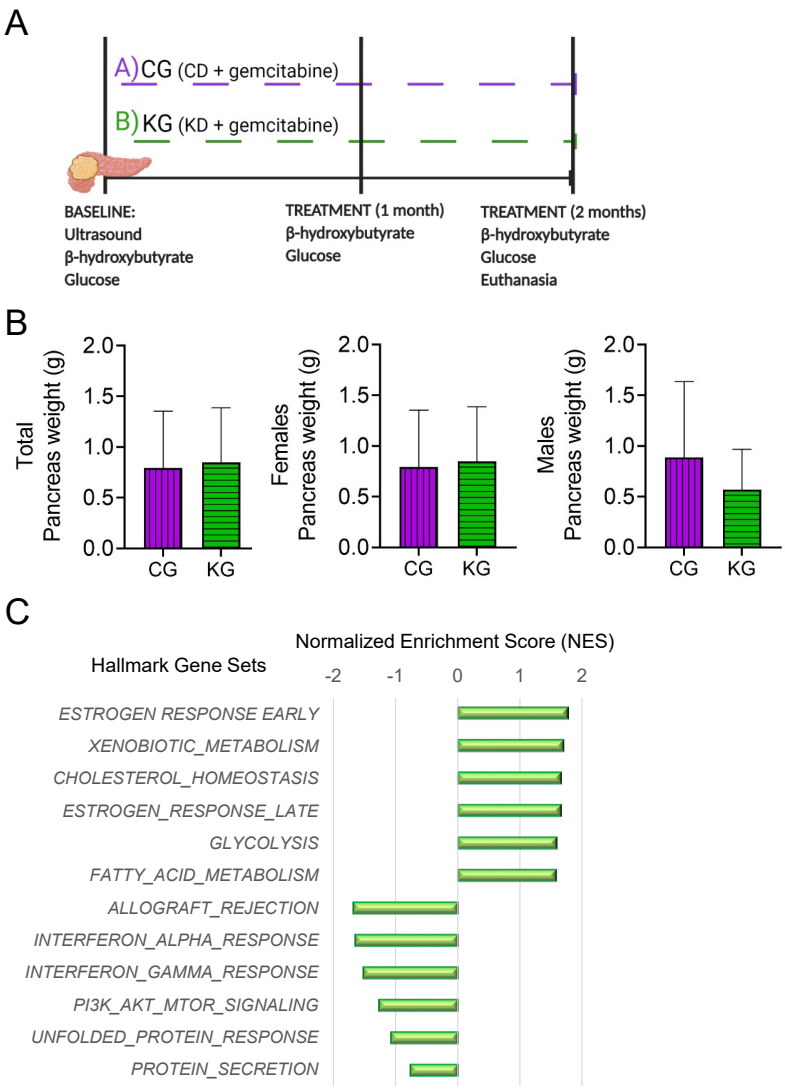

Figure 3. Hallmarks of pathways enriched following a ketogenic diet plus gemcitabine treatment. **A:** Schematic outline of the mechanistic study design. **B:** Pancreatic tumor weight in 28 CG and KG groups following 2 months of treatment. **C:** Top 6 hallmark gene sets identified as increased or decreased in pancreatic tumors isolated from KG-treated mice compared with pancreatic tumors isolated from CG-treated mice, using all differentially expressed genes (DEG; $p < 0.05$).

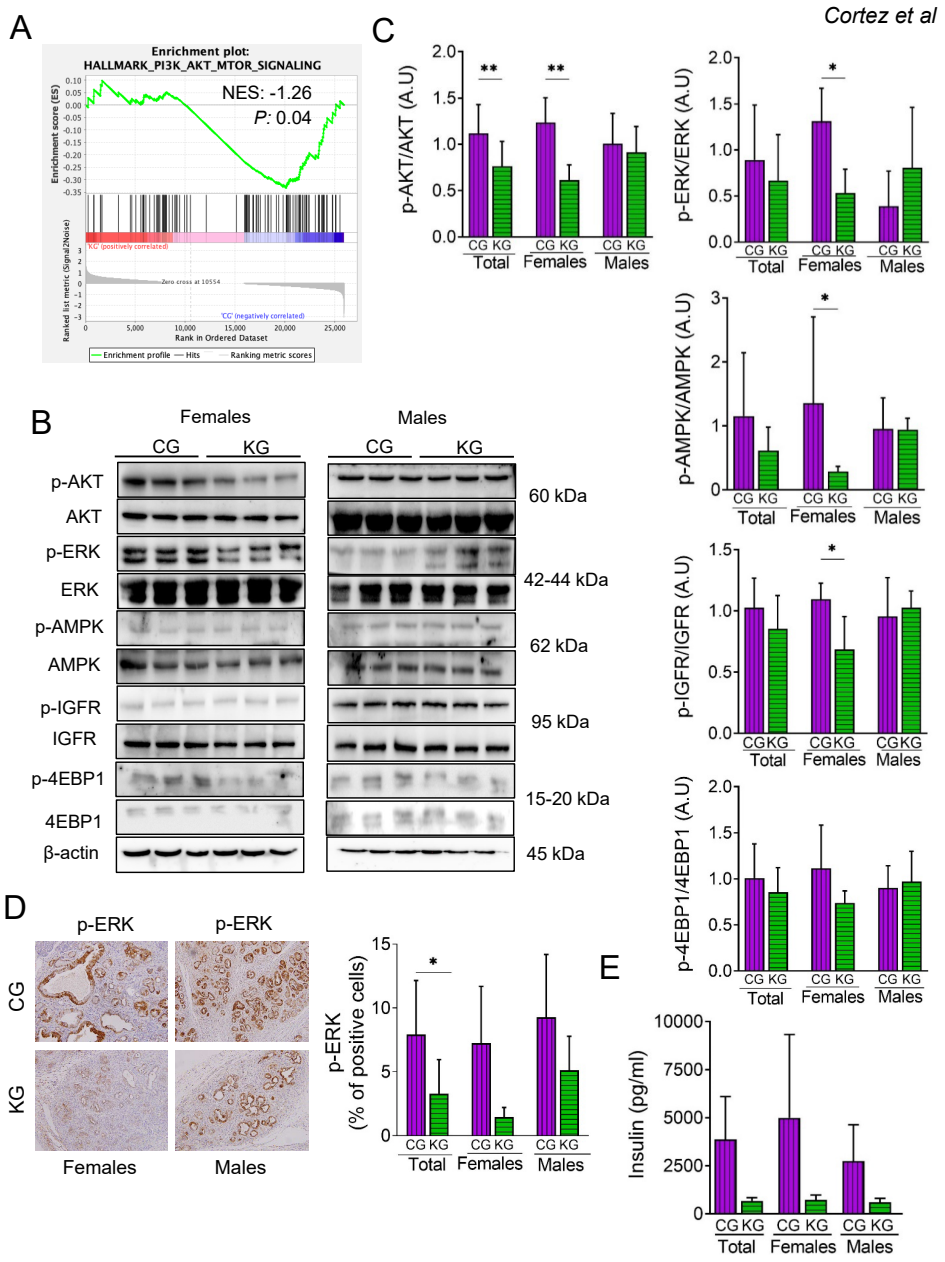

Figure 4. A ketogenic diet plus gemcitabine reduces AKT and ERK activation in KPC mice.
A: Gene set enrichment analysis (GSEA) was conducted on RNA-seq data obtained from pancreatic tumors of KG- and CG-treated KPC mice. The enrichment plot for the PI3K_AKT_MTOR gene set down-regulated by KG treatment (relative to CG) is depicted. Normalized enrichment score (NES) and nominal p value (p) were provided according to GSEA **B:** Immunoblots of p-AKT, AKT, p-ERK, ERK, p-AMPK, AMPK, p-IGFR, IGFR, p-4EBP1 and 4EBP1 in pancreatic tumor homogenates isolated from CG- and KG-treated KPC mice following 2 months of treatment. Loading control: β-actin. **C:** Bands were quantified and results are expressed as % control; *p<0.05. *p<0.01. **D:** Immunohistochemistry for p-ERK were performed on KPC tumor sections and photographs were taken at 20x magnification Representative images are shown. Results were expressed as percent of p-ERK+ cells ± SD per x20 field.*p<0.05. **E:** Insulin levels were measured in serum obtained from KPC mice fed a CG or KG for 2 months.

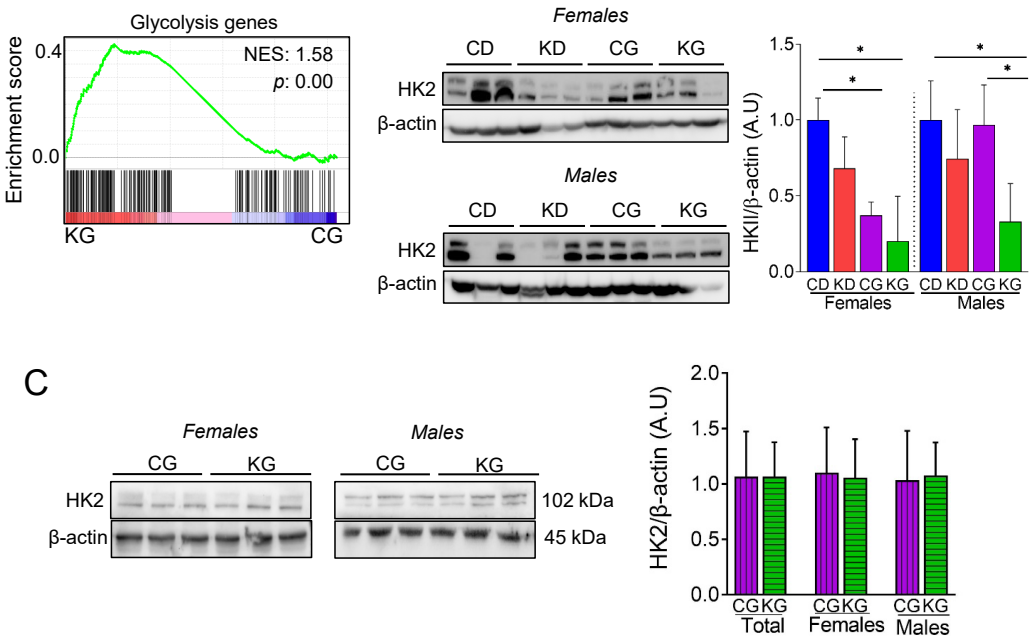

Figure 5. Effect of a ketogenic diet plus gemcitabine on glycolytic pathway in pancreatic tumors. **A:** Gene set enrichment analysis (GSEA) was conducted on RNA-seq data obtained from pancreatic tumors of KG- and CG-treated KPC mice. The enrichment plot for the Glycolysis gene set up-regulated by KG treatment (relative to CG) is depicted. Normalized enrichment score (NES) and nominal p value (p) were provided according to GSEA. **B:** Immunoblots of hexokinase 2 (HK2) in pancreatic tumor homogenates isolated from CD, KD, CG and KG treated KPC mice at endpoint. Loading control: β-actin. Bands were quantified and results are expressed as % control; *p<0.05. **C:** Immunoblots of HK2 in pancreatic tumor homogenates isolated from CG- and KG-treated KPC mice following 2 months of treatment. Loading control: βactin. Bands were quantified and results are expressed as % control.

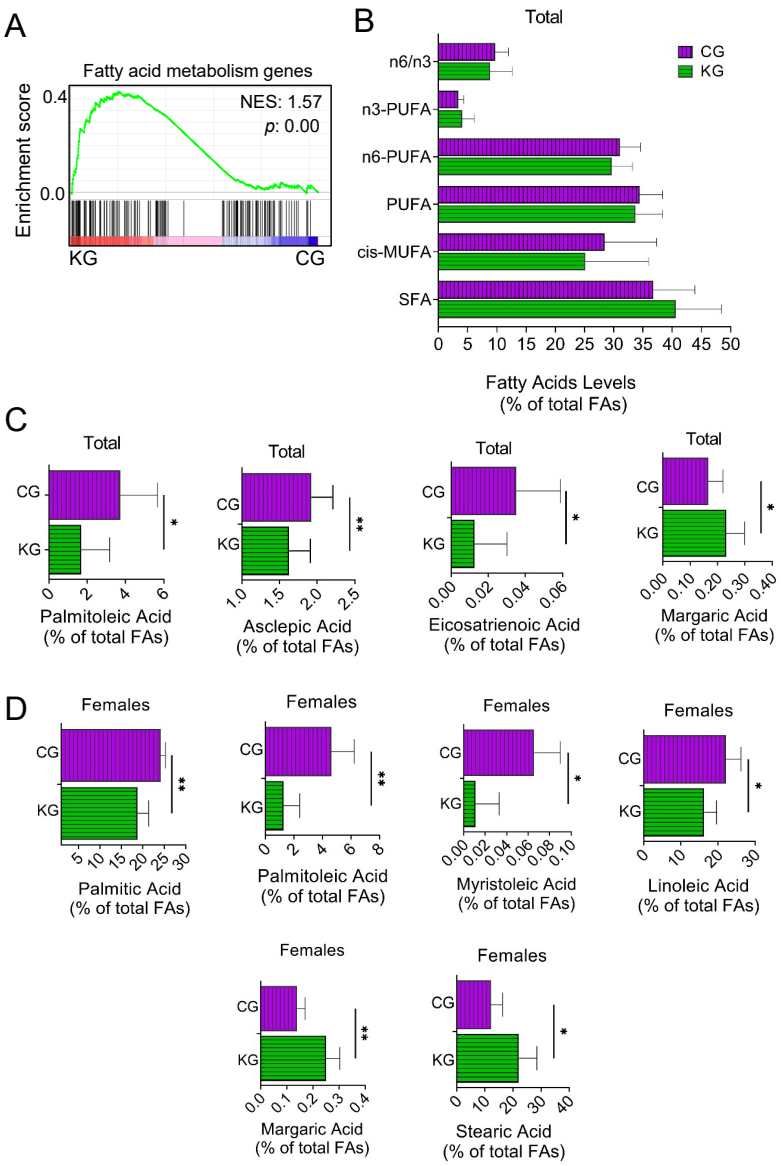

Figure 6. Effect of a ketogenic diet plus gemcitabine on lipid metabolism in pancreatic tumors. **A:** Gene set enrichment analysis (GSEA) was conducted on RNA-seq data obtained from pancreatic tumors of KG- and CG-treated KPC mice. The enrichment plot for the Lipid_Metabolism gene set up-regulated by KG treatment (relative to CG) is depicted. Normalized enrichment score (NES) and nominal p value (p) were provided according to GSEA. **B:** Levels of saturated fatty acids (SFA), MUFA monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), as well as the n-6/n-3 fatty acid ratio in pancreatic tumors isolated from CG- and KG-treated KPC mice following 2 months of treatment. **C:** Concentrations (% of total fatty acids) of selected fatty acids (asclepic acid, palmitoleic acid, margaric acid and eicosatrienoic acid) in pancreatic tumors homogenates isolated from CG- and KG-treated KPC mice (male and females combined) following 2 months of treatment. **D:** Concentrations (% of total fatty acids) of selected fatty acids (palmitic acid, margaric acid, myristoleic acid, palmitoleic acid, linoleic acid, and stearic acid) in pancreatic tumors homogenates isolated from CG- and KG-treated KPC female mice following 2 months of treatment. *p<0.05. **p<0.01.

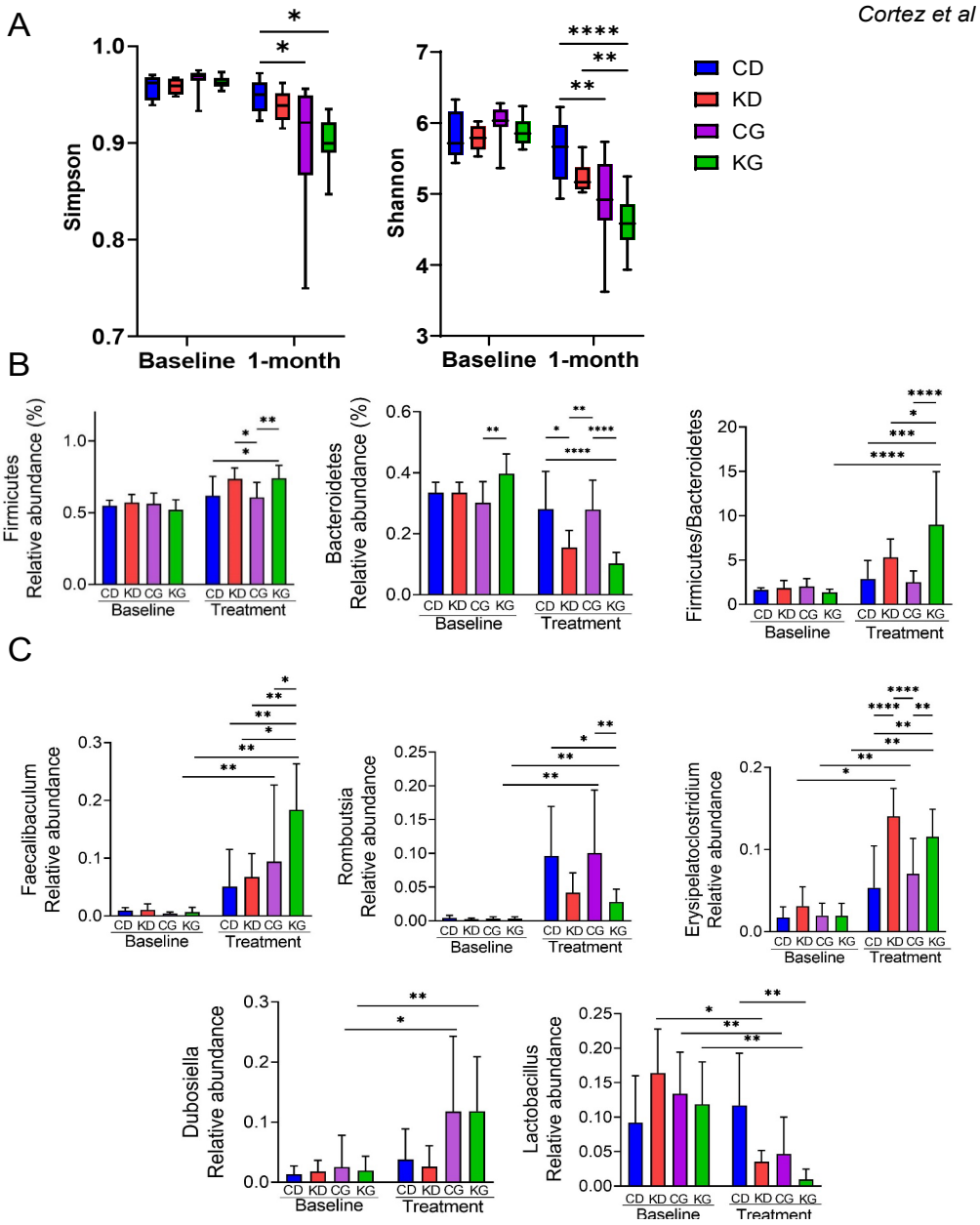

Figure 7. A ketogenic diet plus gemcitabine affects the gut microbiota.
A: Shannon and Simpson indexes were determined in control diet (CD), ketogenic diet (KD), control diet plus gemcitabine (CG) or ketogenic diet plus gemcitabine (KG) groups to evaluate the gut microbiota community diversity and richness among groups. *p<0.05. **p<0.01. **B:** Levels of Firmicutes, Bacteroidetes and the Ratio between Firmicutes and Bacteroidetes at baseline and after 1 month of treatment. *p<0.05. **p<0.01.**C:** Levels of *Faecalibaculum Romboutsia, Erysipelatoclostridium,* and *Dubosiella.* **D:** Distribution histogram of the LDA score determined by effect size (LEfSe). Bacterial taxa specifically enriched in groups with an LDA score > 3.6 are shown in the histogram. Comparisons were made between KG and KD, as well as KG to CG, to highlight the bacteria taxa differentially enriched in KG-treated mice

SUPPLEMENTAL FIGURES

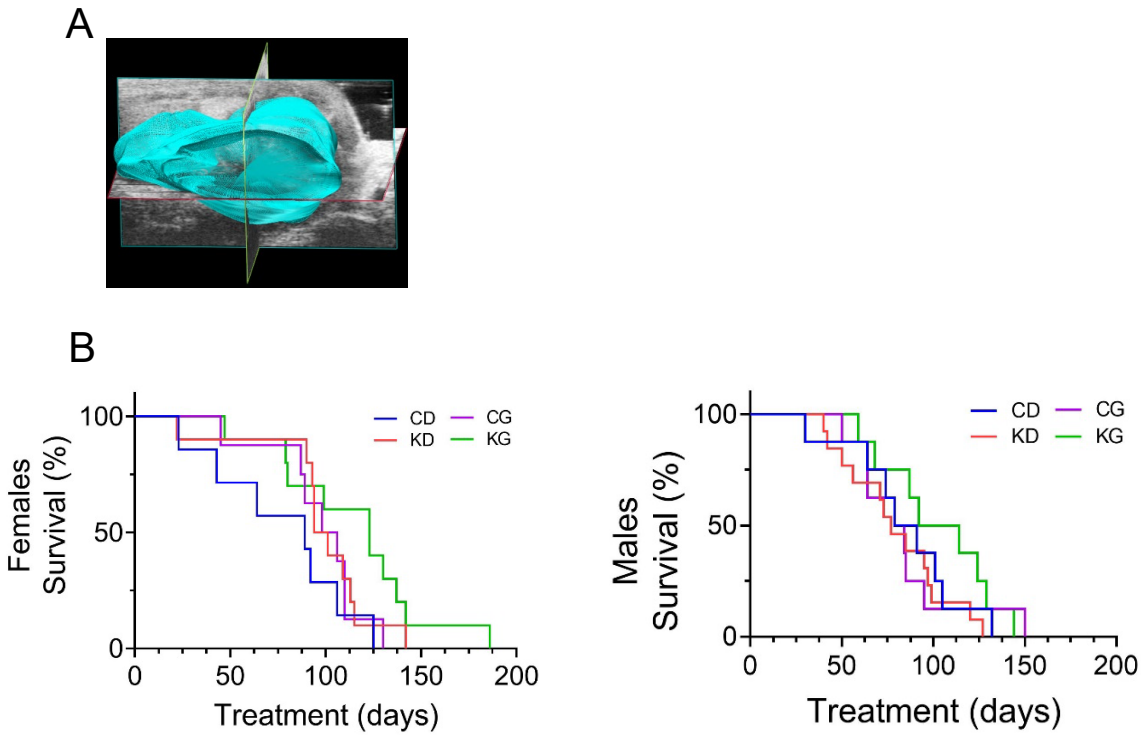

Figure S1. A ketogenic diet plus gemcitabine extends median overall survival in KPC mice. **A**: Representative ultrasound image of a pancreatic tumor in a KPC mouse at enrollment. **B:** Kaplan-Meier survival curves of male and female KPC mice fed a control diet (CD), ketogenic diet (KD), control diet plus gemcitabine (CG) or ketogenic diet plus gemcitabine (KG).

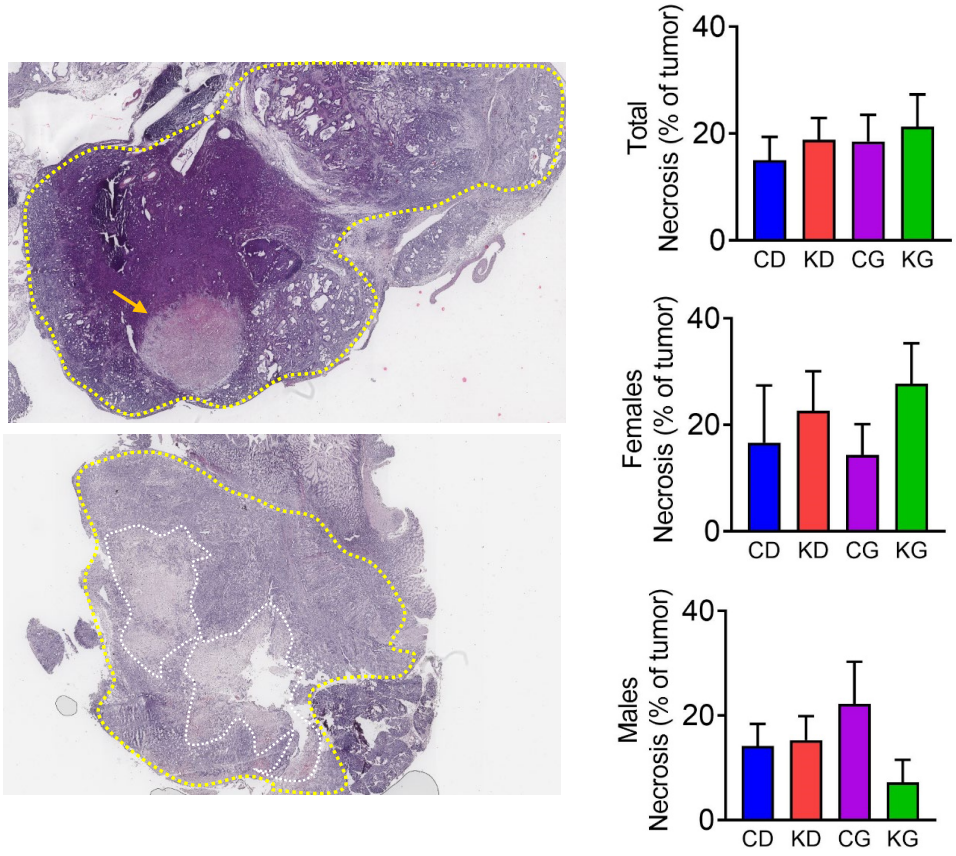

Figure S2: Histopathological analysis of necrosis in pancreatic tumor isolated from female and male KPC mice treated with CD, KD, CG or KD. In the ketogenic diet treated groups, more tumor necrosis was noted overall. Representative images: (top) Control diet, (Bottom) Ketogenic diet. Yellow dotted line delineates total tumor surface area. Orange arrow highlights a discrete focus of tumor necrosis. White dotted line delineates more irregular and more extensive tumor necrosis. Hematoxylin and eosin-stained sections. All images digitally scanned at 20X original magnification.

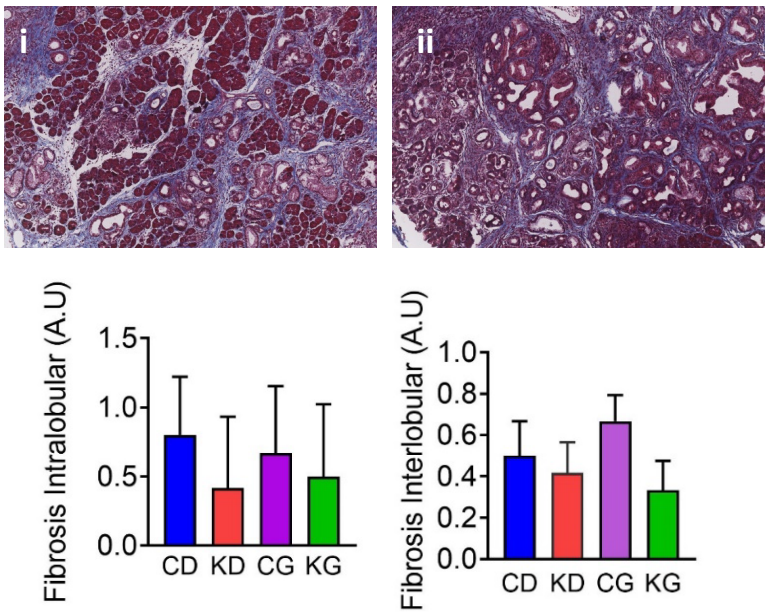

Figure S3: Histopathological analysis of fibrosis in pancreatic tumor isolated from female and male KPC mice treated with CD, KD, CG or KD. In all treatment groups, both intra- and interlobular fibrosis was observed in the background non-neoplastic pancreas. Representative images of Masson trichrome-stained sections: (i) Control diet, (ii) Ketogenic diet.. All images digitally scanned at 20X original magnification.

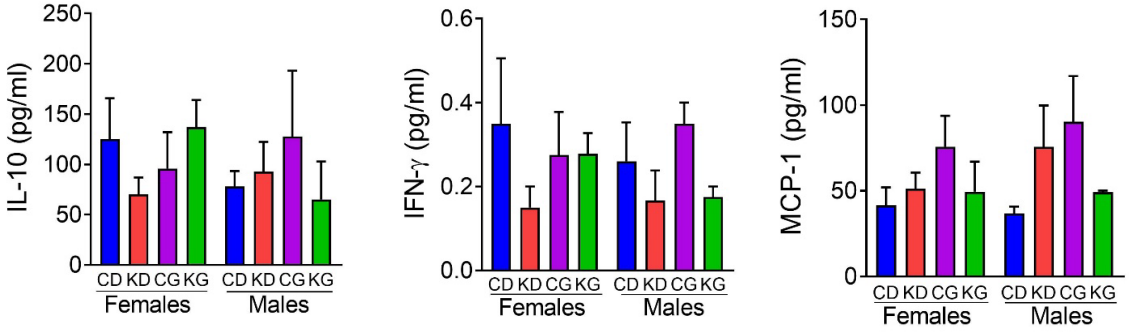

Figure S4: IL-10, IFN-γ and MCP-1 levels were measured in serum obtained from female and male KPC mice fed a CD, KD, CG or KG at euthanasia. Results are expressed as mean ± SD.

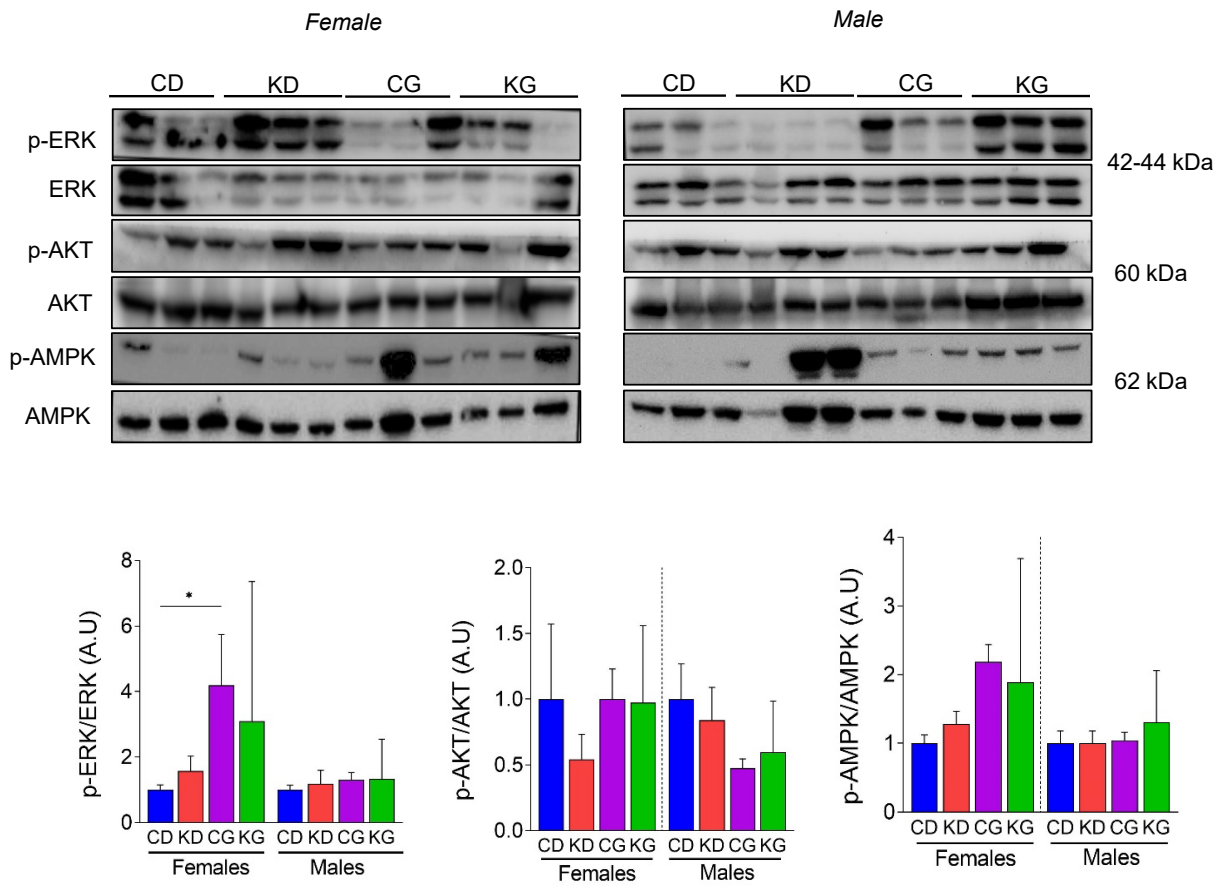

Figure S5. Effect of a ketogenic diet plus gemcitabine on AKT and ERK activation at endpoint. Immunoblots for p-AKT, AKT, p-ERK, ERK, p-AMPK and AMPK, in pancreatic tumor homogenates isolated from CD-, KD-, CG- and KG-treated female and male KPC mice when endpoint was reached. Bands were quantified and results are expressed as % control; *$p<0.05$.

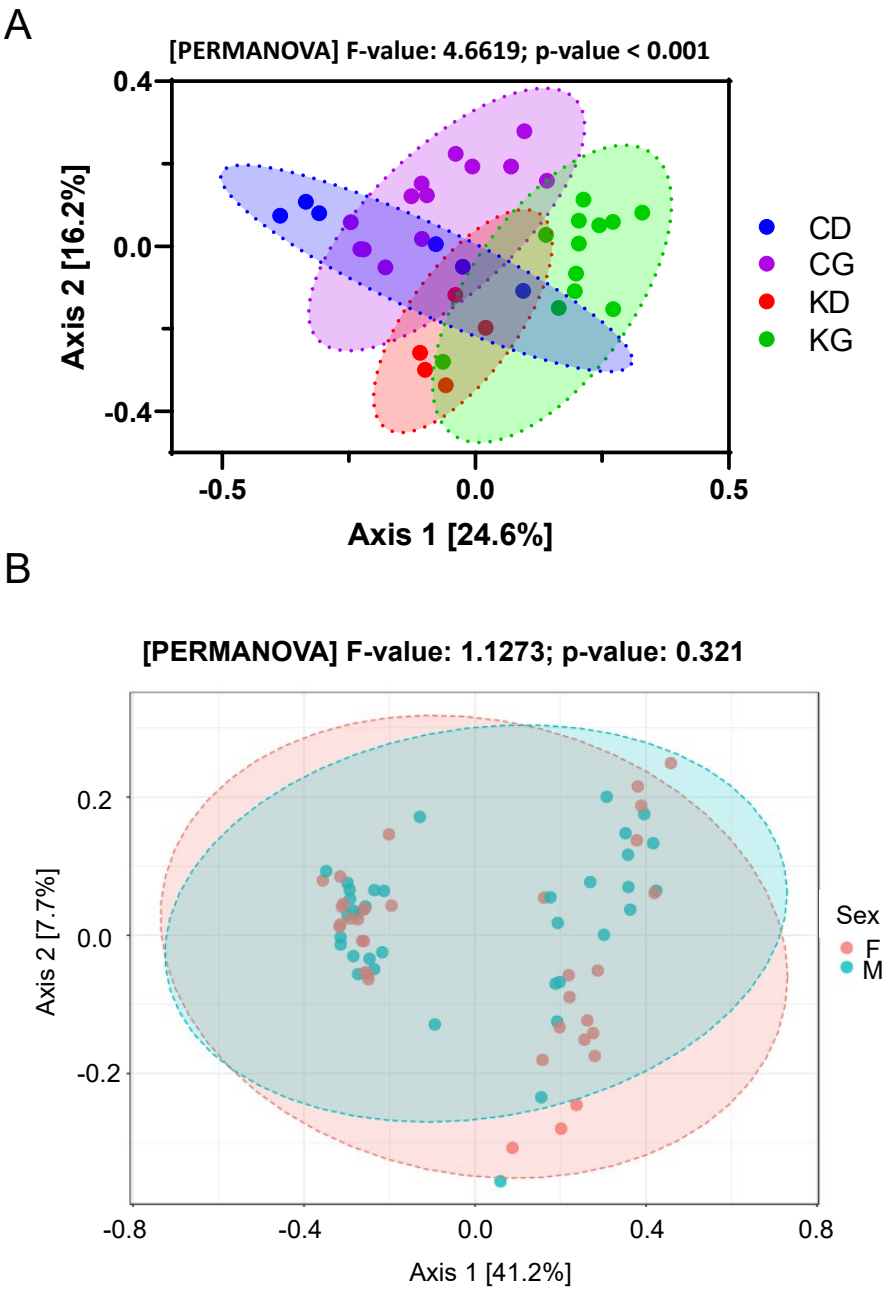

Figure S7: A ketogenic diet plus gemcitabine affects the gut microbiota. A-B: Principal component analysis (PCA). PC1 (axis 1) and PC2 (axis 2) represent the two most principal factors characterizing the bacterial profile among the 4 groups (**A**) or by sex (**B**) and their contribution rates (%) are shown on the axes.

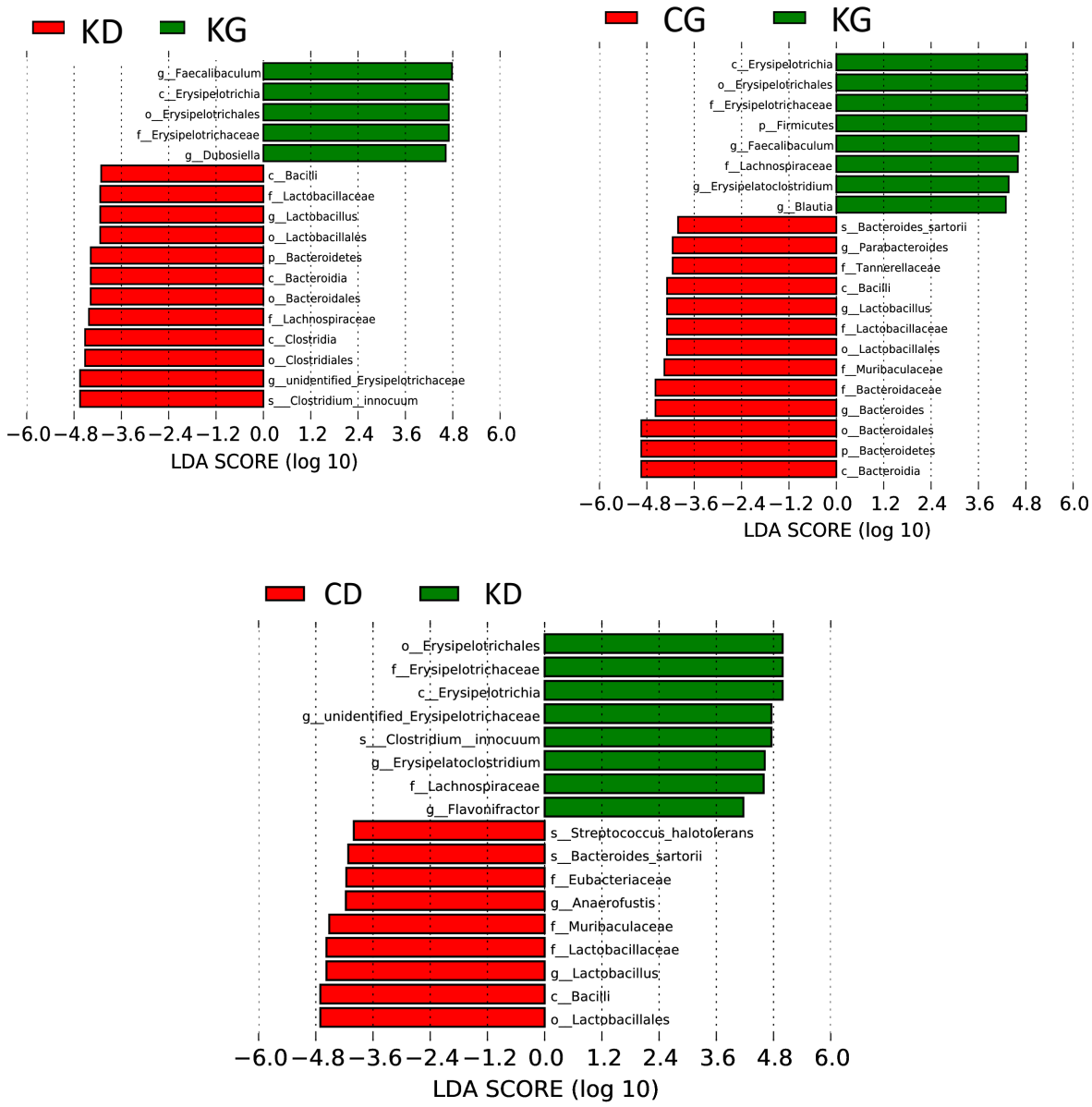

Figure S8: A ketogenic diet affects the gut microbiota. Distribution histogram of the LDA score determined by effect size (LEfSe). Bacterial taxa specifically enriched in groups with an LDA score > 3.6 are shown in the histograms. Comparisons after 1 month of diet/treatment were made between KG and KD (*upper left*), between KG to CG (*upper right*), and between KD and CD (*lower*) to highlight the bacteria taxa differentially enriched in KG-treated mice and in KD-treated mice.

TABLES

Table 1. Parameter estimates from the linear regression model for survival days since treatment to death.

Model Term	Estimate (SE)	P-value
Intercept	70.35 (8.83)	< 0.001
Effect of Keto vs. Control	13.83 (7.01)	0.052
Effect of gemcitabine vs. No gemcitabine	25.76 (7.95)	0.002
Effect of Age at the start of treatment (in days, centered at 90)	-0.25 (0.30)	0.406
Effect of Male vs. Female	-13.19 (7.07)	0.066

Note: Linear regression for survival days since treatment to death was adjusted by sex and age at the start of treatment. There is no censoring in data. Age at the start of treatment is centered at 90 days; thus, the intercept can be interpreted as the predicted survival days since treatment for a female animal taking conventional diet and no gemcitabine, with treatment starting at 90-day old. The estimate for age at the start of treatment can be interpreted as the average increase in outcomes for 1-day increase in age at the start of treatment. Interaction between diet and gemcitabine was excluded from models due to non-significance. The 2-way and 3-way interactions between sex and treatments (diet, gemcitabine) are non-significant.

SUPPLEMENTAL TABLES

Supplemental Table 1. Experimental diets composition

Ingredient (g/kg diet)	Control	Ketogenic
Carbohydrates, of which	685	27.5
Sucrose	100	---
Maltodextrin	130	---
Corn starch	400	7.5
Vitamin Mix	20	20
Mineral Mix	35	---
Protein, of which	162.6	270.6
Casein	162	268
DL-methionine	2.6	2.6
Fat, of which	87.97	571.6
Lard	---	480
Soybean oil	87	90
**Fat in casein*	0.97	1.6
Cellulose	64	---
Tert-butylhydroquinone	0.014	0.130
Mineral Mix	---	20
Calcium phosphate dibasic	---	19
Calcium carbonate	---	8

Chapter 3. A ketogenic diet in combination with gemcitabine mitigates pancreatic cancer associated cachexia in male and female KPC mice

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Running title: Beneficial effect of a ketogenic diet in PDAC-associated cachexia

ABSTRACT

A critical contributor to pancreatic ductal adenocarcinoma (PDAC) mortality is cancer associated cachexia (CAC), a multifactorial disorder characterized by the involuntary and ongoing wasting of skeletal muscle with or without loss of adipose tissue. Unfortunately, to date, there are no effective strategies recognized to reverse or manage CA. Thus, there is an urgent need for new therapeutics for PDAC and PDAC-associated cachexia; and the exploration of dietary interventions is a critical component. In this study, we evaluated whether feeding a strict KD alone or in combination with gemcitabine in the autochthonous LSL-KrasG12D/+; LSL-Trp53 R172H/+; Pdx1-Cre (KPC) mouse model mitigates CAC and potential mechanisms involved. For this purpose, both male and female pancreatic tumor-bearing KPC mice were allocated to a control diet (CD; %kcal: 70% carb, 14% protein, 16% fat), a KD (%kcal: 14% protein, 1% carb, 85% fat), a CD + gemcitabine (CG), or a KD + gemcitabine (KG) group. Furthermore, we evaluated the role of sex on cachexia outcomes. In our survival study, we observed that a KD concomitant with gemcitabine resulted in the mitigation of muscle strength decline over time. Moreover, female KPC mice fed a KD showed higher muscle weights compared CD fed ones. We observed that anorexia, assessed by caloric intake, was diminished in the KG groups when compared to CG and lessened in KG treated female mice. We showed sex-specific differential effects of KG treatment, like inhibition of autophagy in KPC female mice, plus higher total acetyl-lysine levels and phosphorylation of eIF2α in the KG group when compared to CG, particularly in females. Our data suggests that a KD results in preservation of skeletal muscle mass, and that such response to a KD differs between females and males.

Keywords: cachexia, pancreatic cancer, ketogenic diet

INTRODUCTION

Cancer-associated cachexia (CAC) is a complex metabolic disorder characterized by the unintentional and ongoing wasting of skeletal muscle (SKM), with or without loss of adipose tissue [1, 2]. Patients with pancreatic ductal adenocarcinoma (PDAC) have among the highest incidence of CAC, estimated at approximately 80% [3, 4], and with around one third of mortality directly associated to it [5]. Paradoxically, the bidirectional interaction of cachexia with chemotherapy further complicates the scenario. While CAC is a critical contributor to chemotherapy toxicity, chemotherapeutic agents can in turn aggravate cachexia [6, 7]. Hence, the loss of SKM mass, while receiving PDAC-treatment is considered a prognostic factor for worse survival [8]. Unfortunately, to date, there are no approved therapeutics recognized to effectively reverse or manage CAC [2]. Therefore, therapies that support the preservation of muscle mass during PDAC-associated cachexia are warranted. Evidence on the effects of nutritional support is limited [9], but beneficial effects of enteral nutrition support on weight and lean body mass stability in pancreatic cancer patients with cachexia have shown promise [10]. Furthermore, ketogenic diets (KDs), which are high fat/low carbohydrate dietary regimens aimed to produce ketosis, have been shown to exert a protective effect against muscle mass loss [11]. In aging mice, KDs exhibited muscle function preservation and sarcopenia mitigation [12]. The metabolic alterations induced by KDs, particularly the production of ketone bodies, are associated with less degradation of SKM's proteins, plus decreased secretion of pro-inflammatory cytokines and metabolites involved in the pathogenesis of CAC [13, 14]. However, the impact of a KD in PDAC-associated cachexia is limited, with only one study, showing that muscle wasting in an PDAC orthotopic model was improved with a KD [15].

In this study, we evaluated the impact of feeding a strict KD alone or in combination with gemcitabine in the autochthonous LSL-Kras$^{G12D/+,}$ LSL-Trp53$^{R172H/+,}$ Pdx1-Cre (KPC)

genetically engineered mouse model of pancreatic cancer. KPC mice develop pancreatic tumors that recapitulate many of the histopathological, genomic and clinical features of human PDAC. These mice also develop progressive cachexia which makes them an appropriate model to evaluate strategies to mitigate this complication of PDAC [16]. Although sex-specific mechanisms appear to be modulators of PDAC-associated cachexia, the limited research in this area further restricts the development of effective treatments [17]. Therefore, we also evaluated the impact of sex in response to a KD adjuvant to chemotherapy during PDAC-associated cachexia in both male and female KPC mice.

We showed that KD in combination with gemcitabine preserves muscle strength in KPC mice by decreasing autophagy and increasing cellular response and acetylation, with enhanced beneficial effects in female KPC mice.

MATERIALS and METHODS

Animal Studies: All animal use procedures were approved by the University of California, Davis Animal Care and Use Committee.

Genetically Engineered Transgenic Mice: The genetically engineered LSL-$Kras^{LSL\text{-}G12D/+}$$Trp53^{R172H/+}$Pdx-1-Cre (KPC) mice model of PDAC was bred at the UC Davis Animal Facility in Meyer Hall. The KPC mice were generated from three mouse parental strains (LSL-$Kras^{G12D/+}$; LSL-$Trp53^{R172H/+;}$ and Pdx-1-Cre) obtained from National Cancer Institute (NCI) mouse repository and following established procedures described by Hingorani and colleagues [18]. After weaning, rodents were individually housed in polycarbonate cages in a room with controlled temperature (22-24°C) and humidity (40-60%), maintained on a 12 hr light-dark cycle, and fed a chow diet ad libitum (LabDiet 5001, LabDiet, Saint Louis, MO) until enrolled in the studies.

Survival Study: Enrollment of KPC mice was based on tumor size, measured by ultrasound when KPC mice were around 3-4 months old. Briefly, pancreatic tumors were detected

using a high-resolution ultrasound imaging of the pancreas with the Vevo 2100 System with a 35MHz RMV scan-head (Visual Sonics, Inc.). Mice were fasted for two hours and anesthetized with isoflurane. Imaging was obtained and tumor volumes measured following previously published guides [19, 20]. Once tumor size was assessed, male and female KPC mice were assigned randomly to one of the following groups: a control diet (CD), ketogenic diet (KD), a control diet plus gemcitabine (CG) or a ketogenic diet plus gemcitabine (KG).

Throughout the survival study, mice were observed daily for signs of significant weight loss; hemorrhagic ascites; and for other indications of advanced disease including loss of thermoregulation, inactivity, and presence of malignant ascites. Endpoint criteria included the development of abdominal ascites, weight loss exceeding 20% of the initial weight, or extreme weakness or inactivity. When an animal reached the endpoint criteria, it was euthanized by carbon dioxide asphyxiation, blood was collected and tissues, including pancreatic tumors were dissected, weighed, and then stored in liquid nitrogen, RNA later and 10% buffered formalin.

Dietary Interventions. Following tumor size determination, male and female KPC mice were allocated to either a control diet (CD; %kcal: 70% carb, 14% protein, 16% fat), a KD (%kcal: 14% protein, <1% carb, 85% fat), a CD + chemotherapeutic agent gemcitabine, or a KD + gemcitabine group. Mice were fed ad libitum at all times, food was changed and food intake was recorded three times per week. Diets were adapted from the study by Roberts et al [12]. The Envigo (Indianapolis, IN) mineral mix TD94046 was used for the control diets and the TD79055 was used for the ketogenic diets due to their lower carbohydrate contents. For both diets, TD40060 (vitamin mix) was used. The composition of the experimental diets is shown in Table 1.

Chemotherapy Treatment. Gemcitabine (>99% 2'-Deoxy-2',2'-difluorocytidine; dFdC; Gemzar; LY-188011) from Fisher Scientific (Hampton, NH) was administered to the CG and KG groups at 100 mg/kg by i.p, injection two times per week for 3.5 weeks (7 total injections).

Forelimb Grip Strength Test. A forelimb grip strength dynamometer with electronic sensor (Columbus Instruments, Columbus, OH) was used. Mice were positioned to grab the metal grid with forelimbs only and then pulled horizontally by the tail by the test administrator with even force until the animal let go of the bar. Two rounds of three trials each were performed with minimal rest between trials (1min) and 30 min. rest between rounds. The grip strength average of the six total trials was used for analysis. Force was measured in kg. Tests were performed at baseline and then monthly thereafter.

Blood Glucose and Ketones. Non-fasting glucose levels were measured using a glucometer (Easy Plus II, Home Aid Diagnostics Inc, Deerfield Beach, FL), and ketone levels were measured using the Precision Xtra glucose and ketone monitoring system (Abbott) according to the manufacturer's instructions. Blood was obtained by tail-tip cut.

Body Weight and Composition. Body weight was measured weekly. Body composition was assessed using NMR relaxometry (EchoMRI-100H, EchoMRI LLC, Houston, TX) at baseline and then monthly thereafter.

Mechanistic Study: A cohort of mice was allocated to either the CG or the KG groups after tumor detection and euthanized at two months post-interventions. At the end of the 2 months, gastrocnemius was dissected, weighed, sectioned and then stored in liquid nitrogen, RNA later or 10% buffered formalin. Of note, we collected right GTN in order to evaluate potential mechanisms involved in muscle strength preservation.

Metabolic Measurements: Blood samples were collected via cardiac puncture and serum was isolated after centrifugation at 3,000 x g for 10 min at room temperature. Insulin and leptin were assayed using the V-PLEX mouse metabolic kit and mouse leptin kit. Inflammation-related biomarkers were assayed using the V-PLEX Proinflammatory panel I kit (Meso Scale Discovery).

Histology: After necropsy, gastrocnemius was fixed in 10% buffered formalin overnight at 4°C. Tissues were processed and embedded by routine methods. Tissues sections (5 μm) were stained with hematoxylin and eosin, or Masson's Trichrome (Chromaview, Thermo Scientific), and analyzed for blinded histological examination.

Tissue homogenization and western blotting. Gastrocnemius tissue was powdered on liquid nitrogen using a hammer and pestle. Two scoops of powder were then aliquoted into 1.5 mL Eppendorf tubes and homogenized in 250 μL of sucrose lysis buffer [1M Tris, pH 7.5, 1M sucrose, 1mM EDTA, 1mM EGTA, 1% Triton X-100, and 1X protease inhibitor complex]. The solution was set on a shaker for 60 min at 4°C, spun at 8,000g for 10 min, supernatants were transferred to new Eppendorf tubes and protein concentrations were determined using the DC protein assay (Bio-Rad, Hercules, CA). Equal aliquots of 500 μg of protein were diluted in 4X Laemmli sample buffer (LSB) (final volume 200uL) and boiled for 5 min at 100°C. 10μL of protein sample was loaded onto a Criterion TGX Stain-Free Precast Gel and run for 45 min at a constant voltage of 200V. Proteins were then transferred to an Immobilon-P PVDF membrane, after it was activated in methanol and normalized in transfer buffer, at a constant voltage of 100V for 30 min. Membranes were blocked in 1% Fish Skin Gelatin (FSG) in TBST (Tris-buffered saline w/ 0.1% Tween) and incubated overnight at 4°C with the appropriate primary antibody diluted in TBST at 1:1,000. The next day, membranes were washed with TBST for 5 minutes, and successively incubated at room temperature with peroxidase-conjugated secondary antibodies in a 0.5% Nonfat Milk TBST solution at 1:10,000. Bound antibodies were detected using a chemiluminescence HRP substrate detection solution (Millipore, Watford, UK). Imaging and band quantification were determined using BioRad Image Lab Software

26S proteasome activity assay. Briefly, pulverized gastrocnemius muscle samples (20 mg aliquot) were homogenized in 26S proteasome lysis buffer (50 mM Tris, 150 mM sodium chloride (NaCl), 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM magnesium chloride

(MgCl2), 1 mM DTT (freshly added) [pH 7.5]) with a hand-held Potter-Elvehjem homogenizer on ice. The supernatant containing the proteasomes were separated after centrifugation at 12,000 xg for 15 min at 4 °C and were quantified and diluted to 1 µg/µL concentration with proteasome lysis buffer. The β5 (chymotrypsin-like), β2 (trypsin-like) and β1 (caspase-like) activities were assayed using 20 µg of protein. The assays were carried out in a total volume of 100 µL per well in a black 96-well plate. The adenosine triphosphate (ATP)-dependent 26 S assays were performed using 100 µM ATP in the absence and presence of a specific proteasome inhibitor: 10 µM bortezomib (β5 activity) and 100 µM bortezomib (β1 and β2 activities). The assays were initiated by the addition of specific fluorescence-tagged substrates for each proteasomal β subunits (Enzo Life Sciences, NY, USA): Z-LLE-AMC for β1, Boc-LSTR-AMC for β2 and Suc-LLVY-AMC for β5 [21-24]. The final concentration of substrate in each assay was 100 µM. Proteasomal activities were measured every 15 min up to 120 min at an excitation wavelength of 390 nm and an emission wavelength of 460 nm using an Infinite M1000 PRO fluorometer.

Statistical Analysis. The data, obtained from at least three independent experiments, were expressed as the mean ± SEM. Statistical evaluation was performed by t-test or one-factor analysis of variance (ANOVA) followed by the Tukey test adjusted for multiple comparisons. Analyses were performed by GraphPad (Prism version 9.2) and R version 4.0.4. Two-sided $p<0.05$ was regarded as statistically significant.

RESULTS

Effect of a KD alone and in combination with gemcitabine on food intake, body weight and composition in KPC mice

We conducted a survival study in the autochthonous KPC mouse model to evaluate the effect of feeding a strict KD alone or in combination with gemcitabine as a treatment protocol. For this purpose, once tumors were detected, male and female KPC bearing-tumor mice were

randomized into control diet (CD), ketogenic diet (KD), CD + gemcitabine (CG), or KD + gemcitabine (KG) groups (16-23 mice/group) until they reached their endpoint (Fig.1A).

To determine whether feeding a KD increases blood ketone levels, we initially assessed blood β-hydroxybutyrate levels in KPC mice. As expected, after one month on the diet, mice fed a KD, alone or in combination with gemcitabine had increased blood β-hydroxybutyrate (β-HB) levels compared to mice fed a CD or a CG (Fig. 1B). This increase was observed in both female and male mice fed a KD or KG (Fig. S1).

Throughout the study, we measured food intake three times per week. Although weekly mean intake in grams (g/wk) was significantly higher for CD (26.4 $\pm$ 1.54) and CG (24.1 $\pm$ 0.92) than for KD (18.9 $\pm$ 1.5) and KG (17.3 $\pm$ 0.5), the weekly caloric intake (Kcal/wk) was higher for KD (114.8 $\pm$ 4.5) and KG (118.2 $\pm$ 5.9) than for CD (100.5 $\pm$ 5.9) and CG (95.2 $\pm$ 5.2) (Fig. 1C). Moreover, the caloric intake was significantly different for CG group when compared to KD or KG ($p<0.05$). When data was disaggregated by sex, such differences were observed in females but not in males (Fig. S2).

To investigate the effects of a CD and a KD ± gemcitabine on physiologic changes, we measured the total body weight (BW) and the body composition of mice at baseline and on a monthly basis. One month after intervention, treatment with gemcitabine led to a reduction in BW (Fig. 1D). Mice in the CG and KG groups had lower weights one-month post-treatment compared to those in the CD and KD groups but recovered by two months with no BW differences among the four groups. When data was disaggregated by sex, after 1 month of treatment, a significantly higher BW was observed in female KD mice compared to CD and both gemcitabine-treated groups. In males, KG was lower than CD one-month after treatment, but not after two months. The BW of KD-fed mice was significantly higher than CG and KG at two months only in males.

Regarding body composition, a significant decrease in lean mass was observed in the CG and KG groups compared to both non-gemcitabine groups only at one month (Fig.1E). When

analyzed by sex, such effect persisted in females, but in males a significant decrease was observed only in the CG group and not in the KG group when compared to non-gemcitabine groups. In contrast, a significant increase in fat mass was observed in the KD group when compared to all other groups, after two months of treatment (Fig. 1F). In females, the fat mass of KD and KG was higher than CD after two months of treatment. In males, fat mass in KD was significantly higher than both gemcitabine groups at two months.

A KD alone or combined with gemcitabine, preserves muscle strength in KPC mice

We next assessed the impact of a KD ± gemcitabine on muscle strength in male and female KPC mice throughout a survival study, using the forelimb grip-strength test. KPC mice fed a CD, either alone or in combination with gemcitabine, showed muscle strength weakening after two months of treatment, compared to baseline levels (Fig. 2C). In contrast, KPC mice fed a KD, either alone or combined with gemcitabine, significantly maintained muscle strength after 2 months, compared with mice fed a CD. When analyzed by sex, grip strength force was significantly higher in the CD males than in KD at baseline, yet after two months on the diet the opposite was observed, with KD fed mice having higher muscle strength force. In females, both KD and KG had significantly higher grip strength force than CD-fed groups. Moreover, the mass of the gastrocnemius (GTN) at survival endpoint was higher in KPC mice treated with KD or KG and CG when compared to CD-fed mice (Fig. 2B). When separated by sex, such effect was only observed in females.

We then conducted linear regression models for grip strength adjusted by sex and age at the start of treatment and observed a significant effect of sex on grip strength performance ($p< 0.001$). To investigate whether a KD ± gemcitabine's effect increases over time, we conducted linear mixed-effects regression models. Both ketone bodies and grip strength determinations were fitted for repeatedly measured outcomes (measured at baseline, and 1 and 2 months after the start of the intervention), which included fixed effects for diet, drug, time, and their 2-way and 3-

way interactions. As shown in Table 2, a significant diet-time interaction is detected, indicating that the diet effect increases over time. However, the estimated effect of gemcitabine is the same over time and across diet groups.

A ketogenic diet in combination with gemcitabine induces metabolic changes in KPC mice

In the survival study, KPC mice fed a KD alone or combined with gemcitabine significantly maintained muscle strength at 2 months of dietary intervention compared with mice fed a CD, plus a significantly higher GTN mass was observed in mice fed a KD. Thus, to elucidate potential mechanisms underlying the beneficial effects of a KD on skeletal muscle when combined with gemcitabine, we conducted a mechanistic study in which three-month-old male and female KPC mice bearing pancreatic tumors were fed and treated with CG or a KG for 2 months (Fig. 3A).

To ensure the mice in the mechanistic study were in nutritional ketosis, we measured β-hydroxybutyrate levels (Fig. 3B). KG-treated mice had increased blood β-HB levels compared to CG mice. In contrast, no significant differences in non-fasted glucose levels were observed between CG and KG groups (Fig. 3C). After 2 months of treatment, no differences were observed in BW nor lean mass of KG and CG mice at baseline, one month and two months (Fig. 3D-E). In the CG group, fat mass was higher at one month, with no differences at two months among groups. In addition, the GTN weight was similar between the CG and KG groups (Fig. 3G). Moreover, heart weight was obtained as a marker of cachexia and no significant differences were observed between CG and KG-treated mice (Fig.3H).

Given the association of leptin with regulating appetite and weight, we measured leptin levels in serum from KG- and CG-treated mice and compared leptin levels (pg/ml) in KG-females (1693 ± 354) to KG-males (3216 ±1312) ($p=0.24$) (Fig.3I). No significant differences were observed.

A ketogenic diet in combination with gemcitabine induces autophagy and enhances protein acetylation in the gastrocnemius of KPC mice

To assess the effect of a KD on autophagy-related markers involved in protein breakdown, we measured LC3B-II and LC3B-I levels in the GTN of CG- and KG-KPC mice treated for 2 months. A significant reduction in the LC3B-II to LC3B-I ratio was observed in female KG-treated mice when compared to KG-males, suggesting a sex-specific inhibition of autophagy (Fig. 4A). Moreover, to determine the effect of diet and sex on cellular stress response, we measured the level of a key endoplasmic reticulum (ER) protein. The phosphorylation of eIF2α (p-eIF2α) in GTN was significantly increased in the KG group when compared to CG, particularly in females.

Because β-HB can inhibit histone deacetylases (HDACs) [25, 26], we examined the effect of a KD on muscle protein stability by measuring total acetyl-lysine in GTN. After two months of treatment, a trend for higher total acetyl-lysine levels was observed only in female KG-treated mice, compared to female CG mice (p=0.057) (Fig. 4B). Of note, no significant differences in the protein levels of histone acetyltransferase p300 were observed between the groups (Fig. S4).

We then evaluated the effect of KG on protein degradation through the ubiquitin–proteasome system (UPS), by measuring the activities of 3 catalytic active beta subunits of the core unit of the proteasome involved in degradation of oxidized proteins: β1 (caspase-like), β2 (trypsin-like) and β5 (chymotrypsin-like). In male mice, treatment with KG led to an increase in the activity of β1 subunit and a decrease in β2 subunit activity when compared to CG group, whereas no differences in β5 activity was observed between the groups (Fig. 4C).

As a marker of muscle membrane stability, we determined whether KG affected dystrophin levels. Although not significant, a trend to higher dystrophin levels in GTN was observed in females in the KG group when compared to males (p=0.18) and to CG-treated mice (Fig. 4D) (p=0.40). Moreover, to determine the effect of KD and sex on muscle atrophy, we assessed the levels of muscle atrophy-related gene (atrogin-1) and Muscle RING finger 1 (MuRF1). No changes were detected in atrogin-1 and MuRF1 levels between KG and CG groups nor by sex (Fig. S4).

Effect of a ketogenic diet in combination with gemcitabine on skeletal muscle inflammatory markers and oxidative metabolism

To further elucidate potential mechanisms underlying the beneficial effects of a KD on muscle preservation, we examined key signaling proteins known to regulate muscle mass during cachexia, including mTOR NF-κB, AKT and p38 [14, 27-31]. No significant differences were observed in the GTN levels of p-4EBP1, p-p65, p-AKT or p-p38 between the KG and CG groups, and between males and females (Fig. 5A).

To examine the effect of a KG and sex on oxidative metabolism, we investigated the level of enzymes involved in oxidative phosphorylation. No significant differences were observed in the level of several proteins within the electron transfer chain (oxidative phosphorylation; OXPHOS) nor on the antioxidant protein SOD2 (Fig. 5B-C). In addition, no differences in the expression of the ketone body metabolic enzymes succinyl CoA: 3-oxoacid CoA transferase (OXCT1), 3-hydroxybutyrate dehydrogenase 1 (BDH1), and acetyl-CoA acetyltransferase 1 (ACAT1) were observed between groups (Fig. S6).

A ketogenic diet in combination with gemcitabine does not affect serum cytokine levels in KPC mice.

Finally, given that cytokines may drive the cachexia process [32], we assessed the levels of several cytokines involved in inflammation in serum of male and female KPC mice treated with KG or CG for two months. As shown in Figure 6, no significant changes were observed in serum IL-6, TNF-α, IL-1β, IFN-γ, IL-10, KC/GRO nor MCP-1 levels between the groups. No differences were observed when stratifying by sex either (Fig. 6A).

DISCUSSION

Cancer-associated cachexia is a major complication for PDAC patients, as it impairs treatment tolerance, diminishes quality of life and reduces overall survival [33]. Thus, there is an active search for interventions that may reduce PDAC-associated cachexia. In this study, we evaluated whether a KD, with or without gemcitabine, mitigates CAC in tumor-bearing KPC mice, which replicates the progressive detriment of muscle strength characteristic of PDAC-associated cachexia [34]. Furthermore, we evaluated the role of sex on cachexia outcomes.

Previous studies indicate that a KD could be beneficial for preserving muscle strength [12]. For example, in aged mice, Wallace et al. observed that a KD resulted in preservation of SKM and increased markers of mitochondrial biogenesis, oxidative metabolism, and oxidative stress response, while decreasing protein synthesis and proteasome activity [35]. In an orthotopic xenograft model of pancreatic cancer, a KD fed for three weeks to female nude mice led to significantly diminished cachexia [15]. Although such findings were promising, no studies had explored the effects of a long-term KD in clinically relevant models of PDAC-associated cachexia in combination with chemotherapy. In our survival study, we observed that a KD concomitant with gemcitabine resulted in the mitigation of muscle strength decline over time. Consistent with our findings, a KD previously demonstrated increased muscle function and weight preservation in old mice [35] . In a model of colon cancer, muscle weight was also maintained when mice were fed a ketogenic formula [36]. Similar to those observations, female KPC mice fed a KD showed higher muscle weights compared CD fed ones. Nevertheless, the KD did not affect the gastrocnemius weight when gemcitabine was added. However, there are conflicting reports showing that a KD could lead to muscle atrophy in mice [37]. The differences in outcomes could be due to the varieties in KD compositions, the length of dietary treatments, the pathological features and/or the type of models used.

Cachexia is, in part, driven by reduced appetite and a consequent decrease in caloric intake, so ameliorating cachexia-related anorexia is an important aspect of PDAC treatment [38]. We observed that anorexia, assessed by caloric intake, was diminished in the KG groups when compared to CG. Anorexia was lessened in KG treated female mice, which could be a factor in the maintenance of muscle function. Interestingly, Koutnik et al. reported anti-anorexic effects of ketone diesters that are consistent with our data [39].

Although sex may play a role in cancer cachexia development and progression, preclinical studies elucidating sex differences in cancer cachexia are scarce [40]. Our data suggests that a KD results in preservation of skeletal muscle mass, and that such response to a KD differs between females and males. Consistently, reduction of grip strength was shown to differ between female and male cachectic patients [41]. Both in animal models and human subjects, it has been suggested that cancer cachexia affects females and males differently. In a colorectal cancer model, female mice exhibited more severe reductions in body weight and muscle mass compared with male mice [42]. In general, females appear to be more susceptible to disuse induced muscle wasting, yet protected from inflammation induced muscle wasting compared to males [43].

Identifying drivers of cancer-associated muscle loss holds significant promise for improving cancer patient survival and quality of life. Several cellular mechanisms regulating muscle protein synthesis and degradation might explain the beneficial effects of a KD in PDAC-associated cachexia, including, autophagy, mTOR/FoxO, NF-κB, pro-inflammatory cytokines and histone acetylation [44].

Autophagy is essential in maintaining mitochondrial function in SKM [45]. Nevertheless, findings regarding markers of autophagy after KD intake are inconsistent, from reports showing that KD had no effect [35] , to others describing that a KD increases autophagy [37]. In our KPC mouse cohort, we observed a trend for higher LC3B-II to LC3B-I ratio in the muscle of KG-treated male mice, while the opposite effect occurred in KG-treated females. Since excessive autophagic

degradation plays a role in the onset of muscle depletion in CAC [46], inhibition of autophagy observed in our KPC female mice might be a factor in the sex-specific beneficial effects of KG treatment. Moreover, we demonstrated that phosphorylation of eIF2α increases with KG treatment, particularly in females. The levels of phosphorylated eIF2α, which lead to reduced protein synthesis, have been shown to be decreased in tumor-bearing mice [47, 48]. Interestingly, Wallace et al. also observed that p-eIF2α was higher in KD fed mice when compared to CD, and proposed that a KD decreases translation initiation, which could potentially slow protein synthesis, decrease unfolded proteins and mitigate ER stress [35].

Moreover, although the UPS is activated in SKM atrophy and muscle-wasting conditions, it also removes damaged proteins and helps maintain homeostasis [49]. We measured enzyme activity in male muscles to compare the proteasome function between CG and KG treatments. Our data suggests that UPS activation together with autophagy may present a strategy in which ketogenic diet combined with gemcitabine helps mitigate errors associated with protein breakdown and synthesis.

The regulation of gene expression in cachectic SKM can also be controlled by epigenetic mechanisms, through acetylation and deacetylation of histones, which are modified in a post-translational manner through histone acetyltransferases (HATs) and HDACs [50]. Since BHB can inhibit HDACs [25, 26], we measured total acetyl-lysine in muscle. Roberts et al. showed that after one-month total acetyl-Lys levels increased in mice fed a KD when compared to CD [12]. We also observed higher total acetyl-lysine levels in our KPC mice on the KG group when compared to CG, specifically in females.

Cachexia is also driven by the interplay of multiple cellular mediators, including AKT, mTOR, NF-κB and pro-inflammatory cytokines. For instance, the mTOR signaling pathway is a major anabolic pathway regulating protein synthesis in skeletal muscle [27], and is a key pathway modulated during cachexia [27-29]. Moreover, during the development of cancer cachexia, there

is an increase in the chronic production and secretion of pro-inflammatory cytokines as the disease progresses [32]. For example, circulating IL-6 levels are associated with suppressed muscle protein synthesis and mTORc1 signaling [29]. Moreover, NF-κB pathway, a major catabolic pathway, plays a key role in muscle cachexia, and can be activated by different proinflammatory cytokines (TNF-α and IL-6). However, the KD when combined with gemcitabine had no significant effects on the levels of proinflammatory cytokines, nor in the activation of mTOR, AKT and NF-κB pathways. Our findings are in disagreement with some previous reports in which a KD activated mTOR signaling in GTN of old mice [12]. This discrepancy, which requires further investigation, could be due to differences in the composition of the KD (fat type and quantity; protein type and quantity), the time at disease progression when the KD was started, the length of time KD was given, the animal model, and/or the additional effect of gemcitabine.

In summary, our findings indicate that there is a beneficial effect of a ketogenic diet in combination with gemcitabine in the preservation of skeletal muscle function in pancreatic tumor bearing mice. The mechanisms of the favorable effect appears to be multifaceted, including decreased autophagy and increased cellular response and acetylation. Additional research is warranted to further investigate the mechanisms through which a KD together with gemcitabine mitigate the detrimental effects of cachexia and how such diet-treatment combination can be optimized for clinical advantage.

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FIGURES

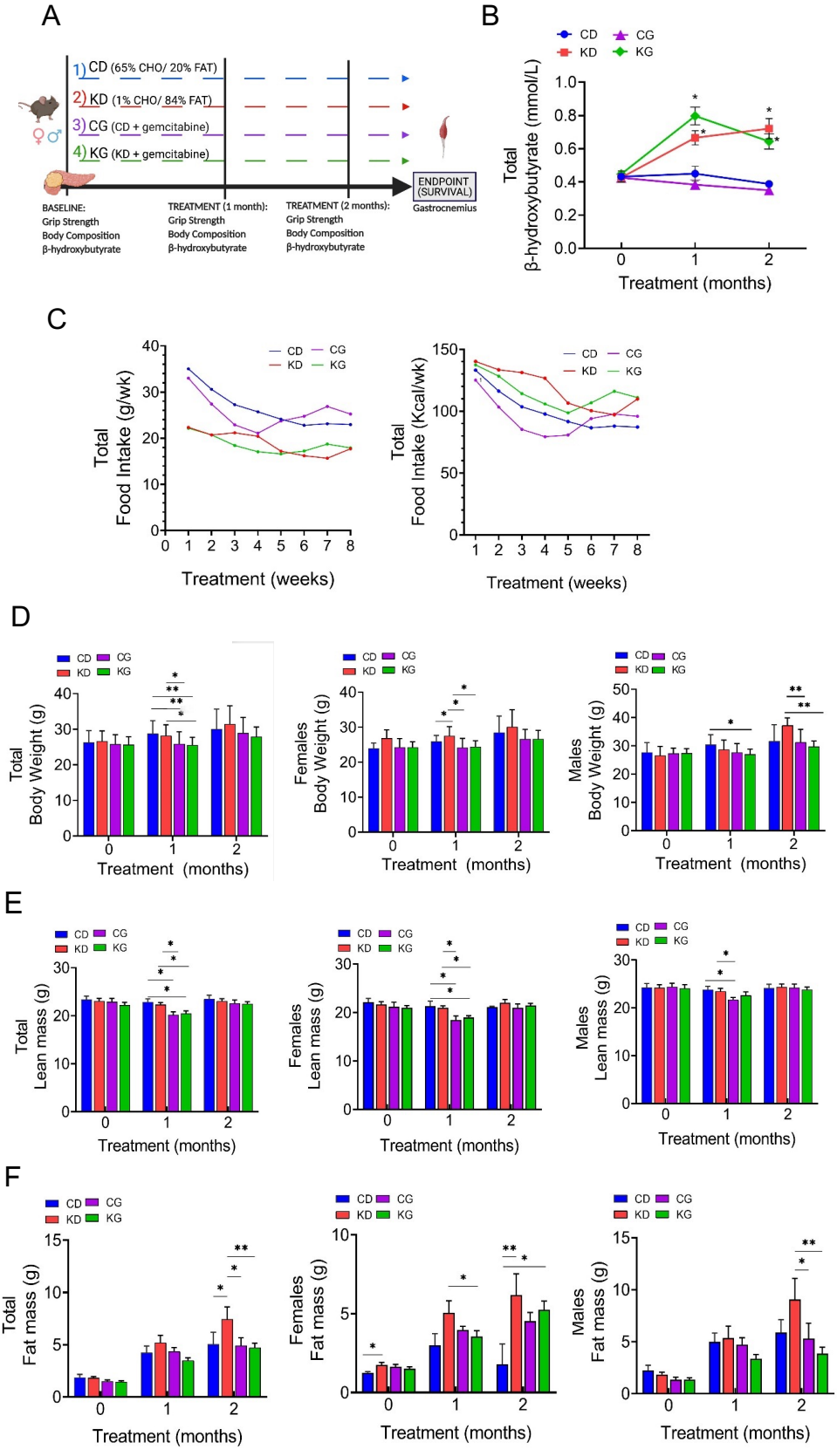

A
1) CD (65% CHO/ 20% FAT)
2) KD (1% CHO/ 84% FAT)
3) CG (CD + gemcitabine)
4) KG (KD + gemcitabine)
ENDPOINT (SURVIVAL)
Gastrocnemius
BASELINE:
Grip Strength
Body Composition
β-hydroxybutyrate
TREATMENT (1 month):
Grip Strength
Body Composition
β-hydroxybutyrate
TREATMENT (2 months):
Grip Strength
Body Composition
β-hydroxybutyrate
B
CD
CG
KD
KG
Total β-hydroxybutyrate (mmol/L)
Treatment (months)
C
Total Food Intake (g/wk)
Treatment (weeks)
Total Food Intake (Kcal/wk)
Treatment (weeks)
D
Total Body Weight (g)
Females Body Weight (g)
Males Body Weight (g)
Treatment (months)
E
Total Lean mass (g)
Females Lean mass (g)
Males Lean mass (g)
Treatment (months)
F
Total Fat mass (g)
Females Fat mass (g)
Males Fat mass (g)
Treatment (months)

Figure 1. Effect of a KD alone and in combination with gemcitabine on food intake, body weight and composition in KPC mice. A: Schematic outline of the survival study design. Once pancreatic tumors were detected, female and male KPC mice were randomized to a control diet (CD), ketogenic diet (KD), CD plus gemcitabine (CG) or KD plus gemcitabine (KG) (n=16-23/group) until endpoint. **B:** Non-fasted blood β-hydroxybutyrate levels at baseline, one and two months after diet initiation. **C:** Food intake in grams per week (g/wk) and kilocalories per week (Kcal/wk) during 8 weeks of treatment. **D:** Body weight progression **E:** lean mass and **F:** fat mass of KPC mice fed a control diet (CD), ketogenic diet (KD), CD plus gemcitabine (CG) or KD plus gemcitabine (KG) are shown at baseline, one and two months after diet initiation for total cohort study (*left*), females only (*center*) and males only (*right*). Values are expressed as mean ± SEM; *p<0.05, **$p < 0.01$.

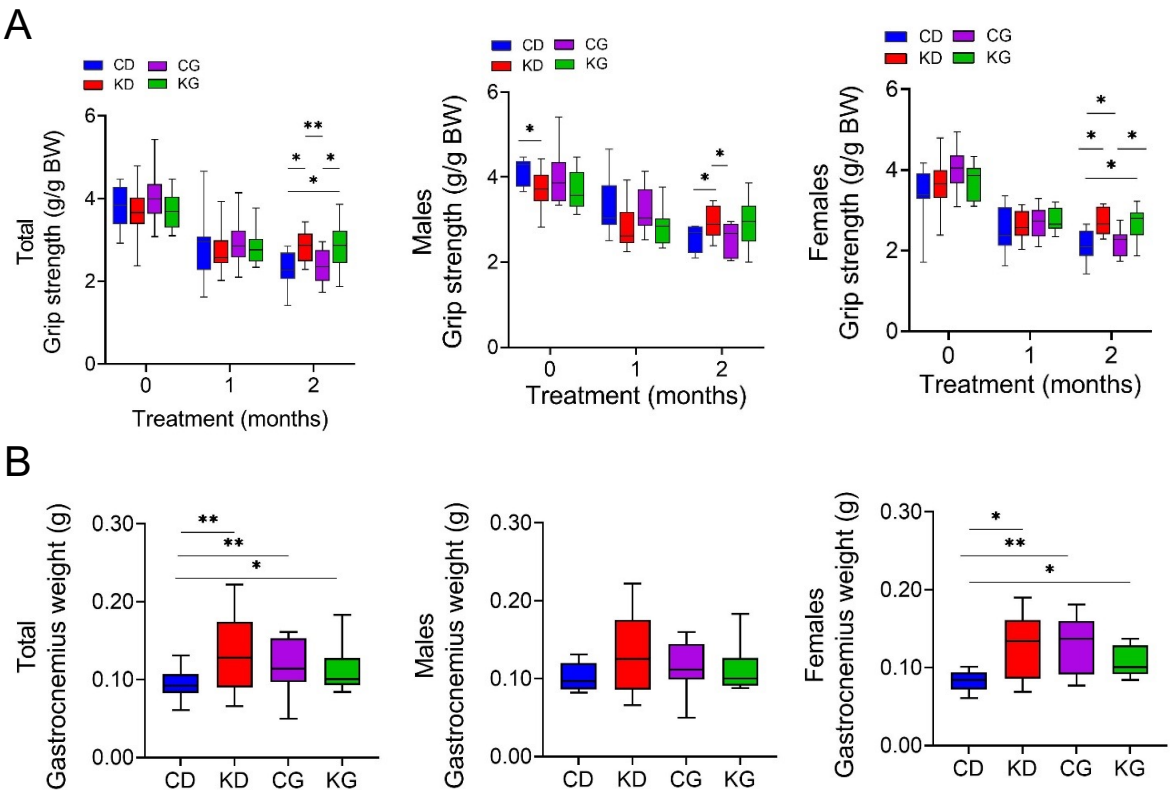


Figure 2. A ketogenic diet alone and in combination with gemcitabine preserves muscle strength and weight in KPC mice. **A:** Relative forelimb-grip strength force at baseline, one and two months after diet initiation and **B:**gastrocnemius weight are shown for total survival study cohort (left), females only (center) and males only (right) of KPC mice fed a control diet (CD), ketogenic diet (KD), CD plus gemcitabine (CG) or KD plus gemcitabine (KG) at survival endpoint; Values are expressed as mean ± SEM; *p<0.05, **$p < 0.01$.

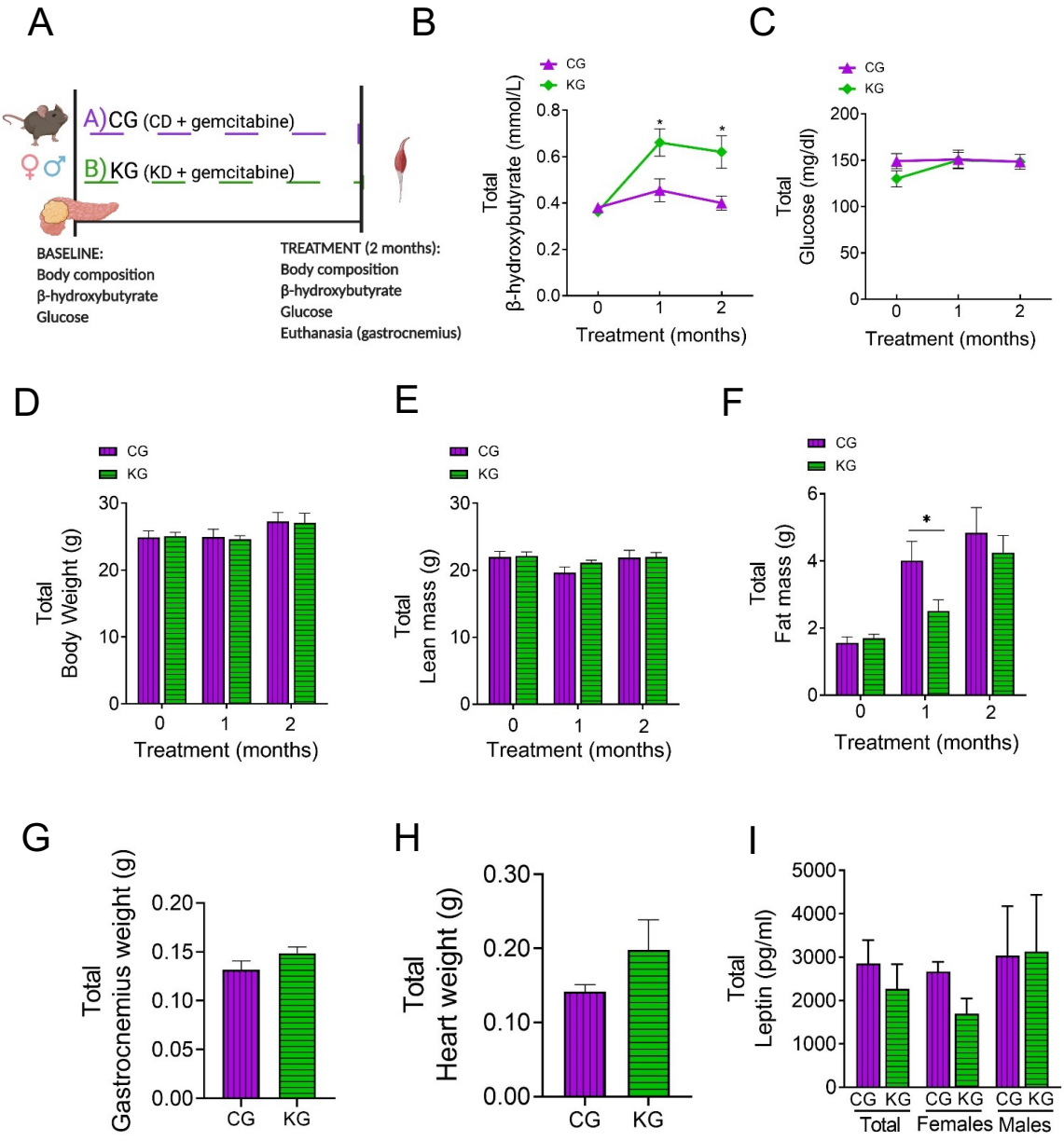


Figure 3. A ketogenic diet in combination with gemcitabine induces metabolic changes in KPC mice. A: Schematic outline of the mechanistic study design. Female and male mice were randomized to a control diet plus gemcitabine (CG) or to a ketogenic diet plus gemcitabine (KG) and euthanized after 2 months; *n*=4 per sex/group. **B:** Non-fasted blood β-hydroxybutyrate levels, **C:** circulating levels of non-fasting glucose, **D:** body weight, **E:** lean mass and **F:** fat mass progression are shown at baseline, one and two months after diet plus gemcitabine initiation of KPC mice treated with CG or KG. **G:** Gastrocnemius weight, **H:** heart weight and **I:**serum leptin levels at euthanasia shown for CG- and KG-treated female and male KPC mice. Values are expressed as mean ± SEM; *p<0.05.

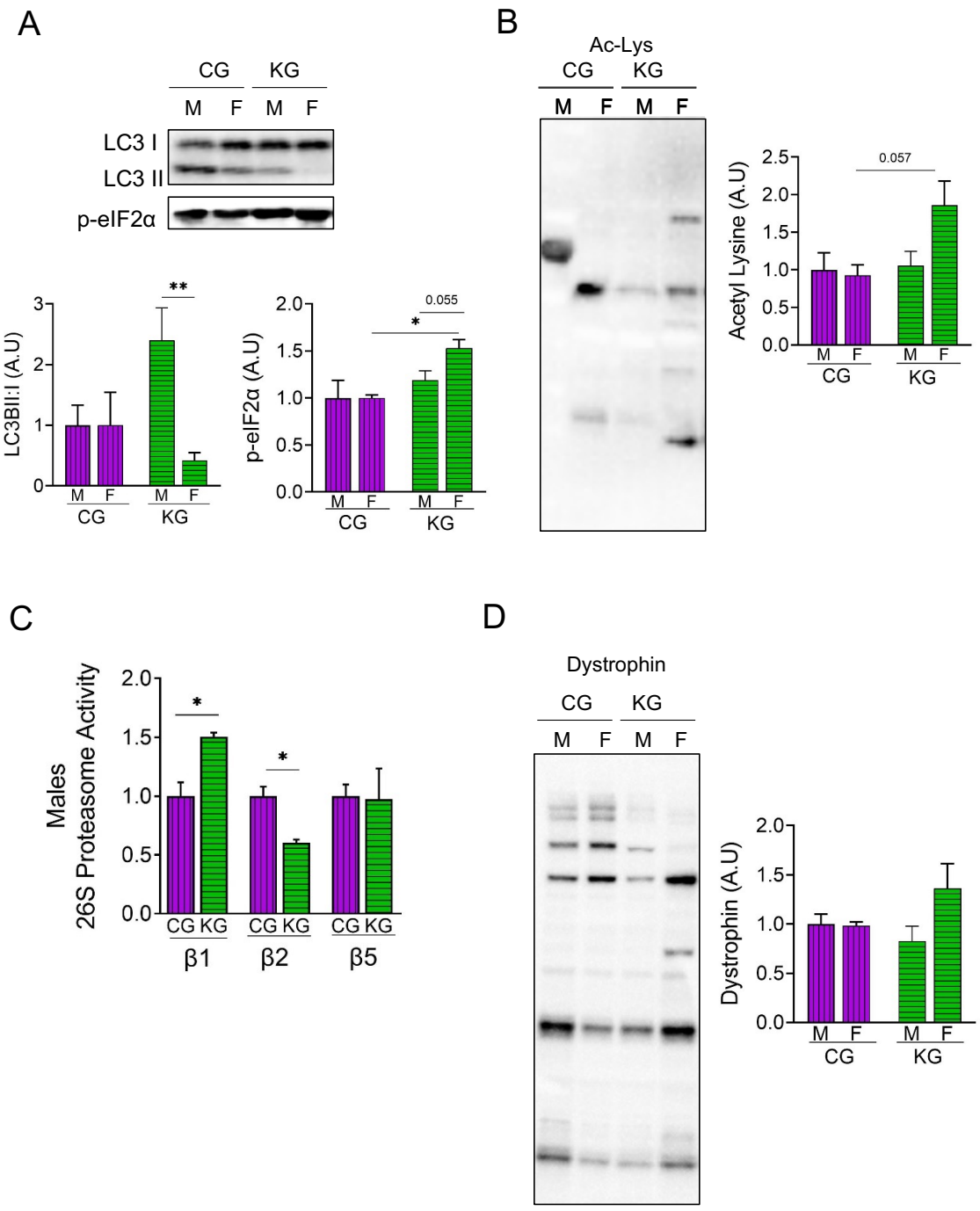


Figure 4. **A ketogenic diet in combination with gemcitabine induces autophagy and enhances protein acetylation in the gastrocnemius of KPC mice.** Immunoblots of **A:** LC3B-II, LC3B-I and p-eIF2α, **B:** acetyl-lysine and **C:** dystrophin in gastrocnemius homogenates isolated from CG- and KG-treated KPC mice following 2 months of treatment. **D:** ATP-dependent (26S) proteasome activity assay of subunit β1, β2, and β5 in gastrocnemius homogenates isolated from CG- and KG-treated female and male KPC mice following 2 months of treatment. Values are expressed as means ± SEM; *n*= 2-5 per sex/group. Values are expressed as mean ± SEM; *p < 0.05; **p < 0.01.

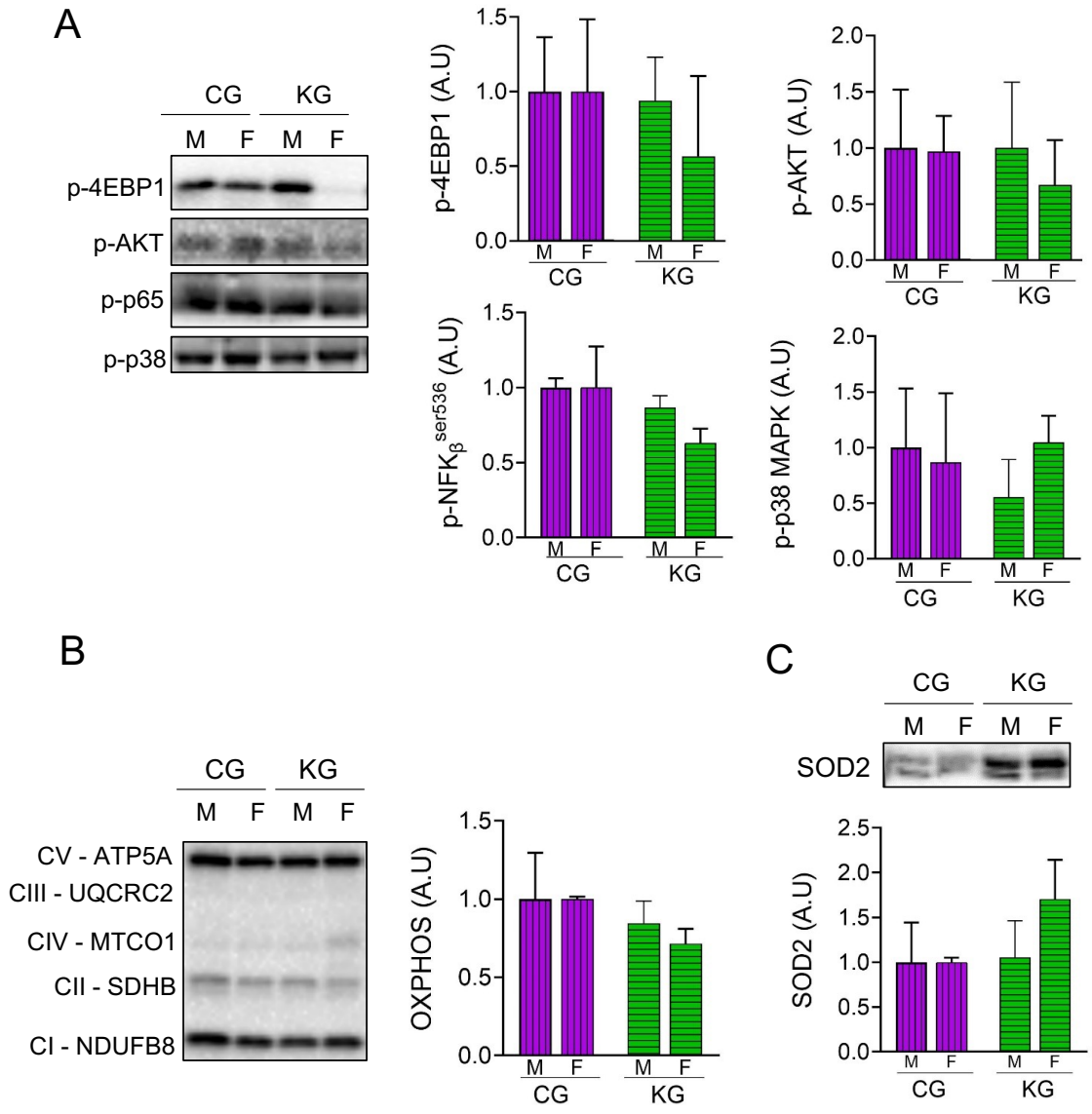


Figure 5. Effect of a ketogenic diet in combination with gemcitabine on skeletal muscle inflammatory markers and oxidative metabolism. Immunoblots of **A:** p-4EBP1, p-AKT, p-p65, p-p38, **B:** oxidative phosphorylation (OXPHOS) proteins from each complex (C-I to C-V), and **C:** SOD2 in gastrocnemius homogenates isolated from CG- and KG-treated female and male KPC mice following 2 months of treatment. *n*= 2-5 per sex/group; Values are expressed as mean ± SEM.

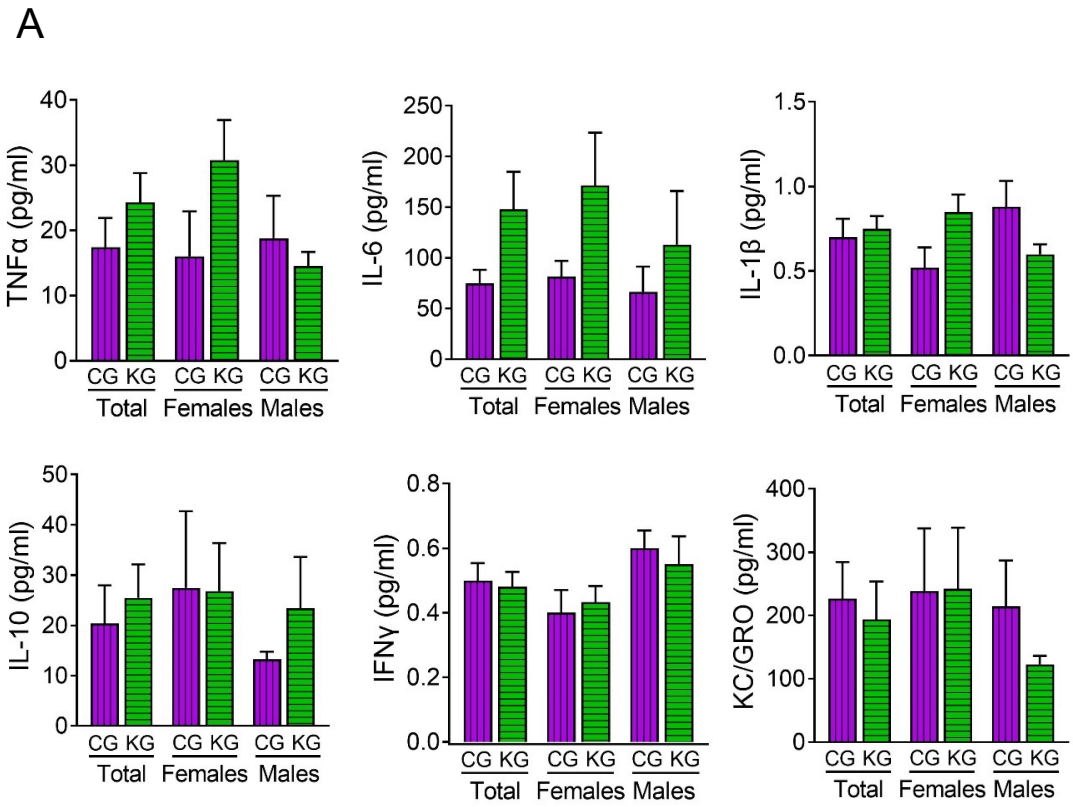
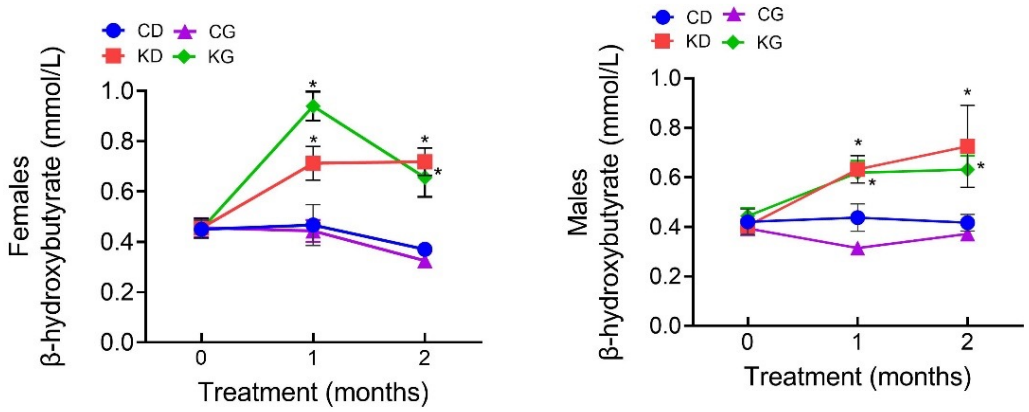
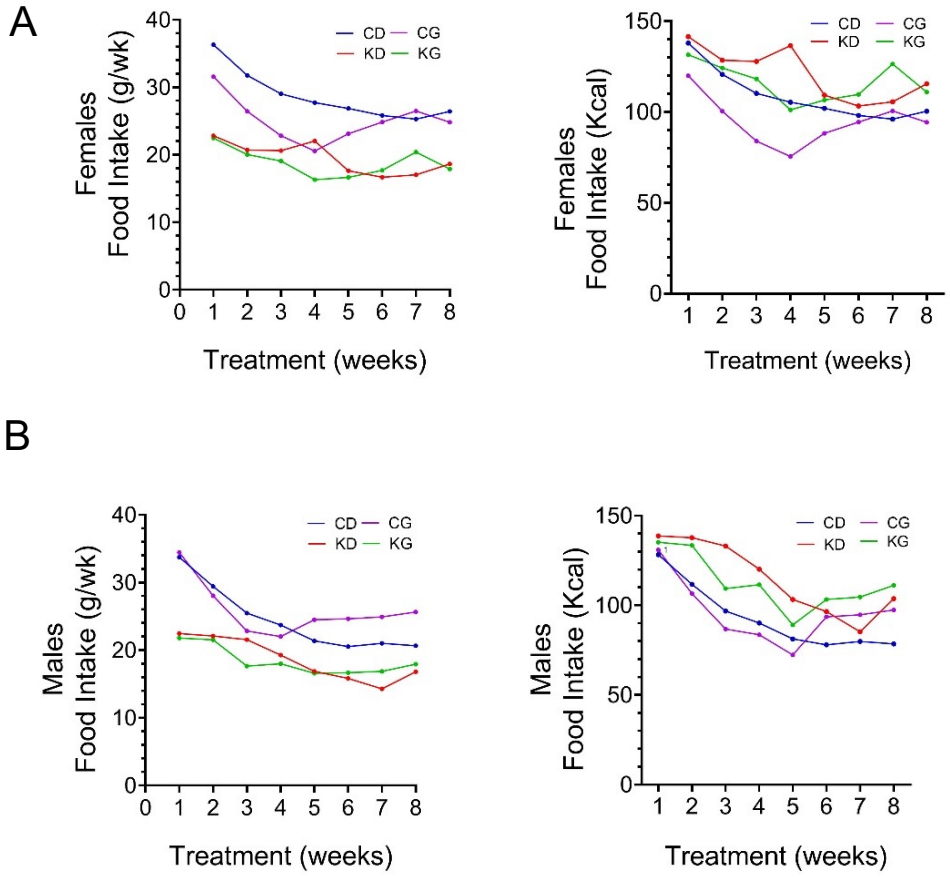


Figure 6. Effect of a ketogenic diet in combination with gemcitabine on serum cytokine levels in KPC mice. Pro-inflammatory cytokines TNFα, IL-6, IL-1β, IL-10, IFN-γ and KC/GRO were determined in serum obtained from CG- and KG-treated female and male KPC mice following 2 months of treatment; *n*=4 per sex/group; Values are expressed as mean ± SEM.

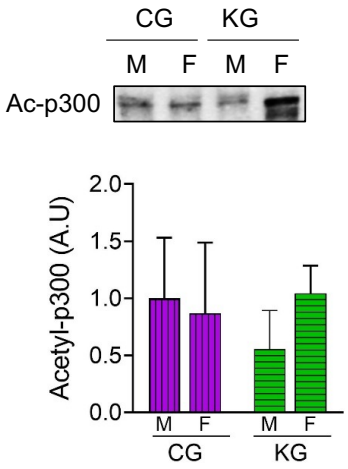
SUPPLEMENTAL FIGURES



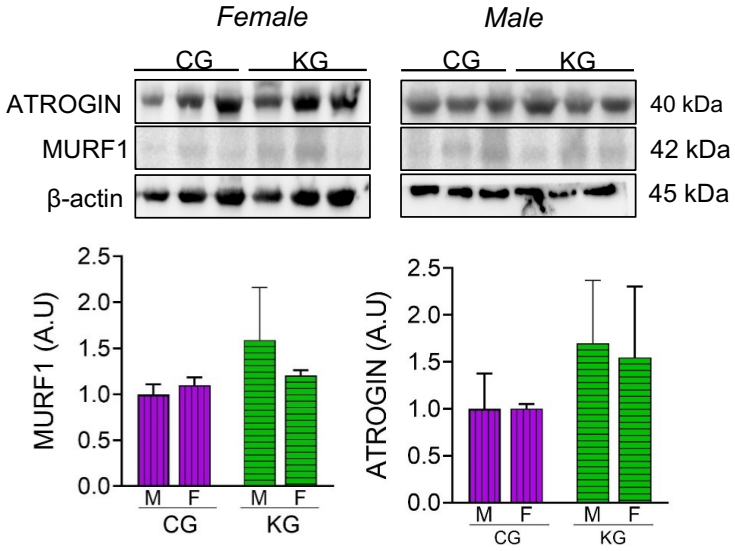
Supplementary Figure 1. Effect of a KD alone and in combination with gemcitabine on β-hydroxybutyrate levels in female and male KPC mice. Non-fasted blood β- hydroxybutyrate levels at baseline, one and two months after diet initiation are shown in KPC mice from the survival study cohort randomized to a control diet (CD), ketogenic diet (KD), CD plus gemcitabine (CG) or KD plus gemcitabine (KG); Values are expressed as means ± SEM; *p<0.05, ***p* < 0.01.



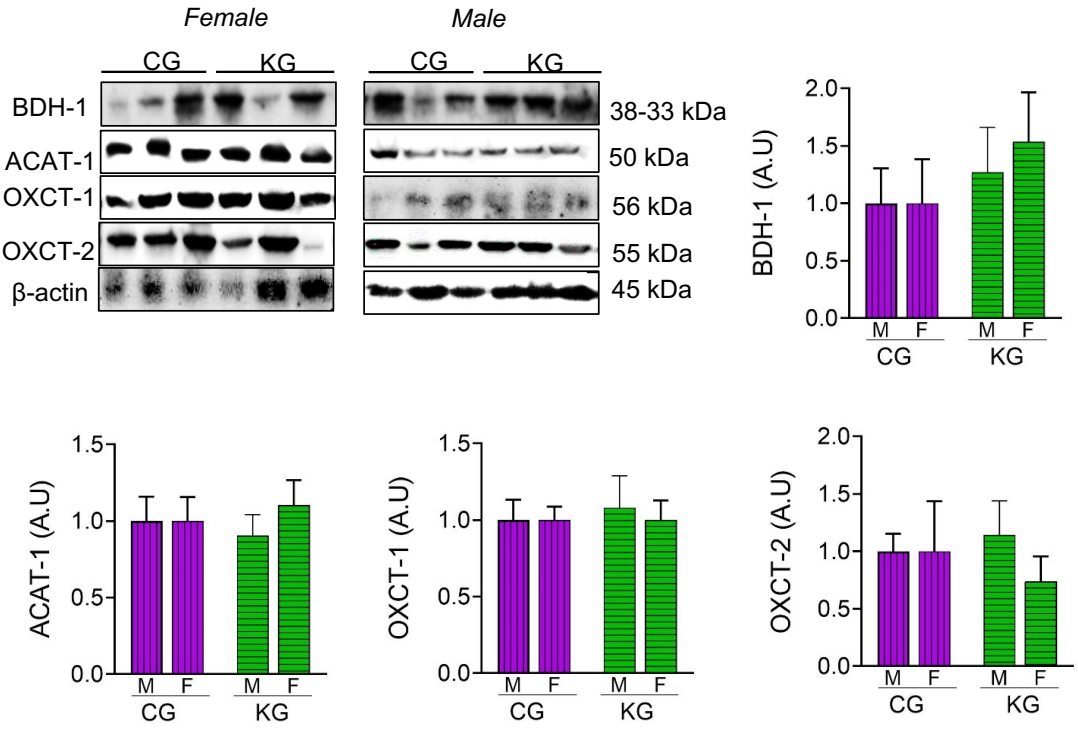
Supplementary Figure 2. Effect of a KD alone and in combination with gemcitabine on food intake in female and male KPC mice. Food intake in grams per week (g/wk) and kilocalories per week (Kcal/wk) shown for **A:** females and **B:** males **of the** KPC mice from the survival study cohort randomized to a control diet (CD), ketogenic diet (KD), control diet plus gemcitabine (CG) or ketogenic diet plus gemcitabine (KG); Values are expressed as means ± SEM.



Supplemental Figure 4. Effect of a ketogenic diet in combination with gemcitabine on protein lysine acetylation. Immunoblot of Acetyl p-300 signaling from the gastrocnemius (GTN) of KPC mice treated with control diet plus gemcitabine (CG) or ketogenic diet plus gemcitabine (KG) separated by sex; *n*= 2-5 per sex per group. Values are expressed as means ± SEM.



Supplemental Figure 5. Effect of a ketogenic diet in combination with gemcitabine on muscle-specific E3 ubiquitin ligases. Immunoblot of the muscle-specific E3 ubiquitin ligases atrophy-related gene (ATROGIN-1) and muscle RING finger 1 (MURF-1) signaling from the gastrocnemius (GTN) of KPC mice treated with control diet plus gemcitabine (CG) or ketogenic diet plus gemcitabine (KG) separated by sex; *n*= 2-5 per sex per group. Values are expressed as means ± SEM.



Supplemental Figure 6. Effect of a ketogenic diet in combination with gemcitabine on ketone body metabolic enzymes. Immunoblots of the ketone body metabolic enzymes succinyl CoA: 3-oxoacid CoA transferase (OXCT1), 3- hydroxybutyrate dehydrogenase 1 (BDH1), and acetyl-CoA acetyltransferase 1 (ACAT1) signaling from the gastrocnemius (GTN) of KPC mice treated with control diet plus gemcitabine (CG) or ketogenic diet plus gemcitabine (KG) separated by sex; *n*= 2-5; Values are expressed as means ± SEM.

Chapter 4. Hepatic safety profile of a ketogenic diet in combination with gemcitabine in pancreatic tumor-bearing mice

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*Contributed equally

Running title: Safety profile of a ketogenic diet plus gemcitabine

ABSTRACT

Ketogenic diets (KDs) are actively being explored for their anticancer effect. Although KDs are generally considered safe, their safety profile when combined with chemotherapy is unknown. In this study, we evaluated the hepatic safety profile of a KD in combination with gemcitabine, as a cancer treatment protocol, in a genetically modified animal model of pancreatic cancer (KPC mice). For this purpose, male and female pancreatic tumor-bearing KPC mice were allocated to a control diet (CD: %kcal: 20% fat, 65% carb, 15% protein) + gemcitabine (CG), or a KD (%kcal: 84% fat, 15% protein, 1% carb) + gemcitabine (KG) group for 2 months. Consumption of a KD in combination with gemcitabine failed to significantly affect mouse body weight, liver weight, serum activity of liver enzymes, including serum aminotransferases [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)]. In addition, KG did not increase markers of liver-lipid accumulation as well as serum cholesterol and triglyceride levels. When examining changes of individual liver fatty acid levels, KG treatment significantly decreased liver levels of saturated fatty acids and increased levels of cis-monounsaturated fatty acids, dihomo-gamma-linolenic acid, α-linolenic acid, eicosapentaenoic acid and docoasapentaenenoic acid compared to CG. Moreover, KG did not affect liver markers of inflammation and oxidative stress, nor liver enzymes involved in ketone bodies and glucose metabolism. Finally, acetylated lysine levels were significantly increased in female KG liver, compared to CG. In summary, a KD in combination with gemcitabine appears safe with no apparent hepatotoxicity. These safety data support the evaluation of a KD as an adjuvant dietary treatment for pancreatic cancer.

Keywords: liver steatosis; liver toxicity; ketogenic diet; gemcitabine; pancreatic cancer.

INTRODUCTION

Ketogenic diets (KDs) are low carbohydrate, adequate protein, high fat diets that mimic fasting and induce ketosis [1]. KDs are well-established, effective, and clinically approved dietary treatments for children with drug-resistant epilepsy; plus, therapeutically used in other neurological conditions like Alzheimer, traumatic brain injury, and stroke [2, 3]. Even though the evidence of successful use of a KD is more limited in oncology, many studies have documented that KDs have benefits as adjuvant therapy in certain types of cancer [4, 5]. Pre-clinical evidence indicates that KDs are safe and feasible as part of cancer treatment [6] and the limited cancer clinical data so far suggests there is no considerable adverse effects [1]. However, due to the lack of evidence demonstrating not only their efficacy, but also their long-term safety, the use of KDs in cancer, including pancreatic cancer, remains controversial.

In Chapter 2, we have documented that a KD enhances the anticancer effect of gemcitabine (2',2'-difluoro-2'-deoxycytidine), an antineoplastic drug commonly used in pancreatic cancer treatment, and improves survival in KPC tumor bearing mice. Nevertheless, this cytotoxic drug has been associated with elevations in indicators of liver disease like serum aminotransferases [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)], and with clinically apparent liver injury in persons with preexisting liver disease [7]. A few case reports have indicated that gemcitabine can cause steatohepatitis [16] and hepatotoxicity [8], but their clinical features are still unclear. Furthermore, while some evidence on the efficacy of KDs in combination with chemotherapy exits [4, 9], little is known regarding their safety with or without concomitant treatment with gemcitabine.

Since the liver metabolizes fatty acids into ketone bodies (i.e., β-hydroxybutyrate, acetoacetate, and acetone) [10], hepatic metabolism plays a major role in substrate availability and metabolic alterations during fasting or with fasting mimicking diets such as KD. In the liver, KDs have shown anti-steatotic effects [11, 12] and inhibition of hepatocellular carcinoma growth in mice [13]. Given the promising role on of KDs as a therapeutic diet for pancreatic cancer,

comprehensive safety studies are needed to validate the use of KDs when combined with chemotherapy. Therefore, the aim of this study was to evaluate the liver safety profile of a KD in combination with gemcitabine in KPC mice bearing pancreatic tumors, with the ultimate goal of advancing this promising treatment strategy to the clinic. Our data shows that in both female and male KPC mice, a KD fed for two months in combination with gemcitabine treatment is safe with no apparent liver toxicity.

MATERIALS and METHODS

Animal studies: All animal use procedures were approved by the University of California, Davis Animal Care and Use Committee.

Genetically engineered transgenic mice: The genetically engineered LSL-*Kras*$^{LSL\text{-}G12D/+}$*Trp53*$^{R172H/+}$*Pdx-1*-Cre (KPC) mouse model of PDAC was bred at the UC Davis Animal Facility in Meyer Hall. The KPC mice were generated from three mouse parental strains (LSL-Kras$^{G12D/+}$; LSL-Trp53$^{R172H/+;}$ and Pdx-1-Cre) obtained from the National Cancer Institute (NCI) mouse repository and following established procedures described by Hingorani and colleagues [14]. After weaning, mice were individually housed in polycarbonate cages in a room with controlled temperature (22-24°C) and humidity (40-60%), maintained on a 12 h light-dark cycle, and fed chow diet ad libitum LabDiet 5001 (LabDiet, Saint Louis, MO) until enrolled in the studies.

Dietary and chemotherapy interventions. Following tumor size determination, male and female KPC mice were allocated to either control diet (%kcal: 20% fat, 65% carb, 15% protein) + chemotherapeutic agent gemcitabine (CG) or the ketogenic diet (%kcal: 84% fat, 15% protein, 1% carb) + chemotherapeutic agent gemcitabine (KG). Gemcitabine (>99% 2'-Deoxy-2',2'-difluorocytidine; dFdC; Gemzar; LY-188011) from Fisher Scientific (Hampton, NH) was administered to the CG and KG groups at 100 mg/kg by i.p, injection two times per week for 3.5 weeks (7 total injections). At the end of the 2 months, liver was dissected, weighed, sectioned,

and then stored in liquid nitrogen, RNA later and 10% buffered formalin. Diets were previously described by our team. The Envigo (Indianapolis, IN) mineral mix TD94046 was used for the control diets and the TD79055 was used for the ketogenic diets due to their lower carbohydrate contents. For both diets, TD40060 (vitamin mix) was used. The composition of the experimental diets and their fatty acid composition is shown in Table 1 and 2, respectively.

Metabolic measurements: Blood samples were collected via cardiac puncture after euthanasia and serum was isolated after centrifugation at 3,000 x g for 10 min at room temperature. Total serum total serum cholesterol (Cat. No: 03039773), triglycerides (Cat. No: 20767107 322), alanine aminotransferase (Cat. No: 20764957 322), aspartate aminotransferase (Cat. No: 20764949 322), alkaline phosphatase (Cat. No: 03333752 190), total bilirubin (Cat. No: 05795397 190), albumin (Cat. No:04469658 190), creatinine (Cat. No: 03263991 190), total protein (Cat. No: 03183734 190), and blood urea nitrogen (Cat. No: 04460715 190) were measured using a COBAS INTEGRA kit (Roche, Indianapolis, IN) according to the manufacturer's instructions.

Histology: After necropsy, liver was fixed in 10% (w/v) buffered formalin overnight at 4°C. Tissues were processed and embedded by routine methods. Tissues sections (5 μm) were stained with hematoxylin and eosin, or Masson's Trichrome (Chromaview, Thermo Scientific), and analyzed for blinded histological examination. Sections were examined using an Olympus BX46 microscope (Olympus America Inc., Center Valley, PA), with 20x and 40x ocular lenses. The obtained images were scored according to Liang et al., depending on the presence of macrovesicular or microvesicular steatosis and hepatocyte hypertrophy in blinded fashion. Macrovesicular steatosis and microvesicular steatosis were both separately scored and the severity was graded, based on the percentage of the total area affected, into the following categories: 0 (less than 5%), 1 (5–33%), 2 (34–66%) and 3 (more than 66%). The difference between macrovesicular and microvesicular steatosis was defined by whether the vacuoles

displaced the nucleus to the side (macrovesicular) or not (microvesicular). Similarly, the level of hepatocellular hypertrophy, defined as cellular enlargement more than 1.5 times the normal hepatocyte diameter, was scored, based on the percentage of the total area affected, into the following categories: 0 (less than 5%), 1 (5–33%), 2 (34–66%) and 3 (more than 66%). For hepatocellular hypertrophy the evaluation was merely based on abnormal enlargement of the cells, irrespective of rounding of the cells and/or changes in cytoplasm or the number of vacuoles and is therefore not a substitute of ballooning. Inflammation was scored based on the number of inflammatory cell clusters (consisting of >=5 lymphocytes) averaged over five fields at 200x magnification, as follows: 0 = <0.5 focus; 1 = 0.5-1.0 focus; 2 = 1.0-2.0 foci, 3 = > 2.0 foci [15].

Western Blot Analysis. Liver tissue homogenates were prepared as previously described [16] and western blots were performed. Aliquots of total homogenates containing 25–40 µg protein were separated by reducing 8%–12.5% (w/v) polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. Membranes were blocked for 1 h in 5% (w/v) nonfat milk and subsequently incubated overnight at 4 °C with the following antibodies: p-ERK1/2 (Thr202/Tyr204) (Cat #4376), ERK1/2 (Cat #9102), p-Akt (Ser473) (Cat #4060), AKT (Cat #9272), Acetylated Lysine (Cat #9441), HKII (Cat #2867), PDH (Cat #3205), PFK (#13123), p-ACC (#) and TLR2 (#12276) from Cell Signaling Technologies (Danvers, MA). HMGCS (sc-373681), SCREBP1 (sc-13551), PPARα (sc-398394), FAS (sc-74540), ACC (sc-), Col1A1 (sc-59772) and TLR4 (sc-293072) from Santa Cruz Biotechnology (Santa Cruz, CA). HMGCL (16898-1-AP) was from Proteintech Group, Inc (Rosemont, IL) and 4-hydroxynonenal (4-HNE) (ab46545) was from Abcam, Inc. (Cambridge, MA). The antibodies were prepared using a 1:1000 dilution. After incubation for 1 h at room temperature in the presence of secondary antibodies (either HRP or biotinylated antibodies, followed by 1 h incubation with streptavidin when a biotinylated antibody was used in a 1:5,000 dilution), the conjugates were visualized and quantified by chemiluminescence detection in a Chemidoc™ Imaging-System, Bio-Rad Laboratories, Inc. β-

actin (Cat #A1978) from Millipore-Sigma, Saint Louis, MO, was used as a loading control. The densitometric analysis was performed using the Image J Program.

Liver fatty acid analysis. Fatty acid content in KPC livers of CG and KG-treated mice was measured using gas chromatography (GC). Briefly, liver samples were freeze-dried and direct-methylated with sodium methoxide as previously described [17]. *Cis*-10-17:1 methyl ester (Nu-Check Prep Inc., Elysian, MN) was added as an internal standard prior to methylating reagent. Fatty acid methyl esters (FAME) were analyzed by GC using a CP-Sil88 column (100 m, 25 µm ID, 0.2 µm film thickness) in a TRACE 1310 gas chromatograph (Thermo Scientific) equipped with a flame-ionization detector (GC-FID, Thermo Scientific). Each sample was analyzed twice by GC using a 175 °C plateau temperature program [17]. The FAME were quantified using chromatographic peak area and internal standard-based calculations.

Statistical Analysis. Data expressed as the mean ± SEM. Statistical evaluation was performed by t-test or two-factor analysis of variance (ANOVA) followed by the Tukey test for multiple comparisons. $p<0.05$ was regarded as statistically significant.

RESULTS

Liver safety profile of a ketogenic diet in combination with gemcitabine in KPC mice

We evaluated the effect of a KD in combination with gemcitabine (KG) in the liver of the autochthonous KPC mouse model to determine the safety profile of such synergistic treatment. Body weight was assessed prior to every gemcitabine injection and at euthanasia. No significant differences in body weight were observed between CG and KG groups throughout the treatment (Fig.1A and B). Moreover, no differences in liver weight were observed between groups (Fig.1C).

To determine whether KG affected normal liver function, we measured serum liver enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP), plus bilirubin in female and male KPC mice at euthanasia (Fig. 1D). All mice

in the CG- and KG-treated groups showed physiological levels of all three liver enzymes, bilirubin, and liver proteins with no significant differences between groups. Moreover, no differences in serum total protein and albumin levels as markers of liver protein metabolism, were observed (Fig. 1E).

Kidney function tests were also assessed, with no differences in creatinine levels between CG and KG groups. In contrast, a higher blood urea nitrogen levels (BUN) were observed in the serum of CG-treated KPC mice when both sexes were analyzed together, compared to the KG-treated group, but still not above normal ranges (Fig. 1F). We also measured the serum lipid profile of CG and KG treated mice, but did not observe any differences in total cholesterol or triglycerides between groups (Fig. 1G).

Effect of a ketogenic diet in combination with gemcitabine on liver lipids accumulation

In previous murine studies, high fat diets induced hepatic steatosis and histopathological changes similar to human NASH[18]. Therefore, we determined if a KD in combination with gemcitabine could promote hepatic lipid accumulation/steatosis. For this purpose, following liver staining with hematoxylin & eosin (Fig. 2A) and Masson's trichrome (Fig. 2B), we evaluated histologically CG and KG-treated livers to assess alterations in hepatocyte injury patterns. Presence of macrovesicular steatosis, microvesicular steatosis and hepatocyte hypertrophy was assessed using a previously established scoring system [19]. Overall, macrovesicular steatosis was rare (2 of 24 cases) and mild (5%); moderate microvesicular steatosis (40%) was seen in a single case (1 of 24 cases); and mild to moderate hepatocyte hypertrophy (5-40%) was observed in 3 of 24 cases. Inflammation scores ranged from 0-3. No differences in macrovesicular steatosis, microvesicular steatosis, hepatocyte hypertrophy, or inflammation were observed between CG- and KG-treated mice (p-value = a; p = b; p = c, p = d, respectively). Additionally, no signs of fibrosis was observed in either group, evidence by a normal morphology after Masson's trichrome staining (Fig. 2B), and no differences in Fibronectin and Col1A1 protein levels (Fig. 2C).

Effect of a ketogenic diet in combination with gemcitabine on hepatic levels of fatty acids.

We then evaluated whether feeding a KD could affect fatty acid composition in the liver. For this purpose, we performed GC/FID analysis in livers isolated from female and male mice treated with a KG or a CG for 2 months. As shown in Fig. 3A, KG significantly reduced the overall total levels of saturated fatty acids (SFA) and increased the levels of cis-monounsaturated fatty acids (*c*-MUFA), compared to CG-treated mice. Moreover, no significant differences were observed in n6-polyunsaturated fatty acids (n6-PUFA), n3-PUFA levels, or the n6/n3 fatty acid ratio in the liver of KG mice compared to CG mice.

When examining changes of individual liver fatty acid levels between KG and CG, we observed that the increase in *c*-MUFA levels in the KG group was driven primarily by a significant increase in palmitoleic acid (*cis*9-16:1), accompanied by increases in margarolic acid (*cis*9-17:1), eicosanoic/gondoleic acid (*cis*9-20:1) and asclepic acid (*cis*11-18:1) (Fig. 3B). Other fatty acids that were increased following KG treatment included dihomo-gamma-linolenic acid (20:3n-6), α-linolenic acid (ALA; 18:3n-3), eicosapentaenoic acid (EPA) (20:5n-3) and docoasapentaenenoic acid (DPA) (22:5n-3). On the other hand, the decreased levels of SFA in the KG group were mainly driven by the decrease in stearic acid (18:0) and margaric acid (17:0). Other fatty acids decreased following KG treatment included arachidonic acid (20:4n-6) and docosahexaenoic acid (DHA) (22:6n-3) (Fig. 3C).

Effect of a ketogenic diet in combination with gemcitabine on de novo lipogenesis

The transcription factor sterol regulatory element binding protein (SREBP) and the enzymes Fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) are central in the regulation of *de novo* lipogenesis [20]. Therefore, we next evaluated if KG could influence SREBP1 and FAS protein levels and ACC phosphorylation in liver. While no differences were observed neither in SREBP1 protein levels nor ACC activation between CG and KG treated mice, KG treatment significantly

reduced levels of FAS in the liver of male mice, compared to CG group (Fig. 4) Peroxisome proliferator-activated receptor alpha (PPARα) is a nuclear receptor mainly express in liver that also modulates the *de novo* lipogenesis pathway [21]. PPARα protein levels were also explored in liver from CG- and KG-treated mice, no differences were observe between groups (Fig. 4)

AMPK and Akt can stimulate de novo lipid synthesis by activating SREBP [22, 23], with the activity of both of these kinases being affected by dietary intake of fat [24]. Thus, we explored whether KG could affect AMPK and AKT phosphorylation. No significant differences in AMPK and AKT phosphorylation liver levels were observed between CK and KG-treated mice (Fig. 4).

Effects of a ketogenic diet in combination with gemcitabine on ketogenesis and glucose metabolism

Given that the liver is a major organ in the metabolism of glucose and ketone bodies [25], we investigated the regulation of enzymes involved in ketogenesis and glucose metabolic pathway following KG treatment. No differences in HMGCL, HMGCS, HK2, PFK and PDH hepatic levels were observed between CG and KG-treated mice (Fig. 5A-B).

Acetylation may play a key role in the coordination of different metabolic pathways in response to extracellular conditions, including nutrient availability [26]. In addition to the glycolytic enzymes, the liver is highly exposed to lysine acetylation [27]. Therefore, we also examined whether a KD in combination with gemcitabine affected lysine acetylation in the liver. After 2 months of treatment, we observed a significant increased acetylation in the liver of female, but not male, KG-fed mice (Fig. 5C).

Effect of a ketogenic diet in combination with gemcitabine on markers of inflammation and oxidative stress in the liver of KPC mice

Activation of Toll-like receptor (TLR) signaling is key in liver inflammation process, with TLR4 and TLR2 playing a crucial role in the progression of NASH [28]. Therefore, we explored if

KG could affect the activation of this two TLRs. Moreover, since the TLRs cascade can lead to the activation of NF-κB and mitogen-activated protein kinases (MAPKs) pathways, which are central players of inflammation, we measured the phosphorylation levels of IKBα, p65 and ERK1/2 (Fig. 6A). No differences in TLR4, TLR2 levels nor in their downstream IKBα and ERK1/2 phosphorylation levels were observed in livers of KG- and CG-treated male and female mice. However, a significant increase in p65 phosphorylation was observed in KG males when compared to KG females (Fig. 6B).

Given that oxidative stress plays a major role in the development of liver injury [29], we next measured 4-hydroxynonenal (4-HNE), a main product of lipid peroxidation that displays increased levels with oxidative stress [30]. No differences were observed in 4-HNE levels between KG and CG-treated groups (Fig. 6C).

DISCUSSION

Understanding the safety profile of a KD is critical for its clinical applications. Adverse effects of long-term KD therapy include dyslipidemias, kidney stones, and/or gastrointestinal issues like constipation, which are generally manageable [31, 32]. Data on the overall safety and feasibility of KDs indicates that this diets can be tolerated by cancer patients [33]. Nevertheless, the long-term safety of a KD when administered with standard cancer treatment modalities needs to be assessed in pre-clinical models prior to suggesting moving forward with clinical trials. In Chapter 2, we demonstrated that a KD acts synergistically with the chemotherapeutic agent gemcitabine, increasing overall survival in the autochthonous-clinically relevant KPC mouse model. In this study, we evaluated the liver safety profile of a long-term KD in combination with gemcitabine (KG) in KPC tumor-bearing mice. To our knowledge, this is the first study exploring the liver safety of a KD in combination with the chemotherapy agent gemcitabine.

Gemcitabine is a main chemotherapeutic agent in pancreatic cancer treatment [34]. Although it is considered generally safe, gemcitabine can still cause hepatic adverse events such

as elevations in serum ALT, ALP and bilirubin [35, 36]. Of note, a case report described that gemcitabine monotherapy caused hepatic failure in an advanced pancreatic patient after pancreaticoduodenectomy [37]. In this study, we assessed multiple parameters of liver toxicity with KG. Overall, the addition of a KD to a gemcitabine regimen was safe with mice showing no body weight change, nor changes in liver enzymes nor markers of kidney function. Consistent with our findings, normal liver and kidney function tests were observed in rats after KD intake for 60 days [38]. Moreover, the effect of a KD on serum lipids has also been different among studies. In healthy KD-fed male mice, cholesterol and triglycerides levels were increased; plus signs of hepatic steatosis were observed after 22 weeks in the diet [39]. On the other hand, KD has led to improvements in total cholesterol and triglycerides in overweight women [40]. In our study, a KD in combination with gemcitabine did not affect serum cholesterol nor triglyceride levels in KPC mice.

High-fat diets can induce liver steatosis, which is a precursor of nonalcoholic fatty liver disease (NAFLD) [41]. Given the high fat content of a KD, this might be a concern. Nevertheless, some controversy exists on whether a KD predisposes to NAFDL development. In patients with NAFLD, a KD given for 6 days improved metabolic abnormalities [11]. Consistent with our findings, normal liver and kidney function tests were observed in mice after KD intake [38]. Moreover, KD prevented the development of steatosis in obese mice [42]. In our study, a KD in combination with gemcitabine did not induce lipid accumulation in the liver, nor did it affect the protein or enzyme levels related to de novo lipogenesis, ketogenesis or glucose metabolism. Finally, a KD in combination with gemcitabine did not affect inflammatory and oxidative stress markers, two known factors that can contribute to the pathogenesis of NAFLD [43, 44].

In addition, we observed significant differences in the composition of numerous intrahepatic FAs following KG-treatment, with higher MUFA levels, but lower SFAs. Interestingly, several mono- or poly-unsaturated fatty acids [MUFAs and PUFAs, e.g. oleic acid, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)] have been shown to be

beneficial to prevent the development of NAFLD [45]. Diverse fat compositions of KDs, and their effects on intrahepatic FAs and chemotherapeutic interactions should be further explored.

Evidence has shown that lysine acetylation on enzymes plays a role in metabolic processes during liver disease development. In addition, members of histone acetylase (HAT) and histone deacetylase (HDAC) families are aberrantly expressed in hepatocarcinomas, with some HDACs inhibitors considered candidates for clinical HCC treatment [46]. Remarkably, β-hydroxybutyrate is a known HDAC inhibitor [47]. In our study, we observed that a KD combined with gemcitabine significantly increased liver lysine acetylation when compared to CG-fed female mice. Our data is in accordance with Hutfles et al., that reports an increased cytosolic and mitochondrial acetylation in male C57Bl/6J livers mice under a KD in contrast to standard diet [48]. Consistent, in aging mice, protein acetylation was increased in the liver of KD-fed mice, which had longer overall median survival that control diet fed animals [36]. These data suggest that protein acetylation may play an important role in liver metabolism.

In summary, our findings indicate that a KD in combination with gemcitabine appears safe in mice bearing pancreatic tumors. It is important to stress that we evaluated the liver safety profile of a specific KD with a specific dose of gemcitabine over two months. Differences in diet composition (macronutrient distribution and/or amount and type of fats), length of the treatment, and type of combination drug could alter the safety when incorporating a KD to the treatment strategy. Future studies should focus on identifying a standardized treatment protocol that includes the composition, length and regimen for a KD [49], which would assist translating this promising dietary treatment strategy safely to the clinic.

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FIGURES

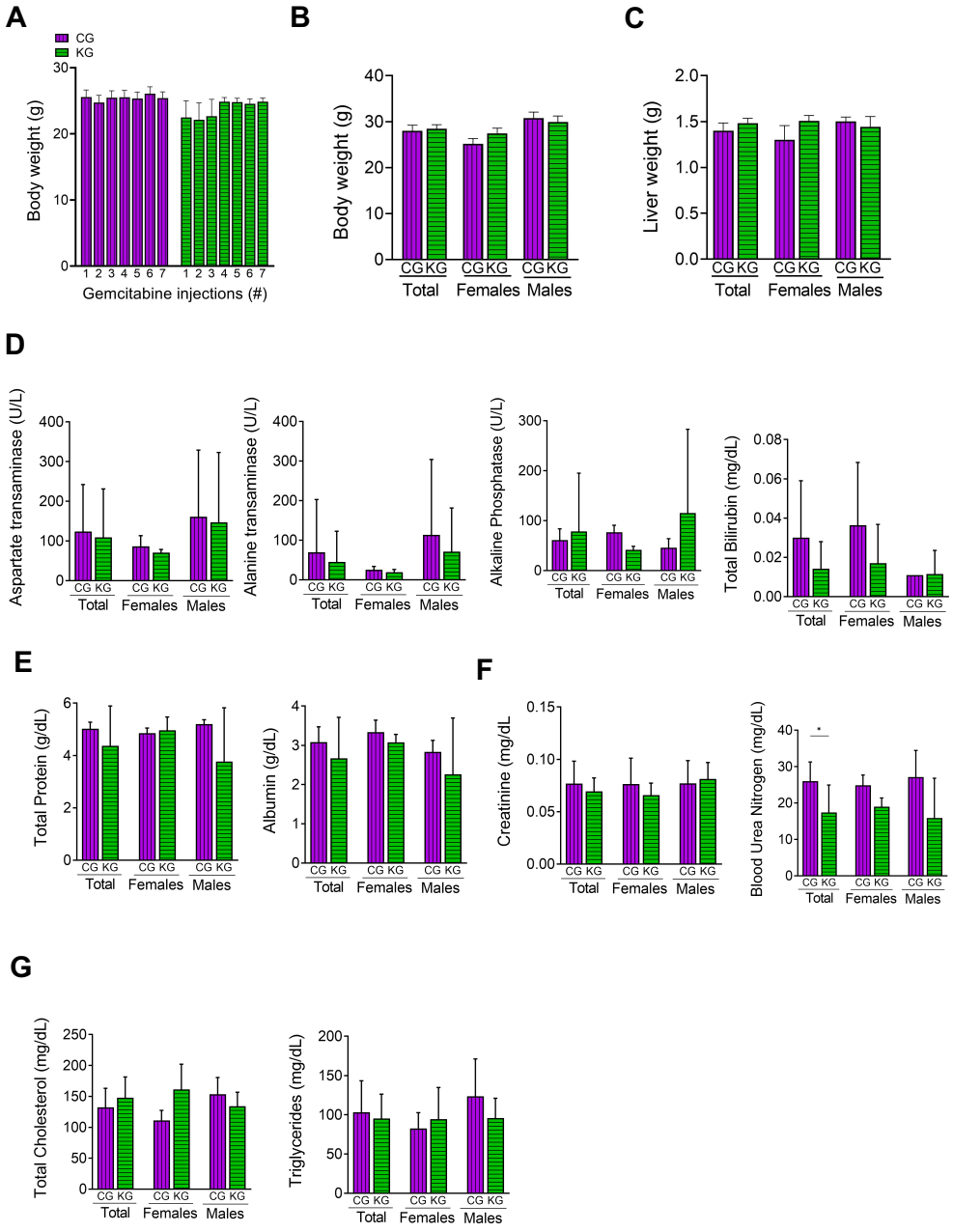

Figure 1. Effect of a ketogenic diet alone or in combination with gemcitabine in body weight, liver weight, liver enzymes, kidney markers and serum lipids in KPC mice.
A: Body weight at each gemcitabine injection. **B:** Final body weight. **C:** Liver weight, at euthanasia. **D:** Serum levels of liver enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) and bilirubin levels. **(E)** Serum total protein and albumin levels. **F:** Serum creatinine and blood urea nitrogen levels. **(G)** Serum cholesterol and triglycerides levels are shown for total cohort (*left*), females only (*center*) and males only (*right*) CG- and KG-treated KPC mice. Values are expressed as mean ± SEM; *p<0.05 of 8 animals per group.

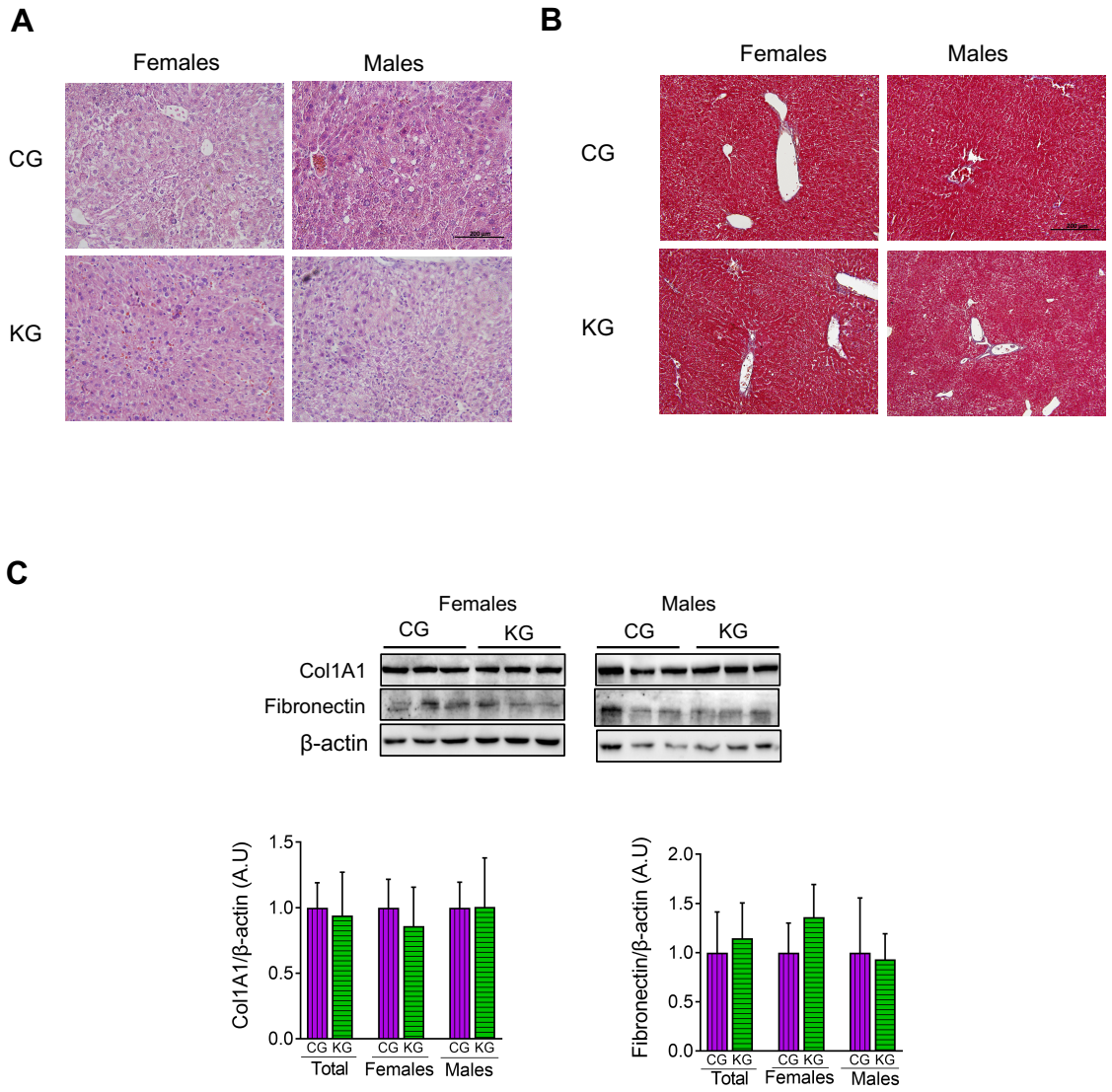

Figure 2. A ketogenic diet alone and in combination with gemcitabine has no effect on liver lipid accumulation. **A:** Hematoxylin/eosin stainning images and **B:** Mason Trichome staining images are shown for liver isolated from female and male KPC mice treated with CG and KG. All images digitally scanned at 20X original magnification. **C:** Immunoblots for Fibronectin and Col1A1 from liver homogenates from CG- and KG-treated female and male KPC mice following 2 months of treatment. Loading control: β-actin. Bands were quantified, and results are expressed as % control. Values are expressed as mean ± SEM of 8 animals per group.

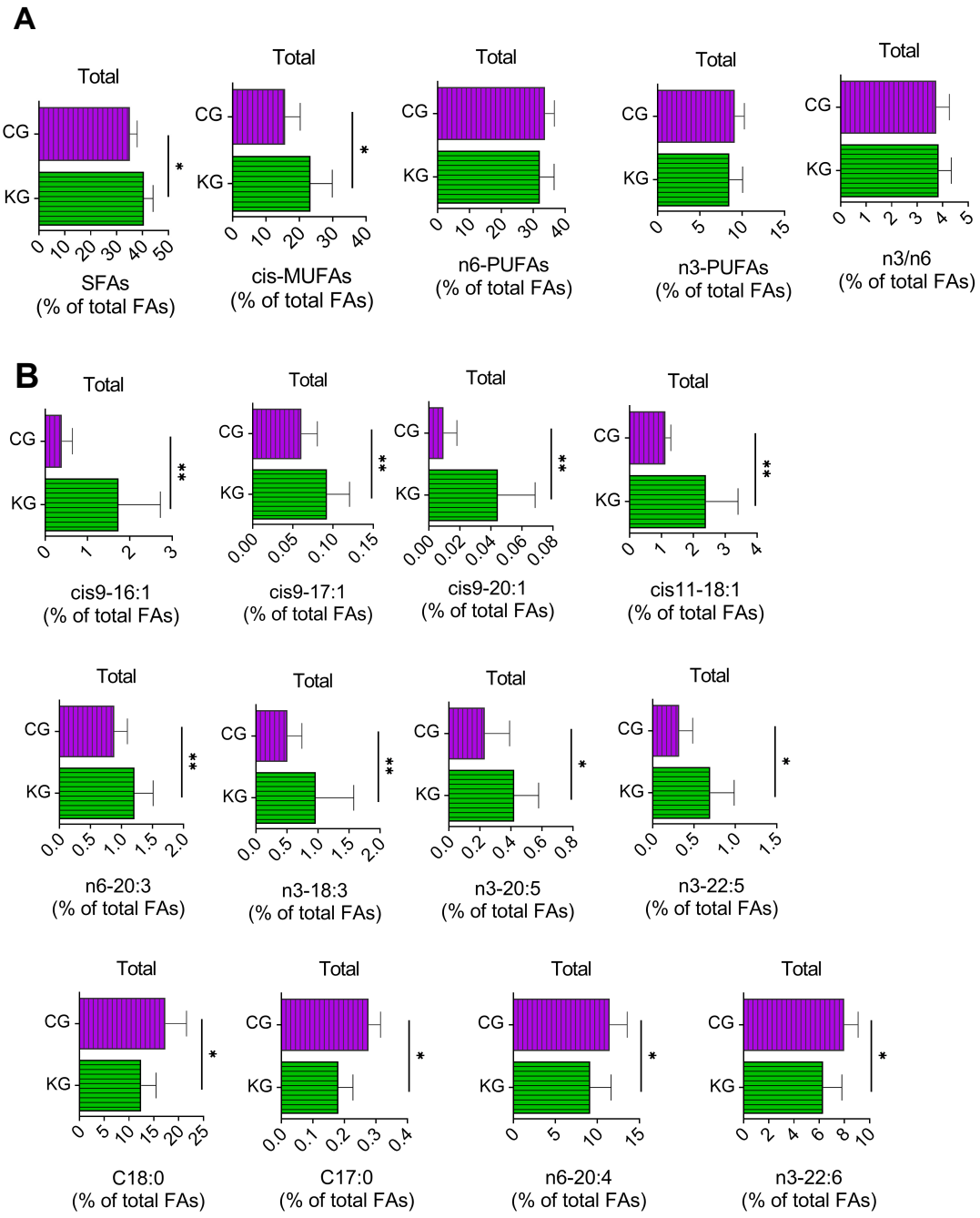

Figure 3. Effect of a ketogenic diet in combination with gemcitabine on hepatic levels of fatty acids. A: Levels of saturated fatty acids (SFA), MUFA monounsaturated fatty acids (MUFA), n-6 and n-3 polyunsaturated fatty acids (PUFA), as well as the n-6/n-3 fatty acid ratio, **B:** Concentrations (% of total fatty acids) of select fatty acids: palmitoleic acid (*cis*9-16:1), asclepic acid (*cis*11-18:1), margarolic acid (*cis*9-17:1), eicosanoic/gondoleic acid (*cis*9-20:1), dihomo-gamma-linolenic acid (20:3n-6), α-linolenic acid (18:3n-3), eicosapentaenoic acid (20:5n-3), docoasapentaenenoic acid (22:5n-3), stearic acid (18:0), margaric acid (17:0), arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3), in liver homogenates isolated from CG- and KG-treated KPC female and male mice following 2 months of treatment. **p<0.05, **p<0.01.* Values are expressed as mean ± SEM of 8 animals per group.

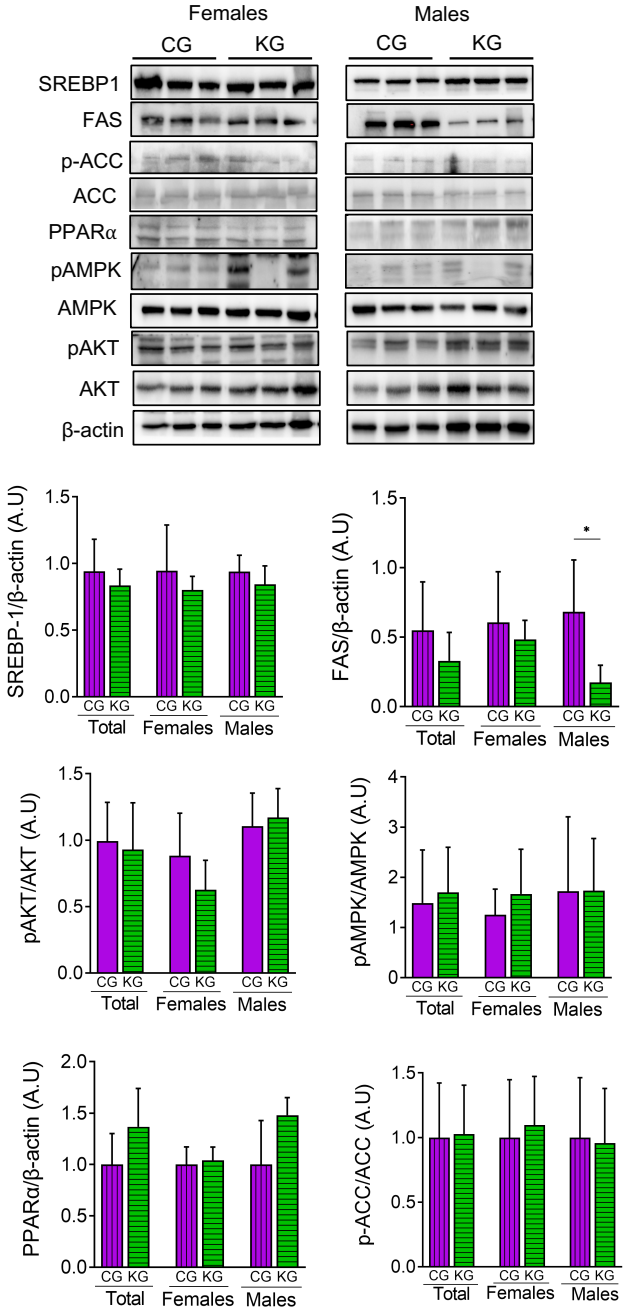

Figure 4. Effect of a ketogenic diet in combination with gemcitabine on enzymes involved in *de novo* fatty acids synthesis. Immunoblots of SREBP-1, FAS, p-ACC/ACC, PPARα pAKT/AKT, and pAMPK/AMPK from liver homogenates from CG- and KG-treated female and male KPC mice following 2 months of treatment. Loading control: β-actin. Bands were quantified, and results are expressed as % control; *$p<0.05$. Values are expressed as mean ± SEM of 8 animals per group.

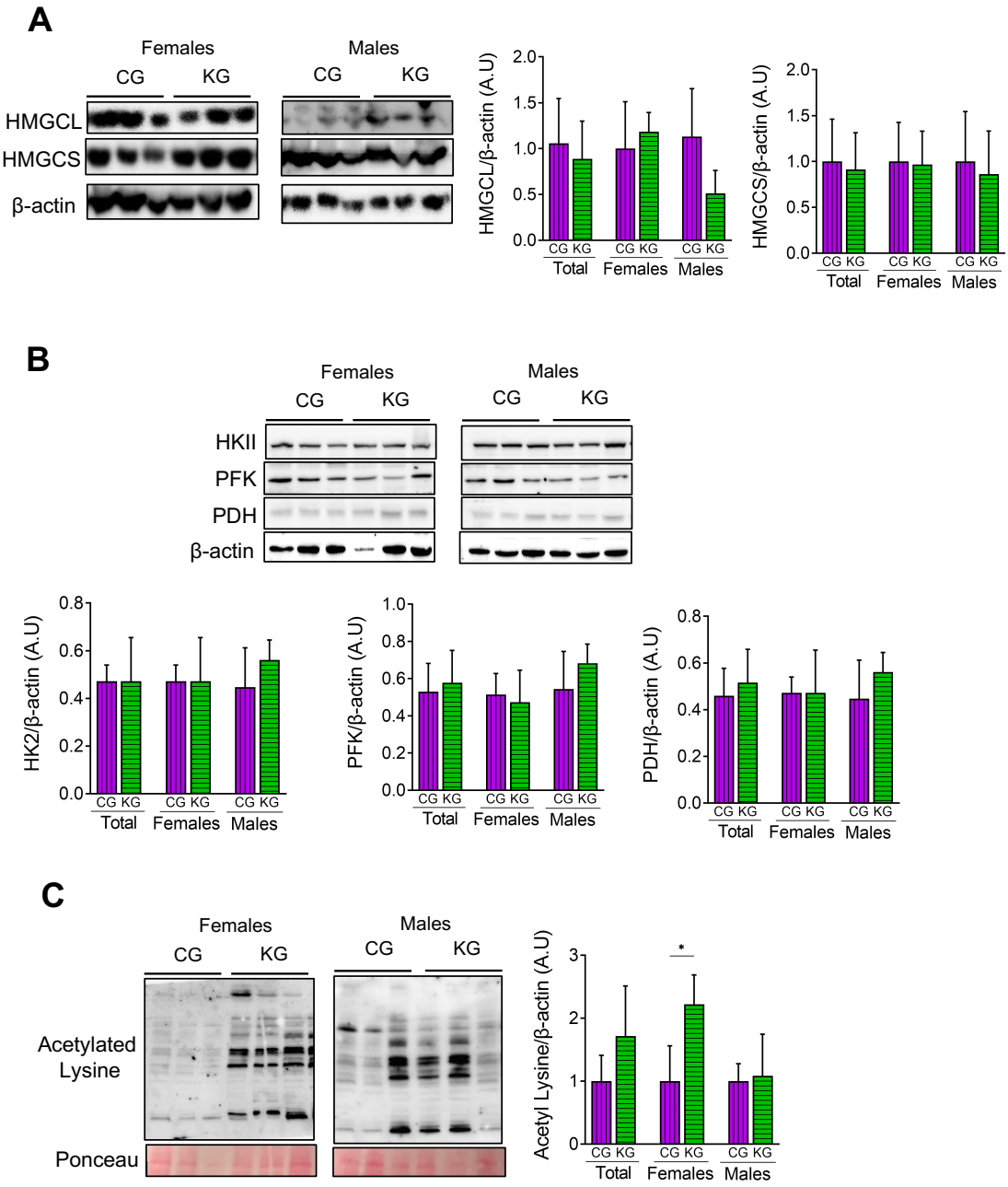

Figure 5. Effect of a ketogenic diet in combination with gemcitabine on ketogenesis and glucose metabolism. Immunoblots of **A:** HMGCL and HMGCS, **B:** HKII, PFH and PDH and **C:** acetylated lysine from liver homogenates from CG- and KG-treated female and male KPC mice following 2 months of treatment. Loading control: β-actin. Bands were quantified, and results are expressed as % control; *$p<0.05$. Values are expressed as mean ± SEM of 8 animals per group.

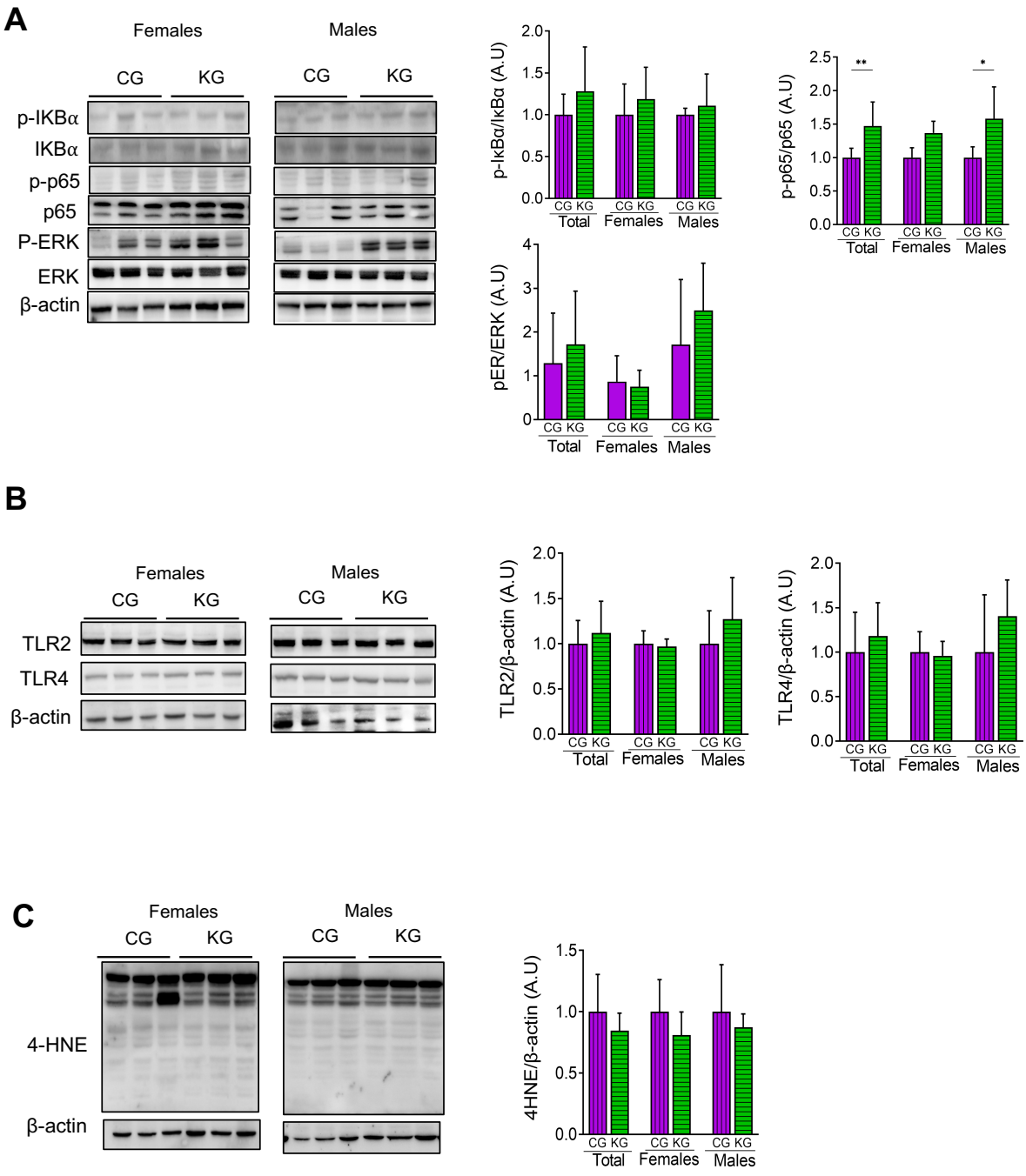

Figure 6. Effect of a ketogenic diet in combination with gemcitabine on markers of inflammation and oxidative stress. Immunoblots of **A:** p-IKBα, IKBα, p-p65, p65, p-ERK and ERK, **B:** 4-HNE and **C:** TLR2 and TLR4 from liver homogenates isolated from CG- and KG-treated female and male KPC mice following 2 months of treatment. Loading control: β-actin. Bands were quantified and values normalized to **A:** the non-phosphorylated protein and **B, C:** β-actin levels. Results are expressed as % control; **p<0.05, **p<0.01.* Values are expressed as mean ± SEM of 8 animals per group.

CONCLUSIONS

In this thesis project, I evaluated the effect of a KD alone or in combination with the chemotherapeutic drug gemcitabine (GEM) on morbidity and mortality in a clinically relevant genetically engineered *LSL-Kras*$^{G12D/+}$*; LSL-Trp53*$^{R172H/+}$*;Pdx1-Cre* (KPC) mouse model of PDAC. The specific aims of this thesis were to determine whether a KD plus GEM increases overall survival and mitigates cachexia, and to elucidate potential mechanisms underlying the beneficial effects of a KD. Overall, this thesis contributes to the notion that a KD may provide an efficient therapeutic strategy when given along with chemotherapy to treat PDAC and its associated cachexia, and adds to the evidence that KDs are safe when fed long term to KPC mice.

An initial observation was that KPC mice fed a strict KD that contained 85% fat and 15% protein had significantly higher ketone bodies than CD-fed mice at one month post intervention, which was maintained two months after the start of the diets. These results are evidence that KDs could be adjusted to a lower fat, higher protein/carbohydrate content, while still inducing ketosis and its associated beneficial effects. When exploring the effects of KD with or without GEM in survival and in the pancreatic tumor, this thesis provides evidence that KD in combination with GEM exhibited a significant increase in overall median survival when compared to mice fed a CD. Since treatment with a KD alone did not have a significant effect on overall median survival, we can conclude that dietary changes themselves were insufficient to cause the tumor responses, and that it is the synergistic effect between the specific diet and chemotherapeutic agent what improved the prognosis of the KPC mice. Moreover, in this work sex-specific differences were evaluated. Although the disaggregated data between females and males showed that the effect of a KD plus GEM was significant in female KPC mice and not in males, our linear regression model indicated that the effect of a KD plus GEM is not sex dependent.

In order to elucidate the cellular mechanisms underlying the beneficial effects of a KD plus GEM (KG) treatment on pancreatic tumors, a mechanistic study in which 3-month-old KPC mice

bearing pancreatic tumors were treated with either CG or KG for 2 months was conducted. The RNA-Seq analysis followed by HALLMARK gene set enrichment analysis (GSEA) on female pancreatic tumors obtained from KG or CG mice indicated that PI3K-AKT-MTOR and fatty acid metabolism signatures were two pathways differentially affected in the KG group compared to CG. Moreover, in pancreatic tumors, a KD inhibited ERK, AKT and IGFR, which are signaling pathways involved in cancer metabolism and drug resistance; thus, a KD may contribute to the effectiveness of GEM and, thus, improve survival. Moreover, the exploration of the modulation of KG-treatment in the gut microbiota and in the intrapancreatic composition of FAs had not been previously done in any model. An intratumor reduction of fatty acids like palmitic acid, myristoleic acid, palmitoleic acid, asclepic acid and linoleic was evidenced following KG treatment. When comparing bacterial changes from baseline to one month after treatment, KG treatment led to increased relative abundance of *Faecalibaculum* (shown to inhibit tumor growth in breast cancer models) and reduction of *Lactobacillus* (shown to promoting tumor growth), which provides a partial explanation of the beneficial effects of KD in combination with gemcitabine observed in KPC. In summary, the main contribution of this thesis findings is that KD works synergistically with GEM to improve median survival of KPC mice, and that the mechanisms of this beneficial response are multifactorial, including inhibition of ERK and AKT pathways, and the regulation of fatty acid metabolism in the pancreas, plus the modulation of gut microbiota.

The second specific aim of this thesis was to evaluate whether a feeding a strict KD, with or without gemcitabine, mitigates cancer-associated cachexia (CAC) in tumor-bearing KPC mice and to explore several cellular mechanisms regulating muscle protein synthesis and degradation might explain the beneficial effects of a KD in PDAC-associated cachexia. Furthermore, we evaluated the role of sex on cachexia outcomes.

In the survival study, a KD concomitant with GEM resulted in the mitigation of muscle strength decline over time. Moreover, female KPC mice fed a KD showed higher muscle weights compared CD fed ones. In addition, anorexia (assessed by caloric intake) was diminished in the

KG groups when compared to CG and lessened in KG treated female mice, which could be a factor in the maintenance of muscle function. Mechanistically, our findings evidence higher total acetyl-lysine levels in our KPC mice on the KG group when compared to CG, specifically in females. This work also presents evidence about some sex-specific differential effects of KG treatment at the muscle level, including the inhibition of autophagy in KPC female mice, the higher total acetyl-lysine levels, and the phosphorylation of eIF2α in the KG group compared to CG, particularly in females. In summary, our findings indicate that a KD in combination with GEM is beneficial in the preservation of skeletal muscle function in pancreatic tumor bearing mice and that such response to a KD differs between females and males.

Finally, this thesis contributes to the evidence that a KD in combination with GEM appears to be safe showing no adverse effects on liver physiopathology or function. For the first time, a complete safety profile was evaluated to probe that KG treatment over two months is safe in KPC mice. Feeding a strict KD in combination with GEM failed to significantly affect body weight, liver weight, liver aminotransferases, liver markers of inflammation and oxidative stress, or liver enzymes involved in ketone bodies and glucose metabolism in KPC mice. In addition, KG did not increase markers of liver-lipid accumulation nor serum cholesterol and triglyceride levels. In summary, a KD in combination with GEM appears safe with no apparent liver toxicity. These safety data support the evaluation of a KD as an adjuvant dietary treatment for pancreatic cancer in the clinical setting.

Overall, our results suggest KD as a potential beneficial adjuvant dietary treatment for PDAC and CAC. Additional research is warranted to further investigate the mechanisms through which a KD together with gemcitabine improves survival and mitigates the detrimental effects of CAC and how such diet-treatment combination can be optimized for clinical advantage in PDAC-patients.

LIMITATIONS

One of the major limitations of our study was that the breeding strategy to generate KPC mice and to maintain the colony is highly time and resource consuming, so it took us several years to reach a relevant sample size for the survival study. Once the mechanistic aspect of the study was incorporated, the inclusion of animals was restricted due to time and resources. In addition, primary lung tumors also arise spontaneously in KPC mice, yet remain undetectable until euthanasia, so our final sample size numbers for analysis were hampered by the removal of several animals who presented with lung tumors and do not faithfully represent the PDAC model. Finally, the COVID-19 pandemic halted the possibility of including more animals to the mechanistic study.

Another important aspect to consider is that although pancreatic tumors in the KPC model arise spontaneously, they develop very quickly and generate a locally invasive dense tumor by 3 months of age. In humans, the median age of diagnosis of PDAC is over 70 years old. Furthermore, we used gemcitabine monotherapy to be given concomitantly with the ketogenic diet in our research model, yet a combination of chemotherapeutics is much more used in the clinical setting. Therefore, the aging difference between our pre-clinical model and PDAC-patients, plus the type of chemotherapies used, are factors that could impact the translation of our pre-clinical findings into the clinical setting.

Overall, financial restrictions were a boundary to the scope of this thesis. For example, a key benefit of the KPC model is their competent immune system but were not able to explore the impact of the KD/KG treatments on the immune microenvironment and/or their immune-biological effects due to time, personnel, and budget limitations. Moreover, we were only able to send a very limited number of samples for RNA-Seq analysis and only female pancreatic tumors obtained from KG or CG mice after 2 months of treatment were analyzed. Additionally, although we collected weekly fecal samples for all animals in both the survival and mechanistic studies, only

a small subset of samples from baseline and one month post treatment was sent for 16S rRNA sequencing analysis of the microbiota.

Finally, because of the unpredictable nature of the survival study and the impact the COVID-19 pandemic had during the mechanistic study, the personnel able to assist during surgeries was narrowed to one or 2 individuals. Therefore, the amount of tissue collected, particularly of muscle, was limited, which in turn restricted the potential experiments to be done.

FUTURE DIRECTIONS

Our data in the autochthonous and clinically relevant KPC mouse model strongly suggests that a KD should be evaluated concomitant to chemotherapeutic treatment in the clinical setting. Nevertheless, KD composition (macronutrient distribution and/or amount and type of fats), length of the treatment, and type of combination drugs, plus the differential effects on survival, tumor microenvironment, cachexia and safety that such changes could cause should be researched. The KD used in our study was mainly prepared with lard, so it would be relevant to evaluate whether a KD from other less saturated fat sources provides an additional beneficial effect. Additional studies are warranted to validate whether one or more of these fatty acids could explain, in part, the beneficial effect of a KD in PDAC, and whether the modifying the type of fat used in the KD could lead a higher tumor inhibitor effect.

It should be considered that due to the high prevalence of pancreatic enzyme insufficiency in patients with PDAC, pancreatic enzyme replacement therapy (PERT) is usually recommended. Future studies should evaluate the effect of KD with PERT concomitant to chemetherapeutic treatments in PDAC patients. Finally, besides body composition and gut microbiome composition, genotype, epigenetics, immunobiology, xenobiotic metabolizing enzymes and the interplay of all factors, should be investigated further to better understand the effect of a KD in PDAC.

Additional research is warranted to further investigate the mechanisms through which a KD together with GEM mitigate the detrimental effects of cachexia and how such diet-treatment

combination can be optimized. Future studies should focus on identifying a standardized treatment protocol that includes the composition, length, and regimen for a KD, which would assist translating this promising dietary treatment strategy safely to the clinic.

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