### Utililizing a ketogenic diet to target cachexia syndrome in pancreatic cancer

### Background

Pancreatic cancer is the fourth leading causes of cancer-related deaths in United States(1). Pancreatic ductal adenocarcinoma (PDAC) accounts for 95% of all pancreatic cancer cases(2). Despite advances in understanding of pancreatic cancer biology, effective chemotherapeutic modalities for the treatment of patients remain yet to be developed. In addition to the aggressive pathogenesis, around 83% pancreatic cancer patients also demonstrate cancer-induced cachexia, which significantly contributes in all cancer-related deaths(3). Thus inhibition of cachexia along with cancer cell growth may be an effective strategy for the management of pancreatic cancer.

Cancer cells exhibit reprogramming of several metabolic pathways along with multiple genetic, epigenetic and growth signalling alterations(4, 5). Most of the cancer cells demonstrate increased glucose uptake, higher rate of glycolysis and increased secretion of lactate despite the presence of oxygen, a phenomenon known as Warburg effect(6). Aerobic glycolysis plays an important role in rapid cellular growth as it provides several intermediates required for biomass synthesis by routing the carbon flux through pentose phosphate pathway(7). Increased lactate production in aerobic glycolysis, due to the increased conversion of pyruvate in to lactate, leads to acidosis in tumor microenvironments that helps in invasion and metastasis of cancer cells(8). Along with glucose uptake and enhanced aerobic glycolysis, cancer patients also present glucose intolerance and increased hepatic glucose production(9). Increased requirement of glucose might be the important stimulus for enhanced hepatic glucose production *i.e.* gluconeogenesis. As gluconeogenesis is energy consuming process and utilizes muscle and fat breakdown molecules as substrate, it is potentially the most significant contributor to cancer-induced cachexia(10).

The Ketogenic diet is high fat and low carbohydrate diet which leads to elevated circulating levels of ketone bodies, e.g. acetoacetatae,  $\beta$ -hydroxybutyrate and acetone and provides an alternative energy source(11). Ketogenic diets possess anticonvulsant and anti-inflammatory activity(12, 13). It has been proposed that ketogenic diet induces metabolic alterations(14). Keeping in view the significant role of inflammation and metabolic alterations in cancer, a ketogenic diet may provide an efficient therapeutic strategy. Furthermore, most of the cancer cells lack key mitochondrial enzymes which metabolize ketone bodies to ATP while myocytes retain this ability(15). Hence, along with the anti-inflamatory action, a ketogenic diet may act against the cancer-induced cachexia.

The overall objectives of this project were to study the effect of a ketogenic diet on pancreatic cancer-induced cachexia and characterize the molecular mechanism(s) by which caloric restriction leads to metabolic alterations in pancreatic cancer cells.

#### Results

# Treatment with ketone bodies leads to growth inhibition and induces apoptosis

We studied the effect of different doses of sodium-hydroxy-butyrate and lithium aceto-acetate (1mM -20mM) on the survival of Capan1 and S2-013 pancreatic cancer cell lines. We observed that both the ketone bodies inhibit cell survival in a dose-dependent manner. They significantly inhibited the cell growth at 10mM and 20mM concentration after 72h treatment (Fig 1A and B). Furthermore, we investigated the effect of ketone bodies on five different pancreatic cancer cell lines and observed that cell growth is significantly inhibited in a dose-dependent fashion (Fig 1C).

#### Treatment with ketone bodies results in altered metabolism of pancreatic cancer cells

We examined the effect of treatment with ketone bodies on glucose uptake and lactate release in Capan 1 and S2-013 cells. Treatment with ketone bodies resulted in a decrease in glucose uptake (Fig 2A and B) as well as lactate release (Fig 2C and D) in both the cell lines. We also studied the effect of ketone bodies on glutamine uptake. Our results indicate reduced glutamine uptake by Capan 1 and S2-013 pancreatic cancer cells under treatment with ketone bodies (Fig 2E and F).



**Figure1**. Treatment with ketone-bodies inhibits growth and induces apoptosis in pancreatic cancer cell lines. (A) Capan1 & (B) S2-013 cells were treated with different concentration of sodium-3-hydroxy butyrate and lithium aceto-acetate for 72 h and cell viability was determined by MTT assay.(C) (F) Different pancreatic cancer cell lines were treated with 10 mM and 20 mM concentration of sodium-3-hydroxy butyrate and lithium aceto-acetate for 72h and cell viability was determined by MTT assay.

# Treatment with ketone-bodies reduces expression of glycolytic enzymes

To determine the mechanism of reduced glucose uptake and lactate release upon treatment of pancreatic cancer cells with ketone bodies, we next examined if ketone body-mediated changes were due to alterations at the gene expression level. Hence, we evaluated the expression of genes encoding glucose transporter-1 (GLUT1) and glycolytic enzymes hexokinase II (HKII) lactate and dehydrogenase A (LDHA) in ketone body-treated and control cells. We observed reduced expression of GLUT1 and LDHA but no significant change in HKII expression (Fig 3 A and B) in both Capan1 and S2-013 cells. Furthermore, we analyzed the protein expression levels of GLUT1 and HKII in S2-013 and Capan1 cells under treatment with ketone bodies or control. Our results indicate reduced expression of GLUT1 but almost



**Figure2.** Treatment with ketone-bodies induces metabolic alterations in pancreatic cancer cell lines. (A) S2-013 and (B) Capan1 cells were treated with different concentration of concentration of sodium-3-hydroxy butyrate and lithium aceto-acetate for 24h and glucose uptake was determined by using <sup>3</sup>H-2DG uptake assay. Lactate release was determined by calorietric assay using culture medium of S2-013 (C) and Capan1 (D) cells treated with different concentration of sodium-3-hydroxy butyrate and lithium aceto-acetate for 24h. Glutamine uptake (E) S2-013 and Capan1 (F) cells was determined under similar conditions using <sup>6</sup>C<sub>5</sub>-Glutamine.



**Figure 3.** Ketone-bodies repress the expression of key-glycolytic enzymes. (A) Capan 1 and (B) S2-013 cells treated with 10 mM and 20 mM concentration of sodium-3-hydroxy butyrate and lithium aceto-acetate for 24 h. Total RNA was isolated from treated as well as control cell and relative mRNA level of different genes were determined using qRT-PCR.  $\beta$ -Actin was used as internal control. Protein expression of GLUT1 and HK II was determined by immunoblotting using total cell lysate of S2-013(C) and Capan1 (D) after 48h treatment. Tubulin was used as internal control.

**Ketone-bodies** inhibits cell-conditioned tumor medium induce muscle fiber and adipocyte degradationStudies indicate that malignant cells lack key mitochondrial enzymes required for metabolizing ketone bodies to produce ATP. while muscle cells retain this capacity(15). Of beta-hydroxy-butyrate note, improves body weight, while reducing proteolysis in muscle cells(16). Hence, we developed a cell culture-based system to evaluate the effect of ketone bodies on muscle fibers and adipose deposits. achieved This was by differentiating C2C12 premyocytes into myotubes and 3T3L1 pre-adipocytes into differentiated adipocytes. Treatment of these differentiated cells with cancer cell-conditioned medium (CCCM)-induced degradation of differentiated C2C12 myotubes and depletion of adipose deposits 3T3L1 adipocytes. in Treatment with ketone bodies demonstrated significant protection of myotube (Fig 4A and B) and adipocytes (Fig 4D and E) against CCCM. Furthermore, we analyzed the gene expression levels of MuRF1 and atrogin in myotubes fibers, and ZAG and hormone sensitive lipase (HSL) in adipocytes treated with CCCM under treatment with ketone bodies or control. These molecules get upregulated during cancerinduced cachexia(17). Ketone bodies significantly lowered MuRF1 and atrogin mRNA levels even in the presence of CCCM (Fig 4C). Similarly, we also observed significant decrease in ZAG1 and HSL expression upon treatment



Figure 4. Ketone-bodies inhibits tumor cells conditioned medium mediated degradation of myofibres and adipolysis. Differentiated C2C12 cells were grown in S2-013(A) and (B) Capan1 cells conditioned medium with or without 10 mM and 20 mM sodium-3-hydroxy butyrate and lithium aceto-acetate for 72h and bright-field images were represented for individual treatments. (C) Differentiated C2C12 cells were grown in Capan1and S2-013 cells conditioned medium with or without 10 mM and 20 mM sodium-3-hydroxy butyrate and lithium aceto-acetate for 24h. Total RNA was isolated and relative mRNA level of MuRF1 and atrogin was determined by using qRT-PCR. β-Actin was used as internal control. Differentiated 3T3L1 cells were grown in S2-013(E) and (F) S2-013 cells conditioned medium with or without 10 mM and 20 mM sodium-3hydroxy butyrate and lithium aceto-acetate for 72h and stained with nile-red and images for individual treatment were presented. (G) Differentiated 3T3L1 cells were grown in Capan1and S2-013 cells conditioned medium with or without 10 mM and 20 mM sodium-3-hydroxy butyrate and lithium aceto-acetate for 24h. Total RNA was isolated and relative mRNA level of ZAG and HSL was determined by using qRT-PCR. β-Actin was used as internal control.

with ketone bodies (Fig 4F). Hence, our results indicate the utility of ketone bodies in inhibiting cancer-induced cachexia.

Ketogenic diet reduces tumor growth and cachetic phenotype in animal models

To determine the effect of a ketogenic diet on tumor growth, we implanted S2-013 pancreatic cancer cells orthotopically into the pancreas of athymic nude mice. One week later, S2-013 implanted mice were fed ad libitum on a ketogenic diet or normal chow for three weeks. After three weeks on the diets, mice were sacrificed and tumor weight, tumor volume, muscle weight and carcass weight was recorded. S2-013-tumorbearing mice fed on the ketogenic diet demonstrated reduced tumor weight and tumor volume (Fig 5A and B). The ketogenic diet also reduced desmoplasia as observed by Masson's trichrome stain (Fig 5C). Furthermore, we investigated the effect of the ketogenic diet on tumor cell proliferation by performing Ki67 staining on tumor sections. We observed that the diet reduced tumor ketogenic cell proliferation as indicated by the decreased percentage of cells with Ki67-positive staining, in comparison to the tumor cells from mice fed on a control chow (Fig 5C). Overall, our results indicate that a ketogenic diet diminishes tumor growth and proliferation in mice and inhibits cancerinduced cachexia.

#### **Future prospects**

We will further explore the molecular mechanism of ketone bodies-mediated metabolic alterations in pancreatic cancer cells, which may provide potential therapeutic targets. We will also investigate the molecular mechanism of ketone bodiesmediated inhibition of cachetic factor secretion and regulation of myotube and adipocytes physiology. As we have



Figure 5. Ketogenic diet reduces tumor growth and proliferation inhibits cachetic phenotype. Effect of ketogenic diet on tumor weight in S2-013-tumor-bearing mice. $0.5 \times 10^6$  S2-013cells were orthotopically implanted in athymic nude mice. After one week of implantation mice were divided in two group and fed with normal diet (ND) and ketogenic diet (KD). After 3 week of treatment mice were sacrificed and tumor weight (A) and tumor volume (B) were measured.(C)Masson,s trichrome staining and immunohistochemistry images of normal diet and ketogenic diet fed mice tumor sections.

demonstrated that a ketogenic diet leads to reduced tumor growth and cachetic phenotype, it can be utilized in combination with other therapeutic agents.

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