DOCTORAL THESIS

Metabolic Reprogramming Accompanying the Development of Colorectal Cancer

Leenu Reinsalu

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

Leenu Reinsalu

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Kolorektaalvähi arenguga kaasnev metaboolne ümberprogrammeerimine

LEENU REINSALU

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List of Publications

The thesis has been prepared based on the following three publications (I-III) and a submitted manuscript that is not yet accepted. The full texts of the publications and the manuscript can be found in the Appendices 1-4.

I	Rebane-Klemm, E; Truu, L; **Reinsalu, L**; Puurand, M; Shevchuk, I; Chekulayev, V; Timohhina, N; Tepp, K; Bogovskaja, J; Afanasjev, V; Suurmaa, K; Valvere, V; Käämbre, T (2020). Mitochondrial Respiration in KRAS and BRAF Mutated Colorectal Tumors and Polyps. Cancers, 12 (4):815. doi: 10.3390/cancers12040815.
II	**Reinsalu, L**; Puurand, M; Chekulayev, V; Miller, S; Shevchuk, I; Tepp, K; Rebane-Klemm, E; Timohhina, N; Terasmaa, A; Kaambre, T (2021). Energy Metabolic Plasticity of Colorectal Cancer Cells as a Determinant of Tumor Growth and Metastasis. Frontiers in Oncology, 11:698951. doi: 10.3389/fonc.2021.698951.
III	Rebane-Klemm, E*, **Reinsalu, L***, Puurand, M, Shevchuk, I, Bogovskaja, J, Suurmaa, K, Valvere, V, Moreno-Sanchez, R, & Kaambre, T (2023). Colorectal polyps increase the glycolytic activity. *Frontiers in oncology*, *13*, 1171887. https://doi.org/10.3389/fonc.2023.1171887
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* equal contribution

Author’s Contribution to the Publications

Contribution to the papers in this thesis are:

I	The author prepared the tissues for and performed the oxygraphic measurements, analysed the data, created visualisation graphs and figures, and edited the original draft.
II	The author contributed to the conceptualisation, visualisation, writing of the original draft, and editing of the publication.
III	The author contributed to the study’s design, planned and performed the experiments (oxygraphic measurements and real-time quantitative polymerase chain reaction), analysed the data, prepared the figures, and wrote the original draft.
Manuscript	The author participated in designing the study, performing the experiments, analysing the data, preparing the graphs, and writing and editing the original draft.

Introduction

Colorectal cancer ranks as the third most common cancer type globally, accounting for about 1,9 million new cases annually. Its high mortality rate highlights the urgent need for earlier detection. The progression of colorectal cancer is generally a long process that starts with benign growths called polyps, which can then develop into a malignant tumour through the accumulation of multiple genetic alterations. The rapid cell growth and proliferation associated with cancer development significantly amplify energy demands in malignant cells.

Metabolic reprogramming is one of the hallmarks of cancer, showing that cancer cells alter their energy metabolism to sustain rapid proliferation and survival in the tumour microenvironment. The famous theory from Otto Warburg suggests that cancer cells have impaired mitochondria and favour glycolytic energy production even in sufficient oxygen concentrations. However, new studies demonstrate high rates of oxidative mitochondrial respiration in various cancer types. Furthermore, cancer cells can simultaneously activate both glycolysis and oxidative phosphorylation, thus exhibiting a hybrid phenotype that contributes to the plasticity of metabolism. A comprehensive understanding of metabolic reprogramming during colorectal cancer development could lead to the discovery of novel diagnostic biomarkers.

Traditionally, metabolic studies focus on single targets, such as genes or proteins. Moreover, these studies often use 2D cell cultures, which unfortunately do not always accurately represent in vivo conditions, as cell metabolism depends on the surrounding microenvironment. This thesis employs a range of methods that allow the evaluation of metabolic differences at several levels. The methods include high-resolution respirometry to study the functional changes in mitochondrial outer membrane permeability and quantitative reverse transcription polymerase chain reaction to assess differences in transcript levels. All experiments were performed on postoperative clinical material to replicate natural conditions best.

Abbreviations

AK	adenylate kinase
CK	creatine kinase
CK-BB	brain type creatine kinase
Cr	creatine
CRC	colorectal cancer
cyt c	cytochrome c
ERK	mitogen-activated protein kinase
G6P	glucose-6-phosphate
hCINAP	human coilin-interacting nuclear ATPase protein
HIF-1	hypoxia-inducible factor 1
HK	hexokinase
K_m(ADP)	Michaelis-Menten constant for exogenous ADP
GLUT	glucose transporter
LDH	lactate dehydrogenase
MCT	monocarboxylate transporter
MEK	mitogen-activated protein kinase kinase
MIM	mitochondrial inner membrane
MOM	mitochondrial outer membrane
MSI	microsatellite instability
mtCK	mitochondrial creatine kinase
OXPHOS	oxidative phosphorylation
PCr	phosphocreatine
ROS	reactive oxygen species
SSPs	sessile serrated polyps
UQ	coenzyme Q
VDAC	voltage-dependent anion channel
V_{max}	maximal ADP-induced respiration rate

1 Literature Review

1.1 Colorectal Cancer Development

Colorectal cancer (CRC) is a malignant tumour type resulting from upregulated cell proliferation in the colon or rectum. It is the third most prevalent cancer type globally, accounting for approximately 1,9 million new cases annually (World Health Organization, 2023). CRC typically exhibits a long, asymptomatic development during its early stages, leading to late-stage diagnoses when cancer has metastasized to distant organs, complicating treatment efforts (Kuipers et al., 2015; Simon, 2016). As a result, CRC ranks second in cancer-related mortality, making it one of the leading causes of cancer death (World Health Organization, 2023).

The development of CRC tends to be a slow, multistep process involving a series of morphological, histological, and genetic alterations that accumulate over time (Figure 1A) (Frank, 2007). It typically begins from benign, precancerous growths called polyps. Polyps derive from cells within the intestinal mucosa and form through the aggregation of abnormal cells that finally protrude into the intestinal lumen. Two types of polyps have a malignant potential – adenomas and sessile serrated polyps (SSPs). Adenomatous or neoplastic polyps are the more common cause of CRC, with around 60-70% of all CRCs developing from adenomas, while the remaining 25-35% originate from SSPs (Conteduca et al., 2016). The likelihood of polyps developing into CRC increases as they grow in size, and genetic mutations and epigenetic changes start accumulating in the growth. Finally, these alterations enable the polyps to invade nearby tissues. The transformation from adenomatous polyps into carcinoma is known as the adenoma-to-carcinoma sequence (Ahnen, 2011; Shussman & Wexner, 2014). The cumulative genetic alterations contributing to this transformation can be categorized into three subgroups:

a) mutations in proto-oncogenes;
b) mutations or deletions reducing the activity of tumour suppressor genes;
c) mutations damaging genes involved in the DNA repair system (Shussman & Wexner, 2014).

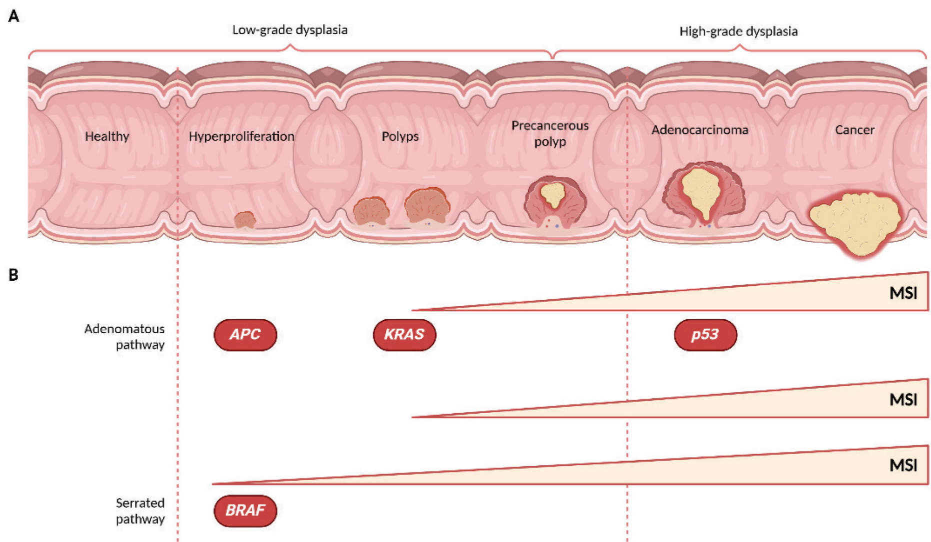


Figure 1: Genetic alterations initiating the development of colorectal cancer. (A) Colorectal cancer starts with hyperproliferation of cells in the colon or rectal wall, forming benign polyps. As polyps grow, the cells develop high-grade dysplasia and can finally penetrate the colon wall and invade nearby tissues. (B) Different genetic alterations affect the development of colorectal cancer from adenomas or sessile serrated polyps. The adenomatous pathway involves mainly mutations in the APC, KRAS, and p53 genes, while BRAF mutations often initiate the serrated pathway. Tumourigenesis is frequently accompanied by microsatellite instability (MSI) caused by impaired DNA mismatch repair. Created with BioRender.com.

Adenomas exhibit low-grade dysplasia, presenting minimal cellular and structural atypia. The accumulation of genetic alterations accompanying the hyperproliferation of cells in polyps can lead to a higher-grade dysplasia, triggering the transformation into invasive carcinoma (Frank, 2007). This malignant tissue can then protrude through the walls of the colon and rectum and invade nearby tissues. In addition, the malignant growth often upregulates vascularisation, promoting the possible spread of cancerous cells to distant organs (Ahnen, 2011; Shussman & Wexner, 2014; Simon, 2016).

1.1.1 Oncogenic Mutations in Colorectal Cancer Development

Adenomas and SSPs generally follow different genetic pathways when developing into carcinomas (Figure 1B) (Bateman, 2014). Adenomas are associated with the chromosomal instability pathway, characterized by an accumulation of genetic mutations. The pathway typically begins with mutations within the tumour suppressor gene *APC*, the most common mutation in CRC. Mutations in *APC* lead to subsequent mutations in several proto-oncogenes, mainly in the *KRAS* oncogene. Finally, these mutations collectively result in the loss of function of the *p53* gene, one of the primary tumour suppressor genes, which normally prevents uncontrolled cell growth and proliferation (Pino & Chung, 2010). However, the development of SSPs usually starts with mutations in the *BRAF* oncogene (Bateman, 2014; Yamane et al., 2014).

Point mutations in *KRAS* and *BRAF* oncogenes are some of the most well-characterized genetic alterations in CRC development and are used in clinical settings as prognostic and

predictive biomarkers (Kocarnik et al., 2015). For a long time, mutations in these genes were considered mutually exclusive; however, recent evidence suggests that, although rarely, they can occur concomitantly in some patients (Midthun et al., 2019).

The *KRAS* oncogene belongs to the Ras superfamily, which comprises more than 160 small GTPases in humans (Rojas et al., 2012). RAS proteins are crucial in several cellular processes, including the cell cycle and survival, making them essential in cancer development (Colicelli, 2004). Mutations in *RAS* genes are found in more than 30% of human cancers (Cicenas et al., 2017). The Ras superfamily has five subfamilies, with the Ras subfamily being the most prominent in cancer research. One of the first *RAS* genes discovered was *KRAS* (Chang et al., 1982), which is now also one of the most studied oncogenes. The KRAS protein is vital in cellular signalling pathways that control proliferation, anti-apoptosis, survival, and differentiation (Karnoub & Weinberg, 2008). Point mutations in the *KRAS* gene can affect cancer initiation and progression through three pathways (Figure 2) (Cicenas et al., 2017):

1) The most important and well-characterised is the role of RAS proteins in the MAPK pathway, where GTP-bound activated RAS proteins interact with and activate the RAF proteins. These RAF proteins, in turn, will phosphorylate mitogen-activated protein kinase kinases (MEK), which subsequently will activate mitogen-activated protein kinases (ERK). The pathway determines the activation of several transcription factors that control and regulate cell division, and therefore, *KRAS* mutations can cause continuous cell proliferation (Marais et al., 1995).
2) RAS proteins also activate the PI3K/AKT/mTOR pathway by directly interacting with and activating PI3K. This interaction leads to the phosphorylation of several target proteins, including AKT. AKT kinases are responsible for critical cellular processes, such as proliferation, survival, and cell growth, making their constant activation potentially cause tumourigenesis (Rodriguez-Viciana et al., 1994).
3) Lastly, RAS proteins can activate RAS-related RAL proteins, which regulate cellular processes like gene expression, cell migration, proliferation, and suppression of apoptosis through the NF-κB pathway (Cascone et al., 2008).

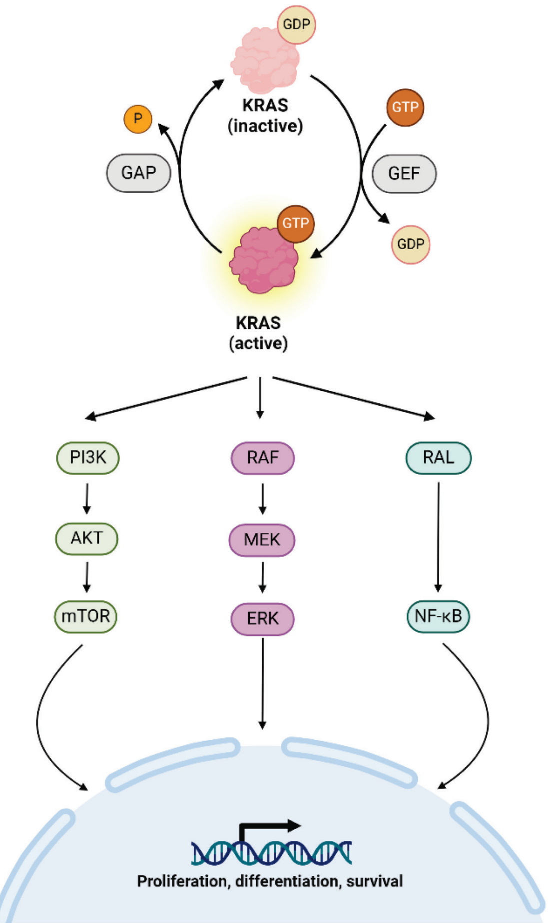


Figure 2: The three tumourigenic pathways downstream of KRAS mutation. Point mutations in the KRAS gene promote cell proliferation, differentiation and survival by activating the PI3K/AKT/mTOR pathway, the MAPK pathway, or the NF-κB pathway. Created with BioRender.com.

KRAS mutation is one of CRC's most prevalent genetic alterations, accounting for approximately 40% of all diagnosed incidences (Muzny et al., 2012). The most frequent are activating missense mutations in codons 12 and 13, affecting the GTPase functions and leading to constant RAF activation (Scheffzek et al., 1997). *BRAF* mutations are not as common, being detected in around 11% of all CRCs (Muzny et al., 2012). Most commonly found are V600E mutations, altering the Valine 600, which BRAF requires to retain the inactive conformation without RAS activation. Therefore, the mutation causes continuous signalling of the RAF-MEK-ERK pathway (Wan et al., 2004).

1.2 Mitochondrial Respiration

Mitochondria are the double membrane organelles in cells. Although they are mainly known as the powerhouses of the cell, mitochondria have many distinct responsibilities and contribute to several cellular processes. In addition to being a site for oxidative phosphorylation (OXPHOS), mitochondria are also a location for many anabolic pathways.

Furthermore, mitochondria regulate apoptosis and Ca^{2+} homeostasis while contributing to cellular signalling pathways through reactive oxygen species (ROS) production (Herst et al., 2018; Simula et al., 2017).

ATP synthesis in the cell generally starts from glycolysis, which takes place in the cytosol, where several enzymatic steps finally convert glucose into pyruvate. The voltage-dependent anion channel (VDAC) and mitochondrial pyruvate carrier then transport pyruvate into the mitochondria, where it undergoes two subsequent metabolic processes – tricarboxylic acid cycle (TCA cycle) and OXPHOS. Cellular respiration theoretically produces a total of 36 ATP molecules per glucose molecule, with the majority (34 molecules) generated through mitochondrial processes (Bonora et al., 2012; Cox, 2013; Siekevitz & Potter, 1955).

Mitochondria contain major enzymatic systems that produce energy using oxygen. Each part of mitochondria has its specific role and carries out different steps of ATP production. The TCA cycle, or the Krebs cycle, takes place in the mitochondrial matrix. Carbohydrates enter the mitochondria as pyruvate, which is then converted into acetyl-CoA. The TCA cycle consists of seven biochemical reactions to release energy through acetyl-CoA oxidation. As a result of the TCA cycle, NADH and $FADH_2$ will carry the free energy as electrons to the electron transport chain (ETC) (Cox, 2013; Fernie et al., 2004).

The mitochondrial outer membrane (MOM) separates mitochondria from the cytosol while allowing the transport of metabolites through the VDAC (Colombini et al., 1996) and the transport of nuclear-encoded proteins through MOM translocase (Pfanner & Wiedemann, 2002). However, the mitochondrial inner membrane (MIM) is the primary location for OXPHOS. MIM forms the functional structures of mitochondria, called the cristae, to expand the surface area of the membrane and thus increase the OXPHOS efficiency (Mannella et al., 1994; Palade, 1952). The ETC carries out OXPHOS in cristae (Gilkerson et al., 2003). ETC contains five enzyme complexes and two electron carriers carrying redox reactions to transfer electrons from electron donors to electron acceptors (Chance & Williams, 1955). The first complex (CI), NADH dehydrogenase, and the second complex (CII), succinate dehydrogenase, catalyse the transfer of electrons from reducing agents to coenzyme Q (UQ). CI uses NADH as its substrate, while CII uses succinate via FAD (Hatefi, 1985). The lipophilic UQ then carries the electrons to cytochrome c (cyt c) oxidoreductase, the third complex (CIII) in the respiratory chain (Kröger & Klingenberg, 1973). CIII subsequently transfers the electrons from the reduced UQ to the hydrophilic heme protein cyt c located on the outer surface of the MIM (Margoliash et al., 1973). This protein then conveys the electrons to the fourth ETC complex (CIV), which catalyses the transfer of electrons from cyt c to molecular oxygen. CI, CIII, and CIV generate a proton gradient across the MIM. This proton gradient is then utilized by the fifth ETC complex, ATP synthase, to synthesize ATP from ADP and inorganic phosphate (Burke, 2017; Cox & Nelson, 2000).

1.2.1 The Role of Mitochondria in Tumourigenesis

Due to its diverse roles in bioenergetics, biosynthesis, and signalling, mitochondria are the crucial mediators of tumourigenesis, and some groups have even proposed that cancer is a mitochondrial metabolic disease (Vyas et al., 2016). Mitochondria generate the vast majority of ROS in the cells. However, ROS production is often enhanced even further in cancers, which can contribute to genomic instability, cause genetic mutations, and promote tumourigenesis (Shadel & Horvath, 2015; Sullivan & Chandel, 2014).

Additionally, evidence shows that ROS-mediated oncogenic signalling can promote tumour cell migration and, therefore, cancer metastasis (Porporato et al., 2014).

The primary source for ROS production in mitochondria is the ETC, and there is much evidence showing that the activity of the ETC is often deregulated in cancer cells. For example, the mtDNA mutations of CI can initiate cancer by enhancing the metastatic potential through ROS (Ishikawa et al., 2008; Koshikawa et al., 2017). Additionally, CI deregulation could induce tumourigenesis by limiting apoptosis (Park et al., 2009). The role of CIII in carcinogenesis is similar to CI as it contributes to ROS production. The upregulation of CIII has been shown in several human tumours, like lung adenocarcinoma and breast cancer (Gao et al., 2016; Y. Han et al., 2019). Additionally, a subunit of CIII, UQCR2, negatively regulates one of the most important tumour suppressor genes, p53, by inducing its degradation and, therefore, promoting tumourigenesis (Raimondi et al., 2020).

The subunits of CII act as tumour suppressors. Even though the exact mechanism remains unclear, research has reported that the accumulation of succinate due to compromised CII activity contributes to a higher hypoxia-inducible factor (HIF) activity, which in turn activates the transcription of various genes promoting tumourigenesis (Tseng et al., 2018). Studies have shown decreased CII activity in renal cell cancer (Hoekstra & Bayley, 2013; Ricketts et al., 2008) and breast cancer (Kim et al., 2013). However, in CRC, the CII is clearly active (Kaldma et al., 2014).

CIV does not directly participate in ROS production but can promote tumourigenesis through collaboration with the BCL-2 oncogene family. BCL-2 proteins bind to pro-apoptotic proteins on the MIM and thus inhibit apoptosis (Campbell & Tait, 2018; Raimondi et al., 2020). *BCL-2* overexpression has been presented in several human tumour types, like breast cancer, prostate cancer, and colorectal adenocarcinomas (Campbell & Tait, 2018; Chen & Pervaiz, 2007; Suvarna et al., 2019).

Mitochondria also support the rapid proliferation of cancer cells by housing several anabolic pathways within the mitochondrial matrix. For example, the TCA cycle and folate-mediated one-carbon metabolism enable cells to convert carbohydrates and amino acids into lipids, non-essential amino acids, nucleotides, glutathione, heme, and other cellular components necessary for new cellular building blocks (Ahn & Metallo, 2015). Due to their plasticity, mitochondria self-regulate and constantly adapt their mass according to the cell's metabolic needs. In cancer cells, mitochondrial biogenesis is often upregulated, while mitophagy regulates the turnover of damaged and dysfunctional mitochondria (Herst et al., 2018; Vyas et al., 2016). Additionally, normal cells maintain the mitochondrial mass through the balance of fission and fusion. However, studies show that cancer cells increase fission while decreasing fusion, leading to a fragmented mitochondrial network (Senft & Ronai, 2016). This altered mitochondrial dynamics is an important characteristic of *KRAS*-mutated cancer cells. KRAS stimulates mitochondrial fragmentation by regulating the phosphorylation of Drp1 via the MAPK pathway. The remodelling of mitochondrial networks in *KRAS*-mutated tumour cells led to impaired cell growth and mitochondrial function, demonstrating the tumourigenic effect of mitochondrial fragmentation (Serasinghe et al., 2015).

1.3 Metabolic Reprogramming in Cancer

Cancer as a neoplastic disease is characterised by deregulated cell growth and proliferation in addition to suppression of cell death. Cancer cells must adapt their energy production pathways to fuel abnormal growth and proliferation. In their second version of Hallmarks of Cancer in 2011, Hanahan and Weinberg (Hanahan & Weinberg, 2011) proposed reprogramming energy metabolism as one of the emerging hallmarks characterising cancer cells. While the general idea of metabolic reprogramming is not new, the exact process remains largely unknown.

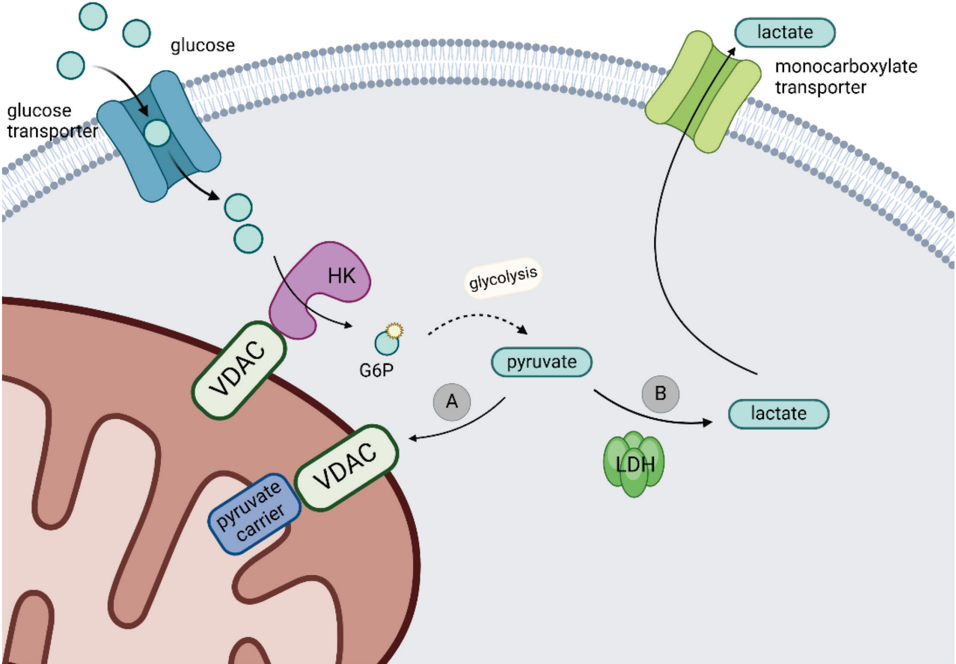


Figure 3: The reprogramming of glucose metabolism in cancer cells. First, the glucose transporter transports glucose into the cell, where it is then phosphorylated into glucose-6-phosphate (G6P) by voltage-dependent anion channel (VDAC) bound hexokinases (HK). Pyruvate, the end product of glycolysis, can then either (A) enter the mitochondrial matrix through VDAC and pyruvate carrier or (B) go through the fermentation into lactate via lactate dehydrogenase (LDH). *Lactate exits the cell through monocarboxylate transporters*, lowering the pH of the extracellular environment, *which in turn promotes tumourigenesis. Created with BioRender.com.*

1.3.1 Warburg Effect

The oldest and one of the most well-known theories on the metabolic reprogramming of cancer cells is their switch toward aerobic glycolysis. In healthy human cells, glucose metabolism generally goes through glycolysis and OXPHOS and ends in CO_2. Only in anaerobic conditions is glucose fermented to produce lactate. Otto Warburg proposed in the 1920s that cancer cells use more glucose for energy production than other tissues, and therefore, the phenomenon is now known as the Warburg effect (Warburg, 1925). Warburg extended his hypothesis by proposing that the reason behind increased glycolysis is dysfunctional mitochondria, which he believed to be the primary cause of

cancer (Warburg, 1956). Decades later, many groups presented evidence that cancer cells have respiratory capability and that the fundamental initiating role of tumourigenesis relies upon oncogenes. Nowadays, there is a lot of scientific evidence showing that both anaerobic and aerobic glycolysis can operate simultaneously in cancer cells (Figure 3), and furthermore, tumour cells can exhibit high rates of OXPHOS (Herst et al., 2018; Liberti & Locasale, 2016). However, the Warburg effect remains a hot topic, and the possible benefits of high glycolytic activity have been thoroughly explored.

One big question regarding the Warburg effect is why cancer cells with increased energy needs would shift toward a glycolytic pathway if the ATP production through OXPHOS is much more efficient. Following are the three possible explanations for the phenomenon:

1) ATP synthesis through glycolysis is 10-100 times faster than the complete process of OXPHOS in mitochondria, thus compensating for the theoretical 18 times smaller net ATP production (Shestov et al., 2014). The increased glycolytic activity might give the rapidly proliferating cells, such as cancer cells, an advantage in competing for limited energy resources (Pfeiffer et al., 2001; Slavov et al., 2014). For example, the high glucose uptake by cancer cells limits nutrient availability for tumour-infiltrating lymphocytes, helping cancer evade the immune system (Chang et al., 2015; Ho et al., 2015).
2) Uncontrollably dividing cells require the upregulation of anabolic processes to support biosynthesis. By increasing glucose consumption, cancer cells have a higher availability of carbon, which is then directed into synthesising nucleotides, lipids, and proteins (Cairns et al., 2011; DeBerardinis et al., 2008; Vander Heiden et al., 2009). Additionally, there is a hypothesis that rapidly proliferating cells, such as cancer cells, may require more reducing equivalents in the form of NADPH. Increasing glycolytic activity enables significantly higher NADPH synthesis, which cells then use in reductive biosynthesis, mainly *de novo* lipid synthesis (Vander Heiden et al., 2009).
3) The elevated lactate secretion accompanying increased glycolysis might benefit the multicellular tumour microenvironment. For instance, the decreased pH of the surrounding microenvironment supports cell migration and invasion by disrupting the cell-cell adhesions (Han et al., 2013). Additionally, the acidified microenvironment could contribute to M2 tumour-associated macrophage polarization, which produces immunosuppressive cytokines and promotes cancer cell motility and metastasis (Colegio et al., 2014).

1.3.2 The Role of Hexokinases in the Warburg Effect

Glucose metabolism begins with glucose entering the cell through glucose transporters (GLUTs) in the cell membrane. Inside the cell, the glycolytic pathway starts with the phosphorylation of glucose into glucose-6-phosphate (G6P), catalysed by the hexokinase (HK) (Moreno-Sánchez et al., 2014). In mammals, five isoforms of HKs have been characterised: HK1-HK4 (Katzen & Schimke, 1965; Wilson, 2003) and hexokinase domain-containing protein-1 (Zapater et al., 2022), each varying in their subcellular locations, functions, and kinetics. This thesis will focus on HK1 and HK2, both of which have a high affinity for glucose and are responsible for a large portion of G6P synthesis. HK1 is found ubiquitously in all mammalian tissues, while HK2 is predominantly expressed in tissues with high energy needs, such as the heart and skeletal muscles, as well as several cancer types (Wilson, 2003).

The overexpression of *HK2* in many tumour types has provoked the hypothesis that it plays a crucial role in the Warburg effect. In the 1970s, Bustamante and Pedersen showed (Bustamante & Pedersen, 1977) that cells with high glycolytic activity tend to overexpress *HK2*. They later proposed that the binding of HK2 to MOM through VDAC is the critical switch toward the Warburg effect in cancer cells. According to this model, mitochondrially produced ATP is preferentially directed to VDAC-bound HK and glycolysis while VDAC remains in the open state, allowing exogenous ADP a free access to mitochondria through MOM (Pedersen, 2007; Pedersen et al., 2002).

In addition to mediating the permeability of MOM and increasing the glycolytic activity, the binding between VDAC and HK provides other benefits for cancer cells. Firstly, increased glucose metabolism promotes cell survival in hypoxic conditions (Infantino et al., 2021). Secondly, VDAC-bound HK provides resistance against apoptosis by preventing the release of cyt c (Pastorino et al., 2002; Zaid et al., 2005). Thirdly, since G6P is a vital precursor molecule for most building blocks necessary for new cells, the increased activity of HK2, and thus increased production of G6P, vigorously promotes the growth and proliferation of cancer cells (Rao et al., 2015).

While the roles of the other HK isoforms have been studied less extensively, some groups have shown the overexpression of *HK1* and its binding to VDAC (Duan et al., 2019; Neumann et al., 2010). However, there are three possible reasons for tumours to upregulate HK2 selectively: (1) Along with HK1 and HK3, HK2 has a high affinity towards glucose (Pedersen, 2007; Wilson, 1995; Wilson, 2003), which is favourable from a metabolic standpoint; (2) HK2 has an N-terminal hydrophobic domain that facilitates the binding to VDAC, which in turn increases the enzyme's affinity for ATP fivefold (Bustamante & Pedersen, 1977); (3) compared to HK1, HK2 has a catalytic affinity in both of its glucokinase-equivalent domains (Ardehali et al., 1999; Tsai & Wilson, 1996).

1.3.3 Metabolic Plasticity

Inspired by Otto Warburg, many research groups have explored cancer metabolism, revealing that metabolic reprogramming in cancer cells extends beyond just a shift toward glycolysis. Evidence shows that tumour cells have fully functional mitochondria; in some cancer cells, mitochondrial respiration could even be upregulated (Herst et al., 2018).

The cancer microenvironment is highly heterogeneous, necessitating the adaptation of tumour cell metabolism to support rapid growth and proliferation under both well-oxygenated and hypoxic conditions. This concept has led to the current hot topic in cancer metabolism: metabolic plasticity (Fendt et al., 2020; Niu et al., 2023; Palm, 2021). Metabolic plasticity allows cancer cells to quickly adapt to changing conditions during cancer progression and metastasis. As previously described, tumourigenesis is a multistep process that provides new metabolic challenges to malignant cells. Furthermore, as tumourigenesis progresses and vascularisation occurs, the availability of nutrients and oxygen dynamically changes, requiring cancer cells to adapt to ensure sustained growth and proliferation continuously.

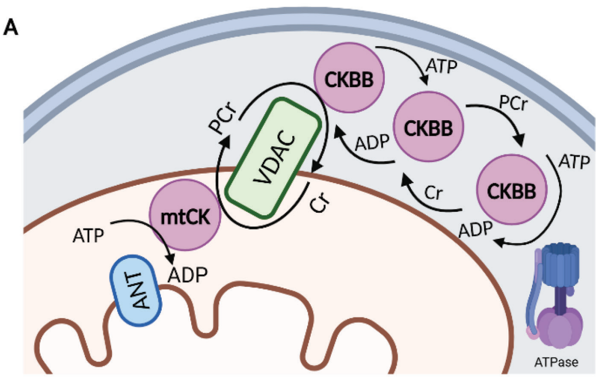
A model proposed by Jia and colleagues (Jia et al., 2019) demonstrates that metabolic plasticity in cancer cells arises from a hybrid metabolic state in which both glycolysis and OXPHOS are both activated. In this model, cytosolic and mitochondrial ROS mediate the complex interplay between HIF-1 and AMP-activated protein kinase (AMPK) (Vander Heiden et al., 2009). Hypoxia stabilises HIF-1α and HIF-2α, transcription factors that regulate several genes promoting cell survival in low-oxygen conditions. For example,

the stabilisation of HIF-1 upregulates several glycolytic enzymes and glucose uptake, as well as a vascular endothelial growth factor, which promotes angiogenesis (Berridge et al., 2010; Landis et al., 2020). As described above, HIF-1α activity can also be induced by the accumulation of succinate in the case of ETC CII downregulation, which causes pseudohypoxia (Hayashi et al., 2019). The precise mechanism regulating the hybrid metabolic phenotype and metabolic plasticity remains largely unknown. However, understanding these mechanisms could uncover new diagnostic or therapeutic targets for cancer treatment.

1.4 Phosphotransfer Pathways

Efficient ATP production is undeniably essential to support the rapid growth and proliferation of cancer cells. However, the effective transport of ATP to the energy-consuming sites within the cell is equally important. This transport is facilitated by energy transport pathways, such as the creatine kinase (CK) and adenylate kinase (AK) systems (Figure 4) that carry the energy-rich phosphate group (Dzeja et al., 2007; Saks et al., 2006). These pathways are fundamental in cells and tissues that require a lot of energy for functioning, such as muscles, the brain, and cancer cells (Watts, 1973).

A



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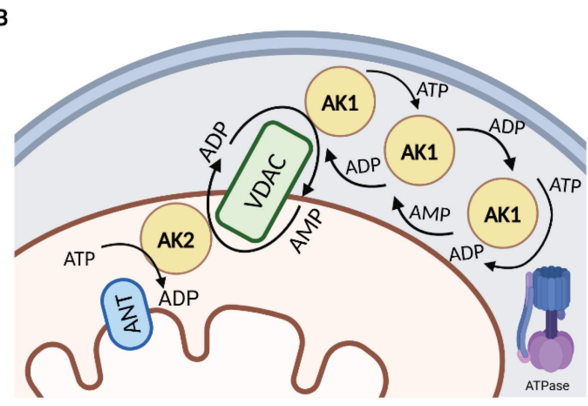

Figure 4: Intracellular phosphotransfer pathways. Creatine kinases (CKs) and adenylate kinases (AKs) form the phosphotransfer networks to carry ATP to energy-consuming sites. (A) Mitochondrial CK isoforms (mtCK) and cytosolic brain isoform (CKBB) catalyse the reversible transfer of phosphate from ATP to creatine (Cr), creating phosphocreatine (PCr) and ADP. (B) Cancer cells often upregulate the AK network, which catalyses the interconversion of adenosine nucleotides. ANT – adenine nucleotide translocase; VDAC – voltage-dependent anion channel. Created with BioRender.com.

1.4.1 Creatine Kinase System

CKs are responsible for maintaining ATP homeostasis in cells by catalysing the reversible conversion of creatine (Cr) and ATP into phosphocreatine (PCr) and ADP (Bessman & Geiger, 1981; Wallimann & Hemmer, 1994). The diffusion of ATP and ADP alone in cells is generally insufficient to maintain ATP homeostasis (Jacobus, 1985). Additionally, PCr and Cr can move through MOM much faster than ADP and ATP (Kuznetsov et al., 1996; Timohhina et al., 2009; Wallimann et al., 2011). The CK system consists of five isoforms encoded by four genes. The three cytosolic isoforms are CK-BB, CK-MM, and CK-MB. While CK-MM and CK-MB are predominantly specific for skeletal and heart muscles, the brain-type CK-BB is also expressed in other tissues like the brain, kidneys, and neuronal cells (Schlattner et al., 2006). The two mitochondrial CK isoforms are the ubiquitous mtCK1 and the sarcomeric mtCK2. These mtCK isoforms are functionally connected to

adenine nucleotide translocase (ANT) in MIM to facilitate the movement of adenine nucleotides near the ATP production site. The cytosolic isoforms are close to ATPases to regenerate *in situ* ATP from the PCr pool at the ATP-consuming site.

Given the high energy demand in malignant cells, many groups have studied the role of CKs in tumourigenesis and metabolic reprogramming. The results from these numerous studies vary significantly, as CK isoforms can be either upregulated or downregulated in cancer. While the precise mechanism underlying the role of the CK system in tumourigenesis remains unknown, several hypotheses describe the relationship. Firstly, many groups have demonstrated that cancer cells require CK isoforms to fight against metabolic stress and inhibit apoptosis (Li et al., 2013; Qian et al., 2012). Secondly, cytosolic CKs can associate with glycolytic enzymes to enhance the conversion of ATP generated by glycolysis to PCr (Gerlach & Hofer, 1986; Kraft et al., 2000; Li et al., 2013). Thirdly, CKs may promote cell motility and metastasis by modulating ATP homeostasis required for cytoskeletal rearrangements (Mulvaney et al., 1998; Yan, 2016). Normal colon epithelium exhibits a high expression of CKs. Therefore, a decrease in CK activity could be necessary for the dedifferentiation of normal cells into tumour cells (Kaldma et al., 2014). Instead, the growth of CRC is associated with the upregulation of the AK system.

1.4.2 Adenylate Kinase System

Like the CK system, the AK network plays a vital role in cellular energy homeostasis. However, AKs catalyse a reversible interconversion of adenine nucleotides (AMP, ADP, ATP). The AK family consists of nine isoforms, each varying in subcellular location and kinetic parameters (Carrasco et al., 2001; Panayiotou et al., 2014). The thesis focuses on isoforms AK1, AK2, AK4, and AK6.

AK1 is a cytosolic isoform found in most human tissues but is predominantly expressed in tissues with a high energy demand (Dzeja & Chung, 2011). Researchers have proposed that *AK1* downregulation may have a tumour-initiating effect. For example, decreased *AK1* expression has been linked to haemolytic anaemia (Matsuura et al., 1989) and the transformation of mouse embryonic fibroblasts into tumour cells (Vasseur et al., 2005). Conversely, studies have shown AK1 upregulation in breast cancer cell lines, suggesting its role in tumourigenesis initiation (Choong & Lim, 2010).

AK2 is in the intermembrane space of mitochondria, mediating the transfer of high-energy phosphoryl group between mitochondria and the cytosol (Dzeja & Terzic, 2009). Studies propose that the interplay between AK2 and mtCKs in the mitochondrial intermembrane space could play a role in malignant transformation (Klepinin et al., 2020). As mtCKs get downregulated in several cancer types, AK2 seems to be upregulated in response (Kaldma et al., 2014; Klepinin et al., 2016; Ounpuu et al., 2017; Klepinin et al., 2020). Furthermore, AK2 could provide a prognostic and therapeutic value in lung adenocarcinomas; its knockdown suppresses proliferation, migration, and invasion while inducing apoptosis and autophagy in human lung adenocarcinoma cells (Liu et al., 2019).

AK4, another mitochondrial isoform, is located in the mitochondrial matrix and actively regulates the TCA cycle and OXPHOS (Liu et al., 2009). Research suggests that AK4's regulation of mitochondrial respiration could have a strong tumourigenic effect. According to one model, AK4 interacts with ANT, VDAC, and HK to form a transmembrane complex, supporting high glycolytic activity in hypoxic conditions by enabling efficient ADP recycling between mitochondrial ATP synthesis and glucose phosphorylation by HK (Fujisawa et al., 2016; Pedersen, 2007). AK4 has been extensively studied in lung cancer,

where its high expression is associated with poor clinical outcomes. By enhancing HIF-1 stability, *AK4* overexpression could promote lung cancer metastasis (Jan et al., 2019; Jan et al., 2019). AK4 upregulation can also promote cell proliferation and invasion in breast and bladder cancer (Xin et al., 2019; Zhang et al., 2019).

AK6, localised in the nucleus, provides energy for nuclear processes such as gene transcription, DNA damage response, and genome stability (Ren et al., 2005). Also known as the human coilin interacting nuclear ATPase protein (hCINAP), AK6 is a unique isoform because it functions as an AK and an ATPase (Xu et al., 2021). hCINAP affects the metabolic reprogramming in CRC by binding to lactate dehydrogenase (LDH), causing a hyperactive Warburg effect (Ji et al., 2017). Additionally, cancer cells may increase AK6 activity in hypoxic conditions via HIF-1α signalling, protecting against apoptosis (Zhang et al., 2020). By regulating rRNA processing and ribosome assembly, AK6 strongly induces cancer-associated gene translation, thus promoting tumourigenesis (Bai et al., 2016). AK6 upregulation has been observed in several solid tumours, including breast cancer (Bai et al., 2016) and CRC (Ji et al., 2017), suggesting its significant role in cancer initiation.

By regulating cellular AMP levels, AKs directly affect AMP signalling. AMP acts as a secondary messenger, activating the energy stress-responsive AMPK. As a master regulator of cellular energy homeostasis, AMPK controls various cellular processes, such as glycolysis (Marsin et al., 2000) and the TCA cycle (Cai et al., 2020). By detecting changes in adenine nucleotide ratios, AMPK activates catabolic processes while shutting down anabolic pathways, thereby upregulating energy production and limiting energy consumption during energy deficits (Hsu et al., 2022). AMPK can function as both a tumour suppressor and an oncogene. As a tumour suppressor, AMPK inhibits the mTORC1 signalling, which is responsible for protein synthesis, cell proliferation, and growth (Gwinn et al., 2008). Furthermore, AMPK has several other downstream targets through which it controls cell proliferation, differentiation, and cell cycle progression (Jones et al., 2005; Liang et al., 2007; Shen et al., 2013). However, increasing evidence supports its role as an oncogene, promoting cancer cell survival by maintaining NADH homeostasis (Jeon et al., 2012) and contributing to cancer metastasis via involvement in the EGF/AKT signalling pathway (Han et al., 2018). Therefore, AMPK's precise role in carcinogenesis remains unknown despite its obvious alterations in various cancer types.

2 Aims of the Thesis

Few published studies are exploring the energy metabolism in colorectal cancer clinical material, and the metabolic reprogramming of colorectal polyps remains an even bigger mystery. However, assessing the metabolic phenotype of premalignant tumours would allow us to better understand the entire carcinogenesis of colorectal cancer and consequently provide us with information that could potentially lay a foundation for earlier diagnosis. Therefore, the aims of this thesis are:

- to explore and characterise the efficacy of mitochondrial respiration in colorectal polyps and colorectal cancer tissue and compare it with healthy control colon tissue;
- to describe the reprogramming of glycolytic pathway in benign and malignant tumours by assessing the changes in the transcript levels of several glycolytic genes;
- to determine the role of phosphotransfer pathways in tumourigenesis by evaluating the changes in creatine kinase and adenylate kinase systems;
- to study if and how *KRAS* and *BRAF* mutational status could affect the direction of metabolic reprogramming during the development of colorectal cancer;
- to explore whether the metabolic phenotype of a colorectal cancer sample could be a prognostic marker for a more aggressive disease.

3 Materials and Methods

To characterize the complex metabolic network of human colorectal cancer and polyps, this study employed the following methods, which are described in detail in the respective publications:

- Clinical material – Publications I, III, and Manuscript
- Preparation of tumour samples and permeabilisation for high-resolution respirometry – Publications I, III, and Manuscript
- Calibration of ADP stock solutions – Publication III
- Oxygraphic measurements – Publication I, III, and Manuscript
- DNA extraction – Publications I and III
- KRAS and BRAF mutation analysis – Publications I and III
- RNA extraction – Publication III and Manuscript
- cDNA synthesis and real-time quantitative polymerase chain reaction – Publication III and Manuscript

4 Results

4.1 The Permeability of the Mitochondrial Outer Membrane for ADP is Different in Control Colorectal Tissue Compared to Polyps and Cancer Tissue

4.1.1 The Functional Parameters for ADP-dependent Mitochondrial Respiration (Publication III)

To study the changes in the kinetics of OXPHOS and, therefore, determine the possible reprogramming of metabolism in colorectal polyps and CRC, we applied high-resolution respirometry on permeabilised postoperative tissues, as described in Publications I, III, and the Manuscript. We calculated the values for the maximal ADP-induced respiration rate (V_{max}) and the apparent Michaelis-Menten constant for ADP (K_m(ADP)), which characterises the MOM affinity to exogenous ADP.

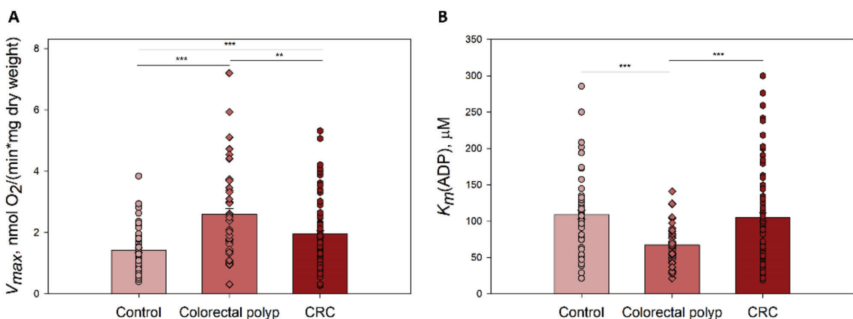

*Figure 5: Kinetic parameters of mitochondrial respiration in control colorectal tissue, colorectal polyps, and colorectal cancer (CRC) tissue. Comparative analysis of (A) the maximal ADP-stimulated respiratory rate (V_{max}); and (B) apparent Michaelis-Menten constant values for ADP (K_m(ADP)) in control tissue (n=46), colorectal polyps (n=42), and CRC tissue (n=57). Data is presented as mean ± SEM. ** p < 0,01, *** p < 0,005 (unpaired t-test).*

Both colorectal polyps and CRC had significantly increased V_{max} values compared to the control tissue (Figure 5A), suggesting an increased OXPHOS capacity in the disease. Interestingly, polyps had an even higher V_{max} value than CRC, showing that the average maximal respiration was higher in benign polyps than malignant tumours. However, K_m(ADP) values were significantly lower in polyps compared to control and cancer (Figure 5B). We showed no difference in K_m(ADP) values between control and CRC. Furthermore, the catalytic efficacy of OXPHOS determined by V_{max}/K_m(ADP) was highest in the polyps (0,039 min^{-1} mg^{-1} mL)) while being similar in cancer tissue (0,019) and the control tissue (0,013).

4.1.2 Mitochondrial Outer Membrane Permeability in *KRAS*- and *BRAF*-mutated Tissues (Publications I and III)

We determined the mutations in *KRAS* codon 12 and 13 of exon 2 and *BRAF* codon 600 of exon 15 (V600E) in polyp and cancer samples using the high-resolution melt analysis described in Publications I and III. We then stratified the samples into three groups based on their mutation status and compared the kinetic parameters V_{max} and K_m(ADP) between the groups.

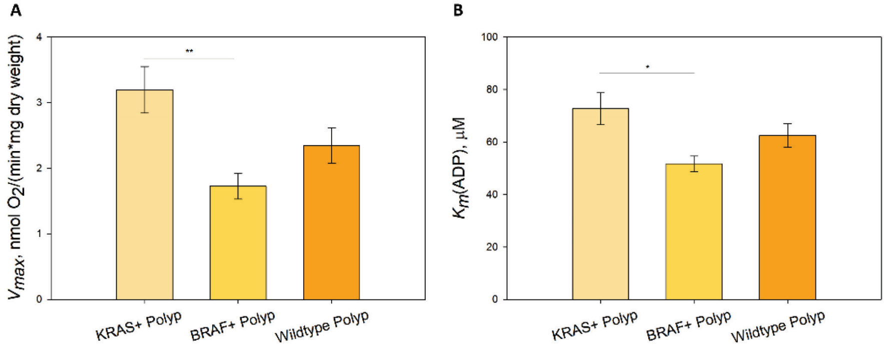

*Figure 6: Kinetic parameters of mitochondrial respiration in KRAS- and BRAF-mutated and wild-type polyps. Comparative analysis of (A) the maximal ADP-induced respiratory rate (V_{max}), and (B) apparent Michaelis-Menten constant values for ADP (K_m(ADP)) in KRAS-mutated polyps (n=14), BRAF-mutated polyps (n=6), and wild-type polyps (n=21). Data is presented as mean ± SEM. * p < 0,05, ** p < 0,01 (unpaired t-test).*

While kinetic profiles were similar between *KRAS*-mutated and wild-type polyps, *BRAF*-mutated polyps had lower V_{max} and K_m(ADP) values than *KRAS*-mutated polyps (Figure 6). The observed changes in kinetic parameters indicate that *BRAF V600E* mutation could already promote metabolic reprogramming at the early steps of CRC development.

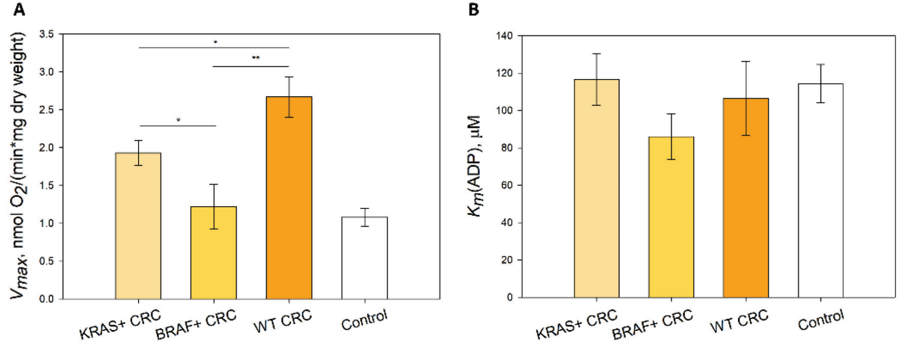

*Figure 7: Kinetic parameters of mitochondrial respiration in KRAS- and BRAF-mutated and wild-type colorectal cancer (CRC). Comparative analysis of (A) the maximal ADP-induced respiratory rate (V_{max}), and (B) apparent Michaelis-Menten constant values for ADP (K_m(ADP)) in KRAS-mutated cancer (n=19), BRAF-mutated cancer (n=7), and wild-type cancer (n=16). Data is presented as mean ±SEM. * p < 0,05, ** p < 0,01 (unpaired t-test).*

We observed no statistically significant differences in K_m(ADP) values between the molecular subtypes of CRC and control tissue (Figure 7B). However, the average V_{max} values showed a significant diversity. Similarly to polyps, *BRAF*-mutated CRC demonstrated the lowest V_{max} value among the studied groups, which is interestingly similar to the control tissue (Figure 7A). The V_{max} values in *KRAS*-mutated tumours were higher than those with *BRAF* mutation. Furthermore, we demonstrated the highest maximal respiration in wild-type CRC samples. These results suggest that the metabolic phenotype of the tumour can largely depend on the driving oncogenic mutation.

4.1.3 The Kinetic Parameters for Mitochondrial Respiration as Prognostic Markers (Manuscript)

We collected disease progression data for 57 patients since 2017 and compared their average V_{max} and K_m(ADP) values. We observed a total of 12 fatalities, out of which 10 had been initially diagnosed with stage 0-II CRC and two with stage III-IV CRC. There was a clear difference in V_{max} and K_m(ADP) values in patients who were still alive and who had succumbed to the disease (Figure 8). The average V_{max} value was 1,39 ± 0,83 for alive patients and 2,83 ± 0,20 for the lethal group (p < 0,001) (Figure 8A). Additionally, we noted a significantly lower K_m(ADP) in the patients who had passed away since the initial analysis (Figure 8B). The catalytic efficiency for the alive group was 0,012 min $^{-1}$ mg $^{-1}$ mL, while in the lethal group, it was 0,042, indicating a more effective OXPHOS system in patients with a more aggressive disease.

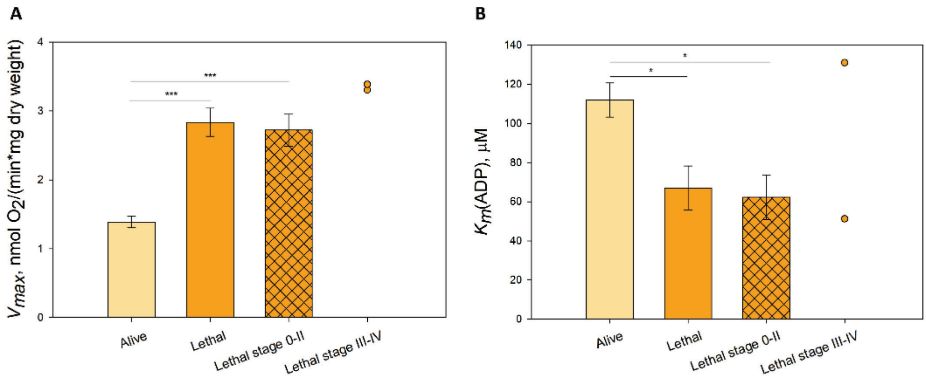

*Figure 8: Kinetic parameters of mitochondrial respiration in alive and dead patients. Comparative analysis of (A) the maximal ADP-induced respiratory rate (V_{max}) and (B) apparent Michaelis-Menten constant for exogenously added ADP (K_m(ADP)) in patients that are alive (n=45) or have passed away (n=12; stage 0-II n=10 and stage III-IV n=2) since their tumour samples were collected. Data is presented as mean ±SEM. * p < 0,05, *** p < 0,001 (unpaired t-test).*

4.2 The Genes of the Glycolytic Pathway Are Deregulated in Polyps and Cancer

4.2.1 mRNA Levels of Glucose Transporter 1 and Hexokinases (Publication III)

To explore the possible upregulation of glycolysis in polyps and CRC, we measured the mRNA levels of *SLC2A1*, coding for GLUT1, *HK1* and *HK2* using the RT-qPCR as described in Publication III. The GLUT family proteins facilitate the glucose transport into the cell through the plasma membrane. The family contains fourteen transporter proteins, among which GLUT1 is the predominant isoform studied in tumourigenesis (Carvalho et al., 2011; Young et al., 2011; Zambrano et al., 2019). Our results demonstrated increased *GLUT1* transcript levels in both benign and malignant tumours compared to control tissue (Figure 9A), suggesting a higher glucose uptake in the diseased than in the healthy tissue.

As described in the literature review, several groups have hypothesised that HK-VDAC binding could promote the Warburg effect and affect VDAC permeability (Bustamante & Pedersen, 1977; Luo et al., 2019). To observe the regulations of HKs in CRC and polyps,

we measured the mRNA levels of HK1 and HK2. Polyps demonstrated a two-fold increase in the average *HK1* transcript level compared to control tissue, while the expression of *HK2* was similar in these two groups (Figure 9B). Interestingly, in CRC, both *HK1* and *HK2* showed a significant decrease in mRNA levels compared to control tissue. The data indicates a higher glycolytic activity in polyps but not in CRC.

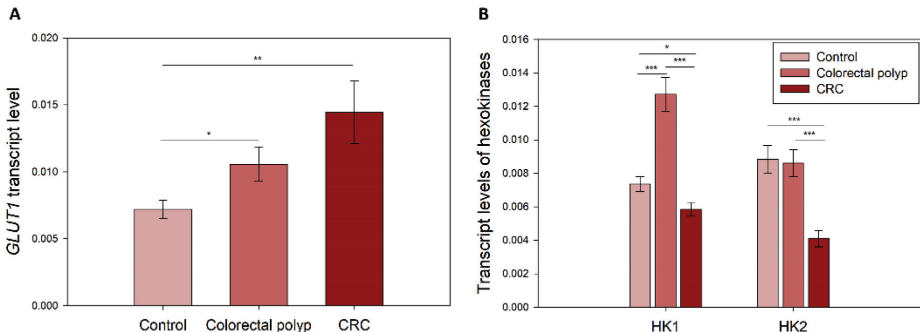

*Figure 9: The characteristics of glucose transporter 1 (GLUT1) and hexokinases (HKs) in colorectal polyps and colorectal cancer (CRC) tissue. The transcript levels of (A) GLUT1 and (B) HK1 and HK2 in control colon tissue (n=27), polyps (n=9), and colorectal cancer (CRC) tissue (n=27). Data is presented as mean ±SEM. * p < 0,05, ** p < 0,01, *** p < 0,001 (unpaired t-test).*

4.2.2 Transcript Levels of Genes Coding for Essential but Non-Controlling Steps of Glycolysis (Publication III)

We studied the deregulation of LDHA further to assess the reprogramming of glycolysis during cancer development. LDHA is an essential enzyme in the glycolytic pathway, converting pyruvate into lactate. Therefore, it marks the point of metabolism where the cell chooses whether pyruvate enters preferentially mitochondria or stays in the cytosol, where it is fermented into lactate.

The results demonstrated a significant increase in *LDHA* transcript levels in polyps, while CRC and control tissue had similar average values for mRNA levels (Figure 10). The high transcript level of *LDHA* in polyps once again suggests a shift towards a glycolytic metabolism in polyps.

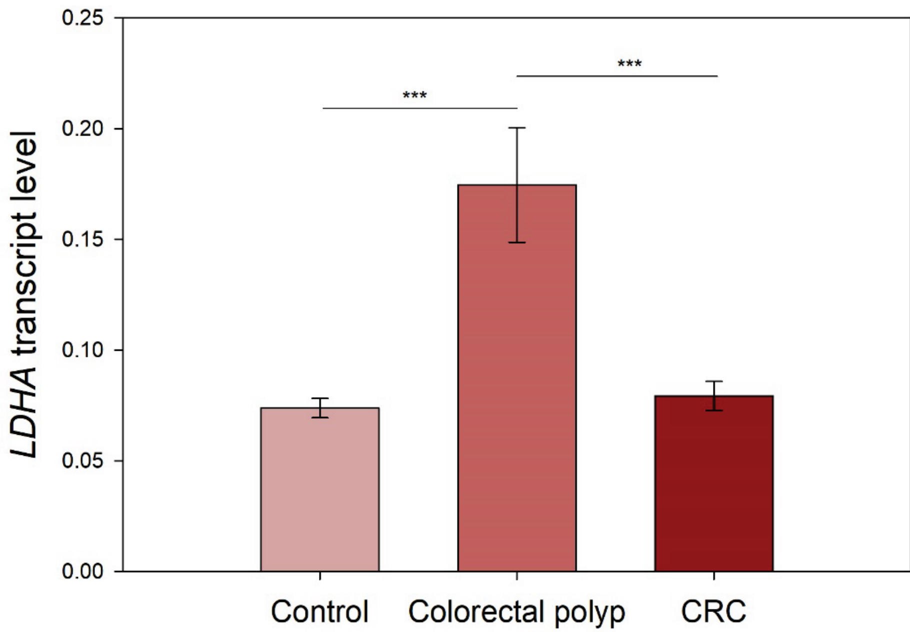

*Figure 10: The transcript levels of LDHA in control colon tissue (n=16), colorectal polyps (n=8), and colorectal cancer tissue (n=9). Data is presented as mean ±SEM. *** p < 0,001 (unpaired t-test).*

Lactate is transported out of the cell through monocarboxylate transporters (MCTs). The MCT family contains 14 transmembrane isoforms that mediate the transport of short-chain monocarboxylates. Only four isoforms – MCT1, MCT2, MCT3, and MCT4 – have a high affinity for lactate (Halestrap & Wilson, 2012), so we explored all of them except MCT3 here.

We measured the mRNA levels of *SLC16A1*, *SLC16A7,* and *SLC16A3,* which encode the previously mentioned isoforms, respectively. The transcript levels of *MCT1* were similar in control and polyp tissue, but the transcription of *MCT2* and *MCT4* showed upregulation in polyps. However, in cancer tissue, the expression of *MCT1* and *MCT2* was downregulated, while *MCT4* demonstrated overexpression compared to control tissue (Figure 11).

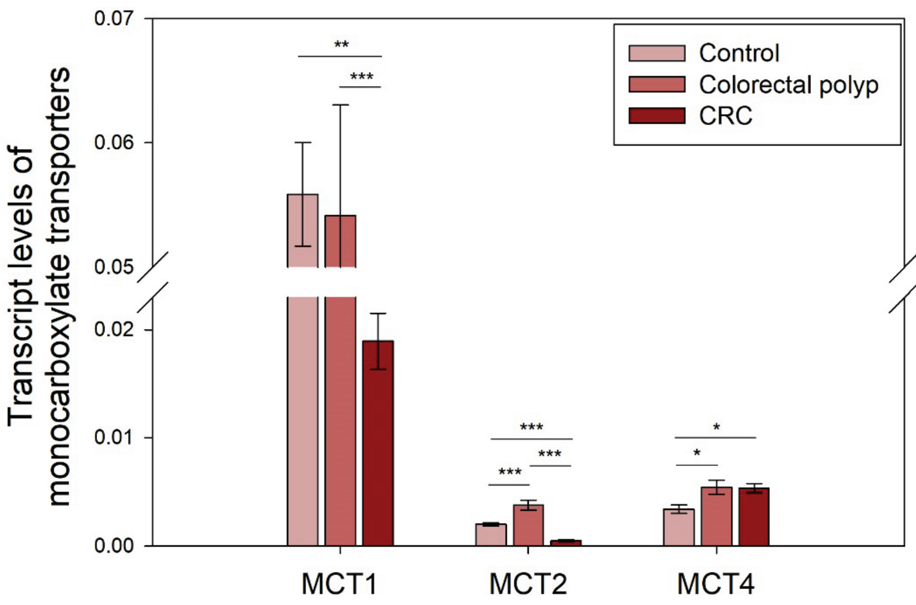

*Figure 11: The expression levels of MCT1 and MCT2 in healthy tissue (n=16), colorectal polyps (n=8), and colorectal cancer (CRC) tissue (n=9), and expression levels of MCT4 in healthy tissue (n=24), colorectal polyps (n=8), and CRC tissue (n=22). Data is presented as mean ± SEM. * p < 0,05, ** p < 0,01, *** p < 0,001 (unpaired t-test).*

4.3 Intracellular Phosphotransfer Pathways (Publication III, Manuscript)

Alterations in intracellular phosphotransfer pathways, e.g., CK and AK systems, are characteristic of many cancer types, including CRC. We determined the transcript levels of several CK and AK isoforms using qRT-PCR to explore the reprogramming of these pathways during the development of CRC.

CKs are crucial in cells with high energy needs, such as cancer cells. We measured the transcript levels of CK isoform *CKBB*, *mtCK1*, and *mtCK2* in control colon tissue, polyps and CRC (Figure 12A). The results demonstrated a high expression of all three isoforms in polyps. Both mitochondrial isoforms presented significant upregulation in polyps compared to a healthy colon. However, cancer tissue showed a strong downregulation of *CK-BB* and *mtCK1* compared to control, suggesting that CKs are not crucial in maintaining energy homeostasis in CRC. Interestingly, CRC's *mtCK2* transcript levels were higher, although its overall expression remains low. AK system could compensate for the downregulation of the CK system in CRC.

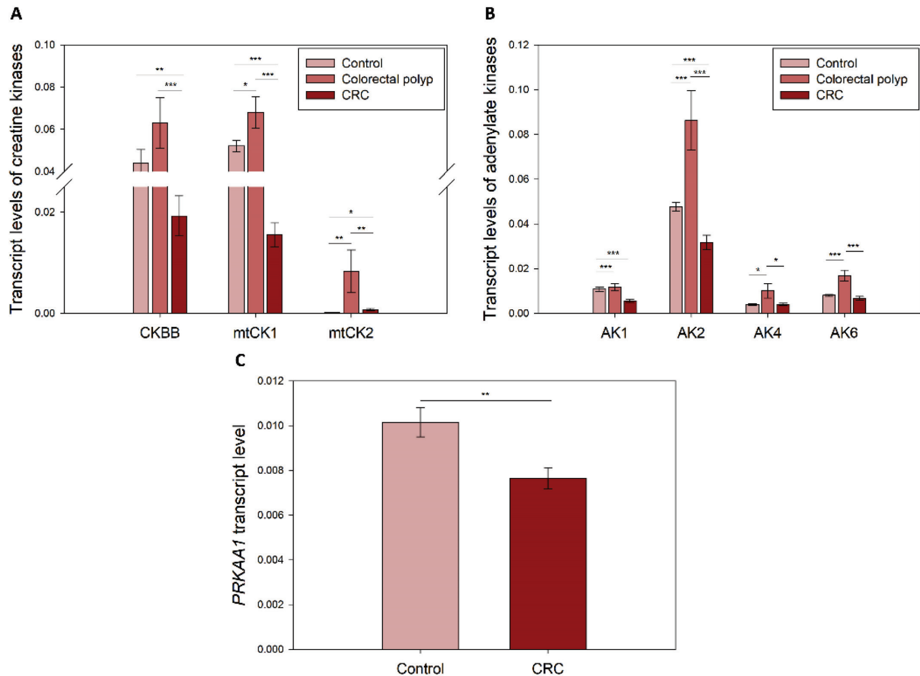

*Figure 12: The characteristics of phosphotransfer pathways in colorectal polyps and colorectal cancer (CRC) tissue. The transcript levels of (A) creatine kinase isoform CKBB, mtCK1, and mtCK2 and (B) adenylate kinase isoforms AK1, AK2, AK4, and AK6 in control colon tissue (n=19), colorectal polyps (n=9), and colorectal cancer (CRC) tissue (n=15). (C) The transcript levels of 5'-AMP-activated protein kinase catalytic subunit alpha-1 (PRKAA1) in control colon tissue (n=24) and CRC tissue (n=24). Data is presented as mean ±SEM. * p < 0,05, ** p < 0,01, *** p < 0,001 (unpaired t-test).*

We studied the transcript levels of isoforms AK1, AK2, AK4, and AK6 (Figure 12B). Interestingly, polyps again increased the expression of three out of four isoforms. Therefore, polyps could upregulate two pathways simultaneously. In CRC, we expected to see the upregulation of AKs in compensation to the downregulation of CKs. However, while *AK4* and *AK6* expression levels were similar in control and CRC, *AK1* and *AK2* were even downregulated.

AKs regulate cellular AMP levels and, therefore, can affect AMP signalling in the cell through AMPK. AMPK is a master regulator of cellular energy that is activated in high concentrations of AMP, which is caused by nutrient deprivation, hypoxia, or oxidative stress. As described in the literature review, it can act as both a tumour suppressor and an oncogene. To study its role in CRC tumourigenesis, we measured the transcript level of *5'-AMP-activated protein kinase catalytic subunit alpha-1* (*PRKAA1*) in control colon tissue and CRC (Figure 12C). The results showed downregulation in cancer, suggesting that AMPK could act as a tumour suppressor in colon cells.

5 Discussion

5.1 The Mitochondrial Outer Membrane Permeability is Different in Colorectal Polyps and Cancer

The flux of ADP, ATP, and inorganic phosphorus through MOM acts through VDAC, as described in the literature review. Therefore, the permeability of VDAC essentially regulates the efficacy of OXPHOS. We applied high-resolution respirometry to study the regulation of OXPHOS and assess the movement of adenine nucleotides through VDAC. It has been shown before that measuring the K_m(ADP) could easily reflect the restrictions in VDAC permeability, as the limitations to ADP movement are visible in permeabilised cells and not in isolated mitochondria (Kay et al., 1997).

When applying high-resolution respirometry, the mitochondrial O_2 consumption upon increasing ADP concentration follows the Michaelis-Menten kinetics. Therefore, by describing the affinity of MOM for ADP, K_m(ADP) characterises the permeability of MOM for adenine nucleotides and, thus, the VDAC permeability (**Publication II**). The previous work has shown significant differences in K_m(ADP) values between oxidative and glycolytic muscles (Kuznetsov et al., 1996), where glycolytic tissue presents a much lower K_m(ADP), indicating less structural and functional restrictions for adenine nucleotide movement through VDAC. In addition, the maximal ADP-dependent oxygen consumption (V_{max}) can characterise OXPHOS efficacy and indicate the tissue's mitochondrial content.

We found significant differences in K_m(ADP) and V_{max} values between control tissue, polyps and CRC, suggesting different metabolic profiles in the groups. Both disease groups demonstrated higher V_{max} values than control colon tissue, indicating an increased number of mitochondria in the tissue that facilitate the extra energy production needed by rapidly proliferating cells. The observation that CRC tissue had a higher V_{max} value than control was in agreement with previous studies (Chekulayev et al., 2015; Kaldma et al., 2014; Koit et al., 2017). Interestingly, polyps had a higher V_{max} value than cancer. The reason could be that while cancer cells have had time to regulate their energy production pathways to work most efficiently, polyps upregulate several pathways to release the proliferative stress and ensure sufficient ATP production. The K_m(ADP) values were similar in control and cancer. On the other hand, polyps demonstrated a much lower average value, suggesting fewer restrictions in adenine nucleotide movement through VDAC and a potential shift towards a more glycolytic metabolic phenotype. However, since the previous studies have measured the K_m(ADP) of 10 μM for glycolytic muscle, the K_m(ADP) of 67 μM in polyps together with the high V_{max} strongly suggests that OXPHOS remains active, and the mitochondria in the tissue stay functional while having fewer restrictions in VDAC permeability.

Furthermore, the catalytic efficiency (V_{max}/K_m(ADP)) was highest in the polyp group (0.039 min^{-1} mg^{-1} mL), while it was similar and lower in cancer and healthy tissue (0,019 and 0,013, respectively). Therefore, the results suggest that during the first steps of cancer development, cells have the highest OXPHOS efficiency while simultaneously increasing the glycolytic activity. This hybrid metabolism, in turn, could show that polyps can quickly adapt and readily use several metabolic pathways according to different tumour microenvironments and, therefore, are characterised by metabolic plasticity.

5.1.1 The Role of Different Oncogenic Mutations in Metabolic Reprogramming

Point mutations in the *KRAS* and *BRAF* oncogenes often drive CRC development by activating the MAPK pathway (Muzny et al., 2012). Studies show that *KRAS* mutations can promote metabolic reprogramming by stimulating glucose metabolism and channelling glycolytic intermediates into anabolic pathways (Bryant et al., 2014; White, 2013). Similarly, *BRAF* mutations are linked to increased glycolytic activity in various cancer types (Soumoy et al., 2020).

The results demonstrated that metabolic reprogramming already accompanies the early stages of cancer development, suggesting that oncogenic mutations might affect mitochondrial respiration. Therefore, we determined the presence of *KRAS* and *BRAF* mutations to analyse whether these mutations have a different effect on metabolic reprogramming by comparing the average V_{max} and K_m(ADP) values. In both cancer and polyps, *BRAF*-mutated samples exhibited significantly lower V_{max} values than *KRAS*-mutated samples, suggesting a more efficient mitochondrial energy production in tumours with *KRAS* mutation. Interestingly, *BRAF*-mutated polyps also had a significantly lower average K_m(ADP) value than those with *KRAS* mutation, indicating lower diffusion of adenine nucleotides through MOM in polyps with *BRAF* mutations. The cells with *BRAF* V600E mutation could potentially display more active glycolysis with a parallel moderate downregulation of OXPHOS. However, no statistically significant difference was observed despite a general trend towards a lower K_m(ADP) in *BRAF*-mutated cancer samples. Unfortunately, based on this data, it remains unclear whether these oncogenic mutations directly cause metabolic programming or if cancer cells adjust energy production in response to increased proliferation caused by the mutations.

5.1.2 Kinetic Markers as Hallmarks of a More Aggressive Disease

Earlier work by Koit suggested that patients with a more aggressive disease tend to initially exhibit higher V_{max} values, while no differences in K_m(ADP) were shown (Koit et al., 2017). This study included 32 patients. To validate these results further, we selected 57 patients for whom K_m(ADP) and V_{max} values had been calculated, and we requested follow-up data from the treating hospital. The data confirmed the earlier finding, showing significant differences in V_{max} values between deceased and alive patients. Thus, a higher V_{max} value (and V_{max}/K_m(ADP) ratio) could be an indicator of a more aggressive disease. One patient in the surviving group had a V_{max} of 4,1 and was excluded from the calculations as an outlier. However, monitoring this patient's disease progression could provide valuable insights into whether a high V_{max} could serve as a prognostic marker.

Additionally, the results revealed that patients who succumbed to the disease had initially lower average K_m(ADP) values. This suggests that aggressive tumours might possess high OXPHOS capacity concurrently with an upregulated glycolysis. Such metabolic plasticity could explain their ability to thrive, proliferate, grow, and metastasize more easily.

5.2 Exploring the Glycolytic Pathway

Otto Warburg showed in the 1920s that cancer cells upregulate glycolysis even in aerobic conditions (Warburg, 1925), and researchers applied this idea of metabolic reprogramming for all cancers. However, nowadays, it is clear that although cancer cells could increase glycolytic activity, OXPHOS remains active in most cancer types. Our results from high-resolution respirometry demonstrated that CRC might have a slight upregulation of

OXPHOS efficacy while having similar MOM permeability as control tissue. However, the decreased K_m(ADP) values indicated a possible increase of glycolytic activity in polyps. The mechanism underlying metabolic reprogramming during the tumourigenesis of CRC remains largely unknown. Therefore, we aimed to additionally explore the glycolytic pathway on the transcript level.

GLUT1 and its role in tumourigenesis have been widely studied in many cancer types (Carvalho et al., 2011; Zambrano et al., 2019) to see whether cells promote glucose uptake to support the Warburg effect. GLUT1 upregulation could be a significant determinant of metabolic reprogramming of CRC since healthy colonocytes do not primarily depend on glucose for energy supply. The results revealed an increase in *GLUT1* expression in disease. Colon polyps had slightly but statistically significantly higher transcript levels than healthy colon, but in CRC, the expression was around two times higher than in control tissue. These data suggest that glucose uptake by cells might increase throughout the tumourigenesis to support the elevated need for energy. However, glucose uptake is not a determinant of aerobic glycolysis or Warburg effect.

HK is the key enzyme that starts the glycolytic pathway by phosphorylating the glucose in the cytosol into G6P. The HK family in humans consists of five isozymes that vary in their subcellular location and kinetics (Wilson, 2003). The most common and well-studied isoforms are HK1 and HK2, which are essential in metabolic reprogramming in cancer cells. Many groups, including Pedersen and colleagues (Bustamante & Pedersen, 1977; Pedersen et al., 2002), have demonstrated HK-VDAC binding in cancer cells and hypothesised that the coupling between HK2 and VDAC could be pivotal in promoting the Warburg effect by directing the mitochondrially produced ATP preferentially towards glycolysis. In the current study, we showed increased *HK1* transcript levels in polyps compared to control tissue, while *HK2* mRNA levels remained similar in control and polyp tissues. Interestingly, both isoforms were downregulated in cancer tissue compared to normal colon and polyps. The higher transcript levels of *HK1* and *HK2* in polyps, together with lower K_m(ADP) values, could suggest a higher activity of the HK system in benign tumours and their possible role in increasing glycolytic activity. However, contrary to the previous belief that CRC is a glycolytic cancer type where HK is upregulated, the results presented lower *HK* transcript levels in CRC tissue, thus suggesting that they might not promote the glycolytic phenotype in this cancer type.

In healthy cells, the end-product of glycolysis is generally pyruvate, which then enters the mitochondria. Only in anaerobic conditions is pyruvate converted into lactate by LDHA. However, according to the Warburg effect, cancer cells would upregulate lactic acid fermentation even in aerobic conditions (Warburg, 1925, 1956). Research has demonstrated increased *LDHA* expression in many cancer types (Claps et al., 2022). Patients with CRC often have elevated blood LDH levels (Xie et al., 2022). We showed increased transcript levels of *LDHA* in polyps but not in cancer tissue. Based on decreased K_m(ADP) values and increased *HK* transcript levels, we proposed that colon polyps might increase glycolytic activity. The elevated *LDHA* mRNA levels suggest that lactate production in polyps increases and further supports the hypothesis above.

Increased lactate production and its release to extracellular space can have a tumour-promoting effect. By decreasing the pH of the tumour microenvironment, elevated lactate levels support the immunosuppression and, therefore, help cancer cells avoid the host immune response (Colegio et al., 2014; Kim et al., 2007). An acidic environment can also break cell-cell adhesions, such as cadherins (Takeichi, 1993), promoting cancer cell migration. The kinetic parameters suggested a shift towards

glycolytic metabolism in more aggressive cancers. Therefore, we can assume that one benefit of stimulating glycolytic activity is increasing lactate production, which makes cancer more aggressive by promoting cell migration and, thus, metastasis.

Lactate export is carried out by MCT proteins, which facilitate the transport of monocarboxylates, such as lactate and pyruvate, as well as short-chain fatty acids like butyrate. Butyrate is the most critical molecule for energy supply in healthy colonocytes. While most cells in the human body preferably use carbohydrates or fatty acids, around 60-70% of the energy produced by cells in the colon is derived from butyrate (Brahe et al., 2013). The microbiota largely influences the metabolism of a healthy colon. By breaking down dietary fibres through microbial fermentation, bacteria located in the lower intestinal tract produce several short-chain fatty acids (Liu et al., 2018), including butyrate. MCT1 carries out butyrate uptake (Lambert et al., 2002). The transcript level of *MCT1* was similar to control in polyps but downregulated in cancer, indicating that while polyps keep using butyrate as a nutrient, cancer cells have reprogrammed their metabolism and do not depend on butyrate. The decrease in butyrate uptake could be complemented by increased glucose uptake in cancer cells, as suggested by the almost two-fold increase in *GLUT1* mRNA levels in CRC.

Polyps upregulated the transcription of both *MCT2* and *MCT4*. MCT2 has a high affinity towards pyruvate (Lin et al., 1998), suggesting its primary role in pyruvate transport. MCT4, however, has a low affinity towards pyruvate and a high affinity towards lactate. Furthermore, it has a higher affinity for intracellular lactate than extracellular lactate (Pinheiro et al., 2010). The increased mRNA levels of *MCT2* and *MCT4,* together with a high *MCT1,* could suggest that polyps upregulate several metabolic pathways simultaneously to enhance nutrient availability and ensure highly effective energy production. CRC cells downregulated *MCT2* transcription but upregulated *MCT4*. MCT4 is upregulated by HIF-1α in hypoxia, which is often a characteristic of CRC and could explain increased transcript levels. Studies show MCT4 relocation to mitochondria in breast cancer cell lines (Hussien & Brooks, 2010). A recent study by Cai and colleagues showed that lactate can enter the mitochondrial matrix and stimulate mitochondrial ETC (Cai et al., 2023). Therefore, it is possible that by relocating to the mitochondrial membrane, MCT4 facilitates lactate transport into the mitochondria in malignant tissue.

5.3 The Rearrangements of Energy Transfer Pathways in Benign and Malignant Tumours

Cells with high energy requirements rely considerably on phosphotransfer pathways to maintain rapid intracellular energy transport and production. These highly organised pathways mediate communication between ATP-consuming and ATP-producing sites, thus maintaining ATP homeostasis in the cell. Considering the increased energy requirements, malignant cells are expected to adapt CK and AK systems to improve metabolic efficacy.

The CK system consists of cytosolic isoforms and mitochondrial isoforms. The cytosolic isoforms can promote the Warburg effect by interacting with glycolytic enzymes (Gerlach & Hofer, 1986; Li et al., 2013). We showed increased transcript levels of cytosolic *CKBB* in colon polyps. As discussed above, polyps upregulate the transcription of several glycolytic transporters and enzymes, such as *GLUT*, *HK*s, and *LDHA*. The increased mRNA levels of *CKBB* suggest that it could promote glycolysis in polyps by interacting with glycolytic enzymes and enhance the consumption of ATP produced by glycolysis by

converting it to PCr. The *CKBB* mRNA level was lower in cancer than in healthy colon; therefore, it is probably less important in cancer cells.

As MOM permeability for adenine nucleotides changes, the functional relationship between mtCKs and ANT becomes more significant. The movement of Cr and PCr through MOM is significantly more convenient than that of adenine nucleotides (Kuznetsov et al., 1996; Timohhina et al., 2009). Therefore, mtCKs facilitate the recycling of ADP and ATP in the mitochondrial matrix and MIM and transport the energy into the cytosol as PCr. Polyps demonstrated increased transcription of both *mtCK* isoforms. Upregulation of CKs could promote tumour development already at the benign stage by inducing proliferation and modulating ATP homeostasis required for cytoskeletal rearrangements (Yan, 2016). CRC tissue downregulated *mtCK1* while upregulating *mtCK2*. However, the total expression of CKs in cancer remains much lower than in control tissue, which is in correlation with previous studies by our group. Kaldma (Kaldma et al., 2014) and Chekulayev (Chekulayev et al., 2015) demonstrated that the enzymatic activity of CKs decreases more than two-fold in CRC compared to normal tissue. Both publications presented the compensatory increase in the efficacy of the AK system by showing higher AK enzymatic activity and measuring AK coupling in permeabilised tissues using high-resolution respirometry.

The AK family consists of nine isoenzymes that vary in function and subcellular location (Carrasco et al., 2001). We studied the mRNA content of cytosolic AK1, mitochondrial AK2 and AK4, and nucleus-located AK6. The results did not show the expected upregulation of the AK system in CRC. Instead, *AK1* and *AK2* transcript levels were lower in cancer tissue than in control. Considering the increased activity of AKs presented in other studies, further research is needed to understand the functional regulation of AKs. They may interact with different protein complexes, like glycolytic or mitochondrial enzymes, to improve localized AMP/ADP/ATP conversion. Interestingly, polyps increased the transcription of *AK* isoform, suggesting that both energy transfer pathways are upregulated at early stages of tumour development to ensure efficient energy transfer in new stress conditions.

As discussed earlier, AKs are crucial in intracellular energy sensing and metabolic signalling. By regulating the conversion between adenine nucleotides, they are able to amplify a slight change in the ADP/ATP ratio into relatively significant changes in AMP concentration. AMP, however, is a secondary messenger, and its high concentrations activate AMPK. The role of AMPK in tumourigenesis remains unclear since it can act as an oncogene or a tumour suppressor (Liang & Mills, 2013). We observed decreased transcript levels of *PRKAA1*, a gene coding for the catalytic subunit of AMPK in cancer tissue, suggesting its role as a tumour suppressor in CRC. An increase in AMP concentrations, and thus AMPK activation, is usually a result of oxidative stress, nutrient deprivation, or hypoxia. When activated, AMPK shuts off the ATP-consuming anabolic process to preserve energy (Hsu et al., 2022). Therefore, it acts as a tumour suppressor by inhibiting protein synthesis, cell growth, and proliferation.

While we did not explore AMPK regulation in polyps, it could potentially provide exciting insights into AMPK's role in CRC development. The highly elevated expression of *AK*s in polyps could subsequently promote AMPK activation. Colon epithelium is generally characterized by hypoxia since it is exposed to a relatively low O_2 concentration (Colgan & Taylor, 2010). Since vascularisation is usually promoted later during tumourigenesis, the microenvironment in benign tumours could be even more hypoxic. Hypoxia, in turn, activates the transcription factor HIF-1α, which regulates several vital

proteins in the glycolytic pathway (Landis et al., 2020). According to the model described in the literature review (Jia et al., 2019), cells with active HIF-1 and AMPK display a hybrid metabolic pathway where glycolysis and OXPHOS work simultaneously, thus having metabolic plasticity. Metabolic plasticity would also explain why polyps have high V_{max}, characteristic of increased oxidative metabolism, and low K_m(ADP), which suggests decreased diffusion restrictions in VDAC permeability and is usually a determinant for glycolytic metabolism (**Publication II**). However, the interplay between HIF-1α and AMPK in colorectal polyps and CRC needs further exploration as it could provide novel insights into how metabolic plasticity is regulated and how it benefits the cells during the multiple steps of cancer development.

5.4 Limitations of the Study

The study has several limitations that could be addressed in future research. Firstly, the sample size, especially for polyps, was limited due to difficulties obtaining material from hospitals. Increasing the number of samples could lead to stronger statistically significant differences between groups and provide more conclusive evidence. It would also allow stratification of samples into smaller groups based on their clinicopathological factors to analyse correlations between metabolic phenotype and a specific tumour type.

Secondly, while measuring the mRNA levels offers valuable insights into the metabolic changes, the transcript levels alone may not be sufficient to draw strong conclusions and fully describe the extent of metabolic reprogramming. Complementary analyses, such as protein quantification and enzymatic assays, are necessary to confirm that the observed transcriptomic alterations lead to functional changes at the protein level. Although the Manuscript demonstrates some differences in protein levels between cancer and control samples, these results were not included in the thesis due to the small sample size and the lack of polyp samples in the dataset.

Thirdly, the thesis primarily focuses on glycolysis and OXPHOS. Expanding the scope to include other metabolic pathways, such as the pentose phosphate pathway or lipid metabolism, could offer a more comprehensive understanding of CRC metabolism. Addressing these limitations in future studies will enhance our understanding of the metabolic reprogramming accompanying the development of CRC.

5.5 Concluding Remarks

The primary aim of the thesis was to explore the metabolic reprogramming that accompanies the development of CRC. The goal was to use comprehensive methodology to explore the complex process of metabolic deregulation represented by changes in transcript levels, as well as differences in kinetic parameters measured by high-resolution respirometry.

A majority of energy metabolism studies are conducted on 2D cell cultures. However, the microenvironment strongly influences cell metabolism, e.g., the media's oxygen and glucose concentrations. Therefore, the current study aimed to utilise postoperative clinical material that could provide valuable insights by representing the in vivo conditions.

Historically, CRC has been considered a hypoxic tumour type that upregulates the glycolytic pathway and presents elevated glucose consumption and lactate production rates. The current study demonstrated that CRC is not fully glycolytic; in fact, the results suggest that it might prefer oxidative mitochondrial respiration.

While the metabolism of cancer cells has been studied quite thoroughly, our knowledge about polyps remains very limited. The thesis revealed several crucial aspects of metabolic reprogramming in these benign tumours. Firstly, the upregulation of glucose transporters and several glycolytic enzymes suggest that colorectal polyps increase glycolytic activity. This notion was supported by decreased K_m(ADP) values that indicate fewer restrictions in MOM permeability. Secondly, polyps demonstrated a high V_{max} value, suggesting higher mitochondrial count and elevated OXPHOS capacity. Therefore, polyps might be characterised by metabolic plasticity, meaning that energy production works simultaneously through glycolytic and oxidative metabolism. Thirdly, polyps showed upregulation of CK and AK systems, suggesting that both pathways are activated at early stages of CRC development to rapidly regulate ATP homeostasis and ensure highly effective energy transport.

In conclusion, the research provides valuable insights into the metabolic reprogramming during the development of CRC. The integral approach of combining different methods and studying changes at several levels unveiled aspects of energy metabolism that have not been described before. By enhancing our knowledge about the early steps of metabolic reprogramming, the results could provide us with possible targets for developing new biomarkers for diagnostics or determining a correct treatment strategy.

6 Conclusions

- The bioenergetic profiles of colorectal polyps and cancer are significantly different from healthy colon, determined by high-resolution respirometry:
  - Colorectal polyps demonstrate increased glycolytic activity while preserving oxidative metabolism (Publication III).
  - Contrary to an old belief, colorectal cancer preferably utilises mitochondrial respiration and is not a highly hypoxic glycolytic cancer type (Publication III).
  - Mutations in the *BRAF* oncogene can induce a shift towards glycolytic energy production already at the early stages of tumourigenesis (Publication I).
  - A high V_{max} value could be an indicator of a more aggressive disease (Manuscript).
- Both benign and malignant tumours present deregulation of the glycolytic pathway, determined by differences in transcript levels measured by qRT-PCR:
  - Polyps upregulate the transcription of *GLUT1*, *HK1*, *LDHA*, *MCT2*, and *MCT*4 (Publication III).
  - Cancer cells upregulate the transcription of *GLUT1* and *MCT4* but downregulate *HK1*, *HK2*, *MCT1*, and *MCT2* (Publication III).
- Creatine kinase and adenylate kinase pathways are modified during the development of colorectal cancer.
  - Colorectal polyps upregulate creatine kinases and adenylate kinases simultaneously (Publication III).
  - Colorectal cancer demonstrated downregulation of both phosphotransfer systems (Publication III).
- Colorectal polyps exhibit characteristics of a hybrid metabolism and, therefore, can be considered metabolically plastic (Publication II).

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References

Ahn, C. S., & Metallo, C. M. (2015). Mitochondria as biosynthetic factories for cancer proliferation. *Cancer & Metabolism*, *3*(1). doi:10.1186/s40170-015-0128-2

Ahnen, D. J. (2011, February). The American College of gastroenterology emily couric lecture the adenoma-carcinoma sequence revisited: Has the era of genetic tailoring finally arrived. *American Journal of Gastroenterology*. doi:10.1038/ajg.2010.423

Ardehali, H., Printz, R. L., Whitesell, R. R., May, J. M., & Granner, D. K. (1999). Functional interaction between the N- and C-terminal halves of human hexokinase II. *Journal of Biological Chemistry*, *274*(23), 15986–15989. doi:10.1074/jbc.274.23.15986

Bai, D., Zhang, J., Li, T., Hang, R., Liu, Y., Tian, Y., … Zheng, X. (2016). The ATPase hCINAP regulates 18S rRNA processing and is essential for embryogenesis and tumour growth. *Nature Communications*, *7*. doi:10.1038/ncomms12310

Bateman, A. C. (2014, October 1). Pathology of serrated colorectal lesions. *Journal of Clinical Pathology*. BMJ Publishing Group. doi:10.1136/jclinpath-2014-202175

Berridge, M. V., Herst, P. M., & Tan, A. S. (2010, November). Metabolic flexibility and cell hierarchy in metastatic cancer. *Mitochondrion*. doi:10.1016/j.mito.2010.08.002

Bessman, S. P., & Geiger, P. J. (1981). Transport of Energy in Muscle: The Phosphorylcreatine Shuttle. *Science*, *211*(4481), 448–452. doi:10.1126/science.6450446

Bonora, M., Patergnani, S., Rimessi, A., de Marchi, E., Suski, J. M., Bononi, A., … Pinton, P. (2012, September). ATP synthesis and storage. *Purinergic Signalling*. doi:10.1007/s11302-012-9305-8

Brahe, L. K., Astrup, A., & Larsen, L. H. (2013). Is butyrate the link between diet, intestinal microbiota and obesity-related metabolic diseases? *Obesity Reviews*, *14*(12), 950–959. doi:https://doi.org/10.1111/obr.12068

Bryant, K. L., Mancias, J. D., Kimmelman, A. C., & Der, C. J. (2014). KRAS: feeding pancreatic cancer proliferation. *Trends in Biochemical Sciences*, *39*(2), 91–100. doi:https://doi.org/10.1016/j.tibs.2013.12.004

Burke, P. J. (2017, December 1). Mitochondria, Bioenergetics and Apoptosis in Cancer. *Trends in Cancer*. Cell Press. doi:10.1016/j.trecan.2017.10.006

Bustamante, E., & Pedersen, P. L. (1977). High aerobic glycolysis of rat hepatoma cells in culture: Role of mitochondrial hexokinase. *Proceedings of the National Academy of Sciences*, *74*(9), 3735–3739. doi:10.1073/pnas.74.9.3735

Cai, X., Ng, C. P., Jones, O., Fung, T. S., Ryu, K. W., Li, D., & Thompson, C. B. (2023). Lactate activates the mitochondrial electron transport chain independently of its metabolism. *Molecular Cell*, *83*(21), 3904-3920.e7. doi:10.1016/j.molcel.2023.09.034

Cai, Z., Li, C. F., Han, F., Liu, C., Zhang, A., Hsu, C. C., … Lin, H. K. (2020). Phosphorylation of PDHA by AMPK Drives TCA Cycle to Promote Cancer Metastasis. *Molecular Cell*, *80*(2), 263-278.e7. doi:10.1016/j.molcel.2020.09.018

Cairns, R. A., Harris, I. S., & Mak, T. W. (2011, February). Regulation of cancer cell metabolism. *Nature Reviews Cancer*. doi:10.1038/nrc2981

Campbell, K. J., & Tait, S. W. G. (2018). Targeting BCL-2 regulated apoptosis in cancer. *Open Biology*, *8*(5), 180002. doi:10.1098/rsob.180002

Carrasco, A. J., Dzeja, P. P., Alekseev, A. E., Pucar, D., Zingman, L. V, Abraham, M. R., … Terzic, A. (2001). Adenylate kinase phosphotransfer communicates cellular energetic signals to ATP-sensitive potassium channels. *Proceedings of the National Academy of Sciences*, *98*(13), 7623–7628. doi:10.1073/pnas.121038198

Carvalho, K. C., Cunha, I. W., Rocha, R. M., Ayala, F. R., Cajaíba, M. M., Begnami, M. D., … Soares, F. A. (2011). GLUT1 expression in malignant tumors and its use as an immunodiagnostic marker. *Clinics*, *66*(6), 965–972. doi:https://doi.org/10.1590/S1807-59322011000600008

Cascone, I., Selimoglu, R., Ozdemir, C., Del Nery, E., Yeaman, C., White, M., & Camonis, J. (2008). Distinct roles of RalA and RalB in the progression of cytokinesis are supported by distinct RalGEFs. *The EMBO Journal*, *27*(18), 2375-2387–2387. doi:https://doi.org/10.1038/emboj.2008.166

Chance, B., & Williams, G. R. (1955). A Method for the Localization of Sites for Oxidative Phosphorylation. *Nature*, *176*(4475), 250–254. doi:10.1038/176250a0

Chang, C.-H., Qiu, J., O'Sullivan, D., Buck, M. D., Noguchi, T., Curtis, J. D., … Pearce, E. L. (2015). Metabolic Competition in the Tumor Microenvironment Is a Driver of Cancer Progression. *Cell*, *162*(6), 1229–1241. doi:https://doi.org/10.1016/j.cell.2015.08.016

Chang, E. H., Gonda, M. A., Ellis, R. W., Scolnick, E. M., & Lowy, D. R. (1982). Human genome contains four genes homologous to transforming genes of Harvey and Kirsten murine sarcoma viruses. *Proceedings of the National Academy of Sciences*, *79*(16), 4848–4852. doi:10.1073/pnas.79.16.4848

Chekulayev, V., Mado, K., Shevchuk, I., Koit, A., Kaldma, A., Klepinin, A., … Kaambre, T. (2015). Metabolic remodeling in human colorectal cancer and surrounding tissues: alterations in regulation of mitochondrial respiration and metabolic fluxes. *Biochemistry and Biophysics Reports*, *4*, 111–125. doi:https://doi.org/10.1016/j.bbrep.2015.08.020

Chen, Z. X., & Pervaiz, S. (2007). Bcl-2 induces pro-oxidant state by engaging mitochondrial respiration in tumor cells. *Cell Death & Differentiation*, *14*(9), 1617–1627. doi:10.1038/sj.cdd.4402165

Choong Lee Yee AND Lim, S. A. N. D. C. P. K. A. N. D. W. C. Y. A. N. D. S. N. A. N. D. L. Y. P. (2010). Proteome-Wide Profiling of the MCF10AT Breast Cancer Progression Model. *PLOS ONE*, *5*(6), 1–8. doi:10.1371/journal.pone.0011030

Cicenas, J., Tamosaitis, L., Kvederaviciute, K., Tarvydas, R., Staniute, G., Kalyan, K., … Valius, M. (2017). KRAS, NRAS and BRAF mutations in colorectal cancer and melanoma. *Medical Oncology*, *34*(2), 26. doi:10.1007/s12032-016-0879-9

Claps, G., Faouzi, S., Quidville, V., Chehade, F., Shen, S., Vagner, S., & Robert, C. (2022). The multiple roles of LDH in cancer. *Nature Reviews Clinical Oncology*, *19*(12), 749–762. doi:10.1038/s41571-022-00686-2

Colegio, O. R., Chu, N.-Q., Szabo, A. L., Chu, T., Rhebergen, A. M., Jairam, V., … Medzhitov, R. (2014). Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature*, *513*(7519), 559–563. doi:10.1038/nature13490

Colgan, S. P., & Taylor, C. T. (2010). Hypoxia: an alarm signal during intestinal inflammation. *Nature Reviews Gastroenterology & Hepatology*, *7*(5), 281–287. doi:10.1038/nrgastro.2010.39

Colicelli, J. (2004). Human RAS Superfamily Proteins and Related GTPases. *Science's STKE*, *2004*(250), re13–re13. doi:10.1126/stke.2502004re13

Colombini, M., Blachly-Dyson, E., & Forte, M. (1996). VDAC, a Channel in the Outer Mitochondrial Membrane. In T. Narahashi (Ed.), *Ion Channels* (pp. 169–202). Boston, MA: Springer US. doi:10.1007/978-1-4899-1775-1_5

Conteduca, V., Sansonno, D., Russi, S., & Dammacco, F. (2013). Precancerous colorectal lesions (Review). *Int J Oncol*, *43*(4), 973–984. doi:10.3892/ijo.2013.2041

Cox, M., & Nelson, D. (2000). *Lehninger Principles of Biochemistry (Vol. 5)*.

Cox, S. E. (2013). Energy Metabolism. In B. Caballero (Ed.), *Encyclopedia of Human Nutrition (Third Edition)* (pp. 177–185). Waltham: Academic Press. doi:https://doi.org/10.1016/B978-0-12-375083-9.00091-X

DeBerardinis, R. J., Lum, J. J., Hatzivassiliou, G., & Thompson, C. B. (2008). The Biology of Cancer: Metabolic Reprogramming Fuels Cell Growth and Proliferation. *Cell Metabolism*, *7*(1), 11–20. doi:https://doi.org/10.1016/j.cmet.2007.10.002

Duan, Q., Li, D., Xiong, L., Chang, Z., & Xu, G. (2019). SILAC Quantitative Proteomics and Biochemical Analyses Reveal a Novel Molecular Mechanism by Which ADAM12S Promotes the Proliferation, Migration, and Invasion of Small Cell Lung Cancer Cells through Upregulating Hexokinase 1. *Journal of Proteome Research*, *18*(7), 2903–2914. doi:10.1021/acs.jproteome.9b00208

Dzeja, P. P., Chung, S., & Terzic, A. (2007). Integration of Adenylate Kinase and Glycolytic and Glycogenolytic Circuits in Cellular Energetics. In *Molecular System Bioenergetics: Energy for Life* (pp. 265–301). doi:10.1002/9783527621095.ch8

Dzeja, P., & Terzic, A. (2009). Adenylate Kinase and AMP Signaling Networks: Metabolic Monitoring, Signal Communication and Body Energy Sensing. *International Journal of Molecular Sciences*, *10*(4), 1729–1772. doi:10.3390/ijms10041729

Dzeja Petras P. AND Chung, S. A. N. D. F. R. S. A. N. D. B. A. A. N. D. T. A. (2011). Developmental Enhancement of Adenylate Kinase-AMPK Metabolic Signaling Axis Supports Stem Cell Cardiac Differentiation. *PLOS ONE*, *6*(4), 1–12. doi:10.1371/journal.pone.0019300

Fendt, S.-M., Frezza, C., & Erez, A. (2020). Targeting Metabolic Plasticity and Flexibility Dynamics for Cancer Therapy. *Cancer Discovery*, *10*(12), 1797–1807. doi:10.1158/2159-8290.CD-20-0844

Fernie, A. R., Carrari, F., & Sweetlove, L. J. (2004). Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. *Current Opinion in Plant Biology*, *7*(3), 254–261. doi:https://doi.org/10.1016/j.pbi.2004.03.007

Frank, S. A. (2007). *Incidence, Inheritance, and Evolution*. Princeton University Press. doi:10.2307/j.ctv301gwh

Fujisawa, K., Terai, S., Takami, T., Yamamoto, N., Yamasaki, T., Matsumoto, T., … Sakaida, I. (2016). Modulation of anti-cancer drug sensitivity through the regulation of mitochondrial activity by adenylate kinase 4. *Journal of Experimental & Clinical Cancer Research*, *35*(1), 48. doi:10.1186/s13046-016-0322-2

Gao, F., Liu, Q., Li, G., Dong, F., Qiu, M., Lv, X., … Guo, Z. (2016). Identification of ubiquinol cytochrome c reductase hinge (UQCRH) as a potential diagnostic biomarker for lung adenocarcinoma. *Open Biology*, *6*(6), 150256. doi:10.1098/rsob.150256

Gerlach, G., & Hofer, H. W. (1986). Interaction of immobilized phosphofructokinase with soluble muscle proteins. *Biochimica et Biophysica Acta (BBA) - General Subjects*, *881*(3), 398–404. doi:https://doi.org/10.1016/0304-4165(86)90032-2

Gilkerson, R. W., Selker, J. M. L., & Capaldi, R. A. (2003). The cristal membrane of mitochondria is the principal site of oxidative phosphorylation. *FEBS Letters*, *546*(2–3), 355–358. doi:https://doi.org/10.1016/S0014-5793(03)00633-1

Gwinn, D. M., Shackelford, D. B., Egan, D. F., Mihaylova, M. M., Mery, A., Vasquez, D. S., … Shaw, R. J. (2008). AMPK Phosphorylation of Raptor Mediates a Metabolic Checkpoint. *Molecular Cell*, *30*(2), 214–226. doi:https://doi.org/10.1016/j.molcel.2008.03.003

Halestrap, A. P., & Wilson, M. C. (2012). The monocarboxylate transporter family—Role and regulation. *IUBMB Life*, *64*(2), 109–119. doi:https://doi.org/10.1002/iub.572
Han, F., Li, C.-F., Cai, Z., Zhang, X., Jin, G., Zhang, W.-N., … Lin, H.-K. (2018). The critical role of AMPK in driving Akt activation under stress, tumorigenesis and drug resistance. *Nature Communications*, *9*(1), 4728. doi:10.1038/s41467-018-07188-9
Han, T., Kang, D., Ji, D., Wang, X., Zhan, W., Fu, M., … Wang, J.-B. (2013). How does cancer cell metabolism affect tumor migration and invasion? *Cell Adhesion & Migration*, *7*(5), 395–403. doi:10.4161/cam.26345
Han, Y., Wu, P., Wang, Z., Zhang, Z., Sun, S., Liu, J., … Gao, Q. (2019). Ubiquinol-cytochrome C reductase core protein II promotes tumorigenesis by facilitating p53 degradation. *EBioMedicine*, *40*, 92–105. doi:https://doi.org/10.1016/j.ebiom.2019.01.002
Hanahan, D., & Weinberg, R. A. (2011, March 4). Hallmarks of cancer: The next generation. *Cell*. doi:10.1016/j.cell.2011.02.013
Hatefi, Y. (1985). The mitochondrial electron transport and oxidative phosphorylation system. *Annual Review of Biochemistry*, *54*(1), 1015–1069.
Hayashi, Y., Yokota, A., Harada, H., & Huang, G. (2019). Hypoxia/pseudohypoxia-mediated activation of hypoxia-inducible factor-1α in cancer. *Cancer Science*, *110*(5), 1510–1517. doi:https://doi.org/10.1111/cas.13990
Herst, P. M., Grasso, C., & Berridge, M. V. (2018). Metabolic reprogramming of mitochondrial respiration in metastatic cancer. *Cancer and Metastasis Reviews*, *37*(4), 643–653. doi:10.1007/s10555-018-9769-2
Ho, P.-C., Bihuniak, J. D., Macintyre, A. N., Staron, M., Liu, X., Amezquita, R., … Kaech, S. M. (2015). Phosphoenolpyruvate Is a Metabolic Checkpoint of Anti-tumor T Cell Responses. *Cell*, *162*(6), 1217–1228. doi:10.1016/j.cell.2015.08.012
Hoekstra, A. S., & Bayley, J.-P. (2013). The role of complex II in disease. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1827*(5), 543–551. doi:https://doi.org/10.1016/j.bbabio.2012.11.005
Hsu, C.-C., Peng, D., Cai, Z., & Lin, H.-K. (2022). AMPK signaling and its targeting in cancer progression and treatment. *Seminars in Cancer Biology*, *85*, 52–68. doi:https://doi.org/10.1016/j.semcancer.2021.04.006
Hussien, R., & Brooks, G. A. (2010). Mitochondrial and plasma membrane lactate transporter and lactate dehydrogenase isoform expression in breast cancer cell lines. *Physiological Genomics*, *43*(5), 255–264. doi:10.1152/physiolgenomics.00177.2010
Infantino, V., Santarsiero, A., Convertini, P., Todisco, S., & Iacobazzi, V. (2021). Cancer Cell Metabolism in Hypoxia: Role of HIF-1 as Key Regulator and Therapeutic Target. *International Journal of Molecular Sciences*, *22*(11). doi:10.3390/ijms22115703
Ishikawa, K., Takenaga, K., Akimoto, M., Koshikawa, N., Yamaguchi, A., Imanishi, H., … Hayashi, J.-I. (2008). ROS-Generating Mitochondrial DNA Mutations Can Regulate Tumor Cell Metastasis. *Science*, *320*(5876), 661–664. doi:10.1126/science.1156906
Jacobus, W. E. (1985). Theoretical support for the heart phosphocreatine energy transport shuttle based on the intracellular diffusion limited mobility of ADP. *Biochemical and Biophysical Research Communications*, *133*(3), 1035–1041. doi:https://doi.org/10.1016/0006-291X(85)91240-9
Jan, Y.-H., Lai, T.-C., Yang, C.-J., Huang, M.-S., & Hsiao, M. (2019). A co-expressed gene status of adenylate kinase 1/4 reveals prognostic gene signature associated with prognosis and sensitivity to EGFR targeted therapy in lung adenocarcinoma. *Scientific Reports*, *9*(1), 12329. doi:10.1038/s41598-019-48243-9

Jan, Y.-H., Lai, T.-C., Yang, C.-J., Lin, Y.-F., Huang, M.-S., & Hsiao, M. (2019). Adenylate kinase 4 modulates oxidative stress and stabilizes HIF-1α to drive lung adenocarcinoma metastasis. *Journal of Hematology & Oncology*, *12*(1), 12. doi:10.1186/s13045-019-0698-5

Jeon, S.-M., Chandel, N. S., & Hay, N. (2012). AMPK regulates NADPH homeostasis to promote tumour cell survival during energy stress. *Nature*, *485*(7400), 661–665. doi:10.1038/nature11066

Ji, Y., Yang, C., Tang, Z., Yang, Y., Tian, Y., Yao, H., … Zheng, X. (2017). Adenylate kinase hCINAP determines self-renewal of colorectal cancer stem cells by facilitating LDHA phosphorylation. *Nature Communications*, *8*(1), 15308. doi:10.1038/ncomms15308

Jia, D., Lu, M., Jung, K. H., Park, J. H., Yu, L., Onuchic, J. N., … Levine, H. (2019). Elucidating cancer metabolic plasticity by coupling gene regulation with metabolic pathways. *Proceedings of the National Academy of Sciences*, *116*(9), 3909–3918. doi:10.1073/pnas.1816391116

Jones, R. G., Plas, D. R., Kubek, S., Buzzai, M., Mu, J., Xu, Y., … Thompson, C. B. (2005). AMP-Activated Protein Kinase Induces a p53-Dependent Metabolic Checkpoint. *Molecular Cell*, *18*(3), 283–293. doi:https://doi.org/10.1016/j.molcel.2005.03.027

Kaldma, A., Klepinin, A., Chekulayev, V., Mado, K., Shevchuk, I., Timohhina, N., … Kaambre, T. (2014). An in situ study of bioenergetic properties of human colorectal cancer: The regulation of mitochondrial respiration and distribution of flux control among the components of ATP synthasome. *The International Journal of Biochemistry & Cell Biology*, *55*, 171–186. doi:https://doi.org/10.1016/j.biocel.2014.09.004

Karnoub, A. E., & Weinberg, R. A. (2008). Ras oncogenes: split personalities. *Nature Reviews Molecular Cell Biology*, *9*(7), 517–531. doi:10.1038/nrm2438

Katzen, H. M., & Schimke, R. T. (1965). Multiple forms of hexokinase in the rat: tissue distribution, age dependency, and properties. *Proceedings of the National Academy of Sciences*, *54*(4), 1218–1225. doi:10.1073/pnas.54.4.1218

Kay, L., Li, Z., Mericskay, M., Olivares, J., Tranqui, L., Fontaine, E., … Saks, V. A. (1997). Study of regulation of mitochondrial respiration in vivo: An analysis of influence of ADP diffusion and possible role of cytoskeleton. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1322*(1), 41–59. doi:https://doi.org/10.1016/S0005-2728(97)00071-6

Kim, R., Emi, M., & Tanabe, K. (2007). Cancer immunoediting from immune surveillance to immune escape. *Immunology*, *121*(1), 1–14. doi:https://doi.org/10.1111/j.1365-2567.2007.02587.x

Kim, S., Kim, D. H., Jung, W.-H., & Koo, J. S. (2013). Succinate dehydrogenase expression in breast cancer. *SpringerPlus*, *2*(1), 299. doi:10.1186/2193-1801-2-299

Klepinin, A., Ounpuu, L., Guzun, R., Chekulayev, V., Timohhina, N., Tepp, K., … Kaambre, T. (2016). Simple oxygraphic analysis for the presence of adenylate kinase 1 and 2 in normal and tumor cells. *Journal of Bioenergetics and Biomembranes*, *48*(5), 531–548. doi:10.1007/s10863-016-9687-3

Klepinin, A., Zhang, S., Klepinina, L., Rebane-Klemm, E., Terzic, A., Kaambre, T., & Dzeja, P. (2020, May 19). Adenylate Kinase and Metabolic Signaling in Cancer Cells. *Frontiers in Oncology*. Frontiers Media S.A. doi:10.3389/fonc.2020.00660

Kocarnik, J. M., Shiovitz, S., & Phipps, A. I. (2015). Molecular phenotypes of colorectal cancer and potential clinical applications. *Gastroenterology Report*, *3*(4), 269–276. doi:10.1093/gastro/gov046

Koit, A., Shevchuk, I., Ounpuu, L., Klepinin, A., Chekulayev, V., Timohhina, N., … Kaambre, T. (2017). Mitochondrial Respiration in Human Colorectal and Breast Cancer Clinical Material Is Regulated Differently. *Oxidative Medicine and Cellular Longevity*, *2017*. doi:10.1155/2017/1372640

Koshikawa, N., Akimoto, M., Hayashi, J.-I., Nagase, H., & Takenaga, K. (2017). Association of predicted pathogenic mutations in mitochondrial ND genes with distant metastasis in NSCLC and colon cancer. *Scientific Reports*, *7*(1), 15535. doi:10.1038/s41598-017-15592-2

Kraft, T., Hornemann, T., Stolz, M., Nier, V., & Wallimann, T. (2000). Coupling of creatine kinase to glycolytic enzymes at the sarcomeric I-band of skeletal muscle: a biochemical study in situ. *Journal of Muscle Research & Cell Motility*, *21*(7), 691–703. doi:10.1023/A:1005623002979

Kröger, A., & Klingenberg, M. (1973). The Kinetics of the Redox Reactions of Ubiquinone Related to the Electron-Transport Activity in the Respiratory Chain. *European Journal of Biochemistry*, *34*(2), 358–368. doi:https://doi.org/10.1111/j.1432-1033.1973.tb02767.x

Kuipers, E. J., Grady, W. M., Lieberman, D., Seufferlein, T., Sung, J. J., Boelens, P. G., … Watanabe, T. (2015). Colorectal cancer. *Nature Reviews Disease Primers*, *1*(1), 15065. doi:10.1038/nrdp.2015.65

Kuznetsov, A. V, Tiivel, T., Sikk, P., Kaambre, T., Kay, L., Daneshrad, Z., … Saks, V. A. (1996). Striking Differences Between the Kinetics of Regulation of Respiration by ADP in Slow-Twitch and Fast-Twitch Muscles In Vivo. *European Journal of Biochemistry*, *241*(3), 909–915. doi:https://doi.org/10.1111/j.1432-1033.1996.00909.x

Lambert, D. W., Wood, I. S., Ellis, A., & Shirazi-Beechey, S. P. (2002). Molecular changes in the expression of human colonic nutrient transporters during the transition from normality to malignancy. *British Journal of Cancer*, *86*(8), 1262–1269. doi:10.1038/sj.bjc.6600264

Landis, C. J., McConathy, J., Darley-Usmar, V., & Hjelmeland, A. B. (2020). The Role of Metabolic Plasticity in Blood and Brain Stem Cell Pathophysiology. *Cancer Research*, *80*(1), 5–16. doi:10.1158/0008-5472.CAN-19-1169

Li, X.-H., Chen, X.-J., Ou, W.-B., Zhang, Q., Lv, Z.-R., Zhan, Y., … Zhou, H.-M. (2013). Knockdown of creatine kinase B inhibits ovarian cancer progression by decreasing glycolysis. *The International Journal of Biochemistry & Cell Biology*, *45*(5), 979–986. doi:https://doi.org/10.1016/j.biocel.2013.02.003

Liang, J., & Mills, G. B. (2013, May 15). AMPK: A contextual oncogene or tumor suppressor? *Cancer Research*. doi:10.1158/0008-5472.CAN-12-3876

Liang, J., Shao, S. H., Xu, Z.-X., Hennessy, B., Ding, Z., Larrea, M., … Mills, G. B. (2007). The energy sensing LKB1–AMPK pathway regulates p27kip1 phosphorylation mediating the decision to enter autophagy or apoptosis. *Nature Cell Biology*, *9*(2), 218–224. doi:10.1038/ncb1537

Liberti, M. V, & Locasale, J. W. (2016). The Warburg Effect: How Does it Benefit Cancer Cells? *Trends in Biochemical Sciences*, *41*(3), 211–218. doi:https://doi.org/10.1016/j.tibs.2015.12.001

Lin, R.-Y., Vera, J. C., Chaganti, R. S. K., & Golde, D. W. (1998). Human Monocarboxylate Transporter 2 (MCT2) Is a High Affinity Pyruvate Transporter*. *Journal of Biological Chemistry*, *273*(44), 28959–28965. doi:https://doi.org/10.1074/jbc.273.44.28959

Liu, Hu, Wang, J., He, T., Becker, S., Zhang, G., Li, D., & Ma, X. (2018). Butyrate: A Double-Edged Sword for Health? *Advances in Nutrition*, *9*(1), 21–29. doi:https://doi.org/10.1093/advances/nmx009

Liu, Huibin, Pu, Y., Amina, Q., Wang, Q., Zhang, M., Song, J., … Mardan, M. (2019). Prognostic and therapeutic potential of Adenylate kinase 2 in lung adenocarcinoma. *Scientific Reports*, *9*(1), 17757. doi:10.1038/s41598-019-53594-4

Liu, R., Ström, A.-L., Zhai, J., Gal, J., Bao, S., Gong, W., & Zhu, H. (2009). Enzymatically inactive adenylate kinase 4 interacts with mitochondrial ADP/ATP translocase. *The International Journal of Biochemistry & Cell Biology*, *41*(6), 1371–1380. doi:https://doi.org/10.1016/j.biocel.2008.12.002

Luo, F., Li, Y., Yuan, F., & Zuo, J. (2019). Hexokinase II promotes the Warburg effect by phosphorylating alpha subunit of pyruvate dehydrogenase. *Chinese Journal of Cancer Research*, *31*(3), 521–532. doi:10.21147/j.issn.1000-9604.2019.03.14

Mannella, C. A., Marko, M., Penczek, P., Barnard, D., & Frank, J. (1994). The internal compartmentation of rat-liver mitochondria: Tomographic study using the high-voltage transmission electron microscope. *Microscopy Research and Technique*, *27*(4), 278–283. doi:https://doi.org/10.1002/jemt.1070270403

Marais, R., Light, Y., Paterson, H. F., & Marshall, C. J. (1995). Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. *The EMBO Journal*, *14*(13), 3136–3145. doi:10.1002/j.1460-2075.1995.tb07316.x

Margoliash, E., Ferguson-Miller, S., Tulloss, J., Kang, C. H., Feinberg, B. A., Brautigan, D. L., & Morrison, M. (1973). Separate Intramolecular Pathways for Reduction and Oxidation of Cytochrome c in Electron Transport Chain Reactions. *Proceedings of the National Academy of Sciences*, *70*(11), 3245–3249. doi:10.1073/pnas.70.11.3245

Marsin, A.-S., Bertrand, L., Rider, M. H., Deprez, J., Beauloye, C., Vincent, M. F., … Hue, L. (2000). Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia. *Current Biology*, *10*(20), 1247–1255. doi:https://doi.org/10.1016/S0960-9822(00)00742-9

Matsuura, S., Igarashi, M., Tanizawa, Y., Yamada, M., Kishi, F., Kajii, T., … Nakazawa, A. (1989). Human adenylate kinase deficiency associated with hemolytic anemia. A single base substitution affecting solubility and catalytic activity of the cytosolic adenylate kinase. *The Journal of Biological Chemistry*, *264*(17), 10148–10155. doi:10.1016/s0021-9258(18)81779-3

Midthun, L., Shaheen, S., Deisch, J., Senthil, M., Tsai, J., & Hsueh, C.-T. (2019). Concomitant KRAS and BRAF mutations in colorectal cancer. *Journal of Gastrointestinal Oncology*, *10*(3), 577.

Moreno-Sánchez, R., Marín-Hernández, A., Saavedra, E., Pardo, J. P., Ralph, S. J., & Rodríguez-Enríquez, S. (2014). Who controls the ATP supply in cancer cells? Biochemistry lessons to understand cancer energy metabolism. *The International Journal of Biochemistry & Cell Biology*, *50*, 10–23. doi:https://doi.org/10.1016/j.biocel.2014.01.025

Mulvaney, P. T., Stracke, M. L., Nam, S. W., Woodhouse, E., O'Keefe, M., Clair, T., … Schiffmann, E. (1998). Cyclocreatine inhibits stimulated motility in tumor cells possessing creatine kinase. *International Journal of Cancer*, *78*(1), 46–52. doi:https://doi.org/10.1002/(SICI)1097-0215(19980925)78:1<46::AID-IJC9>3.0.CO;2-X

Muzny, D. M., Bainbridge, M. N., Chang, K., Dinh, H. H., Drummond, J. A., Fowler, G., … group, T. source sites and disease working. (2012). Comprehensive molecular characterization of human colon and rectal cancer. *Nature*, *487*(7407), 330–337. doi:10.1038/nature11252

Neumann, D., Bückers, J., Kastrup, L., Hell, S. W., & Jakobs, S. (2010). Two-color STED microscopy reveals different degrees of colocalization between hexokinase-I and the three human VDAC isoforms. *PMC Biophysics*, *3*(1), 4. doi:10.1186/1757-5036-3-4

Niu, N., Ye, J., Hu, Z., Zhang, J., & Wang, Y. (2023). Regulative Roles of Metabolic Plasticity Caused by Mitochondrial Oxidative Phosphorylation and Glycolysis on the Initiation and Progression of Tumorigenesis. *International Journal of Molecular Sciences*, *24*(8). doi:10.3390/ijms24087076

Ounpuu, L., Klepinin, A., Pook, M., Teino, I., Peet, N., Paju, K., … Kaambre, T. (2017). 2102Ep embryonal carcinoma cells have compromised respiration and shifted bioenergetic profile distinct from H9 human embryonic stem cells. *Biochimica et Biophysica Acta (BBA) - General Subjects*, *1861*(8), 2146–2154. doi:https://doi.org/10.1016/j.bbagen.2017.05.020

Palade, G. E. (1952). The fine structure of mitochondria. *The Anatomical Record*, *114*(3), 427–451. doi:https://doi.org/10.1002/ar.1091140304

Palm, W. (2021). Metabolic plasticity allows cancer cells to thrive under nutrient starvation. *Proceedings of the National Academy of Sciences*, *118*(14), e2102057118. doi:10.1073/pnas.2102057118

Panayiotou, C., Solaroli, N., & Karlsson, A. (2014). The many isoforms of human adenylate kinases. *The International Journal of Biochemistry & Cell Biology*, *49*, 75–83. doi:https://doi.org/10.1016/j.biocel.2014.01.014

Park, J. S., Sharma, L. K., Li, H., Xiang, R., Holstein, D., Wu, J., … Bai, Y. (2009). A heteroplasmic, not homoplasmic, mitochondrial DNA mutation promotes tumorigenesis via alteration in reactive oxygen species generation and apoptosis. *Human Molecular Genetics*, *18*(9), 1578–1589. doi:10.1093/hmg/ddp069

Pastorino, J. G., Shulga, N., & Hoek, J. B. (2002). Mitochondrial Binding of Hexokinase II Inhibits Bax-induced Cytochrome c Release and Apoptosis*. *Journal of Biological Chemistry*, *277*(9), 7610–7618. doi:https://doi.org/10.1074/jbc.M109950200

Pedersen, P. L. (2007). Warburg, me and Hexokinase 2: Multiple discoveries of key molecular events underlying one of cancers' most common phenotypes, the "Warburg Effect", i.e., elevated glycolysis in the presence of oxygen. *Journal of Bioenergetics and Biomembranes*, *39*(3), 211–222. doi:10.1007/s10863-007-9094-x

Pedersen, P. L., Mathupala, S., Rempel, A., Geschwind, J. F., & Ko, Y. H. (2002). Mitochondrial bound type II hexokinase: a key player in the growth and survival of many cancers and an ideal prospect for therapeutic intervention. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1555*(1), 14–20. doi:https://doi.org/10.1016/S0005-2728(02)00248-7

Pfanner, N., & Wiedemann, N. (2002). Mitochondrial protein import: two membranes, three translocases. *Current Opinion in Cell Biology*, *14*(4), 400–411. doi:https://doi.org/10.1016/S0955-0674(02)00355-1

Pfeiffer, T., Schuster, S., & Bonhoeffer, S. (2001). Cooperation and Competition in the Evolution of ATP-Producing Pathways. *Science*, *292*(5516), 504–507. doi:10.1126/science.1058079

Pinheiro, C., Reis, R. M., Ricardo, S., Longatto-Filho, A., Schmitt, F., & Baltazar, F. (2010). Expression of Monocarboxylate Transporters 1, 2, and 4 in Human Tumours and Their Association with CD147 and CD44. *BioMed Research International*, *2010*(1), 427694. doi:https://doi.org/10.1155/2010/427694

Pino, M. S., & Chung, D. C. (2010). The Chromosomal Instability Pathway in Colon Cancer. *Gastroenterology*, *138*(6), 2059–2072. doi:https://doi.org/10.1053/j.gastro.2009.12.065

Porporato, P. E., Payen, V. L., Pérez-Escuredo, J., De Saedeleer, C. J., Danhier, P., Copetti, T., … Sonveaux, P. (2014). A Mitochondrial Switch Promotes Tumor Metastasis. *Cell Reports*, *8*(3), 754–766. doi:https://doi.org/10.1016/j.celrep.2014.06.043

Qian, X.-L., Li, Y.-Q., Gu, F., Liu, F.-F., Li, W.-D., Zhang, X.-M., & Fu, L. (2012). Overexpression of ubiquitous mitochondrial creatine kinase (uMtCK) accelerates tumor growth by inhibiting apoptosis of breast cancer cells and is associated with a poor prognosis in breast cancer patients. *Biochemical and Biophysical Research Communications*, *427*(1), 60–66. doi:https://doi.org/10.1016/j.bbrc.2012.08.147

Raimondi, V., Ciccarese, F., & Ciminale, V. (2020). Oncogenic pathways and the electron transport chain: a dangeROS liaison. *British Journal of Cancer*, *122*(2), 168–181. doi:10.1038/s41416-019-0651-y

Rao, X., Duan, X., Mao, W., Li, X., Li, Z., Li, Q., … Yi, W. (2015). O-GlcNAcylation of G6PD promotes the pentose phosphate pathway and tumor growth. *Nature Communications*, *6*(1), 8468. doi:10.1038/ncomms9468

Ren, H., Wang, L., Bennett, M., Liang, Y., Zheng, X., Lu, F., … Su, X.-D. (2005). The crystal structure of human adenylate kinase 6: An adenylate kinase localized to the cell nucleus. *Proceedings of the National Academy of Sciences*, *102*(2), 303–308. doi:10.1073/pnas.0407459102

Ricketts, C., Woodward, E. R., Killick, P., Morris, M. R., Astuti, D., Latif, F., & Maher, E. R. (2008). Germline SDHB Mutations and Familial Renal Cell Carcinoma. *JNCI: Journal of the National Cancer Institute*, *100*(17), 1260–1262. doi:10.1093/jnci/djn254

Rodriguez-Viciana, P., Warne, P. H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M. J., … Downward, J. (1994). Phosphatidylinositol-3-OH kinase direct target of Ras. *Nature*, *370*(6490), 527–532. doi:10.1038/370527a0

Rojas, A. M., Fuentes, G., Rausell, A., & Valencia, A. (2012). The Ras protein superfamily: Evolutionary tree and role of conserved amino acids. *Journal of Cell Biology*, *196*(2), 189–201. doi:10.1083/jcb.201103008

Saks, V., Dzeja, P., Schlattner, U., Vendelin, M., Terzic, A., & Wallimann, T. (2006). Cardiac system bioenergetics: metabolic basis of the Frank-Starling law. *The Journal of Physiology*, *571*(2), 253–273. doi:https://doi.org/10.1113/jphysiol.2005.101444

Scheffzek, K., Ahmadian, M. R., Kabsch, W., Wiesmüller, L., Lautwein, A., Schmitz, F., & Wittinghofer, A. (1997). The Ras-RasGAP Complex: Structural Basis for GTPase Activation and Its Loss in Oncogenic Ras Mutants. *Science*, *277*(5324), 333–339. doi:10.1126/science.277.5324.333

Schlattner, U., Tokarska-Schlattner, M., & Wallimann, T. (2006). Mitochondrial creatine kinase in human health and disease. *Biochimica et Biophysica Acta*, *1762*(2), 164–180. doi:10.1016/j.bbadis.2005.09.004

Senft, D., & Ronai, Z. A. (2016). Regulators of mitochondrial dynamics in cancer. *Current Opinion in Cell Biology*, *39*, 43–52. doi:https://doi.org/10.1016/j.ceb.2016.02.001

Serasinghe, M. N., Wieder, S. Y., Renault, T. T., Elkholi, R., Asciolla, J. J., Yao, J. L., … Chipuk, J. E. (2015). Mitochondrial Division Is Requisite to RAS-Induced Transformation and Targeted by Oncogenic MAPK Pathway Inhibitors. *Molecular Cell*, *57*(3), 521–536. doi:https://doi.org/10.1016/j.molcel.2015.01.003

Shadel, G. S., & Horvath, T. L. (2015). Mitochondrial ROS Signaling in Organismal Homeostasis. *Cell*, *163*(3), 560–569. doi:10.1016/j.cell.2015.10.001

Shen, C.-H., Yuan, P., Perez-Lorenzo, R., Zhang, Y., Lee, S. X., Ou, Y., … Zheng, B. (2013). Phosphorylation of BRAF by AMPK Impairs BRAF-KSR1 Association and Cell Proliferation. *Molecular Cell*, *52*(2), 161–172. doi:https://doi.org/10.1016/j.molcel.2013.08.044

Shestov, A. A., Liu, X., Ser, Z., Cluntun, A. A., Hung, Y. P., Huang, L., … Locasale, J. W. (2014). Quantitative determinants of aerobic glycolysis identify flux through the enzyme GAPDH as a limiting step. *ELife*, *3*, e03342. doi:10.7554/eLife.03342

Shussman, N., & Wexner, S. D. (2014). Colorectal polyps and polyposis syndromes. *Gastroenterology Report*, *2*(1), 1–15. doi:10.1093/gastro/got041

Siekevitz, P., & Potter, V. R. (1955). Biochemical structure of mitochondria: I. Intramitochondrial components and oxidative phosphorylation. *Journal of Biological Chemistry*, *215*(1), 221–235. doi:https://doi.org/10.1016/S0021-9258(18)66031-4

Simon, K. (2016). Colorectal cancer development and advances in screening. *Clinical Interventions in Aging*, 967–976.

Simula, L., Nazio, F., & Campello, S. (2017). The mitochondrial dynamics in cancer and immune-surveillance. *Seminars in Cancer Biology*, *47*, 29–42. doi:https://doi.org/10.1016/j.semcancer.2017.06.007

Slavov, N., Budnik, B. A., Schwab, D., Airoldi, E. M., & van Oudenaarden, A. (2014). Constant Growth Rate Can Be Supported by Decreasing Energy Flux and Increasing Aerobic Glycolysis. *Cell Reports*, *7*(3), 705–714. doi:https://doi.org/10.1016/j.celrep.2014.03.057

Soumoy, L., Schepkens, C., Krayem, M., Najem, A., Tagliatti, V., Ghanem, G. E., … Journe, F. (2020). Metabolic Reprogramming in Metastatic Melanoma with Acquired Resistance to Targeted Therapies: Integrative Metabolomic and Proteomic Analysis. *Cancers*, *12*(5). doi:10.3390/cancers12051323

Sullivan, L. B., & Chandel, N. S. (2014). Mitochondrial reactive oxygen species and cancer. *Cancer & Metabolism*, *2*(1), 17. doi:10.1186/2049-3002-2-17

Suvarna, V., Singh, V., & Murahari, M. (2019). Current overview on the clinical update of Bcl-2 anti-apoptotic inhibitors for cancer therapy. *European Journal of Pharmacology*, *862*, 172655. doi:https://doi.org/10.1016/j.ejphar.2019.172655

Takeichi, M. (1993). Cadherins in cancer: implications for invasion and metastasis. *Current Opinion in Cell Biology*, *5*(5), 806–811. doi:https://doi.org/10.1016/0955-0674(93)90029-P

Timohhina, N., Guzun, R., Tepp, K., Monge, C., Varikmaa, M., Vija, H., … Saks, V. (2009). Direct measurement of energy fluxes from mitochondria into cytoplasm in permeabilized cardiac cells in situ: some evidence for mitochondrial interactosome. *Journal of Bioenergetics and Biomembranes*, *41*(3), 259–275. doi:10.1007/s10863-009-9224-8

Tsai, H. J., & Wilson, J. E. (1996). Functional Organization of Mammalian Hexokinases: Both N- and C-Terminal Halves of the Rat Type II Isozyme Possess Catalytic Sites. *Archives of Biochemistry and Biophysics*, *329*(1), 17–23. doi:https://doi.org/10.1006/abbi.1996.0186

Tseng, P.-L., Wu, W.-H., Hu, T.-H., Chen, C.-W., Cheng, H.-C., Li, C.-F., … Chang, W.-T. (2018). Decreased succinate dehydrogenase B in human hepatocellular carcinoma accelerates tumor malignancy by inducing the Warburg effect. *Scientific Reports*, *8*(1), 3081. doi:10.1038/s41598-018-21361-6

Vander Heiden, M. G., Cantley, L. C., & Thompson, C. B. (2009). Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. *Science*, *324*(5930), 1029–1033. doi:10.1126/science.1160809

Vasseur, S., Malicet, C., Calvo, E. L., Dagorn, J. C., & Iovanna, J. L. (2005). Gene expression profiling of tumours derived from rasV12/E1A-transformed mouse embryonic fibroblasts to identify genes required for tumour development. *Molecular Cancer*, *4*(1), 4. doi:10.1186/1476-4598-4-4

Vyas, S., Zaganjor, E., & Haigis, M. C. (2016). Mitochondria and Cancer. *Cell*, *166*(3), 555–566. doi:https://doi.org/10.1016/j.cell.2016.07.002

Wallimann, T., & Hemmer, W. (1994). Creatine kinase in non-muscle tissues and cells. *Cellular Bioenergetics: Role of Coupled Creatine Kinases*, 193–220.

Wallimann, T., Tokarska-Schlattner, M., & Schlattner, U. (2011). The creatine kinase system and pleiotropic effects of creatine. *Amino Acids*, *40*(5), 1271–1296. doi:10.1007/s00726-011-0877-3

Wan, P. T. C., Garnett, M. J., Roe, S. M., Lee, S., Niculescu-Duvaz, D., Good, V. M., … Marais, R. (2004). Mechanism of Activation of the RAF-ERK Signaling Pathway by Oncogenic Mutations of B-RAF. *Cell*, *116*(6), 855–867. doi:10.1016/S0092-8674(04)00215-6

Warburg, O. (1925). The metabolism of carcinoma cells. *The Journal of Cancer Research*, *9*(1), 148–163.

Warburg, O. (1956). On the Origin of Cancer Cells. *Science*, *123*(3191), 309–314. doi:10.1126/science.123.3191.309

Watts, D. C. (1973). 12 Creatine Kinase (Adenosine 5′-Triphosphate-Creatine Phosphotransferase). In *The enzymes* (Vol. 8, pp. 383–455). Elsevier.

White, E. (2013, October 1). Exploiting the bad eating habits of Ras-driven cancers. *Genes and Development*. Cold Spring Harbor Laboratory Press. doi:10.1101/gad.228122.113

Wilson, J E. (1995). Hexokinases. In *Reviews of Physiology, Biochemistry and Pharmacology, Volume 126* (pp. 65–198). Berlin, Heidelberg: Springer Berlin Heidelberg. doi:10.1007/BFb0049776

Wilson, John E. (2003). Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function. *Journal of Experimental Biology*, *206*(12), 2049–2057. doi:10.1242/jeb.00241

World Health Organization. (2023, July 11). Colorectal Cancer. Retrieved 6 July 2024, from https://www.who.int/news-room/fact-sheets/detail/colorectal-cancer

Xie, Z., Zhou, H., Wang, L., & Wu, Y. (2022). The Significance of the preoperative lactate dehydrogenase/albumin Ratio in the Prognosis of Colon Cancer: a retrospective study. *PeerJ*, *10*, e13091. doi:10.7717/peerj.13091

Xin, F., Yao, D.-W., Fan, L., Liu, J.-H., & Liu, X.-D. (2019). Adenylate kinase 4 promotes bladder cancer cell proliferation and invasion. *Clinical and Experimental Medicine*, *19*(4), 525–534. doi:10.1007/s10238-019-00576-5

Xu, R., Yang, Y., & Zheng, X. (2021). Unique structural features of the adenylate kinase hCINAP/AK6 and its multifaceted functions in carcinogenesis and tumor progression. *FEBS Letters*, *595*(16), 2071–2084. doi:https://doi.org/10.1002/1873-3468.14158

Yamane, L., Scapulatempo-Neto, C., Reis, R. M., & Guimarães, D. P. (2014). Serrated pathway in colorectal carcinogenesis. *World Journal of Gastroenterology: WJG*, *20*(10), 2634.

Yan, Y.-B. (2016). Creatine kinase in cell cycle regulation and cancer. *Amino Acids*, *48*(8), 1775–1784. doi:10.1007/s00726-016-2217-0

Young, C. D., Lewis, A. S., Rudolph, M. C., Ruehle, M. D., Jackman, M. R., Yun, U. J., … Anderson, S. M. (2011). Modulation of Glucose Transporter 1 (GLUT1) Expression Levels Alters Mouse Mammary Tumor Cell Growth In Vitro and In Vivo. *PLOS ONE*, *6*(8), e23205-. Retrieved from https://doi.org/10.1371/journal.pone.0023205

Zaid, H., Abu-Hamad, S., Israelson, A., Nathan, I., & Shoshan-Barmatz, V. (2005). The voltage-dependent anion channel-1 modulates apoptotic cell death. *Cell Death & Differentiation*, *12*(7), 751–760. doi:10.1038/sj.cdd.4401599

Zambrano, A., Molt, M., Uribe, E., & Salas, M. (2019). Glut 1 in Cancer Cells and the Inhibitory Action of Resveratrol as A Potential Therapeutic Strategy. *International Journal of Molecular Sciences*, *20*(13). doi:10.3390/ijms20133374

Zapater, J. L., Lednovich, K. R., Khan, Md. W., Pusec, C. M., & Layden, B. T. (2022). Hexokinase domain-containing protein-1 in metabolic diseases and beyond. *Trends in Endocrinology & Metabolism*, *33*(1), 72–84. doi:10.1016/j.tem.2021.10.006

Zhang, J., Yin, Y.-T., Wu, C.-H., Qiu, R.-L., Jiang, W.-J., Deng, X.-G., & Li, Z.-X. (2019). AK4 Promotes the Progression of HER2-Positive Breast Cancer by Facilitating Cell Proliferation and Invasion. *Disease Markers*, *2019*(1), 8186091. doi:https://doi.org/10.1155/2019/8186091

Zhang, Y., Jiang, L., Qin, N., Cao, M., Liang, X., & Wang, R. (2020). hCINAP is potentially a direct target gene of HIF-1 and is required for hypoxia-induced EMT and apoptosis in cervical cancer cells. *Biochemistry and Cell Biology*, *99*(2), 203–213. doi:10.1139/bcb-2020-0090

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Abstract

Metabolic Reprogramming Accompanying the Development of Colorectal Cancer

Approximately 1,9 million people annually are diagnosed with colorectal cancer (CRC), making it the third most common cancer type worldwide. A long asymptomatic development of CRC leads to a high number of late diagnoses, which complicates cancer treatment and contributes to high mortality. There is a massive need for novel methods to allow earlier CRC detection. CRC development is a long multi-step process that generally starts with precursor lesions called polyps, which will finally develop into cancer via the accumulation of several genetic alterations, e.g. point mutations in *KRAS* and *BRAF* oncogenes.

All living cells need a constant supply of energy. However, cancer cells are highly proliferative and have enhanced growth, significantly increasing their energy requirements. Therefore, they undergo metabolic reprogramming to sustain the uncontrolled cell division and growth. Otto Warburg's famous theory from the 1920s states that all cancer cells use aerobic glycolysis because of damaged mitochondria and cannot produce energy through oxidative phosphorylation (OXPHOS). Although many cancer types can upregulate glycolytic activity, nowadays, there is increasing evidence showing that cancer cells can exhibit high rates of OXPHOS. Furthermore, cancer cells can adapt a hybrid metabolic phenotype by dynamically switching between the two metabolic pathways depending on the tumour microenvironment. This phenomenon is called metabolic plasticity.

The aim of the thesis was to characterize the metabolic reprogramming that accompanies CRC development. Most cancer metabolism studies use 2D cell cultures, which is not the best as the metabolic phenotype of cultured cells depends significantly on the conditions in which they were grown. The current thesis used a comprehensive approach of combining several methods, and all experiments were conducted on postoperative clinical material. The primary method was high-resolution respirometry, which allowed the measurements of mitochondrial oxygen consumption and the rate of ATP synthesis. In addition, the transcript levels were assessed using the quantitative reverse transcription polymerase chain reaction.

The results suggested that metabolic reprogramming starts at the early stages of CRC development as polyps demonstrated a distinct metabolic profile compared to control and cancer tissue. Furthermore, the mutations in *KRAS* and *BRAF* affect metabolic reprogramming differently; polyps and cancer with *BRAF* mutations presented a possible shift towards glycolysis, suggested by decreased V_{max} and K_m(ADP) values.

Polyps upregulated several genes from glycolytic pathways, such as *GLUT1*, *HK1*, *LDHA*, *MCT2*, and *MCT4.* They also had a lower K_m(ADP) value than the control tissue, indicating less structural restrictions for adenine nucleotide movement through the mitochondrial outer membrane, a characteristic of glycolytic tissues. However, they also had a higher average V_{max} than the control colon tissue, indicating elevated mitochondrial oxygen consumption and a highly active OXPHOS system. Polyps also upregulated transcription of creatine kinases and adenylate kinases that facilitate intracellular energy transfer and help maintain the ATP homeostasis in the cell. Therefore, colorectal polyps exhibit characteristics of a hybrid metabolism where glycolysis and OPXHOS are simultaneously used for energy production. This metabolic plasticity in benign tumours

could support cell proliferation and growth in the dynamically changing tumour microenvironment and promote cancer development.

To sum it up, the results clearly showed that the development of CRC is characterized by metabolic reprogramming. Furthermore, the data demonstrate that benign polyps have a distinct metabolic profile that is substantially different from that of a healthy colon. These insights can provide us with possible targets for developing novel diagnostic methods.

Lühikokkuvõte

Kolorektaalvähi arenguga kaasnev metaboolne ümberprogrammeerimine

Aastas diagnoositakse kolorektaalvähk ehk jämesoolevähk maailmas umbes 1,9 miljonil inimesel, mis teeb sellest kolmanda kõige levinuma vähitüübi. Kolorektaalvähk võib areneda pikalt ilma sümptomiteta, mistõttu avastatakse see tihti liiga hilja. Hiline vähi avastamine teeb aga ravi keeruliseks ning seetõttu on jämesoolevähile omane kõrge suremus. Sellest tulenevalt on kliiniliselt suur vajadus uute diagnostika meetodite järele, mis võimaldaksid kolorektaalvähi varasemat diagnoosimist. Jämesoolevähi areng on pikk mitmeastmeline protsess, mis algab enamasti healoomulistest kasvajatest ehk polüüpidest. Polüübi kasvamisel hakkavad kogunema erinevad geneetilised muutused, näiteks mutatsioonid *KRAS* ja *BRAF* geenides, mis lõpuks soodustavad polüübi arenemist vähiks.

Kõik rakud vajavad toimimiseks pidevat energia tootmist. Vähirakke iseloomustab aga väga kiire kasv ja jagunemine, mistõttu on nende energiavajadus veelgi suurem. Et toetada neile omast kontrollimatut jagunemist, läbivad vähirakud metaboolse ümberprogrammeerimise. Otto Warburg väitis oma kuulsas artiklis 1920. aastatest, et kõik vähirakud eelistavad kasutada aeroobset glükolüüsi, sest nende mitokondrid ei ole funktionaalsed ja seetõttu ei saa nad energia tootmiseks kasutada oksüdatiivset fosforüülimist. Kuigi mitmed vähitüübid tõepoolest võivad glükolüüsi aktiivsust suurendada, on tänapäeval üha rohkem tõestusmaterjali selle kohta, et vähirakke võib iseloomustada ka väga kõrgelt aktiivne oksüdatiivne fosforüülimine. Vähirakud teeb veelgi erilisemaks see, et nad võivad omandada niiöelda hübriidfenotüübi, mille korral on glükolüüs ja oksüdatiivne fosforüülimine paralleelselt aktiveeritud ning rakud saavad valida endale sobiva metaboolse raja vastavalt kasvaja mikrokeskkonnale. Sellist nähtust nimetatakse metaboolseks plastilisuseks.

Käesoleva töö eesmärk on iseloomustada kolorektaalvähi arenguga kaasnevat rakkude metaboolset ümberprogrammeerimist. Suur hulk vähi metabolismi uurivaid teadustöid kasutab 2D rakukultuure. Rakukultuuri metaboolne fenotüüp sõltub aga suuresti tingimustest, milles neid rakke on kasvatatud ning seetõttu ei peegelda see ilmtingimata inimorganismis toimuvat. Antud töös on kombineeritud mitmeid erinevaid meetodeid ning kõik eksperimendid on sooritatud kliinilisel materjalil. Alustuseks mõõdeti kudede ATP sünteesi kineetilised parameetrid ning seejärel määrati mitmete geenide ekspressioonitase.

Töö tulemused näitavad, et metaboolne ümberprogrammeerimine algab juba kolorektaalvähi arengu varajastases staadiumites. Seda ilmestab polüüpide väga eriline metaboolne profiil, mis erineb selgelt kontroll- ja vähikoest. Lisaks leiti, et *KRAS* ja *BRAF* mutatsioonidel on erinev roll metabolismi mõjutamisel. *BRAF* mutatsiooniga polüüpides ja kasvajamaterjalis on madalamad V_{max} ja K_m(ADP) väärtused, mis viitavad metabolismi nihkumisele glükolüüsi suunas.

Polüüpides tõuseb mitmete glükolüütilise raja geenide, nagu *GLUT1*, *HK1*, *LDHA*, *MCT2* ja *MCT4*, ekspressioon. Lisaks mõõdeti polüüpides madalam K_m(ADP) väärtus kui kontrollkoel, mis näitab, et adeniinnukleotiitide liikumisel läbi mitokondri välismembraani on vähem struktuurilisi piiranguid. See on tavaliselt omane glükolüütilistele kudedele. Samas oli polüüpidel ka kõrgem maksimaalne hingamiskiirus kui tervel soolekoel, mis omakorda viitab võimalikule aktiivsele oksüdatiivsele

fosforüülimisele. Lisaks on polüüpides üleekspresseeritud ka mitmed kreatiinkinaaside ja adenülaatkinaaside isovormid, mis hõlbustavad rakusisest energia transporti ning aitavad säilitada ATP tasakaalu rakus. Seega on soolepolüüpidele omane metabolismi hübriidne fenotüüp, kus on paralleelselt energia tootmiseks kasutuses nii glükolüüs kui ka oksüdatiivne fosforüülimine. Selline metaboolne plastilisus healoomulistes kasvajates võib toetada rakkude kiiret jagunemist ja kasvu dünaamiliselt muutuvas kasvaja mikrokeskkonnas ja seeläbi soodustada vähi arengut.

Antud tööst järeldub, et kolorektaalvähi arengut iseloomustab kasvaja metabolismi ümberprogrammeerimine. Seejuures on eriti oluline, et just healoomulisi polüüpe iseloomustab väga eriline metaboolne profiil, mis erineb selgelt tervest soolekoest. Need uued teadmised võivad anda aluse leidmaks võimalikke sihtmärke uute diagnostikameetodite väljatöötamiseks.

Appendix 1

Publication I

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Article

Mitochondrial Respiration in *KRAS* and *BRAF* Mutated Colorectal Tumors and Polyps

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Abstract: This study aimed to characterize the ATP-synthesis by oxidative phosphorylation in colorectal cancer (CRC) and premalignant colon polyps in relation to molecular biomarkers KRAS and BRAF. This prospective study included 48 patients. Resected colorectal polyps and postoperative CRC tissue with adjacent normal tissue (control) were collected. Patients with polyps and CRC were divided into three molecular groups: *KRAS* mutated, *BRAF* mutated and *KRAS/BRAF* wild-type. Mitochondrial respiration in permeabilized tissue samples was observed using high resolution respirometry. ADP-activated respiration rate (V_{max}) and an apparent affinity of mitochondria to ADP, which is related to mitochondrial outer membrane (MOM) permeability, were determined. Clear differences were present between molecular groups. *KRAS* mutated CRC group had lower V_{max} values compared to wild-type; however, the V_{max} value was higher than in the control group, while MOM permeability did not change. This suggests that *KRAS* mutation status might be involved in acquiring oxidative phenotype. *KRAS* mutated polyps had higher V_{max} values and elevated MOM permeability as compared to the control. *BRAF* mutated CRC and polyps had reduced respiration and altered MOM permeability, indicating a glycolytic phenotype. To conclude, prognostic biomarkers KRAS and BRAF are likely related to the metabolic phenotype in CRC and polyps. Assessment of the tumor mitochondrial ATP synthesis could be a potential component of patient risk stratification.

Keywords: energy metabolism; colorectal cancer; colorectal polyps; mitochondria; oxidative phosphorylation; *KRAS*; *BRAF*

1. Introduction

Colorectal cancer (CRC) is the leading cause of premature cancer death worldwide, prompting the urgent need to develop more effective treatment strategies. CRC is a heterogeneous disease and presents distinct subtypes with different molecular and pathological features. The majority of sporadic CRC typically develops progressively from premalignant precursor lesions, known as polyps, to malignant tumors. Most colorectal polyps are harmless, but some can develop (by not fully understood mechanisms) into malignant invasive adenocarcinomas. According to modern concepts, CRC is triggered by various molecular events in several proto-oncogenes (such as the *PIK3CA*, *p53*, *KRAS*, *BRAF* and *c-MYC* genes) and tumor suppressor genes (such as the APC, PTEN, SMAD4 genes) [1–3]. The malignant transformation of cells, including colon epithelium, is accompanied by strong alterations (reprogramming) of metabolic pathways involved in energy production and biosynthesis that promote tumor growth and metastasis [4–6]. A better understanding of the pathogenesis of CRC, the metabolic heterogeneity of emerging polyps and potential drivers is very important to develop new prognostic markers and successful agents for the prevention and treatment of this disease.

Transcriptome-based classification has been used in CRC as it can better describe the behavior of the tumors. The international CRC Subtyping Consortium classifies CRC into four consensus molecular subtypes (CMSs), each with distinct features: CMS1 (hypermutated, microsatellite instability (MSI), *BRAF* mutation, and immune infiltration and activation); CMS2 (epithelial, WNT and MYC signaling pathway activation); CMS3 (metabolic dysregulation, *KRAS* mutations); and CMS4 (transforming growth factor beta activation, stromal invasion, TGFβ activation, and angiogenesis) [7]. Although transcriptome profiles are not associated with specific mutations, the frequency of *KRAS* mutation varies among the CRC subtypes (23% in CMS1, 28% in CMS2, 68% in CMS3, and 38% in CMS4), these data suggest mutations may drive distinct programs of metabolism gene expression [7]. Mutations in *KRAS* or *BRAF* genes appear to play an important role in the regulation of metabolic reprogramming in multiple cancers, including CRC [8–11]. In this study, two established and common prognostic biomarkers in CRC were investigated: *KRAS* and *BRAF* mutation status. Mutation in *BRAF* codon 600 of exon 15 (V600E) is associated with unfavorable prognosis [12]. Activating *KRAS* mutations in codon 12 and 13 of exon 2, which is common in CRC (30–50% of tumors), are associated with poorer survival and response to chemotherapeutics [13,14]. Our study aims to contribute to understanding how prognostic biomarkers KRAS and BRAF are correlating to cellular metabolic phenotypes in the course of CRC carcinogenesis.

The metabolism of cancer cells is specially adapted to meet their needs to survive and proliferate in both well oxygenated and hypoxic microenvironments. To date, transcriptomics and metabolomics studies have shown the coexistence of three distinct cellular metabolic phenotypes that exist in cancer cells, which are characterized by the following predominant states: glycolytic (aerobic glycolysis, so called Warburg phenotype [15]), oxidative (energy production relying mainly on oxidative phosphorylation, OXPHOS), and hybrid (both OXPHOS and glycolysis can be active simultaneously). Normal cells exhibit only glycolytic and oxidative states [16–18]. Premalignant polyps and arising adenocarcinomas are still regarded as highly glycolytic tumors of the Warburg phenotype [19–21]. Previous studies indicate that although polyps have higher inclination to aerobic glycolysis, the metastatic carcinomas maintain high rates of O_2 consumption (much more than adjacent normal tissues) and exhibit obvious signs of stimulated mitochondrial biogenesis [6,22–24]. In this regard, we assume that upon malignant transformation, there is a selection of specific cell clones that have stimulated mitochondrial biogenesis and, as a result, have elevated aggressiveness. Among patients with CRC, a high level of mitochondrial respiration of tumor samples have been found to be associated with reduced survival [25].

As part of cancer bioenergetic studies, analysis of OXPHOS with high-resolution respirometry can be applied to study the mechanisms of this key element in cellular bioenergetics. Investigating the dependency of adenosine diphosphate (ADP)-dependent respiration rate on ADP concentration in tissue samples can provide two fundamental characteristics for OXPHOS: a maximal ADP-activated

respiration rate (V_{max}), and an apparent affinity of mitochondria for exogenous ADP expressed as apparent Michaelis–Menten constant Km (K_m(ADP)). Our previous experiments showed that the V_{max} value for CRC cells is significantly higher than in cells in healthy colorectal control tissue showing more active ATP-synthesis by OXPHOS. This finding corresponds well with differences in the content of mitochondria in these cells (the number of mitochondria in CRC is almost two times higher than in healthy tissue) [6,25]. The changes in K_m(ADP) show changes in tissue-specific intracellular complexity in terms of energy transport and regulation of mitochondrial outer membrane (MOM) permeability. For the operation of OXPHOS, the flux of respiratory substrates, ATP, ADP and Pi through MOM is regulated by the voltage-dependent anion channel (VDAC) permeability control. In the closed state, VDAC is impermeable to adenine nucleotides [26,27]. Several studies have shown that during carcinogenesis the VDAC permeability for ADP is altered [22,28–30]. The cell-specific differences in K_m(ADP) are likely due to specific structural and functional organization of energy metabolism. For example, cells with a low K_m(ADP) value (~10 μM) like glycolytic muscle, possess less structural and functional obstacles for movement ADP/ATP though MOM as compared to the oxidative muscles (~300 μM) [31]. Known K_m(ADP) values for CRC measured for tumor tissue are about 100 μM [22,25], implying existence of some restrictions for ADP passing VDAC. The sensitivity of the mitochondrial respiration for exogenous ADP in cell cultures is very high (low K_m(ADP) values) and is similar to isolated mitochondria [25,28,32–34], which suggests the need to investigate cancer energy metabolism directly in fresh clinical material. To our knowledge, there is no data on the rate of OXPHOS and its regulation in colon polyps. Assessment of OXPHOS status of this pathology enhances our understanding of colon carcinogenesis.

Thus, the main goal of our study was to characterize the functional activity of mitochondrial OXPHOS among premalignant polyps and CRC, taking into account their *KRAS* and *BRAF* mutation status. To date, it has been shown that *KRAS* and *BRAF* mutations increase the glycolytic capacity of tumor cells and their glutaminolysis [8,35]. In our work, the function of the OXPHOS system was analyzed by means of high-resolution respirometry using freshly prepared postoperative tissue samples.

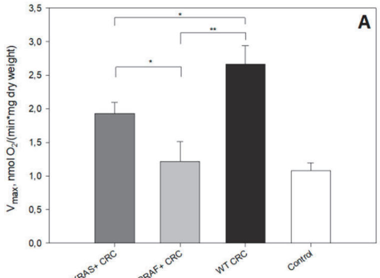
2. Results and Discussion

Cancer metabolism profoundly differs from normal cellular metabolism, and interrelated connections between cancer mitochondrial respiration and oncogenic driver genes like *KRAS* and *BRAF* are relatively unexplored. Somatic mutations involving the GTP-ase RAS protein family and its downstream serine/threonine-protein kinase BRAF lead to loss of cell cycle regulation at key checkpoints and are the main driver mutations for colorectal carcinogenesis [36]. *KRAS* mutations are detected in approximately 40% of all CRC patients, suggesting the importance of *KRAS* in tumor development [37]. The *KRAS* mutation is an early event in CRC and most *KRAS* mutations are located in codons 12 and 13. However, at least 5–10% of CRCs are believed to initiate via acquiring activating mutations in the *BRAF* oncogene [38]. Mutations of *KRAS* and *BRAF* are usually mutually exclusive. Although the existence of intertumoral heterogeneity in CRC is well established and illustrated by molecular subtyping [7], pure genome or transcriptome data are not sufficient to describe the final in situ modifications and the final outcomes of pathways or cellular processes [25]. The purpose of this study was to determine the activity of ATP production by OXPHOS in human tissues during the development of CRC from normal colon tissue to polyps and cancer, depending on the status of *BRAF* and *KRAS* mutations.

To characterize ATP-synthesis by OXPHOS during CRC carcinogenesis we used high resolution respirometry to measure the rate of maximal ADP-activated respiration (V_{max}). We also used apparent K_m values for exogenously added ADP (K_m(ADP)) using permeabilized postoperative tissue (CRC, colon polyps and normal colon tissue). Our previous studies showed that OXPHOS can be a significant supplier of ATP in CRC because its V_{max} values (corresponding to the number of mitochondria) were almost two times higher than in surrounding normal tissues [6,39,40]. Among all the studied groups, the wild-type tumor showed the highest V_{max}, while these values measured for *BRAF* or *KRAS* mutated

tumors were significantly lower (Figure 1A, Tables S1 and S2). This reveals involvement of oncogenic *KRAS* and *BRAF* in metabolic reprogramming of colon mucosa and confirms their role in shifting CRC metabolism to a more glycolytic type. Furthermore, in contrast to the results from an in vitro study conducted by Yun et al.—done with CRC cell cultures where oxygen consumption in cells with mutant *KRAS* or *BRAF* alleles was similar to that in cells with wild type alleles of these genes [41]—we saw a difference in V_{max} values between *BRAF* mutated and *KRAS* mutated tumors (Figure 1A, Tables S1 and S2). Interestingly, the V_{max} of *BRAF* mutated tumors was similar to that in control tissues. These results suggest a distinct role of mutated *KRAS* and *BRAF* in affecting mitochondrial biogenesis and likely tissue differentiation as well.

In colorectal polyps, the V_{max} pattern largely followed that of the respective tumors. The respiration rates in polyps in *KRAS* mutated and wild-type molecular groups showed remarkably higher V_{max} values than the control tissue (V_{max} values 2.19 ± 0.19 and 1.95 ± 0.28 for *KRAS* mutated and wild-type group, respectively, $p < 0.001$ and $p = 0.004$ as compared to the control group (Tables S1 and S2). Polyps that had acquired the *BRAF* mutation showed a tendency to have lower OXPHOS rates (V_{max} 1.41 ± 0.27) than in mutated *KRAS* and wild-type groups. Similar to the *BRAF* tumor group, polyps with mutated *BRAF* did not show a difference with the control tissue (Figure 1, Tables S1 and S2). This suggests that alterations in mitochondrial biogenesis is a very early event and already happens in the pre-malignant stage.



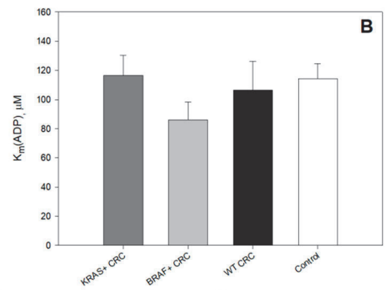


Figure 1. Regulation of mitochondrial respiration in *KRAS*+, *BRAF*+ and wild-type tumors and control. (**A**) Comparative analysis of maximal ADP-activated respiratory rate (V_{max}) and (**B**) the apparent Michaelis–Menten constant (K_m(ADP)) values for ADP. *KRAS*+: *KRAS* mutated; *BRAF*+: *BRAF* mutated; WT: wild type; CRC: colorectal cancer; Control: control tissue. * $p < 0.05$; ** $p < 0.01$.

Maintaining high functional activity of OXPHOS may be necessary because cancer cells with a very low respiration rate cannot form tumors [42]. At the same time, a certain reduction in respiration may be useful for the functioning of signaling molecules, the synthesis of anabolic precursors and other typical aspects of cancer phenotypes [43]. Thus, functional OXPHOS is important in both proliferating and non-proliferating cells, but each situation will emphasize its unique functional aspects [42]. It has been shown that the metabolic profile of cancer cells in culture can have significant variations as a consequence of the culture conditions [25]. In general, cells growing in a glucose-free medium display relatively high rates of oxygen consumption, whereas cultivation in a high-glucose medium results in hyperglycolytic cells together with declined respiratory flux [44–48]. Therefore, for the study of OXPHOS in human tumors, the use of postoperative tissue material is likely to be a more suitable approach.

To investigate possible regulatory alterations affecting OXPHOS during carcinogenesis, we estimated apparent affinity mitochondria for ADP. In all CRC and polyp groups, the corresponding K_m(ADP) value was determined and the measured values (Figure 1B, Tables S1 and S2) were found to be 4 to 8 times higher than in isolated mitochondria (15 µM, measured by Chance and Williams [49,50]). This finding points to the existence of restrictions for the movement of ADP through mitochondrial membranes. The OXPHOS system is located in the inner mitochondrial membrane

and the ADP/ATP carrier has the function of crossing the adenine nucleotides through the membrane into the mitochondrial matrix. In our previous study, we applied metabolic control analysis on ATP-synthasome which consisted of the respiratory system, ATP-synthase, ATP/ADP carrier and Pi transporter, all in CRC tissue. In the framework of metabolic control analysis and by using specific inhibitors, the rate of effect each enzyme has in a pathway (flux control coefficients) can be determined. This analysis showed that the main control over ATP-synthesis by OXPHOS (the highest flux control coefficients) in CRC relied on respiratory complexes I and III and Pi transporter. Inhibition of the ADP/ATP carrier had no major rate-limiting effect on ATP synthesis by OXPHOS [26]. Thus, we assumed that the considerable control over ability of exogenous ADP to influence respiration was mainly dependent on ADP passage through MOM in CRC. The comparison of K_m(ADP) values for *KRAS* mutated, *BRAF* mutated and wild-type tumors did not reveal any substantial differences. In all CRC groups the Km(ADP) values for tumor and control tissue were similar. Our previous study showed that we can distinguish two different populations of mitochondria in control tissue—what we believe could be a mucosal population with lower K_m(ADP) (75 ± 4 µM), and the smooth muscle population with a much higher K_m(ADP) value (362 ± 60 µM) [25]. This is in good agreement with our preliminary results obtained from separately measured colon smooth muscle and mucosa (259 ± 35 µM and 118 ± 11 µM, respectively). To estimate the percentage of mitochondria with highly regulated (oxidative) and unregulated (glycolytic) MOM permeability, we applied the mathematical model used for muscle cells and adapted it to tissues studied by us. According to the model proposed earlier [51], the hypothetical percentage of low oxidative capacity mitochondria in tissue is calculated from the K_m(ADP) value as an inverse asymptotic dependence. Percent of low oxidative capacity of mitochondrion demonstrates the metabolic shift to glycolytic state in all colon polyps, but not in *KRAS* mutated and wild-type tumors compared to control tissue (Table 1, Tables S1 and S2). The changes in glycolytic markers have been observed in the early premalignant colorectal mucosal field and these changes would be expected to promote increased glycolysis [19]. The K_m(ADP) values in polyp molecular groups were 55.3 ± 7.4 µM, 52.5 ± 4.7 µM and 60.1 ± 6.3 µM for *KRAS* mutated, *BRAF* mutated and wild-type group, respectively. These were lower than in control tissue (Tables S1 and S2), which indicates significant changes in regulation MOM permeability. Interestingly, despite the similar V_{max} values in *KRAS* mutated polyp and CRC groups, the difference in K_m(ADP) between these groups was significant, $p = 0.014$ (Tables S1, S2 and Figure S1). Our findings of the relatively low K_m value for ADP for colorectal polyps suggest an early metabolic reprogramming towards the glycolytic phenotype with functional OXPHOS.

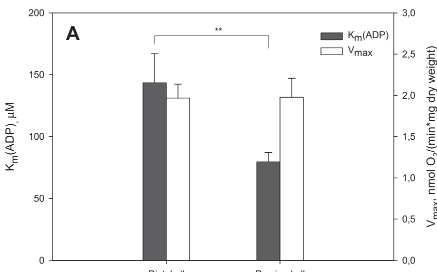
Table 1. Modelled percentage of low oxidative capacity of mitochondrion in KRAS+, BRAF+ and wild-type tumors and controls.

Sample	% of Low Oxidative Capacity of Mitochondrion
KRAS tumors	28.1
KRAS polyps	65.9
BRAF tumors	43.0
BRAF polyps	68.6
Wild-type tumors	32.4
Wild-type polyps	61.7
All controls	29.0

The results of the current study confirm our previous findings, indicating that in cancer tissues, the regulation of MOM permeability to adenine nucleotides is different from that in normal cells [25,28,29]. Proteins that could regulate the VDAC permeability for adenine nucleotides in colonocytes and corresponding cancer cells are still unknown. There are two possible mechanisms proposed for this regulation. According to the first model, cancer cells due to overexpression of

mitochondrially-bound hexokinase 2 support high permeability of the VDAC to adenine nucleotides and direct the ATP formed in mitochondria to the glycolytic pathway. As a consequence, the aerobic glycolysis is facilitated and malignant metabolic reprogramming occurs [52,53]. The second model involves the inhibition of VDAC by free tubulin to limit mitochondrial metabolism in cancer cells [30,54]. The possible candidates are βIII–tubulin and γ-tubulin. βIII–tubulin acts as a marker of cancer aggressiveness, and γ-tubulin formed meshwork has been shown to be associated with mitochondrial membranes [29,55,56]. However, the regulation of energy metabolism through control over metabolites and energy fluxes that pass through the MOM is only one aspect of the possible role of VDAC influencing carcinogenesis. VDAC1—the major mitochondrial protein expressed in mammals and functions in metabolism, Ca^{2+} homeostasis, apoptosis and other activities—is regulated via its interaction with many proteins associated with cell survival and cellular death pathways. VDAC1 is overexpressed in many cancers and represents a promising cancer drug target (reviewed in [57,58]). The mechanistic understanding behind the changes in K_m(ADP) during CRC carcinogenesis observed in the current study and connections with other functions of VDAC require further investigation.

Further, we analyzed whether the observed changes in V_{max} and K_m(ADP) values are related to tumor location. CRC is more frequently observed in the distal colon (left colon, from splenic flexure to rectum) than in the proximal side (right colon, from the cecum to transverse colon [59]). In the current study, the distal and proximal tumors were presented almost equally—20 and 24 samples, respectively. Studies have shown that tumors arising from the left and right colon are distinct in their epidemiology, biology, histology and microbial diversity [59,60]. In the current study, comparing all the distal and proximal tumors showed differences in K_m(ADP) but not in V_{max} values (Figure 2A). A study including 57,847 patients showed proximal patients had better outcomes than those with distal CRC in several subgroups including stage II disease, patients aged >70 years and mucinous adenocarcinoma [61]. Inside the *KRAS* mutated group, proximal and distal tumors were compared to see the potential effect of cancer location on metabolic changes. No statistically significant difference between V_{max} and K_m(ADP) values comparing proximal and distal tumors in the *KRAS* mutated group (Figure 2B) was seen. The location of a tumor did not have an effect on the mitochondrial respiration in the *KRAS* mutated group and all observed alterations were related to the *KRAS* status of the tumor. All *BRAF* mutated tumors were located in the proximal side.



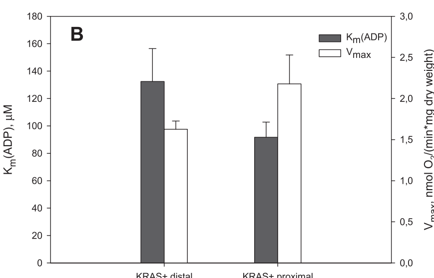


Figure 2. (**A**) In the current study, a comparison of all distal and proximal tumors showed a difference in K_m(ADP) values, but not in V_{max}. (**B**) V_{max} and K_m(ADP) values comparing proximal and distal tumors in the *KRAS* mutated group. ** Significant difference, $p < 0.01$.

All together, we found that colon polyps and colon tumors had higher rates of maximal ADP-activated respiration (a marker of mitochondrial mass) than normal colon tissue (Figure 1A, Tables S1 and S2). *BRAF* mutant tumors and polyps exhibited lower V_{max} values than *KRAS* mutated lesions and they had a relatively high percentage of mitochondria with low control over the movement adenine nucleotides through MOM (Table 1). Therefore, it is most likely that lesions with *BRAF* mutations have higher glycolytic activity, which is confirmed by some published data [62]. In contrast to the *BRAF* mutated lesions, *KRAS* mutated polyps showed signs of stimulated mitochondrial

biogenesis and upon progression could give highly metastatic malignant tumors (i.e., polyps with this energetic phenotype can be more prone to tumor formation). This was unexpected, since the transformed cells carrying the *KRAS* gene mutations were characterized by an increased glycolytic flow associated with the over-expression of glucose transporter 1 (GLUT1) and hexokinase 2 and reduced oxygen consumption due to mitochondrial dysfunction in cell cultures [41,63,64]. Our previous studies demonstrated that the oxygen consumption in vitro significantly differed compared to what occurred in vivo [25]. Moreover, the rate of oxidative ATP production of the tumor seems to be a prognostic marker for cancer survival and metastatic potential [22]. The estimation of *KRAS* or *BRAF* mutation status in colorectal pre- and neoplastic lesions could be a predictor of their response to drugs affecting the OXPHOS. Recently, a new class of anticancer drugs called "mitocans" was proposed. These affect different mitochondrial-associated activities including ATP/ADP carrier, hexokinase, electron transport/respiratory chain inhibitors, and others [65].

3. Materials and Methods

3.1. Reagents

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich Chemical Com. (St. Louis, MO, USA) and were used directly without further purification.

3.2. Clinical Material

All tumor patients examined (n = 33 with ages ranging from 38 to 91 years) had local or locally advanced disease (T2-4 N0-1, M0-1). The patients in the study had not received prior radiation or chemotherapy (Table 2). All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Medical Research Ethics Committee (National Institute for Health Development, Tallinn, Estonia) of nr.1728.

Table 2. Clinicopathological patient characteristics of the colon cancer and polyps cohort.

Characteristics	*n*
Total patients	48
Females	19
Males	29
Age at diagnosis	
Mean	72
Median	74
Range	38–91
Stage of tumor	
I-II	15
III-IV	9
Unknown	9
Molecular subtype of tumor	
KRAS mutated	13
BRAF mutated	6
KRAS and *BRAF* wild-type	14
Molecular subtypes of polyps	
KRAS mutated	4
BRAF mutated	2
KRAS and *BRAF* wild-type	9

CRC post operational and normal tissue samples (0.1–0.5 g) were provided by the Oncology and Hematologic Clinic at the North Estonia Medical Centre (NEMC, Tallinn, Estonia). Pathology reports were obtained by the NEMC for each tissue sample. Only primary tumor samples were examined. All investigations were approved by the Medical Research Ethics Committee (National Institute for Health Development, Tallinn, Estonia) and were in accordance with Helsinki Declaration and Convention of the Council of Europe on Human Rights and Biomedicine.

Normal tissue samples were taken from the same location at sites distant from the tumor and they were evaluated for presence of malignant cells. The adjacent control tissues consisted of colonocytes and smooth muscle cells.

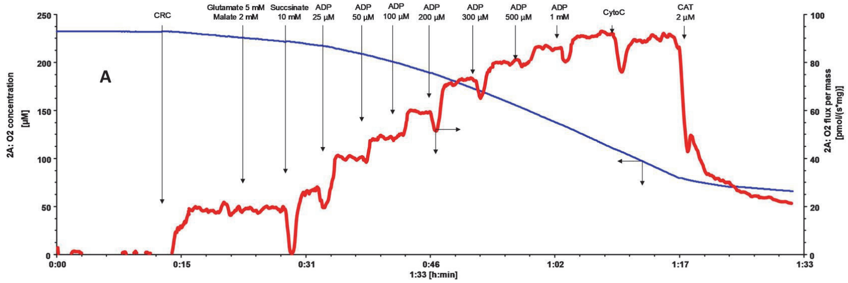
Patients with colorectal polyps (n = 15) (Table 2) were consecutive patients undergoing a colonoscopy for resection of the polyps at the West Tallinn Central Hospital. After removal, tissue samples were immediately placed in medium B, which consisted of the following: 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 3 mM KH_2PO_4, 110 mM sucrose, 0.5 mM dithiothreitol, 20 mM HEPES, 5 µM leupeptin, 2 mg/mL fatty acids free bovine serum albumin (BSA), pH 7.1. All polyps were analyzed immediately after the colonoscopy with quick cancer tests. Only part of the cancer negative polyps was subjected to further analysis for OXPHOS. Due to the limited amount of fresh tissue, *KRAS* and *BRAF* mutation analyses were performed using Formalin-Fixed Paraffin-Embedded (FFPE) samples.

3.3. Preparation of Skinned Tumor Fibers and Permeabilization Procedure

Immediately after the surgery, the tissue samples were placed into pre-cooled (4 °C) medium A, which consisted of 20 mM imidazole, 3 mM KH_2PO_4, 0.5 mM dithiothreitol, 20 mM taurine, 4 mM $MgCl_2$, 100 mM 2-morpholinoethanesulfonic acid, 2.74 mM K_2Ca-EGTA, 4.72 mM K_2-EGTA, 5 µM leupeptin and 2 mg/mL BSA [39]. The samples were dissected into small fiber bundles (10–20 mg) and permeabilized in the same medium with 50 µg/mL of saponin. They were mildly stirred for 30 min at 4 °C [39,66]. The obtained permeabilized (skinned) fibers were then washed three times for 5 min in pre-cooled medium B (without leupeptin). After that, samples were kept in medium B at 4 °C until use. The typical dimension of skinned fibers was about 2 × 2 × 2 mm, and one of these pieces was used in oxygraphic experiments.

3.4. Oxygraphic Measurements

Mitochondrial respiration of permeabilized tissue samples was measured at 25 °C in medium B supplemented with 5 mM glutamate, 2 mM malate and 10 mM succinate, with respiratory substrates using a high-resolution respirometer Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) as described previously [66,67]. The solubility of oxygen at 25 °C was taken as 240 nmol/mL [68]. All respiration rates were normalized per mg dry weight of tissue. To determine the apparent affinity of mitochondria to exogenous ADP (K_m(ADP)), the dependence of respiration rate on exogenous ADP was measured (Figure 3A). The obtained data were plotted as rates of O_2 consumption (the basal respiration rate of respiration was subtracted) versus ADP concentration and K_m(ADP) and V_{max} values were calculated from these plots by nonlinear regression using Michaelis–Menten equation [69,70] (Figure 3B). Additionally, plotting the data to double reciprocal plot gives information about presence of different mitochondrial populations with differently regulated MOM.



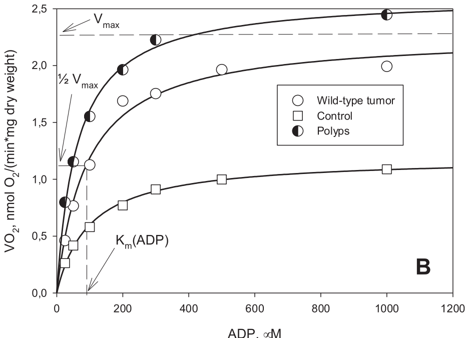


Figure 3. Different kinetics of regulation of mitochondrial respiration by exogenous ADP in colon tissue. (**A**) Recording of original traces of O_2 consumption by permeabilized colorectal cancer (CRC) tissue upon additions of increasing concentrations of ADP. CAT stands for carboxyatractyloside; CyoC stands for cytochrome C. **(B)** The measured respiration rates were plotted vs ADP concentrations, and from this plot corresponding V_{max} and K_m(ADP) values were calculated by nonlinear regression using Michaelis–Menten equation. There was a marked difference in ADP kinetics between wild-type CRC, colon polyps and normal colon tissue (control).

3.5. DNA Extraction

DNA from formalin-fixed paraffin-embedded tissue (FFPE) samples was extracted using ZYMO Quick-DNA™ FFPE Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. DNA concentrations and quality were measured using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

3.6. KRAS and BRAF Mutation Analysis

Mutations in *BRAF* codon 600 of exon 15 (V600E) and *KRAS* codon 12 and 13 of exon 2 were screened using High-Resolution Melt (HRM) analysis. Briefly, a 10 µl reaction mix contained 1x HOT FIREPol® EvaGreen® HRM Mix (Solis BioDyne, Estonia), 250 nM of sense and antisense primers (*KRAS*-antisense, 5′- AAATGACTGAATATAAACTTGTGGTAGT-3′; *KRAS*-sense, 5′- TGAATTAGCTGTATCGTCAAGGCACT-3′; *BRAF*-antisense wild-type, 5′-cgccgcgcgccAAAATAG GTGATTTTGGTCT-3′; *BRAF*-antisense mutation, 5′-TAAAAATAGGTGATTTTGGTCTAGCTACA-3′; *BRAF*-sense, 5′- CCACAAAATGGATCCAGAC AACTG 3′) and 100× dilution of PCR amplification product. PCR amplification and HRM analysis were performed with Rotor-Gene 6000 (QIAGEN) and consisted of an initial 15 min denaturation step at 95 °C, followed by 45 cycles at 95 °C for 10 s, 54 °C for 10 s and 72 °C for 15 s, with a final extension at 72 °C for 3 min. The resulting PCR products were heated at 95 °C for 1 min and cooled to 40 °C to facilitate heteroduplex formation. HRM analysis was

performed from 62 °C to 92 °C with a 0.1 °C step. The results were analyzed using Rotor-Gene 6000 software and unknown samples were compared to control samples with known genotypes.

3.7. Data Analysis

Data in the text, tables and figures are presented as mean ± standard error (SEM). Results were analyzed by Student's *t*-test and *p*-values < 0.05 were considered statistically significant. Apparent K_m values for ADP were measured by fitting experimental data to a non-linear regression (according to a Michaelis–Menten model equation, as shown in Figure 3).

4. Conclusions

While many studies have characterized the metabolic phenotype of CRC cell lines, it is important to understand the metabolic reprogramming in clinical material. Our findings confirm that early changes in mitochondria respiration occur in CRC carcinogenesis and precede the development of pre-cancerous lesions. Mitochondrial respiration differs in *KRAS*, *BRAF* mutated and wild-type tumor groups, confirming that oncogenes may affect the metabolic requirements of cancer cells. In common polyps, it still remains unclear whether the specific metabolic requirement of tumor cells is dictated by oncogenes or if they change dynamically during tumor evolution. Mitochondrial biogenesis, involved in mitochondrial respiration rate, may be developed to be the prognostic marker for cancer prognosis. As there are profound differences in mitochondrial respiration, the assessment of the metabolic profile of CRC polyps and tumors has the potential to become a component of patient risk stratification.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/12/4/815/s1, Figure S1: Regulation of mitochondrial respiration in KRAS+, BRAF+ and wild-type tumors and controls, Table S1: The maximal ADP-activated respiration rates (Vmax) comparison by molecular groups. Respiration rates are given in nmol O2/(min×mg dry weight), Table S2: Km comparison by molecular groups.

Author Contributions: Conceptualization, E.R.-K., L.T. and T.K.; data curation, I.S.; formal analysis, E.R.-K., L.T., LR. and I.S.; funding acquisition, T.K.; investigation, E.R.-K., L.T., L.R., M.P., V.C., N.T. and K.T.; methodology, E.R.-K., L.T. and T.K.; project administration, K.S., V.V. and T.K.; resources, J.B., V.A., K.S. and V.V.; supervision, T.K.; visualization, L.R. and I.S.; writing—original draft, E.R.-K., L.T. and M.P.; writing—review & editing, I.S., V.C. and T.K. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

ADP	adenosine diphosphate
CMS	consensus molecular subtype
CRC	colorectal cancer
K_m	Michaelis–Menten constant
K_m(ADP)	apparent affinity of mitochondria for exogenous ADP
OXPHOS	oxidative phosphorylation
MOM	outer mitochondrial membrane
VDAC	voltage-dependent anion channel
V_{max}	maximal-ADP-activated respiration rate

References

1. Vogelstein, B.; Kinzler, K.W. Cancer genes and the pathways they control. *Nat. Med.* **2004**, *10*, 789–799. [CrossRef]
2. Boutin, A.T.; Liao, W.T.; Wang, M.; Hwang, S.S.; Karpinets, T.V.; Cheung, H.; Chu, G.C.; Jiang, S.; Hu, J.; Chang, K.; et al. Oncogenic kras drives invasion and maintains metastases in colorectal cancer. *Genes Dev.* **2017**, *31*, 370–382. [CrossRef]

3. Hao, Y.; Samuels, Y.; Li, Q.; Krokowski, D.; Guan, B.J.; Wang, C.; Jin, Z.; Dong, B.; Cao, B.; Feng, X.; et al. Oncogenic pik3ca mutations reprogram glutamine metabolism in colorectal cancer. *Nat. Commun.* **2016**, *7*, 11971. [CrossRef]
4. Hanahan, D.; Weinberg, R.A. Hallmarks of cancer: The next generation. *Cell* **2011**, *144*, 646–674. [CrossRef]
5. Schwitalla, S. Tumor cell plasticity: The challenge to catch a moving target. *J. Gastroenterol.* **2014**, *49*, 618–627. [CrossRef]
6. Kaldma, A.; Klepinin, A.; Chekulayev, V.; Mado, K.; Shevchuk, I.; Timohhina, N.; Tepp, K.; Kandashvili, M.; Varikmaa, M.; Koit, A.; et al. An in situ study of bioenergetic properties of human colorectal cancer: The regulation of mitochondrial respiration and distribution of flux control among the components of atp synthasome. *Int. J. Biochem. Cell Biol.* **2014**, *55*, 171–186. [CrossRef]
7. Guinney, J.; Dienstmann, R.; Wang, X.; de Reynies, A.; Schlicker, A.; Soneson, C.; Marisa, L.; Roepman, P.; Nyamundanda, G.; Angelino, P.; et al. The consensus molecular subtypes of colorectal cancer. *Nat. Med.* **2015**, *21*, 1350–1356. [CrossRef]
8. Hutton, J.E.; Wang, X.; Zimmerman, L.J.; Slebos, R.J.; Trenary, I.A.; Young, J.D.; Li, M.; Liebler, D.C. Oncogenic kras and braf drive metabolic reprogramming in colorectal cancer. *Mol. Cell. Proteom.* **2016**, *15*, 2924–2938. [CrossRef]
9. Corazao-Rozas, P.; Guerreschi, P.; Andre, F.; Gabert, P.E.; Lancel, S.; Dekiouk, S.; Fontaine, D.; Tardivel, M.; Savina, A.; Quesnel, B.; et al. Mitochondrial oxidative phosphorylation controls cancer cell's life and death decisions upon exposure to mapk inhibitors. *Oncotarget* **2016**, *7*, 39473–39485. [CrossRef]
10. Ruocco, M.R.; Avagliano, A.; Granato, G.; Vigliar, E.; Masone, S.; Montagnani, S.; Arcucci, A. Metabolic flexibility in melanoma: A potential therapeutic target. *Semin. Cancer Biol.* **2019**, *59*, 187–207. [CrossRef]
11. Pupo, E.; Avanzato, D.; Middonti, E.; Bussolino, F.; Lanzetti, L. Kras-driven metabolic rewiring reveals novel actionable targets in cancer. *Front. Oncol.* **2019**, *9*, 848. [CrossRef] [PubMed]
12. Yokota, T.; Ura, T.; Shibata, N.; Takahari, D.; Shitara, K.; Nomura, M.; Kondo, C.; Mizota, A.; Utsunomiya, S.; Muro, K.; et al. Braf mutation is a powerful prognostic factor in advanced and recurrent colorectal cancer. *Br. J. Cancer* **2011**, *104*, 856–862. [CrossRef] [PubMed]
13. Phipps, A.I.; Buchanan, D.D.; Makar, K.W.; Win, A.K.; Baron, J.A.; Lindor, N.M.; Potter, J.D.; Newcomb, P.A. Kras-mutation status in relation to colorectal cancer survival: The joint impact of correlated tumour markers. *Br. J. Cancer* **2013**, *108*, 1757–1764. [CrossRef]
14. De Roock, W.; Claes, B.; Bernasconi, D.; De Schutter, J.; Biesmans, B.; Fountzilas, G.; Kalogeras, K.T.; Kotoula, V.; Papamichael, D.; Laurent-Puig, P.; et al. Effects of kras, braf, nras, and pik3ca mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: A retrospective consortium analysis. *Lancet Oncol.* **2010**, *11*, 753–762. [CrossRef]
15. Warburg, O. On the origin of cancer cells. *Science* **1956**, *123*, 309–314. [CrossRef] [PubMed]
16. Paudel, B.B.; Quaranta, V. Metabolic plasticity meets gene regulation. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 3370–3372. [CrossRef]
17. Jia, D.; Lu, M.; Jung, K.H.; Park, J.H.; Yu, L.; Onuchic, J.N.; Kaipparettu, B.A.; Levine, H. Elucidating cancer metabolic plasticity by coupling gene regulation with metabolic pathways. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 3909–3918. [CrossRef]
18. Yu, L.; Lu, M.; Jia, D.; Ma, J.; Ben-Jacob, E.; Levine, H.; Kaipparettu, B.A.; Onuchic, J.N. Modeling the genetic regulation of cancer metabolism: Interplay between glycolysis and oxidative phosphorylation. *Cancer Res.* **2017**, *77*, 1564–1574. [CrossRef]
19. Cruz, M.D.; Ledbetter, S.; Chowdhury, S.; Tiwari, A.K.; Momi, N.; Wali, R.K.; Bliss, C.; Huang, C.; Lichtenstein, D.; Bhattacharya, S.; et al. Metabolic reprogramming of the premalignant colonic mucosa is an early event in carcinogenesis. *Oncotarget* **2017**, *8*, 20543–20557. [CrossRef]
20. Grassetto, G.; Capirci, C.; Marzola, M.C.; Rampin, L.; Chondrogiannis, S.; Musto, A.; Crepaldi, G.; Minicozzi, A.M.; Massaro, A.; Rubello, D. Colorectal cancer: Prognostic role of 18f-fdg-pet/ct. *Abdom. Imaging* **2012**, *37*, 575–579. [CrossRef]
21. Graziano, F.; Ruzzo, A.; Giacomini, E.; Ricciardi, T.; Aprile, G.; Loupakis, F.; Lorenzini, P.; Ongaro, E.; Zoratto, F.; Catalano, V.; et al. Glycolysis gene expression analysis and selective metabolic advantage in the clinical progression of colorectal cancer. *Pharm. J.* **2017**, *17*, 258–264. [CrossRef] [PubMed]

22. Chekulayev, V.; Mado, K.; Shevchuk, I.; Koit, A.; Kaldma, A.; Klepinin, A.; Timohhina, N.; Tepp, K.; Kandashvili, M.; Ounpuu, L.; et al. Metabolic remodeling in human colorectal cancer and surrounding tissues: Alterations in regulation of mitochondrial respiration and metabolic fluxes. *Biochem. Biophys. Rep.* **2015**, *4*, 111–125. [CrossRef]
23. Ou, J.; Miao, H.; Ma, Y.; Guo, F.; Deng, J.; Wei, X.; Zhou, J.; Xie, G.; Shi, H.; Xue, B.; et al. Loss of abhd5 promotes colorectal tumor development and progression by inducing aerobic glycolysis and epithelial-mesenchymal transition. *Cell Rep.* **2014**, *9*, 1798–1811. [CrossRef] [PubMed]
24. Ounpuu, L.; Truu, L.; Shevchuk, I.; Chekulayev, V.; Klepinin, A.; Koit, A.; Tepp, K.; Puurand, M.; Rebane-Klemm, E.; Kaambre, T. Comparative analysis of the bioenergetics of human adenocarcinoma caco-2 cell line and postoperative tissue samples from colorectal cancer patients. *Biochim. Biol. Cell.* **2018**, *96*, 808–817. [CrossRef] [PubMed]
25. Koit, A.; Shevchuk, I.; Ounpuu, L.; Klepinin, A.; Chekulayev, V.; Timohhina, N.; Tepp, K.; Puurand, M.; Truu, L.; Heck, K.; et al. Mitochondrial respiration in human colorectal and breast cancer clinical material is regulated differently. *Oxid. Med. Cell. Longev.* **2017**, *2017*, 1372640. [CrossRef]
26. Colombini, M. The vdac channel: Molecular basis for selectivity. *Biochim. Biophys. Acta (BBA) Mol. Cell Res.* **2016**, *1863*, 2498–2502. [CrossRef]
27. Noskov, S.Y.; Rostovtseva, T.K.; Chamberlin, A.C.; Teijido, O.; Jiang, W.; Bezrukov, S.M. Current state of theoretical and experimental studies of the voltage-dependent anion channel (vdac). *Biochim. Biophys. Acta* **2016**, *1858*, 1778–1790. [CrossRef]
28. Klepinin, A.; Ounpuu, L.; Mado, K.; Truu, L.; Chekulayev, V.; Puurand, M.; Shevchuk, I.; Tepp, K.; Planken, A.; Kaambre, T. The complexity of mitochondrial outer membrane permeability and vdac regulation by associated proteins. *J. Bioenerg. Biomembr.* **2018**, *50*, 339–354. [CrossRef]
29. Puurand, M.; Tepp, K.; Timohhina, N.; Aid, J.; Shevchuk, I.; Chekulayev, V.; Kaambre, T. Tubulin betaii and betaiii isoforms as the regulators of vdac channel permeability in health and disease. *Cells* **2019**, *8*, 239. [CrossRef]
30. Maldonado, E.N.; Sheldon, K.L.; DeHart, D.N.; Patnaik, J.; Manevich, Y.; Townsend, D.M.; Bezrukov, S.M.; Rostovtseva, T.K.; Lemasters, J.J. Voltage-dependent anion channels modulate mitochondrial metabolism in cancer cells: Regulation by free tubulin and erastin. *J. Biol. Chem.* **2013**, *288*, 11920–11929. [CrossRef]
31. Varikmaa, M.; Bagur, R.; Kaambre, T.; Grichine, A.; Timohhina, N.; Tepp, K.; Shevchuk, I.; Chekulayev, V.; Metsis, M.; Boucher, F.; et al. Role of mitochondria-cytoskeleton interactions in respiration regulation and mitochondrial organization in striated muscles. *Biochim. Biophys. Acta* **2014**, *1837*, 232–245. [CrossRef] [PubMed]
32. Anmann, T.; Guzun, R.; Beraud, N.; Pelloux, S.; Kuznetsov, A.V.; Kogerman, L.; Kaambre, T.; Sikk, P.; Paju, K.; Peet, N.; et al. Different kinetics of the regulation of respiration in permeabilized cardiomyocytes and in hl-1 cardiac cells. Importance of cell structure/organization for respiration regulation. *Biochim. Biophys. Acta* **2006**, *1757*, 1597–1606. [CrossRef] [PubMed]
33. Monge, C.; Beraud, N.; Tepp, K.; Pelloux, S.; Chahboun, S.; Kaambre, T.; Kadaja, L.; Roosimaa, M.; Piirsoo, A.; Tourneur, Y.; et al. Comparative analysis of the bioenergetics of adult cardiomyocytes and nonbeating hl-1 cells: Respiratory chain activities, glycolytic enzyme profiles, and metabolic fluxes. *Can. J. Physiol. Pharmacol.* **2009**, *87*, 318–326. [CrossRef] [PubMed]
34. Klepinin, A.; Chekulayev, V.; Timohhina, N.; Shevchuk, I.; Tepp, K.; Kaldma, A.; Koit, A.; Saks, V.; Kaambre, T. Comparative analysis of some aspects of mitochondrial metabolism in differentiated and undifferentiated neuroblastoma cells. *J. Bioenerg. Biomembr.* **2014**, *46*, 17–31. [CrossRef]
35. Kawada, K.; Toda, K.; Sakai, Y. Targeting metabolic reprogramming in kras-driven cancers. *Int. J. Clin. Oncol.* **2017**, *22*, 651–659. [CrossRef]
36. Oikonomou, E.; Koustas, E.; Goulielmaki, M.; Pintzas, A. Braf vs ras oncogenes: Are mutations of the same pathway equal? Differential signalling and therapeutic implications. *Oncotarget* **2014**, *5*, 11752–11777. [CrossRef]
37. Vaughn, C.P.; Zobell, S.D.; Furtado, L.V.; Baker, C.L.; Samowitz, W.S. Frequency of kras, braf, and nras mutations in colorectal cancer. *Genes Chromosomes Cancer* **2011**, *50*, 307–312. [CrossRef]
38. Lai, E.; Pretta, A.; Impera, V.; Mariani, S.; Giampieri, R.; Casula, L.; Pusceddu, V.; Coni, P.; Fanni, D.; Puzzoni, M.; et al. Braf-mutant colorectal cancer, a different breed evolving. *Expert Rev. Mol. Diagn.* **2018**, *18*, 499–512. [CrossRef]

39. Kaambre, T.; Chekulayev, V.; Shevchuk, I.; Karu-Varikmaa, M.; Timohhina, N.; Tepp, K.; Bogovskaja, J.; Kutner, R.; Valvere, V.; Saks, V. Metabolic control analysis of cellular respiration in situ in intraoperational samples of human breast cancer. *J. Bioenerg. Biomembr.* **2012**, *44*, 539–558. [CrossRef]
40. Kaambre, T.; Chekulayev, V.; Shevchuk, I.; Tepp, K.; Timohhina, N.; Varikmaa, M.; Bagur, R.; Klepinin, A.; Anmann, T.; Koit, A.; et al. Metabolic control analysis of respiration in human cancer tissue. *Front. Physiol.* **2013**, *4*, 151. [CrossRef]
41. Yun, J.; Rago, C.; Cheong, I.; Pagliarini, R.; Angenendt, P.; Rajagopalan, H.; Schmidt, K.; Willson, J.K.; Markowitz, S.; Zhou, S.; et al. Glucose deprivation contributes to the development of kras pathway mutations in tumor cells. *Science* **2009**, *325*, 1555–1559. [CrossRef]
42. Hubackova, S.; Magalhaes Novais, S.; Davidova, E.; Neuzil, J.; Rohlena, J. Mitochondria-driven elimination of cancer and senescent cells. *Biol. Chem.* **2019**, *400*, 141–148. [CrossRef]
43. Lu, J.; Tan, M.; Cai, Q. The warburg effect in tumor progression: Mitochondrial oxidative metabolism as an anti-metastasis mechanism. *Cancer Lett.* **2015**, *356*, 156–164. [CrossRef]
44. Gnaiger, E.; Kemp, R.B. Anaerobic metabolism in aerobic mammalian cells: Information from the ratio of calorimetric heat flux and respirometric oxygen flux. *Biochim. Biophys. Acta* **1990**, *1016*, 328–332. [CrossRef]
45. Gstraunthaler, G.; Seppi, T.; Pfaller, W. Impact of culture conditions, culture media volumes, and glucose content on metabolic properties of renal epithelial cell cultures. Are renal cells in tissue culture hypoxic? *Cell Physiol. Biochem.* **1999**, *9*, 150–172. [CrossRef]
46. Sherr, C.J.; DePinho, R.A. Cellular senescence: Mitotic clock or culture shock? *Cell* **2000**, *102*, 407–410. [CrossRef]
47. Jose, C.; Rossignol, R. Rationale for mitochondria-targeting strategies in cancer bioenergetic therapies. *Int. J. Biochem. Cell Biol.* **2013**, *45*, 123–129. [CrossRef]
48. Swerdlow, R.H.; Lezi, E.; Aires, D.; Lu, J. Glycolysis-respiration relationships in a neuroblastoma cell line. *Biochim. Biophys. Acta* **2013**, *1830*, 2891–2898. [CrossRef]
49. Chance, B.; Williams, G.R. Respiratory enzymes in oxidative phosphorylation. Ii. Difference spectra. *J. Biol. Chem.* **1955**, *217*, 395–407.
50. Chance, B.; Williams, G.R. Respiratory enzymes in oxidative phosphorylation. Vi. The effects of adenosine diphosphate on azide-treated mitochondria. *J. Biol. Chem.* **1956**, *221*, 477–489.
51. Saks, V.A.; Veksler, V.I.; Kuznetsov, A.V.; Kay, L.; Sikk, P.; Tiivel, T.; Tranqui, L.; Olivares, J.; Winkler, K.; Wiedemann, F.; et al. Permeabilized cell and skinned fiber techniques in studies of mitochondrial function in vivo. *Mol. Cell. Biochem.* **1998**, *184*, 81–100. [CrossRef]
52. Mathupala, S.P.; Ko, Y.H.; Pedersen, P.L. Hexokinase-2 bound to mitochondria: Cancer's stygian link to the "warburg effect" and a pivotal target for effective therapy. *Semin. Cancer Biol.* **2009**, *19*, 17–24. [CrossRef]
53. Pedersen, P.L. Warburg, me and hexokinase 2: Multiple discoveries of key molecular events underlying one of cancers' most common phenotypes, the "warburg effect", i.E., elevated glycolysis in the presence of oxygen. *J. Bioenerg. Biomembr.* **2007**, *39*, 211–222. [CrossRef]
54. Maldonado, E.N.; Patnaik, J.; Mullins, M.R.; Lemasters, J.J. Free tubulin modulates mitochondrial membrane potential in cancer cells. *Cancer Res.* **2010**, *70*, 10192–10201. [CrossRef]
55. Draberova, E.; Sulimenko, V.; Vinopal, S.; Sulimenko, T.; Sladkova, V.; D'Agostino, L.; Sobol, M.; Hozak, P.; Kren, L.; Katsetos, C.D.; et al. Differential expression of human gamma-tubulin isotypes during neuronal development and oxidative stress points to a gamma-tubulin-2 prosurvival function. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **2017**, *31*, 1828–1846. [CrossRef]
56. Lindstrom, L.; Li, T.; Malycheva, D.; Kancharla, A.; Nilsson, H.; Vishnu, N.; Mulder, H.; Johansson, M.; Rossello, C.A.; Alvarado-Kristensson, M. The gtpase domain of gamma-tubulin is required for normal mitochondrial function and spatial organization. *Commun. Biol.* **2018**, *1*, 37. [CrossRef]
57. Shoshan-Barmatz, V.; Krelin, Y.; Shteinfer-Kuzmine, A.; Arif, T. Voltage-dependent anion channel 1 as an emerging drug target for novel anti-cancer therapeutics. *Front. Oncol.* **2017**, *7*, 154. [CrossRef]
58. Shoshan-Barmatz, V.; Maldonado, E.N.; Krelin, Y. Vdac1 at the crossroads of cell metabolism, apoptosis and cell stress. *Cell Stress* **2017**, *1*, 11–36. [CrossRef]
59. Missiaglia, E.; Jacobs, B.; D'Ario, G.; Di Narzo, A.F.; Soneson, C.; Budinska, E.; Popovici, V.; Vecchione, L.; Gerster, S.; Yan, P.; et al. Distal and proximal colon cancers differ in terms of molecular, pathological, and clinical features. *Ann. Oncol.* **2014**, *25*, 1995–2001. [CrossRef]

60. Drewes, J.L.; Housseau, F.; Sears, C.L. Sporadic colorectal cancer: Microbial contributors to disease prevention, development and therapy. *Br. J. Cancer* **2016**, *115*, 273–280. [CrossRef]
61. De Palma, F.D.E.; D'Argenio, V.; Pol, J.; Kroemer, G.; Maiuri, M.C.; Salvatore, F. The molecular hallmarks of the serrated pathway in colorectal cancer. *Cancers (Basel)* **2019**, *11*, 1017. [CrossRef]
62. Fang, S.; Fang, X. Advances in glucose metabolism research in colorectal cancer. *Biomed. Rep.* **2016**, *5*, 289–295. [CrossRef]
63. Iwamoto, M.; Kawada, K.; Nakamoto, Y.; Itatani, Y.; Inamoto, S.; Toda, K.; Kimura, H.; Sasazuki, T.; Shirasawa, S.; Okuyama, H.; et al. Regulation of 18f-fdg accumulation in colorectal cancer cells with mutated kras. *J. Nucl. Med.* **2014**, *55*, 2038–2044. [CrossRef]
64. Wang, P.; Song, M.; Zeng, Z.L.; Zhu, C.F.; Lu, W.H.; Yang, J.; Ma, M.Z.; Huang, A.M.; Hu, Y.; Huang, P. Identification of ndufaf1 in mediating k-ras induced mitochondrial dysfunction by a proteomic screening approach. *Oncotarget* **2015**, *6*, 3947–3962. [CrossRef]
65. Ralph, S.J.; Low, P.; Dong, L.; Lawen, A.; Neuzil, J. Mitocans: Mitochondrial targeted anti-cancer drugs as improved therapies and related patent documents. *Recent Pat. Anticancer Drug Discov.* **2006**, *1*, 327–346. [CrossRef]
66. Kuznetsov, A.V.; Veksler, V.; Gellerich, F.N.; Saks, V.; Margreiter, R.; Kunz, W.S. Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. *Nat. Protoc.* **2008**, *3*, 965–976. [CrossRef]
67. Kuznetsov, A.V.; Tiivel, T.; Sikk, P.; Kaambre, T.; Kay, L.; Daneshrad, Z.; Rossi, A.; Kadaja, L.; Peet, N.; Seppet, E.; et al. Striking differences between the kinetics of regulation of respiration by adp in slow-twitch and fast-twitch muscles in vivo. *Eur. J. Biochem.* **1996**, *241*, 909–915. [CrossRef]
68. Gnaiger, E. Oxygen solubility in experimental media. *OROBOROS Bioenerg. News* **2001**, *6*, 1–6.
69. Puurand, M.; Tepp, K.; Klepinin, A.; Klepinina, L.; Shevchuk, I.; Kaambre, T. Intracellular energy-transfer networks and high-resolution respirometry: A convenient approach for studying their function. *Int. J. Mol. Sci.* **2018**, *19*, 2933. [CrossRef]
70. Timohhina, N.; Guzun, R.; Tepp, K.; Monge, C.; Varikmaa, M.; Vija, H.; Sikk, P.; Kaambre, T.; Sackett, D.; Saks, V. Direct measurement of energy fluxes from mitochondria into cytoplasm in permeabilized cardiac cells in situ: Some evidence for mitochondrial interactosome. *J. Bioenerg. Biomembr.* **2009**, *41*, 259–275. [CrossRef]

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Appendix 2

Publication II

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REVIEW
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Energy Metabolic Plasticity of Colorectal Cancer Cells as a Determinant of Tumor Growth and Metastasis

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Metabolic plasticity is the ability of the cell to adjust its metabolism to changes in environmental conditions. Increased metabolic plasticity is a defining characteristic of cancer cells, which gives them the advantage of survival and a higher proliferative capacity. Here we review some functional features of metabolic plasticity of colorectal cancer cells (CRC). Metabolic plasticity is characterized by changes in adenine nucleotide transport across the outer mitochondrial membrane. Voltage-dependent anion channel (VDAC) is the main protein involved in the transport of adenine nucleotides, and its regulation is impaired in CRC cells. Apparent affinity for ADP is a functional parameter that characterizes VDAC permeability and provides an integrated assessment of cell metabolic state. VDAC permeability can be adjusted *via* its interactions with other proteins, such as hexokinase and tubulin. Also, the redox conditions inside a cancer cell may alter VDAC function, resulting in enhanced metabolic plasticity. In addition, a cancer cell shows reprogrammed energy transfer circuits such as adenylate kinase (AK) and creatine kinase (CK) pathway. Knowledge of the mechanism of metabolic plasticity will improve our understanding of colorectal carcinogenesis.

Keywords: tumor energy metabolism, aerobic glycolysis, oxidative phosphorylation, VDAC, creatine kinase, adenylate kinase, mitochondria

INTRODUCTION

Analysis of mitochondrial function is central to the study of intracellular energy metabolism and pathophysiological mechanisms of various human diseases, including cancer. The metabolism of cancer cells is adapted to meet their needs to survive and proliferate in a hypoxic and also in a well-oxygenated microenvironment and thus must acquire metabolic flexibility. At the molecular level,

Abbreviations: ADP, adenosine diphosphate; AMPK, adenosine 5'-monophosphate-activated protein kinase; AK, adenylate kinase; ANT, adenine nucleotide translocator; CK, creatine kinase; CRC, colorectal cancer; HK, hexokinase; HIF, hypoxia-inducible factor; ISC, iron-sulfur clusters; OMM, outer mitochondrial membrane; TCA, tricarboxylic acid; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; VDAC, voltage-dependent anion channel.

metabolic flexibility relies on the configuration of metabolic pathways, which are regulated by key metabolic enzymes and transcription factors. Reprogramming of cellular energetics is recognized as a distinctive hallmark of cancer (1). The first theory on the peculiarities of cancer metabolism was formulated by Otto Warburg in the early 20th century. He concluded that tumors, unlike normal cells, obtain their energy mainly from aerobic glycolysis, while normal cells usually favor oxidative phosphorylation (OXPHOS), which is much more efficient in terms of ATP gain. This observation is coined as the Warburg effect (2, 3) and became the central model for oncobioenergetics for most of the 20th century. The glycolytic part of the Warburg hypothesis was firmly and thoroughly confirmed for many cancer types, in contrast to the OXPHOS part, which was and still is a matter of intense research and controversy. Verified evidence indicates that in reality, both anaerobic (glucose to lactate) and aerobic (glucose to pyruvate) glycolysis operate in cancer cells simultaneously like in normal cells, although at higher rates than in non-tumor cells (4). In addition, tumor cells often exhibit high rates of OXPHOS (5, 6). Transcriptomics and end-product metabolites analyses of complex molecular pathways converge into a three-node minimum regulatory network consisting of hypoxia-inducible factor 1 (HIF-1), adenosine monophosphate-activated protein kinase (AMPK), and reactive oxygen species (ROS). Therefore, the coexistence of three distinct cellular metabolic phenotypes is revealed in cancer cells: 1) glycolytic, characterized by high activity of HIF-1α and high activity of the glycolytic pathway; 2) OXPHOS state, characterized by high activity of AMPK and high activity of OXPHOS pathways such as glucose oxidation and fatty acid oxidation; 3) hybrid metabolic state, characterized by high activity of AMPK and HIF-1α and concomitant functioning of glycolysis and OXPHOS pathways. In contrast, normal cells exhibit only two metabolic states, namely, glycolytic and OXPHOS, and lack the hybrid state (7, 8). In this regulatory network, HIF-1 and AMPK are the master regulators of glycolysis and OXPHOS, respectively (9), and both cytosolic and mitochondrial ROS mediate the complex interplay between AMPK and HIF-1. Accordingly, the hybrid metabolic state in cancer cells can be promoted by the stabilization of HIF-1α and elevated production of mitochondrial ROS. Hypoxia activates glycolysis *via* stabilization of HIF-1α and HIF-2α, which in turn upregulates the activity of several members of the glycolytic pathway and increases glucose uptake (10, 11). In addition, the elevation of HIF-1α levels could be induced by high concentrations of succinate (pseudohypoxia) (12). A striking feature of cancer cells is their ability to switch their metabolic phenotypes to glycolysis or OXPHOS in response to changes in their microenvironment or inhibition of one of these pathways, giving survival advantage during tumor progression (8, 13). This metabolic plasticity is promoted by the hybrid phenotype of cancer cells and is linked with metastasis and chemoresistance (14). However, it is still largely unknown how cancer cells regulate gene expression to maintain their hybrid metabolic state and metabolic plasticity.

Implementation of the hybrid metabolism paradigm may reveal new therapeutic targets and opportunities for the treatment of cancer. It was previously shown that administration of glycolytic inhibitors alone may be ineffective to eradicate tumors, and targeting the hybrid state to eliminate metabolic plasticity could be a new therapeutic strategy to eliminate cancer aggressiveness (15, 16). We review the changes in OMM permeability and intracellular energy transfer pathways in connection with the metabolic plasticity of CRC cells.

METABOLIC REPROGRAMMING OF COLORECTAL CANCER

Colorectal cancer has been regarded as a purely hypoxic tumor of the Warburg phenotype for many years. This was confirmed by increased expression of several glycolytic enzymes, pentose phosphate pathway, and glucose transporters associated with elevated rates of glucose consumption and lactate production as compared with normal surrounding tissues (17–25). Normal colonocytes use the OXPHOS system as the primary energy source (26, 27). Short-chain fatty acids undergo β-oxidation to form acetyl-CoA, which enters into the tricarboxylic acid (TCA) cycle to yield citrate, NADH, and finally ATP. But, unlike normal colonocytes, colorectal carcinomas cannot utilize butyrate as an energy source and carbon donor (26, 28), implying the truncated TCA cycle in CRC. Importantly, some metabolites of the TCA cycle, such as succinate, fumarate, and α-ketoglutarate, act as "oncometabolites" that support tumor growth *via* oncogenic signaling, inter alia *via* upregulation and stabilization of HIF-1α (29).

Metabolic reprogramming during large intestine carcinogenesis is largely mediated by (a) altered expression of several oncogenes and a loss of tumor suppressor genes, encoding usually various transcriptional factors and protein kinases (30, 31), (b) adaptation to nutrient and oxygen availability in the local tumor microenvironment (metabolic plasticity) (32), and (c) metabolic cross-talk with stromal, adipose tissue and immune cells (31, 33–37).

Data on molecular mechanisms of the metabolic reprogramming of CRC are mostly obtained from studies using cell culture models, while the number of functional studies using clinical material is limited. Moreover, cell culture conditions have variations that could significantly affect the metabolic profile of the cells. For example, cells grown in glucose-free medium display a relatively high rate of oxygen consumption, while cultivation of cells in a high-glucose medium results in hyperglycolytic profile and declined respiratory flux (38–42). Our recent studies revealed remarkable differences in the regulation of outer mitochondrial membrane (OMM) permeability between cultured tumor cells and clinical material from cancer patients (5, 43). Comparative analysis of the biopsy or surgical cancer material and surrounding healthy tissue showed almost unchanged glycolytic activity and upregulation of OXPHOS in CRC, which is inconsistent with the data obtained by using cell culture (43–47). In addition, two widely

used breast cancer cell lines MCF7 and MCF-MDA-231 failed to replicate mitochondrial function in respect to metabolic activity and OXPHOS as seen in respective human samples (43, 46).

Why the CRC cells shift their metabolism in favor of OXPHOS? Perhaps, under normal conditions, the amount of ATP produced through aerobic glycolysis is insufficient to support cell proliferation and migration. There is a growing body of evidence that CRC is characterized by stimulated mitochondrial biogenesis expressed as an increase in mitochondrial DNA copy number (48) and elevated ADP-dependent oxygen consumption in CRC tissue (5, 6, 43–45). Activated mitochondrial biogenesis can be an adaptive response of tumor cells to overcome the chronic energy crisis caused by glucose starvation or defects in the function of their respiratory enzymes due to pathogenic nuclear or mtDNA mutations (49–51). The elevated lactate level may act as a signaling molecule to affect genes and proteins known to be involved in mitochondrial biogenesis (52), *via* upregulation of AMPK- and SIRT1-associated PGC-1α activation (53). Nuclear Respiratory Factor 1 (NRF1) (54) and some cytokines, IL-6/8 (55, 56), activate the AMPK signaling pathway as well as apoptotic resistance of cancer cells (56–58). Some types of tumor cells support their high rates of OXPHOS and drug resistance by transferring mtDNA or even the entire mitochondria from surrounding healthy tissues; this intercellular mitochondrial transfer may occur through exosomes or tunnel nanotubes (59, 60). The signaling pathways responsible for the stimulation of mitochondrial biogenesis can have both intracellular and external origins.

THE ROLE OF VDAC AND THE REGULATION OF OUTER MITOCHONDRIAL MEMBRANE PERMEABILITY IN METABOLIC PLASTICITY

The flux of water-soluble metabolites into and out of the mitochondria occurs through a variety of inner mitochondrial membrane (IMM) carriers, but the flux of ATP, ADP, and Pi across the OMM occurs through a single pathway, the VDAC, and therefore the regulation of OXPHOS is largely mediated by the VDAC permeability control (61). Based on studies of muscle permeabilized fibers, cellular respiration and associated ATP synthesis are regulated by a protein complex called Mitochondrial Interactosome (MI), which is located at the junction of mitochondrial membranes (62, 63). Restrictions for adenine nucleotides in VDAC are evident by measuring an apparent affinity of mitochondria for exogenous ADP [Km (ADP)] in permeabilized cells and tissues by using high-resolution respirometry (64, 65). These barriers appear only in permeabilized cells and not in isolated mitochondria and disappear during mild proteolytic treatment with trypsin (66). Therefore, the metabolic plasticity of cancer cells is associated with the protein-mediated control of VDAC permeability towards ADP.

Cancer Metabolic Plasticity Is Functionally Defined by Changes in ADP Dependent Oxygen Consumption

Analysis of respirometry data provides instant functional profiling of metabolic plasticity. Dependence of mitochondrial O_2 consumption upon ADP concentration follows Michaelis-Menten kinetics and allows evaluation of apparent Michaelis-Menten constant for ADP Km(ADP) in different tissues, cancers, and cell cultures (**Figure 1**). Determined in permeabilized cells and tissues, Km(ADP) is the affinity of the mitochondria for exogenous ADP and characterizes permeability of OMM for adenine nucleotides and, thus, VDAC permeability. Measured Km(ADP) values for human colon mucosa is ~110 μM (47), ~100 μM for CRC (5, 44, 47), ~60 μM for colon polyps (47), and ~40 μM for Caco2 CRC cell line (43), indicating the alteration of control mechanisms over VDAC permeability and OXPHOS during the progression of CRC. Thus, the regulation of OMM permeability to adenine nucleotides in cancer tissues is different from that in normal cells (5, 67, 68). Notably, Km(ADP) values measured in cell cultures are much lower than in tissue biopsies and are similar to Km(ADP) values for isolated mitochondria (69). This illustrates the shortcomings of cell culture studies and highlights the importance of using clinical material for the evaluation of the mechanism of cancer metabolic plasticity.

The cell-specific differences in Km(ADP) are likely caused by the specific structural and functional organization of energy metabolism. For example, cells with a low Km(ADP) value (~10 μM), like glycolytic muscle, possess less structural and functional restrictions for ADP/ATP movement through OMM as compared to the oxidative muscles (Km(ADP) ~300 μM) (64). Thus, relatively low Km(ADP) for colorectal polyps indicates a metabolic reprogramming towards the glycolytic phenotype with functional OXPHOS (as in glycolytic muscle), and an increase in Km values in the CRC reflects a shift to OXPHOS phenotype with increased intracellular complexity (analogy with oxidative muscle). Hence, Km(ADP) value is an important parameter describing metabolic plasticity. According to the model proposed by Saks V. et al, the proportion of mitochondria with low oxidative capacity in the tissue can be inferred from the Km (ADP) value (70). For example, the proportion of mitochondria with high oxidative capacity is 67% in CRC tumors and only 38% in colorectal polyps (47).

In addition to Km(ADP), the maximal ADP-dependent oxygen consumption (V_{max}) is a defining characteristic of metabolic plasticity and is correlated to mitochondrial content (density) in the tissue. V_{max} values are higher in CRC than in normal colon tissue (5, 6, 47), indicating a vigorous metabolic activity. Moreover, V_{max} values in biopsy material from patients that succumbed to colon cancer were significantly higher than in patients staying in remission (5). However, the extent to which high V_{max} values correlate with tumor aggressiveness needs to be confirmed in further studies.

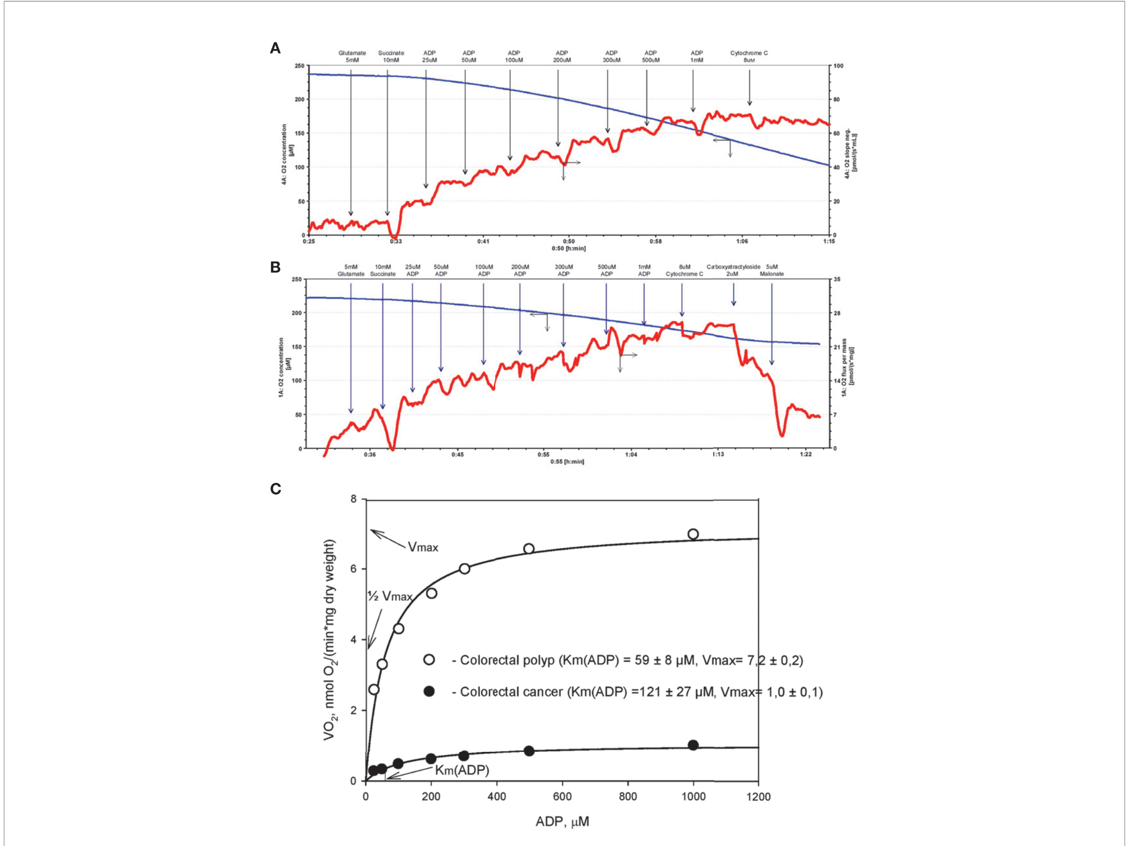

FIGURE 1 | Michaels-Menten kinetics of ADP-dependent respiration of human colorectal cancer and polyp biopsy material. Representative tracing of adenosine diphosphate (ADP)-activated oxygen consumptions rates in human permeabilized tissue of **(A)** colorectal polyp and **(B)** and colorectal cancer. **(C)** Corresponding Km (ADP) and V_{max} values were calculated by non-linear regression using the Michaelis-Menten equation.

The Possible Mechanisms of VDAC Permeability Regulation

Several studies show that VDAC isoform 1 (VDAC1) is the dominant isoform in most malignant tumors including CRC (44, 71, 72). VDAC1 is crucial in communication between the mitochondria and the cytosol. Cancer cells display high levels of metabolic flexibility combined with apoptosis resistance, which provides a survival advantage for these cells. VDAC1 is well recognized as a metabolic checkpoint at the crossroad of these two processes (72, 73). VDAC mediates and regulates the transport of metabolites, ions, and ROS across OMM. Thus, VDAC1 plays a major role in the control of mitochondrial function. Transport of ADP through OMM is mediated *via* VDAC1 and through the inner membrane *via* ANT. Metabolic control analysis of the OXPHOS system of CRC revealed that ANT does not exert exclusive control over the mitochondrial ADP-dependent oxygen consumption (5, 43). Therefore, the rate-limiting step of ADP transport into the mitochondria appears to be VDAC. Therefore, the alteration of Km(ADP) value depends on the changes in interactions of VDAC1 with other proteins or on the modification of VDAC1 itself.

As the name implies, VDAC is regulated by a change of membrane potential. Studies of isolated VDAC1 reconstituted into planar lipid bilayers reveal sharp and symmetrical voltage dependence of VDAC1 permeability (72, 74, 75). At membrane potentials close to zero (between −20 to +20 mV), VDAC1 is open and displays low anionic selectivity. At more positive or more negative membrane potentials (+30.+60 mV or −30.−60 mV), VDAC1 shows diminished permeability to large anions and becomes more selective to small cations (72). However, it is unknown whether the voltage dependence of VDAC1 is relevant in physiological conditions, as the value of membrane potential across OMM is unknown. It is generally believed that any membrane potential generated at OMM will be offset by a relatively undisturbed movement of small ions across OMM. However, there is a theoretical possibility that OMM can be polarized to potentials large enough to alter the permeability of VDAC1 (2, 3). Although the role of OMM potential in the

regulation of VDAC1 permeability is unlikely, it remains to be investigated whether potential across OMM changes in CRC and whether such change can alter Km(ADP).

Hexokinase-VDAC Interaction Regulates the Permeability of VDAC to Adenine Nucleotides

Although the VDAC-hexokinase (HK) binding was demonstrated by several groups using different experimental approaches, it still remains somewhat speculative, and there are different hypothesis on its functional consequences. Research activities of Prof. Pedersen and his colleagues resulted in the discovery of the binding of HK-II to VDAC with the conclusion that this phenomenon could play a pivotal role in the "Warburg Effect" (76–80). Review paper of V. Shoshan-Barmatz et al. proposed the hypothesis that HK-II binds to VDAC and promotes VDAC closing (81). Neumann et al. demonstrated the binding of the cytosolic protein HK-I to VDAC by two-color STED microscopy (82). Our group showed the colocalization of VDAC1 and hexokinase II in cell cultures and clinical cancer samples by confocal microscopy imaging (6, 67). Based on these studies, two models of VDAC permeability control have been proposed. The model proposed by Pedersen et al. states that the binding of HK-II to VDAC plays a pivotal role in maintaining the Warburg phenotype in cancer cells (77, 83). In such a setting, mitochondrial ATP is preferentially directed to glycolysis (HK reaction) and the produced ADP is channeled back to the OXPHOS (**Figure 2**). At the same time, VDAC is assumed to be in an open state and mitochondria have free access to exogenous ADP (84, 85), thus low Km(ADP) values are expected. Glucose-stimulated increase of mitochondrial respiration shows the amount of ADP released in the HK reaction that passes through VDAC and is utilized in mitochondrial ATP synthesis (86). Such glucose effect comprises a fraction of total ADP-stimulated respiration and is higher in cancer cells as compared to normal cells. Accordingly, the glucose effect is about 20% for CRC tissue, about 12% for normal colon tissue samples (6), and about 48% for Caco-2 CRC cell line (43). These results show that the lower affinity of mitochondria for ADP could be related to the weaker ability for glucose to stimulate respiration. CRC displays elevated levels of VDAC1 as compared with surrounding healthy tissues (43), and this is in good agreement with the fact that V_{max} for ADP-dependent respiration is higher in CRC (44). The total HK activity and expression levels of HK1 and HK2 in CRC do not differ from that of normal tissue (6, 44). In both the normal mucosa and the CRC, HK2 is colocalized with VDAC (6, 43). The interaction of HK1 or HK2 with VDAC1 gives numerous advantages to cancer cells: (1) it mediates the increased permeability of the OMM to adenine nucleotides; (2) it increases the rate of aerobic glycolysis and thereby allows the cells to adapt to hypoxic conditions; (3) it mediates elevated resistance to apoptosis and protection from oxidative stress as VDAC1-bound HK acts as an anti-apoptotic protein (73, 87–89). VDAC-HK interaction is reversed with inhibitors of HK2 (e.g., 3-bromopyruvate), and agents that disrupt the VDAC-HK interaction have been tested as anticancer drugs (73, 90–93). It was also reported that silencing of VDAC1 expression by siRNA inhibited the proliferation of several cancer cell lines (including CRC) (94).

Free Beta-Tubulins Controlling VDAC Permeability in CRC

According to the free-tubulin model, the binding of free tubulin blocks VDAC and thereby regulates respiration (95). The

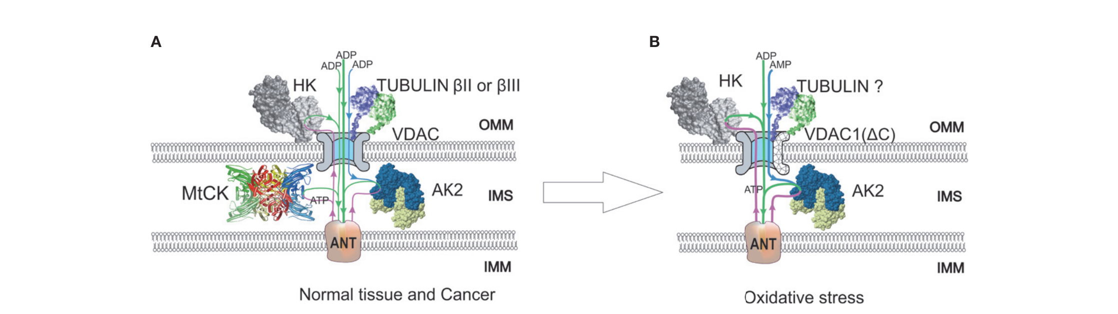

FIGURE 2 | A model of regulation of outer mitochondrial membrane (OMM) permeability for adenine nucleotides in normal and colorectal cancer (CRC) cells. Voltage-dependent anion channel (VDAC) is the pore through which adenine nucleotides move into and out of the mitochondria. **(A)** In normal and possibly some cancer cells, a minor amount of hexokinase (HK) is bound to VDAC and utilizes mitochondrial ATP to initiate glycolysis. Produced ADP is channeled back to the mitochondrial matrix *via* VDAC and adenine nucleotide translocase (ANT) for use in oxidative phosphorylation (OXPHOS). VDAC permeability is also regulated by tubulin binding. As a result of beta-tubulin-VDAC interaction, the VDAC is less permeable to adenine nucleotides. This in turn promotes cells to use creatine kinase (CK) and adenylate kinase (AK) energy transfer networks for intracellular distribution of high-energy phosphates. Mitochondrial intermembrane space (IMS)-residing mitochondrial CK (MtCK) is functionally coupled to ANT, turning OXPHOS to be dependent on ADP originating from MtCK reaction. Mitochondrial AK isoform AK2 uses AMP passing through VDAC and ATP passing through ANT to produce ADP, which stimulates OXPHOS. These energy transport systems provide feedback between ATP consumption and synthesis. **(B)** Redox stress may induce an increased amount of HK bound to VDAC. In addition, VDAC can be truncated at C-terminus by proteases activated in response to oxidative stress. The role of tubulin in the regulation of VDAC permeability remains unclear, as the interaction of truncated VDAC with tubulin might be impaired. The AK2 activity in cancer cells is increased, resulting in enhanced utilization of extra-mitochondrial AMP to OXPHOS. IMM, inner mitochondrial membrane; IMS, intermembrane space.

rationale behind this model is the observation that proliferating cancer cells have high levels of free tubulin for mitotic spindle formation. Free tubulin dimers bound to VDAC induce a closed state of VDAC (**Figure 2**) and cause a suppression of mitochondrial metabolism; thus, aerobic glycolysis will become the main source of energy. Maldonado and Lemasters's group shows at HepG2, A549, and UM-SCC-1 cells that tubulin binding closes the VDAC channel (95). It sounds like the hypothesis in this review contradicts Maldonado's publications (95, 96). However, in fact, the results of both works are in agreement. The amount of dimeric and polymerized tubulin in cells is nearly constant, but the ratio could change significantly. In both cases it is dimeric tubulin, which affects VDAC permeability, but this effect depends on the polymerization state. Also, it should be definitely noted that the regulation of VDAC permeability is tissue specific. Unlike striated muscles, where the main regulator of VDAC is beta-II tubulin (97), in CRC the VDAC and beta-II tubulin colocalization is absent (6). Instead, beta-III tubulin (TUBB3) could be the partner of VDAC in CRC cells. Beta-III tubulin overexpression has been reported in several intestinal cancers like carcinoids of the small intestine and rectal carcinoids (98), gastric cancer (99), colon neoplasias like polyps, and CRC (6, 100). *TUBB3* expression has been associated with the resistance to drugs perturbing the microtubule dynamics (e.g., paclitaxel) and studied as a prognostic biomarker in various cancers (101, 102). It has been demonstrated that in non-small-cell lung cancer, the expression of beta-III tubulin decreases the dependence of cells on glycolysis and thus improves the tumor's ability to cope with the changing nutrient supply in the microenvironment (103). From a functional analysis of the network of proteins forming disulfide bonds with beta-III tubulin, it appears that some of them are involved in oxidative stress and glucose deprivation response (104). It was shown that hypoxia *via* HIF-1α can induce the expression of *TUBB3* (105). Beta-III tubulin is likely part of a complex pathway induced by hypoxia and shortage of nutrients (101). However, our recent study revealed that microtubule destabilizing (colchicine) and stabilizing (taxol) agents do not affect the Km(ADP) in glioblastoma and sarcoma cells (67). Hence, the actual role of beta-tubulins in cancer metabolism and mitochondrial respiratory control needs further investigation.

Regulation of VDAC1 by Protein-Protein Interactions and Redox Stress

In addition to the two previous models, the modifications of VDAC1 protein induced by oxidative stress could be responsible for alterations of apparent value of Km(ADP). Tumor cells are well adapted to a hypoxic environment, and VDAC1 is regulated by oxygen tension in HIF-1α-dependent manner at the levels of transcription and protein modification. Transcription of the *VDAC1* gene is regulated by HIF-1α and NRF-1 (nuclear respiratory factor 1), which leads to increased levels of VDAC1 in response to hypoxia or nutrient deprivation of the cells (106). Along with VDAC1 expression regulation, HIF-1α is also involved in the cleavage of VDAC1, resulting in a truncated form of VDAC1 (107). In normoxic conditions, VDAC1 is expressed as a full-length protein of molecular weight of approximately 30 kDa, while in response to hypoxia, there is a larger proportion of a shorter VDAC1 variant lacking C-terminal part (VDAC1-ΔC) with a molecular weight of approximately 25 kDa (107). The shorter variant is a product of the cleavage of VDAC1 at asparagine 214 by the asparagine endopeptidase Legumain (LGMN), which in turn is activated in a HIF-1α-dependent way upon hypoxia (107). The electrophysiological properties of VDAC1-ΔC are similar to full-length protein; however, its permeability is slightly reduced (107). Levels of VDAC1-ΔC were higher in late-stage lung tumors (107), and it was suggested that HIF-1α mediated induction of VDAC1-ΔC provides protection from apoptosis and enhances cell survival in hypoxia (107, 108). Hypoxia-induced VDAC1-ΔC lacks a phosphorylation site at serine 215, and therefore its interaction with tubulin is impaired (108). Notably, *HIF-1α* overexpression was significantly associated with higher CRC-specific mortality in a cohort of 731 patients (109). Consequently, inhibition of HIF-1α is proposed as a possible treatment strategy for CRC (110). Moreover, the expression of endopeptidase LGMN is elevated in CRC and is associated with a poor prognosis (111). Furthermore, a meta-analysis revealed the overexpression of *LGMN* to be correlated with the aggressiveness of different cancer types, with higher levels of LGMN in late-stage tumors (112).

It is currently unknown whether VDAC1-ΔC is present in CRC cells and whether truncation-induced impairment of VDAC1 interaction with tubulin affects apparent affinity for ADP (**Figure 2**). Given the role of tubulin in the regulation of VDAC1 and the discovery of VDAC1-ΔC in lung cancer, VDAC1 truncation may also play a role in metabolic alterations of CRC. Future studies should reveal whether the truncated form of VDAC1 plays a role in metabolic adaptations of CRC.

Recent studies indicate a link between iron-sulfur cluster (ISC) synthesis and regulation of VDAC1. Biogenesis of ISC is an ancient process, and ISCs are important redox-sensitive cofactors for many enzymes involved in energy homeostasis. Synthesis of ISC starts within the mitochondrial matrix, and depletion of proteins involved in mitochondrial ISC assembly leads to accumulation of VDAC1-ΔC in normoxic conditions independent of HIF-1α (113). Depletion of the iron-sulfur cluster containing protein CISD2 also resulted in the accumulation of truncated VDAC1-ΔC (113). Therefore, mitochondria-associated membrane-localized Fe-S protein CISD2 acts as a link between ISC machinery and accumulation of VDAC1-ΔC (113).

Another iron-sulfur cluster protein, mitoNEET, was found to interact with VDAC1 in a redox-sensitive way (114). MitoNEET harbors [2Fe-2S] cluster and binds to VDAC1 when its cluster is oxidized, thus inhibiting VDAC1 conductivity. Such interaction does not occur when mitoNEET-bound ISC cluster is reduced (114). Therefore, mitoNEET governs VDAC1 permeability in a redox-sensitive way, inhibiting VDAC1 in high redox stress conditions. Oxidative stress is increased in CRC (115); thus, the interaction of mitoNEET with VDAC1 can be altered in CRC.

It remains to be investigated whether such redox-sensitive mitoNEET-VDAC1 interaction can alter the apparent Km (ADP) value and is involved in the metabolic plasticity of CRC.

There is a large number of proteins that were found to interact with VDAC1 and are therefore potentially able to modulate VDAC permeability. Interacting partners of VDAC1 are involved in the regulation of apoptosis (Bax, Bcl2, Bak, etc.), energy metabolism (HK1, HK2, ACSL, CPT1, ANT, etc.), cytoskeletal organization (Tubulin, actin, dynein, etc.), and other cellular functions [Parkin, alpha-synuclein, APP, gamma-secretase) [reviewed in (116)]. However, the role of these interactions in the modulation of cellular respiration needs to be further investigated.

ENERGY TRANSPORT PATHWAYS IN CRC CELLS—THE PARTICIPANTS IN THE METABOLIC PLASTICITY

In addition to the altered transport of adenine nucleotides through OMM alterations of energy transport circuits formed from creatine kinase (CK) and adenylate kinase (AK) isoenzymes are also involved in the development of metabolic plasticity. Cancer cells have uncontrolled cell division, which is accompanied by a high energy need for anabolic processes and large cell structure rearrangements. Therefore, it is hypothesized that energy transport pathways are also reprogrammed in cancer cells to meet these demands. Previous data show downregulation of the CK pathway and mitochondrial CK (MtCK) in CRC cells, which results in functional uncoupling between the CK circuit and OXPHOS (6, 44). In contrast, total AK activity is higher in CRC than in normal intestinal tissue, and it also reflects enhanced coupling between AK and OXPHOS (i.e., AMP can affect the rate of oxygen consumption) (**Figure 2**) (6, 44). This is in agreement with the observation that expression of AK mitochondrial isoform AK2 is increased in several cancers including lung adenocarcinoma (117) and breast cancer (118, 119). Also, there is evidence that another mitochondrial isoform, AK4, is involved in the regulation of mitochondrial metabolism in cancer cells. In HeLa cells, AK4 forms complexes with ANT, VDAC, and HK2 for the efficient recycling of ADP (120). Further, AK4 expression is induced by hypoxia, and protein complex AK4-ANT-VDAC-HK2 complex supports the high glycolytic activity of cancer cells (120). Intestinal cells are able to switch off the CK circuit and turn on the AK pathway to establish metabolic plasticity. Such flexibility of phosphotransfer networks in Caco2 CRC cell lines depends on the availability of key metabolic substrates and is associated with the cell differentiation state (121). The abovementioned data indicate a possible role of the phosphotransfer networks related to the regulation of VDAC permeability for adenine nucleotides and metabolic plasticity.

The function of energy transfer pathways is well characterized in striated muscle cells where its role is to overcome the diffusion restrictions for ATP and ADP, thereby directing the energy-rich phosphate groups to the CK, AK, and glycolytic energy transfer circuits. This way of energy transfer allows the formation of micro-compartments at energy consumption sites where high ATP/ADP levels are maintained for maximal performance. Similarly, in the compartment where energy is produced (e.g., mitochondrial membranes), favorable levels of ADP are maintained to ensure efficient ATP synthesis [reviewed in (65, 122)]. In the case of CRC, downregulation of MtCK leads to the inability to produce phosphocreatine and a loss of functional coupling between the VDAC-MtCK-ANT complex, accompanied by the formation of other regulating combinations like VDAC-HK-ANT. In this aspect, more studies are required to determine the profile of HK, AK, ANT, and VDAC isoform expression in human CRC.

In addition to their role in energy transfer among cellular processes, AKs are an integral part of intracellular energy sensing and metabolic signaling (123, 124). Due to its catalytic reaction (2ADP ↔ AMP + ATP), it can amplify a small change in the ATP/ADP ratio into relatively large changes in AMP concentration. This relates AKs to the activation of cellular AMP-sensitive components like AMPK. In general, activation of AMPK switches on catabolic pathways that generate ATP, while switching off biosynthetic pathways and cell-cycle progress (125). The role of AMPK in cancer is controversial; it has been recognized as a tumor suppressor in some cancers (126–129) and in some cases described as a contextual oncogene, as the AMPK activation promotes tumor progression and chemoresistance (130–132). Downregulation of AK→ AMP→ AMPK signaling could lead to loss of control over the cell cycle, growth, and proliferation (124). A recent in-depth review about AKs and metabolic signaling in cancer cells by Klepinin et al. (124) highlights the role of suppression of AK phosphotransfer and signaling through AMPK as a potential target for cancer metabolism. How different AK isoforms are distributed in CRC cells and how their activities affect AMPK activation and metabolic plasticity need further investigation.

Adenylate kinases network promotes cancer growth and metastasis through participating in AMPK metabolic signaling and regulating mitochondrial adenine nucleotide exchange.

CONCLUSION AND PROSPECTS

Metabolic plasticity is a defining characteristic of the cancer cells that allow undisturbed proliferation in changing environment. At the functional level, different metabolic states of the cancer cells can be identified and characterized by measuring the dependence of mitochondrial respiration upon ADP concentration using the classical Michaelis-Menten kinetic model. The apparent affinity of ADP provides an integrated assessment of cell metabolic state, which is functionally determined by the permeability of VDAC1. Regulation of VDAC1 involves many protein-protein interactions, as well as hypoxia- and redox-sensitive mechanisms. The regulation of OMM permeability for adenine nucleotides is presumably more complex than the binding between the VDAC1 channel and some single type of protein molecule. Unraveling the molecular mechanisms of metabolic plasticity will reveal new therapeutic targets for the development of novel cancer treatments. This knowledge combined with relatively simple

functional evaluation of cancer metabolism in biopsy material can form a new prospect for personalized medicine.

AUTHOR CONTRIBUTIONS

Conceptualization, LT, MP, AT, and TK. Funding acquisition, TK. Project administration, AT and TK. Visualization, LR and IS. Writing—original draft, MP, AT, VC, and TK. Writing—review and editing, LR, SM, ER-K, NT, KT, IS, and TK. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. *Cell* (2011) 144(5):646–74. doi: 10.1016/j.cell.2011.02.013
2. Lemeshko SV, Lemeshko VV. Metabolically Derived Potential on the Outer Membrane of Mitochondria: A Computational Model. *Biophys J* (2000) 79 (6):2785–800. doi: 10.1016/S0006-3495(00)76518-0
3. Lemeshko VV. Model of the Outer Membrane Potential Generation by the Inner Membrane of Mitochondria. *Biophys J* (2002) 82(2):684–92. doi: 10.1016/S0006-3495(02)75431-3
4. Moreno-Sanchez R, Marin-Hernandez A, Saavedra E, Pardo JP, Ralph SJ, Rodriguez-Enriquez S. Who Controls the ATP Supply in Cancer Cells? Biochemistry Lessons to Understand Cancer Energy Metabolism. *Int J Biochem Cell Biol* (2014) 50:10–23. doi: 10.1016/j.biocel.2014.01.025
5. Koit A, Shevchuk I, Ounpuu L, Klepinin A, Chekulayev V, Timohhina N, et al. Mitochondrial Respiration in Human Colorectal and Breast Cancer Clinical Material Is Regulated Differently. *Oxid Med Cell Longev* (2017) 2017:1372640. doi: 10.1155/2017/1372640
6. Kaldma A, Klepinin A, Chekulayev V, Mado K, Shevchuk I, Timohhina N, et al. An *in Situ* Study of Bioenergetic Properties of Human Colorectal Cancer: The Regulation of Mitochondrial Respiration and Distribution of Flux Control Among the Components of ATP Synthasome. *Int J Biochem Cell Biol* (2014) 55:171–86. doi: 10.1016/j.biocel.2014.09.004
7. Yu L, Lu M, Jia D, Ma J, Ben-Jacob E, Levine H, et al. Modeling the Genetic Regulation of Cancer Metabolism: Interplay Between Glycolysis and Oxidative Phosphorylation. *Cancer Res* (2017) 77(7):1564–74. doi: 10.1158/0008-5472.CAN-16-2074
8. Paudel BB, Quaranta V. Metabolic Plasticity Meets Gene Regulation. *Proc Natl Acad Sci USA* (2019) 116(9):3370–2. doi: 10.1073/pnas.1900169116
9. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. *Science* (2009) 324(5930):1029–33. doi: 10.1126/science.1160809
10. Libby CJ, McConathy J, Darley-Usmar V, Hjelmeland AB. The Role of Metabolic Plasticity in Blood and Brain Stem Cell Pathophysiology. *Cancer Res* (2020) 80(1):5–16. doi: 10.1158/0008-5472.CAN-19-1169
11. Berridge MV, Herst PM, Tan AS. Metabolic Flexibility and Cell Hierarchy in Metastatic Cancer. *Mitochondrion* (2010) 10(6):584–8. doi: 10.1016/j.mito.2010.08.002
12. Garrigue P, Bodin-Hullin A, Balasse L, Fernandez S, Essamet W, Dignat-George F, et al. The Evolving Role of Succinate in Tumor Metabolism: An (18)F-FDG-Based Study. *J Nucl Med* (2017) 58(11):1749–55. doi: 10.2967/jnumed.117.192674
13. Lin Y, Ma C, Bezabeh T, Wang Z, Liang J, Huang Y, et al. 1h NMR-Based Metabolomics Reveal Overlapping Discriminatory Metabolites and Metabolic Pathway Disturbances Between Colorectal Tumor Tissues and Fecal Samples. *Int J Cancer* (2019) 145(6):1679–89. doi: 10.1002/ijc.32190
14. Jia D, Park JH, Jung KH, Levine H, Kaipparettu BA. Elucidating the Metabolic Plasticity of Cancer: Mitochondrial Reprogramming and Hybrid Metabolic States. *Cells* (2018) 7(3):21. doi: 10.3390/cells7030021
15. Jia D, Lu M, Jung KH, Park JH, Yu L, Onuchic JN, et al. Elucidating Cancer Metabolic Plasticity by Coupling Gene Regulation With Metabolic Pathways. *Proc Natl Acad Sci* (2019) 116(9):3909–18. doi: 10.1073/pnas.1816391116
16. Sotgia F, Ozsvari B, Fiorillo M, De Francesco EM, Bonuccelli G, Lisanti MP. A Mitochondrial Based Oncology Platform for Targeting Cancer Stem Cells (CSCs): MITO-ONC-Rx. *Cell Cycle* (2018) 17(17):2091–100. doi: 10.1080/15384101.2018.1515551
17. Altenberg B, Greulich KO. Genes of Glycolysis Are Ubiquitously Overexpressed in 24 Cancer Classes. *Genomics* (2004) 84(6):1014–20. doi: 10.1016/j.ygeno.2004.08.010
18. Kawada K, Toda K, Nakamoto Y, Iwamoto M, Hatano E, Chen FS, et al. Relationship Between F-18-FDG PET/CT Scans and KRAS Mutations in Metastatic Colorectal Cancer. *J Nucl Med* (2015) 56(9):1322–7. doi: 10.2967/jnumed.115.160614
19. Iwamoto M, Kawada K, Nakamoto Y, Itatani Y, Inamoto S, Toda K, et al. Regulation of 18F-FDG Accumulation in Colorectal Cancer Cells With Mutated KRAS. *J Nucl Med* (2014) 55(12):2038–44. doi: 10.2967/jnumed.114.142927
20. Rubie C, Kempf K, Hans J, Su T, Tilton B, Georg T, et al. Housekeeping Gene Variability in Normal and Cancerous Colorectal, Pancreatic, Esophageal, Gastric and Hepatic Tissues. *Mol Cell Probes* (2005) 19(2):101–9. doi: 10.1016/j.mcp.2004.10.001
21. Shonk CE, Arison RN, Koven BJ, Majima H, Boxer GE. Enzyme Patterns in Human Tissues. 3. Glycolytic Enzymes in Normal and Malignant Tissues of the Colon and Rectum. *Cancer Res* (1965) 25:206–13.
22. Hennipman A, Smits J, van Oirschot B, van Houwelingen JC, Rijksen G, Neyt JP, et al. Glycolytic Enzymes in Breast Cancer, Benign Breast Disease and Normal Breast Tissue. *Tumor Biol* (1987) 8(5):251–63. doi: 10.1159/000217529
23. Hennipman A, van Oirschot BA, Smits J, Rijksen G, Staal GEJ. Glycolytic Enzyme Activities in Breast Cancer Metastases. *Tumor Biol* (1988) 9(5):241–8. doi: 10.1159/000217568
24. Groheux D, Cochet A, Humbert O, Alberini J-L, Hindié E, Mankoff D. 18f-FDG PET/CT for Staging and Restaging of Breast Cancer. *J Nucl Med* (2016) 57(Supplement 1):17S–26S. doi: 10.2967/jnumed.115.157859
25. Hirayama A, Kami K, Sugimoto M, Sugawara M, Toki N, Onozuka H, et al. Quantitative Metabolome Profiling of Colon and Stomach Cancer Microenvironment by Capillary Electrophoresis Time-of-Flight Mass Spectrometry. *Cancer Res* (2009) 69(11):4918–25. doi: 10.1158/0008-5472.CAN-08-4806
26. Donohoe DR, Garge N, Zhang X, Sun W, O'Connell TM, Bunger MK, et al. The Microbiome and Butyrate Regulate Energy Metabolism and Autophagy in the Mammalian Colon. *Cell Metab* (2011) 13(5):517–26. doi: 10.1016/j.cmet.2011.02.018
27. Fleming SE, Fitch MD, DeVries S, Liu ML, Kight C. Nutrient Utilization by Cells Isolated From Rat Jejunum, Cecum and Colon. *J Nutr* (1991) 121 (6):869–78. doi: 10.1093/jn/121.6.869
28. Donohoe DR, Collins LB, Wali A, Bigler R, Sun W, Bultman SJ. : The Warburg Effect Dictates the Mechanism of Butyrate-Mediated Histone Acetylation and Cell Proliferation. *Mol Cell* (2012) 48(4):612–26. doi: 10.1016/j.molcel.2012.08.033
29. Martinez-Reyes I, Chandel NS. Mitochondrial TCA Cycle Metabolites Control Physiology and Disease. *Nat Commun* (2020) 11(1):102. doi: 10.1038/s41467-019-13668-3
30. Rodriguez-Enriquez S, Marin-Hernandez A, Gallardo-Perez JC, Pacheco-Velazquez SC, Belmont-Diaz JA, Robledo-Cadena DX, et al. Transcriptional Regulation of Energy Metabolism in Cancer Cells. *Cells* (2019) 8(10):1225. doi: 10.3390/cells8101225

31. Neitzel C, Demuth P, Wittmann S, Fahrer J. Targeting Altered Energy Metabolism in Colorectal Cancer: Oncogenic Reprogramming, the Central Role of the TCA Cycle and Therapeutic Opportunities. *Cancers (Basel)* (2020) 12(7):1731. doi: 10.3390/cancers12071731
32. Epstein T, Gatenby RA, Brown JS. The Warburg Effect as an Adaptation of Cancer Cells to Rapid Fluctuations in Energy Demand. *PloS One* (2017) 12 (9):e0185085–e0185085. doi: 10.1371/journal.pone.0185085
33. Brown RE, Short SP, Williams CS. Colorectal Cancer and Metabolism. *Curr Colorectal Cancer Rep* (2018) 14(6):226–41. doi: 10.1007/s11888-018-0420-y
34. Gandhi N, Das MG. Metabolic Reprogramming in Breast Cancer and Its Therapeutic Implications. *Cells* (2019) 8(2):89. doi: 10.3390/cells8020089
35. Dai C, Arceo J, Arnold J, Sreekumar A, Dovichi NJ, Li J, et al. Metabolomics of Oncogene-Specific Metabolic Reprogramming During Breast Cancer. *Cancer Metab* (2018) 6:5. doi: 10.1186/s40170-018-0175-6
36. D'Esposito V, Ambrosio MR, Giuliano M, Cabaro S, Miele C, Beguinot F, et al. Mammary Adipose Tissue Control of Breast Cancer Progression: Impact of Obesity and Diabetes. *Front Oncol* (2020) 10(1554):1554. doi: 10.3389/fonc.2020.01554
37. Tian W, Zhang W, Zhang Y, Zhu T, Hua Y, Li H, et al. FABP4 Promotes Invasion and Metastasis of Colon Cancer by Regulating Fatty Acid Transport. *Cancer Cell Int* (2020) 20(1):512. doi: 10.1186/s12935-020-01582-4
38. Swerdlow RH, L E, Aires D, Lu J. Glycolysis-Respiration Relationships in a Neuroblastoma Cell Line. *Biochim Biophys Acta* (2013) 1830(4):2891–8. doi: 10.1016/j.bbagen.2013.01.002
39. Jose C, Rossignol R. Rationale for Mitochondria-Targeting Strategies in Cancer Bioenergetic Therapies. *Int J Biochem Cell Biol* (2013) 45(1):123–9. doi: 10.1016/j.biocel.2012.07.005
40. Gnaiger E, Kemp RB. Anaerobic Metabolism in Aerobic Mammalian Cells: Information From the Ratio of Calorimetric Heat Flux and Respirometric Oxygen Flux. *Biochim Biophys Acta* (1990) 1016(3):328–32. doi: 10.1016/0005-2728(90)90164-y
41. Gstraunthaler G, Seppi T, Pfaller W. Impact of Culture Conditions, Culture Media Volumes, and Glucose Content on Metabolic Properties of Renal Epithelial Cell Cultures. Are Renal Cells in Tissue Culture Hypoxic? *Cell Physiol Biochem* (1999) 9(3):150–72. doi: 10.1159/000016312
42. Sherr CJ, DePinho RA. Cellular Senescence: Mitotic Clock or Culture Shock? *Cell* (2000) 102(4):407–10. doi: 10.1016/s0092-8674(00)00046-5
43. Ounpuu L, Truu L, Shevchuk I, Chekulayev V, Klepinin A, Koit A, et al. Comparative Analysis of the Bioenergetics of Human Adenocarcinoma Caco-2 Cell Line and Postoperative Tissue Samples From Colorectal Cancer Patients. *Biochem Cell Biol* (2018) 96(6):808–17. doi: 10.1139/bcb-2018-0076
44. Chekulayev V, Mado K, Shevchuk I, Koit A, Kaldma A, Klepinin A, et al. Metabolic Remodeling in Human Colorectal Cancer and Surrounding Tissues: Alterations in Regulation of Mitochondrial Respiration and Metabolic Fluxes. *Biochem Biophys Rep* (2015) 4:111–25. doi: 10.1016/j.bbrep.2015.08.020
45. Kaambre T, Chekulayev V, Shevchuk I, Karu-Varikmaa M, Timohhina N, Tepp K, et al. Metabolic Control Analysis of Cellular Respiration *in Situ* in Intraoperational Samples of Human Breast Cancer. *J Bioenerg Biomembr* (2012) 44(5):539–58. doi: 10.1007/s10863-012-9457-9
46. Koit A, Timohhina N, Truu L, Chekulayev V, Gudlawar S, Shevchuk I, et al. Metabolic and OXPHOS Activities Quantified by Temporal Ex Vivo Analysis Display Patient-Specific Metabolic Vulnerabilities in Human Breast Cancers. *Front Oncol* (2020) 10:1053. doi: 10.3389/fonc.2020.01053
47. Rebane-Klemm E, Truu L, Reinsalu L, Puurand M, Shevchuk I, Chekulayev V, et al. Mitochondrial Respiration in KRAS and BRAF Mutated Colorectal Tumors and Polyps. *Cancers (Basel)* (2020) 12(4):815. doi: 10.3390/cancers12040815
48. Feng S, Xiong LL, Ji ZN, Cheng W, Yang HJ. Correlation Between Increased Copy Number of Mitochondrial DNA and Clinicopathological Stage in Colorectal Cancer. *Oncol Lett* (2011) 2(5):899–903. doi: 10.3892/ol.2011.322
49. Richter C, Gogvadze V, Laffranchi R, Schlapbach R, Schweizer M, Suter M, et al. Oxidants in Mitochondria: From Physiology to Diseases. *Biochim Biophys Acta* (1995) 1271(1):67–74. doi: 10.1016/0925-4439(95)00012-S
50. Lee HC, Yin PH, Chi CW, Wei YH. Increase in Mitochondrial Mass in Human Fibroblasts Under Oxidative Stress and During Replicative Cell Senescence. *J BioMed Sci* (2002) 9(6 Pt 1):517–26. doi: 10.1007/BF02254978
51. Lee HC, Yin PH, Lu CY, Chi CW, Wei YH. Increase of Mitochondria and Mitochondrial DNA in Response to Oxidative Stress in Human Cells. *Biochem J* (2000) 348 Pt 2:425–32. doi: 10.1042/bj3480425
52. Genders AJ, Martin SD, McGee SL, Bishop DJ. A Physiological Drop in pH Decreases Mitochondrial Respiration, and HDAC and Akt Signaling, in L6 Myocytes. *Am J Physiol Cell Physiol* (2019) 316(3):C404–14. doi: 10.1152/ajpcell.00214.2018
53. Canto C, Auwerx J. PGC-1alpha, SIRT1 and AMPK, an Energy Sensing Network That Controls Energy Expenditure. *Curr Opin Lipidol* (2009) 20 (2):98–105. doi: 10.1097/MOL.0b013e328328d0a4
54. Das KJ, Felty Q, Poppiti R, Jackson MR, Roy D. Nuclear Respiratory Factor 1 Acting as an Oncoprotein Drives Estrogen-Induced Breast Carcinogenesis. *Cells* (2018) 7(12):234. doi: 10.3390/cells7120234
55. Wang G, Wang Q, Huang Q, Chen Y, Sun X, He L, et al. Upregulation of mtSSB by Interleukin-6 Promotes Cell Growth Through Mitochondrial Biogenesis-Mediated Telomerase Activation in Colorectal Cancer. *Int J Cancer* (2018) 144:2516–28. doi: 10.1002/ijc.31978
56. Kumari N, Dwarakanath BS, Das A, Bhatt AN. Role of Interleukin-6 in Cancer Progression and Therapeutic Resistance. *Tumor Biol* (2016) 37 (9):11553–72. doi: 10.1007/s13277-016-5098-7
57. Kumari N, Das A, Bhatt AN. Interleukin-6 Confers Radio-Resistance by Inducing Akt-Mediated Glycolysis and Reducing Mitochondrial Damage in Cells. *J Biochem* (2019) 167(3):303–14. doi: 10.1093/jb/mvz091
58. Ham I-H, Oh HJ, Jin H, Bae CA, Jeon S-M, Choi KS, et al. Targeting Interleukin-6 as a Strategy to Overcome Stroma-Induced Resistance to Chemotherapy in Gastric Cancer. *Mol Cancer* (2019) 18(1):68. doi: 10.1186/s12943-019-0972-8
59. Berridge MV, McConnell MJ, Grasso C, Bajzikova M, Kovarova J, Neuzil J. Horizontal Transfer of Mitochondria Between Mammalian Cells: Beyond Co-Culture Approaches. *Curr Opin Genet Dev* (2016) 38:75–82. doi: 10.1016/j.gde.2016.04.003
60. Sahinbegovic H, Jelinek T, Hrdinka M, Bago JR, Turi M, Sevcikova T, et al. Intercellular Mitochondrial Transfer in the Tumor Microenvironment. *Cancers (Basel)* (2020) 12(7):1787. doi: 10.3390/cancers12071787
61. Lemasters JJ, Holmuhamedov E. Voltage-Dependent Anion Channel (VDAC) as Mitochondrial Governator–Thinking Outside the Box. *Biochim Biophys Acta* (2006) 1762(2):181–90. doi: 10.1016/j.bbadis.2005.10.006
62. Guzun R, Kaambre T, Bagur R, Grichine A, Usson Y, Varikmaa M, et al. Modular Organization of Cardiac Energy Metabolism: Energy Conversion, Transfer and Feedback Regulation. *Acta Physiol (Oxf)* (2015) 213(1):84–106. doi: 10.1111/apha.12287
63. Saks V, Guzun R, Timohhina N, Tepp K, Varikmaa M, Monge C, et al. Structure-Function Relationships in Feedback Regulation of Energy Fluxes *In Vivo* in Health and Disease: Mitochondrial Interactosome. *Biochim Biophys Acta* (2010) 1797(6-7):678–97. doi: 10.1016/j.bbabio.2010.01.011
64. Kuznetsov AV, Tiivel T, Sikk P, Kaambre T, Kay L, Daneshrad Z, et al. Striking Differences Between the Kinetics of Regulation of Respiration by ADP in Slow-Twitch and Fast-Twitch Muscles *In Vivo*. *Eur J Biochem* (1996) 241(3):909–15. doi: 10.1111/j.1432-1033.1996.00909.x
65. Puurand M, Tepp K, Klepinin A, Klepinina L, Shevchuk I, Kaambre T. Intracellular Energy-Transfer Networks and High-Resolution Respirometry: A Convenient Approach for Studying Their Function. *Int J Mol Sci* (2018) 19 (10):2933. doi: 10.3390/ijms19102933
66. Kay L, Li Z, Mericskay M, Olivares J, Tranqui L, Fontaine E, et al. Study of Regulation of Mitochondrial Respiration *In Vivo*. An Analysis of Influence of ADP Diffusion and Possible Role of Cytoskeleton. *Biochim Biophys Acta* (1997) 1322(1):41–59. doi: 10.1016/s0005-2728(97)00071-6
67. Klepinin A, Ounpuu L, Mado K, Truu L, Chekulayev V, Puurand M, et al. The Complexity of Mitochondrial Outer Membrane Permeability and VDAC Regulation by Associated Proteins. *J Bioenerg Biomembr* (2018) 50 (5):339–54. doi: 10.1007/s10863-018-9765-9
68. Puurand M, Tepp K, Timohhina N, Aid J, Shevchuk I, Chekulayev V, et al. Tubulin betaII and betaIII Isoforms as the Regulators of VDAC Channel Permeability in Health and Disease. *Cells* (2019) 8(3):239. doi: 10.3390/cells8030239

69. Saks VA, Belikova YO, Kuznetsov AV. *In Vivo* Regulation of Mitochondrial Respiration in Cardiomyocytes: Specific Restrictions for Intracellular Diffusion of ADP. *Biochim Biophys Acta* (1991) 1074(2):302–11. doi: 10.1016/0304-4165(91)90168-g
70. Saks VA, Veksler VI, Kuznetsov AV, Kay L, Sikk P, Tiivel T, et al. Permeabilized Cell and Skinned Fiber Techniques in Studies of Mitochondrial Function *In Vivo*. *Mol Cell Biochem* (1998) 184(1-2):81–100. doi: 10.1023/A:1006834912257
71. Messina A, Reina S, Guarino F, De Pinto V. VDAC Isoforms in Mammals. *Biochim Biophys Acta (BBA) - Biomembr* (2012) 1818(6):1466–76. doi: 10.1016/j.bbamem.2011.10.005
72. Shoshan-Barmatz V, Mizrachi D. VDAC1: From Structure to Cancer Therapy. *Front Oncol* (2012) 2:164. doi: 10.3389/fonc.2012.00164
73. Shoshan-Barmatz V, Ben-Hail D, Admoni L, Krelin Y, Tripathi SS. The Mitochondrial Voltage-Dependent Anion Channel 1 in Tumor Cells. *Biochim Biophys Acta (BBA) - Biomembr* (2015) 1848(10, Part B):2547–75. doi: 10.1016/j.bbamem.2014.10.040
74. Mangan PS, Colombini M. Ultrasteep Voltage Dependence in a Membrane Channel. *Proc Natl Acad Sci* (1987) 84(14):4896. doi: 10.1073/pnas.84.14.4896
75. Colombini M, Mannella CA. VDAC, The Early Days. *Biochim Biophys Acta (BBA) - Biomembr* (2012) 1818(6):1438–43. doi: 10.1016/j.bbamem.2011.11.014
76. Mathupala SP, Ko YH, Pedersen PL. Hexokinase II: Cancer's Double-Edged Sword Acting as Both Facilitator and Gatekeeper of Malignancy When Bound to Mitochondria. *Oncogene* (2006) 25(34):4777–86. doi: 10.1038/sj.onc.1209603
77. Pedersen PL. Warburg, Me and Hexokinase 2: Multiple Discoveries of Key Molecular Events Underlying One of Cancers' Most Common Phenotypes, the "Warburg Effect", I.e., elevated glycolysis in the presence of oxygen. *J Bioenerg Biomembr* (2007) 39(3):211–22. doi: 10.1007/s10863-007-9094-x
78. Pedersen PL. The Cancer Cell's "Power Plants" as Promising Therapeutic Targets: An Overview. *J Bioenerg Biomembr* (2007) 39(1):1–12. doi: 10.1007/s10863-007-9070-5
79. Mathupala SP, Ko YH, Pedersen PL. Hexokinase-2 Bound to Mitochondria: Cancer's Stygian Link to the "Warburg Effect" and a Pivotal Target for Effective Therapy. *Semin Cancer Biol* (2009) 19(1):17–24. doi: 10.1016/j.semcancer.2008.11.006
80. Mathupala SP, Pedersen PL. Voltage Dependent Anion Channel-1 (VDAC-1) as an Anti-Cancer Target. *Cancer Biol Ther* (2010) 9(12):1053–6. doi: 10.4161/cbt.9.12.12451
81. Shoshan-Barmatz V, Maldonado EN, Krelin Y. VDAC1 at the Crossroads of Cell Metabolism, Apoptosis and Cell Stress. *Cell Stress* (2017) 1(1):11–36. doi: 10.15698/cst2017.10.104
82. Neumann D, Buckers J, Kastrup L, Hell SW, Jakobs S. Two-Color STED Microscopy Reveals Different Degrees of Colocalization Between Hexokinase-I and the Three Human VDAC Isoforms. *PMC Biophys* (2010) 3(1):4. doi: 10.1186/1757-5036-3-4
83. Pedersen PL, Mathupala S, Rempel A, Geschwind JF, Ko YH. Mitochondrial Bound Type II Hexokinase: A Key Player in the Growth and Survival of Many Cancers and an Ideal Prospect for Therapeutic Intervention. *Biochim Biophys Acta* (2002) 1555(1-3):14–20. doi: 10.1016/S0005-2728(02)00248-7
84. Rostovtseva TK, Tan W, Colombini M. On the Role of VDAC in Apoptosis: Fact and Fiction. *J Bioenerg Biomembr* (2005) 37(3):129–42. doi: 10.1007/s10863-005-6566-8
85. Majewski N, Nogueira V, Bhaskar P, Coy PE, Skeen JE, Gottlob K, et al. Hexokinase-Mitochondria Interaction Mediated by Akt Is Required to Inhibit Apoptosis in the Presence or Absence of Bax and Bak. *Mol Cell* (2004) 16(5):819–30. doi: 10.1016/j.molcel.2004.11.014
86. Eimre M, Paju K, Pelloux S, Beraud N, Roosimaa M, Kadaja L, et al. Distinct Organization of Energy Metabolism in HL-1 Cardiac Cell Line and Cardiomyocytes. *Biochim Biophys Acta* (2008) 1777(6):514–24. doi: 10.1016/j.bbabio.2008.03.019
87. Ahmad A, Ahmad S, Schneider BK, Allen CB, Chang LY, White CW. Elevated Expression of Hexokinase II Protects Human Lung Epithelial-Like A549 Cells Against Oxidative Injury. *Am J Physiol Lung Cell Mol Physiol* (2002) 283(3):L573–84. doi: 10.1152/ajplung.00410.2001
88. Zaid H, Abu-Hamad S, Israelson A, Nathan I, Shoshan-Barmatz V. The Voltage-Dependent Anion Channel-1 Modulates Apoptotic Cell Death. *Cell Death Differ* (2005) 12(7):751–60. doi: 10.1038/sj.cdd.4401599
89. Pastorino J, Hoek J. Regulation of Hexokinase Binding to VDAC. *J Bioenerg Biomembr* (2008) 40(3):171–82. doi: 10.1007/s10863-008-9148-8
90. Wei L, Zhou Y, Dai Q, Qiao C, Zhao L, Hui H, et al. Oroxylin A Induces Dissociation of Hexokinase II From the Mitochondria and Inhibits Glycolysis by SIRT3-Mediated Deacetylation of Cyclophilin D in Breast Carcinoma. *Cell Death Dis* (2013) 4:e601. doi: 10.1038/cddis.2013.131
91. Ikeda S, Abe F, Matsuda Y, Kitadate A, Takahashi N, Tagawa H. Hypoxia-Inducible Hexokinase-2 Enhances Anti-Apoptotic Function via Activating Autophagy in Multiple Myeloma. *Cancer Sci* (2020) 111:4088–101. doi: 10.1111/cas.14614
92. Fan T, Sun G, Sun X, Zhao L, Zhong R, Peng Y. Tumor Energy Metabolism and Potential of 3-Bromopyruvate as an Inhibitor of Aerobic Glycolysis: Implications in Tumor Treatment. *Cancers (Basel)* (2019) 11(3):317. doi: 10.3390/cancers11030317
93. Woldetsadik AD, Vogel MC, Rabeh WM, Magzoub M. Hexokinase II–derived Cell-Penetrating Peptide Targets Mitochondria and Triggers Apoptosis in Cancer Cells. *FASEB J* (2017) 31(5):2168–84. doi: 10.1096/fj.201601173R
94. Arif T, Vasilkovsky L, Refaely Y, Konson A, Shoshan-Barmatz V. Silencing VDAC1 Expression by siRNA Inhibits Cancer Cell Proliferation and Tumor Growth *In Vivo*. *Mol Ther - Nucleic Acids* (2014) 3:e159. doi: 10.1038/mtna.2014.9
95. Maldonado EN, Patnaik J, Mullins MR, Lemasters JJ. Free Tubulin Modulates Mitochondrial Membrane Potential in Cancer Cells. *Cancer Res* (2010) 70(24):10192–201. doi: 10.1158/0008-5472.CAN-10-2429
96. Maldonado EN, Lemasters JJ. ATP/ADP Ratio, the Missed Connection Between Mitochondria and the Warburg Effect. *Mitochondrion* (2014) 19 Pt A:78–84. doi: 10.1016/j.mito.2014.09.002
97. Varikmaa M, Bagur R, Kaambre T, Grichine A, Timohhina N, Tepp K, et al. Role of Mitochondria-Cytoskeleton Interactions in Respiration Regulation and Mitochondrial Organization in Striated Muscles. *Biochim Biophys Acta* (2014) 1837(2):232–45. doi: 10.1016/j.bbabio.2013.10.011
98. Jirasek T, Mandys V, Viklicky V. Expression of Class III Beta-Tubulin in Neuroendocrine Tumours of Gastrointestinal Tract. *Folia Histochem Cytobiol* (2002) 40(3):305–10.
99. Urano N, Fujiwara Y, Doki Y, Kim SJ, Miyoshi Y, Noguchi S, et al. Clinical Significance of Class III Beta-Tubulin Expression and Its Predictive Value for Resistance to Docetaxel-Based Chemotherapy in Gastric Cancer. *Int J Oncol* (2006) 28(2):375–81. doi: 10.3892/ijo.28.2.375
100. Oztop S, Isik A, Guner G, Gurdal H, Karabulut E, Yilmaz E, et al. Class III Beta-Tubulin Expression in Colorectal Neoplasms Is a Potential Predictive Biomarker for Paclitaxel Response. *Anticancer Res* (2019) 39(2):655–62. doi: 10.21873/anticanres.13160
101. Mariani M, Karki R, Spennato M, Pandya D, He S, Andreoli M, et al. Class III Beta-Tubulin in Normal and Cancer Tissues. *Gene* (2015) 563(2):109–14. doi: 10.1016/j.gene.2015.03.061
102. Mariani M, Shahabi S, Sieber S, Scambia G, Ferlini C. Class III Beta-Tubulin (TUBB3): More Than a Biomarker in Solid Tumors? *Curr Mol Med* (2011) 11(9):726–31. doi: 10.2174/156652411798062368
103. Parker AL, Turner N, McCarroll JA, Kavallaris M. betaIII-Tubulin Alters Glucose Metabolism and Stress Response Signaling to Promote Cell Survival and Proliferation in Glucose-Starved Non-Small Cell Lung Cancer Cells. *Carcinogenesis* (2016) 37(8):787–98. doi: 10.1093/carcin/bgw058
104. Cicchillitti L, Penci R, Di Michele M, Filippetti F, Rotilio D, Donati MB, et al. Proteomic Characterization of Cytoskeletal and Mitochondrial Class III Beta-Tubulin. *Mol Cancer Ther* (2008) 7(7):2070–9. doi: 10.1158/1535-7163.MCT-07-2370
105. Raspaglio G, Filippetti F, Prislei S, Penci R, De Maria I, Cicchillitti L, et al. Hypoxia Induces Class III Beta-Tubulin Gene Expression by HIF-1alpha Binding to its 3' Flanking Region. *Gene* (2008) 409(1-2):100–8. doi: 10.1016/j.gene.2007.11.015
106. Guarino F, Zinghirino F, Mela L, Pappalardo XG, Ichas F, De Pinto V, et al. NRF-1 and HIF-1alpha Contribute to Modulation of Human VDAC1 Gene Promoter During Starvation and Hypoxia in HeLa Cells. *Biochim Biophys Acta Bioenerg* (2020) 1861(12):148289. doi: 10.1016/j.bbabio.2020.148289

107. Brahimi-Horn MC, Ben-Hail D, Ilie M, Gounon P, Rouleau M, Hofman V, et al. Expression of a Truncated Active Form of VDAC1 in Lung Cancer Associates With Hypoxic Cell Survival and Correlates With Progression to Chemotherapy Resistance. *Cancer Res* (2012) 72(8):2140–50. doi: 10.1158/0008-5472.CAN-11-3940
108. Meyenberg Cunha-de Padua M, Fabbri L, Dufies M, Lacas-Gervais S, Contenti J, Voyton C, et al. Evidences of a Direct Relationship Between Cellular Fuel Supply and Ciliogenesis Regulated by Hypoxic VDAC1-DeltaC. *Cancers (Basel)* (2020) 12(11):3484. doi: 10.3390/cancers12113484
109. Baba Y, Nosho K, Shima K, Irahara N, Chan AT, Meyerhardt JA, et al. HIF1A Overexpression is Associated With Poor Prognosis in a Cohort of 731 Colorectal Cancers. *Am J Pathol* (2010) 176(5):2292–301. doi: 10.2353/ajpath.2010.090972
110. Ioannou M, Paraskeva E, Baxevanidou K, Simos G, Papamichali R, Papacharalambous C, et al. HIF-1alpha in Colorectal Carcinoma: Review of the Literature. *J BUON* (2015) 20(3):680–9.
111. Haugen MH, Boye K, Nesland JM, Pettersen SJ, Egeland EV, Tamhane T, et al. High Expression of the Cysteine Proteinase Legumain in Colorectal Cancer - Implications for Therapeutic Targeting. *Eur J Cancer* (2015) 51 (1):9–17. doi: 10.1016/j.ejca.2014.10.020
112. Zhen Y, Chunlei G, Wenzhi S, Shuangtao Z, Na L, Rongrong W, et al. Clinicopathologic Significance of Legumain Overexpression in Cancer: A Systematic Review and Meta-Analysis. *Sci Rep* (2015) 5:16599. doi: 10.1038/srep16599
113. Ferecatu I, Canal F, Fabbri L, Mazure NM, Bouton C, Golinelli-Cohen MP. Dysfunction in the Mitochondrial Fe-S Assembly Machinery Leads to Formation of the Chemoresistant Truncated VDAC1 Isoform Without HIF-1alpha Activation. *PloS One* (2018) 13(3):e0194782. doi: 10.1371/journal.pone.0194782
114. Lipper CH, Stofleth JT, Bai F, Sohn YS, Roy S, Mittler R, et al. Redox-Dependent Gating of VDAC by mitoNEET. *Proc Natl Acad Sci USA* (2019) 116(40):19924–9. doi: 10.1073/pnas.1908271116
115. Basak D, Uddin MN, Hancock J. The Role of Oxidative Stress and Its Counteractive Utility in Colorectal Cancer (CRC). *Cancers (Basel)* (2020) 12 (11):3336. doi: 10.3390/cancers12113336
116. Caterino M, Ruoppolo M, Mandola A, Costanzo M, Orru S, Imperlini E. Protein-Protein Interaction Networks as a New Perspective to Evaluate Distinct Functional Roles of Voltage-Dependent Anion Channel Isoforms. *Mol Biosyst* (2017) 13(12):2466–76. doi: 10.1039/c7mb00434f
117. Liu H, Pu Y, Amina Q, Wang Q, Zhang M, Song J, et al. Prognostic and Therapeutic Potential of Adenylate Kinase 2 in Lung Adenocarcinoma. *Sci Rep* (2019) 9(1):17757. doi: 10.1038/s41598-019-53594-4
118. Klepinin A, Ounpuu L, Guzun R, Chekulayev V, Timohhina N, Tepp K, et al. Simple Oxygraphic Analysis for the Presence of Adenylate Kinase 1 and 2 in Normal and Tumor Cells. *J Bioenerg Biomembr* (2016) 48(5):531–48. doi: 10.1007/s10863-016-9687-3
119. Speers C, Tsimelzon A, Sexton K, Herrick AM, Gutierrez C, Culhane A, et al. Identification of Novel Kinase Targets for the Treatment of Estrogen Receptor-Negative Breast Cancer. *Clin Cancer Res* (2009) 15(20):6327–40. doi: 10.1158/1078-0432.CCR-09-1107
120. Fujisawa K, Terai S, Takami T, Yamamoto N, Yamasaki T, Matsumoto T, et al. Modulation of Anti-Cancer Drug Sensitivity Through the Regulation of Mitochondrial Activity by Adenylate Kinase 4. *J Exp Clin Cancer Res* (2016) 35:48. doi: 10.1186/s13046-016-0322-2
121. Klepinina L, Klepinin A, Truu L, Chekulayev V, Vija H, Kuus K, et al. Colon Cancer Cell Differentiation by Sodium Butyrate Modulates Metabolic Plasticity of Caco-2 Cells *via* Alteration of Phosphotransfer Network. *PloS One* (2021) 16(1):e0245348. doi: 10.1371/journal.pone.0245348
122. Dzeja PP, Terzic A. Phosphotransfer Networks and Cellular Energetics. *J Exp Biol* (2003) 206(Pt 12):2039–47. doi: 10.1242/jeb.00426
123. Dzeja P, Terzic A. Adenylate Kinase and AMP Signaling Networks: Metabolic Monitoring, Signal Communication and Body Energy Sensing. *Int J Mol Sci* (2009) 10(4):1729–72. doi: 10.3390/ijms10041729
124. Klepinin A, Zhang S, Klepinina L, Rebane-Klemm E, Terzic A, Kaambre T, et al. Adenylate Kinase and Metabolic Signaling in Cancer Cells. *Front Oncol* (2020) 10:660. doi: 10.3389/fonc.2020
125. Hardie DG, Ross FA, Hawley SA. AMPK: A Nutrient and Energy Sensor That Maintains Energy Homeostasis. *Nat Rev Mol Cell Biol* (2012) 13 (4):251–62. doi: 10.1038/nrm3311
126. Wang W, Guan KL. AMP-Activated Protein Kinase and Cancer. *Acta Physiol* (2009) 196(1):55–63. doi: 10.1111/j.1748-1716.2009.01980.x
127. Faubert B, Boily G, Izreig S, Griss T, Samborska B, Dong Z, et al. AMPK Is a Negative Regulator of the Warburg Effect and Suppresses Tumor Growth *In Vivo*. *Cell Metab* (2013) 17(1):113–24. doi: 10.1016/j.cmet.2012.12.001
128. Rehman G, Shehzad A, Khan AL, Hamayun M. Role of AMP-Activated Protein Kinase in Cancer Therapy. *Archiv der Pharm* (2014) 347(7):457–68. doi: 10.1002/ardp.201300402
129. Hardie DG, Ross FA, Hawley SA. AMP-Activated Protein Kinase: A Target for Drugs Both Ancient and Modern. *Chem Biol* (2012) 19(10):1222–36. doi: 10.1016/j.chembiol.2012.08.019
130. Park HU, Suy S, Danner M, Dailey V, Zhang Y, Li H, et al. AMP-Activated Protein Kinase Promotes Human Prostate Cancer Cell Growth and Survival. *Mol Cancer Ther* (2009) 8(4):733–41. doi: 10.1158/1535-7163.MCT-08-0631
131. Khan AS, Frigo DE. Regulation, Role and Therapeutic Targeting of AMPK in Prostate Cancer. *Nat Rev Urol* (2017) 14(3):164–80. doi: 10.1038/nrurol.2016.272
132. Huang X, Li X, Xie X, Ye F, Chen B, Song C, et al. High Expressions of LDHA and AMPK as Prognostic Biomarkers for Breast Cancer. *Breast* (2016) 30:39–46. doi: 10.1016/j.breast.2016.08.014

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Appendix 3

Publication III

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Colorectal polyps increase the glycolytic activity

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In colorectal cancer (CRC) energy metabolism research, the precancerous stage of polyp has remained rather unexplored. By now, it has been shown that CRC has not fully obtained the glycolytic phenotype proposed by O. Warburg and rather depends on mitochondrial respiration. However, the pattern of metabolic adaptations during tumorigenesis is still unknown. Understanding the interplay between genetic and metabolic changes that initiate tumor development could provide biomarkers for diagnosing cancer early and targets for new cancer therapeutics. We used human CRC and polyp tissue material and performed high-resolution respirometry and qRT-PCR to detect changes on molecular and functional level with the goal of generally describing metabolic reprogramming during CRC development. Colon polyps were found to have a more glycolytic bioenergetic phenotype than tumors and normal tissues. This was supported by a greater *GLUT1*, *HK*, *LDHA*, and *MCT* expression. Despite the increased glycolytic activity, cells in polyps were still able to maintain a highly functional OXPHOS system. The mechanisms of OXPHOS regulation and the preferred substrates are currently unclear and would require further investigation. During polyp formation, intracellular energy transfer pathways become rearranged mainly by increasing the expression of mitochondrial adenylate kinase (*AK*) and creatine kinase (*CK*) isoforms. Decreased glycolysis and maintenance of OXPHOS activity, together with the downregulation of the CK system and the most common AK isoforms (*AK1* and *AK2*), seem to play a relevant role in CRC development.

KEYWORDS

metabolic phenotype, energy metabolism, colorectal cancer, colonic adenoma, OXPHOS, Warburg effect

1 Introduction

Colorectal cancer (CRC) is a multifactorial and heterogeneous disease that mostly arises from precursor lesions known as polyps. Two major classes of colorectal polyps are conventional adenomas (tubular, tubulovillous, or villous adenoma) and serrated polyps (hyperplastic polyps, sessile serrated adenoma/polyps, and traditional serrated adenomas) (1), which are believed to arise from distinct etiologic pathways. The current understanding of CRC development suggests that the progressive accumulation of oncogenic changes begins with abnormal growth of colon epithelial cells. Sequence alterations in specific genes, including *APC* and *KRAS*, contribute to the development of early precancerous lesions (2) and metabolic reprogramming towards a glycolytic phenotype. Over time, adenomas develop increasingly dysplastic features and eventually acquire malignant potential. However, most adenomas stabilize their growth progression or even regress (3). Although genetic events in colonic polyps are quite well characterized (4, 5), the reprogramming of metabolic pathways has not been widely investigated.

Metabolic reprogramming is one of the hallmarks of cancer (6). However, metabolic alterations in the precancerous stage and colorectal carcinogenesis are not well understood. Almost 100 years ago, Otto Warburg first described that cancer cells metabolize glucose directly to lactic acid even in the presence of high oxygen. This modified glucose metabolism is known as the "Warburg effect" (7). Warburg proposed that the increased rate of aerobic glycolysis was due to irreversible injury of mitochondrial oxidative phosphorylation (OXPHOS), the main pathway providing energy for eukaryotic cells, and generating more adenosine triphosphate (ATP) than glycolysis. Nowadays, it has become clear that glycolysis is upregulated in many tumors without mitochondrial dysfunction.

Several *in vivo* studies have demonstrated up-regulation of the components of the OXPHOS system in certain types of cancer cells (8), which is accompanied by increased mitochondrial respiration and OXPHOS flux. The OXPHOS machinery in most cancer cells seems to be fully functional. Moreover, cells can switch between OXPHOS and aerobic glycolysis or even perform them simultaneously, depending on the availability of substrates (including oxygen) (9). This metabolic plasticity is defined as the ability of cancer cells to reprogram their metabolic pathways to fulfill energetic and anabolic needs in a changing extracellular microenvironment during the various steps of disease progression.

Another important aspect of energy metabolism and metabolic plasticity is the interplay between energy transfer pathways in cancer cells. The isoenzymes of hexokinase (HK), adenylate kinase (AK), and creatine kinase (CK) support specific cellular processes ranging from muscle contraction and cell motility to mitochondrial/nuclear energetics (10). Indeed, it has been proposed that some AK and HK isoenzymes may be targets for antitumor therapy (11, 12). A complete spectrum of HK, AK, and CK isoforms in clinically well-defined patient groups may inform us about the changes in the maintenance of energy homeostasis of tumor cells. Several high-resolution respirometry studies performed on different permeabilized tissues and cells show that there is specificity on how adenosine diphosphate (ADP) may regulate OXPHOS at the level of the mitochondrial outer membrane (MOM). The basis of this last premise is the structurally different intracellular arrangement of functional units; such complexity of the intracellular environment determines the need for the use of energy transfer pathways. Moreover, the differences between cancer and non-cancer cells in the composition of cytoskeleton proteins and their interaction with mitochondria are related to the prevalent type of metabolism, facilitating metabolic plasticity (13, 14).

The present study was aimed to identify and characterize metabolic reprogramming in colon polyps by assessing mitochondrial respiratory rates and gene expression of selected metabolic markers. The precise contribution of different metabolic pathways to the adenoma-carcinoma sequence is not known yet. Understanding the relationship between genetic and metabolic changes, as well as the role of these interactions in tumor initiation, is essential for designing efficient therapeutic approaches targeting the metabolism of tumors.

2 Materials and methods

2.1 Clinical material

All experiments were performed with human tissue samples. The present research protocol was approved by the Medical Research Ethics Committee (National Institute for Health Development, Tallinn, Estonia) by decisions number KK557 and KK558, and was following the Helsinki Declaration and Convention of the Council of Europe on Human Rights and Biomedicine. Research subjects were fully informed about the study and gave their consent.

Tumor and control tissue samples were obtained from the North Estonia Medical Centre. All patients (n=56 with ages ranging from 38 to 101) showed local or locally advanced disease (T2-4, N0-2), and only primary tumors were used. Normal tissue samples were taken from the same location at sites distant from the tumor and were checked for malignancies. Colorectal polyps were resected from patients (n=28, with ages ranging from 50 to 84) undergoing a colonoscopy at the West Tallinn Central Hospital. Only non-cancerous polyps were used. To maintain physiological conditions during geographical displacement, samples were placed immediately after removal into medium B (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 3 mM KH_2PO_4, 110 mM sucrose, 0.5 mM dithiothreitol, 20 mM HEPES, 5 µM leupeptin, 2 mg/mL fatty acids free bovine serum albumin, pH 7.1). Additionally, a small amount of tissue was transported in RNALater Stabilization Solution (Qiagen).

2.2 Preparation of skinned tumor samples and permeabilization procedure

Upon arriving, samples were placed into pre-cooled (4°C) medium A consisting of 3 mM KH_2PO_4, 20 mM taurine, 5.7 mM ATP, 15 mM PCr, 9.5 mM $MgCl_2$, 49 mM MES, 7.23 mM K_2EGTA, and 2.77 mM $K_2CaEGTA$, pH 7.1). Fat and blood vessels were removed from the tissue samples, which were then dissected into

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small samples (5-15 mg). These were permeabilized in medium A containing 50 μg/mL of saponin for 30 min at 4°C. The permeabilized samples were then washed three times for 5 min in pre-cooled medium B without leupeptin and kept at 4°C until use in oxygraphic analysis.

2.3 Oxygraphic measurements

The mitochondrial respiration of permeabilized tissue samples was measured in medium B at 25°C using a high-resolution respirometer Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria). The medium was supplemented with 5 mM glutamate, 2 mM malate, and 10 mM succinate to fully activate respiratory chain complexes 1 and 2 (15). ADP was added in increasing concentrations to measure the dependence of respiration rate on exogenous ADP (Supplementary S1) and then calculate the apparent affinity of mitochondria to exogenous ADP (K_m(ADP)). The obtained data were plotted as rates of O_2 consumption (the basal respiration rate of respiration was subtracted) *versus* ADP concentration and K_m(ADP) and V_{max} values were calculated from these plots by nonlinear regression using Michaelis–Menten equation.

2.3.1 Calibration of ADP stock solutions

To calibrate the concentration of ADP stock solution, the absorbance of NADH was determined using spectrophotometry. The reaction mixture contained a high K^+ concentration medium (120 mM KCl, 20 mM MOPS, 1 mM EGTA, pH 7.2), 5 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 2,5 IU/mL lactate dehydrogenase, 3.75 IU/mL pyruvate kinase, and 0.15 mM NADH. The reaction was initiated by adding 1 μL of ADP stock and the concentration of ADP stock was defined as the decrease of NADH concentration. The extinction coefficient for NADH (6.22 x 10^3 M^{-1} cm^{-1}) was used to convert its absorbance to molar concentration. K_m(ADP) values were corrected accordingly.

2.4 RNA extraction

Tissue samples from patients were transported in RNALater solution (Qiagen) to protect cellular RNA until it was frozen in liquid nitrogen and stored at -80°C. The frozen tissue samples were homogenized by using the TRIzol reagent (Ambion). For RNA isolation, the RNeasy Mini Kit (Qiagen) was used by following the protocol by Untergasser (16). Genomic DNA was removed by using RNase-free DNase I solution (Qiagen). RNA was eluted in 30 μL of RNase-free water and the total concentration of RNA was measured by a BioSpec-Nano spectrophotometer (Shimadzu). Isolated RNA was stored at -80°C.

2.5 cDNA synthesis and real-time quantitative polymerase chain reaction

For cDNA synthesis and qRT-PCR, all reagents used were by Applied Biosynthesis. cDNA was synthesized from 2 μg of RNA by using a High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor following the manufacturer's instructions. Reverse transcription was performed with Eppendorf® 5332 Mastercycler thermocycler.

qRT-PCR was performed with LightCycler 480 II (Roche) and by using the TaqMan Gene Expression Master Mix (Thermo Fisher Scientific). To detect gene expression levels, FAM-labeled TaqMan probes were used: actin-β (Hs01060665_g1), AK1 (Hs0017 6119_m1), AK2 (Hs01123132_g1), AK4 (Hs03405743_g1), AK6 (Hs00360444_g1), CK-BB (Hs00176483_m1), CK-MT1 (Hs00 179727_m1), CK-MT2 (Hs00176502_m1), HK1 (Hs00175976_ m1), HK2 (Hs00606086_m1), GLUT1 (Hs00892681_m1), LDHA (Hs03405707_g1), MCT1 (Hs00161826_m1), MCT2 (Hs043327 06_m1), and MCT4 (Hs00358829_m1). MQ was used as a negative control.

2.6 DNA extraction

DNA was extracted from tissue samples using Invitrogen™ PureLink™ Genomic DNA Mini Kit following the instructions provided by the manufacturer. DNA concentrations and quality were measured using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.7 *KRAS* and *BRAF* mutation analysis

High-Resolution Melt (HRM) analysis was performed to detect the mutations in *KRAS* codon 12 and 13 of exon 2 and *BRAF* codon 600 of exon 15 (V600E). The reaction mix contained 1x HOT FirePol® EvaGreen® HRM Mix (Solis BioDyne, Estonia), 250 nM of sense and antisense primers (Supplementary Table S2), and 100x dilution of PCR amplification product. PCR amplification and HRM analysis were carried out with Rotor-Gene 6000 (QIAGEN) and consisted of an initial 15 min denaturation step at 95°C, followed by 45 cycles at 95°C for 10 s, 54°C for 10 s, and 72°C for 15 s, with a final extension at 72°C for 3 min. The obtained PCR products were heated at 95°C for 1 min and cooled down to 40°C to facilitate the formation of heteroduplex. HRM analysis was performed from 62°C to 92°C with a 0.1°C step. The results were analyzed using Rotor-Gene 6000 software and unknown samples were compared to control samples with known genotypes.

2.8 Data analysis

The authors confirm that the data supporting the findings of this study are available within the article and its Supplementary Materials. Data in text, figures, and tables are presented as mean ± standard error (SEM). Bar charts with individual data points were made by using SigmaPlot 11.0. The results from oxygraphic analysis and qRT-PCR were analyzed by Student's *t*-test and p-values <0.05 were considered statistically significant. Apparent K_m(ADP) values were measured by fitting experimental data to non-linear regression.

3 Results and discussion

3.1 Mitochondrial outer membrane permeability for ADP is different in healthy colon, polyps, and cancer tissue

To identify the changes in OXPHOS activity during the development of CRC, we applied high-resolution respirometry on permeabilized postoperative tissues (CRC, colon polyps, and healthy colon tissue). We determined the rate of maximal ADP-activated respiration (V_{max}) and calculated the apparent Michaelis-Menten constant values for exogenously added ADP (K_m(ADP)), to estimate the coupling of mitochondrial oxygen consumption to OXPHOS and the permeability of voltage-dependent anion channel (VDAC) for exogenous ADP, respectively.

Tissue or cell-specific tuning of OXPHOS activity through regulation of creatine, creatine-phosphate and adenine nucleotides movement *via* VDAC resulting in a certain K_m(ADP) value could be a suitable indicator of the specific complexity of the intracellular organization, which is dealt with *ad hoc* isoforms of CK and AK for catalysis and intracellular energy transfer. In this regard, orders of magnitude different K_m(ADP) values have been found between glycolytic and oxidative striated muscles (tissue-specific K_m (ADP) values are higher in oxidative tissues), which have different metabolic features (17, 18). Thus, the determination of V_{max} and K_m (ADP) values for cellular oxygen consumption rates could provide relevant information about the activity of OXPHOS key components, the type of metabolism, and the complexity of the internal organization of the cells in the three different tissues included in the present study.

There were significant differences in oxygen consumption V_{max} and K_m(ADP) values between polyps and tumors, suggesting that polyps and tumors have different bioenergetic profiles and demands for energy (Figure 1). The observation that V_{max} for tumors was higher than for healthy colon tissue (Figure 1A; Supplementary Table S3) was in agreement with our previous studies (19–21). Interestingly, V_{max} for colon polyps exceeded that in tumors and was two times higher than V_{max} for healthy tissue. Determination of K_m(ADP) values revealed that colon polyps have a significantly lower K_m(ADP) compared to both cancerous and healthy tissue (Figure 1B). At the same time, healthy tissue and tumors showed similar K_m(ADP) values, indicating a lower affinity for ADP than in polyps. The V_{max}/K_m(ADP) ratio was 0.039 min^{-1} mg^{-1} mL for colon polyps, whereas this ratio was similar and lower (0.019 and 0.013, respectively) for tumor and healthy tissue, indicating a more catalytically efficient system in polyps. Additionally, by calculating the % of mitochondrion with low oxidative capacity using the model developed by Saks and colleagues (22), polyps were characterized by a higher % of mitochondrion with low control over the movement of adenine nucleotides through MOM (Supplementary Table S4) compared to both healthy tissue and malignant tumors. This suggests that polyps have higher glycolytic capacity. These observations suggested a metabolic shift towards a more glycolytic type of metabolism while maintaining OXPHOS functionality in polyps, which was indicated respectively by the increased affinity for ADP on MIM and the high ADP-induced respiration level.

3.2 Polyps with *BRAF* mutation demonstrate a higher glycolytic activity together with some down-regulation of OXHOS

The malignant transformation of cells, including colon epithelium, is accompanied by metabolic reprogramming of energy production and biosynthesis pathways that promote tumor growth and metastasis (23). Mutations in *KRAS* or *BRAF* genes appear to play a significant role in the transcriptional regulation of metabolic reprogramming in multiple cancers, including CRC (21, 24–27). The potential effect of *KRAS* and *BRAF* mutations on mitochondrial respiration was investigated in the colorectal polyp group (Figure 2).

Polyps with *KRAS* mutation showed higher V_{max} values compared to those of polyps with *BRAF* mutation (Figure 2A; Supplementary Table S5). This pattern was similar to that obtained when comparing *BRAF* and *KRAS* mutations in CRC, with the difference that the V_{max} of non-mutated CRC was higher than that in the tissue with *KRAS* and *BRAF* mutations (21). Mitochondria in *KRAS* mutated polyps showed lower affinity for exogenous ADP compared to that of *BRAF* mutated polyp group (Figure 2B). There

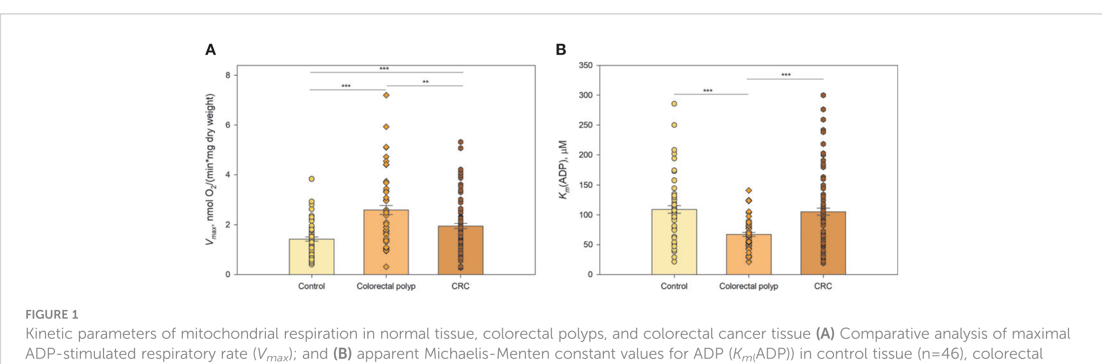

FIGURE 1
Kinetic parameters of mitochondrial respiration in normal tissue, colorectal polyps, and colorectal cancer tissue **(A)** Comparative analysis of maximal ADP-stimulated respiratory rate (V_{max}); and **(B)** apparent Michaelis-Menten constant values for ADP (K_m(ADP)) in control tissue (n=46), colorectal polyps (n=42), and colorectal cancer (CRC) tissue (n=57) ** p < 0,01, *** p < 0,001 (t-test).

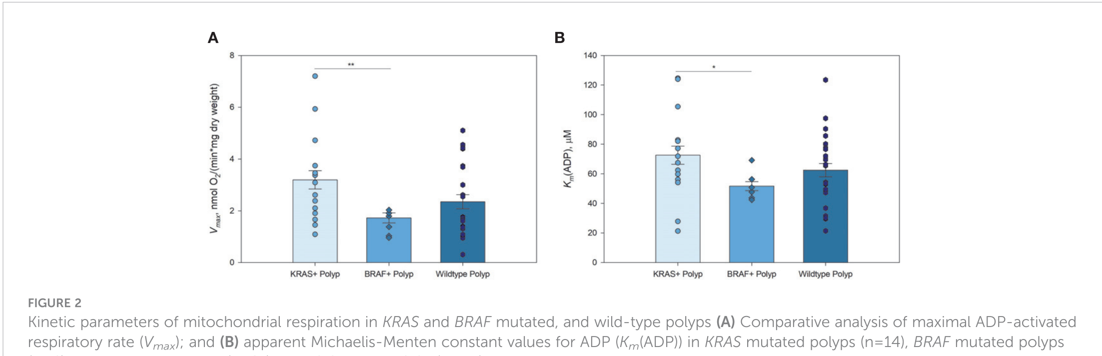

FIGURE 2
Kinetic parameters of mitochondrial respiration in *KRAS* and *BRAF* mutated, and wild-type polyps **(A)** Comparative analysis of maximal ADP-activated respiratory rate (V_{max}); and **(B)** apparent Michaelis-Menten constant values for ADP (K_m(ADP)) in *KRAS* mutated polyps (n=14), *BRAF* mutated polyps (n=6), and wild-type polyps (n=21) * p < 0,05, ** p < 0,01 (t-test).

were no significant differences in V_{max} and K_m(ADP), nor V_{max}/K_m (ADP) ratios, between *KRAS* or *BRAF* mutated and wild-type polyps. However, due to the significantly lower V_{max} in both polyps and tumors with *BRAF* mutation, it may be assumed that cells with this mutation display a more active glycolysis with a parallel moderate down-regulation of OXPHOS. This metabolic profile of *KRAS* and *BRAF* mutated polyps suggested that energy metabolism during the transformation of polyps to colorectal cancer remain relatively unchanged. These results clearly need further assessment by using a larger study group.

To unveil respiratory rate kinetic parameters dependence on clinicopathological characteristics, possible relationships were analyzed of V_{max} and K_m(ADP) of polyps, tumors, and healthy colon tissue with age, gender, location, size, histological type, and molecular group (Supplementary Table S5). No relationship of V_{max} and K_m(ADP) values of healthy tissue, polyps, and CRC groups with clinicopathological factors was found. One exception was the location of polyps and tumors, which rendered different V_{max} values. However, sample sizes among groups were unequal as CRC is more frequently observed in the distal than in the proximal area (28) and genetic architectures of proximal and distal CRC are partly distinct (29). Again, these results clearly require further assessment by using a larger study group.

3.3 Different gene expression levels show changes in energy metabolism during CRC tumorigenesis

Numerous genes and proteins essential for glucose uptake and glycolysis are upregulated in CRC and colon polyps (30–33). In the present study, RT-qPCR was performed to detect mRNA of genes coding for the glycolysis-controlling steps *GLUT1*, *HK1*, and *HK2* (8), as well as for the essential but not controlling steps *MCT1*, *MCT2*, *MCT4*, and *LDHA* and analyze their involvement in metabolic reprogramming in colon polyps. Absence of essential genes or proteins completely stops the functioning of the cellular process/function whereas fractional removal or inhibition of a controlling step brings about a corresponding decrease in the analyzed cellular process/function.

3.3.1 Higher expression levels of genes coding for flux-controlling steps of glucose metabolism indicate an increased glycolytic activity in polyps

The first step in glucose metabolism is the entrance of glucose into the cell, which relies on glucose transport proteins (GLUTs). GLUTs belong to a homologous family of fourteen uniporter transporter proteins. Among these, GLUTs 1-4 have been extensively studied and shown to be upregulated in cancers (34). There is an increasing number of studies identifying GLUT1 (glucose low affinity isoform) and GLUT3 (glucose high affinity isoform) as preeminent actors in accelerated glucose metabolism. High expression of *GLUT1* is associated with poor survival in most cancer types, including colorectal cancer (35). The lower *GLUT1* expression levels in control colon tissue (Figure 3A) compared to diseased states were consistent with the notion that glucose provides a smaller fraction of the energy requirements for the healthy colonic epithelium. The expression of GLUT1 in colorectal polyps was significantly higher than in normal tissue, suggesting an increased demand for glucose. The CRC group also showed an increased level of *GLUT1* expression compared to the healthy colon tissue. The polyp group showed a tendency towards a lower expression of *GLUT1* than the CRC group but there was no significant difference (p=0.136). An increase in glucose uptake may indicate significant changes in energy metabolism as well as in anabolic precursors demand such as glucose-6-phosphate for pentose phosphate pathway, dihydroacetonephosphate for triacylglyceride and phospholipid syntheses, and 3-phosphoglycerate for serine, cysteine and glycine syntheses occurring in the tissue at early events of carcinogenesis.

Hexokinase (HK) is a flux-controlling step of glycolysis catalyzing ATP-dependent phosphorylation of glucose into glucose-6-phosphate (8). Four major HK isoforms, encoded by separated genes, are expressed in human tissues – HK1-4 (36). HKs help to sustain cellular glucose levels by regulating the entry and utilization of glucose and influencing the magnitude and the direction of glucose flux within cells (37). HK1 is the predominant HK isoform in most tissues, is a glucose high affinity isoform and is more abundant than HK2. HK2 is a glucose low affinity isoform and the main isoenzyme in insulin-sensitive organs such as heart, skeletal muscle, and adipose tissue, and in a wide range of tumors.

HK1 and HK2 can also dock to mitochondria through an N-terminal motif absent in the other isoforms. When bound to

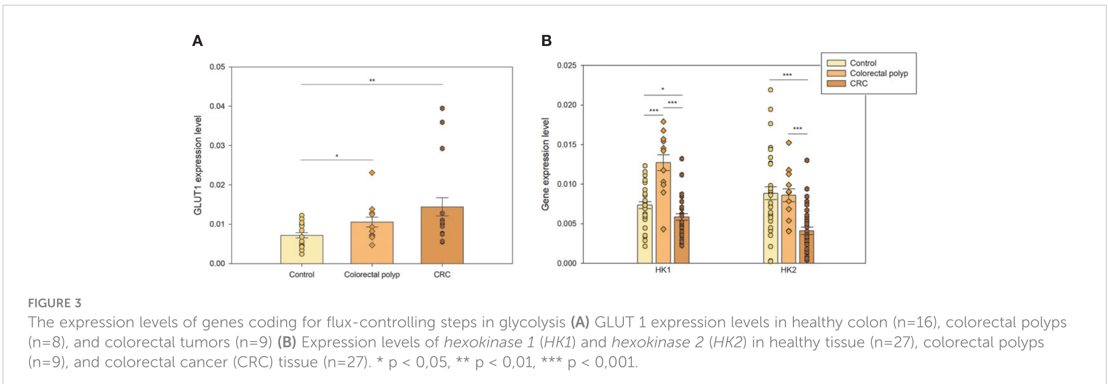

FIGURE 3
The expression levels of genes coding for flux-controlling steps in glycolysis **(A)** GLUT 1 expression levels in healthy colon (n=16), colorectal polyps (n=8), and colorectal tumors (n=9) **(B)** Expression levels of *hexokinase 1* (*HK1*) and *hexokinase 2* (*HK2*) in healthy tissue (n=27), colorectal polyps (n=9), and colorectal cancer (CRC) tissue (n=27). * p < 0,05, ** p < 0,01, *** p < 0,001.

mitochondria, HK1 and HK2 exert cytoprotective effects in healthy and neoplastic cells and increase their efficiency in glucose usage (38). Pedersen proposed that HK2 promotes the Warburg effect by binding to VDAC (11). This interaction leads VDAC to redirect mitochondrial ATP to HK2 to be used in glycolysis. Thus, HK has been proposed to regulate the MOM permeability in glycolytic cancer cells (14, 39). The high expression level and activity of *HK2* together with that of GLUTs in glycolytic cancers are indirectly revealed by ^{18}FDG-PET imaging (38).

Expression of *HK1* and *HK2* was analyzed in healthy colon tissue, colorectal polyp, and CRC groups. *HK1* expression level in polyps was twice as high as in healthy tissue and CRC group, while their *HK2* expression was similar to that of healthy tissue group (Figure 3B). Distinct differences in the regulation of mitochondrial respiration in polyps (Figure 1), specifically lower K_m(ADP) values, suggested that polyps have a more glycolytic type of regulation of energy metabolism than CRC and healthy tissue. *HK2* overexpression has been previously shown in CRC cells, in comparison to normal cells (33, 40). However, our data did not reveal *HK2* overexpression in the CRC group. In fact, it was significantly lower than that of healthy tissue (p<0.001). Then, the low *HK2* expression levels in the CRC group suggested that energy metabolism in CRC cells was not entirely glycolytic and that OXPHOS system was an important energy provider.

In turn, higher expression levels of *HK1* and *HK2* in polyps compared to the CRC group suggested that glycolytic metabolism played a more essential role in polyps than in CRC. Considering the moderate *GLUT1* expression levels and the ensuing moderate glucose uptake in polyps (Figure 3A), then *HK* overexpression seemed counterproductive. We speculate that there was no need to remarkably increase the glucose uptake mediated by GLUT1 in polyps, because highly expressed *HK*s, and perhaps GLUT3, were able to drive an enhanced glycolytic flux.

3.3.2 Expression levels of genes coding for essential but non-controlling steps support the conclusion that polyps increase glycolytic activity

Lactate dehydrogenase A (LDHA) is an essential enzyme in the glycolytic pathway that catalyzes the conversion of pyruvate to lactic acid using NADH and recycling NAD+. Elevated levels of this protein have been found in several cancer types (41, 42), supporting cancer cell proliferation and survival. The low K_m(ADP) value (Figure 1B) and high expression levels of *HK*s (Figure 3B) suggested that cells in colon polyps developed an increased dependence on the glycolytic pathway. This was further supported by the elevated levels of *LDHA* expression in polyps compared to the healthy colon tissue (Figure 4A), suggesting a rise in lactate production. Although LDH is not a flux-controlling step of glycolysis since it is one of the fastest pathway steps, it exerts full control on the pyruvate and lactate levels and hence on the cytosolic redox balance (the Pyr/Lac ratio is tightly linked to the NADH/NAD+ ratio by the overexpressed high LDH activity).

Moreover, increased production of lactate and its further release together with H^+ promotes malignant progression by lowering extracellular pH, which helps cancer cells to overcome host immune response (43). In addition, aerobic glycolysis does not only supply ATP, but also yields metabolic precursors for nucleotides, amino acids, and lipids biosynthesis for cell proliferation (44). Therefore, the high expression of *LDHA* in polyps helps promoting the disease progression to malignancy. There was no significant difference between the expression levels of *LDHA* between the CRC and the healthy tissue group, suggesting that CRC cells do not need to increase lactate production and the ensuing external acidification further because they mainly increase OXPHOS for energy supply and expression of glycolytic controlling steps for anabolic precursors and pyruvate provision.

Healthy colonocytes derive 60-70% of their energy supply from short-chain carboxylic acids, particularly butyrate. Butyrate is transported across the luminal membrane of the colonic epithelium *via* a monocarboxylate transporter (MCT1) (45). MCT1 is a member of the monocarboxylate transporter family, of which 14 isoforms have been identified. In the present study, the expression of *MCT1*, *MCT2*, and *MCT4* was analyzed. Healthy colon tissue showed higher expression level of *MCT1* compared to the tumor (Figure 4B), which is aligned with the fact that butyrate is the main source of energy for colonic epithelial cells (46). Decreased *MCT1* expression in cancer tissue could indicate that cancer cells use less butyrate, displaying metabolic plasticity and making them less dependent on this nutrient.

MCT1 has a high affinity for extracellular lactate and has been shown to transport lactate to sustain energy production in malignant cells (47). Therefore, its low expression level in tumors suggested that

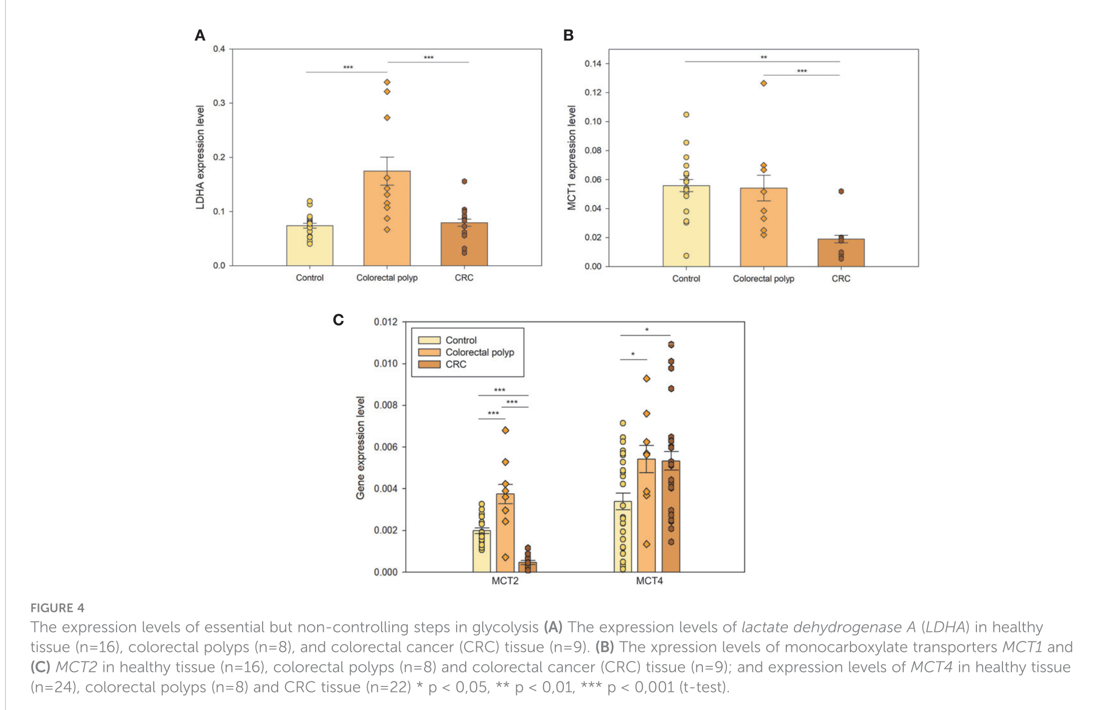

FIGURE 4
The expression levels of essential but non-controlling steps in glycolysis **(A)** The expression levels of *lactate dehydrogenase A* (*LDHA*) in healthy tissue (n=16), colorectal polyps (n=8), and colorectal cancer (CRC) tissue (n=9). **(B)** The xpression levels of monocarboxylate transporters *MCT1* and **(C)** *MCT2* in healthy tissue (n=16), colorectal polyps (n=8) and colorectal cancer (CRC) tissue (n=9); and expression levels of *MCT4* in healthy tissue (n=24), colorectal polyps (n=8) and CRC tissue (n=22) * p < 0,05, ** p < 0,01, *** p < 0,001 (t-test).

CRC cells did not depend on lactate as a metabolic fuel. *MCT1* was expressed in colon polyps similarly to healthy tissue but the expression levels of isoforms *MCT2* and *MCT4* were increased (Figure 4C). In highly glycolytic cancer cells, MCT2 has been shown to localize mainly in the cytosol (48). Decreased expression of *MCT2* in the CRC group compared to the healthy colon tissue group again supported the idea that CRC did not acquire a typical Warburg effect. MCT4 has a low affinity for extracellular lactate and high affinity for intracellular lactate, as well as very high activity for lactate transport and a very low affinity for pyruvate (48), meaning that pyruvate is rather converted to lactate than transported out of the cell whereas internal lactate can be actively expelled.

Similar expression level of *MCT1* (Figure 4B) in control and polyp groups suggested that colon polyps kept using short-chain carboxylic acids, and perhaps other substrates (*e.g.* glutamine). However, polyps exhibited higher *MCT4* expression (Figure 4C) than control indicating that they simultaneously increased glycolytic activity. *MCT4* is upregulated by hypoxia and hypoxia-inducible factor 1alpha (HIF-1*alpha*) (49). It has been shown that *HIF-1alpha* levels are increased in colon polyps and CRC (50). There is a steep oxygen gradient from the anaerobic lumen of the intestine across the epithelium into the highly vascularized sub-epithelium. Epithelial cells lining the mucosa are exposed to a relatively low O_2 tension environment that has been described as "physiological hypoxia (51, 52)." From this perspective, it is perhaps not surprising to see overexpression of *MCT4* in colon polyps as energy demand increases while there is still a low level of oxygenation.

3.3.3 Intracellular phosphotransfer pathways are upregulated in colon polyps

Adenylate kinase (AK) and creatine kinase (CK) play an important role in adjusting mitochondrial ATP synthesis to cellular ATP consumption by forming phosphotransfer circuits, which connect sites of ATP production (glycolysis and OXPHOS) with subcellular sites of ATP utilization (ATPases) to support robust metabolic homeostasis (53–55).

AKs catalyze the reversible interconversion of adenine nucleotides (AMP, ADP, ATP), and they represent the main mediator of intracellular nucleotide exchange and AMP metabolic signaling (56). Suppression of AK phosphotransference and AMP generation in cancer cells, and consequently signaling through AMPK, might be a triggering factor in the initiation of malignant transformation, unleashing uncontrolled cell cycle turnover and proliferation (57). Nine different adenylate kinase isoenzymes (AK1-9) have been identified and characterized so far in human tissues, displaying different organ and subcellular distributions.

In the present study, the gene expression level of *AK1*, *AK2*, *AK4*, and *AK6* was analyzed. AK1 is expressed in the cytosol at high levels in brain, heart, skeletal muscles, and erythrocytes (58). Previous studies have shown that AK activity in CRC tumor tissue is higher than in normal mucosa (59). AK1 has been proposed to be a negative regulator of colorectal cancer development. Its expression level in the polyp group was like that found in healthy tissue and significantly higher compared to the CRC group (Figure 5A).

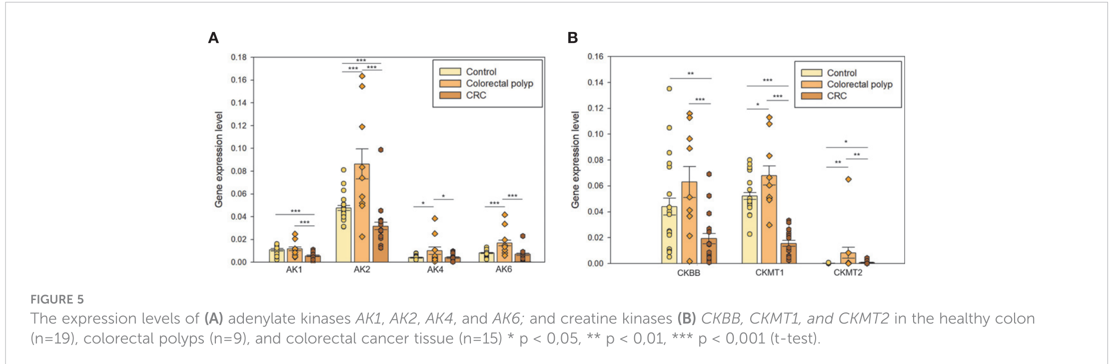

FIGURE 5
The expression levels of **(A)** adenylate kinases *AK1, AK2, AK4*, and *AK6;* and creatine kinases **(B)** *CKBB, CKMT1, and CKMT2* in the healthy colon (n=19), colorectal polyps (n=9), and colorectal cancer tissue (n=15) * p < 0,05, ** p < 0,01, *** p < 0,001 (t-test).

AK2 isoform is localized in the mitochondrial intermembrane space and regulates the ATP/ADP transference rate between the cytosol and mitochondrial matrix (56). Changes in the regulation of AK2 have been observed in several human cancers. *AK2* overexpression has been observed in lung adenocarcinoma, triple-negative breast cancer cells, and neuroblastoma cell lines and it could be related to the aggressive nature of these cancer types (60–62). The expression of AK2 was upregulated in the polyp group compared to CRC and healthy tissue groups (Figure 5A). The CRC group showed a significantly lower *AK2* expression than the control group suggesting that fundamental rearrangements in the energy-related communication networks between cytosol and mitochondria take place during progression to cancer. Although the potential role of AK2 in tumorigenesis has been reported for a long time already, its underlying mechanism is still unclear.

AK4 is expressed in the mitochondrial matrix and may indirectly modulate the mitochondrial membrane permeability *via* its interactions with the ADP/ATP translocase (ANT) (58). Previous studies have demonstrated the involvement of AK4 in the progression of different cancer types, as well as in the resistance to radiation therapy and multiple chemotherapeutic agents (63–65). Indeed, the expression level of *AK4* was significantly higher in the polyp group compared to the healthy tissue group and CRC. AK4 has been demonstrated to promote a glycolytic shift (66), which is aligned with our observation of the glycolytic phenotype in the polyp group.

AK6, renamed as human coilin interacting nuclear ATPase protein (HCINAP), is localized in nucleus and cytosol, and is ubiquitously expressed in different tissues and cell types (67, 68). *AK6* expression was higher in polyps than in control tissue and CRC groups (Figure 5A). AK6 is a glycolysis regulator *via* phosphorylation of LDHA and a modulator of invasion and metastatic activity of cancer stem cells (69). However, *AK6* became upregulated already at the polyp stage and it may support the glycolytic activity in benign tumors. Higher *LDHA* expression level in polyps than CRC (Figure 4A) correlates with a similar pattern in *AK6* expression. It can be hypothesized that AK6 is required to support cell division and that in polyps with active anaerobic glycolysis AK6 could be preferentially located in the cytosol.

Moreover, AKs may regulate intracellular AMP levels and thus directly affect AMPK metabolic signaling. The elevated levels of AK expression in polyps could support AMPK activation and OXPHOS in conditions of intense competition for cytosolic ADP (glycolysis has a high affinity for ADP). In this regard, a model has been developed where the bioenergetics signatures combine the metabolic networks of AMPK and HIF-1 alpha activity (70). The activity of these two regulators defines the metabolic states as follows: a glycolytic state is established by high HIF-1 and low AMPK, an oxidative state is characterized by low HIF-1 and high AMPK, and in a hybrid state both regulators are active (70). As both proteins are important determinants of cell metabolism and fate, understanding the interplay between different AKs in their various locations and their regulation might uncover new targets for cancer treatments or biomarkers for cancer occurrence and prognosis.

Creatine kinase (CK) has a crucial role in cell bioenergetics to efficiently regenerate ATP from phosphocreatine and is over-expressed in cells with high energy requirements such as skeletal, cardiac and smooth muscle, kidney, and brain (71). There are two genes for cytosolic CK subunit isoforms forming three types of dimers (CKMM, CKBB, and CKMB) and two mitochondrial creatine kinase (mtCK) isoenzymes (the *ubiquitous* form – gene *CKMT1* and the *sarcomeric* form – gene *CKMT2* (72). The interplay between cytosolic and mitochondrial CK isoenzymes depends on a large intracellular pool of creatine/phosphocreatine and prevents a rapid fall in global ATP concentrations (72). This ATP buffering system is known as the phosphocreatine (PCr)-creatine kinase (CK) shuttle, or PCr-CK circuit (53).

Mitochondrial CKs catalyze the interconversion of ATP into PCr at the main ATP-producing sites to store the energy in the form of PCr and facilitate its intracellular diffusion across the different subcellular organelles, whereas cytosolic CKs regenerate *in situ* ATP from the PCr pool at ATP-consuming sites (73, 74). CKs are expressed in colon epithelial cells and are coordinately regulated by HIFs. Such regulation is critical for their barrier function (75). Attenuated expression of CK enzymes in inflammatory bowel disease tissue (75), downregulation of CK-BB functional activity and low expression of *MTCK1* in colon cancer (which is a different feature from other cancer types) (59, 76) suggest that intestinal creatine metabolism and PCr/CK circuit may be compromised in colon polyps as well.

Here, the expression level of *CKBB*, *CKMT1*, and *CKMT2* was assessed. Downregulation of *CKBB* in CRC was observed (Figure 5B). CK isoforms may be up- and down-regulated in tumors depending on the nature of the carcinogenesis (77). Mitochondrial CK transcribed

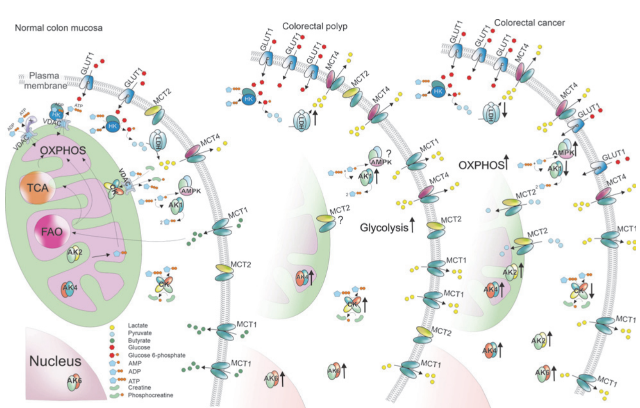

FIGURE 6
Changes of gene expression levels in colorectal polyps and tumors compared to normal colon mucosa. ATP production in normal colon mucosa is presented in the left. Glucose enters the cell through glucose transporters (GLUT1) and is then phosphorylated by hexokinase (HK). The glycolytic product pyruvate enters mitochondria through voltage dependent anion channel (VDAC) in mitochondrial outer membrane and is used to produce ATP through TCA cyle and oxidative phosphorylation (OXPHOS). Some of the pyruvate is converted into lactate by lactate dehydrogenase A (LDHA) and lactate is transported out of the cell using monocarboxylate transporter (MCT) 4. Additionally, colon cells uptake short fatty acids like butyrate using MCT1 and use them in fatty acid oxidation (FAO). To transport ATP and ADP between energy production and energy consumption sites, cells use creatine kinases (CKs) and adenylate kinases (AKs). In colorectal polyps, the energy metabolism is reprogrammed by increasing the glycolytic activity. This phenomenon could be facilitated by higher *GLUT1*, *HK* and *LDHA* expression boosting lactate production. The excessive amounts of lactate are transported out of the cell using MCT1 and MCT4 that were upregulated in polyps. Polyps also displayed higher levels of both CKs and AKs. However, colorectal cancer cells (right) seemed to depend more on OXPHOS suggested by the decreased expression levels of *HK*s and *LDHA*. Poor *MCT* levels indicate that the need for lactate transport is lower. Opposite to polyps, energy transport pathways in cancer cells showed signs of downregulation.

from *CKMT1*, also known as U-MtCK is localized in the inner membrane of mitochondria. CKMT1 may participate in the development of human cancers because of its involvement in several cellular processes such as cell proliferation, migration, and apoptosis (78, 79). Expression of *CKMT1* was significantly lower in the CRC group compared to healthy tissue and polyps (Figure 5B). In this regard, it has been suggested that *MtCK* expression is regulated by the metabolic energy cell status and their expression may represent a mechanism to compensate for a low energy state (72). Thus, high expression of *CKBB* and *CKMT1* in control and polyp, and overexpression of *CKMT2* in polyps is consistent with the observation that polyps are highly glycolytic compared to healthy and cancerous tissue. Whether the changes in intracellular energy transfer are the cause or consequence of CRC and hence how AK and CK energy shuttles may be affected to prevent polyps from becoming malignant remains to be investigated.

4 Conclusions

Although our knowledge on cancer metabolism has increased, the whole process of metabolic reprogramming during tumorigenesis is still rather unexplored. Here we showed that changes in energy production already occur in benign colorectal tumors and the alterations continue throughout the development of colorectal cancer. Colon polyps seem to increase glycolytic activity by overexpressing glucose transporter 1 and hexokinases. The low K_m (ADP) value determined in polyps by high-resolution respirometry as well as their LDHA overexpression added support to the proposal of a glycolytic phenotype for polyps. The higher glycolytic activity may drive cell proliferation in the diseased state (80). On the other hand, while cancer cells seem to upregulate the glycolytic pathway, they still depend highly on mitochondrial respiration. Besides glycolysis, colon polyps upregulate the activity of energy transfer pathways like adenylate kinase and creatine kinase systems. The observations of metabolic reprogramming described in the results are presented in Figure 6. The significant changes in gene expression levels could be used as biomarkers to detect benign tumors in early stages.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Research Ethics Committee of the National Institute for Health Development. The patients/participants provided their written informed consent to participate in this study.

Author contributions

ER-K, LR and MP wrote the main manuscript text. ER-K and LR performed the experiments and analyzed the data. LR and IS prepared

the figures. JB, KS and VV provided the samples. IS prepared the graphical abstract. IS, RM-S and TK reviewed and edited the text. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1171887/full#supplementary-material

References

1. Rex DK, Hassan C, Bourke MJ. The colonoscopist's guide to the vocabulary of colorectal neoplasia: histology, morphology, and management. *Gastrointest Endosc* (2017) 86(2):253–63. doi: 10.1016/j.gie.2017.03.1546

2. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* (1990) 61(5):759–67. doi: 10.1016/0092-8674(90)90186-I

3. Risio M. The natural history of colorectal adenomas and early cancer. *Pathologe* (2012) 33 Suppl 2:206–10. doi: 10.1007/s00292-012-1640-6

4. Rashid A, Houlihan PS, Booker S, Petersen GM, Giardiello FM, Hamilton SR. Phenotypic and molecular characteristics of hyperplastic polyposis. *Gastroenterology* (2000) 119(2):323–32. doi: 10.1053/gast.2000.9361

5. Miller Q, Saeed O, Mesa H. Clinical, pathologic, and molecular-genetic aspects of colorectal polyps. *Gastrointest Endosc Clin N Am* (2022) 32(2):313–28. doi: 10.1016/j.giec.2021.12.007

6. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* (2000) 100(1):57–70. doi: 10.1016/S0092-8674(00)81683-9

7. Warburg O. On the origin of cancer cells. *Science* (1956) 123(3191):309–14. doi: 10.1126/science.123.3191.309

8. Moreno-Sanchez R, Marin-Hernandez A, Saavedra E, Pardo JP, Ralph SJ, Rodriguez-Enriquez S. Who controls the ATP supply in cancer cells? biochemistry lessons to understand cancer energy metabolism. *Int J Biochem Cell Biol* (2014) 50:10–23. doi: 10.1016/j.biocel.2014.01.025

9. Schiliro C, Firestein BL. Mechanisms of metabolic reprogramming in cancer cells supporting enhanced growth and proliferation. *Cells* (2021) 10(5). doi: 10.3390/cells10051056

10. Zhang S, Nemutlu E, Terzic A, Dzeja P. Adenylate kinase isoform network: a major hub in cell energetics and metabolic signaling. In: Aon MA, Saks V, Schlattner U, editors. *Systems biology of metabolic and signaling networks*. Heidelberg, Berlin: Springer (2014). p. 145–62.

11. Pedersen PL. Warburg, me and hexokinase 2: multiple discoveries of key molecular events underlying one of cancers' most common phenotypes, the "Warburg effect", i.e., elevated glycolysis in the presence of oxygen. *J Bioenerg Biomembr* (2007) 39(3):211–22. doi: 10.1007/s10863-007-9094-x

12. Fujisawa K, Terai S, Takami T, Yamamoto N, Yamasaki T, Matsumoto T, et al. Modulation of anti-cancer drug sensitivity through the regulation of mitochondrial activity by adenylate kinase 4. *J Exp Clin Cancer Res* (2016) 35(1):48. doi: 10.1186/s13046-016-0322-2

13. Maldonado EN, Lemasters JJ. Warburg revisited: regulation of mitochondrial metabolism by voltage-dependent anion channels in cancer cells. *J Pharmacol Exp Ther* (2012) 342(3):637–41. doi: 10.1124/jpet.112.192153

14. Puurand M, Tepp K, Timohhina N, Aid J, Shevchuk I, Chekulayev V, et al. Tubulin betaII and betaIII isoforms as the regulators of VDAC channel permeability in health and disease. *Cells* (2019) 8(3). doi: 10.3390/cells8030239

15. Pesta D, Gnaiger E. High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. In: Palmeira CM, Moreno AJ, editors. *Mitochondrial bioenergetics: methods and protocols*. Totowa, NJ: Humana Press (2012). p. 25–58.

16. Untergasser A. *RNAprep - trizol combined with columns* (2008). Available at: http://www.untergasser.de/lab/protocols/rna_prep_comb_trizol_v1_0.htm.

17. Kuznetsov AV, Tiivel T, Sikk P, Kaambre T, Kay L, Daneshrad Z, et al. Striking differences between the kinetics of regulation of respiration by ADP in slow-twitch and fast-twitch muscles in vivo. *Eur J Biochem* (1996) 241(3):909–15. doi: 10.1111/j.1432-1033.1996.00909.x

18. Puurand M, Tepp K, Klepinin A, Klepinina L, Shevchuk I, Kaambre T. Intracellular energy-transfer networks and high-resolution respirometry: a convenient approach for studying their function. *Int J Mol Sci* (2018) 19(10). doi: 10.3390/ijms19102933

19. Chekulayev V, Mado K, Shevchuk I, Koit A, Kaldma A, Klepinin A, et al. Metabolic remodeling in human colorectal cancer and surrounding tissues: alterations in regulation of mitochondrial respiration and metabolic fluxes. *Biochem Biophysics Rep* (2015) 4:111–25. doi: 10.1016/j.bbrep.2015.08.020

20. Koit A, Shevchuk I, Ounpuu L, Klepinin A, Chekulayev V, Timohhina N, et al. Mitochondrial respiration in human colorectal and breast cancer clinical material is regulated differently. *Oxid Med Cell Longev 2017* (2017) p:1372640. doi: 10.1155/2017/1372640

21. Rebane-Klemm E, Truu L, Reinsalu L, Puurand M, Shevchuk I, Chekulayev V, et al. Mitochondrial respiration in KRAS and BRAF mutated colorectal tumors and polyps. *Cancers (Basel)* (2020) 12(4). doi: 10.3390/cancers12040815

22. Veksler VI, Kuznetsov AV, Kay L, Sikk P, Tiivel T, Tranqui L, et al. Permeabilized cell and skinned fiber techniques in studies of mitochondrial function in vivo. *Mol Cell Biochem* (1998) 184:81–100. doi: 10.1023/A:1006834912257

23. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) 144(5):646–74. doi: 10.1016/j.cell.2011.02.013

24. Yun J, Rago C, Cheong I, Pagliarini R, Angenendt P, Rajagopalan H, et al. Glucose deprivation contributes to the development of KRAS pathway mutations in tumor cells. *Science* (2009) 325(5947):1555–9. doi: 10.1126/science.1174229

25. Son J, Lyssiotis CA, Ying H, Wang X, Hua S, Ligorio M, et al. Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway. *Nature* (2013) 496(7443):101–5. doi: 10.1038/nature12040

26. Nagarajah J, Ho AL, Tuttle RM, Weber WA, Grewal RK. Correlation of BRAFV600E mutation and glucose metabolism in thyroid cancer patients: an ^{18}F-FDG PET study. *J Nucl Med* (2015) 56(5):662–7. doi: 10.2967/jnumed.114.150607

27. Faubert B, Solmonson A, DeBerardinis RJ. Metabolic reprogramming and cancer progression. *Science* (2020) 368(6487):eaaw5473. doi: 10.1126/science.aaw5473

28. Missiaglia E, Jacobs B, D'Ario G, Di Narzo AF, Soneson C, Budinska E, et al. Distal and proximal colon cancers differ in terms of molecular, pathological, and clinical features. *Ann Oncol* (2014) 25(10):1995–2001. doi: 10.1093/annonc/mdu275

29. Huyghe JR, Harrison TA, Bien SA, Hampel H, Figueiredo JC, Schmit SL, et al. Genetic architectures of proximal and distal colorectal cancer are partly distinct. *Gut* (2021) 70(7):1325–34. doi: 10.1136/gutjnl-2020-321534

30. Leclerc D, Pham DN, Lévesque N, Truongcao M, Foulkes WD, Sapienza C, et al. Oncogenic role of PDK4 in human colon cancer cells. *Br J Cancer* (2017) 116(7):930–6. doi: 10.1038/bjc.2017.38

31. Azuma M, Shi M, Danenberg KD, Gardner H, Barrett C, Jacques CJ, et al. Serum lactate dehydrogenase levels and glycolysis significantly correlate with tumor VEGFA and VEGFR expression in metastatic CRC patients. *Pharmacogenomics* (2007) 8 (12):1705–13. doi: 10.2217/14622416.8.12.1705

32. Graziano F, Ruzzo A, Giacomini E, Ricciardi T, Aprile G, Loupakis F, et al. Glycolysis gene expression analysis and selective metabolic advantage in the clinical

progression of colorectal cancer. *Pharmacogenomics J* (2017) 17(3):258–64. doi: 10.1038/tpj.2016.13

33. Wang NN, Zhang PZ, Zhang J, Wang HN, Li L, Ren F, et al. Penfluridol triggers mitochondrial-mediated apoptosis and suppresses glycolysis in colorectal cancer cells through down-regulating hexokinase-2. *Anat Rec (Hoboken)* (2021) 304(3):520–30. doi: 10.1002/ar.24464

34. Barron CC, Bilan PJ, Tsakiridis T, Tsiani E. Facilitative glucose transporters: implications for cancer detection, prognosis and treatment. *Metabolism* (2016) 65 (2):124–39. doi: 10.1016/j.metabol.2015.10.007

35. Ancey PB, Contat C, Meylan E. Glucose transporters in cancer - from tumor cells to the tumor microenvironment. *FEBS J* (2018) 285(16):2926–43. doi: 10.1111/febs.14577

36. Robey RB, Hay N. Mitochondrial hexokinases, novel mediators of the antiapoptotic effects of growth factors and akt. *Oncogene* (2006) 25:4683–96. doi: 10.1038/sj.onc.1209595

37. Patra KC, Wang Q, Bhaskar PT, Miller L, Wang Z, Wheaton W, et al. Hexokinase 2 is required for tumor initiation and maintenance and its systemic deletion is therapeutic in mouse models of cancer. *Cancer Cell* (2013) 24(2):213–28. doi: 10.1016/j.ccr.2013.06.014

38. Mathupala SP, Ko YH, Pedersen PL. Hexokinase II: cancer's double-edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria. *Oncogene* (2006) 25(34):4777–86. doi: 10.1038/sj.onc.1209603

39. Klepinin A, Ounpuu L, Mado K, Truu L, Chekulayev V, Puurand M, et al. The complexity of mitochondrial outer membrane permeability and VDAC regulation by associated proteins. *J Bioenerg Biomembr* (2018) 50(5):339–54. doi: 10.1007/s10863-018-9765-9

40. Ho N, Coomber BL. Hexokinase II expression is correlated with colorectal cancer prognosis. *Cancer Treat Commun* (2016) 6:11–6. doi: 10.1016/j.ctrc.2016.02.008

41. Cai H, Li J, Zhang Y, Liao Y, Zhu Y, Wang C, et al. LDHA promotes oral squamous cell carcinoma progression through facilitating glycolysis and epithelial–mesenchymal transition. *Front Oncol* (2019) 9. doi: 10.3389/fonc.2019.01446

42. Sun X, Sun Z, Zhu Z, Guan H, Zhang J, Zhang Y, et al. Clinicopathological significance and prognostic value of lactate dehydrogenase a expression in gastric cancer patients. *PloS One* (2014) 9(3):e91068. doi: 10.1371/journal.pone.0091068

43. Kim R, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. *Immunology* (2007) 121(1):1–14. doi: 10.1016/B978-012372551-6/50066-3

44. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the warburg effect: the metabolic requirements of cell proliferation. *Science* (2009) 324(5930):1029–33. doi: 10.1126/science.1160809

45. Lambert DW, Wood IS, Ellis A, Shirazi-Beechey SP. Molecular changes in the expression of human colonic nutrient transporters during the transition from normality to malignancy. *Br J Cancer* (2002) 86(8):1262–9. doi: 10.1038/sj.bjc.6600264

46. Cummings JH, Pomare EW, Branch WJ, Naylor CP, Macfarlane GT. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* (1987) 28(10):1221–7. doi: 10.1136/gut.28.10.1221

47. Sonveaux P, Végran F, Schroeder T, Wergin MC, Verrax J, Rabbani ZN, et al. Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. *J Clin Invest* (2008) 118(12):3930–42. doi: 10.1172/JCI36843

48. Pinheiro C, Reis RM, Ricardo S, Longatto-Filho A, Schmitt F, Baltazar F. Expression of monocarboxylate transporters 1, 2, and 4 in human tumours and their association with CD147 and CD44. *J Biomedicine Biotechnol* (2010) 2010:427694. doi: 10.1155/2010/427694

49. Ullah MS, Davies AJ, Halestrap AP. The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1 α-dependent mechanism *. *J Biol Chem* (2006) 281(14):9030–7. doi: 10.1074/jbc.M511397200

50. Giles RH, Lolkema MP, Snijckers CM, Belderbos M, van der Groep P, Mans DA, et al. Interplay between VHL/HIF1alpha and wnt/beta-catenin pathways during colorectal tumorigenesis. *Oncogene* (2006) 25(21):3065–70. doi: 10.1038/sj.onc.1209330

51. Colgan SP, Taylor CT. Hypoxia: an alarm signal during intestinal inflammation. *Nat Rev Gastroenterol Hepatol* (2010) 7(5):281–7. doi: 10.1038/nrgastro.2010.39

52. Furuta GT, Turner JR, Taylor CT, Hershberg RM, Comerford K, Narravula S, et al. Hypoxia-inducible factor 1-dependent induction of intestinal trefoil factor protects barrier function during hypoxia. *J Exp Med* (2001) 193(9):1027–34. doi: 10.1084/jem.193.9.1027

53. Wallimann T, Tokarska-Schlattner M, Schlattner U. The creatine kinase system and pleiotropic effects of creatine. *Amino Acids* (2011) 40(5):1271–96. doi: 10.1007/s00726-011-0877-3

54. Dzeja PP, Terzic A. Phosphotransfer networks and cellular energetics. *J Exp Biol* (2003) 206(12):2039–47. doi: 10.1242/jeb.00426

55. Dzeja PP, Zeleznikar RJ, Goldberg ND. Adenylate kinase: kinetic behavior in intact cells indicates it is integral to multiple cellular processes. *Mol Cell Biochem* (1998) 184(1):169–82. doi: 10.1023/A:1006859632730

56. Dzeja P, Terzic A. Adenylate kinase and AMP signaling networks: metabolic monitoring, signal communication and body energy sensing. *Int J Mol Sci* (2009) 10 (4):1729–72. doi: 10.3390/ijms10041729

57. Klepinin A, Zhang S, Klepinina L, Rebane-Klemm E, Terzic A, Kaambre T, et al. Adenylate kinase and metabolic signaling in cancer cells. *Front Oncol* (2020) 10. doi: 10.3389/fonc.2020.00660

58. Panayiotou C, Solaroli N, Karlsson A. The many isoforms of human adenylate kinases. *Int J Biochem Cell Biol* (2014) 49:75–83. doi: 10.1016/j.biocel.2014.01.014

59. Kaldma A, Klepinin A, Chekulayev V, Mado K, Shevchuk I, Timohhina N, et al. An in situ study of bioenergetic properties of human colorectal cancer: the regulation of mitochondrial respiration and distribution of flux control among the components of ATP synthasome. *Int J Biochem Cell Biol* (2014) 55:171–86. doi: 10.1016/j.biocel.2014.09.004

60. Liu H, Pu Y, Amina Q, Wang Q, Zhang M, Song J, et al. Prognostic and therapeutic potential of adenylate kinase 2 in lung adenocarcinoma. *Sci Rep* (2019) 9 (1):17757. doi: 10.1038/s41598-019-53594-4

61. Klepinin A, Ounpuu L, Guzun R, Chekulayev V, Timohhina N, Tepp K, et al. Simple oxygraphic analysis for the presence of adenylate kinase 1 and 2 in normal and tumor cells. *J Bioenerg Biomembr* (2016) 48(5):531–48. doi: 10.1007/s10863-016-9687-3

62. Klepinin A, Miller S, Reile I, Puurand M, Rebane-Klemm E, Klepinina L, et al. Stable isotope tracing uncovers reduced gamma/beta-ATP turnover and metabolic flux through mitochondrial-linked phosphotransfer circuits in aggressive breast cancer cells. *Front Oncol* (2022) 12:892195. doi: 10.3389/fonc.2022.892195

63. Jan YH, Tsai HY, Yang CJ, Huang MS, Yang YF, Lai TC, et al. Adenylate kinase-4 is a marker of poor clinical outcomes that promotes metastasis of lung cancer by downregulating the transcription factor ATF3. *Cancer Res* (2012) 72(19):5119–29. doi: 10.1158/0008-5472.CAN-12-1842

64. Xin F, Yao DW, Fan L, Liu JH, Liu XD. Adenylate kinase 4 promotes bladder cancer cell proliferation and invasion. *Clin Exp Med* (2019) 19(4):525–34. doi: 10.1007/s10238-019-00576-5

65. Lei W, Yan C, Ya J, Yong D, Yujun B, Kai L. MiR-199a-3p affects the multi-chemoresistance of osteosarcoma through targeting AK4. *BMC Cancer* (2018) 18 (1):631. doi: 10.1186/s12885-018-4460-0

66. Jan Y-H, Lai T-C, Yang C-J, Lin Y-F, Huang M-S, Hsiao M. Adenylate kinase 4 modulates oxidative stress and stabilizes HIF-1α to drive lung adenocarcinoma metastasis. *J Hematol Oncol* (2019) 12(1):12. doi: 10.1186/s13045-019-0698-5

67. Ren H, Wang L, Bennett M, Liang Y, Zheng X, Lu F, et al. The crystal structure of human adenylate kinase 6: an adenylate kinase localized to the cell nucleus. *Proc Natl Acad Sci USA* (2005) 102(2):303–8. doi: 10.1073/pnas.0407459102

68. Xu R, Yang Y, Zheng X. Unique structural features of the adenylate kinase hCINAP/AK6 and its multifaceted functions in carcinogenesis and tumor progression. *FEBS Lett* (2021) 595(16):2071–84. doi: 10.1002/1873-3468.14158

69. Ji Y, Yang C, Tang Z, Yang Y, Tian Y, Yao H, et al. Adenylate kinase hCINAP determines self-renewal of colorectal cancer stem cells by facilitating LDHA phosphorylation. *Nat Commun* (2017) 8:15308. doi: 10.1038/ncomms15308

70. Paudel BB, Quaranta V. Metabolic plasticity meets gene regulation. *Proc Natl Acad Sci USA* (2019) 116(9):3370–2. doi: 10.1073/pnas.1900169116

71. Wallimann T, Wyss M, Brdiczka D, Nicolay K, Eppenberger HM. Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. *Biochem J* (1992) 281(Pt 1):21–40. doi: 10.1042/bj2810021

72. Schlattner U, Tokarska-Schlattner M, Wallimann T. Mitochondrial creatine kinase in human health and disease. *Biochim Biophys Acta (BBA) - Mol Basis Dis* (2006) 1762(2):164–80. doi: 10.1016/j.bbadis.2005.09.004

73. Saks V, Kaambre T, Guzun R, Anmann T, Sikk P, Schlattner U, et al. The creatine kinase phosphotransfer network: thermodynamic and kinetic considerations, the impact of the mitochondrial outer membrane and modelling approaches. *Subcell Biochem* (2007) 46:27–65. doi: 10.1007/978-1-4020-6486-9_3

74. Sahlin K, Harris RC. The creatine kinase reaction: a simple reaction with functional complexity. *Amino Acids* (2011) 40(5):1363–7. doi: 10.1007/s00726-011-0856-8

75. Glover LE, Bowers BE, Saeedi B, Ehrentraut SF, Campbell EL, Bayless AJ, et al. Control of creatine metabolism by HIF is an endogenous mechanism of barrier regulation in colitis. *Proc Natl Acad Sci USA* (2013) 110(49):19820–5. doi: 10.1073/pnas.1302840110

76. Balasubramani M, Day BW, Schoen RE, Getzenberg RH. Altered expression and localization of creatine kinase b, heterogeneous nuclear ribonucleoprotein f, and high mobility group box 1 protein in the nuclear matrix associated with colon cancer. *Cancer Res* (2006) 66(2):763–9. doi: 10.1158/0008-5472.CAN-05-3771

77. Yan Y-B. Creatine kinase in cell cycle regulation and cancer. *Amino Acids* (2016) 48(8):1775–84. doi: 10.1007/s00726-016-2217-0

78. Li M, Liu H, Li J, Guo S, Lv Y. Mitochondrial creatine kinase 1 in non-small cell lung cancer progression and hypoxia adaptation. *Respir Res* (2021) 22(1):1–8. doi: 10.1186/s12931-021-01765-1

79. Amamoto R, Uchiumi T, Yagi M, Monji K, Song Y, Oda Y, et al. The expression of ubiquitous mitochondrial creatine kinase is downregulated as prostate cancer progression. *J Cancer* (2016) 7(1):50–9. doi: 10.7150/jca.13207

80. Hsu PP, Sabatini DM. Cancer cell metabolism: warburg and beyond. *Cell* (2008) 134(5):703–7. doi: 10.1016/j.cell.2008.08.021

Appendix 4

Manuscript
Reinsalu, L*; Miller, S*; Auditano, G. L; Puurand, M; Moreno-Sanchez, R; Saavedra, E; Valvere, V; Käämbre, T. Energy Metabolism Profiling of Human Colorectal Tumors. (Submitted).

Energy Metabolism Profiling of Human Colorectal Tumors

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Abstract

Colorectal cancer (CRC) is a significant global health burden and its early detection is crucial. Novel diagnostic and prognostic methods are required for improving patient treatment, survival and quality of life. One promising approach is the analysis and understanding of the metabolic reprogramming undergone by cancer cells. Then, by analyzing in a systematic way the changes in transcript and protein contents, activities, pathway flux and energy metabolite ratios in post operative CRC tumors, in comparison to adjacent healthy tissue, the energy metabolism was characterized at the molecular and functional levels. Greater expression of glucose transporter 1 and lactate dehydrogenase A (LDH), together with increased protein content and activity of LDH, in tumors suggested a higher glycolytic capability. Hexokinase transcripts, protein and activity were rather similar, whereas monocarboxylate transport transcripts and protein contents were rather lower in tumors. The creatine kinase transcripts and the adenylate kinase protein contents were also clearly lower in tumors, indicating a functional decrease in the CRC energy transfer pathway. However, oxidative phosphorylation was fully functional and with higher catalytic efficiency (*Vmax*/*Km*) in tumors, although the cellular energy charge was slightly lower in tumors. Furthermore, higher OxPhos catalytic efficiency with the increase in the CRC clinical stage was observed. The data revealed that CRC exhibits a hybrid energy metabolism phenotype where both glycolysis and oxidative phosphorylation are highly active.

Keywords

Colorectal cancer, energy metabolism, oxidative phosphorylation, glycolysis

Abbreviations:

adenylate kinase, **AK**; AMP-activated protein kinase, **AMPK**; colorectal cancer, **CRC**; creatine kinase, **CK**; energy charge, **EC**; glucose transporter 1, **GLUT1**; hexokinase, **HK**; lactate dehydrogenase, **LDH**; monocarboxylate transporter, **MCT**; Oxidative phosphorylation, **OxPhos**; Quantitative reverse transcription polymerase chain reaction, **RT-qPCR;** voltage-dependent anion channel, **VDAC**.

Introduction

In 2020, 18.1 million people worldwide were diagnosed with cancer, among these, colorectal cancer (CRC) stands as the third most prevalent form and ranks second in cancer-related mortality following lung cancer (1). Only 5-10% of CRC cases occurred by genetic predisposition, emphasizing the relevant influence of environmental factors on cancer development risks. Despite advancements in screening only nearly 33% of all CRC cases are diagnosed at the early localized stage, and around a fifth of all diagnosed incidences already showed distant metastases upon diagnosis (2). Considering the stark contrast in the 5-year survival rates between localized and metastasized CRC (88% *versus* 16%, respectively), there is an imperative and urgent public health need to enable earlier CRC detection. In that regard, metabolomics approaches to identify diagnostic biomarkers in blood or urine have been pursued (3, 4); however, many of the proposed metabolites are not specific of CRC. Therefore, a comprehensive understanding of the metabolic reprogramming undergone during CRC development should be reached, in order to identify enzymes and/or metabolites that are more specific for early detection and prognosis of CRC development.

Living cells rely on a constant supply of energy, which they produce by converting food-sourced fuels into usable cellular energy carrier metabolites (*e.g.*, ATP and creatine-phosphate). In cancer cells, energy metabolism *i.e.*, glycolysis and oxidative phosphorylation (OxPhos) undergoes significant reprogramming to sustain the accelerated cell proliferation and growth typical of neoplastic disease (5). The predominant energy production pathway in different cancer cell types has not been completely established. The most common theory, the Warburg effect, suggests that cancer cells favor glycolytic energy production even in the presence of oxygen (6). However, there is increasing evidence demonstrating that several cancer cell types exhibit high rates of OxPhos (7-9). Furthermore, cancer cells may adopt a hybrid energy metabolic state, simultaneously depending on both glycolysis and OxPhos pathways. This dynamic adaptative capacity of cancer cells has been termed metabolic plasticity (10). Nonetheless, most of the mechanisms underlying this plasticity remain largely unknown.

Understanding the CRC development requires a comprehensive analysis at several levels, including changes in gene expression (transcript levels) and protein levels, enzyme activities, pathway fluxes, isoform profiles, and their roles in cellular function (11). While cancer has been historically considered solely as a genetic disease, it is now evident that extensive alterations occur in several cellular systems involved in functions like protein expression and activities, signaling and metabolic pathways, many of which cannot be solely attributed to genetic causes. Traditionally, molecular biology and biomedical research has focused on the study of single genes, individual protein targets, single metabolites, and specific signaling pathways. However, an integral approach that considers the complex interplay of these components provides a more promising direction for uncovering changes in metabolic networks, diseases, and

understanding the mechanisms of drug effects (11). Furthermore, significant metabolic research is conducted primarily on 2D cell cultures. Cell metabolism is profoundly influenced by the microenvironment. For instance, the metabolic phenotype of cultured cells can be modified by the conditions in which they are grown (12). While these studies may offer valuable insights, they fail to fully represent the clinic situation. Therefore, in the present study, metabolic reprogramming in post-operative tumor material obtained from CRC patients was analyzed.

Methods

Clinical material

Post-operative tissue material was provided by the Oncology and Hematology Clinic at the North Estonian Medical Centre (NEMC, Tallinn, Estonia). Only primary, treatment naive tumors were examined. Fresh samples of CRC tumors and non-cancerous mucosa tissue located at 5 cm from tumor were collected in plastic containers with Mitomedium B solution (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 3 mM KH_2PO_4, 110 mM sucrose, 0.5 mM dithiothreitol, 20 mM HEPES, 5 μM leupeptin, 2 mg/mL fatty acids free bovine serum albumin, pH 7.1), stored on ice and immediately transported to the laboratory. Pathology reports for all the analyzed samples were provided by NEMC. All subjects were informed about the study and their respective signed informed consent letters were obtained. To protect the identity of patients, coded identity protection was applied. All actions concerning human subjects and follow-up protocols have been approved by the Tallinn Medical Research Ethics Committee (decision numbers KK557 and KK558), and are in accordance with Helsinki Declaration and Convention of the Council of Europe on Human Rights and Biomedicine.

RNA extraction

To preserve cellular RNA integrity, a fraction of approximately 40% of each tissue sample was suspended in RNALater solution (Qiagen) for transportation. Upon arrival to the laboratory, the sample was frozen in liquid nitrogen and stored at -80°C. The RNA was later extracted following the protocol by Untergasser (13). Briefly, the frozen tissue samples were homogenized using the TRIzol reagent (Ambion); the RNeasy Mini Kit (Qiagen) was used for RNA isolation. RNase-free DNase I Solution (Qiagen) was used to eliminate DNA. Finally, RNA was eluted from the spin column with 30 μL of RNase-free water and the total concentration of RNA was determined with a BioSpec-Nano spectrophotometer (Shimadzu). The isolated RNA was stored at -80°C.

cDNA synthesis and real-time quantitative polymerase chain reaction

All reagents used for cDNA synthesis and quantitative reverse transcription polymerase chain reaction (RT-qPCR) were from Applied Biosynthesis. cDNA was synthesized from 2 μg of RNA using the High-Capacity cDNA Reverse Transcription Kit with RNase

inhibitor following the protocol provided by the manufacturer. Reverse transcription reaction incubation was performed in an Eppendorf® 5332 Mastercycler thermocycler.

RT-qPCR was performed using a LightCycler 480 II instrument (Roche). The reaction mix contained TaqMan Gene Expression Master Mix (Thermo Fisher Scientific) and the FAM-labeled TaqMan probes: actin-β (Hs01060665_g1), for adenylate kinase AK1 (Hs00176119_m1), AK2 (Hs01123132_g1), AK4 (Hs03405743_g1), AK6 (Hs00360444_g1); for creatine kinase CK-BB (Hs00176483_m1), CK-MT1 (Hs00179727_m1), CK-MT2 (Hs00176502_m1); for hexokinases HK1 (Hs00175976_m1), and HK2 (Hs00606086_m1); for glucose transporter 1 GLUT1 (Hs00892681_m1), for lactate dehydrogenase LDHA (Hs03405707_g1); for MCTs MCT1 (Hs00161826_m1), MCT2 (Hs04332706_m1) and MCT4 (Hs00358829_m1); and for 5'-AMP-activated protein kinase catalytic subunit alpha-1 PRKAA1 (Hs01562315_m1). Milli-Q water was used as a no-template control to check for extraneous nucleic acid contamination.

Protein extraction for Western blot

To extract proteins for Western blotting, the snap-frozen samples were first grinded in liquid nitrogen using mortar and pestle. The resulting powder was transferred into 2 mL Lysing Matrix A tubes (RotaPrep) filled with radioimmunoprecipitation assay (RIPA) buffer consisting of 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS and supplemented with a protease inhibitor cocktail (Roche) following the manufacturer's instructions. The sample was homogenized three times for 10 s using the Monolyzer (RotaPrep) at maximum speed. Subsequently, the homogenates were maintained under constant agitation for 2 hours at 4°C, followed by centrifugation for 20 min at 16 000 x *g* at 4°C. The resulting tissue extracts were collected, aliquoted, and stored at −80 °C until protein quantification using BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's protocol.

Western blot

Proteins (30 µg) from lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by overnight electroblotting onto Immobilon® -P PVDF membranes, pore size 45 µm (Merck Millipore); blocking for 30 min with phosphate buffered saline containing 0.05% Tween 20 (PBS-T) and 5% BSA, at room temperature. Next, the blot was incubated with primary antibodies (Table 1) for 2 h at 4 °C in PBS-T with 2% BSA, washed with PBS-T and incubated with secondary antibodies using either goat anti-rabbit IgG (H+L)-horse radish peroxidase (Invitrogen) diluted 1:2000 or 1:4000, or rabbit anti-mouse IgG H&L -HRP (Abcam) diluted 1:5000 for 1 h at room temperature.

Table 1: Antibodies used for Western blot analysis

Protein	Catalog number	Manufacturer	Dilution	Host species
HK1	PA5-117986	Invitrogen	1:1000	Rabbit
HK2	PA5-29326	Invitrogen	1:2500	Rabbit
LDHA	PA5-27406	Invitrogen	1:2000	Rabbit
MCT1	PA5-72957	Invitrogen	1:500	Rabbit
MCT2	Sc-50322	Santa Cruz Biotechnology	1:500	Rabbit
MCT4	Sc-367101	Santa Cruz Biotechnology	1:500	Mouse
AK1	Sc-365316	Santa Cruz Biotechnology	1:500	Mouse
AK2	PA5-28611	Invitrogen	1:500	Rabbit
AK4	PA5-61978	Invitrogen	1:500	Rabbit
AK6	10544-1-AP	Proteintech	1:200	Rabbit

Chemiluminescence was detected using the Super Signal™ West Femto Maximum Sensitivity substrate (Thermo Fisher Scientific) and imaged with Biospectrum multispectral imaging system. Protein levels were quantified using ImageJ software. The unspecific background signal was subtracted, and the area for each protein blot was determined. The levels of protein of interest were normalized to total protein levels obtained from Ponceau S staining signal. The relative quantity of target in each sample was assessed by comparing normalized target quantity in each sample to normalized target quantity in the reference sample.

Cell Culture

The colorectal adenocarcinoma cell line Caco-2 (ATCC) was cultured in 100 mm diameter Falcon Corning cell culture dishes using Dulbecco's Modification of Eagle's Medium (Corning, 10-013-CV), supplemented with 4.5 g/L glucose, 584 mg/L L-glutamine, and 110 mg/L sodium pyruvate, along with 1% 100x penicillin/streptomycin solution (Capricorn Scientific) and 10% fetal bovine serum (FBS Xtra, sourced from South America, Capricorn Scientific). Cell aliquots stored in liquid nitrogen, were unfrozen for cell growth. Culturing was conducted in a CO_2 incubator maintained at 37°C with 95% air/5% CO_2. Sub-culturing of cells was performed every two days by trypsinization using 1x trypsin-EDTA 0.5% solution (Capricorn Scientific) in DPBS (Dulbecco's Phosphate-Buffered Saline, without calcium and magnesium, Corning).

Subsequently, for harvesting, the cell suspensions were centrifuged at 125 x *g* for 5 min, and the supernatant discarded. Then, cell number was determined using a Bürker-Türk counting chamber; aliquots of $17x10^6$ cells were resuspended in 1 mL of DPBS in 2 mL Eppendorf tubes, and centrifuged as described previously. Following centrifugation, supernatants were discarded, and cell pellets were frozen in liquid nitrogen and stored at -80°C for further analysis.

Enzyme activities

To extract proteins for enzyme activity determinations from intact and saponin-treated human tissue samples (10-100 mg semidried weight) and CaCo2 cells (17 x 10^6), the samples were resuspended in 1 mL of 25 mM Tris-HCl buffer pH 7.6, with 1 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 0.1-0.3% Triton X-100 and 20x dilution of protease inhibitor mix (Roche). The samples were homogenized in a Retsch MM400 ball mill homogenizer with 2 metal beads (3 mm diameter) for 2.5 min at 30 Hz. The homogenized samples were then subjected to liquid nitrogen freezing and warm water bath thawing for 2-3 cycles to further facilitate breaking of cells and membranes. After each procedure step, strong vortexing for 1 min was applied to the cell or tissue suspensions. The samples were then centrifuged at 5000 x *g* for 1 min at 4°C followed by recovering the supernatant and keeping it on ice for immediate enzymatic activity assays. Total protein levels were determined with the BCA protein assay kit (Thermo Fisher Scientific) according to the manufacturer's protocol.

Activities of HK and LDH were determined spectrophotometrically as described before (6) by following the NADP+ reduction and NADH oxidation absorbance at 340 nm, respectively, using a spectrophotometer Cary Bio 100, Varian) at 37°C

Assay for LDH was carried out in 1 mL KME buffer (120 mM KCl, 20 mM MOPS, 1 mM EGTA, pH 7.2) including 0.2 mM NADH, 10-40 μg protein supernatant sample. The reaction was started by adding 1 mM pyruvate or increasing concentrations of pyruvate.

HK assay was carried out in 1 mL KME buffer with 0.6 mM NADP+, 10 mM $MgSO_4$, 1-2 units Glc6PDH, 50-200 μg protein supernatant. 10 mM ATP was added seconds before starting the reaction to avoid unspecific ATP hydrolysis by ATPases in the biological samples. The reaction was started by adding 2 mM glucose or increasing concentrations of glucose.

For the calculations of kinetic parameters (V_{max}, K_m, V_{max}/K_m), all the enzyme activities normalized to protein content at variable substrate concentrations were fitted to the Michaelis-Menten equation by non-linear regression analysis using SigmaPlot 14.0 (Copyright © 2017 Systat Software, Inc.) and using a NAD(P)H extinction coefficient of 6.22 mM^{-1} cm^{-1} at 340 nm and under initial-rate conditions.

Sample preparation for respirometry

Upon arrival to the laboratory (within 60 min after surgery), the tissue samples were placed into pre-cooled (4 °C) medium A (3 mM KH_2PO_4, 20 mM taurine, 5.7 mM ATP, 15 mM phosphocreatine (PCr), 9.5 mM $MgCl_2$, 49 mM MES, 7.23 mM K_2EGTA, and 2.77 mM $K_2CaEGTA$, pH 7.1). Blood vessels and fat were removed from the tissue samples, which were then dissected into small samples (5-15 mg). These were permeabilized in medium A containing 50 µg saponin/mL for 30 min at 4 °C under 360° rotatory mixing. The permeabilized samples were then washed three times for 5 min in

pre-cooled Mitomedium B without leupeptin and kept at 4°C until use in oxygraphic analysis.

Oxygraphic measurements

The mitochondrial respiration of permeabilized tissue samples was measured in Mitomedium B at 25 °C using a high-resolution respirometer Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria). The medium was supplemented with 5 mM glutamate, 2 mM malate, and 10 mM succinate to fully activate respiratory chain complexes 1 and 2. To determine the relationship between respiration rate and exogenous ADP, increasing concentrations of ADP were added into the medium in the oxygraphic chamber. The collected data were then plotted as rates of O_2 consumption or OxPhos (the basal respiration rate of respiration attained in the absence of added ADP was subtracted) *versus* ADP concentration. From these plots, the apparent affinity of OxPhos for exogenous ADP (Km_{ADP}) and maximal respiration level (V_{max}) values were calculated by nonlinear regression using Michaelis–Menten equation. Respirometry medium aliquots were withdrawn to calculate energy charges from adenylate nucleotide quantification.

To activate glycolysis in the samples incubated in the respirometer, 0.1 mM of ATP and 10 mM of glucose were added after glutamate, malate and succinate followed by addition of 1 mM of ADP. To inhibit glycolysis 6 - 20 mM 2-deoxyglucose and 0.5 mM iodoacetate were added before ADP and to inhibit OxPhos 2.5 µM of rotenone, 10 µM of antimycin-A and 2 µg/ml oligomycin were added before ADP.

Adenine nucleotide quantification

2 mL of respiration media from both tumor and control tissue incubations were collected from the oxygraph chambers after the experiments and processed immediately or kept in -80 °C for no longer than 2 weeks before processing. To precipitate proteins, 70% $HClO_4$ was added to the samples to 0.6 M final concentration followed by centrifugation at 17000 x *g* for 10 min at 4°C. The supernatant was collected and neutralized with 2 M $KHCO_3$ (120-600 µL depending on sample) and spun again after which the supernatants were either directly loaded into a UPLC apparatus for measurements, or freeze-dried and resuspended in desired volume and solvent and kept at -20 °C for up to few weeks, before analysis.

Separation and quantification of adenine nucleotides was carried out with an Agilent 1290 Infinity UPLC apparatus using a reverse-phase column Tessek Separon SGX C18 5 µm 3 x 150 mm. The samples were eluted as described before (14). The concentrations of the nucleotides in neutralized oxygraphic samples were calculated from the peak areas accounting for all dilution factors and normalized to wet weight of tissue. The energy charge (EC) was calculated using the formula:

EC=[ATP]+0.5x[ADP] / [ATP]+[ADP]+[AMP].

Data analysis

Data in text and figures are presented as mean ± standard error (SEM). All plots were made by using SigmaPlot 14.0. The results were analyzed by using one way ANOVA and p-values <0.05 were considered significant.

3. Results

3.1 Glycolysis proteins

3.1.1 Glucose transporters

Glucose enters the cells via glucose transporters (GLUTs); among the 14 members of the GLUT family, alterations of GLUT1 have been predominantly shown in various types of tumors (15-17). To investigate whether glucose transport gene transcription could be affected in CRC cells, the transcript levels of the *SLC2A1* gene, which encodes for GLUT1 protein, were assessed using RT-qPCR. The results revealed a more than twofold increase in transcript levels in tumors compared to control colon tissue (Figure 1A), suggesting a higher content of GLUT1 protein and a heightened glucose uptake by cancer cells.

3.1.2 Hexokinases

Hexokinase may couple with VDAC in the outer mitochondrial membrane, thereby modulating adenine nucleotides permeability. The transcript levels of one of the main controlling steps of glycolysis, catalyzed by *HK1* and *HK2* were evaluated (Figure 1B). While *HK1* transcript remained similar in both tissue types, *HK2* expression was 35% lower in CRC tumors. This observation supports our earlier findings showing lower *HK2* expression in CRC compared to healthy colon tissue (18, 19).

To further explore changes in HKs, their protein levels were compared. No significant differences were observed between the CRC and control tissue for each isoform (Figure 1C); however, it is important to note that HK2 was detected in only four tumor samples out of nine (44%) examined, supporting the notion of HK2 downregulation in CRC. Finally, HK activity assays were carried out in both intact and saponin-treated control and tumor tissue, as well as in Caco-2 cells (Figure 1D). However, there were no significant HK activity differences between permeabilized control tissue and tumor intact or permeabilized tissue, and CaCo cells.

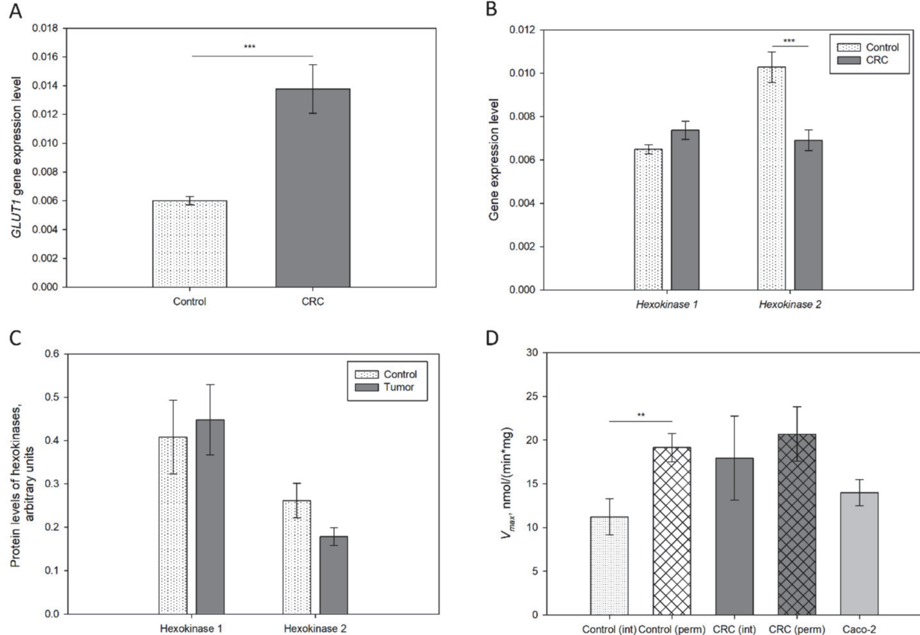

Figure 1: The characteristics of glucose transporter 1 (GLUT1) and hexokinases (HKs) in colorectal tumor tissue. The transcript levels of (A) *GLUT1* and (B) *HK1* and *HK2* were determined by RT-qPCR in control colon tissue (n=24) and CRC tissue (n=24). (C) The protein levels of HK1 and HK2 measured by Western blot in control (n=8 and n=7, respectively) and CRC tissues (n=8 and n=4, respectively). (D) HK activity in intact control tissue (Control (int), n=7), permeabilized control tissue (Control (perm), n=12), intact colorectal cancer tissue (CRC (int), n=7), permeabilized colorectal cancer tissue (CRC (perm), n=12), and Caco-2 cells soluble clarified cell extract (n=4). ** $p<0.01$, *** $p<0.001$ (one-way ANOVA).

3.1.3 Lactate dehydrogenase

To further explore whether CRC has the capability to exhibit an enhanced glycolysis the expression level of the gene encoding lactate dehydrogenase (*LDHA*) was measured. LDHA converts pyruvate into lactate in the cytosol, thus its activity in this compartment may modulate whether pyruvate is preferentially directed toward mitochondria or transformed to lactate in the cytosol. The transcript level of *LDHA* was higher in CRC compared to control tissue (Figure 2A).

Although LDHA protein levels appeared generally higher in tumor tissue, no statistically significant difference was apparent (Figure 2B), due to considerable heterogeneity among clinical samples and a small sample size. Notwithstanding, LDH activity showed significant differences between different tissue types (Figure 2C). Notably, a two-fold increase in LDH activity of intact tumor *vs.* intact control samples was observed, suggesting higher potential of pyruvate processing in CRC. In contrast, no difference in LDH activity between permeabilized tumor and permeabilized control samples was attained. LDH activity in Caco-2 cells was remarkably higher, exceeding that of the clinical samples by approximately 25%, with significant differences observed across all groups.

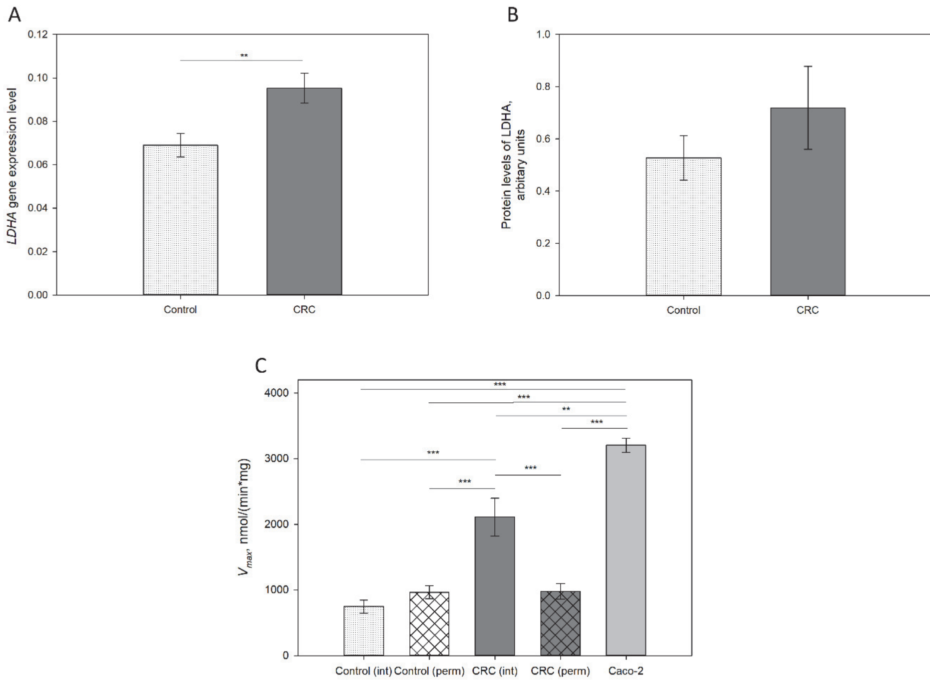

Figure 2: The characteristics of lactate dehydrogenase (LDH) in colorectal tumor. (A) The transcript level of *LDHA* measured by RT-qPCR in control colon tissue (n=24) and CRC tissue (n=24). (B) The protein level of LDHA measured by Western blot in control colon tissue (n=9) and CRC tissue (n=9). (C) LDH activity in intact control tissue (Control (int), n=7), permeabilized control tissue (Control (perm), n=13), intact colorectal cancer tissue (CRC (int), n=7), permeabilized colorectal cancer tissue (CRC (perm), n=13) and Caco-2 cells (n=4). ** p<0.01, *** p<0.001 (one-way ANOVA).

3.1.4 Monocarboxylate transporters

The MCT family consists of 14 transmembrane proteins but only MCT1, MCT2, MCT3, and MCT4 exhibit high affinity for lactate. Therefore, the transcript levels of *SLC16A1, SLC16A7* and *SLC16A3*, encoding for *MCT* 1, 2 and 4 isoforms, respectively, were analyzed (Figure 3A). While the transcripts of *MCT2* and *MCT4* were similar between control and tumor tissues, *MCT1* transcript in cancer was two-fold lower than in control colon tissue. The transcript pattern did not agree with the protein level pattern of MCT1 and MCT4, where MCT1 protein levels were nearly identical between control and CRC tissues, whereas the protein levels of both MCT2 and MCT4 were significantly decreased in CRC (Figure 3B).

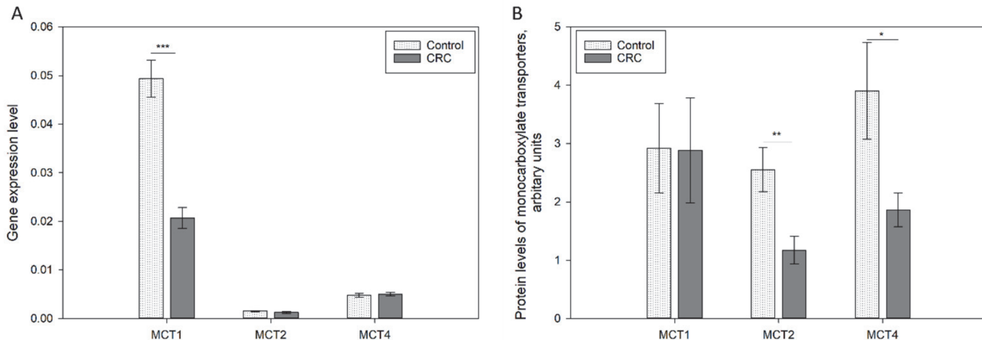

Figure 3: The characteristics of monocarboxylate transporters (MCTs) in colorectal tumor tissue. (A) transcript levels (n=24) and (B) protein levels (n=9) of monocarboxylate transporters 1, 2, and 4 in control colon tissue and colorectal cancer tissue. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (one-way ANOVA).

3.2 Energy Transfer Pathways

Alterations in CK system have been observed in many cancer types (20), including CRC. The transcript levels of three CK isoforms were determined via RT-qPCR (Figure 4A). The results demonstrated significantly decreased levels of *CKBB* and *CKMT1* in CRC compared to control tissue. This last observation was in agreement with previous studies from our group showing that the CKs activity decreases approximately two-fold in CRC compared to normal colon tissue (19). Interestingly, the expression of the second mitochondrial isoform *CKMT2* was higher in CRC tissue, although its overall expression remains low compared to other isoforms.

Downregulation of CKs could be compensated by upregulating the AK system. In this regard, it has been shown that the AKs activity largely increases in CRC compared to normal colon tissue (19). AKs catalyze the reversible transfer of phosphate groups

between ADP, AMP, and ATP. Nine isoforms of AKs have been identified and characterized in mammalian tissues so far (21). Four isoforms were analyzed – cytosolic AK1, mitochondrial AK2 and AK4, and nucleus-located AK6. Contrary to the hypothesis, cancer cells did not show any increased transcripts of *AK*s (Figure 4B). Furthermore, while no statistically significant differences were observed in protein levels of AK isoforms (Figure 4C), there appears to be a trend of lower AK1 levels in cancer tissue, supporting the similar finding in transcript levels.

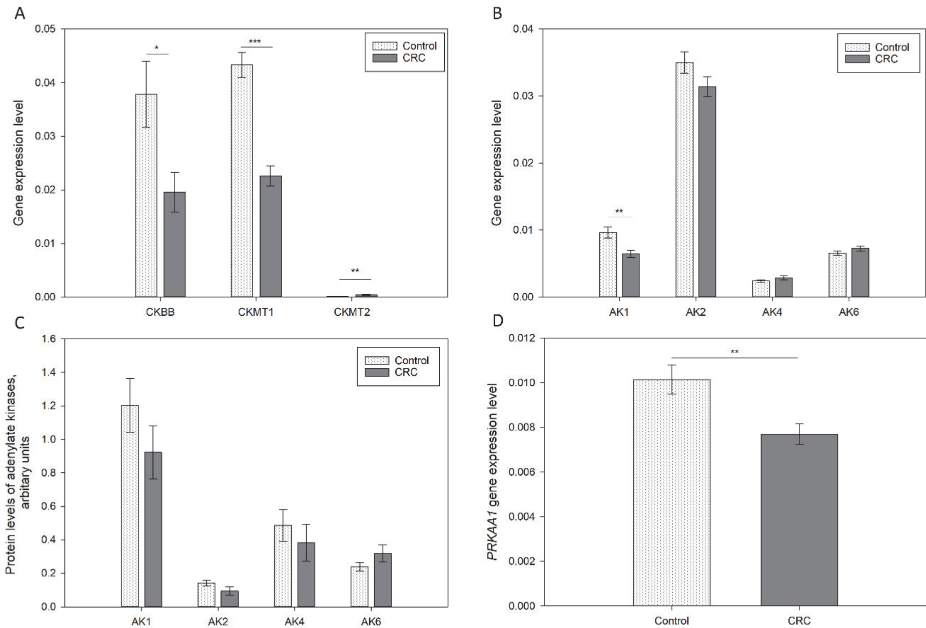

Figure 4: The characteristics of energy transfer pathways in colorectal tumors.

The transcript levels of (A) creatine kinase isoforms *CKBB, CKMT1,* and *CKMT2* and (B) adenylate kinase isoforms *AK1*, *AK2*, *AK4*, and *AK6* in control colon tissue (n=24) and CRC tissue (n=24). (C) The protein levels of adenylate kinase isoforms AK1, AK2, AK4, and AK6 in control colon tissue (n=8) and CRC tissue (n=8). (D) The transcript level of *5'-AMP-activated protein kinase catalytic subunit alpha-1* (*PRKAA1*) in control colon tissue (n=24) and CRC tissue (n=24). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (one-way ANOVA).

In addition to AKs, another central regulator of cellular energy homeostasis is AMP-activated protein kinase (AMPK). AMPK is activated by low ATP and high AMP concentrations, conditions that could be caused by nutrient deprivation, hypoxia, or

oxidative stress. Once activated, AMPK shifts metabolism towards catabolism by phosphorylating proteins in multiple pathways (22). Its role in cancer is still unclear. Here, the transcript level of *5'-AMP-activated protein kinase catalytic subunit alpha-1* (*PRKAA1*) was assessed and shown to be downregulated (Figure 4D) suggesting that in colon cells AMPK may act as tumor suppressor.

3.3 Oxidative phosphorylation

Next, high-resolution respirometry was applied to explore the possible reprogramming of metabolism in CRC by analyzing the kinetics of the OxPhos flux: the values for maximal ADP-stimulated respiration (V_{max}), and the apparent Michaelis-Menten constant for exogenously added ADP (Km_{ADP}) were determined.

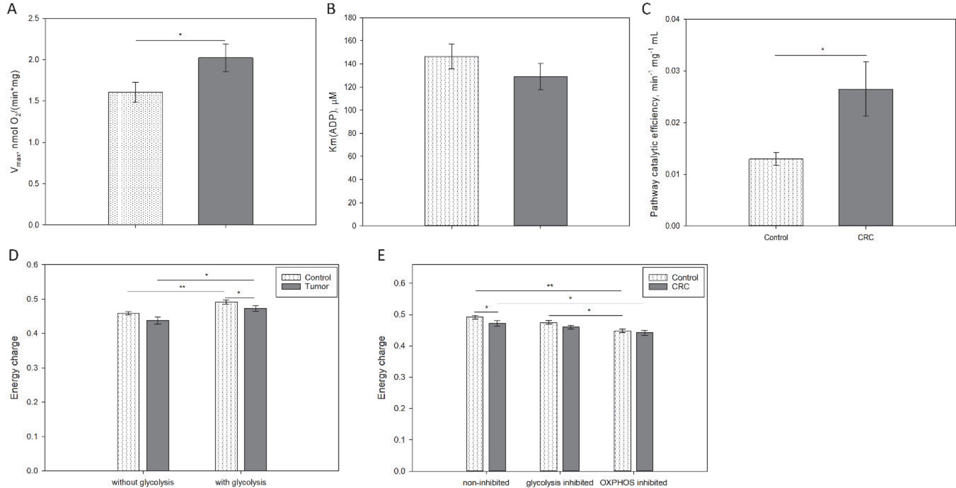

Figure 5: **The functional characteristics of oxidative phosphorylation in colorectal tumors.** Comparative analysis of (A) maximal ADP-stimulated respiratory rate (V_{max}) and (B) apparent Michaelis-Menten constant values for ADP (Km_{ADP}) in control tissue (n=29) and CRC tissue (n=29). (C) The catalytic efficiency of OxPhos pathway represented by V_{max}/Km_{ADP} ratio was calculated from paired samples. (D) Energy charges of control and tumor tissue without (n=15) and with activated glycolysis (n=16), and (E) energy charges of non-inhibited (n=16), glycolysis inhibited (n=17) and OxPhos inhibited (n=10) states in control and tumor tissue. * $p<0.05$, ** $p<0.01$

A statistically significant difference in V_{max} values between control and CRC tissue was found (Figure 5A), but no differences in Km_{ADP} values (Figure 5B). The higher maximal ADP-stimulated respiration rate in CRC suggests an increased OxPhos capacity.

Further, the catalytic efficiency (V_{max}/Km_{ADP}) ratio) of the whole OxPhos pathway (Figure 5C) for control was 0.013 and for CRC was 0.026 min^{-1} mg^{-1} mL, indicating a more catalytically efficient system in tumor tissue.

3.4 Energy charge

Analysis of the adenine nucleotides concentrations revealed that both control and CRC samples experienced a small increase in ECs when glycolysis was active (Figures 5D, 5E). This suggested that reliance on glycolysis indeed affected cell energy status in healthy and cancerous tissue. In contrast, the ATP/ADP ratio (0.2-0.25 value range) showed no differences between tumor and control tissue, with or without glycolysis activated (data not shown).

However, EC in control tissues was significantly impacted by OxPhos inhibition compared to the non-inhibited state (Figure 5E), as well as the ATP/ADP ratios (from 0.23 down to 0.17; data not shown), indicating a substantial reliance on OxPhos for maintaining energy homeostasis in non-cancerous cells. CRC tissues also showed a significant decrease in EC (and ATP/ADP ratios from 0.25 to 0.15) with OxPhos inhibition, also suggesting OxPhos-dependent energy production in cancer cells. A significant decrease was noted in the control group when comparing glycolysis inhibition to OxPhos inhibition, highlighting the distinct contributions of these metabolic pathways to cellular energy status. In contrast, CRC tissues did not demonstrate a significant change under the same conditions, indicating a more pronounced metabolic flexibility or a compensatory mechanism enabling energy maintenance when glycolysis is inhibited.

3.5 OxPhos and Energy charges under CRC progression

Searching for possible prognostic markers for aggressive cancer OxPhos flux of CRC cells was analyzed at different disease stages. When comparing V_{max} and Km_{ADP} values (and ATP/ADP ratios) across different CRC stages, no significant intergroup differences were observed. However, a close resemblance in V_{max} values was noted between control tissues and stage II CRC (Figure 6A). In addition, Km_{ADP} values for stage II CRC were higher than those of other stages (Figure 6B), suggesting a slight decrease in OxPhos activity specifically in stage II of CRC (lower activity and lower affinity for substrate). The OxPhos catalytic efficiencies at different CRC stages were clearly greater, 0.025 for stage 1, 0.031 for stage 2, and 0.021 for stage 3, than in the control colon tissue of 0.013.

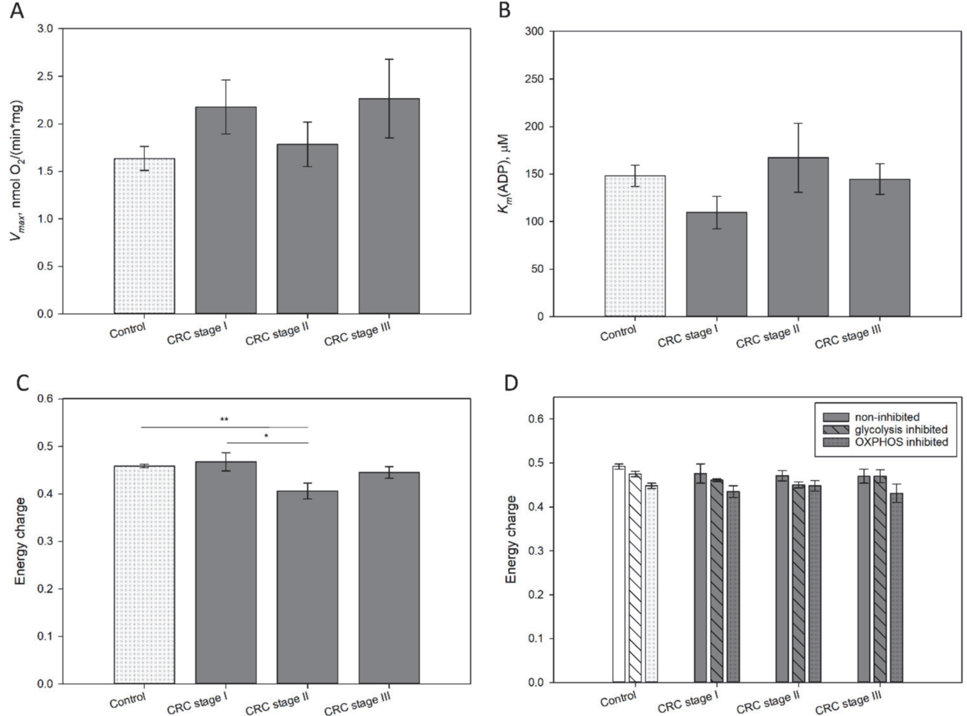

Figure 6: The comparison of oxidative phosphorylation and energy charges between different colorectal cancer stages.

(A) Maximal ADP-induced respiration rate (V_{max}) and (B) apparent Michaelis-Menten constant values for exogenously added ADP (Km_{ADP}) in control and tumor stages I-III. (C) Energy charges of control tissue and tumor stages I-III without glycolysis activation (n=17) and (D) energy charges of tumor stages under non-inhibited (n=16), glycolysis inhibited (n=17) and OxPhos inhibited (n=10) conditions. * $p<0.05$, ** $p<0.01$

Regarding EC without glycolysis activation (Fig. 6C or D), control tissues showed significantly higher values compared to stage II CRC, and there was a notable decrease between stage I and stage II CRC, pointing to a decrease in energy charge as the tumor progresses from stage I to stage II.

Finally, EC evaluations across control and CRC stages I-III, under various metabolic conditions —non-inhibited, glycolysis-inhibited, and OxPhos-inhibited— revealed no significant differences between the groups (Figures 6D, 6E). The lack of significant differences could be attributed to the limited sample sizes available for each CRC stage. This limitation might mask subtle variations in EC that could otherwise be indicative of

stage-specific metabolic alterations. Hence, a larger cohort may be necessary to accurately discern any differences in EC associated with the progression of CRC. Despite these, a similar pattern of EC dependence was observed in control and stage I tissues, whereas in stage II and III the pattern of changes seems different to control.

3.6 OxPhos and Energy charges Profiles of CRC Patients

Previous work showed that CRC patients who had succumbed to the disease presented significantly higher OxPhos V_{max} values (23). This analysis was performed with data on 32 patients. Here, additional disease progression data for all patients after 2017 were gathered and a long-term disease progression analysis was conducted. Among the cohort of 57 patients, a total of 12 fatalities were observed. Out of these 12 patients, 10 had initially been diagnosed with a stage 0-II CRC and 2 with stage III-IV CRC. It is important to note that the very small number of stage III-IV patients is because we only collected samples from patients who had not received any systemic therapy before surgery. Patients with advanced disease mostly require some systemic therapy to achieve a resectable tumor size.

This updated analysis showed again a significantly lower average V_{max} value for alive patients compared to patients who had succumbed to the disease (Figure 7A). In addition, a higher average Km_{ADP} value was noted amongst the alive patients (Figure 7B). The catalytic efficiency for the alive group was 0.012 whereas for the lethal groups was 0.042-0.043, indicating a more efficient OxPhos in the latter patients.

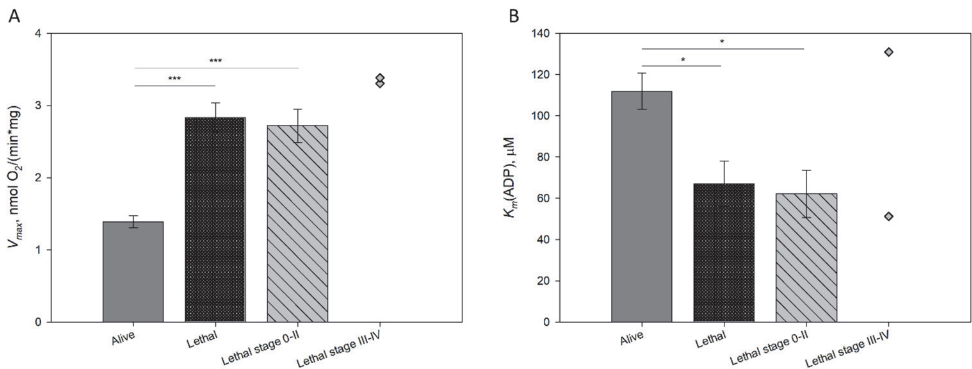

Figure 7: The comparison of (A) maximal ADP-induced respiratory rate (V_{max}) and (B) apparent Michaelis-Menten constant for exogenously added ADP (Km_{ADP}) in patients that are alive (n=45) or have passed away (n=12; stage 0-II n=10 and stage III-IV n=2) since their tumor samples were collected. * p<0.05, *** p<0.001

Discussion

Glycolysis

One crucial enzyme involved in cancer energy metabolism is hexokinase (HK). HK is one of the main rate-controlling steps of cancer glycolysis (24). Moreover, HK2 has been proposed as an independent prognostic factor of CRC (25). Coupling between VDAC and HK2 has been proposed to promote the Warburg effect by channeling mitochondria-generated ATP preferentially towards glycolysis (26). This interaction may render numerous advantages to cancer cells such as increased permeability of the mitochondrial outer membrane (MOM) to adenine nucleotides, elevated levels of aerobic glycolysis, and enhanced resistance to apoptosis. However, co-localization of HK2 with VDAC has been detected in both normal colorectal mucosa and CRC (27, 28). Thus, it is likely that the frequency and abundance of the HK-VDAC interaction depends on the cancer cell type.

However, the changes observed in the present study on the transcript and protein levels of two HK isoforms in colorectal tumor and control colorectal tissue samples were not reflected in changes in whole HK activity. Interestingly, permeabilized control tissue showed higher activity than intact control. Upon permeabilization, cytosolic low molecular weight biomolecules are removed, and presumably only cytosolic proteins like glycolytic enzymes and organelles are left intact. In contrast, tissues that have not undergone permeabilization are expected to contain more peptides and proteins in the cytosol. Then, permeabilized tissues may retain their native active HK isoforms on a diminished overall protein background, thereby exhibiting increased overall enzyme activity *per* mg protein.

Lactate dehydrogenase (LDH) together with the monocarboxylate transporters (MCTs) catalyze the production and transport of lactate, respectively (29). LDH and MCTs are pivotal players in the "lactate shuttle" hypothesis, which involves transporting lactate from highly glycolytic tissues (*e.g.*, muscles) to other organs like the liver or heart for energy production or conversion back to glucose. Elevated LDH levels are often observed in the bloodstream of individuals with colon cancer (30-32). On the other hand, MCTs facilitate the transport of monocarboxylates, glycolytic intermediates like lactate, pyruvate, and short-chain carboxylic acids across the plasma cell membrane (33). In colon tissue, the most important isoform is MCT1 which is primarily responsible for the uptake of butyrate and lactate, and MCT4 which removes lactate produced by glycolysis. The release of lactate into the tumor microenvironment contributes to an acidic pH, fostering tumor growth, invasion, and metastasis. The lactate shuttle facilitated by MCTs may serve as an energy source for metastatic cancer cells in distant organs.

The permeabilization process, which presumably preserves high molecular weight proteins and mitochondria while removing cytosolic low-molecular weight contents, resulted in substantial decrease in LDH activity in permeabilized tumor samples

compared to their intact counterparts. This observation suggested loosely bound LDH to intracellular components like probably microtubules in tumor cells. In addition, the results showed increased transcription of *LDHA* gene together with a trend of higher LDHA protein levels and elevated LDH activity in cancer tissue, suggesting an increased lactate production capability in CRC compared to healthy colon tissue. The higher LDH activity in CaCo2 cells underscored a fundamental difference between cancer cell culture models and actual human tumors.

The metabolism of healthy colon is strongly influenced by the microbiome. While most of the cells in the human body use carbohydrates and fatty acids as the main energy source, colonocytes preferentially consume bacteria-produced butyrate (34). In normal colonocytes, butyrate undergoes β-oxidation, producing acetyl-CoA which subsequently enters the TCA cycle, thus making OxPhos the primary pathway for ATP production. Given that butyrate is primarily transported into the cell via MCT1, its higher transcript level and high protein content lead colon cells to partly depend on short-chain carboxylic acids supply. The higher protein content of MCT1, over those of MCT2 and MCT4, in CRC suggested an increased dependence on such short-chain carboxylic acids supply, which may be linked to increased acetate metabolism in CRC cells (35). The discrepancy between MCT gene and protein levels may be related to their different regulation mechanisms and cautions on over-interpretations of their physiological meaning, suggesting direct determination of activity (11).

We previously demonstrated a substantial decrease in *MCT2* transcript in CRC while *MCT4* transcript was higher in CRC compared to control tissue (18). The current study did not show a similar outcome at the level of mRNA. However, the decreased MCT2 protein level correlated with prior results. MCT2 and MCT4 have been shown to translocate to mitochondria in breast cancer cell lines (36). Therefore, it is possible that the low protein levels presented here are due to our protein extraction protocol not being able to extract proteins from mitochondrial membranes. This hypothesis requires further experimentation. Nevertheless, it is evident that MCTs are dysregulated in cancer tissue.

Energy Transfer Pathway

A highly organized and efficient phosphoryl transfer system is essential to mediate intracellular communication between ATP-consuming and ATP-producing cellular processes for cell maintenance, growth and differentiation. During tumorigenesis, cells develop specific expression profiles of adenylate kinase (AK) and creatine kinase (CK) isoforms that correlate with their oncometabolic phenotype (27, 37). The CK system includes the mitochondrial (CKMTs) and cytosolic isoforms (27, 38). The AK family includes nine isoforms, each exhibiting distinct intracellular localizations and functional properties (21, 39, 40). Similar to CK, the primary role of the AK system is to maintain the ATP/ADP ratio across various intracellular compartments (41). AKs act as metabolic

regulators in both glycolysis and OxPhos, facilitating the interconversion of adenine nucleotides (ATP, ADP, AMP), thereby ensuring a consistent and adequate ATP supply to fulfill cellular energy demands.

It has been demonstrated that cytosolic CK, associated with glycolytic enzymes, may support the Warburg effect by maintaining ATP homeostasis at glycolytic sites (42). However, changes in MOM permeability can influence the interplay between CKMTs and adenine nucleotide translocase. Growth of CRC is associated with upregulation of the AK system and, in parallel, with a decrease in total CK and CKMT activities (43). Indeed, downregulation of the CK genes of colorectal tumors was found, which was accompanied by similar AK transcripts and protein levels than control colorectal tissue.

Furthermore, there is a significant increase in the coupling of mitochondrial AK with OxPhos in CRC (10, 44). Oxidative stress, accompanied by a decline in ATP levels and ADP increase (and hence decreased ATP/ADP ratios) induce increased AK activity leading to an elevation in intracellular AMP. In turn, AMP acting as a secondary messenger may activate the energy stress-responsive AMP-dependent protein kinase (AMPK), that stimulates ATP production through catabolic processes, while inhibiting the ATP-consuming processes involved in growth. The role of AMPK in cancer remains controversial (45). The observed slightly lower AMPK expression in colorectal tumors suggested a slight diminished regulatory role in activating catabolism and inhibiting anabolism.

AMPK has been recognized as a tumor suppressor in certain cancers (46-49) by inhibiting protein synthesis, cell proliferation and growth, since it regulates the mTORC1 pathway. However, AMPK has been also described as a contextual oncogene due to its ability to promote tumor progression, chemoresistance upon activation, and cancer cell survival by maintaining NADPH homeostasis (50-52). AK1 has been shown to have an additional gene product AK1β, the expression of which is regulated by p53 (53). Since *p53* mutation is found in approximately 60% of CRCs (54), this could affect the expression of AK1 or its alternative products. The relationship between *p53* mutation and AK1/AK1β levels should be further investigated on a larger population.

Energy Charge, ATP/ADP ratios and Oxidative Phosphorylation

In 1967, Atkinson and Walton proposed that “energy charge” (EC) derived from the adenine nucleotides contents is a fundamental metabolic control parameter because it provides a quantitative value of the cellular energy state, reflecting the balance between energy availability and demand (55). More recent research has delved into the intricacies of how EC affects cancer cell metabolism and mitochondrial function, suggesting that alterations in EC can disrupt the energy balance in cancer cells, influencing their growth and survival (47). Indeed, colorectal tumors exhibited slightly lower EC values than control tissue. Understanding such metabolic shifts could offer new avenues for cancer treatment by targeting the energy regulation mechanisms (56).

EC values are established to range from 0 to 1, being close to 0 when the adenine nucleotide pool is made of mainly AMP, 0.5 when ADP is the predominant adenine nucleotide, and 1 if ATP is predominant. Previous studies have documented EC values within a narrow range of 0.7 to 0.95, across various organisms and cell types, including liver and muscle cells. These values usually decrease under stressful conditions (57), for instance decreasing from ≥ 0.8 to less than 0.5 under nutrient depletion (58). In contrast, our experiments consistently showed EC values ranging between 0.4 and 0.5. The estimated ATP/ADP ratio values were also lower than those determined for experiments with isolated mitochondria and intact cells and tissues.

The lower and narrower range of EC values and ATP/ADP ratios may be attributed to the use of permeabilized tissues in our experiments, which presumably retain high molecular weight enzymes that are preferentially bound to intracellular structures such as membranes, organelles and microtubules. Thus, leakage of cytosolic proteins (glycolytic enzymes) may be induced by saponin permeabilization, thereby limiting the observable portion of glycolytic activity. In addition, tissue permeabilization may induce increased ATPase activity. Indeed, pooling together data from saponin-treated colorectal tumor and control tissue not subjected to lengthy incubations, revealed ATP/ADP ratios of 0.3 for tumors and 0.2 for control tissues. Future research should include additional experiments using intact tumor and control clinical samples specifically for determination of EC values, ATP/ADP ratios and glycolytic flux.

Our group has previously shown a significant difference in Km_{ADP} values between glycolytic and oxidative muscle tissue (59). The oxidative tissue shows higher Km_{ADP} values indicating a shift in the regulation of the MOM permeability by VDAC. In addition, we have previously demonstrated that while control colon tissue and CRC tend to have similar Km_{ADP} values, which are in agreement with those found in the present study, benign colon polyps showed a significantly lower Km_{ADP} (18), suggesting an early metabolic switch during CRC development.

Moreover, the higher Vmax values and V_{max}/Km_{ADP} ratios in CRC suggested an increased OxPhos capacity and efficiency. These data confirmed the earlier findings (18) suggesting that high OxPhos V_{max} values (and V_{max}/Km_{ADP} ratios) could be a marker for a more aggressive disease. There was one recent CRC patient among the group of alive patients that showed a high V_{max} value of 4.21, which was left out of the calculations as an outlier. However, it could be interesting to follow this patient's disease progression to see if it confirms the hypothesis of high V_{max} being a prognostic marker.

There are several possible explanations to why lethal stage III-IV group did not show a significant difference in Km_{ADP}. Firstly, the group contained only two patients; secondly, advanced stage tumors have grown into nearby tissues including muscle tissue in colon wall meaning that these samples may include some muscle tissue that is characterized by a high Km_{ADP}. As described before, lower Km_{ADP} value could indicate a shift to glycolytic metabolism. High V_{max} may be explained, for example, by advanced vascularization in more aggressive malignant tumors. Interestingly, our previous work

has shown a similar high V_{max} – low Km_{ADP} metabolic profile in colon polyps which are benign growths in colon mucosa (18).

Modern therapeutic strategies for CRC have been standardized for stages I and IV, except for stage II, where the approach remains a subject of considerable debate. Stage II is recognized as a heterogeneous category, with prognoses varying significantly, exhibiting 5-year overall survival rates ranging from 87.5% to 58.4%. In some cases, patients with stage II CRC have a prognosis that is worse than those with low-risk stage III cancer. Standard adjuvant chemotherapy, typically involving 5-fluorouracil and leucovorin, offers a modest improvement in 5-year survival rates, increasing by only 2-5%. The search for more effective therapeutic strategies has led to the exploration of various biomarkers (60). Our results suggested the presence of a critical metabolic anomality within stage II CRC reflecting changes in energetic needs, that warrants further investigation into the specifics of its energy metabolism.

Furthermore, the data suggested that distinct energy metabolism profiles may emerge for the various colorectal tumor stages and their progression from one stage to another. Although the metabolic differences among CRC stages I, II, and III might seem inherently minimal, this may have derived from the limited small sample size examined. Our study did not include cases of high-grade metastatic CRC, which previous research has shown to exhibit a notable increase in EC by approximately 26%, whereas CRC stages I and III have shown negligible changes in EC (61), like the ones described here.

From the results described it is clear that energy metabolism undergoes reprogramming throughout CRC development. We hypothesize that benign colon polyps increase glycolytic activity to support the increased proliferation by mainly suppling anabolic precursors such as Glc6P, Fru6P, DHAP, 3PG, Pyr and helping with the elevated energy demand under hypoxic conditions (8). As the cell number increases and tumor grows in size, the cells become deprived of oxygen and nutrients, consequently, new blood vessels are generated in the tissue to restore the supply. Hanahan and Weinberg have proposed induced angiogenesis as one of the hallmarks of cancer (62). While angiogenesis is usually dormant in adults, it is reinduced in early stages of tumorigenesis to further support the cell growth and proliferation. As the cancer progresses even further, cancer tissue will become more and more heterogeneous. Some cells will persist in hypoxic conditions and rely predominantly on glycolysis, while others, benefiting from sufficient oxygen availability, utilize lactate produced by the former cells as a substrate for ATP production via OxPhos (8, 63, 64).

The data of the present study showed that colorectal human tumors display altered expression, protein contents and enzyme activities of its energy metabolism, including the energy transfer pathway. The contents and activities of some glycolytic proteins, the changes in the energy charge values, and enhanced OxPhos flux and catalytic efficiency suggested that colorectal tumors have highly active glycolysis and OxPhos and hence they have a hybrid energy metabolism phenotype. Moreover, the present study underscores the complex nature of cancer, advocating for an integrated approach

that employs both cell cultures and clinical material. This comprehensive methodology has unveiled instances previously unseen, which might be challenging or even impossible to discern through more conventional or isolated research strategies. The findings highlight the importance of combining diverse experimental models to fully understand the multifaceted biological behaviors of cancer.

Authorship contribution statement

Leenu Reinsalu: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original draft, Visualization. **Sten Miller**: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original draft. **Giuseppe Leonardo Auditano**: Methodology, Formal analysis, Investigation, Writing – Review & Editing. **Marju Puurand**: Investigation, Formal analysis. **Rafael Moreno-Sanchez**: Conceptualization, Writing – Review & Editing, Funding acquisition. **Emma Saavedra**: Writing – Review & Editing. **Vahur Valvere**: Methodology, Resources. **Tuuli Käämbre**: Conceptualization, Writing – Review & Editing, Supervision, Funding acquisition.

Conflict of interest disclosure

The authors confirm that there are no conflicts of interest.

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Ethics statement

The studies involving human participants were reviewed and approved by Research Ethics Committee of the National Institute for Health Development in Estonia (decision numbers KK557 and KK558) and followed the Helsinki Declaration and Convention of the Council of Europe on Human Rights and Biomedicine. All participants were informed about the study and provided their written informed consent to participate in the study.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon request.

References

1. Cancer. In, World Health Organization (2022)
2. U.S Cancer Statistics Colorectal Cancer Stat Bite. In, Centers for Disease Control and Prevention (2023)
3. R. Udo, K. Katsumata, H. Kuwabara, M. Enomoto, T. Ishizaki, M. Sunamura, Y. Nagakawa, R. Soya, M. Sugimoto and A. Tsuchida: Urinary charged metabolite profiling of colorectal cancer using capillary electrophoresis-mass spectrometry. *Scientific Reports*, 10(1), 21057 (2020) doi:10.1038/s41598-020-78038-2
4. F. Di Cesare, A. Vignoli, C. Luchinat, L. Tenori and E. Saccenti: Exploration of Blood Metabolite Signatures of Colorectal Cancer and Polyposis through Integrated Statistical and Network Analysis. In: *Metabolites*. (2023) doi:10.3390/metabo13020296
5. D. Hanahan and Robert A. Weinberg: Hallmarks of Cancer: The Next Generation. *Cell*, 144(5), 646-674 (2011) doi:10.1016/j.cell.2011.02.013
6. O. Warburg: On the Origin of Cancer Cells. *Science*, 123(3191), 309-314 (1956) doi:doi:10.1126/science.123.3191.309
7. R. Moreno-Sanchez, S. Rodriguez-Enriquez, A. Marin-Hernandez and E. Saavedra: Energy metabolism in tumor cells. *FEBS J*, 274(6), 1393-418 (2007) doi:10.1111/j.1742-4658.2007.05686.x
8. R. Moreno-Sanchez, A. Marin-Hernandez, E. Saavedra, J. P. Pardo, S. J. Ralph and S. Rodriguez-*Enriquez: Who Controls the ATP Supply in Cancer Cells? Biochemistry lessons to understand cancer energy metabolism. International Journal of Biochemistry & Cell Biology (2014) doi:S1357-2725(14)00042-9 [pii] 10.1016/j.biocel.2014.01.025*
9. *R. Moreno-Sánchez, D. X. Robledo-Cadena, S. C. Pacheco-Velázquez, J. L. Vargas Navarro, J. A. Padilla-Flores and S. Rodríguez-Enríquez: Estimation of energy pathway fluxes in cancer cells - Beyond the Warburg effect. Archives of Biochemistry and Biophysics, 739, 109559 (2023) doi:https://doi.org/10.1016/j.abb.2023.109559*
10. L. Reinsalu, M. Puurand, V. Chekulayev, S. Miller, I. Shevchuk, K. Tepp, E. Rebane-Klemm, N. Timohhina, A. Terasmaa and T. Kaambre: Energy Metabolic Plasticity of Colorectal Cancer Cells as a Determinant of Tumor Growth and Metastasis. *Front Oncol*, 11, 698951 (2021) doi:10.3389/fonc.2021.698951
11. R. Moreno-Sánchez, E. Saavedra, J. C. Gallardo-Pérez, F. D. Rumjanek and S. Rodríguez-Enríquez: Understanding the cancer cell phenotype beyond the limitations of current omics analyses. *The FEBS Journal*, 283(1), 54-73 (2016) doi:https://doi.org/10.1111/febs.13535
12. M. V. Golikov, V. T. Valuev-Elliston, O. A. Smirnova and A. V. Ivanov: Physiological Media in Studies of Cell Metabolism. *Molecular Biology*, 56(5), 629-637 (2022) doi:10.1134/s0026893322050077
13. A. Untergasser: RNAprep - Trizol combined with Columns, 2023(10.05) (2008)
14. E. K. Seppet, T. Kaambre, P. Sikk, T. Tiivel, H. Vija, M. Tonkonogi, K. Sahlin, L. Kay, F. Appaix, U. Braun, M. Eimre and V. A. Saks: Functional complexes of mitochondria with Ca,MgATPases of myofibrils and sarcoplasmic reticulum in muscle cells. *Biochim Biophys Acta*, 1504(2-3), 379-95 (2001) doi:S0005272800002693 [pii]
15. P.-B. Ancey, C. Contat, G. Boivin, S. Sabatino, J. Pascual, N. Zangger, J. Y. Perentes, S. Peters, E. D. Abel, D. G. Kirsch, J. C. Rathmell, M.-C. Vozenin and E. Meylan: GLUT1 Expression in Tumor-Associated Neutrophils Promotes Lung Cancer Growth and Resistance to Radiotherapy. *Cancer Res*, 81(9), 2345-2357 (2021) doi:10.1158/0008-5472.can-20-2870
16. P.-B. Ancey, C. Contat and E. Meylan: Glucose transporters in cancer – from tumor cells to the tumor microenvironment. *FEBS J*, 285(16), 2926-2943 (2018) doi:https://doi.org/10.1111/febs.14577

17. H. Xiao, J. Wang, W. Yan, Y. Cui, Z. Chen, X. Gao, X. Wen and J. Chen: GLUT1 regulates cell glycolysis and proliferation in prostate cancer. *Prostate*, 78(2), 86-94 (2018) doi:https://doi.org/10.1002/pros.23448
18. E. Rebane-Klemm, L. Reinsalu, M. Puurand, I. Shevchuk, J. Bogovskaja, K. Suurmaa, V. Valvere, R. Moreno-Sanchez and T. Kaambre: Colorectal polyps increase the glycolytic activity. *Front Oncol*, 13 (2023) doi:10.3389/fonc.2023.1171887
19. A. Kaldma, A. Klepinin, V. Chekulayev, K. Mado, I. Shevchuk, N. Timohhina, K. Tepp, M. Kandashvili, M. Varikmaa, A. Koit, M. Planken, K. Heck, L. Truu, A. Planken, V. Valvere, E. Rebane and T. Kaambre: An in situ study of bioenergetic properties of human colorectal cancer: The regulation of mitochondrial respiration and distribution of flux control among the components of ATP synthasome. *Int J Biochem Cell Biol*, 55, 171-186 (2014) doi:https://doi.org/10.1016/j.biocel.2014.09.004
20. J. Joseph, A. Cardesa and J. Carreras: Creatine kinase activity and isoenzymes in lung, colon and liver carcinomas. *Br J Cancer*, 76(5), 600-5 (1997)
21. C. Panayiotou, N. Solaroli and A. Karlsson: The many isoforms of human adenylate kinases. *Int J Biochem Cell Biol*, 49, 75-83 (2014) doi:https://doi.org/10.1016/j.biocel.2014.01.014
22. C.-C. Hsu, D. Peng, Z. Cai and H.-K. Lin: AMPK signaling and its targeting in cancer progression and treatment. *Seminars in Cancer Biology*, 85, 52-68 (2022) doi:https://doi.org/10.1016/j.semcancer.2021.04.006
23. A. Koit, I. Shevchuk, L. Ounpuu, A. Klepinin, V. Chekulayev, N. Timohhina, K. Tepp, M. Puurand, L. Truu, K. Heck, V. Valvere, R. Guzun and T. Kaambre: Mitochondrial Respiration in Human Colorectal and Breast Cancer Clinical Material Is Regulated Differently. *Oxid Med Cell Longev*, 2017, 1372640 (2017) doi:10.1155/2017/1372640
24. D. J. Roberts and S. Miyamoto: Hexokinase II integrates energy metabolism and cellular protection: Akting on mitochondria and TORCing to autophagy. *Cell Death Differ*, 22(2), 248-57 (2015) doi:10.1038/cdd.2014.173
25. M. Katagiri, H. Karasawa, K. takagi, S. Nakayama, S. Yabuuchi, F. Fujishima, T. Naitoh, M. Watanabe, T. Suzuki, M. Unno and H. Sasano: Hexokinase 2 in colorectal cancer: A potent prognostic factor associated with glycolysis, proliferation and migration. *Histology and Histopathology*, 32(4), 351-360 (2017) doi:10.14670/HH-11-799
26. P. L. Pedersen, S. Mathupala, A. Rempel, J. F. Geschwind and Y. H. Ko: Mitochondrial bound type II hexokinase: a key player in the growth and survival of many cancers and an ideal prospect for therapeutic intervention. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1555(1), 14-20 (2002) doi:https://doi.org/10.1016/S0005-2728(02)00248-7
27. A. Kaldma, A. Klepinin, V. Chekulayev, K. Mado, I. Shevchuk, N. Timohhina, K. Tepp, M. Kandashvili, M. Varikmaa, A. Koit, M. Planken, K. Heck, L. Truu, A. Planken, V. Valvere, E. Rebane and T. Kaambre: An in situ study of bioenergetic properties of human colorectal cancer: the regulation of mitochondrial respiration and distribution of flux control among the components of ATP synthasome. *International Journal of Biochemistry & Cell Biology*, 55, 171-86 (2014) doi:10.1016/j.biocel.2014.09.004
28. L. Ounpuu, L. Truu, I. Shevchuk, V. Chekulayev, A. Klepinin, A. Koit, K. Tepp, M. Puurand, E. Rebane-Klemm and T. Kaambre: Comparative analysis of the bioenergetics of human adenocarcinoma Caco-2 cell line and postoperative tissue samples from colorectal cancer patients. *Biochem Cell Biol*, 96(6), 808-817 (2018) doi:10.1139/bcb-2018-0076
29. H. Hatami, A. Sajedi, S. M. Mir and M. Y. Memar: Importance of lactate dehydrogenase (LDH) and monocarboxylate transporters (MCTs) in cancer cells. *Health Sci Rep*, 6(1), e996 (2023) doi:10.1002/hsr2.996
30. Z. Xie, H. Zhou, L. Wang and Y. Wu: The Significance of the preoperative lactate dehydrogenase/albumin Ratio in the Prognosis of Colon Cancer: a retrospective study. *PeerJ*, 10, e13091 (2022) doi:10.7717/peerj.13091

31. A. Casadei-Gardini, E. Scarpi, P. Ulivi, M. A. Palladino, C. Accettura, I. Bernardini, A. Spallanzani, F. Gelsomino, J. Corbelli, G. Marisi, S. Ruscelli, M. Valgiusti, G. L. Frassineti and A. Passardi: Prognostic role of a new inflammatory index with neutrophil-to-lymphocyte ratio and lactate dehydrogenase (CII: Colon Inflammatory Index) in patients with metastatic colorectal cancer: results from the randomized Italian Trial in Advanced Colorectal Cancer (ITACa) study. *Cancer Manag Res*, 11, 4357-4369 (2019) doi:10.2147/CMAR.S198651

32. M. Manerba, L. Di Ianni, M. Govoni, M. Roberti, M. Recanatini and G. Di Stefano: Lactate dehydrogenase inhibitors can reverse inflammation induced changes in colon cancer cells. *Eur J Pharm Sci*, 96, 37-44 (2017) doi:10.1016/j.ejps.2016.09.014

33. V. L. Payen, E. Mina, V. F. Van Hee, P. E. Porporato and P. Sonveaux: Monocarboxylate transporters in cancer. *Mol Metab*, 33, 48-66 (2020) doi:10.1016/j.molmet.2019.07.006

34. P. S. Salvi and R. A. Cowles: Butyrate and the Intestinal Epithelium: Modulation of Proliferation and Inflammation in Homeostasis and Disease. In: *Cells*. (2021) doi:10.3390/cells10071775

35. S. Rodríguez-Enríquez, D. X. Robledo-Cadena, J. C. Gallardo-Pérez, S. C. Pacheco-Velázquez, C. Vázquez, E. Saavedra, J. L. Vargas-Navarro, B. A. Blanco-Carpintero, Á. Marín-Hernández, R. Jasso-Chávez, R. Encalada, L. Ruiz-Godoy, J. L. Aguilar-Ponce and R. Moreno-Sánchez: Acetate Promotes a Differential Energy Metabolic Response in Human HCT 116 and COLO 205 Colon Cancer Cells Impacting Cancer Cell Growth and Invasiveness. *Frontiers in Oncology*, 11 (2021) doi:10.3389/fonc.2021.697408

36. R. Hussien and G. A. Brooks: Mitochondrial and plasma membrane lactate transporter and lactate dehydrogenase isoform expression in breast cancer cell lines. *Physiol Genomics*, 43(5), 255-264 (2011) doi:10.1152/physiolgenomics.00177.2010

37. V. Chekulayev, K. Mado, I. Shevchuk, A. Koit, A. Kaldma, A. Klepinin, N. Timohhina, K. Tepp, M. Kandashvili, L. Ounpuu, K. Heck, L. Truu, A. Planken, V. Valvere and T. Kaambre: Metabolic remodeling in human colorectal cancer and surrounding tissues: alterations in regulation of mitochondrial respiration and metabolic fluxes. Biochemistry and Biophysics Reports, 4, 111-125 (2015) doi:10.1016/j.bbrep.2015.08.020

38. U. Schlattner, M. Tokarska-Schlattner and T. Wallimann: Mitochondrial creatine kinase in human health and disease. Biochim Biophys Acta, 1762(2), 164-80 (2006) doi:S0925-4439(05)00133-X [pii] 10.1016/j.bbadis.2005.09.004

39. M. Amiri, F. Conserva, C. Panayiotou, A. Karlsson and N. Solaroli: The human adenylate kinase 9 is a nucleoside mono- and diphosphate kinase. The International Journal of Biochemistry & Cell Biology, 45(5), 925-931 (2013) doi:http://dx.doi.org/10.1016/j.biocel.2013.02.004

40. P. Dzeja and A. Terzic: Adenylate kinase and AMP signaling networks: Metabolic monitoring, signal communication and body energy sensing. *Int J Mol Sci*, 10(4), 1729-72 (2009) doi:10.3390/ijms10041729

41. D. M. Bai, J. F. Zhang, T. T. Li, R. L. Hang, Y. Liu, Y. L. Tian, D. D. Huang, L. L. Qu, X. F. Cao, J. F. Ji and X. F. Zheng: The ATPase hCINAP regulates 18S rRNA processing and is essential for embryogenesis and tumour growth. Nature Communications, 7 (2016) doi:ARTN 12310 10.1038/ncomms12310

42. Y.-B. Yan: Creatine kinase in cell cycle regulation and cancer. Amino Acids, 48(8), 1775-1784 (2016) doi:10.1007/s00726-016-2217-0

43. S. M. Mooney, K. Rajagopalan, B. H. Williams, Y. Zeng, C. S. Christudass, Y. Li, B. Yin, P. Kulkarni and R. H. Getzenberg: Creatine kinase brain overexpression protects colorectal cells from various metabolic and non-metabolic stresses. *J Cell Biochem*, 112(4), 1066-75 (2011) doi:10.1002/jcb.23020

44. A. Klepinin, S. Zhang, L. Klepinina, E. Rebane-Klemm, A. Terzic, T. Kaambre and P. Dzeja: Adenylate Kinase and Metabolic Signaling in Cancer Cells. *Front Oncol*, 10, 660 (2020) doi:10.3389/fonc.2020.00660

45. J. Liang and G. B. Mills: AMPK: a contextual oncogene or tumor suppressor? *Cancer Res*, 73(10), 2929-35 (2013) doi:10.1158/0008-5472.CAN-12-3876

46. W. Wang and K. L. Guan: AMP-activated protein kinase and cancer. *Acta Physiologica*, 196(1), 55-63 (2009) doi:10.1111/j.1748-1716.2009.01980.x
47. B. Faubert, G. Boily, S. Izreig, T. Griss, B. Samborska, Z. Dong, F. Dupuy, C. Chambers, Benjamin J. Fuerth, B. Viollet, Orval A. Mamer, D. Avizonis, Ralph J. DeBerardinis, Peter M. Siegel and Russell G. Jones: AMPK Is a Negative Regulator of the Warburg Effect and Suppresses Tumor Growth In Vivo. *Cell Metabolism*, 17(1), 113-124 (2013)
48. G. Rehman, A. Shehzad, A. L. Khan and M. Hamayun: Role of AMP-Activated Protein Kinase in Cancer Therapy. *Archiv der Pharmazie*, 347(7), 457-468 (2014) doi:10.1002/ardp.201300402
49. D. G. Hardie, Fiona A. Ross and Simon A. Hawley: AMP-Activated Protein Kinase: A Target for Drugs both Ancient and Modern. *Chemistry & Biology*, 19(10), 1222-1236 (2012) doi:http://dx.doi.org/10.1016/j.chembiol.2012.08.019
50. H. U. Park, S. Suy, M. Danner, V. Dailey, Y. Zhang, H. Li, D. R. Hyduke, B. T. Collins, G. Gagnon, B. Kallakury, D. Kumar, M. L. Brown, A. Fornace, A. Dritschilo and S. P. Collins: AMP-activated protein kinase promotes human prostate cancer cell growth and survival. *Mol Cancer Ther*, 8(4), 733-41 (2009) doi:10.1158/1535-7163.MCT-08-0631
51. A. S. Khan and D. E. Frigo: Regulation, Role and Therapeutic Targeting of AMPK in Prostate Cancer. *Nature reviews. Urology*, 14(3), 164-180 (2017) doi:10.1038/nrurol.2016.272
52. X. Huang, X. Li, X. Xie, F. Ye, B. Chen, C. Song, H. Tang and X. Xie: High expressions of LDHA and AMPK as prognostic biomarkers for breast cancer. *The Breast*, 30, 39-46 (2016) doi:https://doi.org/10.1016/j.breast.2016.08.014
53. L. Collavin, D. Lazarevič, R. Utrera, S. Marzinotto, M. Monte and C. Schneider: wt p53 dependent expression of a membrane-associated isoform of adenylate kinase. *Oncogene*, 18(43), 5879-5888 (1999) doi:10.1038/sj.onc.1202970
54. M. Michel, L. Kaps, A. Maderer, P. R. Galle and M. Moehler: The Role of p53 Dysfunction in Colorectal Cancer and Its Implication for Therapy. In: *Cancers*. (2021) doi:10.3390/cancers13102296
55. D. E. Atkinson and G. M. Walton: Adenosine Triphosphate Conservation in Metabolic Regulation: RAT LIVER CITRATE CLEAVAGE ENZYME. *Journal of Biological Chemistry*, 242(13), 3239-3241 (1967) doi:https://doi.org/10.1016/S0021-9258(18)95956-9
56. L. Iommarini, A. Ghelli, G. Gasparre and A. M. Porcelli: Mitochondrial metabolism and energy sensing in tumor progression. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1858(8), 582-590 (2017) doi:https://doi.org/10.1016/j.bbabio.2017.02.006
57. I. M. De la Fuente, J. M. Cortés, E. Valero, M. Desroches, S. Rodrigues, I. Malaina and L. Martínez: On the Dynamics of the Adenylate Energy System: Homeorhesis vs Homeostasis. *PLOS ONE*, 9(10), e108676 (2014) doi:10.1371/journal.pone.0108676
58. F. J. Passman, J. Schmidt and R. P. Lewis: The relationship between microbial community vitality and ATP bioburden in bottom waters under fuel microcosms. *Access Microbiology*, 5(4) (2023) doi:https://doi.org/10.1099/acmi.0.000411
59. A. V. Kuznetsov, T. Tiivel, P. Sikk, T. Kaambre, L. Kay, Z. Daneshrad, A. Rossi, L. Kadaja, N. Peet, E. Seppet and V. A. Saks: Striking Differences Between the Kinetics of Regulation of Respiration by ADP in Slow-Twitch and Fast-Twitch Muscles In Vivo. *Eur J Biochem*, 241(3), 909-915 (1996) doi:https://doi.org/10.1111/j.1432-1033.1996.00909.x
60. P. Parent, R. Cohen, E. Rassy, M. Svrcek, J. Taieb, T. André and A. Turpin: A comprehensive overview of promising biomarkers in stage II colorectal cancer. *Cancer Treatment Reviews*, 88 (2020) doi:10.1016/j.ctrv.2020.102059
61. Z. Teo, M. K. Sng, J. S. K. Chan, M. M. K. Lim, Y. Li, L. Li, T. Phua, J. Y. H. Lee, Z. W. Tan, P. Zhu and N. S. Tan: Elevation of adenylate energy charge by angiopoietin-like 4 enhances epithelial–mesenchymal transition by inducing 14-3-3γ expression. *Oncogene*, 36(46), 6408-6419 (2017) doi:10.1038/onc.2017.244

62. D. Hanahan and R. A. Weinberg: The Hallmarks of Cancer. *Cell*, 100(1), 57-70 (2000) doi:10.1016/s0092-8674(00)81683-9
63. S. J. Ralph, S. Rodríguez-Enríquez, J. Neuzil, E. Saavedra and R. Moreno-Sánchez: The causes of cancer revisited: “Mitochondrial malignancy” and ROS-induced oncogenic transformation – Why mitochondria are targets for cancer therapy. *Molecular Aspects of Medicine*, 31(2), 145-170 (2010)
64. R. Moreno-Sanchez, E. Saavedra, S. Rodriguez-Enriquez, J. C. Gallardo-Perez, H. Quezada and H. V. Westerhoff: Metabolic control analysis indicates a change of strategy in the treatment of cancer. *Mitochondrion*, 10(6), 626-39 (2010) doi:S1567-7249(10)00105-4 [pii] 10.1016/j.mito.2010.06.002

Curriculum vitae

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Education

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2012–2015	Pärnu Koidula Gymnasium
2003–2012	Pärnu Kuninga Street Basic School

Language competence

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Professional employment

2023–…	Swixx Biopharma, Medical Advisor
2020–…	National Institute of Chemical Physics and Biophysics, Junior Researcher
6/2019–8/2019, 6/2020–8/2020	West Tallinn Central Hospital, Lab Technician
3/2020–6/2020	SYNLAB Eesti OÜ, Lab Technician

Honors and awards

2022	Estonia Academy of Sciences' competition "Science in 3 minutes" – Top 5 and the winner of public voting
2018	Estonian Biochemical Society's student research contest – First prize

Conferences

September, 2023	Baltic Congress of Oncologists and Surgeons, Oral presentation: *Energy metabolism is reprogrammed during colorectal cancer development*
November, 2022	XIV Science Conference of the School of Science, Oral presentation: *Dietary preferences of colorectal cancer*
June, 2022	FEBS3+ Conference of Estonian, Latvian and Lithuanian Biochemical Societies, Poster presentation: *Metabolic plasticity supports colorectal cancer*

September, 2021 EMBO workshop “Systems approaches in cancer”, Poster presentation: *Metabolic reprogramming in colorectal cancer and colon polyps*

Public appearances

November, 2022 TV show Uudishimu tippkeskus – episode on cancer and its treatment

May, 2022 TV show Terevisioon – discussing cancer and cancer metabolism

March, 2022 TV show Laser – episode on colon cancer

February, 2022 Radio show Nutikas – discussing cancer and working as a scientist

Supervised dissertations

Janne Jegorkina, Bachelor's Degree, 2023, (sup) Leenu Reinsalu,
The occurrence of genetic and protein changes in the energy metabolism of colorectal cancer, Tallinn University of Technology, School of Science, Department of Chemistry and Biotechnology.

Betty Abel, Bachelor's Degree, 2022, (sup) Leenu Reinsalu,
Rearrangements in energy metabolism promoting the development of colorectal cancer, Tallinn University of Technology, School of Science, Department of Chemistry and Biotechnology.

Publications

Rebane-Klemm, Egle*; **Reinsalu, Leenu***; Puurand, Marju; Shevchuk, Igor; Bogovskaja, Jelena; Suurmaa, Kulliki; Valvere, Vahur; Moreno-Sanchez, Rafael; Kaambre, Tuuli (2023). Colorectal polyps increase the glycolytic activity. Frontiers in Oncology, 13, 1171887. DOI: 10.3389/fonc.2023.1171887.

Tepp, Kersti; Aid-Vanakova, Jekaterina; Puurand, Marju; Timohhina, Natalja; **Reinsalu, Leenu**; Tein, Karin; Plaas, Mario; Shevchuk, Igor; Terasmaa, Anton; Kaambre, Tuuli; (2022). Wolframin deficiency is accompanied with metabolic inflexibility in rat striated muscles. Biochemistry and Biophysics Reports, 30, 101250. DOI: 10.1016/j.bbrep.2022.101250.

Reinsalu, Leenu (2022). Kasvajate edukus tuleneb nende kavalast energia tootmisest. ERR Novaator.

Reinsalu, Leenu; Puurand, Marju; Chekulayev, Vladimir; Miller, Sten; Shevchuk, Igor; Tepp, Kersti; Rebane-Klemm, Egle; Timohhina, Natalja; Terasmaa, Anton; Kaambre, Tuuli (2021). Energy Metabolic Plasticity of Colorectal Cancer Cells as a Determinant of Tumor Growth and Metastasis. Frontiers in Oncology, 11, ARTN 698951. DOI: 10.3389/fonc.2021.698951.

Rebane-Klemm, Egle; Truu, Laura; **Reinsalu, Leenu**; Puurand, Marju; Shevchuk, Igor; Chekulayev, Vladimir; Timohhina, Natalja; Tepp, Kersti; Bogovskaja, Jelena; Afanasjev, Vladimir; Suurmaa, Külliki; Valvere, Vahur; Käämbre, Tuuli (2020). Mitochondrial Respiration in KRAS and BRAF Mutated Colorectal Tumors and Polyps. Cancers, 12 (4), ARTN 815. DOI: 10.3390/cancers12040815.

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Teaduspreemiad ja tunnustused

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Konverentsid

September, 2023	Baltic Congress of Oncologists and Surgeons, Suuline ettekanne: *Energy metabolism is reprogrammed during colorectal cancer development*
November, 2022	Tallinna Tehnikaülikooli Loodusteaduskonna XIV teaduskonverents Suuline ettekanne: *Dietary preferences of colorectal cancer*
Juuni, 2022	FEBS3+ Conference of Estonian, Latvian and Lithuanian Biochemical Societies, Posterettekanne: *Metabolic plasticity supports colorectal cancer*

September, 2021 EMBO workshop “Systems approaches in cancer”, Posterettekanne: *Metabolic reprogramming in colorectal cancer and colon polyps*

Juhendatud väitekirjad

Janne Jegorkina, bakalaureusekraad, 2023, (juh) Leenu Reinsalu, *Kolorektaalvähi energiametabolismis esinevad geneetilised ja valgulised muutused*, Tallinna Tehnikaülikool, Loodusteaduskond, Keemia ja biotehnoloogia instituut

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Rebane-Klemm, Egle*; **Reinsalu, Leenu***; Puurand, Marju; Shevchuk, Igor; Bogovskaja, Jelena; Suurmaa, Kulliki; Valvere, Vahur; Moreno-Sanchez, Rafael; Kaambre, Tuuli (2023). Colorectal polyps increase the glycolytic activity. Frontiers in Oncology, 13, 1171887. DOI: 10.3389/fonc.2023.1171887.

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Rebane-Klemm, Egle; Truu, Laura; **Reinsalu, Leenu**; Puurand, Marju; Shevchuk, Igor; Chekulayev, Vladimir; Timohhina, Natalja; Tepp, Kersti; Bogovskaja, Jelena; Afanasjev, Vladimir; Suurmaa, Külliki; Valvere, Vahur; Käämbre, Tuuli (2020). Mitochondrial Respiration in KRAS and BRAF Mutated Colorectal Tumors and Polyps. Cancers, 12 (4), ARTN 815. DOI: 10.3390/cancers12040815.

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