THESIS ON NATURAL AND EXACT SCIENCES B228

Metabolic Remodeling of Human Colorectal Cancer: Alterations in Energy Fluxes

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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 any academic degree.

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Soolevähi metaboolne remodelleerimine: muutused energiavoogudes

ANDRUS KALDMA

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Contents

List of publications

Journal articles

Vladimir Chekulayev, Kati Mado, Igor Shevchuk, Andre Koit, Andrus Kaldma, Aleksandr Klepinin, Natalja Timohhina, Kersti Tepp, Manana Kandashvili, Lyudmila Ounpuu, Karoliina Heck, Laura Truu, Anu Planken, Vahur Valvere, Tuuli Kaambre (2015) Metabolic remodeling in human colorectal cancer and surrounding tissues: alterationsin regulation of mitochondrial respiration and metabolic fluxes. Biochemistry and Biophysics Reports 4 (2015) 111–125.

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Klepinin, Aleksandr; Chekulayev, Vladimir; Timohhina, Natalja; Shevchuk, Igor; Tepp, Kersti; Kaldma, Andrus; Koit, Andre; Saks, Valdur; Kaambre, Tuuli (2014). Comparative analysis of some aspects of mitochondrial metabolism in differentiated and undifferentiated neuroblastoma cells. Journal of Bioenergetics and Biomembranes, 46(1), 17 - 31.

Kaambre, Tuuli; Chekulayev, Vladimir; Shevchuk, Igor; Tepp, Kersti; Timohhina, Natalja; Varikmaa, Minna; Bagur, Rafaela; Klepinin, Aleksandr; Anmann, Tiia; Koit, Andre; Kaldma, Andrus; Guzun, Rita; Valvere, Vahur; Saks, Valdur (2013). Metabolic Control Analysis of Respiration in Human Cancer Tissue. Frontiers in Physiology, 4, 1 - 6.

Poster presentations

Kaldma, Andrus; Shevchuk, Igor; Koit, Andre; Chekulayev, Vladimir; Timohhina, Natalja; Tepp, Kersti; Mado, Kati; Khandashvili, Manana; Heck, Karoliina; Truu, Laura; Planken, Anu; Kaambre, Tuuli (2014). Bioenergetic profiling of human colorectal cancer clinical material. Cell Symposia, Hallmarks of Cancer: Asia. Beijing, China.

Kaldma, Andrus; Koit, Andre; Shevchuk, Igor; Chekulayev, Vladimir; Tepp, Kersti; Timohhina, Natalja; Bogovskaja, Jelena; Valvere, Vahur; Saks,

Valdur; Kaambre, Tuuli (2013). Regulation of respiration of human colorectal cancer: application of Metabolic Control Analysis. In: Mitochondrion: Mitochondrial Medicine 2013, Newport Beach, CA, USA.

Shevchuk, Igor; Koit, Andre; Kaldma, Andrus; Timohhina, Natalja; Chekulayev, Vladimir; Tepp, Kersti; Bogovskaja, Jelena; Valvere, Vahur; Saks, Valdur; Kaambre, Tuuli (2013). Comparative aspects of bioenergetic profiling of tumor clinical material and cell cultures. In: Mitochondrion: Mitochondrial Medicine 2013, Newport Beach, CA, USA.

Abbrevations

3-NP	3-nitropropionic acid
ADP	adenosine di phosphate
AMP	adenosine mono phosphate
ANT	adenine nucleotide translocase
APC	adenomatous polyposis coli
ATP	adenosine tri phosphate
ATPase	*adenosine triphosphatase*
AK	adenylate kinase
CAT	carboxyatractyloside
CIMP	CpG island methylator phenotype
CIN	chromosomal instability
CK	creatine kinase
CK BB	brain type creatine kinase isoenzyme
COX	cytochrome-c oxidase
Cr	Creatine monohydrate
CRC	colorectal cancer
DNA	deoxyribonucleic acid
ETC	electron transport chain
FADD	phospho-fas-associated protein with death domain
FADH	flavin adenine dinucleotide
FCC	flux control coefficient
HIF1	hypoxia-inducible factor 1
HK	hexokinase
I_{GLU}	glucose index
K_m	apparent Michaelis-Menten constant
MCA	metabolic control analysis
MI	mitochondrial Interactosome
MIM	Mitochondrial inner membrane
MIN	microsatellite instability
MOM	mitochondrial outer membrane
mRNA	Messenger RNA
MtCK	mitochondrial creatine kinase
NAD+	aldehyde dehydrogenase
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
OXPHOS	oxidative phosphorylation
PCr	phosphocreatine
PET	Positron Emission Tomography
Pi	inorganic phosphor
ROS	reactive oxygen species
RT-PCR	quantitative real-time polymerase chain reaction

SEM	standard error of the mean
TCA cycle	tricarboxylic acid cycle
TMPD	tetramethyl-phenylenediamine
TNM	TNM Classification of Malignant Tumours
uMtCK	ubiquitous mitochondrial creatine kinase
V_0	basal respiration rate
VDAC	voltage-dependent anion channel
V_m	maximal respiration rate

Review of literature

1. Colorectal cancer

1.1. *Incidence, survival worldwide and in Estonia*

Colorectal cancer is one of the most common types of cancer in the world. Number of new cases all over the world has been steadily rising for the last 30 years. There are exceptions like USA where the number of new cases in colorectal cancer has been lowering since the beginning of 90s (Siegel, Miller et al. 2015). For 2008, it was predicted that there will be more than 1,2 million new colorectal cancer cases and around 600,000 deaths (Jemal, Bray et al. 2011). In 2012, there were already 1,4 million new cases of colorectal cancer and 700,000 deaths worldwide (Stewart and Wild 2014). It is third most common cancer in men and second in women (Jemal, Bray et al. 2011, Dassow and Aigner 2013, Saadatian, Masotti et al. 2014).

In Estonia, there is about 700 new diagnoses of colorectal cancer and about 400 deaths annually (Berrino, De Angelis et al. 2007, Suuroja 2009).

1.2. *Definition, etiology*

Colorectal cancer is tumor which develops in colon or rectum part of intestinal tract (**Figure 1**). Colorectal cancer starts developing in the inner part of colon lumen in the mucosa layer of colon wall from some abnormal cells or polyps that have become malignant.

Large intestine is about 1,5 meters in length and 6-7 centimeters in diameter. It goes up from the right side (*ascending colon*) of the abdominal body cavity where it is connected to small intestine, across the top (*transverse colon*) and down (*descending colon*) from left side where it turns towards the center of body (*sigmoid colon*) and then turns again down to *anus*. Large intestine has almost no digestive function. Mainly, it absorbs water and electrolytes from the remaining chyme (Graaff 2001).

The large intestine is made of tissue layers (**Figure 2**) starting with *mucosa* that is innermost colon epithelial tissue. The layer is smooth, but contains tubular intestinal glands (crypts of Lieberkühn) and secrets *mucus* to the lumen of large intestine to protect the colon wall and to lubricate it for food particles. *Lamina propria* has a lot of elements from immune system and nodules of lymphatic tissue. It is made up of connective tissue, plasma cells, mast cells, macrophages, eosinophils and lymphocytes. *Muscularis propria* has two types of contractions: segmentation, which is local and not moving the content, and peristalsis that moves the colon content. *Submucosa* and *serosa* is the layer

connecting colon wall to the other structures. Large blood and lymphatic vessels go through *serosa* layer (Michael H. Ross 2010).

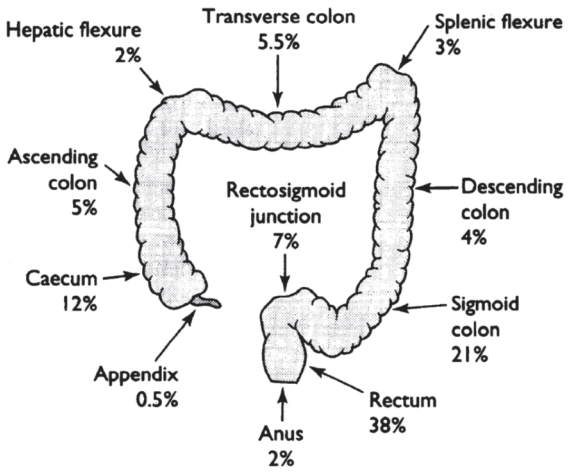

Figure 1. Distribution of sites of cancer in large bowel in United Kingdom ((Austoker 1994), with permission). Small intestine connects with *ileum* to the first part of large intestine called *caecum* in the lower right side of abdomen. From *caecum* travels up ascending colon and connects to transverse colon that travels across the abdomen. Descending colon runs down from the left side of the abdomen and connects to short curvy part called sigmoid colon. Large intestine ends with rectum that connects to anus.

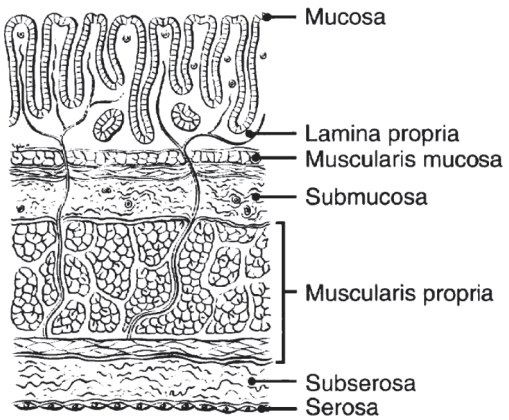

Figure 2. Cross section of colon wall showing the various layers ((AJCC 2002), with permission). Colon tissue is composed of four main layers: mucosa, submucosa, muscular layer and serosa. Mucosa layer folds to increase the surface area and secrets mucus that lubricates and protects the surface. Muscle layer is composed of mainly smooth muscle that helps to move the digested material.

Genetic factors can be recognized in up to 30% of colorectal cancer (CRC) patients (**Figure 3A**) where genetic abnormality is increasing the risk of diagnosis (Stigliano, Sanchez-Mete et al. 2014). Also when close relatives have cancer then the risk to develop same type of cancer increases (National Research Council (US) Committee on Diet 1982, Health. 1989, Shike, Winawer et al. 1990) and estimate on inherited CRC cases is 5-15% (Papadopoulos, Nicolaides et al. 1994, Stewart and Kleihues 2003, Jackson-Thompson, Ahmed et al. 2006, Stigliano, Sanchez-Mete et al. 2014). CRC is mostly over 50 year old people's disease but up to 10% can have the tumor in younger age, even in adolescent age (Stigliano, Sanchez-Mete et al. 2014). Diet full of animal fats and proteins and too low physical activity are also linked to colorectal cancer (**Figure 3B**) (Giovannucci and Willett 1994, Zhu, Gao et al. 2014). There is a lot of data suggesting obesity as risk factor (Schwartz and Yehuda-Shnaidman 2014). Some authors have even shown for the diet to be the cause on as much as 35% of cancer patients (Doll and Peto 1981) (AICR American Institute for Cancer Research and World Cancer Research Fund 2007). Polyps are quite common in older age but few can become malignant. 80% of colorectal cancers can come from polyps that have become malignant (Fearon and Vogelstein 1990, Jass 2007, Leggett and Whitehall 2010, Guarinos, Sanchez-Fortun et al. 2012). In colorectal cancer polyps are histologically classified as neoplastic, hyperplastic, hamartomatous, or inflammatory. Neoplastic polyps (also known as adenomatous polyps or adenomas), are the most likely to develop in to cancerous. 96% of colorectal cancers are adenocarcinomas (Stewart, Wike et al. 2006) and majority of those arise from adenomatous polyps. Adenomatous polyps are common and occurring in 20-40% of people older than 50 years of age in western world (Shussman and Wexner 2014). Less than 10% of adenomatous polyps become cancerous (Levine and Ahnen 2006).

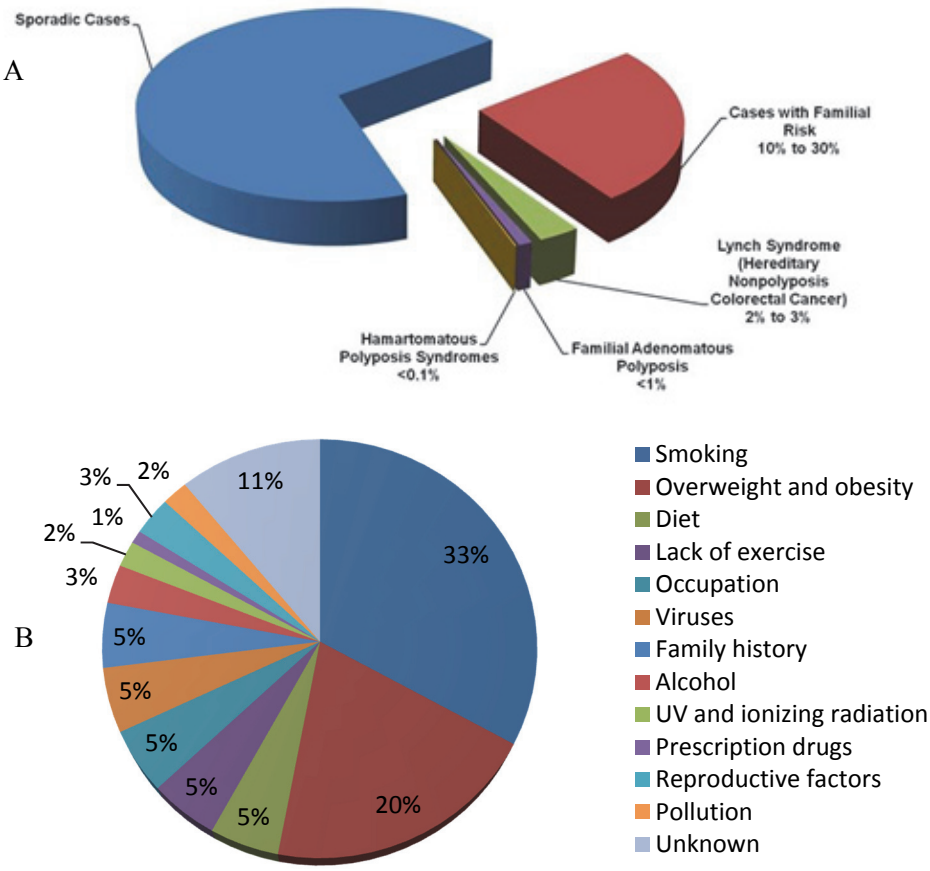

Figure 3. Colon cancer cases arising in various risk settings. A: Colon cancer risk factors from family history settings ((Burt 2000), with permission). B: Causes of cancer. Figure based on the data from Colditz ((Colditz, Wolin et al. 2012), with permission).

Some diseases can count as risk factors to develop colorectal cancer. Among them on women are ovarian-, uterine and mammary gland cancers.

For colorectal cancer 40% of the cases are in developed world where there is only 15% of world's population (Jemal, Bray et al. 2011) this can mainly be associated with dietary and metabolic factors or so called western lifestyle (Chan and Giovannucci 2010).

1.3. *Staging system*

Most common classification system for malignant tumors is TNM staging system (**Table 1**), which is most commonly also used for human colorectal cancer. Staging is used to assess how much cancer is in the body and where is it located. It describes the severity of the cancer, magnitude of the original tumor and the extent of metastasis in the body. Dukes staging system proposed in 1932 (Dukes 1932) and systems based on it have also been used but it is less detailed and is no longer recommended for clinical practices. TNM classification gives information about primary tumor (T), lymph nodes (N) and distant organ metastasis (M). It was developed by Pierre Denoix between 1943 and 1952 for solid tumors. Most medical institutions are presently using American Joint Committee on Cancer (AJCC) Cancer Staging Manual to assess the severity of the tumor.

Table 1. Abbreviations used in Tumor classification. These definitions are used for both clinical and pathologic staging (AJCC 2002). Primary tumor gives information on the tumor itself. Regional lymph nodes give information on how far has the tumor spread through lymph nodes and distant metastasis tells if the tumor has given metastasis to other tissues and organs.

Primary Tumor (T)

TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	*Carcionoma in situ*: intraepithelial or invasion of *lamina proparia*
T1	Tumor invades *submucosa*
T2	Tumor invades *muscularis propria*
T3	Tumor invades through the *muscularis propria* into the *subserosa*, or into non-peritonealized pericolic or perirectal tissues
T4	Tumor directly invades other organs or structures, and/or perforates *visceral peritoneum*

Regional Lymph Nodes (N)

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1 to 3 regional lymph nodes
N2	Metastasis in 4 or more regional lymph nodes

Distant metastasis (M)

MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Colorectal cancer is divided into four stages with additional sub-stages (AJCC 2002).

- Stage 0: Cancer has few abnormal cells that are situated in innermost lining of colon.
- Stage 1: Cancer starts to spread. The tumor invades submucosa and or muscularis propria.
- Stage 2: It has three substages A, B and C. In Stage 2A the tumor invades to pericolorectal tissue. In stage 2B the tumor penetrates to the surface of *visceral peritoneum*. In stage 2C the tumor invades other organs. In stage 2 the cancer has not spread to lymph nodes.
- Stage 3: It has also three substages A, B and C. In Stage 3A tumor invades *submucosa* or *muscularis propria* and gives metastasis in up to 6 local lymph nodes. In stage 3B the tumour invades *submucosa* or *muscularis propria* or into pericolorectal tissue. In stage 3B the tumor can invade up to 7 or more lymph nodes but gives no distant metastasis. In stage 3C the tumor invades into pericolorectal tissue or other organs and gives metastasis in regional lymph nodes.
- Stage 4: Tumor spreads to other parts of the body like liver or lungs or other organs.

1.4. ***Prognosis and treatment***

Prognosis depends on the stage of the cancer. Prognosis is statistical value that is usually given as five-year survival rate. The rate for localized-stage disease is 90%, for diagnosis of regional disease is 70% and for distant stage is 12% (Rick Alteri 2011).

Prognosis could be much better if the cancer is discovered earlier. Screening can help to prevent the cancer because most cancers develop from adenomatous polyps and those can be detected and removed turning the screening. Also, if the cancer has already developed then detecting it at an earlier stage will help to treat it better (Levin, Lieberman et al. 2008).

Colorectal cancer treatment is dependent on the location and stages of the tumor. Treatment for colon cancer can differ from rectal cancer treatment. The stage of the cancer is most important factor on choosing the treatment options. Treatments are simple polyp removal and small surgery to remove the tumor and surrounding tissue in stage 0 and stage 1. In more serious cases of stage 2 chemotherapy may be added but that depends on the exact parameters of stage 2 and usually chemotherapy is not needed. In stage 3 patients will receive chemotherapy and radiation

therapy may also be needed if the tumor is large and invading surrounding tissue of colon. When tumor has progressed to stage 4 and given metastasis then the five year survival rate decreases dramatically and all possible treatment options are open. This includes surgery for colon as well as other organs that may have metastasis such as liver or lungs. To increase survival and relieve symptoms chemotherapy is given. Chemotherapy includes experimental drugs that may have side effects but that may help in case of drug resistance. Also, immunological and radiation therapy can be used (Society 2016).

2. *Molecular pathways and cellular metabolism in colorectal cancer*

2.1. *Cancer development pathways*

Development of human colorectal cancer is known as “adenoma-carcinoma sequence” where normal colonic epithelium transforms in to adenomatous polyp intermediate and then in to adenocarcinoma (Morson 1974). However, not all polyps develop in to carcinomas and not all carcinomas are developed from polyps.

There are several genetic transformations that occur at the same time with the “adenoma-carcinoma sequence”. One of the first changes to occur is mutation in Adenomatous polyposis coli (APC) gene on chromosome 5 (Homfray, Cottrell et al. 1998). Other important changes that have been described include genomic instability’s (**Figure 4**) like the chromosomal instability (CIN), CpG island methylation phenotype (CIMP) pathway and microsatellite instability (MIN).

CIN is the most common type of genomic instability. It results in gain or loss of large parts of chromosomes, change in sequence and chromosome segregation defects (Lengauer, Kinzler et al. 1998).

In CIMP pathway the CpG islands are hyper methylated leading to silencing of respective genes (Goel, Nagasaka et al. 2007). This kind of methylation can occur on several genes simultaneously.

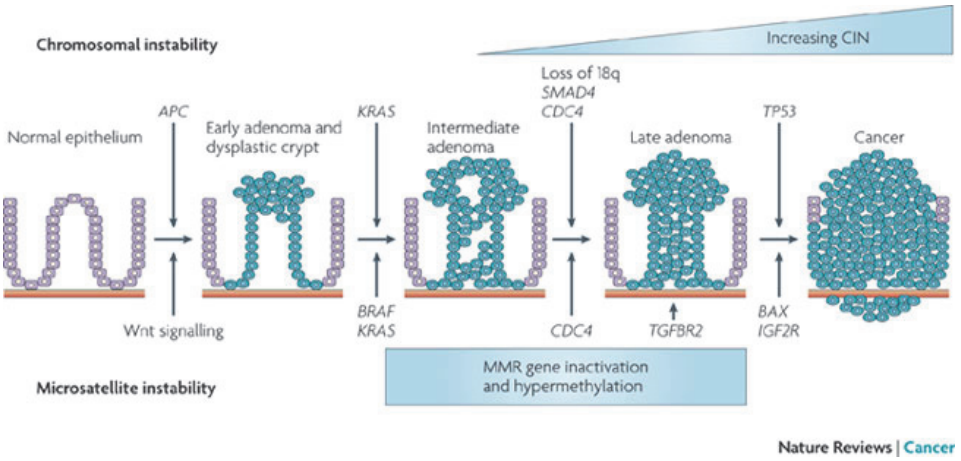

Figure 4. Graphic illustration of chromosomal and microsatellite instability pathway development models with some specific mutations that occur turning the development of those pathways ((Walther, Johnstone et al. 2009), with permission).

MIN pathway is caused by the loss of DNA mismatch repair activity (Boland and Goel 2010). This loss of mismatch repair system can be characterized by small insertions and deletions that are seen on gel electrophoresis as band shifts due to microsatellite length change through those mutations (Boland, Thibodeau et al. 1998). In colorectal cancer the MIN pathway is activated by mutation in *hMSH2, hMLH1, hPMS1, hPMS2*, or *hMSH6* mismatch repair gene (Haydon and Jass 2002).

In serrated neoplastic pathway there are hyperplastic polyps and serrated adenomas which result in the development of cancer. Serrated polyps have in folding of epithelium that is giving them saw-tooth appearance. There are two types of serrated pathways: Sessile serrated pathway and Traditional serrated pathway (Snover 2005). Mutations involved in serrated polyp pathway are BRAF mutations, CpG island methylation and microsatelite instability (Haque, Greene et al. 2014).

In addition to cancer development pathways, there are some oncogenic mutations that can disrupt apoptosis mechanisms, leading to tumor initiation and progression or even to metastasis and those mutations can also decrease sensitivity to apoptotic signals (Lowe and Lin 2000).

In western world the lifetime risk for human colorectal cancer is about 5% (Bretthauer 2011) and from that about 90% are sporadic and about 10% can be traced to originate from hereditary cancer syndromes (Soreide, Janssen et al. 2006). 85% of the sporadic cases have chromosomal instability phenotype and only 15% are originating from microsatellite instability phenotype (Cunningham, Atkin et al. 2010).

2.2. ***Metabolic reprogramming in cancer***

Accumulation of mutations is thought to be responsible for Metabolic reprogramming (Amoedo, Rodrigues et al. 2014). Metabolic reprogramming includes increased glycolysis, glutaminolysis and macromolecule biosynthesis, upregulation of lipid and amino acid metabolism, mitochondrial biogenesis and changes in pentose phosphate pathway (Hanahan and Weinberg 2011).

Increased glycolysis in tumor cells has also increased cells glucose uptake through upregulation of glucose transporters Glut1-Glut5 (Deberardinis, Sayed et al. 2008). This phenomenon is widely used in medicine to locate and visualize the tumor by Positron Emission Tomography (PET) where radiolabeled glucose analogs are used. In healthy cells glucose is processed to acetyl-CoA and is used up in TCA cycle but in tumor cells glucose is converted to lactate. Some of the lactate is converted to NADH and some of it is thrown out of the cell to produce tumor microenvironment. Use of lactate is not the most efficient for biosynthesis and energy production but usually nutrients are not limiting factor in tumor cell proliferation so optimizing the metabolism and ATP

production is not primary requirement (Vander Heiden, Cantley et al. 2009).

Glutaminolysis converts glutamine to glutamate and α-ketoglutarate in TCA cycle. Tumor cells can use intermediates of TCA cycle to produce lipids, amino acids and other essential products and also electrons for the electron transport chain to produce ATP. Reprogramming glutaminolysis can increase the biomass and energy synthesis (DeBerardinis and Cheng 2010).

Pentose phosphate pathway consists of two phases: oxidative and non-oxidative phase. In oxidative phase glycose-6-phosphate is converted to ribulose-5-phosphate that is used for nucleotide synthesis and NADPH that is important for biosynthesis reactions and countering oxidative stress. Non-oxidative phase produces fructose-6-phosphate and glyceraldehyde-3- phosphate. Oxidative phase products are used for biosynthesis, glutathione production and detoxification reactions. Non-oxidative products are used in glycolysis pathway. Upregulation of pentose phosphate pathway helps tumor cells to resist radiation and chemotherapy as well as to promote invasion and metastasis (Riganti, Gazzano et al. 2012).

Control over mitochondrial biogenesis is important aspect for tumor cells as the mitochondria are producing most of the ATP and have important role in biosynthesis. Mitochondria control cellular homeostasis through redox balance and Ca^{2+} concentration. Therefore mitochondria play an important part in tumor cell growth and proliferation (Wallace 2012). In cancer cells mitochondria have been found to be dysfunctional in many aspects compared to normal cells. For example, in cancer cells increased glycolysis promotes tumor cell survival by regulation of cytochrome C and inhibition of apoptosis (Vaughn and Deshmukh 2008). Mitochondria are the main producer of reactive oxygen species (ROS) in the cell and almost all cancers have elevated ROS levels. ROS has been associated with cell cycle progression and proliferation, cell survival and apoptosis, energy metabolism, cell morphology, cell-cell adhesion, cell motility, angiogenesis and maintenance of tumor stemness (Liou and Storz 2010).

In tumor cells lipid biosynthesis is frequently upregulated because proliferating cells are building constantly new organelles and cell membranes. Change in lipid balance is common in cancer cells. It has been shown that lipid composition and amount can contribute to formation or degradation of respiratory chain supercomplexes (Lenaz and Genova 2012). Cellular cholesterol is frequently upregulated to support cell proliferation and tumor progression (Gorin, Gabitova et al. 2012). Also lipid called ganglioside has been shown to be present in many tumors supporting angiogenesis and as immunosuppressor (Llado, Lopez et al. 2014). Lipids are also used in signaling, for energy storage and function

as hormones. Changes in lipid metabolism will affect cell growth and proliferation, motility and differentiation of cells (Santos and Schulze 2012). Tumor cells also have higher accumulation of lipid droplets (Accioly, Pacheco et al. 2008). As mentioned before obesity is a risk factor in cancer development. Increased lipid concentration induces insulin resistance that induces increased insulin and insulin like growth factors secretion which in turn increase cancer proliferation and survival (Rosenzweig and Atreya 2010, Samuel and Shulman 2012).

2.3. *Functionality of OXPHOS in cancer cells*

Mitochondria are important regulators of metabolism and energy production but also in regulating cell death and signaling pathways (Brenner and Grimm 2006).

Mitochondria produce ATP through the oxidative phosphorylation and this process in turn generates most of cellular ROS (Solaini and Harris 2005). ROS is up regulated in cancers and can damage cells macromolecules and modulate mitogenic signaling pathways and through that promote carcinogenesis (Weinberg and Chandel 2009). For cancer cells it is important to adapt their metabolism so that it is able to produce all the proteins, enzymes and energy to promote biomass generation and tumor expansion. Tumor cells have been shown to produce only 40-75% of their energy need through aerobic glycolysis thus 25-60% is still generated through oxidative phosphorylation as well as through the associated tricarboxylic acid cycle (Mathupala, Ko et al. 2010). It seems to be that availability of energy substrate as well as the availability of oxygen and aggressiveness of tumor can modulate the OXPHOS capacity of mitochondria (Solaini, Sgarbi et al. 2011).

Although mitochondrial dysfunction is quite common in cancer cells there is only minimal changes in respiratory chain complexes and ATP synthase compared to healthy cells. Several studies are showing high rates of OXPHOS and minimal or no changes in respiratory complexes and ATP synthase between cancerous tissue compared with healthy tissue (Moreno-Sanchez, Rodriguez-Enriquez et al. 2009, Diers, Broniowska et al. 2012, Kaldma, Klepinin et al. 2014). Although suppression of complex I –dependent respiration has also been shown in some cancers (Putignani, Raffa et al. 2008, Puurand, Peet et al. 2012, He, Zhou et al. 2013)

Oxidative phosphorylation has been shown by Tan and coworkers (Tan, Baty et al. 2015) to be one of the most essential elements for metastasizing cancers. They showed that tumor cells that exhibit deficiency in OXPHOS are able to acquire mitochondrial DNA from bystander cells and become functional (Maiuri and Kroemer 2015, Tan, Baty et al. 2015).

2.4. *Phosphotransfer networks*

Adenine nucleotides do not diffuse freely in cells to avoid ADP/ATP accumulation and through that inhibition of ATPase's. (Saks, Kuznetsov et al. 1995). The sites of energy consumption and production are connected through mitochondrial kinases (**Figure 5**) (Dzeja, Zeleznikar et al. 1998) and thus, the transfer of Pi and returning metabolic signals are instantaneous. This system of energy transfer through different kinase systems, where energy gets passed from one part of the system to the next, works without concentration gradients and is thermodynamically efficient (Dzeja, Zeleznikar et al. 1998). This helps also to explain why there are no changes in adenine nucleotide concentrations although there is observable increase in metabolic flux (Zeleznikar, Dzeja et al. 1995). Flux wave is able to move much faster than diffusion of reactants (Mair and Muller 1996).

There are several intracellular enzymatic energy transport networks to facilitate ATP-utilization and ATP-generation that facilitate broad range of cellular activities. Among those enzymatic networks are creatine kinase (CK) network, adenylate kinase (AK) network, glycolytic enzymes and carbonic anhydrase mediated networks. In tumor cells the regulation of these networks can be up or down regulated to facilitate the growth and energy demand.

Adenylate kinase (AK) catalyzes the reaction between AMP and ATP to generate ADP and vice versa (2ADP ↔ ATP + AMP). AK facilitates transfer and utilization of γ- and β-phosphoryls in the ATP molecule (Dzeja and Terzic 2009).

Through reactions in AK we can report cellular energy status because AMP concentrations change a lot while change in the balance of ADP and ATP are small enabling the cell to respond to different stress situations through AMP sensors (metabolic sensors, AMP sensitive enzymes, etc.) (Hardie 2004). AK can respond to inadequate oxygenation or nutrient supply and physical activity through monitoring of body energy imbalances (Dzeja and Terzic 2009). AK helps cancer cells to adapt to hostile environment and protects cells from cell death (Jan, Tsai et al. 2012). In pancreatic cancer cells cytosolic AK1 transcription downregulation has reported to increases apoptosis (Giroux, Iovanna et al. 2006). AK2 has been reported frequently to be downregulated in human cancer cells with high levels of phospho-fas-associated protein with death domain (FADD) (Kim, Lee et al. 2014). AK 4 has been found to be upregulated and to promote metastasis in lung cancer (Jan, Tsai et al. 2012).

Creatine kinase catalyzes the transfer of energy between ATP and Cr to ADP and PCr (PCr + MgADP + H ↔ Cr + MgATP). It has an important role in energy buffering and phosphoryl energy transfer. CK is found mainly in tissues with high energy demand like skeletal muscles, brain or heart but also in some tumors. CK isoenzyme BB (brain type) has been

found to be upregulated in cancer patients. It has been suggested, that because of tumor cells are growing and proliferating much faster than normal tissue cells they also require large amounts of energy supply (Silverman, Dermer et al. 1979, Zarghami, Giai et al. 1996). CK reactions are an order of magnitude faster on regenerating ATP than OXPHOS and glycolysis are on producing or ATPases consuming the energy (Bittl and Ingwall 1985). CK through its activity keeps the intracellular ADP levels where they can participate in the control of mitochondrial respiration (Chance, Leigh et al. 1985).

Glycolysis is a sequence of reactions for sugar oxidation. Glycolysis occurs in the cytosol and it produces ATP without the involvement of molecular oxygen. It consists of 10 steps where early steps of glycose molecule breakdown consumes 2 molecules of ATP but later steps produces 4 molecules of ATP. Electrons are removed from glycose molecules by NAD^+ producing 2 molecules of NADH per glycose (Alberts, Wilson et al. 2008).

In most animal and plant cells, pyruvate the final product of glycolysis, is transported to mitochondria and further oxidized to acetyl CoA and CO_2 and then further to CO_2 and H_2O (Alberts, Wilson et al. 2008).

In anaerobic organisms the pyruvate is instead converted to ethanol and CO_2 or in muscles in to lactate. This process is known as fermentation (Alberts, Wilson et al. 2008).

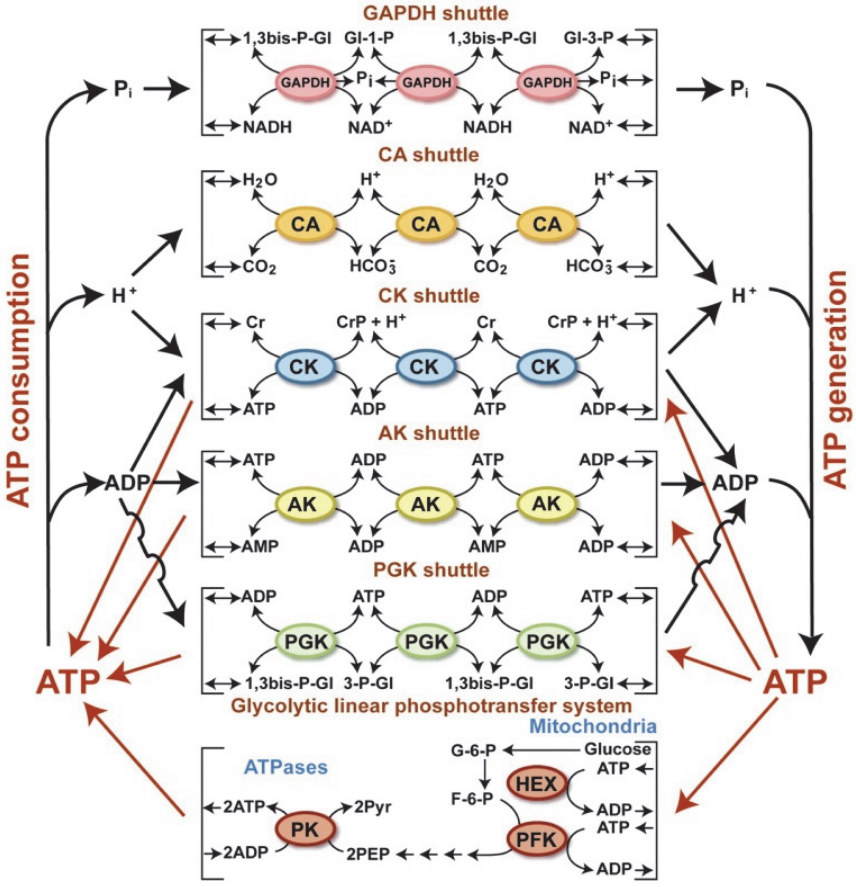

Figure 5. Phosphotransfer pathways from ATP-generation to ATP utilization. Cells use different parallel pathways to transport energy from sites of energy generation to sites of utilization using kinase systems. This allows almost instantaneous movement of energy and removes the accumulation of adenosine phosphates (Reproduced / adapted with permission (Dzeja and Terzic 2003)).

2.5. *Role of tubulins in regulation of energy metabolism in cancer cells*

Tubulin heterodimers can be in the form of free monomers in cells or constitute microtubules which are constantly lengthening and shortening dynamic structures. They take part in many cell processes including cell growth, movement and signaling (Nogales 2000). Microtubules are part of cell cytoskeleton and are made up of α- and β-tubulin heterodimers that form cylindrical hallow structures (Nogales 2000). Microtubules have

special motor proteins and they work as highways for cell components and proteins.

It has been described, that tubulins have alterations in cancer (**Figure 6**). Tubulins can have altered expression of tubulin isotypes, post translational modification changes or microtubule- associated protein expression changes (Kavallaris, Tait et al. 2001). Alterations in microtubules can introduce chemotherapy resistance, promote tumor development and affect cell survival (Kavallaris 2010).

Importantly, from the metabolic regulation side, tubulin is able to bind to VDAC channel and thereby able to block and regulate energy transfer through the channel (Rostovtseva, Sheldon et al. 2008, Maldonado, Sheldon et al. 2013). Mostly βIII-tubulin but also other isoforms have been shown to be associated with glycolysis and TCA cycle enzymes (Cicchillitti, Penci et al. 2008). Tubulins are responsible for regulating metabolic activity and metabolic reprogramming.

Tubulin βIII has been shown to be most dynamic of all tubulin isoforms (Panda, Miller et al. 1994). Further it has been shown that tubulin βIII isoform also increases resistance to chemotherapy drugs (estramustine, taxol, paclitaxel, docetaxel) designed to suppress microtubule assembly (Derry, Wilson et al. 1997). Tubulin βIII has been seen in many different cancers and most of all in those that are aggressive and giving metastasis (Katsetos, Herman et al. 2003).

Soluble free tubulin lowers mitochondrial membrane potential in cancer cells that in return affects OXPHOS and apoptosis (Maldonado, Patnaik et al. 2010).

Hypoxia dramatically remodels microtubules by increasing tumor cell invasion and stabilizing microtubules (Yoon, Shin et al. 2005). Hypoxia-inducible factor 1 (HIF1) is helping the cancer cells to withstand hypoxia and it is up regulated in many solid tumors (Mabjeesh, Escuin et al. 2003). HIF1 is using microtubules to move around the cell and it is important for its activity that it is able to translocate to the nucleus (Carbonaro, Escuin et al. 2012).

Tubulins and microtubules are also responsible for distribution and regulation of mitochondrial motility (Baumann and Murphy 1995).

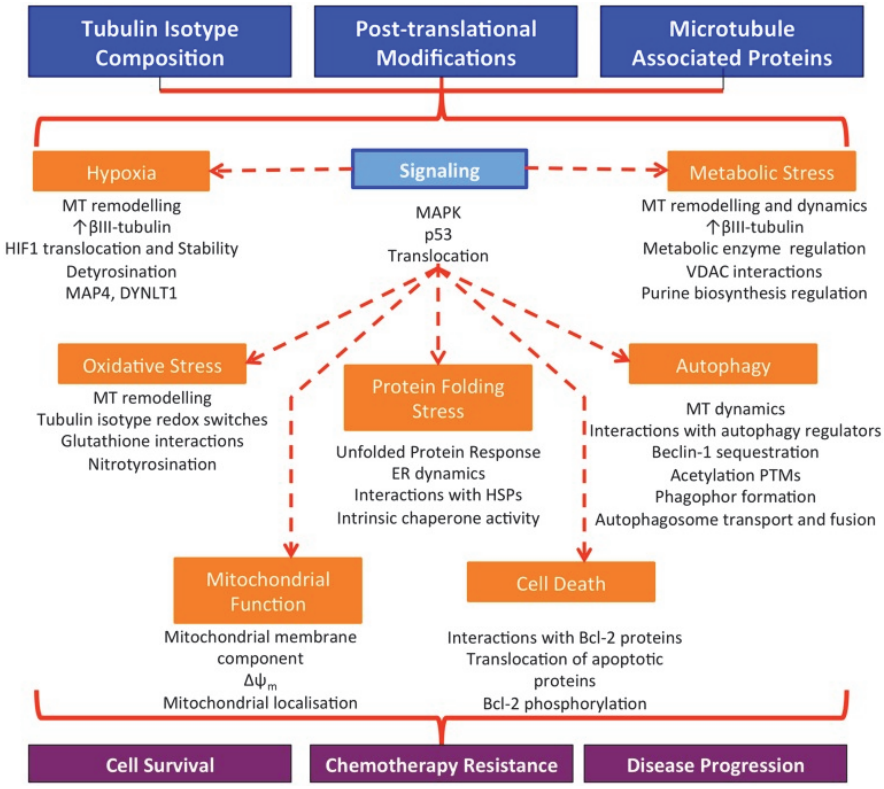

Figure 6. Microtubules are involved in many stress regulation signaling and response pathways in tumor cells. Modifications in microtubules in tumor cells can influence vide variety of cellular functions by promoting cell survival in oxidative and hypoxic or in metabolic and protein stress situations. Microtubules are responsible of organelle transport and protein signaling network functioning. Tubulins and microtubules are in important position in regulating cells functions. (Reproduced with permission under the CC-BY Creative Commons license (Parker, Kavallaris et al. 2014)).

3. Cancer models

3.1. *Mitochondrial Interactosome*

In cells there are two systems for recycling metabolites. ADP/ATP recycling is mainly done in mitochondrial intermembrane and matrix space and Cr/PCr recycling is done in cytoplasm and intermembrane space of mitochondria. The two metabolic cycles are coupled in the mitochondrial intermembrane space by supercomplex called Mitochondrial Interactosome (MI) (Timohhina, Guzun et al. 2009).

MI (**Figure 7**) consists of ATP synthasome, mitochondrial creatine kinase and voltage-dependent anion channel that is bound with regulatory protein tubulin with its linker protein. ATP syntasome is formed by ATP synthase, adenine nucleotide carrier, inorganic phosphate carrier and sometimes in addition the electron transport chain complexes (Pedersen 2007).

ATP syntasome creates ATP from ADP by adding inorganic phosphate in the inner membrane of mitochondria. In the intermembrane space MtCK catalyzes the transfer of Pi from ATP to Cr to form PCr that leaves mitochondria through VDAC. PCr serves as the main energy carrier in a cell. Small amounts of cytosolic ADP moves through VDAC as signaling molecule for ATP synthesis (Timohhina, Guzun et al. 2009).

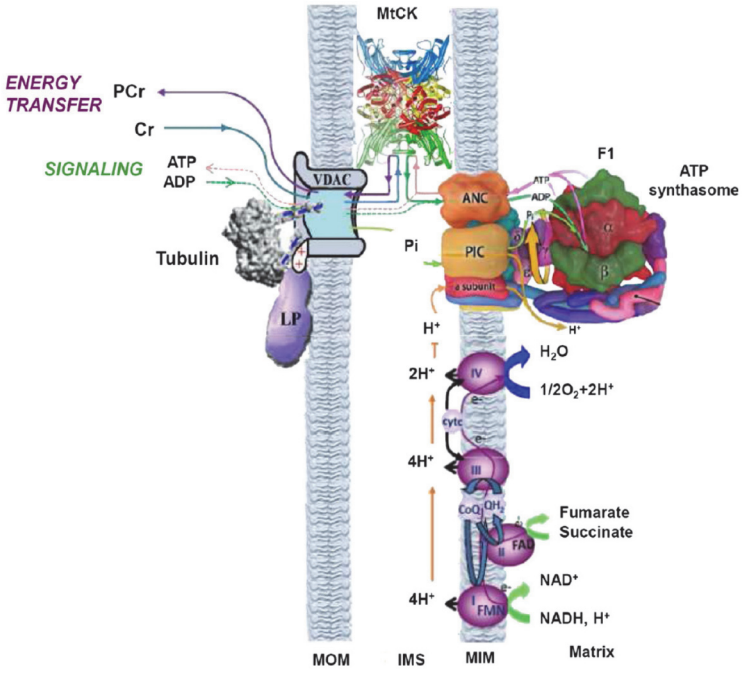

Figure 7. Model of Mitochondrial Interactsome (MI). MI consists of voltage dependent anion channel (VDAC) coupled with linker proteins and tubulin, mitochondrial creatine kinase functionally coupled with ATP synthasome, adenine nucleotide carrier and inorganic phosphate carrier and electron transport chain complexes ((Saks, Guzun et al. 2010) with permission).

3.2. *The Warburg effect*

Pathological alterations in cellular energy metabolism define the difference between cancer and normal cells. Glucose metabolism is the catabolic process, which being proposed, the tumors rely on. In normal cells glucose is metabolized to pyruvate. In oxidative conditions in differentiated tissue mitochondria metabolize most of the pyruvate to CO_2 and in small parts pyruvate is turned to lactate. This is the most effective way to produce ATP (**Figure 8**). In oxygen deprived condition in differentiated tissue all of the pyruvate is turned to lactate, resulting in minimal amounts of ATP.

In the beginning of 20th century Otto Warburg described a glycolysis in the presence of oxygen in tumor cells. This phenomenon is named the “aerobic glycolysis or the Warburg effect”. For his work he received Nobel

Prize in physiology *"for his discovery of the nature and mode of action of the respiratory enzyme"* in 1931. Warburg postulated that this change in metabolism is the fundamental cause for cancer (Warburg, Poesener et al. 1924). In aerobic glycolysis that Warburg observed in tumors, most of the pyruvate is turned to lactate whether the oxygen is present or not. Aerobic glycolysis is not very efficient way for producing ATP but proliferating cells have other important metabolic requirements besides only ATP (Vander Heiden, Cantley et al. 2009). For example, some of the glucose is diverted to biosynthesis to produce biomass of the growing tumor. Also not ATP but reducing agents like NADPH are thought to be the rate limiting products and increased glycolysis that ends with lactate helps to resolve this problem (Lunt and Vander Heiden 2011). Another important aspect that arises from Warburg effect is the acidification of microenvironment through the excretion of lactate (Estrella, Chen et al. 2013). It has also been shown that there are changes in the signaling in the case of Warburg effect compared with healthy cells. For example tumor cells have elevated signals for nutrient uptake and metabolism (Wellen and Thompson 2012).

Conclusive explanation for “Warburg effect” has been eluding scientists for over 90 years.

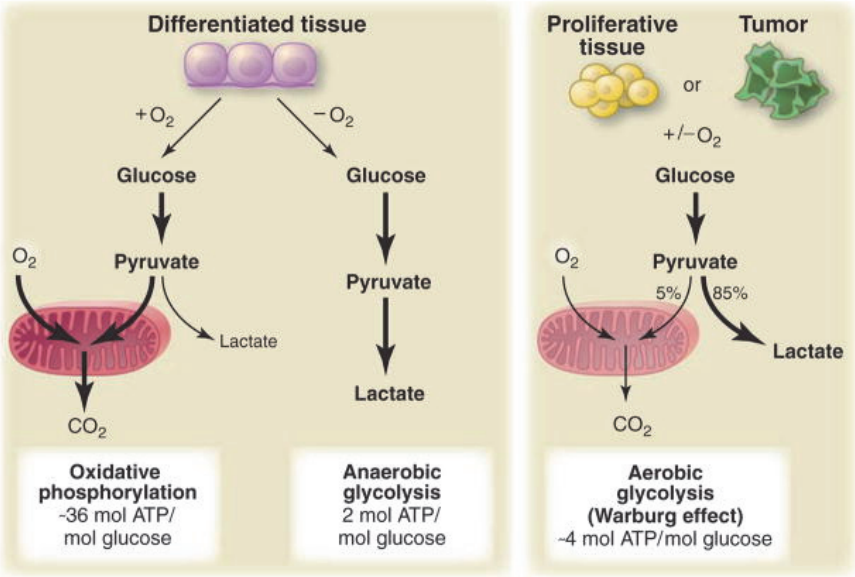

Figure 8. Schematic illustration of oxidative phosphorylation, anaerobic glycolysis and aerobic glycolysis ((Vander Heiden, Cantley et al. 2009), with permission).

3.3. *Pedersen model and dysfunctions in MI*

Pedersen and co-workers showed a model where overexpressed hexokinase 2 is bound to voltage-dependent anion channel in mitochondrial outer membrane (**Figure 9**) and through that regulating oxidative phosphorylation (Pedersen 2007).

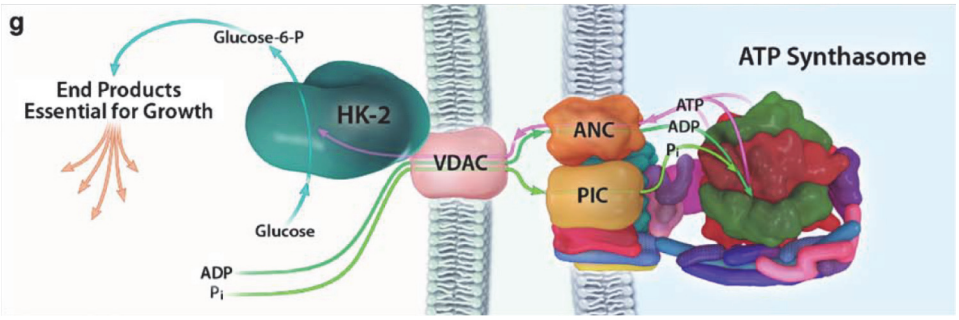

Figure 9. Schematic illustration of Pedersen model. HK-2 binds to VDAC channel and has direct access to ATP generated by ATP synthasome. By binding to VDAC, HK-2 no longer gets inhibited by the end product, Glucose-6-P, and the glycolysis can "jumpstart". Most of the Glycose gets turned to lactate, instead of pyruvate that fuels OXPHOS in healthy cells, and is used up for biomass generation ((Pedersen, 2007) with permission).

Work done in Pedersen laboratory showed that "Warburg effect" resulted mostly from involvement of three factors: overexpression of hexokinase 2, hexokinase 2 binding to voltage-dependent anion channel and the amplification and up regulation of hexokinase 2 gene (Pedersen 2007). In the following years they looked further in to those factors showing that hexokinase plays key role in facilitating high glycolytic activity. Hexokinase is bound to mitochondrial outer membrane and is directly coupled to ATP synthesis in the inner membrane of the mitochondria. Also, this hexokinase that was bound to outer membrane of mitochondria was not inhibited by the glycolysis end product glycose-6-phosphate (Bustamante and Pedersen 1977). Then, it was identified that hexokinase binds to voltage dependent anion channel (Nakashima, Mangan et al. 1986). After that it was identified that the hexokinase isoform bound to mitochondrial outer membrane is heksokinase-2 (Nakashima, Paggi et al. 1988). In addition to work done in Pedersen laboratory, other authors like Altenberg and coworkers (Altenberg and Greulich 2004) showed overexpression of glycolytic pathway isoenzymes in human cancers, Eboli with his coworkers (Eboli, Paradies et al. 1977) showed reduced capacity of tumor cells to oxidize pyruvate and studies by Rose and coworkers (Rose and Warms 1967) showed that hexokinase is bound to mitochondria. Hexokinase 2 via its interactions with mitochondria

suppresses the death of cancer cells and increases cancer cells possibility to give metastasis and leades ultimately to the death of the human host (Mathupala, Ko et al. 2006).

3.4. *Two-compartment tumor model*

Human cancer cells have been shown to use supporting host cells (**Figure 10**), such as adipocytes and fibroblasts as energy source (Sotgia, Martinez-Outschoorn et al. 2011). In that kind of two-compartment metabolism model, energy is being transferred from catabolic adipocytes and fibroblasts to anabolic tumor cells that hyper activate their capacity for OXPHOS by increasing their mitochondrial mass (Sotgia, Martinez-Outschoorn et al. 2012). Nieman et al showed that ovarian cancer cells can have much higher mitochondrial metabolic activity in co-culture with adipocytes (Nieman, Kenny et al. 2011). These findings suggest that microenvironment is extremely important for tumor research and same tumor cells can act completely differently in the presence of supporting host cells compared to pure cultures.

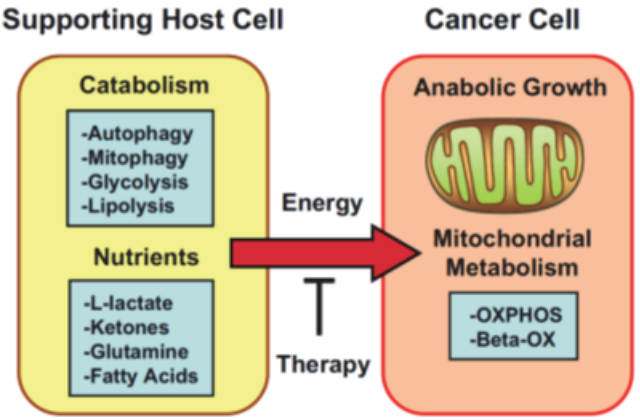

Figure 10. Overview of the two-compartment tumor metabolism model. It has been theorized that tumor is surrounded by supporting host cells that provide the cancer with energy and nutrients. In this model the anabolic cancer cell has functional OXPHOS system and catabolic supporting cells are glycolytic explaining the Warburg mechanism and high OXPHOS capacity seen in some cancers ((Martinez-Outschoorn, Sotgia et al. 2012) with permission).

Epithelial cancer cells can promote oxidative stress in adjacent stromal cells by secreting hydrogen peroxide which causes stromal fibroblast activation and HIF1-α up regulation witch drives autophagy, mitophagy and aerobic glycolysis in the tumor stroma (Martinez-Outschoorn, Pestell

et al. 2011). Glycolytic fibroblasts can provide tumor with L-lactate that is metabolized in tumor cells by OXPHOS. Host cells catabolism can also provide ketone bodies and glutamine for the adjacent cancer cells (Martinez-Outschoorn, Pavlides et al. 2011). This type of multi compartment metabolism is also known as “reverse Warburg effect”.

Co-operation between cells, tissues and organs is quite normal. For example, glycolytic fast-twitch fibers supply slow-twitch fibers with L-lactate for OXPHOS In mitochondria that is known as “lactate shuttle” (Brooks 2009). In the brain astrocytes supply neurons which is known as “neuron-glia metabolic coupling” (Pellerin and Magistretti 1994). Therefore, this type of metabolic coupling is normal physiological process that epithelial tumor cells have adopted and this is also a reason why tumors should not be studied separately from supporting tissue and as pure cancer cultures.

4. Metabolic Control Analysis

Before the development of metabolic control analysis, the understanding was that in any given metabolic pathway the metabolic rate is set by one key rate limiting enzyme responsible for the regulation of the pathway. The assumption was that there is single rate-limiting step and multiple candidates only brought confusion. In the first half of 1970s two groups Kacser & Burns (Kacser and Burns 1973) and Heinrich & Rapoport (Heinrich and Rapoport 1974, Heinrich and Rapoport 1974) described a new concept that later became known as Metabolic Control Analysis. Metabolic control analysis allows identifying different steps in the pathway by rating them with flux control coefficients. Flux control coefficient shows fractional change in the pathway flux caused by a one percent change in the rate of a particular step. Now it is possible to show that there are several rate limiting enzymes or the control can be equally shared among the pathway.

Flux control coefficient (C^{J}_{vi}), which is the degree of control that the rate (V) of a given enzyme (i) exerts on flux (J) (Moreno-Sanchez, Saavedra et al. 2008).

The definition of flux control coefficient is:

$$C_{Vi}^{J} = \frac{dJ}{dV_i} \times \frac{V_{i0}}{J_0} \qquad \textbf{(Equation 1)}$$

where dJ/dv_i is the variation in flux (J) when extremely small change occurs in the enzyme (i) activity or concentration. The scaling factor V_{io}/J_o is applied to obtain normalized and dimensionless values (Moreno-Sanchez, Saavedra et al. 2008).

In a setup where it is not possible to change the amount of the enzyme involved in mitochondrial oxidative phosphorylation, inhibitors can be used to vary their activation. In such a case where irreversible or pseudoirreversible inhibitors are used, the control strength can be calculated from inhibition curve:

$$C_i = -\frac{dJ / J}{dI / I_{\max}} \qquad \textbf{(Equation 2)}$$

were I_{max} is amount of inhibitor required for total inhibition (Groen, Wanders et al. 1982).

The different components of metabolic pathway are not independent of each other. Flux control coefficients and concentration control coefficients are clearly distinguished from each other but at the same time both are subject to summation theorems, that reflect the systemic properties, and connectivity theorems, that relate single enzyme properties to the system behavior (Schuster 1996). The summation theorem states that the sum of all flux control coefficients in a given metabolic network in linear pathway equals one (Kacser and Burns 1973, Heinrich and Rapoport 1974).

$$C^J 1 = 1 \text{ or } \sum_{k=1}^{r} C_{jk}^J = 1 \qquad \textbf{(Equation 3)}$$

Although the sum of flux control coefficients equals one in linear pathway there can be exceptions. It was shown by Westerhoff and Kholodenko that in “channeled” pathway the enzymes can have enzyme-enzyme interactions that can increase the values of flux control coefficients and they tend to be larger than in none channeled or “simple” pathways (Kholodenko, Demin et al. 1993). They showed that flux control by the enzymes and the degree of metabolite channeling can be quantified (Kholodenko and Westerhoff 1993, Peletier, Westerhoff et al. 2003). From those studies, it can be concluded that if the sum of flux control coefficients is greater than one then there is direct channeling in the pathway.

Aims of the thesis

The aim of this PhD thesis is to characterize the changes in mitochondrial function and the regulation of energy fluxes in human large colon clinical material in health and disease. For this purpose, the tissue specimens taken from the human patients undergoing surgery for colorectal cancer were studied.

The objectives of this study were following:

1. Kinetic analysis of the regulation of mitochondrial respiration in permeabilized human colorectal cancer, nearby and healthy colon tissue samples.

2. Analyze of the mitochondrial electron transport chain functionality in permeabilized human colorectal cancer, nearby and healthy colon tissue samples.

3. Quantification of the fluxcontrol of mitochondrial electron transport chain and ATP synthasome complexes in permeabilized human colorectal cancer and healthy colon tissue samples.

4. Determination of functional coupling of OXPHOS with phosphoryltransfer networks in permeabilized human colorectal cancer, nearby and healthy colon tissue samples.

Materials and methods

5. Biological materials

5.1. *Clinical material and patients*

There where total of 55 patients and they varied from age 63-92. They all had local or locally advanced disease (T2-4, N0-1, M0-1) and they had not received any radiation or chemotherapy.

Post-operative normal, nearby and tumor tissue samples (0,1-0,5 g) were obtained from North Estonia Medical Centers Oncology and Hematologic Clinic (NEMEC, Tallinn). Normal tissue samples were obtained from site distant of 5 cm from the tumor. Normal tissue consisted of colonocytes and smooth muscle cells. Nearby tissue samples were obtained next to the tumor tissue (2 cm distant). Tumor, nearby and normal control tissue samples were placed in to pre-cooled modified Mitomedium-B solution originally from Kuznetsov (Kuznetsov, Veksler et al. 2008) (0,5 mM EGTA, 3 mM $MgCl_2*6H_2O$, 3 mM KH_2PO_4, 20 mM taurine, 20 mM HEPES, 110 mM sucrose, 0,5 mM DTT, 60 mM K-lactobionate, 5 mg/ml BSA and leupeptine) and transported on ice to Bioenergetics laboratory in National Institute of Chemical Physics and Biophysics where the testing was performed on the tissue samples.

All patients understood and signed consent for tissue samples to be used in research. All investigations were approved by the Medical Research Ethics Committee (National Institute for Health Development, Tallinn) and were in accordance with Helsinki Declaration and Convention of the Council of Europe on Human Rights and Biomedicine.

5.2. *Preparation of skinned tumor fibers*

Skinned fibers were prepared according to methods described by Kuznetsov and coworkers (Kuznetsov, Veksler et al. 2008): tissue samples were dissected on ice into small (25-35 mg) fiber bundles and permeabilized in pre-cooled solution-A (20 mM imidazole, 20 mM taurine, 3 mM KH_2PO_4, 5,7 mM ATP, 15 mM PCr, 9,5 mM $MgCl_2*6H_2O$, 49 mM K-MES, 2,77 mM K_2Ca EGTA, 7,23 mM K2 EGTA, 1 µM leupeptine and 50 µg/ml saponin) for 30 minutes at 4°C. The permabilized fibers were then washed three times for 5 minutes in pre-cooled mitomedium-B solution (0,5 mM EGTA, 3 mM $MgCl_2*6H_2O$, 3 mM KH_2PO_4, 20 mM taurine, 20 mM HEPES, 110 mM sucrose, 0,5 mM DTT 60 mM K-lactobionate and 5 mg/ml BSA, pH 7.1). After washing the samples were kept at 4°C in mitomedium-B.

5.3. *Reagents*

Chemicals were purchased from Sigma-Aldrich Chemical Company. Primary and secondary antibodies were purchased from Santa Cruz Biotechnology Inc. or Abcam PLC. Rabbit polyclonal antibodies for VDAC were kindly donated by Dr. Catherine Brenner from Paris-Sud University, France.

6. Methods

6.1. *Oxygraphic measurements*

The rates of oxygen uptake where measured with high-resolution respirometer Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) in mitomedium-B solution with glutamate and malate (0,5 mM EGTA, 3 mM $MgCl_2*6H_2O$, 3 mM KH_2PO_4, 20 mM taurine, 20 mM HEPES, 110 mM sucrose, 0,5 mM DTT, 60 mM K-lactobionate, 5 mg/ml BSA, 5 mM glutamate and 2 mM malate) at 25°C to slow down the Oxygen updake and to be able to use the tissue longer. All respiration rates were normalized per mg dry weight of tissue.

6.2. *Analysis of OXPHOS coupling with adenylate kinase*

Modified protocols by Gruno (Gruno, Peet et al. 2006) where used to measure OXPHOS coupling with AK by respirometry. Mitochondrial state II respiration was activated by glutamate (at 5mM concentration), malate (at 2 mM concentration) and succinate (at 10 mM concentration) in the medium B. To stimulate mitochondria, 100 µM ATP was added to produce endogenous ADP. Adenylate kinase system coupled with ANT was activated with addition of AMP (2 mM). Cytochrome c was added to assess the intactness of mitochondrial outer membrane and AK reaction was inhibited by addition of diadenosin pentaphosphate (AP5A; at 0,2 mM concentration).

To characterize the AK coupling with OXPHOS system AK index was calculated by using the equation: ($I_{AK}=(V_{AMP}-V_{AP5A})/V_{AP5A}$). Where V_{AMP} is a normalized rate of AMP activated mitochondrial respiration and V_{AP5A} is the respiration after total inhibition of AK by AP5A (Klepinin, Ounpuu et al. 2016).

6.3. *Analysis of OXPHOS coupling with creatine kinase (MtCK)*

The steady state kinetics of MtCK reaction coupled to oxidative phosphorylation via ANT in permeabilized CRC and control tissue *in situ* was studied using the protocol shown by (Guzun, Timohhina et al. 2009). Respiration was activated by addition of MgATP at different fixed concentrations. Then 20 U/ml pyruvate kinase and 5 mM PEP were added. MtCK reaction was activated by adding creatine in increasing concentrations.

For analysis only creatine dependent respiration rates were used. These were obtained by subtracting the respiration rates after PK-PEP addition from the respiration rates in the presence of ATP and creatine.

6.4. *Immunofluorescence and confocal microscopy*

Studies of immunofluorescence and confocal microscopy where carried out as described in our previous work (Kaldma, Klepinin et al. 2014).

Inverted laser scanning confocal microscope Olympus FV10i-W equipped with a 60X water immersion objective was used. Laser excitation for secondary antibodies was for DyLight-488 488 nm and for DyLight-549 561 nm.

Immunostaining and confocal microscopy was applied to skinned fibers and paraffin-embedded sections of normal and cancer samples of human colorectal tissues. Intracellular localization of mitochondria (via VDAC immunolabeling), HK-2, and βII-tubulin where assessed.

Skinned fibers were fixed with 4% paraformaldehyde for 15 min at 37°C then treated with an antigen retrieval buffer (100 mM tris buffer with 5%, w/v urea, pH 9,5) and permeabilized with 1,0% triton X-100 for 15 min at room temperature.

Formalin fixed paraffin-embedded tissue sections where rinsed with xylene for 5 min and rehydrated step by step with 100% and 50% ethanol followed by treatment with antigen retrieval buffer at 98°C for 15 min.

Tissue samples where then blocked with 2% BSA solution in PBS and kept overnight at 4°C with primary antibodies. Monoclonal mouse anti tubulin βII (Abcam®, ab92857), polyclonal rabbit antibody for VDAC (donated by Dr. Catherine Brenner from Paris-Sud University, France) and polyclonal goat antibodies for HK-2 (SantaCruz Biotechnology Inc., sc-6521) were used. Samples were washed and incubated at room temperature for 2 hours with DyLight-488 goat anti-rabbit IgG (Abcam®, ab96899) or DyLight-549 goat anti-mouse IgG (Abcam®, ab96880)

secondary antibodies. Thereafter ProLong® Gold Antifade reagent with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Life Technologies) was used and samples were deposited between glass coverslips for confocal microscopy.

6.4.1. Quantification of mitochondrial content

The mitochondrial content in the tissue was quantified with selective marking of mitochondrial outer membrane translocase with Tom20 (SantaCruz Biotechnology, sc17764) in paraffin embedded tumor and normal colon tissue samples. The Tom20 fluorescence intensity was normalized against whole β-tubulin (Abcam®, ab6046) fluorescence.

6.5. *Assessment of enzymatic activities*

HK activity was measured as the total glucose phosphorylating capacity of whole cell extracts, using a standard glucose-6-phosphate dehydrogenase (G6PDH)-coupled spectrophotometric assay (Wilson 1989, Robey, Raval et al. 2000). One milliunit (mU) of HK activity was calculated as the amount of enzyme activity required to phosphorylate 1 nmol of glucose in 1 min at 25 °C.

The CK activity was assessed spectrophotometrically at 25 °C in the direction of ATP formation in the presence of di(adenosine-5') pentaphosphate (an adenylate kinase inhibitor (Lienhard and Secemski 1973)), 20 mM phosphocreatine (PCr) and with 2 U/ml G6PDH and 2 U/ml HK as the coupled enzymes (Monge, Beraud et al. 2009).One mU of CK activity represents the formation of 1 nmol of ATP per minute at 25°C.

AK activity of whole-cell extracts was measured at 25°C by a coupled enzyme assay (Dzeja, Vitkevicius et al. 1999). The reaction was initiated with 2 mM ADP, and the arising changes in absorbance at 340 nm were recorded using a Cary 100 Bio UV-visible spectrophotometer. One mU of AK activity represents the formation of 1 nmole of ATP per minute at 25◦C.

All enzymatic activities were normalized per mg of tissue protein. The protein content of tissue extracts was determined by a Pierce BCA Protein Assay Kit according to the manufacturer recommendations using BSA as a standard.

6.6. *RNA isolation and real-time quantitative RT-PCR*

RT –PCR analysis where carried out as described in our previous work (Vladimir Chekulayev 2015).

RNA from 29 human frozen colorectal cancer and normal colon tissue samples was isolated using trizol (Life Technologies) solution, followed by purification using the RNeasy Mini Kit (QIAGEN Sciences) with DNase treatment. Extracted RNA was dissolved in RNase-free water, quality and concentration were measured using Nanodrop and RNA was stored at −80◦C until cDNA synthesis.

For cDNA synthesis 2 g of total RNA was used. cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems). cDNA was used as a template for TaqMan® quantitative RT-PCR (qRT-PCR) analysis in the Roche LightCycler 480 system (Roche). TaqMan® Gene Expression Master Mix and FAM labeled TaqMan® (Applied Biosystems) gene assays were used to detect the mRNA expression level of the gene of interest and of actin as a reference gene. The used TaqMan® probes were the following: actin beta – Hs01060665 g1; tubulin beta 2A –Hs00742533 s1; tubulin beta 2B – Hs00603550 g; tubulin beta 2C– Hs00607181 g1; tubulin beta 3 – Hs00801390 s1; tubulin beta4 – Hs00760066 s1; tubulin beta 1/5 – Hs00742828 s1; hexokinase I – Hs00175976 m1; hexokinase II – Hs00606086 m1; creatine kinase, mitochondrial 1B – Hs00179727 m1; creatine kinase, mitochondrial 2 – Hs00176502 m1. Reactions were carried out in four replicates. Data was analyzed using the 2(−Delta Delta C(T)) method, where the gene expression levels were normalized to the level of actin beta housekeeping gene. The data of studied genes following normal distribution were parametrically tested by unpaired t-test.

6.7. *Metabolic control analysis (MCA) and determination of flux control coefficients*

MCA was performed as described previously (Groen, Wanders et al. 1982, Fell 1997, Moreno-Sanchez, Saavedra et al. 2008, Tepp, Shevchuk et al. 2011, Kaambre, Chekulayev et al. 2012).

Flux control coefficients (FCC) were calculated by using non-linear regression analysis by fitting experimental data to the mathematical model, as described by Gellerich and Small (Gellerich, Kunz et al. 1990, Small and Fell 1990).

The results were also verified by a graphical method (Gellerich, Kunz et al. 1990, Fell 2005).

6.8. *Data analysis*

Data in the text, tables and figures are presented as mean ± standard error (SEM). Results were analyzed by Student's t-test. 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.

Cutoff values were calculated by using of SigmaPlot 11 software (Systat Software,Inc).

Experimental data was fitted to non-linear regression or double reciprocal Lineweaver-Burk plot according to Michaelis-Menten equation.

Results and Discussion

7. Mitochondrial respiration

7.1. *Quality test*

To assess the quality of mitochondria we looked at the intactness of mitochondrial membranes by characterizing the respiratory activity of mitochondria in CRC cells and adjacent normal tissue *in situ* with permeabilized cell technique (**Figure 11**). This allows to study mitochondria in conditions similar to physiological ones (Kuznetsov, Veksler et al. 2008). We permeabilized the CRC and normal colon tissue with saponin and then added 2 mM exogenous ADP to activate respiration. To assess the intactness of outer mitochondrial membrane we added cytochrome-c that had no effect on the respiration of the fibers. To control the intactness of mitochondrial inner membrane we added carboxyatractyloside (CAT). CAT decreased the respiration back to the basal level due to inactivation of ANT (Saks, Veksler et al. 1998, Timohhina, Guzun et al. 2009).

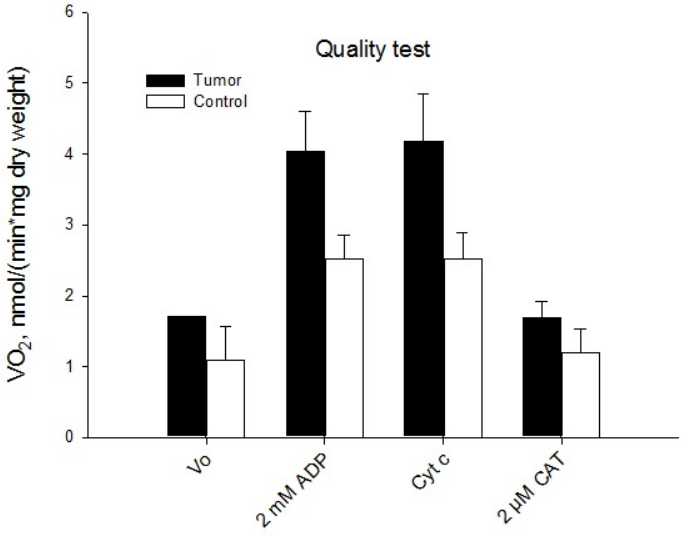

Figure 11. Quality tests of mitochondrial membrane intactness of permeabilized CRC and adjacent normal tissue samples. Experiments were conducted in the presence of respiratory substrates 5 mM glutamate, 2 mM malate and 10 mM succinate in the medium-B solution. Sequentially adding the respiratory substrates respiration was first activated with 2 mM ADP and then 8 μM cytochrome-c was added to assess the intactness of mitochondrial outer membrane. Then addition of 2 μM CAT showed the intactness of inner mitochondrial membrane by decreasing the respiration back to basal level (V_0).

7.2. *Kinetic analysis of ADP-dependent mitochondrial respiration*

To characterize mitochondrial affinity for ADP we found that the apparent Michaelis-Menten constant (K_m) and maximal respiration rate (V_m) for exogenous ADP in CRC nearby and healthy colon tissue (**Table 2**).

Maximal ADP activated respiration (V_m) was found to be much higher in tumor and nearby tissues compared to healthy colon tissue. This about 2-fold difference is most likely related to increased number of mitochondria in CRC cells. We also performed cutoff analysis of V_m values (**Figure 13 C and D**). For control-tumor cutoff value was found to be 0,88 nmol O_2/min per mg dry weight of tissue and for control nearby the value was 0,48 nmol O_2/min per mg dry weight of tissue.

Immunochemistry and confocal microscopy imaging reveled that mitochondrial content differs in CRC and healthy colon tissue about same order of magnitude as in maximal respiration rates (**Figure 12**). Expression of TOM20 was used to estimate the relative expression of mitochondria (**Figure 12 B**). Statistical difference in fluorescence signal was found ($p<0,05$).

Apparent (K_m) values for CRC and nearby tissue were much smaller than in healthy colon tissue (**Table 2**). Also we preformed cut off analysis on the samples (**Figure 13 A** and **B**) and the corresponding values for tumor-control (cutoff > 134 μM) and nearby-control (cutoff > 121 μM) tissue were very close. On the analysis of this data we can conclude that K_m normal values are above120-130 μM and pathological values are below that.

This data shows increased affinity for ADP in mitochondria *in situ* for CRC and nearby tissue samples.

Table 2. Basic respiratory parameters of human CRC, nearby and healthy colon tissue. Table shows basal respiration rate (V_0), maximal respiration rate (V_m) and ADP activated apparent K_m values. * significant difference against healthy colon tissue; [a] all respiratory rates are given as nmol /O2/min/mg dry weight of tissue; [b] The K_m and V_m the values have been determined by fitting experimental data to non-linear regression equation according to Michaelis-Menten model; [c] nearby tissue samples are taken at the border between normal and healthy colon tissue, healthy samples are taken at the site 5 cm distant from tumor.

Tissue	V_0	$K_{m\ ADP}$, μM	V_m
Colorectal cancer [a]	1,99±0,26	93,6±7,7 [b]	3,82±0,32 *
Nearby tisuse [c]	1,64±0,27	84,9±9,9 [b]	2,98±0,34 *
Healthy colon tisuse [c]	1,13±0,12	256±34 [b]	1,92±0,14

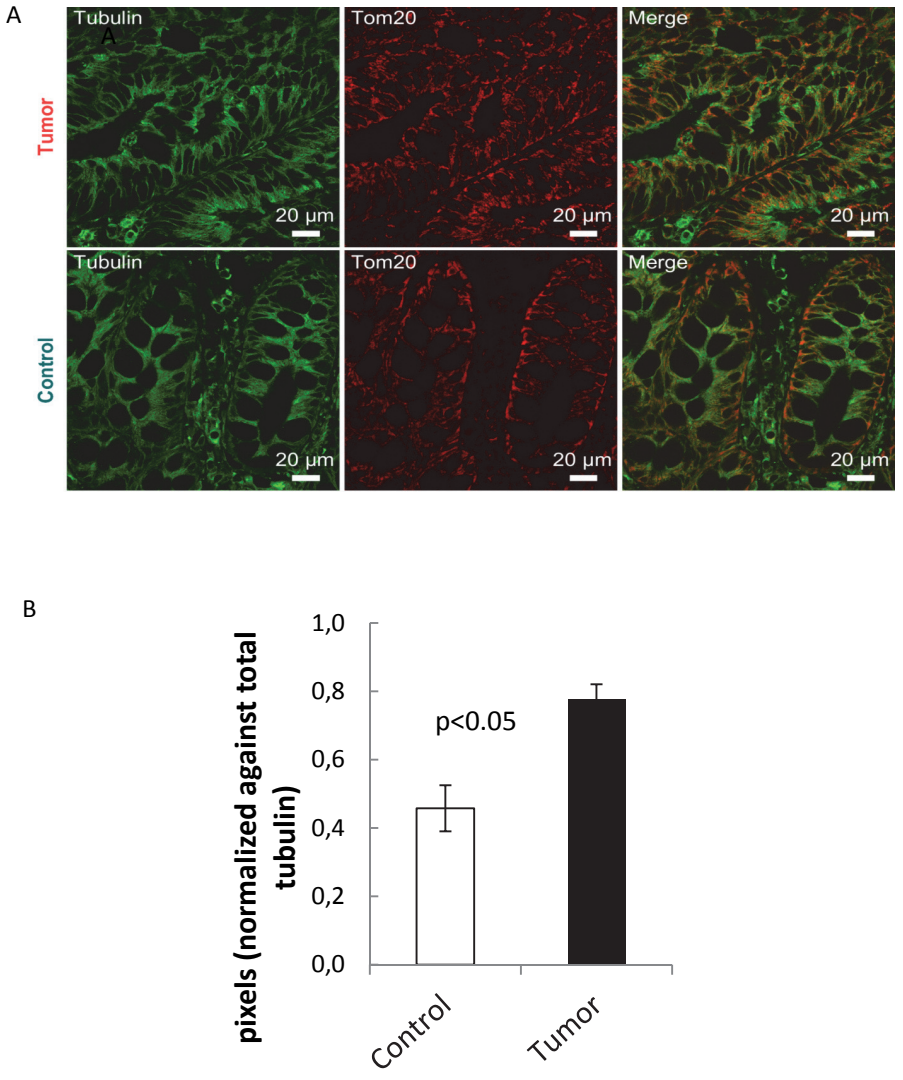

Figure 12. Immunohistochemistry quantification of the total mitochondrial content in postoperative tissue samples (A) Confocal microscopy imaging of CRC and healthy colon tissue samples. Florescence signal intensities where normalized aginst total β-tubuline florescence intensities (B) Graphical representation of signal intensities. Bars are SEM, n7, p<0,05.

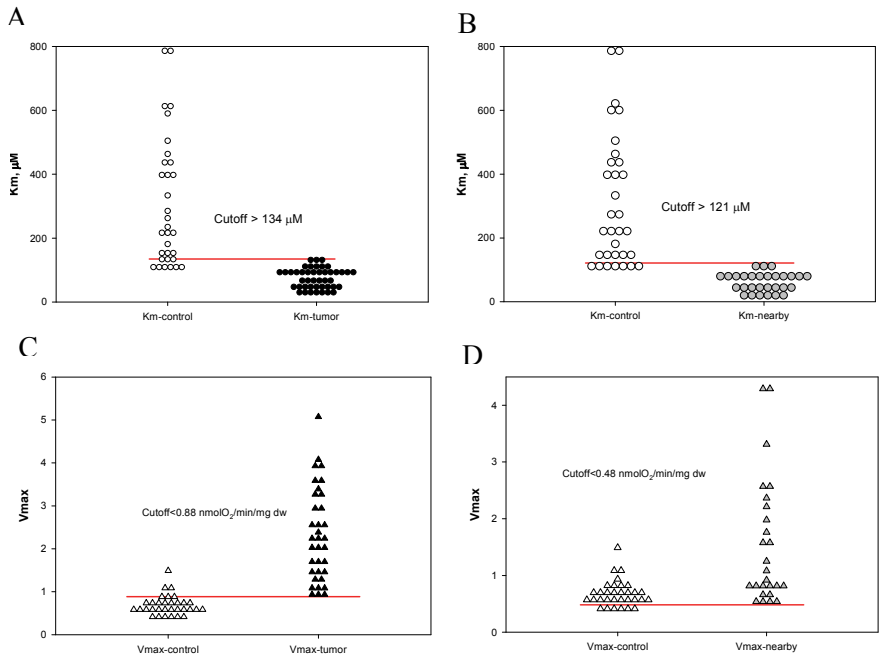

Figure 13. Cutoff analysis of apparent K_m (ADP) values and V_m values. (A) K_m control-tumor (B) K_m control-nearby (C) V_m control-tumor (D) V_m control-nearby.

To determine if our tissue samples had different populations of mitochondria with different OXPHOS capacity we analyzed the kinetics of respiration of skinned fibers with exogenously added ADP as proposed by Saks and coworkers (Saks, Veksler et al. 1998). Double reciprocal Lineweaver-Burk plot analysis showed that there are two subpopulations in healthy colon tissue but CRC and nearby tissue are homogenous (**Figure 14**). One subpopulation of mitochondria in healthy colon tissue has low K_m value of 16,5 ± 5,8 µM and another subpopulation has high K_m value of 288 ± 67 µM. This remarkable difference in K_m values makes it possible to see those 2 different populations. Most probably we are dealing here with different colon wall tissues like mucosa and smooth muscle. In nearby as well as in CRC the apparent K_m value for ADP is 69 ± 10 µM.

Based on these results we concluded that in colorectal carcinogenesis the amount of mitochondria rises and that there are changes in the regulation of MOM permeability for ADP.

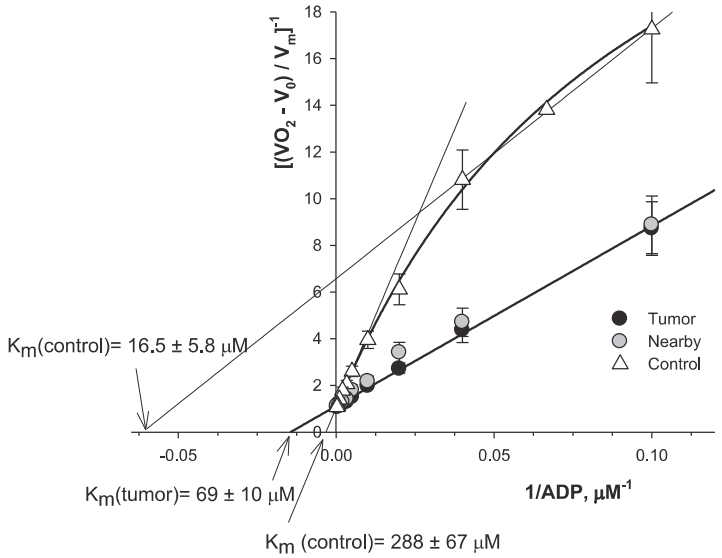

Figure 14. The dependance of the normalized values of respiration rates of permeabilized tissue samples: double reciporal Lineweaver-Burk plots of CRC nearby and healthy colon tissue (n=9)

7.3. ***Functionality of OXPHOS system in CRC and in surrounding tissues***

We analyzed the mitochondrial electron transport chain (ETC) components using saponin- skinned samples in non-neoplastic, CRC and nearby tissue samples (**Figure 15**). We found that nearby tissue has slightly higher state 2 and state 3 respirations then healthy colon tissue. In the presence of respiratory substrates (2 mM malate and 5 mM glutamate) the mean value of V_0 was assayed as 1,59±0,37 nmol O_2/min/mg dry tissue weight. After addition of ADP (2 mM) the respiratory rate was increased by almost 2 times to 2,97±0,53 nmol O_2/min/mg dry tissue weight ($p<0,05$). This increase in respiration is confirming the presence of functionally-active mitochondria. State 3 respiration is ~1, 5 times higher for nearby and CRC tissues compared to healthy colon tissue and also compared to state 2 respiration. Addition of rotenone (20 mM) inhibited

respiration in all studied tissue samples which means that Complex-I is active in all of them. The difference between respiration rates in tissues is statistically not significant. These results show that development of colorectal cancer is not accompanied by suppression of Complex-I related respiration. To look at the Complex-II dependent respiration we added Complex-II substrate succinate (10 mM) which led to increase in respiration suggesting that the Complex-II is functionally active in these tissues. Respiration was inhibited with antimycin-A (10 μM) that is Complex-III inhibitor. Results show that Complex-III is also functional in all studied tissues. To activate cytochrome-c oxidase (COX) we added N,N,N',N'-tetramethyl-phenylenediamine (TMPD) (1 mM) with ascorbate (5 mM) which resulted in increase in respiration of all tissues. In the end cytochrome-c was added to assess the quality of examined tissue samples outer mitochondrial membrane.

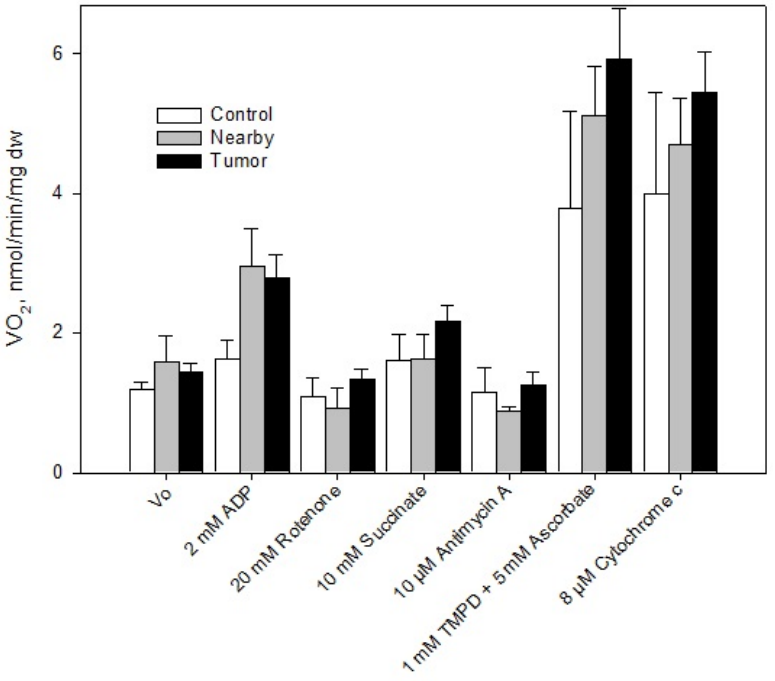

Figure 15. Analysis of the mitochondrial respiratory chain. These measurements were carried out on human CRC, nearby and healthy colon tissue with respiratory substrates 2 mM malate and 5 mM glutamate in the respiratory medium-B. All respiratory substrates and inhibitors where added in sequential order as shown on the x-axis of the figure.

7.4. ***Metabolic control analysis***

To determine the key regulators in mitochondrial respiratory chain we applied MCA method on CRC and healthy colon tissue. Mitochondrial respiration was activated with exogenously added 2 mM ADP after addition of respiratory substrates and then every respiratory chain complex was inhibited with titrating increasing concentrations of complex specific inhibitors. (**Figure 16)** shows an example of O_2 consumption trace in steady state conditions in permeabilized CRC and healthy tissue fibers on increasing concentrations of complex-II inhibitor 3-nitropropionic acid (3-NP).

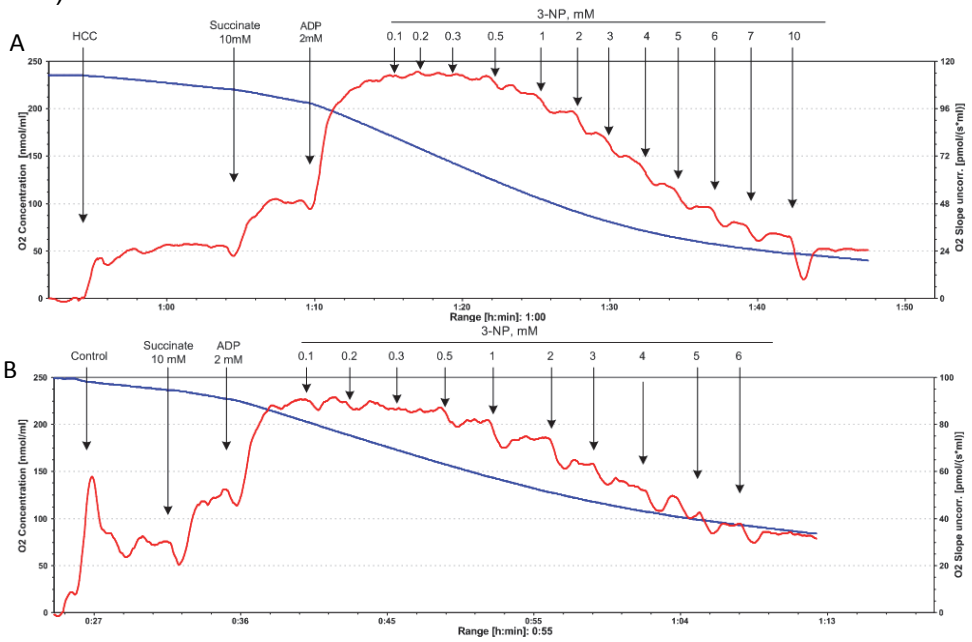

Figure 16. Oxygraphic tracing of changes in the O_2 consumtion (red lines) in CRC (A) and adjecant healthy colon tissue (B) durning theyr titration with 3-nitropropionic acid (Complex-II inhibitor) after activation of respiration with 2 mM ADP in the presence of respiratory substrates.

Similar O_2 consumption traces were obtained for all electron transport chain and ATP synthasome complexes (**Figure 17 A** and **B**). They were plotted as relative rates of O_2 consumption vs. concentration of used inhibitor. The flux control coefficients (FCC) were calculated based on the obtained experimental data, according to the model described by Gellerich and coworkers (Gellerich, Kunz et al. 1990, Wisniewski, Gellerich et al. 1995). FCCs for ETC and ATP synthasome complexes were analyzed as

described before (Tepp, Shevchuk et al. 2011, Kaambre, Chekulayev et al. 2012). We examined NADH dependent electron transfer and FADH dependent electron transfer (**Figure 17 C** and **D**). Most of the FCCs values were similar for CRC and healthy colon tissue with the exception of Complex-III. The sum for FCC values for NADH dependent pathway for CRC are 3,05 ± 0,23 and for healthy tissue 3,15±0,59. And for FADH dependent pathway this values are for CRC 3,03 ± 0,20 and for healthy colon tissue 3,25±0,49. Obtained sum of FCC values exceed significantly the theoretical value for linear system (close to 1). Therefore, we can assume that respiratory chain and ATP synthasome complexes are forming super-complexes and there could also be direct channeling of substrates between the protein complexes.

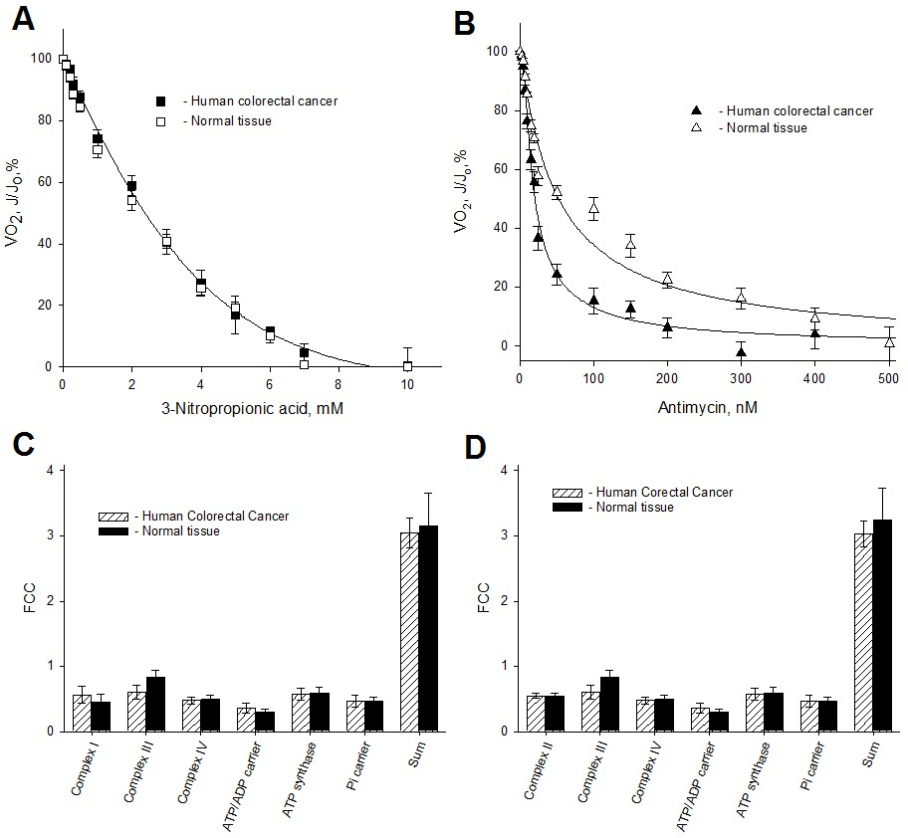

Figure 17. Titration curves of the inhibition of the mitochondrial respiratory chain complexes in human CRC and healthy colon tissue with 3-nitropropionic acid (A) and antimycin (B). Flux control coefficients for MI supercomplex: (C) electron transfer through NADH and (D) succinate dependent electron transfer through FADH. Similar titration curves as shown in Fig. 17 were constructed for other inhibitors of MI components (supplementary figure 4 in (Kaldma, Klepinin et al. 2014)). Every data point was calculated as the mean of 10-15 experiments and bars are SEM.

7.5. *OXPHOS coupling to creatine kinase, adenylate kinase and hexokinase phosphoryltransfer pathways*

We found that total CK activity is much lower (~2,5 times) in nearby and cancer tissue compared with healthy colon tissue (**Table 3**). But at the same time qRT-PCR show that sMtCK (CKMT2) mRNA is up regulated by 6,8 fold (p=0,06) and the uMtCK (CKMT1) is down regulated by 2 fold (p=0,009) in tumor tissue (**Figure 18**). General MtCK gene expression level is much higher in tumor tissue then in healthy colon tissue.

Table 3. Enzymatic activities of the human CRC, nearby and healthy colon tissue samples. P values where calculated by Students test vs. control non-tumorous tissue data: numbers of examined samples was 11-16.

Enzyme activities, mU (s) per mg proteiin	**Tissues, mean ± SE**		
	Healthy colon tissue	Nearby	Colorectal cancer
Hexokinase	244±50	172±30 p=0,12	215±40 p=0,33
Creatine kinase	497±142	202±52 p<0,05	204±84 p<0,05
Adenylate kinase	257±35	256±35 p=0,49	411±43 p<0,05

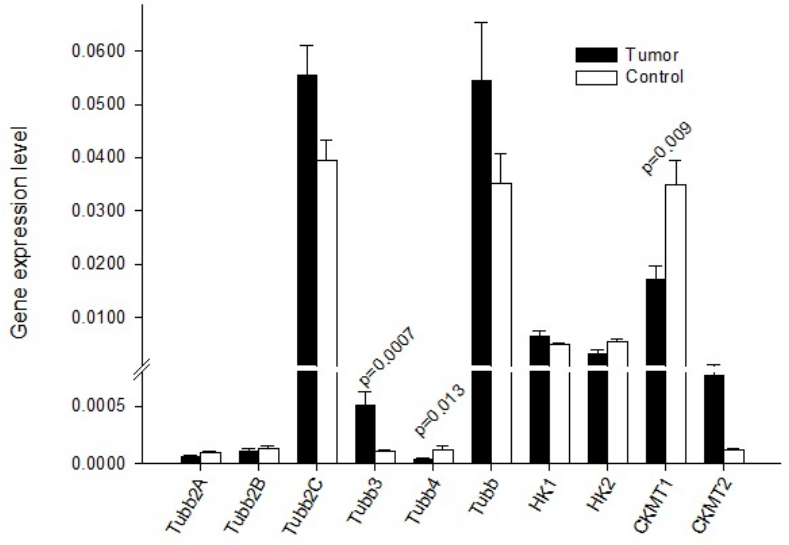

Figure 18. Expression levels of different tubuline heksokinase and creatine kinase isotype mRNA's in human CRC and healthy colon tissue. mRNA levels where assessed by qRT-PCR (bars are SEM, n=29)

Considering the low activity and high expression, we performed a study to estimate the role of MtCK in energy transfer (**Figure 19**). We added respiratory substrates and activated mitochondrial respiration with 0,2 mM ATP. We then added 30 U/ml pyruvate kinase (PYK) and 5 mM phosphoenolpyruvate (PEP) for trapping extramitochondrial ADP to follow only the mitochondrial creatine kinase (MtCK). Addition of 10 mM Cr increased respiration only in healthy colon tissue nearly 60% due do activation of MtCK. These experiments on CK suggest that mitochondria of tumorous as well as nearby tissue have decreased capacity to produce PCr.

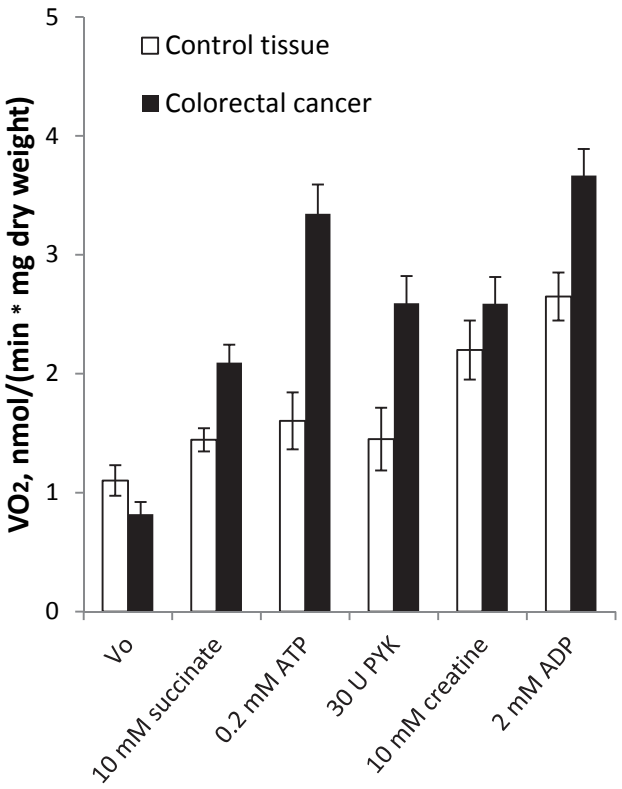

Figure 19. Creatine effect on the OXPHOS by mitochondrial CK through local ADP production in human CRC and healthy colon tissue. Respiratory substrates 5 mM glutamate and 2 mM malate where in medium-B and 10 mM succinate was added before activaiting the respiration with 0,2 mM MgATP that induced production of endogenous ADP in MgATPase reaction. Then PEP-PYK was added to trap all extramitochondrial ADP. Creatine in these conditions activates mitochondrial CK reaction, local MgADP recycling and oxygen consumtion rate to the maximal value. Finally 2 mM ADP was added. Bars are SEM, n=8

Because of decreased activity of CK and absence of MtCK and OXPHOS coupling we decided to look at the AK catalyzed energy transfer processes and coupling with OXPHOS. The total AK activity was about 1,6 times higher in tumorous tissue then in healthy and nearby colon tissues that had similar values (**Table 3**). These results indicate that AK system is up regulated in CRC.

To assess functional coupling of AK catalyzed processes and OXPHOS we tracked the respiration of CRC control and nearby tissues in specific conditions (**Figure 20**). We added respiratory substrates and 0,1 mM Mg-ATP and after that addition of 2 mM AMP resulted in ~40% increase in respiration of CRC fibers. Cytochrome-c had no effect on samples indicating the intactness of MOM. AK inhibitor diadenosine

pentaphosphate (AP5A) at 0,2 mM concentration inhibited respiration to initial level. Therefore, the respiration was mediated by ADP produced in AK reactions. For nearby and healthy colon tissue samples the results were about 1,5 times lower.

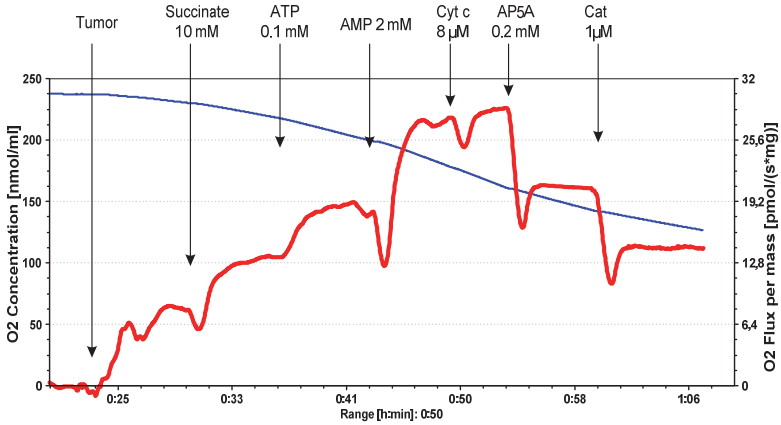

Figure 20. Oxygraphic tracing to assess functional coupling of adenylate kinase catalyzed processes and OXPHOS. Red line represents O_2 consumption. With respiratory substrates in the medium-B the respiration was initiated with 2 mM AMP in the presence of ATP. Addition of AMP activated mitochondrial respiration by formation of ADP in AK reactions. To confirm the AK reactions, specific AK inhibitor AP5A was added. Addition of AP5A resulted in strong decrease in the rate of O_2 consumtion by CRC. Same experiments where preformed with nearby and healthy colon tissue. Cytochrome c was added to confirm the intactness of MOM and in the end CAT a selective inhibitor of adenine nucleotide traslocator was added.

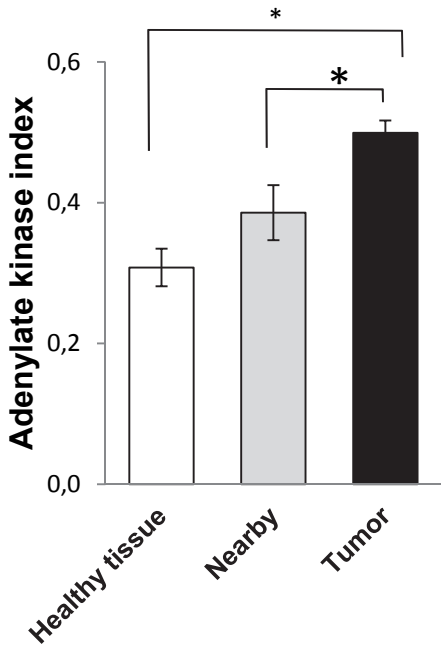

Figure 21. Efficiency of coupling of AK catalyzed processes and OXPHOS was estimated by means of AK index. Bars are SEM, n=10; *p<0,05

To further assess the coupling between OXPHOS and AK we applied the AK index (I_{AK}) method proposed by Gruno et al (Gruno, Peet et al. 2006). I_{AK} for CRC (I_{AK}=0,50±0,02, n=12) is much higher than for healthy colon (I_{AK}=0,31±0,03, n=8) and nearby (I_{AK}=0,39±0,04, n=10) tissue (**Figure 21**). These results indicate AK up regulation and stronger coupling between OXPHOS and AK in tumorous tissue.

Appearance of Warburg effect has been considered as an important change that takes place in tumor cells so we looked at the mechanisms underlying glucose fermentation. We found that hexokinase activity was similar in CRC cells as well as in healthy colon tissue (**Table 3**). Also our qRT-PCR results showed that HK-1 and HK-2 genes are expressed in all tissue types (**Figure 18, Figure 23**). Expression levels are quite similar for HK-1 in CRC cells and in healthy colon tissue but expression levels for HK-2 are much lower for CRC cells (p=0,01) (**Figure 23**). We also looked at the coupling between OXPHOS and HK by oxygraphic analysis (**Figure 22, Figure 24 A**). After addition of 0,1 mM MgATP we added 10 mM glucose that showed some stimulatory effect on CRC fibers and the stimulatory effect was about 30-35% of the 2mM ADP mediated activation. This glucose stimulatory effect was not seen on healthy colon tissue. For

characterization of glucose effect on mitochondria we also calculated the glucose index (I_{GLU}) that allows to compare glucose stimulation in maximally activated respiration (in the presence of 2 mM ADP) (**Figure 24 B**). CRC at I_{GLU} =19,1% and nearby tissue at I_{GLU} =18,7% have very similar values, but healthy colon tissue has I_{GLU} =11,7%

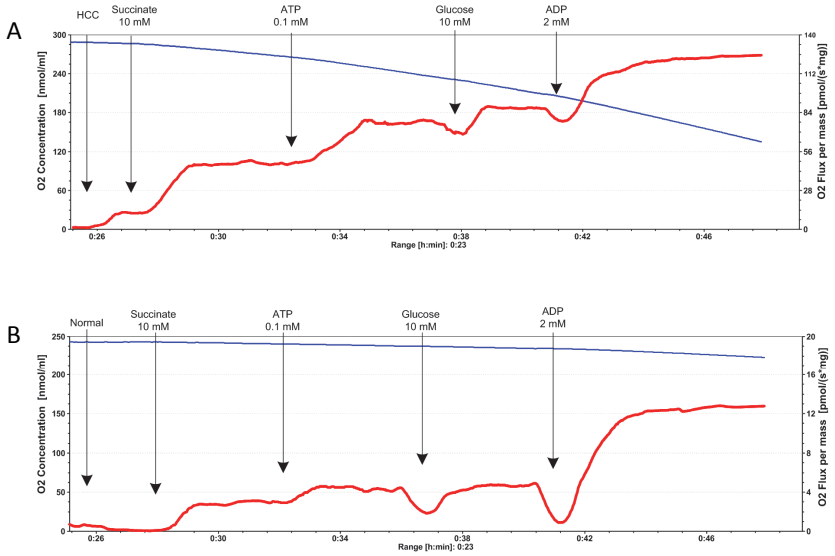

Figure 22. Oxygraphic tracing to analysis coupling of HK to OXPHOS in CRC (A) and healthy colon tissue (B). Red line is O_2 consumption. In the presence of respiratory substrates and 0,1 mM MgATP addition of 10mM glucose resulted a stimulatory effect for CRC tissue fibers but this effect was not observed on healty colon tissue.

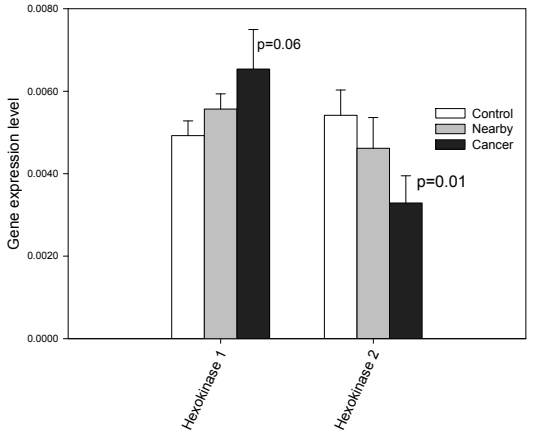

Figure 23. Analysis of mRNA expression levels with Q-PCR for CRC nearby and healthy colon tissue. Bars are SEM; p=0,06 for HK1 and p=0,01 for HK2; n=29.

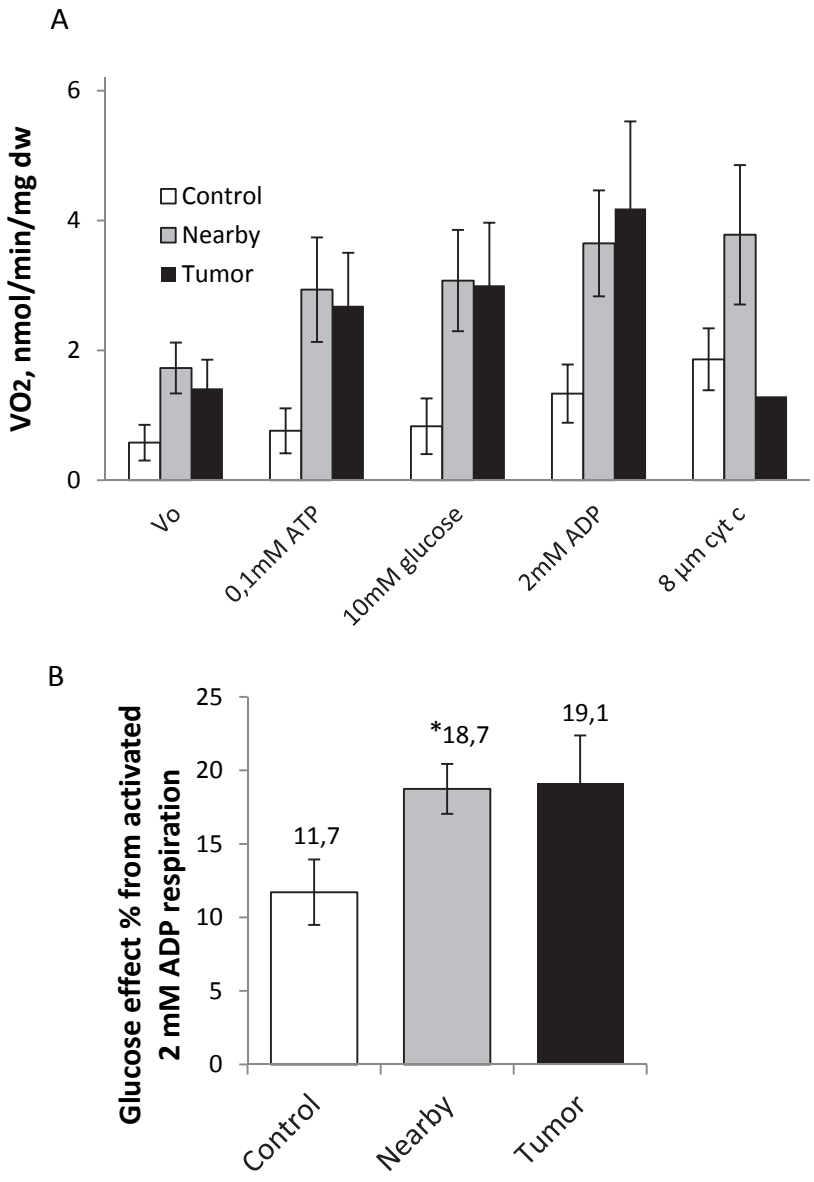

Figure 24. Glucose effect. (A) Oxygraphic analysis of coupling of HK to OXPHOS in CRC nearby and healthy colon tissue. Addition of 10 mM glucose caused an stimulatory effect on CRC and nearby tissue samples. (B) The comparason of glucose index (Glucose effect %=(V_{glu}-V_{ATP})*100/(V_{ADP}-V_{ATP})) on crc nearby and healthy tissue samples. Bars are SEM; n=5; * p<0,05.

8. Discussion

It is widely accepted that tumor cells produce energy predominantly through glycolysis. This idea has originated from the Otto Warburg discovery in the beginning of 20th century, where he describes high rates of glucose uptake in the presence of oxygen and increased lactate production. Number of researchers has shown that not all tumors are solely glycolytic (Zheng 2012, Moreno-Sanchez, Marin-Hernandez et al. 2014), the mitochondrial metabolism is not suppressed and OXPHOS is functionally active in tumor cells. These innovative findings bring up new potential targets in drug therapy associated with OXPHOS for oxidative type tumors (Haq, Shoag et al. 2013, Vazquez, Lim et al. 2013).

Several studies have been carried out in order to understand the regulation of metabolism *in vitro* (cell cultures) mostly characterizing human CRC as glycolytic tumor (Chung, Lee et al. 1999, Huang, Kuo et al. 2013, Abrantes, Tavares et al. 2014, Song, Qin et al. 2014, Amorim, Pinheiro et al. 2015, Zhang, Yu et al. 2015). However, cells grown in cultures differ from *in vivo* conditions. Ertel and colleges (Ertel, Verghese et al. 2006) have shown that there are differences in cellular pathways between cell lines and tumor and normal tissue samples. Comparison of genomic profiles of cell lines and tumor cell samples has revealed that most of the cell lines do not resemble very closely tumor profiles (Domcke, Sinha et al. 2013). For these reasons it is important to study human tissue samples. Little is known about mechanisms underlying the energy production of human CRC cells.

In our study we found that human CRC cells influence the nearby tissue so that energy metabolism in nearby tissue becomes similar to tumor tissue. Nearby tissue samples have, similarly to tumor tissue samples, upregulation of mitochondrial respiration (**Table 2**) with active mitochondria, fully functional respiratory chain (**Figure 15**) and nearby tissue is not with purely glycolytic energy metabolism (**Table 3**). It is unknown how the tumor cells are influencing the nearby tissue and further work on the subject is needed.

There is difference in kinetics of respiration regulation by ADP in CRC and healthy colon tissue. In nearby tissue as well as in CRC tissue the mitochondria show increased affinity for the exogenously added ADP. The apparent K_m value for exogenously added ADP was found to be almost 3 times lower than for healthy colon tissue (**Table 3)**. The cutoff analysis of obtained K_m values further showed strong differences in regulation of mitochondrial respiration by revealing that there is a boundary where above 120-130 μM K_m value for healthy tissue and below that CRC and affected NB tissues (**Figure 13 A and B**). This difference in K_m values shows clearly that there are diffusion restrictions for extracellular ADP in healthy tissue that is missing in tumor and nearby tissue. One explanation

for those changes in regulation of ADP-activated respiration would be that the higher K_m value in healthy tissue could be from utilization of ADP in AK reactions, but this is unlikely as the AK activity was even lower than in tumor tissue (**Table 2**). Also Saks et al (Saks, Belikova et al. 1991) has shown in skinned muscle fibers that complete inhibition of AK has no effect on the affinity of mitochondria for ADP. Another explanation for the diffusion restriction for extracellular ADP in healthy colon tissue could be that there is much better developed cytoskeleton (Vendelin, Eimre et al. 2004, Kaambre, Chekulayev et al. 2012). It has been shown that βII-tubulin could bind to VDAC and through that selectively limits the permeability of the MOM for adenine nucleotides (Rostovtseva, Sheldon et al. 2008, Guzun, Karu-Varikmaa et al. 2011). In that case βII-tubulin could be responsible for higher K_m values in healthy colon tissue. Previous studies have shown βII-tubulin as the potential key regulator of VDAC permeability in adult rat cardiomyotcytes (Gonzalez-Granillo, Grichine et al. 2012, Guzun, Gonzalez-Granillo et al. 2012). It has been shown that there is no binding of βII-tubulin with VDAC in the case of CRC (Kaldma, Klepinin et al. 2014, Vladimir Chekulayev 2015). Also there can be differences in expression patterns and isoforms of VDAC, ANT and also tubulin that could be responsible for the selective permeability of VDAC channel.

Because our results show that OXPHOS could play a key role in ATP generation we carried out MCA inhibition titration studies on Complex I, II, III, IV, ATP synthase, ANT and PIC with respiration activation with exogenously added ADP. All received FCC's were found to be with relatively high values (**Figure 17**). Interestingly, in human CRC as well as in healthy colon tissue the control is distributed rather equally between the ATP synthasome complexes. Key sites in respiration regulation are ATP synthasome and complex III.

The sum of FCC's for ADP activated respiration in CRC and in healthy colon tissue is more then 3 (**Figure 17**) while the theoretical value on a linear system is close to 1 (Kholodenko and Westerhoff 1993). For example in isolated mitochondria and in permeabilized cardiac muscle cells with ADP activated respiration the value of FCC's is close to 1 (Tepp, Shevchuk et al. 2011). High FCC values have been shown in Cr activated respiration in rat heart cells and in permeabilized neuroblastoma cells (Tepp, Shevchuk et al. 2011, Klepinin, Chekulayev et al. 2014). Like described previously the theoretical value in linear system for FCC's should be close to 1, but it can be much higher if there are enzyme-enzyme interactions, direct substrate channeling or recycling within the complexes (Kholodenko, Demin et al. 1993, Kholodenko and Westerhoff 1993). Those high values for FCC's seen in the case of human CRC and healthy colon wall samples in ADP activated respiration indicates that there are some changes in respiratory chain compared with non-proliferating and highly-differentiated cardiac and some skeletal muscle cells.

These results (**Figure 17**) are similar with some other authors that have shown high FCC's and confirming super complexes with electron microscopy, native gel-electrophoresis and single particle image processing in different samples (Bianchi, Genova et al. 2004, Genova, Baracca et al. 2008, Vonck and Schafer 2009, Dudkina, Kouril et al. 2010, Lenaz and Genova 2010, Dudkina, Kudryashev et al. 2011, Quarato, Piccoli et al. 2011). Supramolecular respiratory complexes (respirasomes) seem to be characteristic for cells with high proliferative rates. Therefore, human colon tissue mitochondrial OXPHOS system also seems to contain super complexes with direct substrate channeling.

Respiratory supercomplexes have been suggested to help avoid competition by channeling ubiquinol and cytochrome c from other enzymes, stabilization of respiratory chain complexes and reduction of substrate diffusion times, prevention of superoxide generation and protection from apoptosis by suppression of cytochrome c release in to the cytoplasm (Dudkina, Kouril et al. 2010, Hoefs, Rodenburg et al. 2012).

Our result confirmed that *in situ* human colorectal cancer shows glycolytic activity close to healthy colon tissue (**Table 2)** and up-regulation of OXPHOS. We saw that rate of maximal mitochondrial respiration was much higher in CRC and NB tissue samples then in healthy colon tissue **(Table 2)** and also cutoff analysis for maximal respiration rate reveled similar results for CRC and NB (**Figure 13 C and D**). These results can be associated with increased amount of mitochondria in CRC cells **(Figure 12**). It has been shown that mitochondrial biogenesis may be up-regulated in some cancers (Jose and Rossignol 2013). In colorectal carcinoma there has been shown overexpression of MYC proto-oncogene (Dang, Le et al. 2009) which has been associated with increased mitochondrial biogenesis. In contrast it has been shown that MYC null rat fibroblasts have smaller mitochondrial mass and decreased number of normal mitochondria (Li, Wang et al. 2005).

Higher rates of OXPHOS seen in CRC (**Table 2**) are not in correlation with other authors who have shown that β-F1-ATPase catalytic subunit of the mitochondrial H^+-ATP synthase is downregulated in human CRC (Sanchez-Cenizo, Formentini et al. 2010, Willers, Isidoro et al. 2010) and that is associated with the up regulation of the ATPase inhibitory factor 1 expression (Formentini, Sanchez-Arago et al. 2012). These changes should be suppressing OXPHOS but our results do not confirm this. Instead our results support hypothesis to explain mechanisms underlying Warburg effect by Pedersen and coworkers (Pedersen 2007) who showed that HK-2 could be responsible for increased glycolysis through binding to VDAC channel and consuming most of the energy produced by mitochondria. Our results support this hypothesis as we saw that addition of glucose to human CRC fibers increased their respiration rates (**Figure 24**). Same effect was seen on nearby tissue samples but it was negligible

on the healthy colon tissue samples. With Q-PCR analysis we confirmed that all tissue samples have HK-1 and HK-2 expressed but at slightly different expression levels (**Figure 23**). It has been reported that tumor cells have an increased number of VDAC channels in mitochondria and increased cholesterol amount in MOM and due to that they have elevated binding capacity for HK1 and HK2 (Pastorino and Hoek 2008). We have registered increased levels of VDAC in human CRC tissue cells (Vladimir Chekulayev 2015) and for that reason it is plausible that exogenously added glucose has negligible effect on normal tissue cells (**Figure 24**) although the HK1 expression levels are similar (**Figure 23**). There are several different VDAC isoforms but they have similar kinetic characteristics witch indicates that HK binding differences are due to quantitative differences in binding site availability (Shinohara, Ishida et al. 2000).

In normal colonocytes and muscle cells CK system produces PCr and maintains energy homeostasis but in CRC and nearby tissue this system is switched off or down regulated (**Table 3**). These results are in good agreement with some literature data (Joseph, Cardesa et al. 1997). In normal colon tissue cells we found ubiquitous MtCK to be upregulated (**Figure 18)** and there was a strong coupling between the MtCK and OXPHOS system but this functional coupling was missing in CRC tissue samples (**Figure 19**). These results show that MtCK has only minor role in the cellular energy transfer and functional coupling of MtCK with ANT in CRC cells is weak. Downregulation of brain type CK may play an important role in human colorectal cancer progression by promotion of epithelial to mesenchymal transition in these cells (Mooney, Rajagopalan et al. 2011). Interestingly CRC cells seem to exert a distant effect on the nearby tissue because CK system is also down regulated there (**Table 3**). This down regulation in CRC and nearby tissue samples indicates that both these tissues have lost their ability to synthesize PCr. Other authors have also shown down regulation of CK and PCr system (Kasimos, Merchant et al. 1990).

In contrast AK system regulates cellular adenine nucleotide pool and energy charge of cells. In human colon epithelial cells there are two AK isoenzymes; cytosolic (AK1) and mitochondrial (AK2) isoenzymes (Birkenkamp-Demtroder, Olesen et al. 2005). Little is known about AK system role in the maintenance of human CRC energy homeostasis but in some malignancies expression of AK2 has been found to generate up to 50% of ATP used in the cancer cells (Nelson and Kabir 1985). It has been shown that suppression of CK phosphotransfer system can lead to up regulation of AK phosphotransfer system to compensate the energy need (Dzeja, Zeleznikar et al. 1996). We found that AK activity in CRC is about 1,6 fold higher than in healthy colon tissue and also in nearby tissue (**Table 3**). We looked at the functional coupling of AK catalyzed processes with

OXPHOS in all three tissue samples. In respiratory experiments we saw ~40% increase in oxygen consumption with AMP in CRC samples (**Figure 20),** meaning that respiration was fueled by ADP that was produced in AK reactions. Non tumorous tissues showed about 1,5 times lower stimulatory effect on the addition of exogenous AMP then CRC samples. In addition the AK index (I_{AK}) proposed by Gruno et al (Gruno, Peet et al. 2006) was much higher for CRC then for healthy colon and nearby tissue **(Figure 21**).

Taken together our respiratory study's showed that in CRC tissue the coupling between AK and OXPHOS system is higher than in healthy colon tissue (**Figure 20 Figure 21**).

Our results emphasize the need to use real tissues with their complex surroundings and intertwined relations to surrounding cells instead of homogenous cell cultures. The cancer itself can't be considered as one homogenous mass and all tumors cannot be considered inherently glycolytic. We have shown that there is slight difference between CRC and NB but they are more similar in many aspects to each other than to healthy tissue.

Conclusions

1. It was shown that human colorectal cancer is not hypoxic tumor and the rate of oxygen consumption is even higher than in adjacent healthy colon tissue. There is an increase in the number of mitochondria accompanied with increase in the mitochondrial outer membrane permeability for adenine nucleotides.

2. Direct measurements showed that human colorectal cancer has fully functional electron transport chain.

3. By applying metabolic control analysis, it was quantitatively shown that all the ATP synthasome complexes remain almost unchanged with only minor differences seen in electron transport chain complexes. Sum of all flux control coefficients where found to be much higher than the theoretical value 1 for a linear system suggesting the presence of respiratory chain supercomplexes or direct substrate channeling within the ATP synthasome and ETC complexes.

4. Measurements on CK mediated energy transfer network and AK mediated energy transfer network showed that PCr/CK shuttle is downregulated and AK mediated energy transport system is upregulated in human CRC. Because CK mediated energy transport is replaced with AK mediated energy transport there is also decreased ability to produce PCr.

5. CRC exerts distant effect on the nearby tissue cells. Nearby tissue is similar to tumor tissue with upregulation of mitochondrial respiration and with mitochondria that have functional respiratory chain. CK system is down regulated and permeability of MOM for adenine nucleotides is increased.

Reference:

Abrantes, A. M., L. C. Tavares, S. Pires, J. Casalta-Lopes, C. Mendes, M. Simoes, M. M. Grazina, R. A. Carvalho and M. F. Botelho (2014). "Metabolic effects of hypoxia in colorectal cancer by 13C NMR isotopomer analysis." Biomed Res Int **2014**: 759791.

Accioly, M. T., P. Pacheco, C. M. Maya-Monteiro, N. Carrossini, B. K. Robbs, S. S. Oliveira, C. Kaufmann, J. A. Morgado-Diaz, P. T. Bozza and J. P. Viola (2008). "Lipid bodies are reservoirs of cyclooxygenase-2 and sites of prostaglandin-E2 synthesis in colon cancer cells." Cancer Res **68**(6): 1732-1740.

AICR American Institute for Cancer Research and World Cancer Research Fund (2007). Food, Nutrition, Physical Activity, and the Prevention of Cancer: A Global Perspective. Washington DC.

AJCC, a. j. c. o. c. (2002). AJCC cancer staging manual sixth eddition. USA, Springer.

Alberts, B., J. H. Wilson and T. Hunt (2008). Molecular biology of the cell. New York, Garland Science.

Altenberg, B. and K. O. Greulich (2004). "Genes of glycolysis are ubiquitously overexpressed in 24 cancer classes." Genomics **84**(6): 1014-1020.

Amoedo, N. D., M. F. Rodrigues and F. D. Rumjanek (2014). "Mitochondria: are mitochondria accessory to metastasis?" Int J Biochem Cell Biol **51**: 53-57.

Amorim, R., C. Pinheiro, V. Miranda-Goncalves, H. Pereira, M. P. Moyer, A. Preto and F. Baltazar (2015). "Monocarboxylate transport inhibition potentiates the cytotoxic effect of 5-fluorouracil in colorectal cancer cells." Cancer Lett **365**(1): 68-78.

Austoker, J. (1994). "Screening for colorectal cancer." BMJ **309**(6951): 382-386.

Baumann, O. and D. B. Murphy (1995). "Microtubule-associated movement of mitochondria and small particles in Acanthamoeba castellanii." Cell Motil Cytoskeleton **32**(4): 305-317.

Berrino, F., R. De Angelis, M. Sant, S. Rosso, M. Bielska-Lasota, J. W. Coebergh, M. Santaquilani and E. W. group (2007). "Survival for eight major cancers and all cancers combined for European adults diagnosed in 1995-99: results of the EUROCARE-4 study." Lancet Oncol **8**(9): 773-783.

Bianchi, C., M. L. Genova, G. Parenti Castelli and G. Lenaz (2004). "The mitochondrial respiratory chain is partially organized in a supercomplex assembly: kinetic evidence using flux control analysis." J Biol Chem **279**(35): 36562-36569.

Birkenkamp-Demtroder, K., S. H. Olesen, F. B. Sorensen, S. Laurberg, P. Laiho, L. A. Aaltonen and T. F. Orntoft (2005). "Differential gene

expression in colon cancer of the caecum versus the sigmoid and rectosigmoid." Gut **54**(3): 374-384.
Bittl, J. A. and J. S. Ingwall (1985). "Reaction rates of creatine kinase and ATP synthesis in the isolated rat heart. A 31P NMR magnetization transfer study." J Biol Chem **260**(6): 3512-3517.
Boland, C. R. and A. Goel (2010). "Microsatellite instability in colorectal cancer." Gastroenterology **138**(6): 2073-2087 e2073.
Boland, C. R., S. N. Thibodeau, S. R. Hamilton, D. Sidransky, J. R. Eshleman, R. W. Burt, S. J. Meltzer, M. A. Rodriguez-Bigas, R. Fodde, G. N. Ranzani and S. Srivastava (1998). "A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer." Cancer Res **58**(22): 5248-5257.
Brenner, C. and S. Grimm (2006). "The permeability transition pore complex in cancer cell death." Oncogene **25**(34): 4744-4756.
Bretthauer, M. (2011). "Colorectal cancer screening." J Intern Med **270**(2): 87-98.
Brooks, G. A. (2009). "Cell-cell and intracellular lactate shuttles." J Physiol **587**(Pt 23): 5591-5600.
Burt, R. W. (2000). "Colon cancer screening." Gastroenterology **119**(3): 837-853.
Bustamante, E. and P. L. Pedersen (1977). "High aerobic glycolysis of rat hepatoma cells in culture: role of mitochondrial hexokinase." Proc Natl Acad Sci U S A **74**(9): 3735-3739.
Carbonaro, M., D. Escuin, A. O'Brate, M. Thadani-Mulero and P. Giannakakou (2012). "Microtubules regulate hypoxia-inducible factor-1alpha protein trafficking and activity: implications for taxane therapy." J Biol Chem **287**(15): 11859-11869.
Chan, A. T. and E. L. Giovannucci (2010). "Primary prevention of colorectal cancer." Gastroenterology **138**(6): 2029-2043 e2010.
Chance, B., J. S. Leigh, Jr., B. J. Clark, J. Maris, J. Kent, S. Nioka and D. Smith (1985). "Control of oxidative metabolism and oxygen delivery in human skeletal muscle: a steady-state analysis of the work/energy cost transfer function." Proc Natl Acad Sci U S A **82**(24): 8384-8388.
Chung, J. K., Y. J. Lee, C. Kim, S. R. Choi, M. Kim, K. Lee, J. M. Jeong, D. S. Lee, J. J. Jang and M. C. Lee (1999). "Mechanisms related to [18F]fluorodeoxyglucose uptake of human colon cancers transplanted in nude mice." J Nucl Med **40**(2): 339-346.
Cicchillitti, L., R. Penci, M. Di Michele, F. Filippetti, D. Rotilio, M. B. Donati, G. Scambia and C. Ferlini (2008). "Proteomic characterization of cytoskeletal and mitochondrial class III beta-tubulin." Mol Cancer Ther **7**(7): 2070-2079.

Colditz, G. A., K. Y. Wolin and S. Gehlert (2012). "Applying what we know to accelerate cancer prevention." Sci Transl Med **4**(127): 127rv124.
Cunningham, D., W. Atkin, H. J. Lenz, H. T. Lynch, B. Minsky, B. Nordlinger and N. Starling (2010). "Colorectal cancer." Lancet **375**(9719): 1030-1047.
Dang, C. V., A. Le and P. Gao (2009). "MYC-induced cancer cell energy metabolism and therapeutic opportunities." Clin Cancer Res **15**(21): 6479-6483.
Dassow, H. and A. Aigner (2013). "MicroRNAs (miRNAs) in colorectal cancer: from aberrant expression towards therapy." Curr Pharm Des **19**(7): 1242-1252.
DeBerardinis, R. J. and T. Cheng (2010). "Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer." Oncogene **29**(3): 313-324.
Deberardinis, R. J., N. Sayed, D. Ditsworth and C. B. Thompson (2008). "Brick by brick: metabolism and tumor cell growth." Curr Opin Genet Dev **18**(1): 54-61.
Derry, W. B., L. Wilson, I. A. Khan, R. F. Luduena and M. A. Jordan (1997). "Taxol differentially modulates the dynamics of microtubules assembled from unfractionated and purified beta-tubulin isotypes." Biochemistry **36**(12): 3554-3562.
Diers, A. R., K. A. Broniowska, C. F. Chang and N. Hogg (2012). "Pyruvate fuels mitochondrial respiration and proliferation of breast cancer cells: effect of monocarboxylate transporter inhibition." Biochem J **444**(3): 561-571.
Doll, R. and R. Peto (1981). "The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today." J Natl Cancer Inst **66**(6): 1191-1308.
Domcke, S., R. Sinha, D. A. Levine, C. Sander and N. Schultz (2013). "Evaluating cell lines as tumour models by comparison of genomic profiles." Nat Commun **4**: 2126.
Dzeja, P. and A. Terzic (2009). "Adenylate kinase and AMP signaling networks: metabolic monitoring, signal communication and body energy sensing." Int J Mol Sci **10**(4): 1729-1772.
Dzeja, P. P., R. J. Zeleznikar and N. D. Goldberg (1996). "Suppression of creatine kinase-catalyzed phosphotransfer results in increased phosphoryl transfer by adenylate kinase in intact skeletal muscle." J Biol Chem **271**(22): 12847-12851.
Dzeja, P. P., R. J. Zeleznikar and N. D. Goldberg (1998). "Adenylate kinase: kinetic behavior in intact cells indicates it is integral to multiple cellular processes." Mol Cell Biochem **184**(1-2): 169-182.
Dzeja, P. P. and A. Terzic (2003). "Phosphotransfer networks and cellular energetics." J Exp Biol **206**(Pt 12): 2039-2047.

Dzeja, P. P., K. T. Vitkevicius, M. M. Redfield, J. C. Burnett and A. Terzic (1999). "Adenylate kinase-catalyzed phosphotransfer in the myocardium : increased contribution in heart failure." Circ Res **84**(10): 1137-1143.

Dudkina, N. V., R. Kouril, K. Peters, H. P. Braun and E. J. Boekema (2010). "Structure and function of mitochondrial supercomplexes." Biochim Biophys Acta **1797**(6-7): 664-670.

Dudkina, N. V., M. Kudryashev, H. Stahlberg and E. J. Boekema (2011). "Interaction of complexes I, III, and IV within the bovine respirasome by single particle cryoelectron tomography." Proc Natl Acad Sci U S A **108**(37): 15196-15200.

Dukes, C. E. (1932). "The classification of cancer of the rectum." The Journal of Pathology and Bacteriology **35**(3): 323-332.

Eboli, M. L., G. Paradies, T. Galeotti and S. Papa (1977). "Pyruvate transport in tumour-cell mitochondria." Biochim Biophys Acta **460**(1): 183-187.

Ertel, A., A. Verghese, S. W. Byers, M. Ochs and A. Tozeren (2006). "Pathway-specific differences between tumor cell lines and normal and tumor tissue cells." Mol Cancer **5**(1): 55.

Estrella, V., T. Chen, M. Lloyd, J. Wojtkowiak, H. H. Cornnell, A. Ibrahim-Hashim, K. Bailey, Y. Balagurunathan, J. M. Rothberg, B. F. Sloane, J. Johnson, R. A. Gatenby and R. J. Gillies (2013). "Acidity generated by the tumor microenvironment drives local invasion." Cancer Res **73**(5): 1524-1535.

Fearon, E. R. and B. Vogelstein (1990). "A genetic model for colorectal tumorigenesis." Cell **61**(5): 759-767.

Fell, D. (1997). Understanding the control of metabolism. London, Portland Press.

Fell, D. (2005). Metabolic Control Analysis. Systems Biology. L. Alberghina and H. V. Westerhoff, Springer Berlin Heidelberg. **13:** 69-80.

Formentini, L., M. Sanchez-Arago, L. Sanchez-Cenizo and J. M. Cuezva (2012). "The mitochondrial ATPase inhibitory factor 1 triggers a ROS-mediated retrograde prosurvival and proliferative response." Mol Cell **45**(6): 731-742.

Gellerich, F. N., W. S. Kunz and R. Bohnensack (1990). "Estimation of flux control coefficients from inhibitor titrations by non-linear regression." FEBS Lett **274**(1-2): 167-170.

Genova, M. L., A. Baracca, A. Biondi, G. Casalena, M. Faccioli, A. I. Falasca, G. Formiggini, G. Sgarbi, G. Solaini and G. Lenaz (2008). "Is supercomplex organization of the respiratory chain required for optimal electron transfer activity?" Biochim Biophys Acta **1777**(7-8): 740-746.

Giovannucci, E. and W. C. Willett (1994). "Dietary factors and risk of colon cancer." Ann Med **26**(6): 443-452.

Giroux, V., J. Iovanna and J. C. Dagorn (2006). "Probing the human kinome for kinases involved in pancreatic cancer cell survival and gemcitabine resistance." FASEB J **20**(12): 1982-1991.

Goel, A., T. Nagasaka, C. N. Arnold, T. Inoue, C. Hamilton, D. Niedzwiecki, C. Compton, R. J. Mayer, R. Goldberg, M. M. Bertagnolli and C. R. Boland (2007). "The CpG island methylator phenotype and chromosomal instability are inversely correlated in sporadic colorectal cancer." Gastroenterology **132**(1): 127-138.

Gonzalez-Granillo, M., A. Grichine, R. Guzun, Y. Usson, K. Tepp, V. Chekulayev, I. Shevchuk, M. Karu-Varikmaa, A. V. Kuznetsov, M. Grimm, V. Saks and T. Kaambre (2012). "Studies of the role of tubulin beta II isotype in regulation of mitochondrial respiration in intracellular energetic units in cardiac cells." J Mol Cell Cardiol **52**(2): 437-447.

Gorin, A., L. Gabitova and I. Astsaturov (2012). "Regulation of cholesterol biosynthesis and cancer signaling." Curr Opin Pharmacol **12**(6): 710-716.

Graaff, V. D. (2001). "Van De Graaff: Human Anatomy, Sixth Edition." **6th edition**.

Groen, A. K., R. J. Wanders, H. V. Westerhoff, R. van der Meer and J. M. Tager (1982). "Quantification of the contribution of various steps to the control of mitochondrial respiration." J Biol Chem **257**(6): 2754-2757.

Gruno, M., N. Peet, E. Seppet, L. Kadaja, K. Paju, M. Eimre, E. Orlova, M. Peetsalu, A. Tein, J. Soplepmann, U. Schlattner, A. Peetsalu and E. K. Seppet (2006). "Oxidative phosphorylation and its coupling to mitochondrial creatine and adenylate kinases in human gastric mucosa." Am J Physiol Regul Integr Comp Physiol **291**(4): R936-946.

Guarinos, C., C. Sanchez-Fortun, M. Rodriguez-Soler, C. Alenda, A. Paya and R. Jover (2012). "Serrated polyposis syndrome: molecular, pathological and clinical aspects." World J Gastroenterol **18**(20): 2452-2461.

Guzun, R., M. Gonzalez-Granillo, M. Karu-Varikmaa, A. Grichine, Y. Usson, T. Kaambre, K. Guerrero-Roesch, A. Kuznetsov, U. Schlattner and V. Saks (2012). "Regulation of respiration in muscle cells in vivo by VDAC through interaction with the cytoskeleton and MtCK within Mitochondrial Interactosome." Biochim Biophys Acta **1818**(6): 1545-1554.

Guzun, R., M. Karu-Varikmaa, M. Gonzalez-Granillo, A. V. Kuznetsov, L. Michel, C. Cottet-Rousselle, M. Saaremae, T. Kaambre, M. Metsis, M. Grimm, C. Auffray and V. Saks (2011). "Mitochondria-cytoskeleton interaction: distribution of beta-tubulins in

cardiomyocytes and HL-1 cells." Biochim Biophys Acta **1807**(4): 458-469.
Guzun, R., N. Timohhina, K. Tepp, C. Monge, T. Kaambre, P. Sikk, A. V. Kuznetsov, C. Pison and V. Saks (2009). "Regulation of respiration controlled by mitochondrial creatine kinase in permeabilized cardiac cells in situ. Importance of system level properties." Biochim Biophys Acta **1787**(9): 1089-1105.
Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.
Haq, R., J. Shoag, P. Andreu-Perez, S. Yokoyama, H. Edelman, G. C. Rowe, D. T. Frederick, A. D. Hurley, A. Nellore, A. L. Kung, J. A. Wargo, J. S. Song, D. E. Fisher, Z. Arany and H. R. Widlund (2013). "Oncogenic BRAF regulates oxidative metabolism via PGC1alpha and MITF." Cancer Cell **23**(3): 302-315.
Haque, T., K. G. Greene and S. D. Crockett (2014). "Serrated neoplasia of the colon: what do we really know?" Curr Gastroenterol Rep **16**(4): 380.
Hardie, D. G. (2004). "The AMP-activated protein kinase pathway--new players upstream and downstream." J Cell Sci **117**(Pt 23): 5479-5487.
Haydon, A. M. and J. R. Jass (2002). "Emerging pathways in colorectal-cancer development." Lancet Oncol **3**(2): 83-88.
He, X., A. Zhou, H. Lu, Y. Chen, G. Huang, X. Yue, P. Zhao and Y. Wu (2013). "Suppression of mitochondrial complex I influences cell metastatic properties." PLoS One **8**(4): e61677.
Health., N. R. C. U. C. o. D. a. (1989). Diet and Health: Implications for Reducing Chronic Disease Risk. Diet and Health: Implications for Reducing Chronic Disease Risk. Washington (DC).
Heinrich, R. and T. A. Rapoport (1974). "A linear steady-state treatment of enzymatic chains. Critique of the crossover theorem and a general procedure to identify interaction sites with an effector." Eur J Biochem **42**(1): 97-105.
Heinrich, R. and T. A. Rapoport (1974). "A linear steady-state treatment of enzymatic chains. General properties, control and effector strength." Eur J Biochem **42**(1): 89-95.
Hoefs, S. J., R. J. Rodenburg, J. A. Smeitink and L. P. van den Heuvel (2012). "Molecular base of biochemical complex I deficiency." Mitochondrion **12**(5): 520-532.
Homfray, T. F., S. E. Cottrell, M. Ilyas, A. Rowan, I. C. Talbot, W. F. Bodmer and I. P. Tomlinson (1998). "Defects in mismatch repair occur after APC mutations in the pathogenesis of sporadic colorectal tumours." Hum Mutat **11**(2): 114-120.
Huang, C. Y., W. T. Kuo, Y. C. Huang, T. C. Lee and L. C. Yu (2013). "Resistance to hypoxia-induced necroptosis is conferred by

glycolytic pyruvate scavenging of mitochondrial superoxide in colorectal cancer cells." Cell Death Dis **4**: e622.

Jackson-Thompson, J., F. Ahmed, R. R. German, S. M. Lai and C. Friedman (2006). "Descriptive epidemiology of colorectal cancer in the United States, 1998-2001." Cancer **107**(5 Suppl): 1103-1111.

Jan, Y. H., H. Y. Tsai, C. J. Yang, M. S. Huang, Y. F. Yang, T. C. Lai, C. H. Lee, Y. M. Jeng, C. Y. Huang, J. L. Su, Y. J. Chuang and M. Hsiao (2012). "Adenylate kinase-4 is a marker of poor clinical outcomes that promotes metastasis of lung cancer by downregulating the transcription factor ATF3." Cancer Res **72**(19): 5119-5129.

Jass, J. R. (2007). "Classification of colorectal cancer based on correlation of clinical, morphological and molecular features." Histopathology **50**(1): 113-130.

Jemal, A., F. Bray, M. M. Center, J. Ferlay, E. Ward and D. Forman (2011). "Global cancer statistics." CA Cancer J Clin **61**(2): 69-90.

Jose, C. and R. Rossignol (2013). "Rationale for mitochondria-targeting strategies in cancer bioenergetic therapies." Int J Biochem Cell Biol **45**(1): 123-129.

Joseph, J., A. Cardesa and J. Carreras (1997). "Creatine kinase activity and isoenzymes in lung, colon and liver carcinomas." Br J Cancer **76**(5): 600-605.

Kaambre, T., V. Chekulayev, I. Shevchuk, M. Karu-Varikmaa, N. Timohhina, K. Tepp, J. Bogovskaja, R. Kutner, V. Valvere and V. Saks (2012). "Metabolic control analysis of cellular respiration in situ in intraoperational samples of human breast cancer." J Bioenerg Biomembr **44**(5): 539-558.

Kacser, H. and J. A. Burns (1973). "The control of flux." Symp Soc Exp Biol **27**: 65-104.

Kaldma, A., 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 (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." Int J Biochem Cell Biol **55**: 171-186.

Kasimos, J. N., T. E. Merchant, L. W. Gierke and T. Glonek (1990). "31P magnetic resonance spectroscopy of human colon cancer." Cancer Res **50**(3): 527-532.

Katsetos, C. D., M. M. Herman and S. J. Mork (2003). "Class III beta-tubulin in human development and cancer." Cell Motil Cytoskeleton **55**(2): 77-96.

Kavallaris, M. (2010). "Microtubules and resistance to tubulin-binding agents." Nat Rev Cancer **10**(3): 194-204.

Kavallaris, M., A. S. Tait, B. J. Walsh, L. He, S. B. Horwitz, M. D. Norris and M. Haber (2001). "Multiple microtubule alterations are associated with Vinca alkaloid resistance in human leukemia cells." Cancer Res **61**(15): 5803-5809.

Kholodenko, B. N., O. V. Demin and H. V. Westerhoff (1993). "'Channelled' pathways can be more sensitive to specific regulatory signals." FEBS Lett **320**(1): 75-78.

Kholodenko, B. N. and H. V. Westerhoff (1993). "Metabolic channelling and control of the flux." FEBS Lett **320**(1): 71-74.

Kim, H., H. J. Lee, Y. Oh, S. G. Choi, S. H. Hong, H. J. Kim, S. Y. Lee, J. W. Choi, D. Su Hwang, K. S. Kim, H. J. Kim, J. Zhang, H. J. Youn, D. Y. Noh and Y. K. Jung (2014). "The DUSP26 phosphatase activator adenylate kinase 2 regulates FADD phosphorylation and cell growth." Nat Commun **5**: 3351.

Klepinin, A., V. Chekulayev, N. Timohhina, I. Shevchuk, K. Tepp, A. Kaldma, A. Koit, V. Saks and T. Kaambre (2014). "Comparative analysis of some aspects of mitochondrial metabolism in differentiated and undifferentiated neuroblastoma cells." J Bioenerg Biomembr **46**(1): 17-31.

Klepinin, A., L. Ounpuu, R. Guzun, V. Chekulayev, N. Timohhina, K. Tepp, I. Shevchuk, U. Schlattner and T. Kaambre (2016). "Simple oxygraphic analysis for the presence of adenylate kinase 1 and 2 in normal and tumor cells." J Bioenerg Biomembr.

Kuznetsov, A. V., V. Veksler, F. N. Gellerich, V. Saks, R. Margreiter and W. S. Kunz (2008). "Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells." Nat Protoc **3**(6): 965-976.

Leggett, B. and V. Whitehall (2010). "Role of the serrated pathway in colorectal cancer pathogenesis." Gastroenterology **138**(6): 2088-2100.

Lenaz, G. and M. L. Genova (2010). "Structure and organization of mitochondrial respiratory complexes: a new understanding of an old subject." Antioxid Redox Signal **12**(8): 961-1008.

Lenaz, G. and M. L. Genova (2012). "Supramolecular organisation of the mitochondrial respiratory chain: a new challenge for the mechanism and control of oxidative phosphorylation." Adv Exp Med Biol **748**: 107-144.

Lengauer, C., K. W. Kinzler and B. Vogelstein (1998). "Genetic instabilities in human cancers." Nature **396**(6712): 643-649.

Levin, B., D. A. Lieberman, B. McFarland, R. A. Smith, D. Brooks, K. S. Andrews, C. Dash, F. M. Giardiello, S. Glick, T. R. Levin, P. Pickhardt, D. K. Rex, A. Thorson, S. J. Winawer, G. American Cancer Society Colorectal Cancer Advisory, U. S. M.-S. T. Force and C. American College of Radiology Colon Cancer (2008).

"Screening and surveillance for the early detection of colorectal cancer and adenomatous polyps, 2008: a joint guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology." CA Cancer J Clin **58**(3): 130-160.

Levine, J. S. and D. J. Ahnen (2006). "Clinical practice. Adenomatous polyps of the colon." N Engl J Med **355**(24): 2551-2557.

Li, F., Y. Wang, K. I. Zeller, J. J. Potter, D. R. Wonsey, K. A. O'Donnell, J. W. Kim, J. T. Yustein, L. A. Lee and C. V. Dang (2005). "Myc stimulates nuclearly encoded mitochondrial genes and mitochondrial biogenesis." Mol Cell Biol **25**(14): 6225-6234.

Lienhard, G. E. and Secemski, II (1973). "P 1 ,P 5 -Di(adenosine-5')pentaphosphate, a potent multisubstrate inhibitor of adenylate kinase." J Biol Chem **248**(3): 1121-1123.

Liou, G. Y. and P. Storz (2010). "Reactive oxygen species in cancer." Free Radic Res **44**(5): 479-496.

Llado, V., D. J. Lopez, M. Ibarguren, M. Alonso, J. B. Soriano, P. V. Escriba and X. Busquets (2014). "Regulation of the cancer cell membrane lipid composition by NaCHOleate: effects on cell signaling and therapeutical relevance in glioma." Biochim Biophys Acta **1838**(6): 1619-1627.

Lowe, S. W. and A. W. Lin (2000). "Apoptosis in cancer." Carcinogenesis **21**(3): 485-495.

Lunt, S. Y. and M. G. Vander Heiden (2011). "Aerobic glycolysis: meeting the metabolic requirements of cell proliferation." Annu Rev Cell Dev Biol **27**: 441-464.

Mabjeesh, N. J., D. Escuin, T. M. LaVallee, V. S. Pribluda, G. M. Swartz, M. S. Johnson, M. T. Willard, H. Zhong, J. W. Simons and P. Giannakakou (2003). "2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF." Cancer Cell **3**(4): 363-375.

Mair, T. and S. C. Muller (1996). "Traveling NADH and proton waves during oscillatory glycolysis in vitro." J Biol Chem **271**(2): 627-630.

Maiuri, M. C. and G. Kroemer (2015). "Essential role for oxidative phosphorylation in cancer progression." Cell Metab **21**(1): 11-12.

Maldonado, E. N., J. Patnaik, M. R. Mullins and J. J. Lemasters (2010). "Free tubulin modulates mitochondrial membrane potential in cancer cells." Cancer Res **70**(24): 10192-10201.

Maldonado, E. N., K. L. Sheldon, D. N. DeHart, J. Patnaik, Y. Manevich, D. M. Townsend, S. M. Bezrukov, T. K. Rostovtseva and J. J. Lemasters (2013). "Voltage-dependent anion channels modulate mitochondrial metabolism in cancer cells: regulation by free tubulin and erastin." J Biol Chem **288**(17): 11920-11929.

Martinez-Outschoorn, U. E., S. Pavlides, A. Howell, R. G. Pestell, H. B. Tanowitz, F. Sotgia and M. P. Lisanti (2011). "Stromal-epithelial metabolic coupling in cancer: integrating autophagy and metabolism in the tumor microenvironment." Int J Biochem Cell Biol **43**(7): 1045-1051.

Martinez-Outschoorn, U. E., R. G. Pestell, A. Howell, M. L. Tykocinski, F. Nagajyothi, F. S. Machado, H. B. Tanowitz, F. Sotgia and M. P. Lisanti (2011). "Energy transfer in "parasitic" cancer metabolism: mitochondria are the powerhouse and Achilles' heel of tumor cells." Cell Cycle **10**(24): 4208-4216.

Martinez-Outschoorn, Ubaldo E., F. Sotgia and Michael P. Lisanti (2012). "Power Surge: Supporting Cells "Fuel" Cancer Cell Mitochondria." Cell Metabolism **15**(1): 4-5.

Mathupala, S. P., Y. H. Ko and P. L. Pedersen (2006). "Hexokinase II: cancer's double-edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria." Oncogene **25**(34): 4777-4786.

Mathupala, S. P., Y. H. Ko and P. L. Pedersen (2010). "The pivotal roles of mitochondria in cancer: Warburg and beyond and encouraging prospects for effective therapies." Biochim Biophys Acta **1797**(6-7): 1225-1230.

Michael H. Ross, W. P. (2010). Histology: A Text and Atlas, with Correlated Cell and Molecular Biology, 6th Edition Lippincott Williams & Wilkins, a Wolters Kluwer business. **6th edition**.

Monge, C., N. Beraud, K. Tepp, S. Pelloux, S. Chahboun, T. Kaambre, L. Kadaja, M. Roosimaa, A. Piirsoo, Y. Tourneur, A. V. Kuznetsov, V. Saks and E. Seppet (2009). "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 **87**(4): 318-326.

Mooney, S. M., K. Rajagopalan, B. H. Williams, Y. Zeng, C. S. Christudass, Y. Li, B. Yin, P. Kulkarni and R. H. Getzenberg (2011). "Creatine kinase brain overexpression protects colorectal cells from various metabolic and non-metabolic stresses." J Cell Biochem **112**(4): 1066-1075.

Moreno-Sanchez, R., A. Marin-Hernandez, E. Saavedra, J. P. Pardo, S. J. Ralph and S. Rodriguez-Enriquez (2014). "Who controls the ATP supply in cancer cells? Biochemistry lessons to understand cancer energy metabolism." Int J Biochem Cell Biol **50**: 10-23.

Moreno-Sanchez, R., S. Rodriguez-Enriquez, E. Saavedra, A. Marin-Hernandez and J. C. Gallardo-Perez (2009). "The bioenergetics of cancer: is glycolysis the main ATP supplier in all tumor cells?" Biofactors **35**(2): 209-225.

Moreno-Sanchez, R., E. Saavedra, S. Rodriguez-Enriquez and V. Olin-Sandoval (2008). "Metabolic control analysis: a tool for designing strategies to manipulate metabolic pathways." J Biomed Biotechnol **2008**: 597913.

Morson, B. (1974). "President's address. The polyp-cancer sequence in the large bowel." Proc R Soc Med **67**(6 Pt 1): 451-457.

Nakashima, R. A., P. S. Mangan, M. Colombini and P. L. Pedersen (1986). "Hexokinase receptor complex in hepatoma mitochondria: evidence from N,N'-dicyclohexylcarbodiimide-labeling studies for the involvement of the pore-forming protein VDAC." Biochemistry **25**(5): 1015-1021.

Nakashima, R. A., M. G. Paggi, L. J. Scott and P. L. Pedersen (1988). "Purification and characterization of a bindable form of mitochondrial bound hexokinase from the highly glycolytic AS-30D rat hepatoma cell line." Cancer Res **48**(4): 913-919.

National Research Council (US) Committee on Diet, N., and Cancer. (1982). Diet, Nutrition, and Cancer. Diet, Nutrition, and Cancer. Washington (DC).

Nelson, B. D. and F. Kabir (1985). "Adenylate kinase is a source of ATP for tumor mitochondrial hexokinase." Biochim Biophys Acta **841**(2): 195-200.

Nieman, K. M., H. A. Kenny, C. V. Penicka, A. Ladanyi, R. Buell-Gutbrod, M. R. Zillhardt, I. L. Romero, M. S. Carey, G. B. Mills, G. S. Hotamisligil, S. D. Yamada, M. E. Peter, K. Gwin and E. Lengyel (2011). "Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth." Nat Med **17**(11): 1498-1503.

Nogales, E. (2000). "Structural insights into microtubule function." Annu Rev Biochem **69**: 277-302.

Panda, D., H. P. Miller, A. Banerjee, R. F. Luduena and L. Wilson (1994). "Microtubule dynamics in vitro are regulated by the tubulin isotype composition." Proc Natl Acad Sci U S A **91**(24): 11358-11362.

Papadopoulos, N., N. C. Nicolaides, Y. F. Wei, S. M. Ruben, K. C. Carter, C. A. Rosen, W. A. Haseltine, R. D. Fleischmann, C. M. Fraser, M. D. Adams and et al. (1994). "Mutation of a mutL homolog in hereditary colon cancer." Science **263**(5153): 1625-1629.

Parker, A. L., M. Kavallaris and J. A. McCarroll (2014). "Microtubules and their role in cellular stress in cancer." Front Oncol **4**: 153.

Pastorino, J. G. and J. B. Hoek (2008). "Regulation of hexokinase binding to VDAC." J Bioenerg Biomembr **40**(3): 171-182.

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." J Bioenerg Biomembr **39**(3): 211-222.
Peletier, M. A., H. V. Westerhoff and B. N. Kholodenko (2003). "Control of spatially heterogeneous and time-varying cellular reaction networks: a new summation law." J Theor Biol **225**(4): 477-487.
Pellerin, L. and P. J. Magistretti (1994). "Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization." Proc Natl Acad Sci U S A **91**(22): 10625-10629.
Putignani, L., S. Raffa, R. Pescosolido, L. Aimati, F. Signore, M. R. Torrisi and P. Grammatico (2008). "Alteration of expression levels of the oxidative phosphorylation system (OXPHOS) in breast cancer cell mitochondria." Breast Cancer Res Treat **110**(3): 439-452.
Puurand, M., N. Peet, A. Piirsoo, M. Peetsalu, J. Soplepmann, M. Sirotkina, A. Peetsalu, A. Hemminki and E. Seppet (2012). "Deficiency of the complex I of the mitochondrial respiratory chain but improved adenylate control over succinate-dependent respiration are human gastric cancer-specific phenomena." Mol Cell Biochem **370**(1-2): 69-78.
Quarato, G., C. Piccoli, R. Scrima and N. Capitanio (2011). "Variation of flux control coefficient of cytochrome c oxidase and of the other respiratory chain complexes at different values of protonmotive force occurs by a threshold mechanism." Biochim Biophys Acta **1807**(9): 1114-1124.
Rick Alteri, M. P. B., MS; Durado Brooks, MD, MPH; Vilma Cokkinides, PhD, MSPH; Mary Doroshenk; Ted Gansler, MD; Keona Graves; Eric Jacobs, PhD; Debbie Kirkland; Joan Kramer, MD; Bernard Levin, MD; Adriane Magro; Marji McCullough, ScD, RD; Deepa Naishadham, MS; Brenda McNeal; Mona Shah, MPH; Scott Simpson; Robert Smith, PhD; Kristen Sullivan, MS, MPH; Dana Wagner. (2011). Colorectal Cancer Facts & Figures 2011-2013, American Cancer Society, Inc.
Riganti, C., E. Gazzano, M. Polimeni, E. Aldieri and D. Ghigo (2012). "The pentose phosphate pathway: an antioxidant defense and a crossroad in tumor cell fate." Free Radic Biol Med **53**(3): 421-436.
Robey, R. B., B. J. Raval, J. Ma and A. V. Santos (2000). "Thrombin is a novel regulator of hexokinase activity in mesangial cells." Kidney Int **57**(6): 2308-2318.
Rose, I. A. and J. V. Warms (1967). "Mitochondrial hexokinase. Release, rebinding, and location." J Biol Chem **242**(7): 1635-1645.
Rosenzweig, S. A. and H. S. Atreya (2010). "Defining the pathway to insulin-like growth factor system targeting in cancer." Biochem Pharmacol **80**(8): 1115-1124.

Rostovtseva, T. K., K. L. Sheldon, E. Hassanzadeh, C. Monge, V. Saks, S. M. Bezrukov and D. L. Sackett (2008). "Tubulin binding blocks mitochondrial voltage-dependent anion channel and regulates respiration." Proc Natl Acad Sci U S A **105**(48): 18746-18751.

Saadatian, Z., A. Masotti, Z. Nariman Saleh Fam, B. Alipoor, M. Bastami and H. Ghaedi (2014). "Single-Nucleotide Polymorphisms Within MicroRNAs Sequences and Their 3' UTR Target Sites May Regulate Gene Expression in Gastrointestinal Tract Cancers." Iran Red Crescent Med J **16**(7): e16659.

Saks, V., R. Guzun, N. Timohhina, K. Tepp, M. Varikmaa, C. Monge, N. Beraud, T. Kaambre, A. Kuznetsov, L. Kadaja, M. Eimre and E. Seppet (2010). "Structure-function relationships in feedback regulation of energy fluxes in vivo in health and disease: mitochondrial interactosome." Biochim Biophys Acta **1797**(6-7): 678-697.

Saks, V. A., Y. O. Belikova and A. V. Kuznetsov (1991). "In vivo regulation of mitochondrial respiration in cardiomyocytes: specific restrictions for intracellular diffusion of ADP." Biochim Biophys Acta **1074**(2): 302-311.

Saks, V. A., A. V. Kuznetsov, Z. A. Khuchua, E. V. Vasilyeva, J. O. Belikova, T. Kesvatera and T. Tiivel (1995). "Control of cellular respiration in vivo by mitochondrial outer membrane and by creatine kinase. A new speculative hypothesis: possible involvement of mitochondrial-cytoskeleton interactions." J Mol Cell Cardiol **27**(1): 625-645.

Saks, V. A., V. I. Veksler, A. V. Kuznetsov, L. Kay, P. Sikk, T. Tiivel, L. Tranqui, J. Olivares, K. Winkler, F. Wiedemann and W. S. Kunz (1998). "Permeabilized cell and skinned fiber techniques in studies of mitochondrial function in vivo." Mol Cell Biochem **184**(1-2): 81-100.

Samuel, V. T. and G. I. Shulman (2012). "Mechanisms for insulin resistance: common threads and missing links." Cell **148**(5): 852-871.

Sanchez-Cenizo, L., L. Formentini, M. Aldea, A. D. Ortega, P. Garcia-Huerta, M. Sanchez-Arago and J. M. Cuezva (2010). "Up-regulation of the ATPase inhibitory factor 1 (IF1) of the mitochondrial H+-ATP synthase in human tumors mediates the metabolic shift of cancer cells to a Warburg phenotype." J Biol Chem **285**(33): 25308-25313.

Santos, C. R. and A. Schulze (2012). "Lipid metabolism in cancer." FEBS J **279**(15): 2610-2623.

Schuster, H. (1996). The regulation of cellular systems.

Schwartz, B. and E. Yehuda-Shnaidman (2014). "Putative role of adipose tissue in growth and metabolism of colon cancer cells." Front Oncol **4**: 164.

Shike, M., S. J. Winawer, P. H. Greenwald, A. Bloch, M. J. Hill and S. V. Swaroop (1990). "Primary prevention of colorectal cancer. The WHO Collaborating Centre for the Prevention of Colorectal Cancer." Bull World Health Organ **68**(3): 377-385.

Shinohara, Y., T. Ishida, M. Hino, N. Yamazaki, Y. Baba and H. Terada (2000). "Characterization of porin isoforms expressed in tumor cells." Eur J Biochem **267**(19): 6067-6073.

Shussman, N. and S. D. Wexner (2014). "Colorectal polyps and polyposis syndromes." Gastroenterol Rep (Oxf) **2**(1): 1-15.

Siegel, R. L., K. D. Miller and A. Jemal (2015). "Cancer statistics, 2015." CA Cancer J Clin **65**(1): 5-29.

Silverman, L. M., G. B. Dermer, M. H. Zweig, A. C. Van Steirteghem and Z. A. Tokes (1979). "Creatine kinase BB: a new tumor-associated marker." Clin Chem **25**(8): 1432-1435.

Small, J. R. and D. A. Fell (1990). "Covalent modification and metabolic control analysis. Modification to the theorems and their application to metabolic systems containing covalently modifiable enzymes." Eur J Biochem **191**(2): 405-411.

Snover, D. C. (2005). "Serrated polyps of the large intestine." Semin Diagn Pathol **22**(4): 301-308.

Society, A. C. (2016). "Colorectal cancer." from http://www.cancer.org/cancer/colonandrectumcancer/detailedguide/colorectal-cancer-treating-by-stage-colon.

Solaini, G. and D. A. Harris (2005). "Biochemical dysfunction in heart mitochondria exposed to ischaemia and reperfusion." Biochem J **390**(Pt 2): 377-394.

Solaini, G., G. Sgarbi and A. Baracca (2011). "Oxidative phosphorylation in cancer cells." Biochim Biophys Acta **1807**(6): 534-542.

Song, H. T., Y. Qin, G. D. Yao, Z. N. Tian, S. B. Fu and J. S. Geng (2014). "Astrocyte elevated gene-1 mediates glycolysis and tumorigenesis in colorectal carcinoma cells via AMPK signaling." Mediators Inflamm **2014**: 287381.

Soreide, K., E. A. Janssen, H. Soiland, H. Korner and J. P. Baak (2006). "Microsatellite instability in colorectal cancer." Br J Surg **93**(4): 395-406.

Sotgia, F., U. E. Martinez-Outschoorn, A. Howell, R. G. Pestell, S. Pavlides and M. P. Lisanti (2012). "Caveolin-1 and cancer metabolism in the tumor microenvironment: markers, models, and mechanisms." Annu Rev Pathol **7**: 423-467.

Sotgia, F., U. E. Martinez-Outschoorn, S. Pavlides, A. Howell, R. G. Pestell and M. P. Lisanti (2011). "Understanding the Warburg effect and

the prognostic value of stromal caveolin-1 as a marker of a lethal tumor microenvironment." Breast Cancer Res **13**(4): 213.
Stewart, B. W. and P. Kleihues (2003). World Cancer Report. Lyon, IARCPress.
Stewart, B. W. and C. P. Wild (2014). World Cancer Report 2014, IARC.
Stewart, S. L., J. M. Wike, I. Kato, D. R. Lewis and F. Michaud (2006). "A population-based study of colorectal cancer histology in the United States, 1998-2001." Cancer **107**(5 Suppl): 1128-1141.
Stigliano, V., L. Sanchez-Mete, A. Martayan and M. Anti (2014). "Early-onset colorectal cancer: a sporadic or inherited disease?" World J Gastroenterol **20**(35): 12420-12430.
Suuroja, T. (2009). "Jämesoolevähi käsitlus." Eesti Arst **88**(10): 648−655.
Zarghami, N., M. Giai, H. Yu, R. Roagna, R. Ponzone, D. Katsaros, P. Sismondi and E. P. Diamandis (1996). "Creatine kinase BB isoenzyme levels in tumour cytosols and survival of breast cancer patients." Br J Cancer **73**(3): 386-390.
Zeleznikar, R. J., P. P. Dzeja and N. D. Goldberg (1995). "Adenylate kinase-catalyzed phosphoryl transfer couples ATP utilization with its generation by glycolysis in intact muscle." J Biol Chem **270**(13): 7311-7319.
Zhang, X. H., H. L. Yu, F. J. Wang, Y. L. Han and W. L. Yang (2015). "Pim-2 Modulates Aerobic Glycolysis and Energy Production during the Development of Colorectal Tumors." Int J Med Sci **12**(6): 487-493.
Zheng, J. (2012). "Energy metabolism of cancer: Glycolysis versus oxidative phosphorylation (Review)." Oncol Lett **4**(6): 1151-1157.
Zhu, Q. C., R. Y. Gao, W. Wu, B. M. Guo, J. Y. Peng and H. L. Qin (2014). "Effect of a high-fat diet in development of colonic adenoma in an animal model." World J Gastroenterol **20**(25): 8119-8129.
Tan, A. S., J. W. Baty, L. F. Dong, A. Bezawork-Geleta, B. Endaya, J. Goodwin, M. Bajzikova, J. Kovarova, M. Peterka, B. Yan, E. A. Pesdar, M. Sobol, A. Filimonenko, S. Stuart, M. Vondrusova, K. Kluckova, K. Sachaphibulkij, J. Rohlena, P. Hozak, J. Truksa, D. Eccles, L. M. Haupt, L. R. Griffiths, J. Neuzil and M. V. Berridge (2015). "Mitochondrial genome acquisition restores respiratory function and tumorigenic potential of cancer cells without mitochondrial DNA." Cell Metab **21**(1): 81-94.
Tepp, K., I. Shevchuk, V. Chekulayev, N. Timohhina, A. V. Kuznetsov, R. Guzun, V. Saks and T. Kaambre (2011). "High efficiency of energy flux controls within mitochondrial interactosome in cardiac intracellular energetic units." Biochim Biophys Acta **1807**(12): 1549-1561.
Timohhina, N., R. Guzun, K. Tepp, C. Monge, M. Varikmaa, H. Vija, P. Sikk, T. Kaambre, D. Sackett and V. Saks (2009). "Direct measurement of energy fluxes from mitochondria into cytoplasm in

permeabilized cardiac cells in situ: some evidence for Mitochondrial Interactosome." J Bioenerg Biomembr **41**(3): 259-275.
Wallace, D. C. (2012). "Mitochondria and cancer." Nat Rev Cancer **12**(10): 685-698.
Walther, A., E. Johnstone, C. Swanton, R. Midgley, I. Tomlinson and D. Kerr (2009). "Genetic prognostic and predictive markers in colorectal cancer." Nat Rev Cancer **9**(7): 489-499.
Vander Heiden, M. G., L. C. Cantley and C. B. Thompson (2009). "Understanding the Warburg effect: the metabolic requirements of cell proliferation." Science **324**(5930): 1029-1033.
Warburg, O., K. Poesener and E. Negelein (1924). "Über den Stoffwechsel der Tumoren." Biochem.
Vazquez, F., J. H. Lim, H. Chim, K. Bhalla, G. Girnun, K. Pierce, C. B. Clish, S. R. Granter, H. R. Widlund, B. M. Spiegelman and P. Puigserver (2013). "PGC1alpha expression defines a subset of human melanoma tumors with increased mitochondrial capacity and resistance to oxidative stress." Cancer Cell **23**(3): 287-301.
Vaughn, A. E. and M. Deshmukh (2008). "Glucose metabolism inhibits apoptosis in neurons and cancer cells by redox inactivation of cytochrome c." Nat Cell Biol **10**(12): 1477-1483.
Weinberg, F. and N. S. Chandel (2009). "Reactive oxygen species-dependent signaling regulates cancer." Cell Mol Life Sci **66**(23): 3663-3673.
Wellen, K. E. and C. B. Thompson (2012). "A two-way street: reciprocal regulation of metabolism and signalling." Nat Rev Mol Cell Biol **13**(4): 270-276.
Vendelin, M., M. Eimre, E. Seppet, N. Peet, T. Andrienko, M. Lemba, J. Engelbrecht, E. K. Seppet and V. A. Saks (2004). "Intracellular diffusion of adenosine phosphates is locally restricted in cardiac muscle." Mol Cell Biochem **256-257**(1-2): 229-241.
Willers, I. M., A. Isidoro, A. D. Ortega, P. L. Fernandez and J. M. Cuezva (2010). "Selective inhibition of beta-F1-ATPase mRNA translation in human tumours." Biochem J **426**(3): 319-326.
Wilson, J. E. (1989). "Rapid purification of mitochondrial hexokinase from rat brain by a single affinity chromatography step on Affi-Gel blue." Prep Biochem **19**(1): 13-21.
Wisniewski, E., F. N. Gellerich and W. S. Kunz (1995). "Distribution of flux control among the enzymes of mitochondrial oxidative phosphorylation in calcium-activated saponin-skinned rat musculus soleus fibers." Eur J Biochem **230**(2): 549-554.
Vladimir Chekulayev, K. M., Igor Shevchuk, Andre Koit, Andrus Kaldma, Aleksandr Klepinin, Natalja Timohhina, Kersti Tepp, Manana Kandashvili, Lyudmila Ounpuu, Karoliina Heck, Laura Truu, Anu

Planken, Vahur Valvere, Tuuli Kaambre (2015). "Metabolic remodeling in human colorectal cancer and surrounding tissues: alterations in regulation of mitochondrial respiration and metabolic fluxes." Biochemistry and Biophysics Reports **Volume 4**: 111–125.

Vonck, J. and E. Schafer (2009). "Supramolecular organization of protein complexes in the mitochondrial inner membrane." Biochim Biophys Acta **1793**(1): 117-124.

Yoon, S. O., S. Shin and A. M. Mercurio (2005). "Hypoxia stimulates carcinoma invasion by stabilizing microtubules and promoting the Rab11 trafficking of the alpha6beta4 integrin." Cancer Res **65**(7): 2761-2769.

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Abstract

The aim of this study is to characterize the function of mitochondria and the regulation of energy fluxes in human colorectal cancer (CRC) cells. Results of this work are showing that CRC is not a pure glycolytic tumor and the oxidative phosphorylation (OXPHOS) system may be the main provider of ATP in these tumor cells.

In this study we analyzed quantitatively the cellular respiration in post-operational tissue samples taken from patients with human colorectal cancer.

The method of Metabolic Control Analysis (MCA) has been applied to skinned cancer tissue samples in comparison with healthy tissue of same type. MCA helps to understand how the control is shared between the enzymes and transporters of the pathway and to identify the steps that could be modified to achieve a successful alteration of flux or metabolite concentration in pathways. For every enzyme and transporter flux control coefficient (FCC) is calculated witch quantifies the control that certain reaction exerts on the steady-state flux. If alterations in the pathway can be identified, then they become potential targets for cancer treatment. We are showing that all the ATP synthasome complexes remain almost unchanged with only minor differences seen in electron transport chain complexes and that sum of the FCC was close to 3 in all studied tissues indicating the formation of supercomplexes or direct substrate channeling.

To study the kinetics of regulation of mitochondrial respiration we evaluated the affinity of mitochondria for exogenously added ADP by measuring the apparent Michaelis-Menten constant (K_m) for ADP and assess the permeability of mitochondrial outer membrane, also maximal respiratory rate (V_m) to assess the functionality of OXPHOS.

To determine the functionality of the energy transfer pathways we looked at the enzymatic activities and functional coupling between mitochondrial kinases and OXPHOS in the main energy transfer pathways in the malignant and healthy cells.

Experiments showed that human CRC is not a glycolytic tumor and it has functional OXPHOS system. CRC has increased number of mitochondria and increased permeability of the mitochondrial outer membrane for adenine nucleotides. MCA showed that there are respiratory chain super complexes or direct substrate channeling in the MI and ETC complexes. In energy transfer, CK system is replaced with AK system.

This work shows that there are functional mitochondria in human CRC and identifies differences between healthy colon tissue and cancer thus identifying potential targets in mitochondrial metabolism for human colorectal cancer therapy.

Kokkuvõte

Selle töö eesmärk on iseloomustada jämesoolevähi mitokondrite funktsiooni ja energia voogude regulatsiooni. Antud töö tulemused näitavad, et jämesoolevähk ei ole puhtalt glükolüütiline kasvaja ning tema oksüdatiivse fosforüleerimise süsteem tundub olevat peamine ATP tootja. Selles töös me analüüsisime kvantitatiivselt rakuhingamist soolevähi patsientide operatsioonijärgses materjalis.
Me kasutasime metaboolse voo kontrolli analüüsi (MVKA) meetodit skineeritud kiududel nii vähi kui ka terve koe proovidel. MVKA näitab kuidas jaguneb kontroll erinevate ensüümide ja transporterite vahel metaboolsel rajal ning aitab tuvastada samme, mis mõjutavad voo liikumist antud rajal. Igale sammule antakse voo kontrolli koefitsient, mis näitab kui suurt kontrolli antud reaktsioon avaldab stabiilses olekus metaboolsele voole. Võimalike kõrvalekallete tuvastamine annab potentsiaalse võimaluse uute ravisihtmärkide tuvastamiseks. Antud töö näitab, et kõik Mitokondriaalse Interaktosoomi kompleksid püsivad peaaegu muutumatuna minimaalsete erinevustega elektrontranspordi ahelas Voo kontrolli koefitsientide summa oli uuritud kudedes kolme lähedal, mis viitab superkomplekside moodustumisele antud süsteemis.
Mitokondriaalse hingamise regulatsiooni kineetika hindamiseks vaatlesime mitokondrite afiinsust ADP suhtes ning mõõtsime Michaelis-Menteni konstandi (K_m) ADP suhtes, samuti uurisime oksüdatiivse fosforüleerimise funktsionaalsuse hindamiseks mitokondrite välismembraani läbitavust adenosiin nukleotiididele ja maksimaalse hingamise kiirust (V_m).
Energia ülekande hindamiseks rakkudes vaatasime põhilisi energiaülekande teid ja vastavate ensüümide aktsiivsusi.
Tulemused näitasid, et inimese jämesoolevähk ei ole glükolüütiline kasvaja ja tal on funktsionaalne oksüdatiivse fosforüleerimise süsteem. Jämesoolevähis on kasvanud mitokondrite arv ning tõusnud adenosiin nukleotiidide läbivus. Metaboolse kontrolli analüüs näitas et rakkudes on valgulised hingamisahela superkompleksid või substraatide otse liikumine Mitokondriaalse Interaktosoomi ja elektrontranspordi ahela vahel. Samuti on kreatiinkinaasi põhine energiaülekanne on asendunud adenülaatkinaasi põhisega.
Antud töö näitab, et jämesoolevähis on mitokondrid funktsionaalsed ning esineb mitmeid kõrvalekaldeid tervest koest, mis loovad võimaluse leida potentsiaalseid vähiravi ja diagnostika sihtmärke.

Publication 1

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Metabolic remodeling in human colorectal cancer and surrounding tissues: alterations in regulation of mitochondrial respiration and metabolic fluxes

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ABSTRACT

The aim of the work was to evaluate whether or not there is glycolytic reprogramming in the neighboring cells of colorectal cancer (CRC). Using postoperative material we have compared the functional capacity of oxidative phosphorylation (OXPHOS) in CRC cells, their glycolytic activity and their inclination to aerobic glycolysis, with those of the surrounding and healthy colon tissue cells. Experiments showed that human CRC cannot be considered a hypoxic tumor, since the malignancy itself and cells surrounding it exhibited even higher rates of OXPHOS than healthy large intestine. The absence of acute hypoxia in colorectal carcinomas was also confirmed by their practically equal glucose-phosphorylating capacity as compared with surrounding non-tumorous tissue and by upregulation of VEGF family and their ligands. Studies indicated that human CRC cells *in vivo* exert a strong distant effect on the energy metabolism of neighboring cells, so that they acquire the bioenergetic parameters specific to the tumor itself. The growth of colorectal carcinomas was associated with potent downregulation of the creatine kinase system. As compared with healthy colon tissue, the tumor surrounding cells display upregulation of OXPHOS and have high values of basal and ADP activated respiration rates. Strong differences between the normal and CRC cells in the affinity of their mitochondria for ADP were revealed; the corresponding K_m values were measured as 93.6 ± 7.7 μM for CRC cells and 84.9 ± 9.9 μM for nearby tissue; both these apparent K_m (ADP) values were considerably (by almost 3 times) lower in comparison with healthy colon tissue cells (256 ± 34 μM).

1. Introduction

Colorectal cancer (CRC) is a major cause of cancer death worldwide necessitating new strategies for treatment of this disease. Recent studies show that targeting cancer cell energy metabolism is possibly a new and very effective therapeutic approach for selective ablation of malignancies [39,61,70]. Intracellular ATP levels may be a key determinant of chemoresistance of human CRC cells [110]. There are some indications in literature that mitochondria (the main cell system for ATP generation) could play a supportive or possibly even a triggering role in metastasis of cancer cells [4].

Abbreviations: AK, adenylate kinase; ANT, adenine nucleotide translocator; AP5A, diadenosine pentaphosphate; BSA, bovine serum albumin; BB-CK, – brain type creatine kinase; CAT, carboxyatractyloside; CIMP, CpG island methylator phenotype; COX, cytochrome c oxidase; CK, creatine kinase; CRC, colorectal cancer; ETC, electron transport chain; FDG, 18-fluorodeoxyglucose; HK, hexokinase; K_m, Michaelis–Menten constant; uMtCK, ubiquitous mitochondrial creatine kinase; OXPHOS, oxidative phosphorylation; MI, Mitochondrial Interactosome; MOM, mitochondrial outer membrane; PCr, phosphocreatine; PET, positron emission tomography; PEP, phosphoenolpyruvate; PYK, pyruvate kinase; qPCR, real-time quantitative PCR; TMPD, N,N,N′,N′-tetramethyl-p-phenylenediamine; VEGF, vascular endothelial growth factor; VDAC, voltage dependent anion channel; V_0, basal respiration level; V_m, maximal respiration rate

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The first half of the 20th century led to substantial breakthroughs in bioenergetics and mitochondrial research. During that time, Otto Heinrich Warburg observed abnormally high glycolysis and lactate production in cancer cells even in the presence of oxygen (later named as "aerobic glycolysis"), leading him to suggest that defects in mitochondrial functions are at the heart of malignant cell transformation [117].

The exact mechanisms mediating the strong tendency of some cancers to aerobic glycolysis remain still unclear. Several different hypothesis have been proposed to explain the causes of the Warburg effect, such as: 1) poor tumor vascularization leading to hypoxia-induced dysfunction of mitochondria [95] and stabilization of HIF-1α-a master of regulation of glycolytic fluxes [103], 2) post-translational modifications, 3) glutamine metabolism [26,29]; 4) miRNA expression [42], 5) epigenetic changes [17], 6) nuclear and mitochondrial DNA mutations [10,18] leading to mitochondrial dysfunction in cancer cells [77], and 7) oncogene activation and loss of tumor suppressor genes function [115].

A very attractive hypothesis for explanation of the Warburg phenomenon was proposed by Pedersen and colleagues [75]. They have suggested that in highly glycolytic malignant cells the overexpression of hexokinase-2 (HK-2) associated with its binding to voltage-dependent anion channel (VDAC, located on the outer mitochondrial membrane) plays a crucial role in mediating their high rate of aerobic glycolysis. In tumor cells, the interaction of HK-2 with VDAC induces very rapid phosphorylation of glucose through the use of mitochondrially-generated ATP. Also, the binding of HK-2 to mitochondria strongly (almost 5-fold) increases its affinity for ATP [12]. It is important to note that binding of HK-2 to VDAC maintains this channel in the open state [98] which further facilities the transport of adenine nucleotides across mitochondrial membranes in malignant cells. In cancer tissues, the high glycolytic activity requires an up-regulation of the key glycolytic enzymes including HK(s). Interestingly, the percentage of hexokinase binding to the mitochondria is also significantly increased in some cancer cells. For instance, in AS-20D liver tumor cells, the hexokinase protein level (mainly HK-2) was found to be more than 500 times higher than in normal liver cells, which mainly express HK-IV instead. Furthermore, around 80% of HK-2 is found to be associated with mitochondria [7]. Due to the frequent up-regulation of HK-2 in cancer cells and its important role in glycolytic pathway, this enzyme seems to be an attractive target for anticancer drug development. In line with this, Chen et al. [19] have shown treatment of cancer cells with 3-bromopyruvate (an inhibitor of glycolysis) caused a covalent modification of HK-2 protein that triggered its dissociation from mitochondria, leading to a specific release of apoptosis-inducing factor from the mitochondria to cytosol and cell death.

The regulation of mitochondrial function is a central issue in the bioenergetics of cancer cell. Studies performed during the past decade showed that interaction between cytoskeletal proteins and mitochondria is deeply involved in the regulation of mitochondrial function. A lot of experimental data demonstrate the importance of the structural factors in the intracellular arrangement of mitochondria and in the control of outer mitochondrial membrane permeability [104,118,40,45,47,5,6]. Potential candidates for the key roles in this regulation are the cytoskeletal proteins such as plectin and tubulin [118,45,47,5,6,63]. It was hypothesized that in high-energy demand tissues there is a colocalization of β-tubulin isotype II with mitochondria (through VDAC) and it was suggested that it can be coupled with the adenine nucleotide translocase (ANT), mitochondrial creatine kinase (MtCK) and VDAC. This mitochondrial supercomplex (ANT-MtCK-VDAC) is responsible for the efficient intracellular energy transfer *via* the phosphocreatine (PCr) pathway. It is shown that the localization and function of β-tubulin isotypes varied in different muscle tissues and neoplastic cells [112,118,63,78].

Recent investigations have clarified the benefits and selective advantages of aerobic glycolysis. Although glycolysis yields a lower amount of ATP compared to mitochondrial OXPHOS, several key benefits inherent in aerobic glycolysis drive cancer cells to favor glycolysis over mitochondrial oxidation [28]. Firstly, it was proposed [91] that the high rates, but low yields of ATP production through glycolysis, may give selective advantage under rivalry for shared energy sources. Moreover, the rate of ATP generation may be 100 times faster with glycolysis as compared with OXPHOS [36]. The low efficiency of ATP generation by glycolysis is nevertheless sufficient to meet intracellular demand. Secondly, besides ATP, cancer cells need additional metabolic intermediates and precursors that are decisive for the biosynthesis of macromolecules, the ultimate building blocks necessary to expand the tumor mass during its growth and proliferation [116]. Currently, human CRC is considered as a neoplasm of the Warburg phenotype with deregulated OXPHOS system. Positron emission tomography (PET) with 18-fluorodeoxyglucose (FDG) showed that the malignancy exhibits, as compared to surrounding normal intestine tissue, higher rates of glucose consumption [22] that in turn was associated with increased intratumoral levels of lactic acid [54], overexpression of GLUT-1 [48] and genes encoding glycolytic enzymes such as pyruvate kinase M2 (PKM2) [1], glyceraldehyde-3-phosphate dehydrogenase and enolase-1α [3], LDH5 [62], and HK-2 [52].

It is becoming evident that the upregulation of glycolysis exhibited by some cancer cells does not necessarily imply a strict anaerobic phenotype or a dysfunctional OXPHOS system. Rather, it is believed that the normal interplay between the glycolysis in the cytosol and OXPHOS in the mitochondria becomes disturbed or reprogrammed in tumor cells; the Crabtree effect was observed in cancer cells that exemplifies the intimate connection between glycolysis and the oxidative metabolism [90]. Moreover, recent studies have shown that not all tumor mitochondria display OXPHOS deficiency [111,121,30,60,95]. The OXPHOS system may be the principal ATP producer ($>90\%$) for several malignant tumor cell types under normoxic conditions [111,96,97]. Therefore, drug therapy targeting OXPHOS has emerged as an important alternative for growth arrest of oxidative type tumors [39,82,96].

In our recent study, we clearly showed that CRC cannot be regarded as a tumor of purely Warburg phenotype and that in these cancer cells the OXPHOS system is the main energy source instead of aerobic glycolysis [58]. Although total glycolytic capacity of human CRC cells was found to be similar with normal cells, all their respiratory rates (both basal and ADP-activated) exceeded considerably those of healthy colon tissue samples. Furthermore, our studies indicated that the OXPHOS system may be even up-regulated in CRC cells; the content of mitochondria in human CRC cells was found to be at least 2-times higher than that in healthy colon tissue cells [58].

Recently, a new framework of "Reprogramming the of Tumor Stroma metabolism" or "Reverse Warburg effect" was introduced in experimental oncology [108,123,68]. According to the paradigm, there is metabolic coupling between mitochondria in cancer cells and catabolism in stromal cells that promotes tumor growth and metastasis. In another words, cancer cells can induce the reprogramming of tumor microenvironment (fibroblasts, macrophages and other tumor-associated cells) towards the Warburg phenotype, so they donate the necessary fuels (L-lactate, ketone bodies, glutamine and others) to anabolic cancer cells, which metabolize these *via* the tricarboxylic acid cycle (TCA) and OXPHOS. Pioneering studies showed that such metabolic symbiosis may occur between breast cancer cells and the tumor stromal fibroblasts [107,120,73], and now this paradigm has extended to other malignancies like osteosarcoma, ovarian cancer, head and neck

tumors, and cancer lymph node metastases [106,25,85]. However, in the case of human CRC the concept of "reverse Warburg" effect was not explored yet.

Though the above described form of "parasitic metabolism" in malignancies has only recently been proposed, transfer of energy precursors between cells to fuel growth is actually not a new discovery, but instead reflects the co-optation of normal physiological processes by tumor cells.

Taking into account the information presented above, the aim of the work was to estimate whether or not there is metabolic reprogramming in the human CRC surrounding cells. For this purpose, we used postoperative material, to estimate the glycolytic capacity of human CRC cells, their inclination towards aerobic glycolysis (coupling between HK processes and the OXPHOS system) and compared these parameters with those of healthy colon tissue and the tumor surrounding cells (nearby tissue). In addition, *in situ* experiments with the use of "permeabilized cell" techniques were carried out to compare the bioenergetic function of mitochondria in these tissues as well as the role of adenylate and creatine kinase systems in maintaining energy homeostasis.

2. Materials and methods

2.1. Reagents

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich Chemical Com. (USA). Primary and secondary antibodies were obtained from Santa Cruz Biotechnology Inc. or Abcam PLC, rabbit polyclonal antibodies *vs.* VDAC were kindly donated by Dr. Catherine Brenner from Paris-Sud University, France.

2.2. Clinical materials and patients

CRC and normal tissue samples (0.1–0.5 g) were provided by the Oncology and Hematologic Clinic at the North Estonia Medical Centre, Tallinn. Pathology and histological reports were provided by the oncology clinic for each tissue sample. All patients examined ($n=55$, with ages ranging from 63 to 92 years) had local or locally advanced disease (T2-4, N0-1, M0-1). Only primary samples of adenocarcinoma were examined. The patients in the study had not received prior radiation or chemotherapy. All investigations were approved by the Medical Research Ethics Committee (National Institute for Health Development, Tallinn) in accordance with Helsinki Declaration and Convention of the Council of Europe on Human Rights and Biomedicine. Normal colon tissue samples were taken at sites distant from the tumor by 5 cm and they were controlled for presence of cancer cells. Nearby tissue is the junction area between cancer and normal mucosa. In addition, we performed molecular characterization of tissue samples from 35 patients (both tumor and normal) using microsatellite instability, CpG island methylator phenotype (CIMP) and 5-hydroxymethylation assay. CIMP is one of the mechanisms involved in colorectal carcinogenesis [38]. Our studies showed that both non-tumorous tissue samples (nearby and healthy) had a stable microsatellite profile and no CIMP phenotype. Also, the 5-hydroxymethylation expression was statistically significantly higher in both control and neighboring tissue samples as compared to tumor that was analyzed in 13 patients (Supplementary Fig. 1). 5-hydroxymethylation analysis was carried out according to manufacturer instructions provided with the MethylFlash Hydroxymethylated DNA Quantification Kit (Epigentek, USA).

2.3. Preparation of tumor fibers, permeabilization procedure, and assessment of mitochondrial respiration in situ

Numerous studies have demonstrated that isolated mitochondria behave differently from mitochondria *in situ* with respect to respiratory activity [101]. Therefore, we investigated mitochondrial respiratory activity of tumor and non-tumorous tissues *in situ* using the skinned fiber technique [101,56,58,65], which allows analyzing the function of mitochondria in a cell in their natural surroundings. This technique leaves the links of these organelles with cytoskeletal structures intact. Immediately after surgery, the tissue samples treated as described previously [56] and the respiration of State 2 and ADP activated respiration was measured.

2.4. Mitochondrial respiration in saponin-permeabilized tissue samples

Rates of O_2 consumption by skinned tissue fibers were assayed at 25 °C by an Oxygraph-2k high resolution respirometer (Oroboros Instruments, Innsbruck Austria) in pre-equilibrated respiration buffer (medium-B supplemented with 5 mM glutamate and 2 mM malate or 10 mM succinate as respiratory substrates) as described previously [65]. The solubility of oxygen at 25 °C was taken as 240 nmol/ml [37]. All respiration rates were normalized per mg dry weight of tissue.

2.4.1. Analysis of OXPHOS coupling with hexokinase, adenylate and creatine kinase mediated processes

The coupling of mitochondrially-bound hexokinases with the OXPHOS system in permeabilized tumor and non-tumorous tissues was assayed by oxygraphy through stimulation of mitochondrial respiration by locally-generated ADP [58]. The glucose effect on mitochondrial respiration was expressed by glucose index (I_{GLU}) that was calculated according to the equation: $I_{GLU}(\%)=[(V_{GLU}-V_{ATP})/(V_{ADP}-V_{ATP})]^*100$, where V_{ADP} is the rate of O_2 consumption in the presence of 2 mM, V_{GLU}-respiration rates with 10 mM glucose, and V_{ATP} is respiration rate with 0.1 mM ATP; i.e. this index reflects the degree glucose-mediated stimulation of mitochondrial respiration as compared to maximal ADP-activated rates of O_2 consumption.

The adenylate kinase (AK) coupling with OXPHOS was estimated by respirometry using modified protocols of [43]. Then AK index (I_{AK}) was calculated according to the equation: $I_{AK}=(V_{AMP}-V_{AP5A})/V_{AP5A}$, where V_{AMP} and V_{AP5A} are mitochondrial respiration rates in the presence of 2 mM AMP and 0.2 mM diadenosine pentaphosphate (AP5A, an inhibitor of AK), respectively. This index shows the efficiency of the functional coupling between AK and mitochondrial OXPHOS.

The functional coupling between mitochondrial creatine kinase (CK) and OXPHOS system was estimated by respirometry essentially as described previously [114,56].

2.5. Determination of enzymatic activities

HK activity was measured as the total glucose phosphorylating capacity of whole tissue extracts, using a standard glucose-6-phosphate dehydrogenase coupled spectrophotometric assay [94].

The CK activity was assessed spectrophotometrically at 25 °C in the direction of ATP formation [78]. One mU of CK activity represents the formation of 1 nmole of ATP per minute at 25 °C.

AK activity of whole-tissue extracts was measured at 25 °C by a coupled enzyme assay [33]. All enzymatic activities were normalized per mg of tissue protein. The protein content of tissue extracts was determined by a Pierce BCA Protein Assay Kit according to the manufacturer recommendations using BSA as a standard.

2.6. Western blot analysis of the levels of beta-tubulin isotypes and VDAC expression in postoperative tissue samples

Postoperative tissues were frozen in liquid N_2 and crushed to a powder. The powder was then suspended in 20 volumes of microtubule lysing buffer containing 100 mM PIPES, 5 mM $MgCl_2$, 1 mM EGTA, 30% glycerol, 0.1% IGEPAL, 0.1% Tween-20, 0.1% Triton X-100, 0.1% beta-mercaptoethanol, 1 mM ATP, 0.1 mM GTP and complete protease inhibitor cocktail (Roche); the recipe is according to Cytoskeleton Inc. The lysate was homogenized by Retsch Mixer Mill at 25 Hz for 2 min, and incubated for 30 min at 35 °C. The obtained tissue lysates were then clarified by centrifugation at 21,000 g for 40 min at 35 °C. The protein concentration in lysates was determined using the Pierce BCA Protein Kit. Proteins were separated by 12% SDS-PAGE and transferred onto PVDF membrane by Trans-Blot Semi-Dry Transfer system (Bio-Rad).

For determinations of the presence of beta-tubulin isotypes Abcam mono- and polyclonal antibodies (Anti beta I Tub (ab11312), Anti Tubb2A (ab170931), Anti beta III Tub (ab52901), Anti beta IV (ab11315)) were used. For VDAC detection, rabbit polyclonal antibody, kindly provided by Dr Catherine Brenner (Paris-Sud University, France) was used. Secondary antibodies were accordingly: anti-mouse (ab97046) and anti-rabbit (ab6721) HRP conjugates. After chemoluminescence reaction, the PVDF membranes were stained with Coomassie brilliant blue R250 to measure the total protein amount. The beta tubulin isoforms and VDAC signal intensities were calculated by ImageJ software and normalized to total protein intensities; staining with Coomassie is routinely used as loading control in Western blot analysis. Besides, after enzymatic chemiluminescence reaction and imaging, the PVDF membrane was washed once with Tris-buffered saline, recolored with Coomassie brilliant blue for 5 min, distained and dried completely, and then was imaged again [119].

2.7. RNA isolation and real-time quantitative RT-PCR (qRT-PCR)

The technique of qRT-PCR was applied to estimate the expression of genes encoding main members of the vascular endothelial growth factor family (VEGFs) and their receptors (such as VEGF-A, -B, -C, FLT-1, FLT-4 KDR, NRP-1 and NRP-2) in human CRC, neighboring non-tumorous and healthy colon tissues. Total RNA from frozen postoperative tissue samples ($n=47$) was isolated by means of Trizol (Life Technologies) solution, followed by purification using the RNeasy Mini Kit (QIAGEN Sciences) with DNase treatment. Extracted RNA was dissolved in RNase-free water, quality and concentration were measured using Nanodrop and RNA was stored at −80 °C until cDNA synthesis. For cDNA synthesis 2 μg of total RNA was used. cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems). cDNA was used as a template for TaqMan® quantitative RT-PCR (qRT-PCR) analysis in the Roche LightCycler 480 system (Roche). TaqMan® Gene Expression Master Mix and FAM labeled TaqMan® (Applied Biosystems) gene assays were used to detect the mRNA expression level of the gene of interest and of actin as a reference gene. The following primers were used to determine the relative gene expression using qPCR: vegf-a (Hs00900055_m1), vegf-b (Hs00173634_m1),vegf-c (Hs00153458_m1), flt-1(Hs01052961_m1), flt-4 (Hs01047677_m1), kdr (Hs00911700_m1), nrp-1(Hs00826128_m1), nrp-2 (Hs00187290_m1). Reactions were carried out in four replicates. Data were analyzed by the 2(−Delta Delta C(T)) method [71], where the gene expression levels were normalized to the level of actin beta housekeeping gene. The data of studied genes following normal distribution were parametrically tested by unpaired Student *t*-test.

qRT-PCR analysis of the level of mRNA expression for CK(s), HK-1 and HK-2 was carried out as described in our previous work [58].

2.8. Statistical analysis

All results are presented as a mean value ± standard error (SEM). They 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 or double reciprocal Lineweaver–Burk plot according to a Michaelis–Menten equation. In our studies, cutoff values were calculated by using of available SigmaPlot 11 software, Systat Software, Inc.

3. Results

3.1. Evaluation of the function of OXPHOS system in human CRC and surrounding non-tumorous tissues

Our study was based on the hypothesis that epithelial CRC cells induce the Warburg effect (aerobic glycolysis) in neighboring tissue cells. In this situation, suppression of the mitochondrial function in association with the upregulation of glucose consumption could occur in nearby tissue cells. Perhaps, the increased glucose uptake registered by PET-FDG in CRC patients [22] is largely mediated by the higher glycolytic capacity of the tumor stroma as compared to its parenchymal cells. In addition, our prior studies on the function of OXPHOS system in CRC and healthy colon tissue cells are in line with the concept of reverse Warburg effect. In the current study we found that human CRC cells have fully-active respiratory chain (Fig. 1). CRC cells display higher rates of State 2 and State 3 oxygen consumption as compared with healthy colon tissue [58]; the measured rates of maximal ADP stimulated respiration (V_m) for tumor and normal tissues correlated well with their mitochondrial content [58]. Besides, it was found that both CRC and healthy colon tissues exhibit practically equal total glucose-phosphorylating activity (Table 1) [58]. These findings led us to the conclusion that human CRC is not a glycolytic tumor and in these cancer cells the OXPHOS system may be the

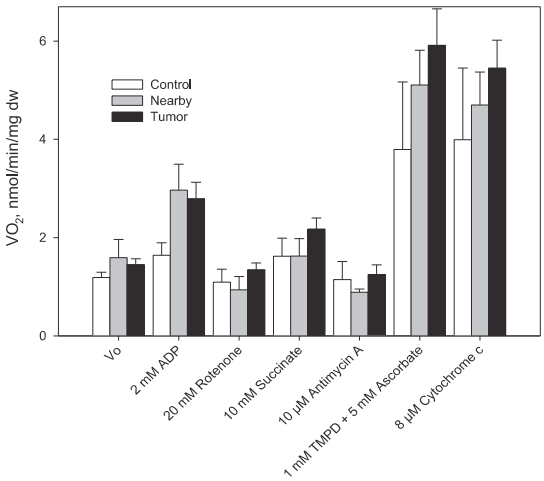

Fig. 1. Analysis of the mitochondrial respiratory chain function in permeabilized human colorectal cancer, junction area between cancer and normal mucosa (nearby) and healthy tissue samples. These studies were carried out in medium-B with 5 mM glutamate and 2 mM malate as respiratory substrates. TMPD is N,N,N′, N′-tetramethyl-phenylenediamine, and asc – ascorbate (bars are SEM, $n=7$, $p<0.05$). All respiratory substrates and inhibitors were added sequentially as indicated in the *X*-axis.

Table 1
Enzymatic activities in human colorectal cancer and surrounding non-tumorous tissue samples.

Enzyme activities, mU (s) per mg protein	Tissues, mean ± SE		
	Healthy colon tissue	Nearby	Colorectal cancer
Hexokinase	244 ± 50	172 ± 30, $p=0.12$	215 ± 40, $p=0.33$
Creatine kinase	497 ± 142	202 ± 52, $p<0.05$	204 ± 84, $p<0.05$
Adenylate kinase	257 ± 35	256 ± 35, $p=0.49$	411 ± 43, $p<0.05$

Notes: *p*-values were calculated by Student test *vs.* control non-tumorous tissue data; number of examined samples was 11 – 16.

main source of ATP. Thus, there is a probability that glycolytic stromal cells support the CRC cells growth by fueling their OXPHOS. But, the functional capacity of mitochondria in neighboring, to the malignancy growth area tissue was remained to be explored. Hereby, we have performed the corresponding comparative studies, and this is very important especially in the light of the concept of "reverse Warburg" effect.

For this purpose, we analyzed the function of the mitochondrial electron transport chain (ETC) components in non-neoplastic, CRC, and nearby tissues using saponin-skinned samples. It was found (Fig. 1) that nearby tissue has relatively high rates of State 2 and ADP-activated respiration; in the presence 2 mM malate and 5 mM glutamate as respiratory substrates, the mean value V_0 (State 2) was assayed as 1.59 ± 0.37 nmol O_2/min/mg dry tissue weight and it was increased after addition of 2 mM ADP by almost 2 times (2.97 ± 0.53 nmol O_2/min/mg dry tissue weight, $p<0.05$), confirming the presence of functionally-active mitochondria. The values of V_0 and the respiration rate in the presence of 2 mM ADP for neighboring tissue exceeded notably (by ~1.5 times) the values obtained for skinned healthy colon tissue fibers. The ADP-activated respiration was strongly inhibited in all tissue samples upon addition of rotenone; this is a characteristic feature of cells with active Complex-I (Fig. 1). The differences of respiration rates between these three types of tissues are not statistically significant and it can be concluded that the development of colorectal cancer was not accompanied with suppression of complex I-dependent respiration as described in gastric cancer [92]. The next step was to estimate the Complex-II dependent respiration that can be achieved by addition of its substrate – succinate. We found that the addition of succinate (10 mM) leads to increase in the rates of O_2 consumption suggesting that Complex-II of the mitochondrial respiratory chain is functionally active in these tissues cells. The ADP stimulated respiration was suppressed by addition of 10 μM antimycin-A (inhibitor for Complex-III); this result shows that the mitochondrial respiratory chain Complex III is also functionally active not only in unaffected tissue, but also in CRC cells. To activate cytochrome-c oxidase (COX) 1 mM N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) jointly with 5 mM ascorbate were added and this resulted in a very strong increase in the rate of O_2 consumption by all examined tissue samples (Fig. 1). The cytochrome c test is used to investigate the quality of the outer mitochondrial membrane after treatment of the tissue. Permeabilization of fibers does not alter the permeability of the outer mitochondrial membrane since addition of exogenous cytochrome c has no effect on respiration of the fibers [101]. The intactness of inner mitochondrial membrane was controlled by carboxyatractyloside (CAT). Upon addition of the inhibitor, the respiration rate decreased back to the basal respiration level due to inactivation of ANT [114].

Respiratory control index (RCI) values for tumor and non-tumorous tissues were the following: ~2.0 for CRC, ~1.8 for the neighboring tissue and ~1.7 for unaffected colon tissue. The maximal rates of ADP-activated respiration (V_m) for cancerous and nearby tissue were found to exceed considerably those of healthy colon tissue (Table 2). This could be largely driven by the difference in the cellular content of mitochondria. In this connection, we estimated the content of mitochondria in tumorous and non-tumorous tissues through the analysis of VDAC protein expression. Western-blot analysis showed (Fig. 2) that CRC and neighboring tissue cells exhibited manifold higher levels of VDAC expression

Table 2
The values of basal respiration rate (V_0), maximal rate of respiration (V_m, was calculated from a titration curve after step-wise addition of ADP, up to 2 mM), apparent K_m values for ADP for permeabilized human colorectal cancer and surrounding tissue samples as well as some healthy rat muscle tissues of different histological type.

Tissue	V_0	K_{mADP}^{app}, μM	V_m	Source
Colorectal cancer[a]	1.99 ± 0.26	93.6 ± 7.7[b]	3.82 ± 0.32[*]	Our data
Nearby tissue[c]	1.64 ± 0.27	84.9 ± 9.9[b]	2.98 ± 0.34[*]	Our data
Healthy colon tissue[c]	1.13 ± 0.12	256 ± 34[b]	1.92 ± 0.14	Our data
Rat heart fibers	6.45 ± 0.19	297 ± 35	28.7 ± 1.1	[56,64]
Rat soleus	2.19 ± 0.30	354 ± 46	12.2 ± 0.5	[56,64]
Rat gastrocnemius white	1.23 ± 0.13	14.4 ± 2.6	7.0 ± 0.5; 4.10 ± 0.25	[56,64]

[*] significant difference *vs.* normal intestinal tissue, $p<0.05$.
[a] All respiratory rates are given as nmol O_2/min/mg dry weight of tissue.
[b] These apparent K_m values were determined by fitting experimental data to a non-linear regression equation (according to a Michaelis–Menten model).
[c] Nearby and healthy intestinal tissue samples were taken at a site distant from the tumor locus by 2 and 5 cm, respectively.

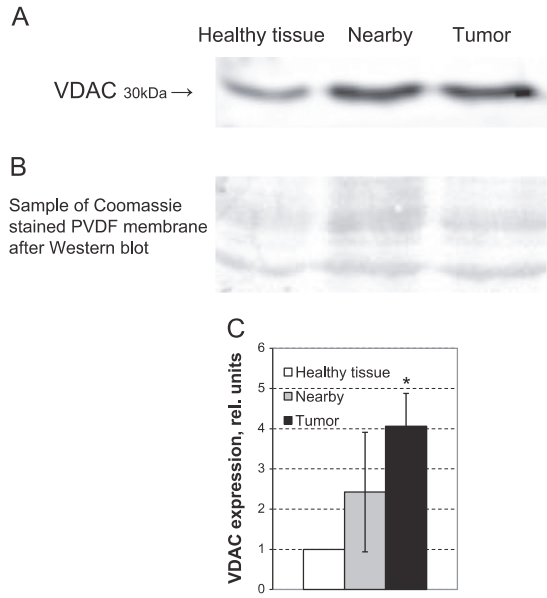

Fig. 2. (A) Western blot analysis of the level of voltage-dependent anion channel (VDAC) porin protein expression in human CRC, nearby and unaffected non-tumorous tissue. The VDAC bands in reference samples correspond to the molecular mass of 30 kDa. (B) Sample of Coomassie-stained PVDF membrane (cutoff between 35 and 63 kDa) after Western blot (loading control). (C) The level of VDAC expression in CRC and nearby tissues was normalized to that in unaffected (control) intestinal tissue. Mean values from 8 patients with clinically-diagnosed CRC; bars are SEM, * $p<0.05$.

and, consequently, the number of mitochondria, as compared with healthy colon tissue, that in turn correlated directly with the measured V_m values (Table 2). In general, our estimations of the mitochondrial content in CRC and healthy intestinal tissues, as shown by increased VDAC expression in Western blot analysis, are similar with those obtained *via* immunocytochemical staining of mitochondrial outer membrane translocase (Tom20) protein [58]. In conclusion, our oxygraphic experiments clearly showed that the tissue neighboring to CRC contains a larger number of mitochondria, exhibits high rates of OXPHOS (close to carcinoma cells) and both these parameters exceed substantially the levels monitored for healthy colon tissue cells.

3.2. Analysis of the coupling of hexokinase reactions with the OXPHOS system in CRC and surrounding tissues

Some tumor cells (e.g., breast cancer [44] along with HK-2 express type-1 hexokinase (HK-1) that like type-2 can bind to VDAC and could mediate their highly glycolytic type [87]. In our prior study, we have shown that human CRC cells are characterized by the presence of mitochondrially-bound HK-2 and that its interaction with VDAC may be responsible for increased rates of aerobic glycolysis in these cancer cells [58].

By using qRT-PCR, we estimated the levels of mRNA expression for HK-1 and -2 in CRC, adjacent and healthy colon tissue. It was found that HK-1 is expressed in each of the tissues examined in comparable levels (Fig. 3), only for HK 2 the statistical difference was significant for tumor and control tissue ($p=0.01$). Further, we performed oxygraphic analysis of the coupling of HK with the OXPHOS system in tumor, nearby and control tissues through the stimulation of OXPHOS by mitochondrially-bound HK(s) due to local generation of ADP. Experiments showed that the addition of 10 mM glucose to CRC and nearby tissue fibers in the presence of 0.1 mM Mg-ATP resulted in activation of mitochondrial respiration, but this HK coupling with OXPHOS was absent in healthy colon tissue (Fig. 4). In order to characterize the glucose effect on mitochondrial respiration, we used the glucose index (I_{GLU}); it allows to compare the degree of stimulatory action of glucose (through ADP released in HK reactions) with maximal ADP (2 mM) activated respiration. As can be seen on Fig. 4B, the tissue neighboring to CRC has similar value of I_{GLU} (18.7%), for the malignancy the index was measured as 19.1%, and healthy colon tissue had

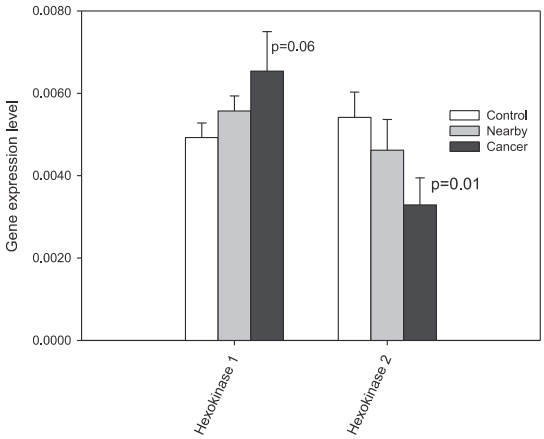

Fig. 3. Q-PCR analysis of the levels of mRNA expression for hexokinase 1 and 2 in human CRC and surroundings tissues; bars are SEM; $p=0.06$ for HK 1 and $p=0.01$ for HK 2. Average results for 29 CRC patients.

$I_{GLU} \approx 11.7\%$. HK activities are similar in CRC, nearby and healthy colon tissues (Table 2), it could be assumed that mitochondria of CRC and nearby tissue cells have a slightly higher capability for hexokinase binding in comparison with normal intestinal tissue cells and, as a consequence, slightly higher inclination to aerobic glycolysis. The revealed bioenergetic difference showing that this type of cancer cells could induce reprogramming of nearby tissue cells towards aerobic glycolysis.

3.3. Western blot analysis of the levels of beta-tubulin isotypes in healthy colon, CRC and the tumor surrounding tissue

To understand possible reasons for the observed large difference between CRC and normal intestinal tissues in their respiratory parameters, we also examined the expression of main beta-tubulin isoforms in these tissues. Prior studies have shown that some β-tubulin isoforms may be involved in the regulation of OXPHOS and cellular cytoarchitecture in muscle cells of oxidative type. HK(s) can compete with tubulin for binding sites on the VDAC. It was already shown that in HL-1 tumor cells the down-regulation of β2-tubulin expression can mediate their Warburg phenotype [45]. In this relation, we examined the spectrum of main beta-tubulin isoforms expression in CRC and surrounding non-tumorous tissues. Western blot analysis showed that there is not any statistically-significant difference in the levels of β-tubulin-I, II and IV expression between CRC, healthy colon and nearby tissues (Supplementary Fig 2). Only a slight (~ 1.5-fold) increase in the content of beta-III tubulin isotype was monitored in CRC in comparison with nearby and control tissues (Fig. 5)

3.4. Interrelationship between expression of vascular endothelial growth factors in CRC adjacent tissues and their respiratory activity

The ability of tumors to initiate the growth of new blood vessels is one of the key molecular events during carcinogenesis. It has been reported that the growth of solid tumors *in vivo* beyond 1–2 mm in diameter requires induction and maintenance of an angiogenetic response [93]. This abnormal vascularization of tumors may result in the development of microenvironments deprived of oxygen and nutrients [50]. As a consequence, hypoxic cancer cells use glycolysis, instead of OXPHOS, as a primary mechanism of ATP production. Moreover, severe prolonged hypoxia may affect OXPHOS or even cause irreversible damage of these organelles in cancer cells. But, our data suggest that human colorectal carcinomas are not hypoxic, since display high rates of OXPHOS (Fig. 1, and Table 2) due probably to extremely strong stimulation of angiogenesis. To check this assumption, we investigated the expression of genes encoding the VEGF family and its receptors (such as VEGF-A, -B, -C, FLT-1, FLT-4, KDR, NRP-1, and NRP-2) in human CRC, neighboring and healthy colon tissues. The role of VEGFs in promoting neovascularization and subsequent growth of tumors is well established [67]. Our experiments showed that the expression of gene encoding VEGF-A was predominant regardless of the examined tissue, since the levels of mRNA for VEGF-B and -C were found to be nearly 2-times lower (Fig. 6A). Among the VEGF receptors, the expression of FLT1 gene was the predominant in tumor, whereas the level of FLT4 is low in all tissue samples. Our studies suggest that a potent activation of angiogenesis, both in CRC and neighboring tissues can occur. The levels of VEGFs (A-C) expression in these tissues were found to be excessive, when the results standardized with respect to the control tissue for each patient separately (Fig. 6B). The ability of CRC cells to produce VEGFs, that will result in development of new blood and lymphatic vessels in the tumor growth area, is in good agreement with previous *in vitro* and *in vivo* studies performed in other laboratories [124,15,51,8,93]. In humans, angiogenesis is

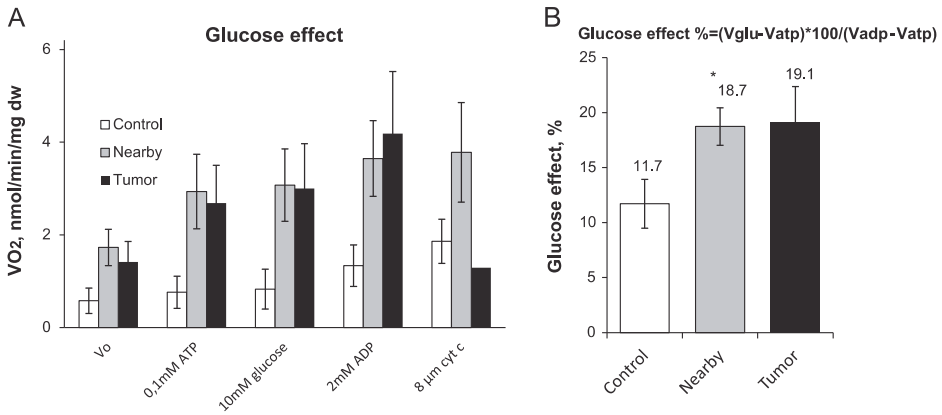

Fig. 4. (A) Oxygraphic analysis of coupling of HK to OXPHOS in permeabilized CRC, nearby and control (healthy large intestine) tissue samples. The addition of 10 mM glucose to CRC fibers in the presence of 0.1 mM Mg-ATP caused a stimulatory effect on mitochondrial respiration in nearby and tumor tissue. (B) The comparison of glucose indexes of all permeabilized tissues samples; bars are SEM, $n=5$, * $p<0.05$.

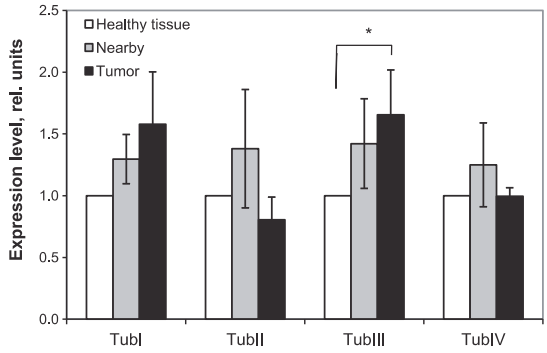

Fig. 5. Western blot analysis of the level of various β-tubulin isotypes: relative expression compared to control. Bars are SEM, $n=7$, * $p<0.05$. Additional data on Supplementary Fig. 2.

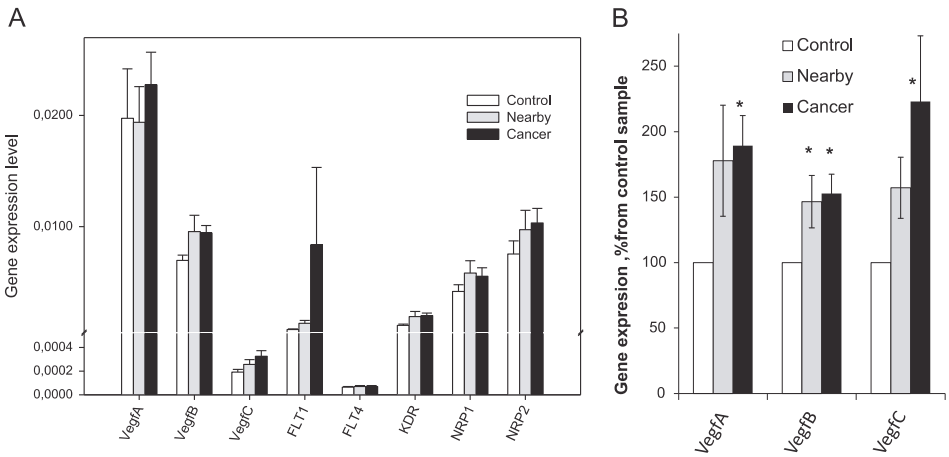

Fig. 6. (A) Q-PCR analysis of the mRNA expression for some vascular endothelial growth factors and their receptors in human CRC and surrounding tissue samples. (B) VEGF (A-C) gene expressions normalized to the unaffected tissue. Bars are SEM, $n=29$, * $p<0.05$.

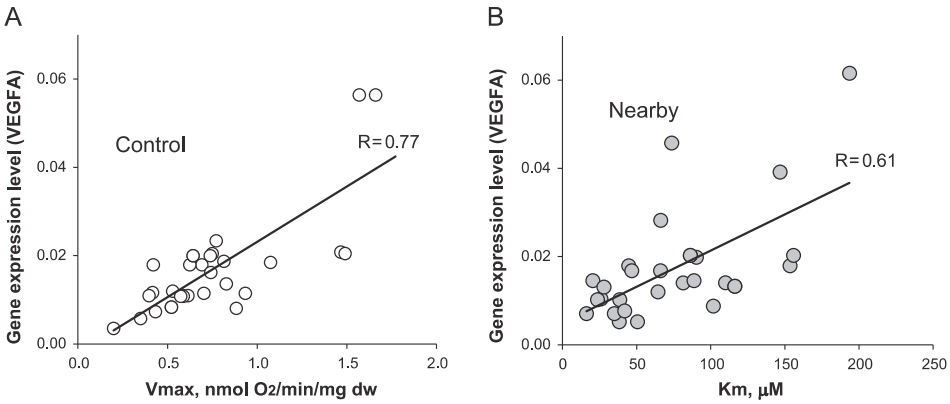

Fig. 7. Interrelationships between the level of VEGF-A expression in tissue samples of CRC patients and their respiratory parameters; rates of maximal ADP-activated respiration (V_m) and K_m values for ADP. (A) The relationship between the level of VEGF-A expression and V_m values for healthy (control) large intestine tissue samples. (B) Correlation between the level of VEGF-A and apparent K_m (ADP) value for nearby non-tumorous tissue fibers. On these figures each data point corresponds to the individual patient. Note: such interrelationships were not registered for colorectal cancer samples.

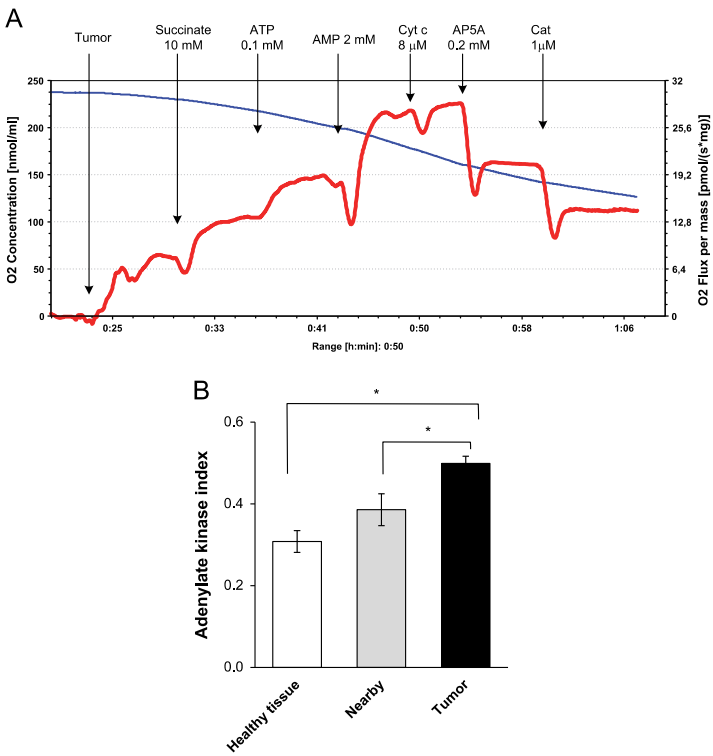

Fig. 8. Oxygraphic analysis of the functional coupling between adenylate kinase (AK) catalyzed processes and OXPHOS in permeabilized CRC, nearby and adjacent healthy intestine tissue samples. (A) Representative tracing of rates of O_2 consumption by CRC fibers; addition of 2 mM AMP in the presence of ATP led to activation of mitochondrial respiration due to formation of ADP in AK reactions. The involvement of AK(s) in stimulation of mitochondrial respiration was confirmed by subsequent addition of diadenosine pentaphosphate (AP5A)-an inhibitor of AK; as can be seen, the administration of AP5A resulted in a strong decrease in the rate of O_2 consumption by CRC samples. Similar experiments were performed with healthy colon and nearby tissue samples. Outer mitochondrial intactness was controlled by effect of exogenously-added cytochrome c (Cyt); usually, the stimulating effect of Cyt on mitochondrial respiration was $< 10\%$. CAT is carboxyatractyloside – a selective inhibitor of adenine nucleotide translocator. (B) Efficiency of the coupling was estimated by means of AK index. Bars are SEM, $n=10$; $^{*}\ p<0.05$.

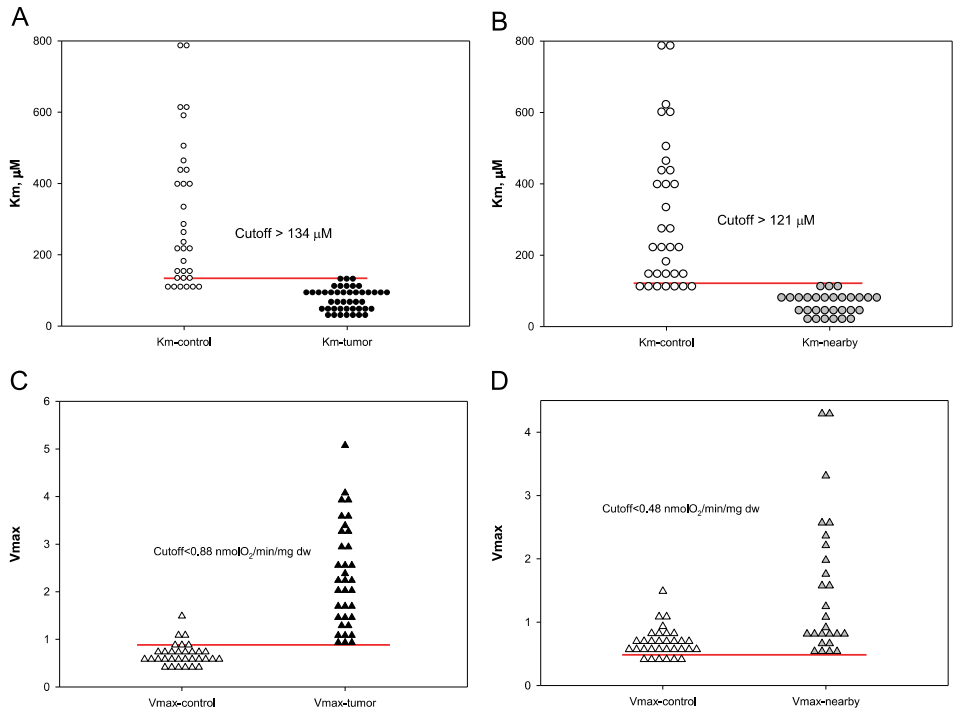

Fig. 9. Cutoff analysis of apparent K_m (ADP) for control-tumor (A), and control-nearby (B) permeabilized samples. V_m values cutoff analysis was also performed for these two datasets: (C) control-tumor and (D) control-nearby. For these calculations samples were taken from 46 patients.

usually absent in most normal differentiated tissues and only very negligible levels of VEGFs can be observed. The relatively high levels of VEGFs expression in healthy intestinal tissue (Fig. 6) could be causally-linked with high regenerative activity of its epithelium.

Besides, our data indicates that the process of neovascularization provides sufficient levels of nutrients and oxygen to support high rates of OXPHOS in CRC and its surrounding non-tumorous tissue cells (Table 2). So, among patients with CRC, a direct interrelationship between the level of VEGF-A expression and apparent K_m (ADP) values was monitored for nearby tissue samples (Fig. 7B). A direct correlation between the level of VEGF-A expression and V_m values was found to occur in normal colon tissue samples (Fig. 7A). However, such correlation was not observed for CRC and nearby tissue samples. There was also no correlation between other vascular endothelial growth factors expression and these bioenergetics parameters.

3.5. *Comparative analysis of regulation of mitochondrial respiration in CRC, nearby and healthy intestinal tissue cells*

3.5.1. *Coupling of OXPHOS with creatine kinase and adenylate kinase systems*

Coupling of spatially separated intracellular ATP producing and ATP-consuming systems is fundamental for the bioenergetics of living organisms, ensuring a fail-safe operation of the energetic system over a broad range of cellular functional activities. The central cellular mechanism of functioning of these organized metabolic pathways is the functional coupling between the isoenzymes of the creatine kinase (CK) and/or adenylate (AK) kinase systems and mitochondrial adenine nucleotide translocase [100,31,32,45,46,55]. Human colonocytes express all CK isoenzymes, can form PCr, and it was found that BB-CK which is characteristic for the brain, is also the predominant isoenzyme in normal colon tissue [55,58,59]. The revealed downregulation of uMtCK mRNA as well as oxygraphic analysis the coupling of MtCK with OXPHOS in CRC tissue let us to conclude that mitochondria of these carcinoma cells have a poor ability to synthesize PCr [58]. Our experiments showed that the nearby tissue is also characterized by downregulation of the CK system: a more than 2-fold fall in the total CK activity was monitored (Table 1). Our *in situ* experiments indicated that mitochondria in nearby tissue cells have a decreased ability for the production of PCr like it was observed for cancer tissue [58].

It is well-known that AK-catalyzed phosphotransfer plays one of the key roles in the maintenance of energy homeostasis in fully differentiated cells with a high-energy demand, such as neural, cardiac and some skeletal muscle cells [31]. To assess the role of AK in CRC cells, the presence of functional coupling between ANT and OXPHOS was investigated. Recently we revealed that in human CRC cells the AK system is up-regulated in comparison with healthy colon tissue, but the function of the system in nearby tissue cells remained to be explored [58].

Experiments showed that the total AK activity of healthy intestine and nearby tissue extracts had similar values and they were ~40% lower as compared with CRC tissue (Table 1). These results indicate that the AK system, in contrast to CK system, is up-regulated in human colorectal carcinomas. Further, we compared the degree of functional coupling of AK-catalyzed processes with the OXPHOS system in CRC, nearby and non-tumor tissue samples. Fig. 8A depicts typical tracings of a change in the rates of O_2 consumption, which were obtained during these studies. The

addition of AMP (in the presence of 0.1 mM Mg-ATP) to permeabilized CRC fibers results in a remarkable (by ~40%) increase in the rate of O_2 consumption by the samples (Fig. 8A). Addition of exogenous cytochrome c had no effect on ADP-dependent respiration indicting the intactness of MOM. Diadenosine pentaphosphate (AP5A, an inhibitor of AK) at a concentration of 0.2 mM suppressed the AMP-stimulated respiration up to initial level indicating thereby that it was largely mediated by ADP produced in AK reactions. Similar stimulatory effects of exogenously-added AMP on mitochondrial respiration were monitored for all nontumorous tissues and they were notably (by about 1.5 times) lower as compared with CRC samples. For more precise estimating of the functional activity of AK processes and their coupling with the OXPHOS system both in cancerous and normal surrounding tissues, we applied the AK index (I_{AK}) proposed by Gruno et al. [43]. The I_{AK} measured for both non-neoplastic tissues (unaffected intestine: $I_{AK}=0.31\pm0.03$; nearby tissue: $I_{AK}=0.39\pm0.04$, $n=10$) were smaller as compared with CRC ($I_{AK}=0.50\pm0.02$, $n=12$) (Fig. 8B). The reason of this difference may be the upregulation of AK system and a stronger interaction between the AK and OXPHOS system in comparison with nontumorous tissue. We assume that this could partly mediate by higher activity of AK2 in carcinoma cells in comparison with non-transformed cells. This will be checked in our further studies.

3.5.2. *Cutoff analysis of rates of maximal respiration, apparent Michaelis–Menten constant values for exogenously added ADP*

For a better understanding of the functioning and regulation of OXPHOS in human CRC cells and surrounding tissues, we measured their rates of maximal respiration as well as apparent K_m values for exogenously-added ADP. The obtained results were also subjected to a cutoff analysis (based on random choice) for studies of distribution of these respiratory parameters in tumor, control and nearby tissues.

The analysis showed that there are strong differences in the regulation of mitochondrial respiration between CRC, nearby and unaffected tissue and it revealed the almost obvious signs the influence of tumor on mitochondrial function in neighboring tissue. Among the patients with CRC, the characteristic values of apparent K_m for ADP for tumor tissue cells were found to be considerably smaller as compared with healthy large intestine (cutoff value > 134 μM) (Fig. 9A). Similarly, decreased K_m (ADP) values (very close to CRC cells) were also registered for nearby non-tumorous tissue cells; upon comparison of the data obtained for healthy and the tumor neighboring tissue the corresponding cutoff value was calculated as > 121 μM (Fig. 9B). The analysis of this dataset led to the conclusion that K_m normal values are above 120–130 μM, but pathological values will remain below this value. The maximal rates of ADP activated respiration V_m measured for permeabilized CRC and nearby tissue fibers were found to exceed significantly that for healthy intestinal cells (Table 2) due most probably to an increased amount of mitochondria in these tissue cells. Appropriate test cutoff values were calculated (Fig. 9C and D) and these could be used for diagnostics of CRC based on the measurements of corresponding respiratory rates in biopsy material. For control-tumor dataset the V_m cutoff value was calculated as 0.88 nmol O_2/min per mg dry weight of tissue (Fig. 9C) This value is similar to the cutoff value found for the control-nearby dataset (0.48 nmol O_2/min per mg dry weight of tissue) (Fig. 9D).

Thus, the obtained data is showing that *in situ* the mitochondria of human CRC and neighboring normal tissue have increased affinity towards adenine nucleotides as compared with healthy colon tissue and these changes may appear in early stages of carcinogenesis. Besides, the results raise the question about the nature of tumor-associated factors which could influence the bioenergetic function of mitochondria in neighboring normal

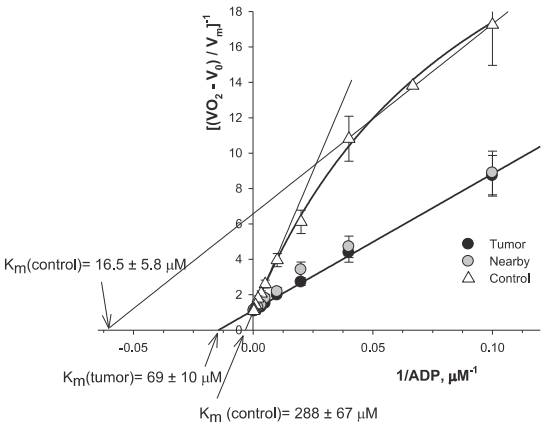

Fig. 10. The dependence of the normalized values of respiration rates of CRC, nearby and unaffected colon tissue permeabilized samples: double reciprocal Lineweaver–Burk plots ($n=9$).

tissue. These factors are not related to hypoxia, since the V_m values characteristic for surrounding CRC tissue were even higher than those for healthy colon tissue (Fig. 9).

3.5.3. *Assessment of the homogeneity of mitochondrial populations in CRC, nearby and control tissues*

In addition, we evaluated heterogeneity of mitochondrial populations in CRC, nearby and normal tissue cells through analyzing the kinetics of respiration of skinned fibers with exogenously-added ADP as proposed by [101]. Distinct mitochondrial subpopulations could be present in these tissue cells and they could differ in their OXPHOS capacity. In our studies, permeabilized fibers were treated with increasing concentrations of ADP, and the measured rates of O_2 consumption were plotted *vs.* ADP concentration in medium as double reciprocal Lineweaver–Burk plots, according to a Michaelis–Menten equation. A linearization approach permits us to calculate corresponding V_m and K_m values. As has been shown by Saks and colleagues [101], the presence of two kinetic phases of respiration regulation on a graph curve is an indicator for the presence of two populations of mitochondria in a cell, which have different affinity for ADP and, correspondingly, various K_m and V_m parameters. Our studies showed that there are not distinct mitochondrial subpopulations in CRC and neighboring tissue cells (Fig. 10). But, in healthy bowel, very clearly two populations of mitochondria with very different properties are revealed in double reciprocal plots. One population of mitochondria is characterized with a low K_m value (16.5 ± 5.8 μM) but K_m for the second population is manifold higher (288 ± 67 μM) (Fig. 10). The large difference in these apparent K_m values for ADP (exceeding the factor of nearly 20 times) explains why in this case two populations of mitochondria are possible to see. These two populations of mitochondria, most probably, represent different types of fibers in this normal colon sample: mucosa and smooth muscle. Such a difference may be a reflection of the heterogeneity of healthy colon tissue cellular composition; high K_m values could be attributed to mitochondria of the tissue cells with high OXPHOS rates. Similar values of K_m for ADP were monitored by us for rat heart cardiomyocytes and soleus muscle cells. Low K_m values for exogenously-added ADP (in the range of 20–40 μM) are usually characteristic for mitochondria of cells with high glycolytic rates, such as gastrocnemius white (see Table 2) or some poorly differentiated or tumor cells [47,60]. In the nearby tissue two populations of mitochondria are absent and the apparent K_m value for

ADP is the same as for cancer tissue (Km = 69 ± 10 μM) (Fig. 10). Thus, we established that during colorectal carcinogenesis the amount of mitochondria rises together with profound changes in the regulation of the mitochondrial outer membrane permeability for ADP.

4. Discussion

Previous studies have shown that during malignant transformation the emerging cancer cells acquire new behavioral features and profound changes in their energy metabolism, which in part resemble undifferentiated embryonic cells, such as: invasive and destructive growth, dissemination, higher glycolytic capacity even in the presence of O_2 [49,76,83]. The arising shifts in bioenergetics of cancer cells provide them with high yields and rates of ATP production, which are sufficient to support high rates of biosynthetic processes and proliferation even in hypoxic environment [36]. Some signaling pathways underlying the Warburg phenotype of malignant tumors are now uncovered and some therapeutic strategies which target glycolysis in tumor cells have also been proposed [115,74,89]. Currently, however, little is known about the features of main energy producing systems and their functioning in human CRC cells, although a deeper understanding of the specificity of bioenergetic processes which occur in the malignant cells, is a prerequisite for the development of more effective treatment strategies. To date, most of the corresponding studies have been carried out on tumor cells grown in cultures, whose proliferative, metabolic and bioenergetic properties may differ cardinally from *in vivo* conditions. CRC is currently regarded as a tumor of the Warburg phenotype [22,54,62], but our recent studies clearly showed that *in situ* human CRC cells have the total glycolytic activity close to healthy tissue cells and that in these malignant cells there is up-regulation of OXPHOS associated with stimulation of mitochondrial biogenesis [58] (Tables 1 and 2). These data were in good agreement with the results of other researchers who showed that tumor cells are not intrinsically glycolytic and that not all tumors have mitochondria with OXPHOS deficiency. The OXPHOS system was reported to be the principal ATP producer (> 90%) for several malignant tumor cell types under normoxic conditions [111,81]. Therefore, drug therapy targeting OXPHOS has emerged as an important alternative for growth arrest of oxidative type tumors [81,83,96].

Recently, Witkiewicz and colleagues proposed a new conceptual model for metabolism of a cancer cell at the tissue level that sheds new light on the significance of aerobic glycolysis, linked with lactic acid production, in fueling tumor growth and metastasis, and acting as a paracrine onco-metabolite [123]. This phenomenon was called as the “reverse Warburg effect”, since aerobic glycolysis occurred in stromal fibroblasts, but not in epithelial cancer cells [123]. In our work, *in situ* experiments were carried out to reveal the presence of “parasitic” energy-transfer interactions between CRC and neighboring tissue cells. For this aim, we compared the glycolytic activity and the OXPHOS capacity of CRC tissue cells with those in neighboring and distant healthy intestinal tissue cells.

Taken together our *in situ* studies indicate that human CRC cells *in vivo* exert a strong effect on the energy metabolism of neighboring tissue cells, so that they acquire the bioenergetic parameters specific to the tumor itself. Nearby tissue samples are characterized with upregulated mitochondrial respiration: values of State 2 and State 3 respiration rates are close to the tumor cells (Table 2). It is also found that nearby tissue is not purely glycolytic (Table 1) and it contains active mitochondria with fully-functional respiratory chain complexes (Fig. 1). Further work is needed to clarify the nature of paracrine factors released by CRC cells, which could alter the energy metabolism in tumor neighboring tissue. These can be various transforming growth factors (TGFs), as a large proportion of colorectal cancers were found to be characterized by elevated TGF-β production [13]. The growth of colorectal carcinomas is also associated with downregulation of the CK system in nearby tissue cells (Table 2) as well as with profound alterations in function of their mitochondria. In nearby tissue the mitochondria exhibit increased affinity to exogenously-added ADP similarly to tumor mitochondria. The corresponding K_m values were measured as 93.6 ± 7.7 μM for CRC cells and 84.9 ± 9.9 μM for nearby tissue; both these apparent K_m (ADP) values were considerably (by almost 3 times) lower in comparison with distant healthy colon tissue cells (256 ± 34 μM) (Table 1).

Our results indicate a very low probability that the nearby tissue cell populations' fuel *via* a lactate shunt the OXPHOS system in CRC cells. We found that the postoperative samples derived from CRC and neighboring tissue displayed practically equal values of the glucose effect (Fig. 4), levels of HK-1 and HK-2 expression (Fig. 3), and total glucose-phosphorylating activity (Table 1).

Our studies demonstrate that human CRC cannot be considered as a pure hypoxic tumor, since the malignancy and its neighboring tissue exhibit high rates of OXPHOS (Table 2). The respiration rates for these tissues are even higher than in unaffected tissue. The possible absence of acute hypoxic nature in colorectal carcinomas is also confirmed by their practically equal glucose-phosphorylating capacity as compared with surrounding non-tumorous tissues (Table 1), although hypoxia is a well-known factor that strongly increases the dependence of cancer cells on glycolytic pathway for ATP generation. Pioneering studies by Folkman demonstrated the crucial role of angiogenesis in tumor growth [34]. In the presented work, we found that there is some upregulation of VEGFs (A, B, and C) and their receptors expression in CRC as well as in the tumor surrounding tissue (Fig. 6). These might support tumor growth by inducing neovascularization, providing thereby O_2 and substrates for cancer cell proliferation. It was already proven that VEGF-B is abundantly expressed in tissues with highly-active energy metabolism, where it could support significant metabolic functions [11]. It has generally been assumed that in cancer cells, the upregulation of VEGFs is caused by a deficiency in oxygen through HIF-1α pathways [27]. Co-expression of HIF-1α with VEGFs has been already monitored in human CRC tissue, and to be associated with poor prognosis [14]. But our findings suggest that in human CRC cells HIF-1α is not the main factor in the induction of VEGFs formation. The opinion [109,86] that HIF-1α negatively regulates mitochondrial biogenesis and O_2 consumption in tumor cells is doubtful, as in our previous work we clearly showed (*via* TOM20 expression) that in human colorectal carcinomas mitochondrial biogenesis is upregulated [58]. Increased levels of VEGFs, as compared to healthy colon, were also registered by us in nearby tissue (Fig. 6B). Recent findings suggest that in some cases the tumor accessory cells, such as macrophages, may be the main source of VEGFs promoting thereby neovascularization and tumor proliferation [24]. In this relation, it was robustly demonstrated that some non-steroid anti-inflammatory drugs (e.g., aspirin) lower risk of colorectal cancer-mediated mortality [16]. Besides, it was reported [27] that cancer-associated fibroblasts can express increased levels of VEGFs, thereby contributing to angiogenesis and tumor progression. Multiple clinical trials using antiangiogenic agents blocking VEGFs have recently demonstrated high efficacy for patients with metastatic colorectal cancer [23]. Our results show a correlation between the level of VEGF-A expression and maximal mitochondrial respiration rate V_m occurring in healthy colon tissue (Fig. 7A), but in tumor nearby tissue this correlation is replaced by a relationship between VEGF-A expression and the mitochondrial outer membrane permeability for ADP (Fig. 7B). The reason for this is not clear yet.

Our study demonstrates that human CRC cells have higher rates of OXPHOS as compared to normal tissue cells (Table 2) [58]. This is surprising, since it has been reported [102,122] that β-F1-ATPase catalytic subunit of the mitochondrial H^+-ATP synthase is down-regulated in human colorectal carcinomas, and this is associated with the upregulation of the ATPase inhibitory factor 1 expression [35]. These changes should be associated with severe suppression of OXPHOS capacity, but our results do not confirm this. Our results support the hypothesis proposed by Pedersen and coworkers [89] that in tumor cells the presence of mitochondrially-bound HK-2 can be responsible for their increased rates of aerobic glycolysis. We found that the addition of glucose to permeabilized fibers (in the presence of exogenous ATP) resulted in some stimulation of mitochondrial respiration, not only in CRC, but also in neighboring samples. This effect, on the contrary, was negligible for healthy colon tissue cells (Fig. 4). The minor effect of glucose on mitochondrial respiration of permeabilized fibers derived from unaffected tissue samples was initially confusing; all examined tissues are characterized by the presence of mitochondrially-bound HK-2 [58], but have different levels of HK-1 and HK-2 expression (Fig. 3). At the same time, it was reported that mitochondria of tumor cells have elevated binding capacity towards HK-1 and -2 as compared with normal cells due to an increased number of VDAC(s) per mitochondria and cholesterol content in the MOM [87]. Elevated levels of VDAC protein (a binding partner for HKs) were registered by us in human CRC tissue cells, as compared to healthy colon tissue (Fig. 2), and this may be a plausible explanation for the negligible stimulating effect of exogenously added glucose on mitochondrial respiration in the normal tissue cells (Fig. 4) in spite of similar with cancerous tissue the levels of HK 1 expression (Fig. 3). Although several isoforms of VDAC are known, they do have similar kinetic characteristics, which indicate that the contribution of VDAC to enhanced HK binding and glucose phosphorylation is due to quantitative differences in binding site availability [105]. The distribution and role of HK isoforms in CRC and surrounding tissues *in situ* need further studies.

CK plays a key role in the energy homeostasis of vertebrate cells. It has been shown by us and other researchers that during carcinogenesis there is a nearly 2 times decrease in the total CK activity (Table 1) associated with loss of BB-CK (predominant isoform of CK in normal intestinal tissue) MM-CK, sMtCK and uMtCK [55,58]. Also, in our prior study [58] we showed that normal intestinal cells have elevated levels of uMtCK expression and these cells are characterized by, on contrary to CRC cells, tight coupling between the CK isoform and OXPHOS system like in cardiac and slow-twitch skeletal muscle cells [114,47,78]. Down-regulation of the CK system (associated with loss of uMtCK) was registered by us and also others tumors with different histological type, such as human breast carcinoma [56], neuroblastoma [60] and sarcoma cells [88,9]. Possible signaling pathways mediating the downregulation of CK system in HCC cells remain still unclear. It was proposed that the downregulation of BB-CK in human CRC cells may play an important role in the tumor progression [80]. In the presented work, we found that colorectal carcinomas exert a distant effect on neighboring cells resulting in downregulation of their CK system (Table 1). The revealed absence of stimulating effect of exogenous creatine on mitochondrial respiration in CRC and nearby samples, contrary to healthy intestinal tissue, shows that mitochondria in both these tissues lose their ability to produce PCr. Our findings, on the poor ability of human CRC cells mitochondria to produce the marked amount of PCr, are in good agreement with literature data [59]. Although during CRC progression the arising malignant cells were found to up-regulate their AK system (Table 1) and there is tight coupling of the system with OXPHOS (Fig. 8, perhaps partly *via* upregulation of mitochondrial AK2), the distant effect of the neoplasm on AK processes in surrounding tissue cells was found to be minor (Table 1 and Fig. 8). We assume that the revealed downregulation of CK system may be casually-linked with upregulation of the AK phosphotransfer system. Indeed, we found that in human CRC cells the total AK activity exceeded significantly from that of normal intestinal tissue cells (Table 1) and it has also been reported by Dzeja et al. [33] that suppression of CK-catalyzed phosphotransfer may result in increased phosphoryl transfer by AK in intact skeletal muscle. But in contrast to our findings concerning CRC, the total AK activity in human lung adenocarcinomas was found to be substantially less as compared with normal tissue [41].

In oxidative type tissues with high energy demand the OXPHOS system is organized into protein supercomplexes, called the Mitochondrial Interactosome (MI) [113,45,79,99]. MI is a large transmembrane complex consisting of ATP-Synthasome, MtCK, VDAC, and protein factors regulating the outer mitochondrial membrane. There also some indication that the mitochondrial supercomplex may include certain HK(s) [74] as well as AK2 and AK4 [69]. From our studies it becomes clear that during carcinogenesis the structure and function of MI in colonocytes undergoes severe and specific alterations. Moreover, our results strongly suggest that colorectal carcinomas exert, by a currently unknown way, a distant effect on the functioning of MI in neighboring tissue cells. These alterations provide the basis for successful proliferation and viability of malignant cells in the hostile environment, characterized by deficiency in oxygen and nutrients. It is important to emphasize that in tumor cells, in addition to the MI, substantial alterations were also found to occur in the organization of mitochondrial respiratory chain: some enzymes of the ETC can form large supercomplexes that in turn may promote the generation of ATP [2,56,57,60]. Indeed, our studies showed that the MOM in CRC and nearby tissue cells has increased permeability towards adenine nucleotides and they display an increased affinity for exogenous ADP. Thus, the apparent K_m values for exogenously-added ADP measured for CRC and the neighboring tissue cells were found to be considerably (by about 3 times) smaller in comparison with that for distant normal tissues samples (Table 2). Our results and an analysis of literature data allowed us to propose several possible explanations for this phenomenon. Firstly, our results indicate that CRC and neighboring tissue cells, as compared to control colon tissue, contain higher levels of VDAC (Fig. 2) and, consequently, they can bind much more HK-2 [87]. The presence of mitochondrially-bound HK-2 in CRC and control intestinal tissues was adjusted by our immunofluorescent studies [58]. This event can mediate lower K_m for ADP, since the binding of HK to VDAC holds this channel in the opened state [98] or then some other unknown protein factors could be involved in this phenomenon. Secondly, the revealed downregulation of MtCK and the absence of creatine kinase energy transfer network in CRC and neighboring tissue cells could be also responsible for increased permeability of the MOM [114,45]. Thirdly, it is known that some ß-tubulin isoforms can bind to the VDAC localized on MOM and suppress its permeability towards adenine nucleotides, mediating thereby higher K_m values for ADP and high rates of PCr production [114,47]. Western blot analysis showed similar levels of ß-tubulin isoforms (I, II, III and IV) expression in CRC, healthy colon and nearby tissue samples (Fig. 5) and no binding of ßII-tubulin to VDAC(s) was also registered in these tissues [58]. This result is not in accordance with the data obtained on pure oxidative cells (rat heart cardiomyocytes) [112,46] and the question arises, what complex is interacting with VDAC and therefore possibly regulating its permeability. This question remains the key in understanding the regulation mechanism of ATP production in mitochondria, not only in cardiac and muscle cells, but also in cells of the CRC. Tubulin and its potential biding partner – VDAC can

undergo a number of various posttranslational modifications [53,87], which may deeply affect their interactions. Fourthly, humans encode three different VDACs; VDAC1, VDAC2, and VDAC3, which were found to display different binding capacity towards tubulin, and the VDAC3 permeability is not sensitive to tubulin regulation [72]. In this relation, we propose that in CRC and neighboring non-tumor tissue cells, having lowered apparent K_m values for ADP (Table 2) the VDAC3 isoform may be predominant. In addition, remarkable differences in the profile of ANT isoforms expression between normal and cancerous tissue cells could be responsible for the increased permeability of MOM in tumor cells. Cancer cells express predominantly ANT2, which interacts with VDAC [20,21], and this could result in a facilitated diffusion adenine nucleotides across the MOM.

The increased level of β-III tubulin expression in colorectal carcinomas (Fig. 5) was also registered in other laboratories, and this may contribute to cancer cell invasion [66,84].

5. Conclusion

This study revealed several important aspects in the bioenergetic metabolism of CRC and surrounding tissues cells. For a long time aerobic glycolysis has been considered the main energy source in CRC cells, but our results strongly suggest that CRC should not be considered a hypoxic tumor, since the malignancy exhibits high (even more than healthy colon tissue) rates of oxygen consumption, increased amount of mitochondria and practically equal glucose-phosphorylating capacity with the surrounding tissues. Obvious signs of stimulated neovascularization in CRC and nearby tissue are also evident. We observed strong differences in the function and regulation OXPHOS between CRC and normal intestinal tissue cells. During carcinogenesis, the amount of mitochondria is increasing in parallel to the change in the regulation of mitochondrial outer membrane permeability and phosphocreatine/creatine kinase shuttle that is completely replaced with AK mediated energy transport. The malignant cells are characterized by downregulation of CK, which is further associated with upregulation of the AK system and this could promote tumor growth and metastasis. The mitochondria of CRC cells lose the ability to produce PCr, reveal possibility of coupling of HK to OXPHOS and have increased affinity for ADP. In this aspect, more studies are required to determine the profile of AK, ANT and VDAC isoforms expression in human colorectal carcinomas. One of the most important findings from our studies is also that CRC cells exert a potent distant effect on the energy metabolism of nearby tissue cells. As compared with healthy colon tissue cells, nearby cells are also characterized by stimulated OXPHOS, downregulation of the CK system, which is associated with the loss of MtCK and increased permeability of the MOM against adenine nucleotides. Our data refused the initial hypothesis according to which the CRC surrounding cells could fuel *via* a lactate shunt the OXPHOS system in the malignancy cells.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.08.020.

References

[1] M. Abdullah, A.A. Rani, M. Simadibrata, A. Fauzi, A.F. Syam, The value of fecal tumor M2 pyruvate kinase as a diagnostic tool for colorectal cancer screening, Acta Med Indones 44 (2012) 94–99.
[2] R. Acin-Perez, J.A. Enriquez, The function of the respiratory supercomplexes: The plasticity model, Biochim. Biophys. Acta (BBA) – Bioenerg. 1837 (2014) 444–450.
[3] B. Altenberg, K.O. Greulich, Genes of glycolysis are ubiquitously overexpressed in 24 cancer classes, Genomics 84 (2004) 1014–1020.
[4] N.D. Amoedo, M.F. Rodrigues, F.D. Rumjanek, Mitochondria: are mitochondria accessory to metastasis? Int. J. Biochem. Cell Biol. 51 (2014) 53–57.
[5] T. Anmann, R. Guzun, N. Beraud, S. Pelloux, A.V. Kuznetsov, L. Kogerman, T. Kaambre, P. Sikk, K. Paju, N. Peet, 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 1757 (2006) 1597–1606.
[6] F. Appaix, A.V. Kuznetsov, Y. Usson, L. Kay, T. Andrienko, J. Olivares, T. Kaambre, P. Sikk, R. Margreiter, V. Saks, Possible role of cytoskeleton in intracellular arrangement and regulation of mitochondria, Exp. Physiol. 88 (2003) 175–190.
[7] K.K. Arora, P.L. Pedersen, Functional significance of mitochondrial bound hexokinase in tumor cell metabolism. Evidence for preferential phosphorylation of glucose by intramitochondrially generated ATP, J Biol Chem *263* (1988) 17422–17428.
[8] L. Bagnasco, D. Piras, S. Parodi, I. Bauer, G. Zoppoli, F. Patrone, A. Ballestrero, Role of angiogenesis inhibitors in colorectal cancer: sensitive and insensitive tumors, Curr. Cancer Drug Targets 12 (2012) 303–315.
[9] S. Bera, T. Wallimann, S. Ray, M. Ray, Enzymes of creatine biosynthesis, arginine and methionine metabolism in normal and malignant cells, FEBS J. 275 (2008) 5899–5909.
[10] M. Brandon, P. Baldi, D.C. Wallace, Mitochondrial mutations in cancer, Oncogene 25 (2006) 4647–4662.
[11] M. Bry, R. Kivela, V.M. Leppanen, K. Alitalo, Vascular endothelial growth factor-B in physiology and disease, Physiol. Rev. 94 (2014) 779–794.
[12] E. Bustamante, P.L. Pedersen, Mitochondrial hexokinase of rat hepatoma cells in culture: solubilization and kinetic properties, Biochemistry 19 (1980) 4972–4977.
[13] A. Calon, E. Espinet, S. Palomo-Ponce, Tauriello, V.F. Daniele, M. Iglesias, Céspedes, V. María, M. Sevillano, C. Nadal, P. Jung, Zhang, H.F. Xiang, et al., Dependency of Colorectal Cancer on a TGF-β-Driven Program in Stromal Cells for Metastasis Initiation, Cancer Cell 22 (2012) 571–584.
[14] D. Cao, M. Hou, Y.S. Guan, M. Jiang, Y. Yang, H.F. Gou, Expression of HIF-1alpha and VEGF in colorectal cancer: association with clinical outcomes and prognostic implications, BMC Cancer 9 (2009) 432.
[15] M. Cecilia Subauste, T. Kupriyanova, E. Conn, V. Ardi, J. Quigley, E. Deryugina, Evaluation of metastatic and angiogenic potentials of human colon carcinoma cells in chick embryo model systems, Clin. Exp. Metastasis 26 (2009) 1033–1047.
[16] A.T. Chan, S. Ogino, C.S. Fuchs, Aspirin use and survival after diagnosis of colorectal cancer, JAMA 302 (2009) 649–658.
[17] D. Chandra, K.K. Singh, Genetic insights into OXPHOS defect and its role in cancer, Biochim. Biophys. Acta (BBA) – Bioenerg. 1807 (2011) 620–625.
[18] A. Chatterjee, E. Mambo, D. Sidransky, Mitochondrial DNA mutations in human cancer, Oncogene 25 (2006) 4663–4674.
[19] Z. Chen, H. Zhang, W. Lu, P. Huang, Role of mitochondria-associated hexokinase II in cancer cell death induced by 3-bromopyruvate, Biochim. Biophys. Acta (BBA) – Bioenerg. 1787 (2009) 553–560.
[20] A. Chevrollier, D. Loiseau, B. Chabi, G. Renier, O. Douay, Y. Malthiery, G. Stepien, ANT2 isoform required for cancer cell glycolysis, J. Bioenerg. Biomembr. *37* (2005) 307–316.
[21] A. Chevrollier, D. Loiseau, P. Reynier, G. Stepien, Adenine nucleotide translocase 2 is a key mitochondrial protein in cancer metabolism, Biochim. Biophys. Acta (BBA) – Bioenerg. 1807 (2011) 562–567.
[22] J.K. Chung, Y.J. Lee, C. Kim, S.R. Choi, M. Kim, K. Lee, J.M. Jeong, D.S. Lee, J. J. Jang, M.C. Lee, Mechanisms related to [18F]fluorodeoxyglucose uptake of human colon cancers transplanted in nude mice, J. Nucl. Med. 40 (1999) 339–346.
[23] J.M. Clarke, H.I. Hurwitz, F. Rangwala, Understanding the mechanisms of action of antiangiogenic agents in metastatic colorectal cancer: a clinician's perspective, Cancer Treat. Rev. 40 (2014) 1065–1072.
[24] O.R. Colegio, N.-Q. Chu, A.L. Szabo, T. Chu, A.M. Rhebergen, V. Jairam, N. Cyrus, C.E. Brokowski, S.C. Eisenbarth, G.M. Phillips, et al., Functional polarization of tumour-associated macrophages by tumour-derived lactic acid, Nature 513 (2014) 559–563.
[25] J.M. Curry, M. Tuluc, D. Whitaker-Menezes, J.A. Ames, A. Anantharaman, A. Butera, B. Leiby, D.M. Cognetti, F. Sotgia, M.P. Lisanti, U.E. Martinez-Outschoorn, Cancer metabolism, stemness and tumor recurrence: MCT1 and MCT4 are functional biomarkers of metabolic symbiosis in head and neck cancer, Cell Cycle 12 (2013) 1371–1384.
[26] D. Daye, K.E. Wellen, Metabolic reprogramming in cancer: Unraveling the

role of glutamine in tumorigenesis, Semin. Cell Dev. Biol. 23 (2012) 362–369.
[27] E.M. De Francesco, R. Lappano, M.F. Santolla, S. Marsico, A. Caruso, M. Maggiolini, HIF-1alpha/GPER signaling mediates the expression of VEGF induced by hypoxia in breast cancer associated fibroblasts (CAFs), Breast Cancer Res. 15 (2013) R64.
[28] A.C. de Souza, G.Z. Justo, D.R. de Araujo, A.D. Cavagis, Defining the molecular basis of tumor metabolism: a continuing challenge since Warburg's discovery, Cell. Physiol. Biochem. 28 (2011) 771–792.
[29] R.J. DeBerardinis, A. Mancuso, E. Daikhin, I. Nissim, M. Yudkoff, S. Wehrli, C. B. Thompson, Beyond aerobic glycolysis: Transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis, Proc. Natl. Acad. Sci. 104 (2007) 19345–19350.
[30] A.R. Diers, K.A. Broniowska, C.F. Chang, N. Hogg, Pyruvate fuels mitochondrial respiration and proliferation of breast cancer cells: effect of monocarboxylate transporter inhibition, Biochem. J. 444 (2012) 561–571.
[31] P. Dzeja, A. Terzic, Adenylate kinase and AMP signaling networks: Metabolic monitoring, signal communication and body energy sensing, Int. J. Mol. Sci. 10 (2009) 1729–1772.
[32] P.P. Dzeja, A. Terzic, Phosphotransfer networks and cellular energetics, J. Exp. Biol. 206 (2003) 2039–2047.
[33] P.P. Dzeja, K.T. Vitkevicius, M.M. Redfield, J.C. Burnett, A. Terzic, Adenylate kinase-catalyzed phosphotransfer in the myocardium: increased contribution in heart failure, Circ. Res. 84 (1999) 1137–1143.
[34] J. Folkman, Tumor angiogenesis: therapeutic implications, N. Engl. J. Med. 285 (1971) 1182–1186.
[35] L. Formentini, M. Sanchez-Arago, L. Sanchez-Cenizo, J.M. Cuezva, The mitochondrial ATPase inhibitory factor 1 triggers a ROS-mediated retrograde prosurvival and proliferative response, Mol. Cell 45 (2012) 731–742.
[36] S. Ganapathy-Kanniappan, J.F. Geschwind, Tumor glycolysis as a target for cancer therapy: progress and prospects, Mol. Cancer 12 (2013) 152.
[37] E. Gnaiger, Oxygen solubility in experimental media, OROBOROS Bioenerg. Newsl. 6 (2001) 1–6.
[38] A. Goel, T. Nagasaka, C.N. Arnold, T. Inoue, C. Hamilton, D. Niedzwiecki, C. Compton, R.J. Mayer, R. Goldberg, M.M. Bertagnolli, C.R. Boland, The CpG island methylator phenotype and chromosomal instability are inversely correlated in sporadic colorectal cancer, Gastroenterology 132 (2007) 127–138.
[39] V. Gogvadze, S. Orrenius, B. Zhivotovsky, Mitochondria as targets for cancer chemotherapy, Semin. Cancer Biol. 19 (2009) 57–66.
[40] M. Gonzalez-Granillo, A. Grichine, R. Guzun, Y. Usson, K. Tepp, V. Chekulayev, I. Shevchuk, M. Karu-Varikmaa, A.V. Kuznetsov, M. Grimm, et al., Studies of the role of tubulin beta II isotype in regulation of mitochondrial respiration in intracellular energetic units in cardiac cells, J. Mol. Cell. Cardiol. 52 (2012) 437–447.
[41] O. Greengard, J.F. Head, S.L. Goldberg, Uridine kinase, adenylate kinase, and guanase in human lung tumors, Cancer Res. 40 (1980) 2295–2299.
[42] L. Gregersen, A. Jacobsen, L. Frankel, J. Wen, A. Krogh, A. Lund, MicroRNA-143 down-regulates Hexokinase 2 in colon cancer cells, BMC Cancer 12 (2012) 232.
[43] M. Gruno, N. Peet, E. Seppet, L. Kadaja, K. Paju, M. Eimre, E. Orlova, M. Peetsalu, A. Tein, J. Soplepmann, et al., Oxidative phosphorylation and its coupling to mitochondrial creatine and adenylate kinases in human gastric mucosa, Am. J. Physiol. Regul. Integr. Comp. Physiol. 291 (2006) R936–R946.
[44] V. Gudnason, S. Ingvarsson, A. Jonasdottir, V. Andresdottir, V. Egilsson, Isoenzyme pattern and subcellular localization of hexokinases in human breast cancer and nonpathological breast tissue, Int. J. Cancer 34 (1984) 63–66.
[45] R. Guzun, M. Gonzalez-Granillo, M. Karu-Varikmaa, A. Grichine, Y. Usson, T. Kaambre, K. Guerrero-Roesch, A. Kuznetsov, U. Schlattner, V. Saks, Regulation of respiration in muscle cells in vivo by VDAC through interaction with the cytoskeleton and MtCK within Mitochondrial Interactosome, Biochim. Biophys. Acta 1818 (2012) 1545–1554.
[46] R. Guzun, T. Kaambre, R. Bagur, A. Grichine, Y. Usson, M. Varikmaa, T. Anmann, K. Tepp, N. Timohhina, I. Shevchuk, et al., Modular organization of cardiac energy metabolism: energy conversion, transfer and feedback regulation, Acta Physiol. 213 (2015) 84–106.
[47] R. Guzun, M. Karu-Varikmaa, M. Gonzalez-Granillo, A.V. Kuznetsov, L. Michel, C. Cottet-Rousselle, M. Saaremae, T. Kaambre, M. Metsis, M. Grimm, et al., Mitochondria-cytoskeleton interaction: distribution of beta-tubulins in cardiomyocytes and HL-1 cells, Biochim. Biophys. Acta 1807 (2011) 458–469.
[48] R.S. Haber, A. Rathan, K.R. Weiser, A. Pritsker, S.H. Itzkowitz, C. Bodian, G. Slater, A. Weiss, D.E. Burstein, GLUT1 glucose transporter expression in colorectal carcinoma: a marker for poor prognosis, Cancer 83 (1998) 34–40.
[49] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell 144 (2011) 646–674.
[50] A.L. Harris, Hypoxia–a key regulatory factor in tumour growth, Nat. Rev. Cancer 2 (2002) 38–47.
[51] X. Huang, B.R. Withers, R.C. Dickson, Sphingolipids and lifespan regulation, Biochim. Biophys. Acta (BBA) – Mol. Cell Biol. Lipids 1841 (2014) 657–664.
[52] K. Izuishi, Y. Yamamoto, T. Sano, R. Takebayashi, Y. Nishiyama, H. Mori, T. Masaki, A. Morishita, Y. Suzuki, Molecular mechanism underlying the detection of colorectal cancer by 18F-2-fluoro-2-deoxy-D-glucose positron emission tomography, J. Gastrointest. Surg.: Off. J. Soc.r Surg. Aliment. Tract 16 (2012) 394–400.
[53] C. Janke, J.C. Bulinski, Post-translational regulation of the microtubule cytoskeleton: mechanisms and functions, Nat. Rev. Mol. Cell. Biol. 12 (2011) 773–786.
[54] B. Jimenez, R. Mirnezami, J. Kinross, O. Cloarec, H.C. Keun, E. Holmes, R. D. Goldin, P. Ziprin, A. Darzi, J.K. Nicholson, 1H HR-MAS NMR spectroscopy of tumor-induced local metabolic "field-effects" enables colorectal cancer staging and prognostication, J. Proteome Res. 12 (2013) 959–968.
[55] J. Joseph, A. Cardesa, J. Carreras, Creatine kinase activity and isoenzymes in lung, colon and liver carcinomas, Br. J. Cancer 76 (1997) 600–605.
[56] T. Kaambre, V. Chekulayev, I. Shevchuk, M. Karu-Varikmaa, N. Timohhina, K. Tepp, J. Bogovskaja, R. Kutner, V. Valvere, V. Saks, Metabolic control analysis of cellular respiration in situ in intraoperational samples of human breast cancer, J. Bioenerg. Biomembr. 44 (2012) 539–558.
[57] T. Kaambre, V. Chekulayev, I. Shevchuk, K. Tepp, N. Timohhina, M. Varikmaa, R. Bagur, A. Klepinin, T. Anmann, A. Koit, et al., Metabolic control analysis of respiration in human cancer tissue, Front. Physiol. 4 (2013) 1–6.
[58] A. Kaldma, A. Klepinin, V. Chekulayev, K. Mado, I. Shevchuk, N. Timohhina, K. Tepp, M. Kandashvili, M. Varikmaa, A. Koit, 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. 55 (2014) 171–186.
[59] J.N. Kasimos, T.E. Merchant, L.W. Gierke, T. Glonek, 31P Magnetic Resonance Spectroscopy of Human Colon Cancer, Cancer Res. 50 (1990) 527–532.
[60] A. Klepinin, V. Chekulayev, N. Timohhina, I. Shevchuk, K. Tepp, A. Kaldma, A. Koit, V. Saks, T. Kaambre, Comparative analysis of some aspects of mitochondrial metabolism in differentiated and undifferentiated neuroblastoma cells, J. Bioenerg. Biomembr. 46 (2014) 17–31.
[61] Y.H. Ko, P.L. Pedersen, J.F. Geschwind, Glucose catabolism in the rabbit VX2 tumor model for liver cancer: characterization and targeting hexokinase, Cancer Lett. 173 (2001) 83–91.
[62] M.I. Koukourakis, A. Giatromanolaki, C. Simopoulos, A. Polychronidis, E. Sivridis, Lactate dehydrogenase 5 (LDH5) relates to up-regulated hypoxia inducible factor pathway and metastasis in colorectal cancer, Clin. Exp. Metastasis 22 (2005) 25–30.
[63] A.V. Kuznetsov, S. Javadov, R. Guzun, M. Grimm, V. Saks, Cytoskeleton and regulation of mitochondrial function: the role of beta-tubulin II, Front. Physiol. 4 (2013) 1–7.
[64] A.V. Kuznetsov, T. Tiivel, P. Sikk, T. Kaambre, L. Kay, Z. Daneshrad, A. Rossi, L. Kadaja, N. Peet, E. Seppet, 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 (1996) 909–915.
[65] A.V. Kuznetsov, V. Veksler, F.N. Gellerich, V. Saks, R. Margreiter, W.S. Kunz, Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells, Nat. Protoc. 3 (2008) 965–976.
[66] L.J. Leandro-García, S. Leskelä, I. Landa, C. Montero-Conde, E. López-Jiménez, R. Letón, A. Cascón, M. Robledo, C. Rodríguez-Antona, Tumoral and tissue-specific expression of the major human β-tubulin isotypes, Cytoskeleton 67 (2010) 214–223.
[67] E.Y. Lin, J.-F. Li, L. Gnatovskiy, Y. Deng, L. Zhu, D.A. Grzesik, H. Qian, X.-n Xue, J.W. Pollard, Macrophages regulate the angiogenic switch in a mouse model of breast cancer, Cancer Res. 66 (2006) 11238–11246.
[68] M.P. Lisanti, U.E. Martinez-Outschoorn, F. Sotgia, Oncogenes induce the cancer-associated fibroblast phenotype: Metabolic symbiosis and "fibroblast addiction" are new therapeutic targets for drug discovery, Cell Cycle 12 (2013) 2740–2749.
[69] R. Liu, A.-L. Ström, J. Zhai, J. Gal, S. Bao, W. Gong, H. Zhu, Enzymatically inactive adenylate kinase 4 interacts with mitochondrial ADP/ATP translocase, Int. J. Biochem. Cell Biol. 41 (2009) 1371–1380.
[70] X.H. Liu, X.F. Zheng, Y.L. Wang, Inhibitive effect of 3-bromopyruvic acid on human breast cancer MCF-7 cells involves cell cycle arrest and apoptotic induction, Chin. Med. J. (Engl.) 122 (2009) 1681–1685.
[71] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ Method, Methods 25 (2001) 402–408.
[72] E.N. Maldonado, K.L. Sheldon, D.N. DeHart, J. Patnaik, Y. Manevich, D. M. Townsend, S.M. Bezrukov, T.K. Rostovtseva, J.J. Lemasters, Voltage-dependent anion channels modulate mitochondrial metabolism in cancer cells: regulation by free tubulin and erastin, J. Biol. Chem. 288 (2013) 11920–11929.
[73] U.E. Martinez-Outschoorn, Z. Lin, C. Trimmer, N. Flomenberg, C. Wang, S. Pavlides, R.G. Pestell, A. Howell, F. Sotgia, M.P. Lisanti, Cancer cells metabolically "fertilize" the tumor microenvironment with hydrogen peroxide, driving the Warburg effect: implications for PET imaging of human tumors, Cell Cycle 10 (2011) 2504–2520.
[74] S.P. Mathupala, Y.H. Ko, P.L. Pedersen, Hexokinase II: cancer's double-edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria, Oncogene 25 (2006) 4777–4786.
[75] S.P. Mathupala, Y.H. Ko, P.L. Pedersen, Hexokinase-2 bound to mitochondria: cancer's stygian link to the "Warburg Effect" and a pivotal target for effective therapy, Semin. Cancer Biol. 19 (2009) 17–24.
[76] A. Mayevsky, Mitochondrial function and energy metabolism in cancer cells: Past overview and future perspectives, Mitochondrion 9 (2009) 165–179.
[77] M. Miettinen, J. Lasota, Succinate dehydrogenase deficient gastrointestinal stromal tumors (GISTs) – a review, Int. J. Biochem. Cell Biol. 53 (2014) 514–519.
[78] C. Monge, N. Beraud, K. Tepp, S. Pelloux, S. Chahboun, T. Kaambre, L. Kadaja, M. Roosimaa, A. Piirsoo, Y. Tourneur, 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. 87 (2009) 318–326.

[79] C. Monge, R. Guzun, K. Tepp, N. Timohhina, M. Varikmaa, P. Sikk, T. Kaambre, V. Saks, Mitochondrial interactosome in health and disease: structural and functional aspects of molecular system bioenergetics of muscle and neuronal cells, in: O.L. Svensson (Ed.), Mitochondria: Structure, Functions and Dysfunctions, Nova Science Pub Inc, New York, 2010, pp. 441–470.

[80] S.M. Mooney, K. Rajagopalan, B.H. Williams, Y. Zeng, C.S. Christudass, Y. Li, B. Yin, P. Kulkarni, R.H. Getzenberg, Creatine kinase brain overexpression protects colorectal cells from various metabolic and non-metabolic stresses, J. Cell. Biochem. 112 (2011) 1066–1075.

[81] R. Moreno-Sanchez, A. Marin-Hernandez, E. Saavedra, J.P. Pardo, S.J. Ralph, S. Rodriguez-Enriquez, Who controls the ATP supply in cancer cells? Biochemistry lessons to understand cancer energy metabolism, Int. J. Biochem. Cell Biol. 50 (2014) 10–23.

[82] R. Moreno-Sanchez, S. Rodriguez-Enriquez, A. Marin-Hernandez, E. Saavedra, Energy metabolism in tumor cells, FEBS J. 274 (2007) 1393–1418.

[83] R. Moreno-Sánchez, S. Rodríguez-Enríquez, E. Saavedra, A. Marín-Hernández, J.C. Gallardo-Pérez, The bioenergetics of cancer: Is glycolysis the main ATP supplier in all tumor cells? BioFactors 35 (2009) 209–225.

[84] S. Mozzetti, C. Ferlini, P. Concolino, F. Filippetti, G. Raspaglio, S. Prislei, D. Gallo, E. Martinelli, F.O. Ranelletti, G. Ferrandina, G. Scambia, Class III beta-tubulin overexpression is a prominent mechanism of paclitaxel resistance in ovarian cancer patients, Clin. Cancer Res. 11 (2005) 298–305.

[85] K.M. Nieman, H.A. Kenny, C.V. Penicka, A. Ladanyi, R. Buell-Gutbrod, M. R. Zillhardt, I.L. Romero, M.S. Carey, G.B. Mills, G.S. Hotamisligil, et al., Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth, Nat. Med. 17 (2011) 1498–1503.

[86] I. Papandreou, R.A. Cairns, L. Fontana, A.L. Lim, N.C. Denko, HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption, Cell Metab. 3 (2006) 187–197.

[87] J. Pastorino, J. Hoek, Regulation of hexokinase binding to VDAC, J. Bioenerg. Biomembr. 40 (2008) 171–182.

[88] S. Patra, S. Bera, S. SinhaRoy, S. Ghoshal, S. Ray, A. Basu, U. Schlattner, T. Wallimann, M. Ray, Progressive decrease of phosphocreatine, creatine and creatine kinase in skeletal muscle upon transformation to sarcoma, FEBS J. 275 (2008) 3236–3247.

[89] P.L. Pedersen, 3-bromopyruvate (3BP) a fast acting, promising, powerful, specific, and effective "small molecule" anti-cancer agent taken from labside to bedside: introduction to a special issue, J. Bioenerg. Biomembr. 44 (2012) 1–6.

[90] H. Pelicano, D.S. Martin, R.H. Xu, P. Huang, Glycolysis inhibition for anticancer treatment, Oncogene 25 (2006) 4633–4646.

[91] T. Pfeiffer, S. Schuster, S. Bonhoeffer, Cooperation and competition in the evolution of ATP-producing pathways, Science 292 (2001) 504–507.

[92] M. Puurand, N. Peet, A. Piirsoo, M. Peetsalu, J. Soplepmann, M. Sirotkina, A. Peetsalu, A. Hemminki, E. Seppet, Deficiency of the complex I of the mitochondrial respiratory chain but improved adenylate control over succinate-dependent respiration are human gastric cancer-specific phenomena, Mol. Cell. Biochem. 370 (2012) 69–78.

[93] J. Rak, Y. Mitsuhashi, L. Bayko, J. Filmus, S. Shirasawa, T. Sasazuki, R.S. Kerbel, Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis, Cancer Res. 55 (1995) 4575–4580.

[94] R.B. Robey, B.J. Raval, J. Ma, A.V. Santos, Thrombin is a novel regulator of hexokinase activity in mesangial cells, Kidney Int. 57 (2000) 2308–2318.

[95] S. Rodriguez-Enriquez, L. Carreno-Fuentes, J.C. Gallardo-Perez, E. Saavedra, H. Quezada, A. Vega, A. Marin-Hernandez, V. Olin-Sandoval, M.E. Torres-Marquez, R. Moreno-Sanchez, Oxidative phosphorylation is impaired by prolonged hypoxia in breast and possibly in cervix carcinoma, Int. J. Biochem. Cell Biol. 42 (2010) 1744–1751.

[96] S. Rodriguez-Enriquez, J.C. Gallardo-Perez, A. Marin-Hernandez, J.L. Aguilar-Ponce, E.A. Mandujano-Tinoco, A. Meneses, R. Moreno-Sanchez, Oxidative phosphorylation as a target to arrest malignant neoplasias, Curr. Med. Chem. 18 (2011) 3156–3167.

[97] S. Rodriguez-Enriquez, M.E. Torres-Marquez, R. Moreno-Sanchez, Substrate oxidation and ATP supply in AS-30D hepatoma cells, Arch. Biochem. Biophys. 375 (2000) 21–30.

[98] T.K. Rostovtseva, W. Tan, M. Colombini, On the role of VDAC in apoptosis: fact and fiction, J. Bioenerg. Biomembr. 37 (2005) 129–142.

[99] V. Saks, R. Guzun, N. Timohhina, K. Tepp, M. Varikmaa, C. Monge, N. Beraud, T. Kaambre, A. Kuznetsov, L. Kadaja, et al., Structure-function relationships in feedback regulation of energy fluxes in vivo in health and disease: mitochondrial interactosome, Biochim. Biophys. Acta 1797 (2010) 678–697.

[100] V. Saks, A. Kuznetsov, T. Andrienko, Y. Usson, F. Appaix, K. Guerrero, T. Kaambre, P. Sikk, M. Lemba, M. Vendelin, Heterogeneity of ADP diffusion and regulation of respiration in cardiac cells, Biophys. J. 84 (2003) 3436–3456.

[101] V.A. Saks, V.I. Veksler, A.V. Kuznetsov, L. Kay, P. Sikk, T. Tiivel, L. Tranqui, J. Olivares, K. Winkler, F. Wiedemann, W.S. Kunz, Permeabilized cell and skinned fiber techniques in studies of mitochondrial function in vivo, Mol. Cell. Biochem. 184 (1998) 81–100.

[102] L. Sanchez-Cenizo, L. Formentini, M. Aldea, A.D. Ortega, P. Garcia-Huerta, M. Sanchez-Arago, J.M. Cuezva, Up-regulation of the ATPase inhibitory factor 1 (IF1) of the mitochondrial H+-ATP synthase in human tumors mediates the metabolic shift of cancer cells to a Warburg phenotype, J. Biol. Chem. 285 (2010) 25308–25313.

[103] G.L. Semenza, Regulation of cancer cell metabolism by hypoxia-inducible factor 1, Semin. Cancer Biol. 19 (2009) 12–16.

[104] E.K. Seppet, M. Eimre, T. Anmann, E. Seppet, N. Peet, T. Kaambre, K. Paju, A. Piirsoo, A.V. Kuznetsov, M. Vendelin, et al., Intracellular energetic units in healthy and diseased hearts, Exp. Clin. Cardiol. 10 (2005) 173–183.

[105] Y. Shinohara, T. Ishida, M. Hino, N. Yamazaki, Y. Baba, H. Terada, Characterization of porin isoforms expressed in tumor cells, Eur. J. Biochem. 267 (2000) 6067–6073.

[106] F. Sotgia, U.E. Martinez-Outschoorn, M.P. Lisanti, The reverse warburg effect in osteosarcoma, Oncotarget 5 (2014) 7982–7983.

[107] F. Sotgia, U.E. Martinez-Outschoorn, S. Pavlides, A. Howell, R.G. Pestell, M. P. Lisanti, Understanding the Warburg effect and the prognostic value of stromal caveolin-1 as a marker of a lethal tumor microenvironment, Breast Cancer Res. 13 (2011) 213.

[108] F. Sotgia, D. Whitaker-Menezes, U.E. Martinez-Outschoorn, N. Flomenberg, R. Birbe, A.K. Witkiewicz, A. Howell, N.J. Philp, R.G. Pestell, M.P. Lisanti, Mitochondrial metabolism in cancer metastasis: visualizing tumor cell mitochondria and the "reverse Warburg effect" in positive lymph node tissue, Cell Cycle 11 (2012) 1445–1454.

[109] H. Zhang, P. Gao, R. Fukuda, G. Kumar, B. Krishnamachary, K.I. Zeller, C. V. Dang, G.L. Semenza, HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity, Cancer Cell 11 (2007) 407–420.

[110] Y. Zhou, F. Tozzi, J. Chen, F. Fan, L. Xia, J. Wang, G. Gao, A. Zhang, X. Xia, H. Brasher, et al., Intracellular ATP Levels Are a Pivotal Determinant of Chemoresistance in Colon Cancer Cells, Cancer Res. 72 (2012) 304–314.

[111] X.L. Zu, M. Guppy, Cancer metabolism: facts, fantasy, and fiction, Biochem. Biophys. Res. Commun. 313 (2004) 459–465.

[112] K. Tepp, K. Mado, M. Varikmaa, A. Klepinin, N. Timohhina, I. Shevchuk, V. Chekulayev, A.V. Kuznetsov, R. Guzun, T. Kaambre, The role of tubulin in the mitochondrial metabolism and arrangement in muscle cells, J. Bioenerg. Biomembr. 46 (2014) 421–434.

[113] K. Tepp, I. Shevchuk, V. Chekulayev, N. Timohhina, A.V. Kuznetsov, R. Guzun, V. Saks, T. Kaambre, High efficiency of energy flux controls within mitochondrial interactosome in cardiac intracellular energetic units, Biochim. Biophys. Acta 1807 (2011) 1549–1561.

[114] N. Timohhina, R. Guzun, K. Tepp, C. Monge, M. Varikmaa, H. Vija, P. Sikk, T. Kaambre, D. Sackett, V. Saks, Direct measurement of energy fluxes from mitochondria into cytoplasm in permeabilized cardiac cells in situ: some evidence for mitochondrial interactosome, J. Bioenerg. Biomembr. 41 (2009) 259–275.

[115] M. Upadhyay, J. Samal, M. Kandpal, O.V. Singh, P. Vivekanandan, The Warburg effect: insights from the past decade, Pharmacol. Ther. 137 (2013) 318–330.

[116] M.G. Vander Heiden, L.C. Cantley, C.B. Thompson, Understanding the warburg effect: the metabolic requirements of cell proliferation, Science 324 (2009) 1029–1033.

[117] O. Warburg, F. Wind, E. Negelein, The metabolism of tumors in the body, J. Gen. Physiol. 8 (1927) 519–530.

[118] M. Varikmaa, R. Bagur, T. Kaambre, A. Grichine, N. Timohhina, K. Tepp, I. Shevchuk, V. Chekulayev, M. Metsis, F. Boucher, et al., Role of mitochondria-cytoskeleton interactions in respiration regulation and mitochondrial organization in striated muscles, Biochim. Biophys. Acta 1837 (2014) 232–245.

[119] C. Welinder, L. Ekblad, Coomassie staining as loading control in western blot analysis, J. Proteome Res. 10 (2011) 1416–1419.

[120] D. Whitaker-Menezes, U.E. Martinez-Outschoorn, Z. Lin, A. Ertel, N. Flomenberg, A.K. Witkiewicz, R.C. Birbe, A. Howell, S. Pavlides, R. Gandara, et al., Evidence for a stromal-epithelial "lactate shuttle" in human tumors: MCT4 is a marker of oxidative stress in cancer-associated fibroblasts., Cell Cycle 10 (2011) 1772–1783.

[121] A. Viale, P. Pettazzoni, C.A. Lyssiotis, H. Ying, N. Sanchez, M. Marchesini, A. Carugo, T. Green, S. Seth, V. Giuliani, et al., Oncogene ablation-resistant pancreatic cancer cells depend on mitochondrial function, Nature 514 (2014) 628–632.

[122] I.M. Willers, A. Isidoro, A.D. Ortega, P.L. Fernandez, J.M. Cuezva, Selective inhibition of beta-F1-ATPase mRNA translation in human tumours, Biochem. J. 426 (2010) 319–326.

[123] A.K. Witkiewicz, D. Whitaker-Menezes, A. Dasgupta, N.J. Philp, Z. Lin, R. Gandara, S. Sneddon, U.E. Martinez-Outschoorn, F. Sotgia, M.P. Lisanti, Using the "reverse Warburg effect" to identify high-risk breast cancer patients: Stromal MCT4 predicts poor clinical outcome in triple-negative breast cancers, Cell Cycle 11 (2012) 1108–1117.

[124] J. Xu, J. Wang, B. Xu, H. Ge, X. Zhou, J.-Y. Fang, Colorectal Cancer Cells Refractory to Anti-VEGF Treatment Are Vulnerable to Glycolytic Blockade due to Persistent Impairment of Mitochondria, Mol. Cancer Ther. 12 (2013) 717–724.

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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

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ABSTRACT

The aim of this study is to characterize the function of mitochondria and main energy fluxes in human colorectal cancer (HCC) cells. We have performed quantitative analysis of cellular respiration in post-operative tissue samples collected from 42 cancer patients. Permeabilized tumor tissue in combination with high resolution respirometry was used.

Our results indicate that HCC is not a pure glycolytic tumor and the oxidative phosphorylation (OXPHOS) system may be the main provider of ATP in these tumor cells. The apparent Michaelis–Menten constant (K_m) for ADP and maximal respiratory rate (V_m) values were calculated for the characterization of the affinity of mitochondria for exogenous ADP: normal colon tissue displayed low affinity ($K_m = 260 \pm 55$ μM) whereas the affinity of tumor mitochondria was significantly higher ($K_m = 126 \pm 17$ μM). But concurrently the V_m value of the tumor samples was 60–80% higher than that in control tissue. The reason for this change is related to the increased number of mitochondria. Our data suggest that in both HCC and normal intestinal cells tubulin β-II isoform probably does not play a role in the regulation of permeability of the MOM for adenine nucleotides.

The mitochondrial creatine kinase energy transfer system is not functional in HCC and our experiments showed that adenylate kinase reactions could play an important role in the maintenance of energy homeostasis in colorectal carcinomas instead of creatine kinase.

Immunofluorescent studies showed that hexokinase 2 (HK-2) was associated with mitochondria in HCC cells, but during carcinogenesis the total activity of HK did not change. Furthermore, only minor alterations in the expression of HK-1 and HK-2 isoforms have been observed.

Metabolic Control analysis showed that the distribution of the control over electron transport chain and ATP synthasome complexes seemed to be similar in both tumor and control tissues. High flux control coefficients point to the possibility that the mitochondrial respiratory chain is reorganized in some way or assembled into large supercomplexes in both tissues.

Abbreviations: AK, adenylate kinase; ANT, adenine nucleotide translocator; BSA, bovine serum albumin; CAT, carboxyatractyloside; COX, cytochrome c oxidase; CK, creatine kinase; ETC, electron transport chain; FDG, 18-fluorodeoxyglucose; FCC, flux control coefficient; HCC, human colorectal cancer; HK, hexokinase; K_m, Michaelis–Menten constant; uMtCK, ubiquitous mitochondrial creatine kinase; MCA, Metabolic Control Analysis; 3-NP, 3-nitropropionic acid; OXPHOS, oxidative phosphorylation; MOM, mitochondrial outer membrane; PCr, phosphocreatine; PET, positron emission tomography; Pi, inorganic phosphate; PIC, inorganic phosphate carrier; PEP, phosphoenolpyruvate; PYK, pyruvate kinase; RCI, respiratory control index; TMPD, N,N,N′,N′-tetramethyl-p-phenylenediamine; VDAC, voltage dependent anion channel; V_0, basal respiration level; V_m, maximal respiration rate.

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1. Introduction

Human colorectal cancer (HCC) is a malignant tumor caused by uncontrolled growth of mutated cells in the colon, rectum or vermiform appendix. It is highly resistant to chemotherapy, has strong inclination to metastasis and is one of the main causes of cancer death worldwide which necessitate for new strategies of the HCC treatment. Studies performed during the past decade demonstrated that targeting cancer cell energy metabolism might be a new and very effective therapeutic approach for selective ablation of malignancies (Geschwind et al., 2004; Gogvadze et al., 2009). Today, however, little is known about the key processes involved in the maintenance of energy homeostasis in HCC cells as well as the bioenergetic function of their mitochondria.

In the 1920s, Warburg et al. (1927) observed that tumor cells consumed a large amount of glucose and converted most of it to lactic acid even in the presence of oxygen which was contrary to Pasteur observation ("Pasteur effect"), who found that in most eukaryotic cells the rate of glycolysis decreases significantly in the presence of oxygen.

According to the model proposed by Pedersen and co-workers (Pedersen, 2008), the interaction of voltage dependent anionic channel (VDAC), located within the outer mitochondrial membrane (MOM) with hexokinase-2 (HK-2) (a key glycolytic enzyme that is usually upregulated in tumor cells), is one of the main pathways mediating the "Warburg effect" in cancer (Pedersen, 2007a,b). It was shown that mitochondrially-bound HK uses exclusively intramitochondrially compartmented ATP (Cesar Mde and Wilson, 1998). It was reported (Majewski et al., 2004) that the binding of HK-2 to mitochondria through VDAC supported this porin complex in an open state. Another consequence of HK–VDAC interaction is that it prevents binding of pro-apoptotic proteins to VDAC (a component of the MPT pore) and thereby protects cell from the induction of apoptosis (Pastorino and Hoek, 2008).

Our recent studies have shown that alterations in the expression profile of some tubulin isotypes in tumor cells could induce their Warburg behavior (Guzun et al., 2011a; Rostovtseva et al., 2008). We found (Guzun et al., 2012) that the localization and function of β-tubulin isotypes varied in different muscle tissues and malignant cells. The absence of βII-tubulin in cancer cells permits binding of HK-2 to VDAC mediating thereby the initiation of the Warburg effect. At the same time, it was shown (Rodriguez-Enriquez et al., 2011; Rodríguez-Enríquez et al., 2006; Zu and Guppy, 2004) that chemotherapeutic strategies using glycolytic inhibitors can often be inefficient in arresting tumor proliferation and the Warburg hypothesis may not be applicable to all existing malignancies. The concept of glycolytic cancer cells has been recently brought into question; Zu and Guppy (2004) concluded that the data from the last 40 years provided no evidence that cancer cells were inherently glycolytic but some tumors might indeed be glycolytic *in vivo* as a result of their adaptation to the hypoxic environment. Mitochondria, besides their role in cellular energy metabolism also play a critical role in many regulatory and signaling events in the response to a multiplicity of physiological and genetic stress factors, inter-organelle communication, cell proliferation and cell death (Goldenthal and Marín-García, 2004). To date, a new concept for cancer treatment focused on targeting tumor cell mitochondria has been elaborated, which may prove to be a very effective therapeutic approach for selective ablation of malignancies (Fulda et al., 2010; Gogvadze et al., 2009).

It has now become obvious that some cancer cells, like normal proliferating cells, can reprogram carbon metabolism by reducing energy production of oxidative phosphorylation (OXPHOS) and simultaneously up-regulating glycolysis. All this drives glycolytic and tricarboxylic acid cycle (TCA) intermediates into biosynthetic pathways (Lunt and Vander Heiden, 2011; Vander Heiden et al., 2009); possible signaling pathways of this phenomenon are the object of intensive studies (Agathocleous and Harris, 2013).

During the last years, the concept of aerobic glycolysis as the framework of tumor cell metabolism has been challenged, as some tumor cells exhibit high rates of OXPHOS (Diers et al., 2012; Klepinin et al., 2014; Moreno-Sánchez et al., 2009). Also, our recent studies on clinical material have shown that human breast cancer is a non-hypoxic oxidative tumor in which the mitochondrial respiration is significantly increased and is sensitive to respiratory chain inhibitors (Kaambre et al., 2012; Sotgia et al., 2012). Our results show that in these carcinoma cells the OXPHOS system, but not glycolysis, is the major source of ATP. Recently, a new two-compartment model has been proposed to understand the Warburg effect in tumor metabolism, which is referred to as "reverse Warburg" effect (Sotgia et al., 2012; Witkiewicz et al., 2012). In this model, glycolytic stromal cells produce mitochondrial fuels which are then transferred into oxidative cancer cells, driving OXPHOS and fueling tumor growth and metastasis (Martinez-Outschoorn et al., 2012; Sotgia et al., 2012; Whitaker-Menezes et al., 2011; Witkiewicz et al., 2012). Although, the first studies on two-compartment tumor metabolism were first performed on fibroblasts and breast cancer cells (Martinez-Outschoorn et al., 2011; Sotgia et al., 2011, 2012; Whitaker-Menezes et al., 2011), this emerging paradigm has already expanded to other malignancies like adipocytes and ovarian cancer cells, head and neck tumors and cancer lymph node metastases (Curry et al., 2013; Nieman et al., 2011; Sotgia et al., 2012).

For better understanding functional centers, which control and regulate the energy fluxes in cancer cells *in vivo*, analytical tools are needed to link the properties of metabolic systems with the kinetic characteristics of the component enzymes and their impact on network function. A potent experimental approach for this is Metabolic Control Analysis (MCA) (Fell, 2005). MCA has been shown to be very helpful for understanding the enzymatic abnormalities in syndromes associated with mitochondrial dysfunction (Kuznetsov et al., 2008). Moreno-Sanchez and colleagues have applied MCA to investigate the control of glycolytic flux and mitochondrial respiration in different types of tumor cells growing in culture. Main conclusion of these studies was that the significance of OXPHOS in bioenergetics of cancer cells should be re-evaluated and experimentally determined for each particular type of neoplasm (Marin-Hernandez et al., 2006; Moreno-Sanchez et al., 2007, 2010; Moreno-Sánchez et al., 2009). Recently in our laboratory, MCA was successfully used to study the bioenergetic function of mitochondria in human breast cancer postoperative samples (Kaambre et al., 2012).

In the present work, MCA was applied to characterize the function of OXPHOS in HCC cells *in situ*. We quantified the control exerted by different components of the respiratory chain and the ATP synthasome complex (a large mitochondrial complex consisting of ATP synthase, adenine nucleotide transporter (ANT) and inorganic phosphate carrier (Pedersen, 2008)) in these carcinoma cells as compared with normal colon tissue. To determine the flux control coefficients, the flux was measured as the rate of O_2 consumption by permeabilized tissue fibers derived from HCC patients when all components of the OXPHOS system were titrated with specific inhibitors to stepwise decrease selected respiration complex activities. It is important to note that the use of MCA and the permeabilization techniques permit to estimate the function of OXPHOS system without isolation of mitochondria and thereby avoiding artifacts linked to their isolation procedure and the loss of components involved in the regulation. Besides, such a methodology preserves intimate interactions between mitochondria within the cell as well as the intactness of their cytoskeletal structures.

The present work also evaluated the significance of creatine kinase (CK) and adenylate kinase (AK) reactions in the maintenance of energy homeostasis in HCC cells.

2. Materials and methods

2.1. Reagents

Chemicals were purchased from Sigma–Aldrich Chemical Com. (USA) and were used directly without further purification. Primary and secondary antibodies were obtained from Santa Cruz Biotechnology Inc. or Abcam PLC, whereas rabbit polyclonal antibodies *vs.* VDAC were kindly donated by Dr. Catherine Brenner from Paris-Sud University, France.

2.2. Clinical materials and patients

All patients examined ($n = 42$, with ages ranging from 63 to 92 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.

HCC postoperational 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). Pathology reports were provided 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) 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 by 5 cm and they were evaluated for presence of malignant cells. The adjacent control tissues consisted of colonocytes and smooth muscle cells. Images of hematoxylin-eosin stained preparations of tumor and surrounding normal tissues are shown in Fig. 1 in supplement. In addition, we performed molecular characterization of tissue samples from 35 patients (both tumor and normal) using microsatellite instability, CpG island methylator (CIMP) phenotype and 5-hydroxymethylation assay. They showed that all control samples had a stable microsatellite profile and no CIMP phenotype. Also the 5-hydroxymethylation expression was statistically significantly higher in normal tissue samples as compared to tumor tissue, as analyzed in 13 patients (Supplementary Fig. 2). 5-Hydroxymethylation analysis was carried out according to manufacturer instructions provided with the MethylFlash Hydroxymethylated DNA Quantification Kit (Epigentek, USA).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2014.09.004.

2.3. Preparation of skinned tumor fibers and permeabilization procedure

Immediately after the surgery the tissue samples were placed into pre-cooled (on melting ice) medium-A (Kaambre et al., 2012), dissected into small fiber bundles (10–20 mg) and permeabilized in the same medium with 50 $\mu g/ml$ saponin upon mild stirring for 30 min at 4 °C (Kaambre et al., 2012; Kuznetsov et al., 2008). The obtained permeabilized (skinned) fibers were then washed three times for 5 min in pre-cooled solution containing: 20 mM imidazole, 3 mM KH_2PO_4, 0.5 mM DTT, 20 mM taurine, 4 mM $MgCl_2$, 100 mM 2-morpholinoethanesulfonic acid, 2.74 mM K_2Ca-EGTA, 4.72 mM K_2-EGTA, and 5 mg/ml fatty acids free bovine serum albumin (BSA); medium-B, pH 7.1. After that samples were kept in medium-B at 4 °C until use. Typical dimension of skinned fibers was about 2 mm × 2 mm × 2 mm, and one of these pieces was used in oxygraphic experiments.

2.4. Oxygraphic measurements

Mitochondrial respiration of tissue samples was measured at 25 °C in medium-B supplemented with 5 mM glutamate, 2 mM malate and 10 mM succinate as respiratory substrates using high-resolution respirometer Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) as described previously (Kuznetsov et al., 2008). The solubility of oxygen at 25 °C was taken as 240 nmol/ml (Gnaiger, 2001). All respiration rates were normalized per mg dry weight of tissue.

2.4.1. Analysis of OXPHOS coupling with adenylate kinase

The adenylate kinase coupling with OXPHOS was measured by respirometry using modified protocols of Gruno et al. (2006). Mitochondrial basal respiration was activated by glutamate, malate and succinate (at a final concentration of 5, 2 and 10 mM, respectively), 100 μM ATP was added to produce a minimum amount of endogenous ADP to stimulate mitochondria, AMP (2 mM) activated the coupled reaction of adenylate kinase system with ANT followed by addition of 0.2 mM diadenosine pentaphosphate (AP5A) to inhibit total AK reaction. Then AK index (IAK): [IAK = (VAMP − VAP5A)/VAP5A] was calculated, where it expressed the strength of the AK functional coupling with OXPHOS. A rate of AMP activated respiration (VAMP) was normalized for the respiration after total inhibition of AK by AP5A (VAP5A).

2.4.2. Analysis of OXPHOS coupling with creatine kinase (MtCK)

The steady state kinetics of MtCK reaction coupled to oxidative phosphorylation *via* ANT in permeabilized HCC and control tissue *in situ* was studied using the protocol described earlier by Guzun et al. (2009) and Gellerich et al. (2002).

2.5. Immunofluorescence and confocal microscopy

Confocal microscopy was applied to immunostained skinned fibers and paraffin-embedded sections of HCC and normal colorectal tissue to assess the presence and intracellular localization of mitochondria (*via* VDAC immunolabeling), HK-2, and βII-tubulin. For immunocytochemistry, skinned fibers were fixed with 4% paraformaldehyde (PFA) for 15 min at 37 °C, treated with an antigen retrieval buffer (ARB, 100 mM Tris buffer with 5%, w/v urea, pH 9.5) and permeabilized with 1.0% Triton X-100 for 15 min at room temperature (RT). For immunohistochemistry, formalin fixed paraffin-embedded tissue sections were rinsed with xylene for 4–5 min, rehydrated step-by-step by ethanol (at 100% and 50%) and treated with ARB at 98 °C for 15 min.

Fibers and tissue sections were then blocked with 2% BSA in PBS and incubated overnight at 4 °C with primary antibodies. Monoclonal mouse anti-tubulin βII (Abcam®, ab92857), polyclonal rabbit antibody *vs.* VDAC and polyclonal goat antibodies *vs.* HK-2 (Santa Cruz Biotechnology Inc., sc-6521) were used. Thereafter fibers were washed and incubated for 2 hours at RT with following secondary antibodies: DyLight-488 goat anti-rabbit IgG (Abcam®, ab96899) or DyLight-549 goat anti-mouse IgG (Abcam®, ab96880). Thereafter fibers and tissue slides were mounted in ProLong® Gold Antifade Reagent supplemented with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Life Technologies), deposited between glass coverslips and observed by confocal microscope. Confocal images were collected using Olympus FV10i-W inverted laser scanning confocal microscope equipped with a 60× water immersion objective. Laser excitation was 488 nm for DyLight-488 and 561 nm for DyLight-549.

2.6. Quantification of mitochondrial content

The mitochondrial content was quantified in paraffin embedded neoplastic and normal colon tissue samples *via* selective marking of mitochondrial outer membrane translocase Tom20 (Santa Cruz Biotechnology, sc17764). The Tom20 fluorescence intensity was normalized against whole β-tubulin (Abcam®, ab6046) fluorescence.

2.7. Assessment of enzymatic activities

HK activity was measured as the total glucose phosphorylating capacity of whole tissue extracts, using a standard glucose-6-phosphate dehydrogenase (G6PDH)-coupled spectrophotometric assay (Robey et al., 2000).

The CK activity was assessed spectrophotometrically at 25 °C in the direction of ATP formation in the presence of di(adenosine-5′) pentaphosphate (an adenylate kinase inhibitor (Lienhard and Secemski, 1973)), 20 mM phosphocreatine (PCr) and with 2 U/ml G6PDH and 2 U/ml HK as the coupled enzymes (Monge et al., 2009). One mU of CK activity represents the formation of 1 nmol of ATP per minute at 25 °C.

AK activity of whole-tissue extracts was measured at 25 °C by a coupled enzyme assay (Dzeja et al., 1999). The reaction was initiated with 2 mM ADP, and the arising changes in absorbance at 340 nm were recorded using a Cary 100 Bio UV-visible spectrophotometer. One mU of AK activity represents the formation of 1 nmole of ATP per minute at 25 °C.

All enzymatic activities were normalized per mg of tissue protein. The protein content of tissue extracts was determined by a Pierce BCA Protein Assay Kit according to the manufacturer recommendations using BSA as a standard.

2.8. RNA isolation and real-time quantitative RT-PCR

RNA from 29 human frozen colorectal cancer and normal colon tissue samples was isolated using Trizol (Life Technologies) solution, followed by purification using the RNeasy Mini Kit (QIAGEN Sciences) with DNase treatment. Extracted RNA was dissolved in RNase-free water, quality and concentration were measured using Nanodrop and RNA was stored at −80 °C until cDNA synthesis.

For cDNA synthesis 2 μg of total RNA was used. cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems). cDNA was used as a template for TaqMan® quantitative RT-PCR (qRT-PCR) analysis in the Roche LightCycler 480 system (Roche). TaqMan® Gene Expression Master Mix and FAM labeled TaqMan® (Applied Biosystems) gene assays were used to detect the mRNA expression level of the gene of interest and of actin as a reference gene. The used TaqMan® probes were the following: actin beta – Hs01060665_g1; tubulin beta 2A – Hs00742533_s1; tubulin beta 2B – Hs00603550_g; tubulin beta 2C – Hs00607181_g1; tubulin beta 3 – Hs00801390_s1; tubulin beta 4 – Hs00760066_s1; tubulin beta 1/5 – Hs00742828_s1; hexokinase I – Hs00175976_m1; hexokinase II – Hs00606086_m1; creatine kinase, mitochondrial 1B – Hs00179727_m1; creatine kinase, mitochondrial 2 – Hs00176502_m1. Reactions were carried out in four replicates. Data was analyzed using the 2(−Delta Delta C(T)) method, where the gene expression levels were normalized to the level of actin beta housekeeping gene. The data of studied genes following normal distribution were parametrically tested by unpaired *t*-test.

2.9. Metabolic control analysis (MCA) and determination of flux control coefficients

MCA was performed as described previously (Fell, 1997; Groen et al., 1982; Kaambre et al., 2012; Moreno-Sanchez et al., 2008; Tepp et al., 2011). Flux control coefficients (FCC) were calculated by using non-linear regression analysis by fitting experimental data to the mathematical model, as described by Gellerich et al. (1990) and Small and Fell (1990). The results were also verified by a graphical method (Fell, 2005; Gellerich et al., 1990).

2.10. Data analysis

Data in the text, tables and figures are presented as mean ± standard error (SEM). Results were analyzed by Student's *t*-test. *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.

3. Results

3.1. Mitochondrial respiration in colorectal carcinomas

3.1.1. Quality test of intactness of mitochondrial membranes

We performed a comparative study to characterize the respiratory activities of mitochondria in HCC cells and adjacent normal tissue *in situ* using the permeabilized cell technique, which allows to study the mitochondrial function under conditions close to physiological ones (Kuznetsov et al., 2008). Fig. 1 shows the quality test of respiration of saponin permeabilized fibers prepared from HCC and normal colon tissue. The mitochondrial respiration activated by 2 mM ADP was not increased after addition of exogenous cytochrome-c, showing the intactness of the outer mitochondrial membrane. Mitochondrial inner membrane intactness was controlled by carboxyatractyloside (CAT) where the respiration rate decreased back to the basal respiration level (V_0) due to inactivation of ANT (Saks et al., 1998; Timohhina et al., 2009).

Respiration rates of human colorectal cancer and normal tissue samples in the absence of saponin treatment were also estimated,

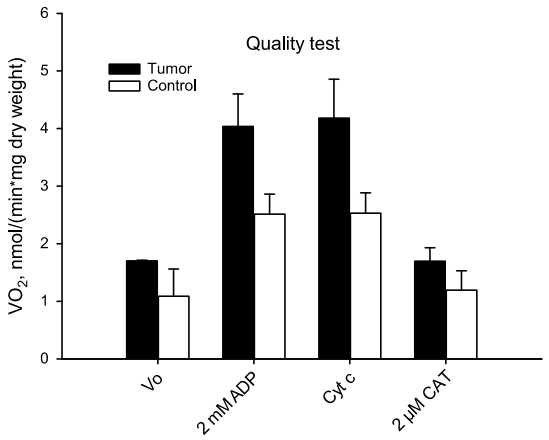

Fig. 1. Quality tests for the intactness of mitochondrial membranes in permeabilized human colorectal cancer and surrounding normal tissue fibers. These experiments were performed in medium-B with 5 mM glutamate, 2 mM malate, and 10 mM succinate as respiratory substrates. Respiration of permeabilized samples was activated by 2 mM ADP and the addition of cytochrome c (Cyt c, final concentration – 8 μM) did not cause any marked increase in the rate of oxygen consumption, indicating the intactness of the outer mitochondrial membrane. Finally, addition of carboxyatractyloside (CAT, final concentration – 2 μM) decreased the respiration rate back to the basal (V_0) level showing that the inner membrane of the mitochondria was intact (bars are SEM, $n = 11$). All respiratory substrates and inhibitors were added sequentially as indicated in the *X*-axis.

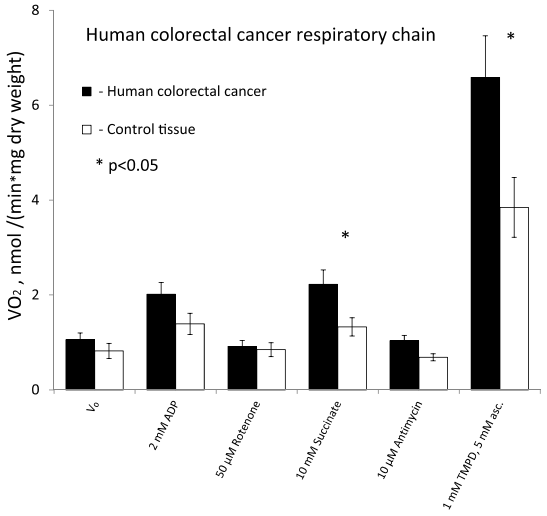

Fig. 2. Evaluation of the respiratory chain functions in permeabilized human colorectal cancer fibers and normal adjacent tissues. The experiment was performed in medium-B with 5 mM glutamate and 2 mM malate as respiratory substrates. TMPD is N,N,N′,N′-tetramethyl-phenylenediamine, and asc – ascorbate (bars are SEM, $n = 7$, $p < 0.05$).

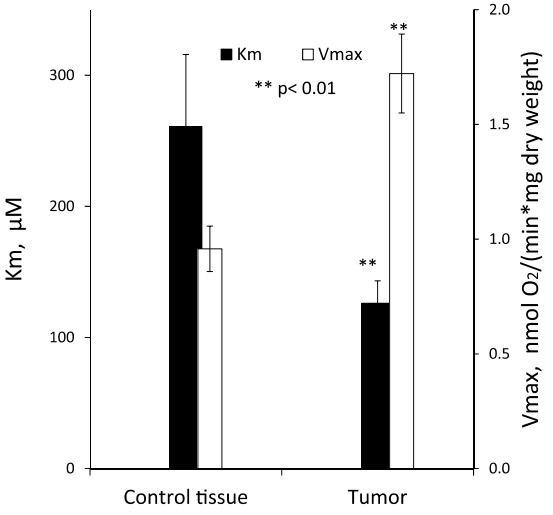

Fig. 3. Comparative analysis of the apparent K_m values for ADP and the maximal respiration rate (V_{max}) for permeabilized skinned HCC and normal colon samples. Bars are SEM, $n = 35$, $p < 0.01$.

and there is only a minor activation in comparison with permeabilized tissue samples (Supplementary Fig. 3). The unambiguous analysis of respiratory parameters is possible solely after permeabilization of the cell membrane with saponin or another suitable detergent, since it is impermeable to the adenine nucleotides.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2014.09.004.

3.1.2. Activities of respiratory chain complexes in HCC and non-neoplastic tissue samples

Activities of segments of the mitochondrial respiratory chain were analyzed by using a specific step-by-step substrate inhibitor titration oxygraphic protocol. It was found (Fig. 2) that the mean value (1.06 ± 0.14 nmol O_2/min/mg dry weight) of basal respiration in skinned fibers of HCC exceeded slightly that in non-tumorous tissues (0.82 ± 0.16 nmol O_2/min/mg dry weight) The addition of 2 mM Mg-ADP resulted in a strong increase in the rates of O_2 consumption by both HCC and normal tissue fibers. The maximal ADP-stimulated (state 3) respiration rate for HCC samples exceed that measured for non-neoplastic tissue; 2.02 ± 0.14 nmol O_2/min/mg *vs.* 1.39 ± 0.22 nmol O_2/min/mg dry weight, correspondingly. The ADP activated respiration was strongly inhibited in both HCC and normal tissue samples by rotenone; this was a characteristic feature of cells with active Complex-I (Fig. 2). Addition of succinate (Complex-II substrate) resulted in reactivation of oxygen consumption showing that the Complex-II of the mitochondrial respiratory chain is functionally active in both normal and tumor tissues. The addition of 10 μM antimycin-A inhibited the electron flow from Complex-III to cytochrome c. Mitochondrial Complex IV was activated by 1.0 mM N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) in the presence of 5 mM ascorbate, and this resulted in a very strong increase in the rate of O_2 consumption in HCC fibers (Fig. 2). Finally, exogenously added 8 μM cytochrome c had no effect on the TMPD ascorbate activated respiration, suggesting that the applied permeabilization procedure did not damage the intactness of the external mitochondrial membrane in HCC cells.

3.2. Regulation of mitochondrial respiration in HCC cells

Fig. 3 summarizes the values of the apparent Michaelis–Menten constant (K_m) and maximal respiration rate (V_m) for exogenous ADP in tumor and adjacent normal colon tissue samples. From these data, the apparent K_m for ADP and V_m were calculated for characterization of the affinity of mitochondria for ADP. For skinned fibers from control tissue the statistical average apparent K_m for ADP is found to be 260 ± 55 μM. The lower K_m value for ADP (126 ± 17 μM) in HCC cells, as compared with control tissue, suggests that in cancer cells the MOM has an increased permeability for adenine nucleotides.

Fig. 3 also demonstrates a striking difference in the values of V_m for ADP between normal and HCC tissue samples. There is an approximately 2-fold difference in mitochondrial respiration between control ($V_m = 0.96 \pm 0.1$ nmol O_2/min per mg dry weight of tissue) and tumor tissues ($V_m = 1.72 \pm 0.13$ nmol O_2/min per mg dry weight of tissue). The reason of this change is probably related with the increased number of mitochondria (Fig. 4). Therefore, mitochondrial content was estimated in HCC samples with immunocytochemistry and confocal microscopic imaging (Fig. 4). Differences in maximal respiration rates in tumor and control tissue were of the same order of magnitude than that in the number of mitochondria (by 60–70%). The immunocytochemical study used for analysis of the relative content of mitochondria in tumor and healthy tissues based on the expression of TOM20 (Fig. 4B) show statistical significance of the differences in obtained, values ($p < 0.05$) of fluorescence signals.

The presented data clearly show that *in situ* there are striking differences between the kinetics of regulation of mitochondrial respiration by ADP in colorectal carcinomas and adjacent healthy tissue.

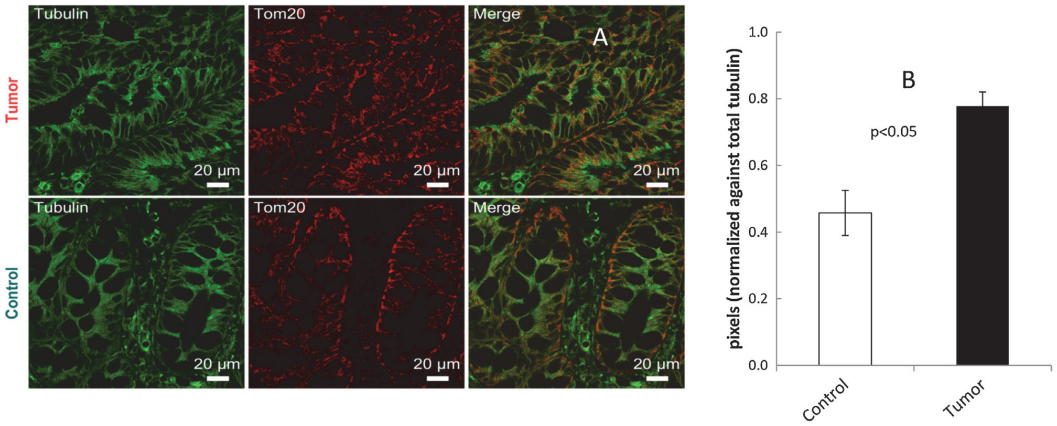

Fig. 4. Immunohistochemistry quantification of the total mitochondrial content in postoperative tissue samples; this was performed *via* measurements of Tom20 expression levels. (A) Representative confocal images of paraffin-embedded sections of HCC and normal colorectal tissue preloaded with anti-tubulin βII and anti Tom20 antibodies.; Fluorescence signal intensities in normal and cancerous colorectal tissue where normalized against total β-tubulin fluorescence intensities (B); bars are SEM, $n = 7$, $p < 0.05$.

3.2.1. Coupling of OXPHOS with creatine and adenylate kinase systems

Decreased permeability of MOM for adenine nucleotides significantly enhances the functional coupling between MtCK and ANT. Thus, adenine nucleotides are compartmented in mitochondrial matrix-inner membrane space where ADP and ATP recycling is ensured between OXPHOS and MtCK (Guzun et al., 2009; Saks et al., 2010). The changes in the CK energy transfer pathway in HCC could be responsible for changes in regulation of mitochondrial respiration during carcinogenesis. Especially CK is a ubiquitous enzyme that catalyses the reversible transphosphorylation reaction between ATP and Cr, generating ADP and phosphocreatine (PCr) (Dzeja and Terzic, 2003). Human colonocytes express all types of CK isoenzymes and are capable to produce PCr. BB-CK, which is characteristic for the brain, is the predominant isoenzyme in normal colon tissue (Joseph et al., 1997; Kasimos et al., 1990).

Our results show that the total CK activity in HCC samples is considerably (by ~2.5 times) lower in comparison with normal colon tissues (Table 1). By means of qRT-PCR, we revealed that at gene level sMtCK (CKMT2) mRNA is remarkably upregulated (by 6.8-fold, $p = 0.06$) in tumor tissue (Fig. 5); the uMtCK (CKMT1) expression is 2-fold higher ($p = 0.009$) in normal colon tissue. The general expression level for this gene is significantly higher than for sMtCK. Therefore, we performed a study to estimate the potential role of MtCK in maintenance of energy homeostasis in HCC tissue as compared with normal colon sample. In these experiments, mitochondrial respiration was activated with 10 mM Cr and MgATP (final concentration, 0.2 mM) in the presence of pyruvate kinase (PYK, 30 U/ml) and phosphoenolpyruvate (PEP, 5 mM) for trapping extramitochondrial ADP to follow control of mitochondrial oxygen consumption only by uMtCK (Gellerich and Saks, 1982). The obtained results showed that addition Cr (in the presence of PYK-PEP system and exogenously added MgATP) to normal colon tissue

Table 1
Enzymatic activities in human colorectal cancer and adjacent normal tissue samples.

Enzyme activities, mU(s) per mg protein	Normal tissue, mean ± SE, $n = 11$	Tumor, mean ± SE, $n = 11$	p-values
Hexokinase	244 ± 50	215 ± 40	0.33
Creatine kinase	497 ± 142	204 ± 84	<0.05
Adenylate kinase	257 ± 35	411 ± 43	<0.05

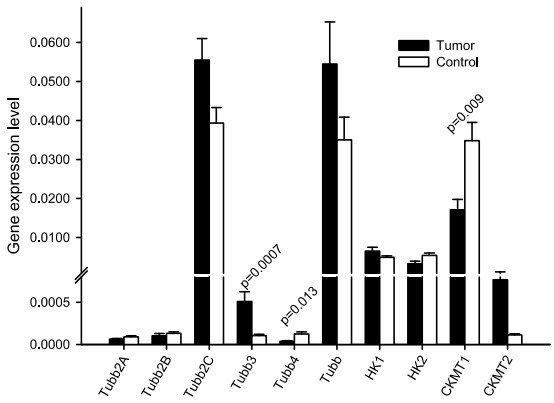

Fig. 5. Expression profile of various β-tubulin isotypes, some creatine kinase and hexokinase isoforms in human colorectal cancer and normal tissue; mRNA levels were assayed by real-time quantitative RT-PCR (bars are SEM, $n = 29$). Here: HK-1 and HK-2 are hexokinase-1 and -2; CKMT1 and CKMT2 are ubiquitous and sarcomeric mitochondrial creatine kinase, respectively.

fibers caused an increase in the rate of ADP-mediated oxygen consumption nearly 60% due to the activation of MtCK and this effect was not observed in cancerous tissues (Fig. 6). These *in situ* experiments indicate that mitochondria in HCC cells have a decreased capacity for the production of PCr which is in a good agreement with some literature data (Kasimos et al., 1990; Monge et al., 2009).

It is well-known that AK-catalyzed phosphotransfer plays one of the key roles in the maintenance of energy homeostasis in fully differentiated cells with a high-energy demand, such as neural, cardiac and some skeletal muscle cells (Ames, 2000; Dzeja and Terzic, 2009). Due to decreased CK activity and the absence of MtCK coupling with OXPHOS in HCC cells, the functional coupling of AK-catalyzed processes with the OXPHOS system was estimated in both tumor and normal colon tissues. The total AK activity (Table 1) of HCC tissue extracts exceeded substantially (by ~40%) that of adjacent normal tissue. In addition, the coupling between AK and OXPHOS was expressed with AK index (IAK) proposed by Gruno et al. (2006). The value of IAK, which is independent of the tissues mitochondrial content, was substantially smaller for

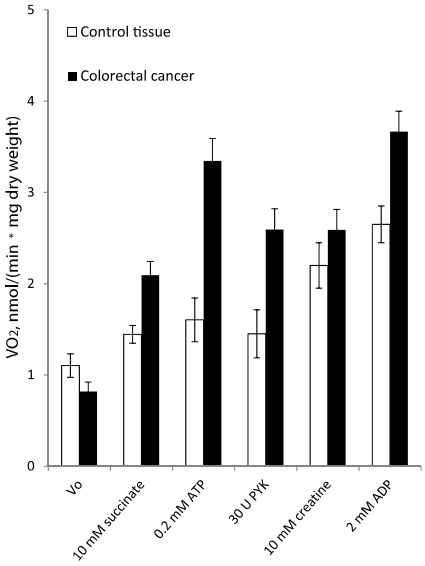

Fig. 6. Efficiency of creatine in the control of OXPHOS by mitochondrial creatine kinase (uMtCK) *via* local ADP production in permeabilized HCC and normal tissue fibers. The experiment was performed in medium-B with 5 mM glutamate and 2 mM malate and 10 mM succinate as respiratory substrates. Creatine under these conditions rapidly activates MtCK reaction and subsequently local MgADP recycling and oxygen consumption rate to the maximal values. First, the respiration is activated by addition of 0.2 mM MgATP after permeabilization of tumor and control samples inducing production of endogenous ADP in MgATPase reaction. Then PEP–PYK is added to trap all extramitochondrial free ADP. Finally, 2 mM exogenous ADP was added to activate maximal respirator rate. Bars are SEM, $n = 8$.

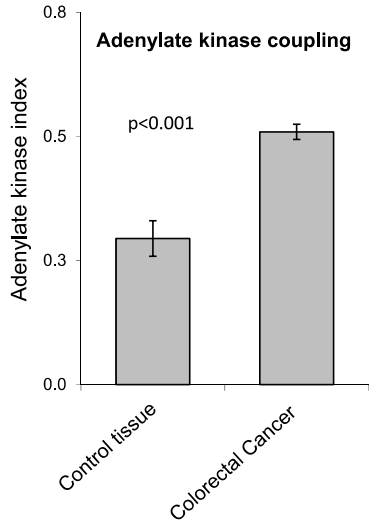

Fig. 7. Adenylate kinase (AK) functional coupling in permeabilized control and HCC tissues. Adenylate kinase coupling with mitochondria was expressed using AK index. Outer mitochondrial intactness was controlled by effect of exogenously-added cytochrome *c* (cytochrome *c* effect <20%). Bars are SEM, $n = 6$, and $p < 0.001$.

non-neoplastic tissue comparing to HCC (Fig. 7). This strong difference between the values of AK indexes indicates that the AK system may be upregulated; *i.e.*, AK and OXPHOS systems are stronger in HCC cells than in normal tissue. This could be partly mediated by higher activity of AK2 in carcinoma cells in comparison with non-transformed cells.

Further work is needed to clarify mechanisms and functional significance of these changes in the interaction and coupling between CK and AK energy transfer systems during carcinogenesis.

3.2.2. *Coupling of OXPHOS with HK reactions, β-tubulin mRNA expression in tumor and normal tissues*

Our qRT-PCR and immunocytochemistry studies have shown that HCC cells express genes encoding HK-1 and 2 (Fig. 5); in these malignant cells HK-2 may be presumably bound to mitochondria. Indeed, in Fig. 9 the colocalization of the HK-2 isoenzyme with VDAC is demonstrated. HK could play an important role in energy metabolism of HCC cells. Since, it has been proposed by Pedersen and colleagues that in tumor cells the binding of HK to the VDAC in the mitochondrial outer membrane (MOM) can mediate their Warburg phenotype (Pedersen, 2008), in this study was therefore investigated coupling between HK and OXPHOS in HCC cells. Glucose (10 mM) exerted some stimulatory effect in HCC mitochondrial respiration at 0.1 mM MgATP, but its effect was negligible in permeabilized fibers derived from normal tissue (Fig. 8). The stimulating effect of glucose on mitochondrial respiration rate in HCC fibers was about 30–35% of the ADP-mediated (2 mM) activation. Consequently, in HCC cells either one or both isoforms of both HK-2 and HK-1 are associated with VDAC in the MOM. Thus, this interaction of HK with mitochondria allows exerting control over OXPHOS so that HK uses mitochondrially-generated ATP for glucose phosphorylation.

Although, in HCC the expression levels of HK-1 and 2 as well as total glucose phosphorylating activity are similar to those in non-tumorous tissue (Fig. 5 and Table 1), we did not registered any stimulatory effect of glucose on mitochondrial respiration in the normal colon tissue (Fig. 8).

Altered profiles of some cytoskeletal protein (β-tubulin isotypes) expression could explain this difference in the coupling of OXPHOS with HK reactions in HCC cells as compared to non-tumorous tissues. Previous studies have shown that the cytoskeletal tubulin–microtubular system has considerable role in the regulation of mitochondrial respiration *in vivo* (Guzun et al., 2009, 2011a,b, 2012). It was demonstrated that tubulin could compete with HK-2 for the binding sites on VDAC. Therefore, in cancer cells decreased levels of tubulin favor the HK binding and thus explain the Warburg effect of increased glycolytic lactate production in these cells (Guzun et al., 2011a, 2012).

We further investigated the spectrum of β-tubulins by comparing the mRNA expression of the five human β-tubulin isotypes encoding class I IIa, IIb, III, IVa, and IVb, in colorectal carcinomas with that in non-tumorous tissue. It was found that in tumor tissues TUBB3 (gene encoding βIII-tubulin) had statistically significantly increased expression levels compared to normal tissue (by 4.8-fold, $p = 0.0007$), TUBB4 (βIVa) was significantly decreased (by 3.4-fold, $p = 0.013$) in tumor tissue, where as the levels of mRNA expression for other tubulin isotypes TUBB2A (II) and TUBB2B (II) TUBB2C (IVb) and TUBB (I) were not significantly changed in both studied tissues (Fig. 5). We found that HCC is characterized by a significant increase in the expression of TUBB3 (Fig. 5) which is the marker cell invasiveness as shown in the literature (Leandro-Garcia et al., 2010; Mozzetti et al., 2005).

The association of βII-tubulin with mitochondria (immunolabeled with anti-VDAC anti-body) was determined by immunohistochemistry and confocal microscopy. In both tissue types, mitochondria were showing frequent alignment along βII-tubulin

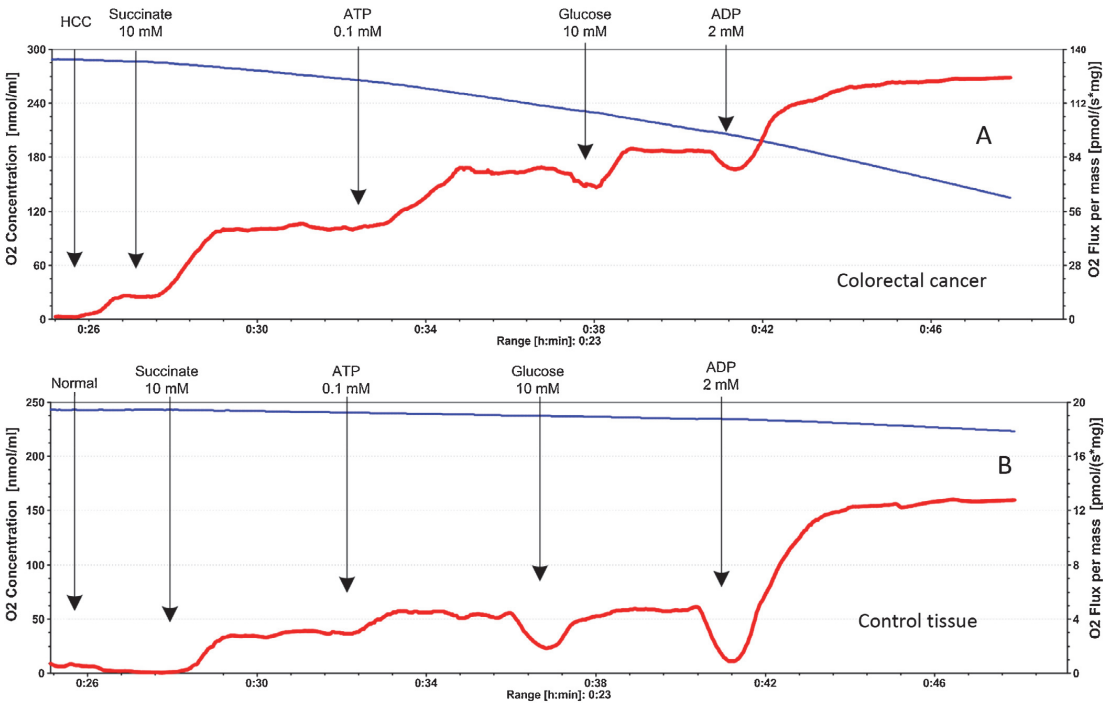

Fig. 8. Oxygraphic analysis of coupling of HK to OXPHOS in permeabilized HCC (A) and normal tissue fibers (B); recording of original traces of O_2 consumption. The addition of 10 mM glucose to HCC fibers in the presence of 0.1 mM Mg-ATP caused a stimulatory effect on mitochondrial respiration, but this HK coupling with OXPHOS was not observed in the case of fibers derived from normal adjacent tissue. Blue line – O_2 concentration, red – rates of O_2 consumption. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

containing microfilaments, but no direct association between the two was noticed (Fig. 10). This was further confirmed with comparison of the pixel intensity profiles of both dye channels, demonstrating that the signal intensity levels of the two were co-varying in random fashion. Thus, in contrast to our previous observations which were made on cardiac cells (Guzun et al., 2011a), βII-tubulin did not seem to be involved in regulation of VDAC conductance in colorectal tissue. Some differences between normal and HCC samples were also observed in respect to βII-tubulin network morphology and nuclei distribution. In particular, in normal colorectal tissue βII-tubulin appeared to form dense microtubular network that spread uniformly throughout the cytoplasm, whereas in HCC, clustering and fragmentation of βII-tubulin filaments was seen, suggesting that some remodeling of its biochemical properties (*e.g.*, dynamic stability) has occurred. In addition, HCC cells stand out also by their enlarged and stratified nuclei, which are one of the main signs of colorectal tissue malignancy. It is impossible to explain the enhanced control of HK-2 over mitochondrial respiration in HCC cells through downregulation of βII-tubulin expression as was observed earlier in tumor cells of another histological type (Guzun et al., 2011a).

3.3. Metabolic control analysis of respiration regulation in HCC and normal non-tumor tissues

The approach of MCA allows to identify the key regulatory complexes of the energy metabolism pathways and to find the best targets for effective antineoplastic treatment (Moreno-Sanchez et al., 2008, 2010). In our MCA studies, the mitochondrial respiration in permeabilized HCC and normal tissue fibers was activated by exogenously added ADP (final concentration, 2 mM). The use of ADP for activation of respiration was mainly mediated by low expression levels of uMtCK and its negligible activity in HCC samples. Fig. 11 shows representative traces of O_2 consumption in permeabilized HCC and non-neoplastic tissue fibers upon their titration with increasing concentrations of 3-NP, an inhibitor of Complex-II (Huang et al., 2006). The respiration rate was registered in steady-state conditions (Fig. 11). The same titration curves were obtained for other inhibitors of the mitochondrial ETC and ATP synthasome complexes (Supplementary Fig. 4). They were plotted as relative rates of O_2 consumption (VO_2, J/J_o) *versus* concentration of used inhibitors (Fig. 12A and B). The FCC(s) for tumor and non-tumorous samples were calculated by fitting experimental data to the mathematical model of Gellerich et al. (Gellerich et al., 1990; Wisniewski et al., 1995). We analyzed distribution of metabolic flux controls for all of mitochondrial respiratory chain and the ATP-Synthasome complexes as described previously in (Kaambre et al., 2012; Small, 1993; Tepp et al., 2011). Two ways of electron transfer were examined, namely, NADH and FADH (succinate dependent) electron transfers (Fig. 12C and D). The calculated values (Gellerich et al., 1990; Small, 1993) FCC(s), were found to be comparable in control and tumor tissues, only FCCs for the mitochondrial respiratory chain Complex-III differ from each slightly. The sums of FCC(s) calculated for HCC cells (NADH dependent pathway, 3.05 ± 0.23; succinate dependent pathway, 3.03 ± 0.20) and normal colonic cells (NADH dependent, 3.15 ± 0.50; succinate dependent, 3.25 ± 0.49) had similar values, and was found to exceed significantly the theoretic value for linear systems (close to 1)

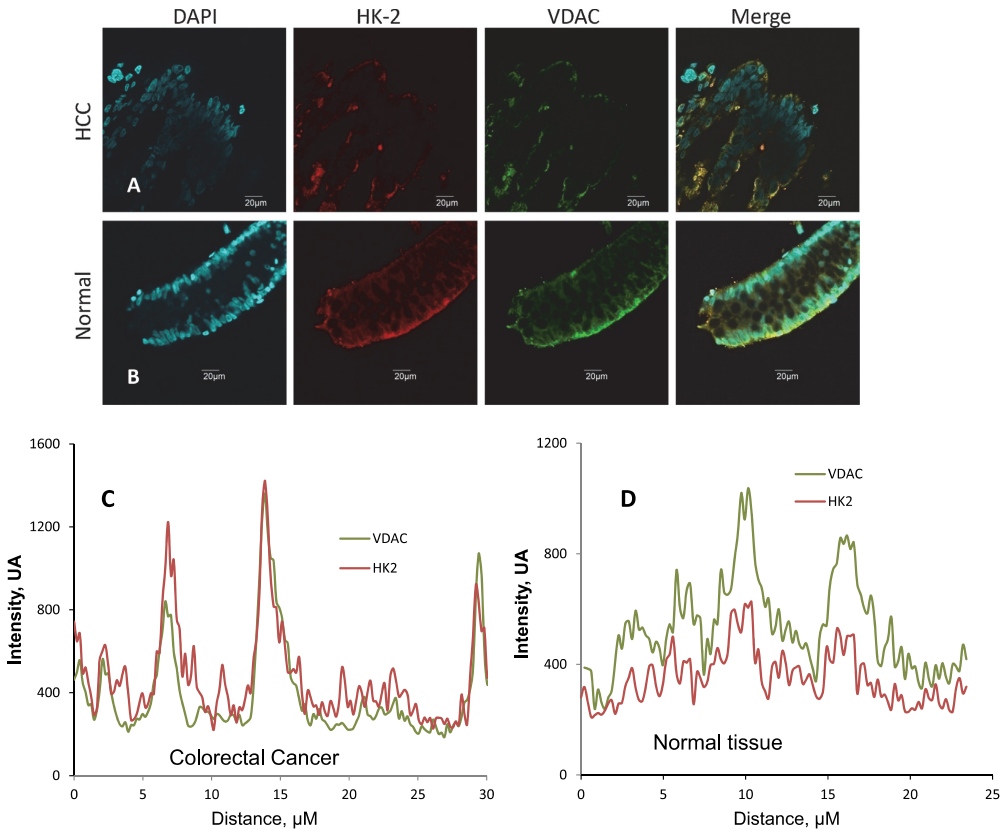

Fig. 9. Confocal imaging immunofluorescence of hexokinase-2 (HK-2), mitochondrial VDAC and their colocalization in saponin-skinned human colorectal cancer (HCC) fibers (A) and adjacent normal tissue (B) obtained by immunocytochemistry: blue fluorescence (nucleus, DAPI), red color indicates the presence of HK-2 (Cy-3 labeled antibody), green fluorescence corresponds to the outer mitochondrial membrane VDAC (DyLight-488) and yellow color is superposition of HK-2 and VDAC. Parts C and D indicate the distribution of fluorescent signals derived from HK-2 and VDAC in tumor and normal tissue samples. The images clearly show that HCC cells contain a number of mitochondria and that in these cells the HK-2 is bound to VDAC. This can mediate, according to the hypothesis of Pedersen and colleagues (Pedersen, 2008), a Warburg phenotype of malignancies.

(Kholodenko and Westerhoff, 1993). These values are higher than for some normal non-proliferative cells with high rates of OXPHOS (for adult rat cardiomyocytes the $\Sigma = 1.33$) (Tepp et al., 2011). It can be concluded that the function of respiratory chain and ATP synthasome complexes in intestinal cells may differ considerably from that in non-proliferative muscle cells of oxidative type. One possible explanation for the increased sum of the FCCs may be due to the existence of direct channeling of substrates between the protein complexes or formation of supercomplexes (Kholodenko and Westerhoff, 1993).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2014.09.004.

4. Discussion

So far, HCC was considered as a tumor of the Warburg phenotype having high rates of glucose consumption associated with the deregulated OXPHOS system (Chung et al., 1999; Dias et al., 2007; Haber et al., 1998; Izuishi et al., 2012; Jun et al., 2011; Koukourakis et al., 2005). Some *in vitro* studies suggest that HCC cells have gone through a metabolic shift from OXPHOS to aerobic glycolysis; so it was reported (Donohoe Dallas et al., 2012) that unlike normal intestinal cells, colorectal carcinomas cannot utilize short-chain fatty acids (butyrate, propionic acid) as an energy source and carbon donor. Instead they utilize glucose and glutamine as the main providers of energy and carbon for biosynthetic processes (Zhdanov et al., 2014).

Our studies indicate that HCC is not a pure glycolytic tumor and that the OXPHOS system is the substantial source of ATP in these malignant cells; it was found that the cells contain an increased, in comparison with normal tissue, amount of mitochondria (Fig. 4), fully-functioning respiratory chain complexes, and display higher rates of basal and ADP-activated respiration (Figs. 1–3). Different authors have shown that the mitochondrial biogenesis may be up-regulated in some cancer types *versus* healthy tissues; *e.g.* (Jose and Rossignol, 2013). Unexpectedly, one of the reasons for this change may be related to stimulated mitochondrial biogenesis. Overexpression of MYC proto-oncogene is characteristic for human colorectal carcinomas (Dang et al., 2009) and this event could lead to a strong increase in the content of mitochondria, since native MYC (encoding a transcription factor c-MYC) is known to play a pivotal role in the regulation of mitochondrial biogenesis. It has been previously reported that MYC null rat fibroblasts have diminished mitochondrial mass and decreased number of normal mitochondria (Li et al., 2005). We found that the rate of

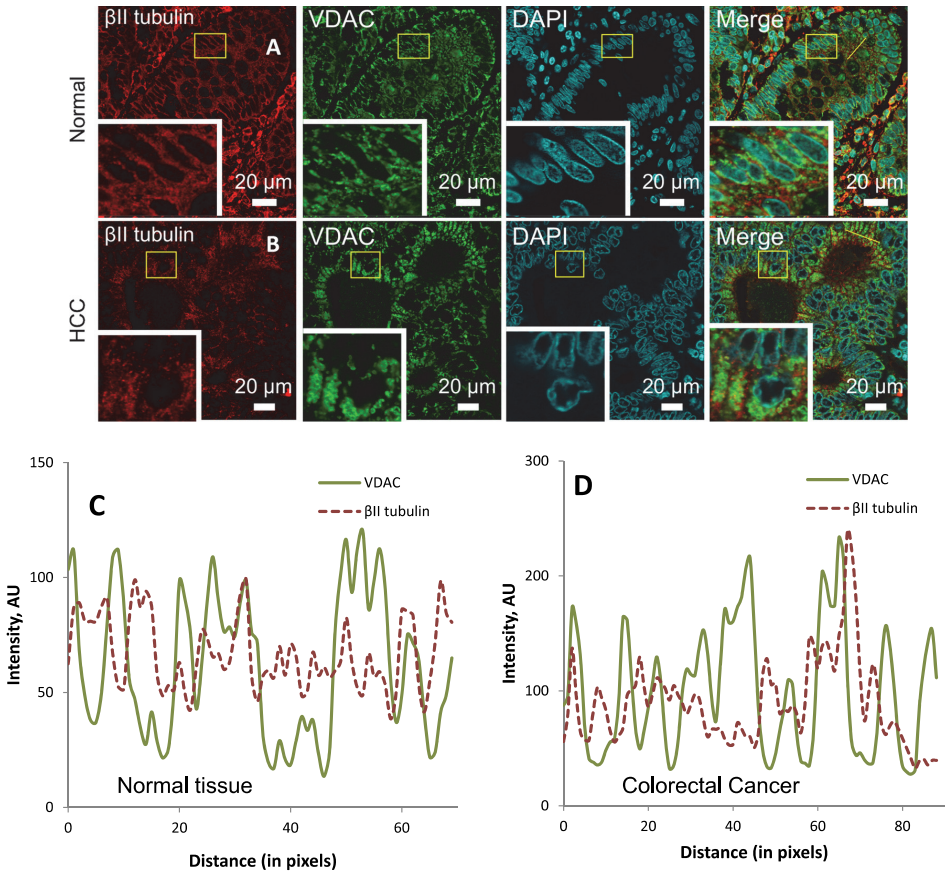

Fig. 10. Confocal imaging immunofluorescence of mitochondrial VDAC, cytoskeletal βII-tubulin, and their colocalization in normal (A) and human colorectal cancer (HCC) (B) tissue slices. Here: blue fluorescence (nucleus, DAPI); red color indicates the presence of βII-tubulin (Dylight-550 labeled Ig, ab96880), green color is the staining for VDAC, and yellow color is superposition of VDAC and βII-tubulin. Parts C and D indicate the distribution of fluorescent signals derived from VDAC and βII-tubulin in tumor and normal tissue samples. Immunofluorescence imaging showed a weak association between βII-tubulin and mitochondrial VDAC in the studied samples.

mitochondrial respiration in HCC fibers is higher than in surrounding normal tissues. It could be also hypothesized that a reverse Warburg effect occurs in colorectal carcinomas; *i.e.*, carcinoma cells cause reprogramming the energy metabolism of normal tissues from OXPHOS towards aerobic glycolysis (Bonuccelli et al., 2010a,b). However more extensive studies are needed to support this hypothesis.

It is clearly shown that there are striking differences between the kinetics of respiration regulation by ADP in colorectal carcinomas and in healthy tissue. In normal tissues, the apparent K_m value for exogenously added ADP was found to exceed nearly 2 times that for tumor tissue (Fig. 3). Low affinity of mitochondria for ADP in control tissue, in comparison with tumor tissue, is obviously a result of diffusion restriction of extracellular ADP. Several possible explanations can be proposed to clarify such changes in the regulation of ADP-activated respiration. The higher K_m value for ADP in normal tissue, as compared with HCC fibers, could be mediated by very rapid utilization of ADP in AK reactions, but this pathway may be excluded since the total AK activity in non-tumorous tissue was even smaller than in cancer (Table 1) and it has been shown that complete inhibition of AK by diadenosine pentaphosphate had no effect on the affinity of mitochondria for ADP in skinned muscle fibers (Saks et al., 1991). The diffusion restrictions for extracellular ADP in non-transformed cells may be due to more dense and developed cytoskeleton which was extensively investigated in our previous works (Kaambre et al., 2012; Kuznetsov et al., 1996; Kuznetsov et al., 2008; Saks et al., 2003; Vendelin et al., 2004). Higher levels of βII-tubulin expression in normal intestinal tissues could be responsible for the observed high K_m value. It was recently shown that in normal oxidative muscle cells, βII-tubulin could bind to VDAC and selectively limit the permeability of the MOM for adenine nucleotides (Guzun et al., 2011a; Rostovtseva et al., 2008) and therefore mediate the high K_m values (about 300–500 μM). The K_m for rat permeabilized cardiac non-beating HL-1 tumor cells was by more than order of magnitude lower (Anmann et al., 2006; Guzun et al., 2011a). Our previous studies of intracellular distribution of different β-tubulin isotypes using immunocytochemistry revealed the role of the βII-tubulin as one of the key potential regulatory proteins of VDAC channel permeability in adult rat cardiomyocytes (Gonzalez-Granillo et al., 2012; Guzun et al., 2012). Surprisingly the levels of βII-tubulin expression in HCC and normal adjacent tissue are comparable in spite of the differences in MOM permeability and βII-tubulin isotype does not display very clear colocalization with VDAC (Fig. 10). One possible explanation

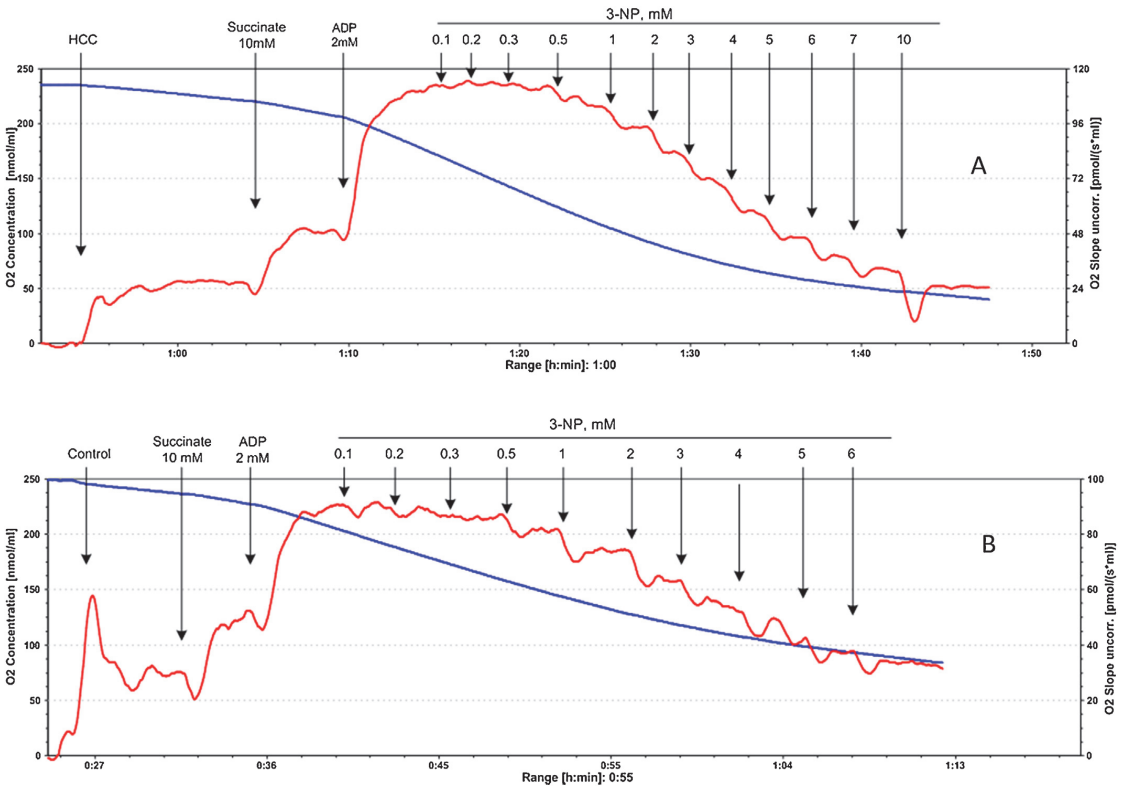

Fig. 11. Representative tracing of change in the rate of O_2 consumption (red lines) by permeabilized human colorectal cancer (A) and adjacent normal tissue fibers (B) during their titration with increasing concentrations of 3-nitropropionic acid (3-NP, a specific inhibitor of Complex-II) in the presence of 10 mM succinate, and direct activation of mitochondrial respiration with 2 mM ADP; on these figures, final concentrations of 3-NP are shown at every steady state. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

for this phenomenon is that some other proteins like actin, desmin, microtubule-associated proteins, plectin, other tubulin isoforms or their posttranslational modifications could be involved in the regulation of permeability of mitochondrial VDAC towards adenine nucleotides (Appaix et al., 2003; Guzun et al., 2012). Furthermore, it is possible that differences in MOM permeability for ADP and ATP could arise from distinct expression patterns of VDAC and/or ANT isoforms. Recently it was found that in some tissues of oxidative type – α-enolase, a glycolytic enzyme – can bind with mitochondrial VDAC1, to display anti-apoptotic activity (Gao et al., 2014). Changed profile of this enzyme isotypes could also influence the VDAC selective permeability for adenine nucleotides.

And last, but not least, in our previous works it was shown that the βII isotype of tubulin is closely associated with mitochondria and co-expressed with mitochondrial creatine kinase (MtCK). MtCK coupled with ATP synthasome, as well as with VDAC, provide functional compartmentation of ATP in mitochondria and energy transfer into cytoplasm *via* phosphotransfer network. Therefore, direct transfer of mitochondrially produced ATP to sites of its utilization is largely avoided under physiological conditions, but may occur in pathology (Gonzalez-Granillo et al., 2012; Saks et al., 2010, 2012; Tepp et al., 2011; Timohhina et al., 2009).

The CK system, playing one of the key roles in production of PCr and maintaining the energy homeostasis in normal colonocytes and muscle cells, is downregulated in HCC cells and this result is in accordance with some literature data (Joseph et al., 1997; Kasimos et al., 1990). Our studies showed that during carcinogenesis total CK activity was strongly decreased (Table 1). Furthermore, in normal intestinal cells elevated levels of uMtCK expressions were noticed together with tight coupling between the MtCK and OXPHOS system, but such functional coupling was absent in HCC cells (Figs. 5 and 6). The same functional coupling had been reported in cardiac and slow twitch skeletal muscle cells (Guzun et al., 2009; Monge et al., 2009; Seppet et al., 2001; Timohhina et al., 2009). Possible signaling pathways mediating the downregulation of CK system in HCC cells remain still unclear. These results lead to the conclusion that uMtCK plays a minor role in the cellular energy transfer and the functional coupling of MtCK with ANT in HCC cells. It was recently reported that in HCC cells BB-CK has an unusual profile of intracellular distribution, where BB-CK is expressed not only in the cytoplasm (characteristic for normal cells) but predominantly in the cell nucleus (Balasubramani et al., 2006). It was also suggested that the downregulation of BB-CK in HCC cells may play an important role in the tumor progression (Mooney et al., 2011).

AK isoenzymes are important regulators of the composition of the cellular adenine nucleotide pool and determinants of the energy charge of cells. The presence of nine AK isoenzymes have been reported in vertebrates (Panayiotou et al., 2011). Proteomics studies have shown that both cytosolic (AK1) and mitochondrial (AK2) isoenzymes are expressed in human colon epithelial cells (Birkenkamp-Demtroder et al., 2005; Li et al., 2004). But according to literature, little is known about the expression profile of

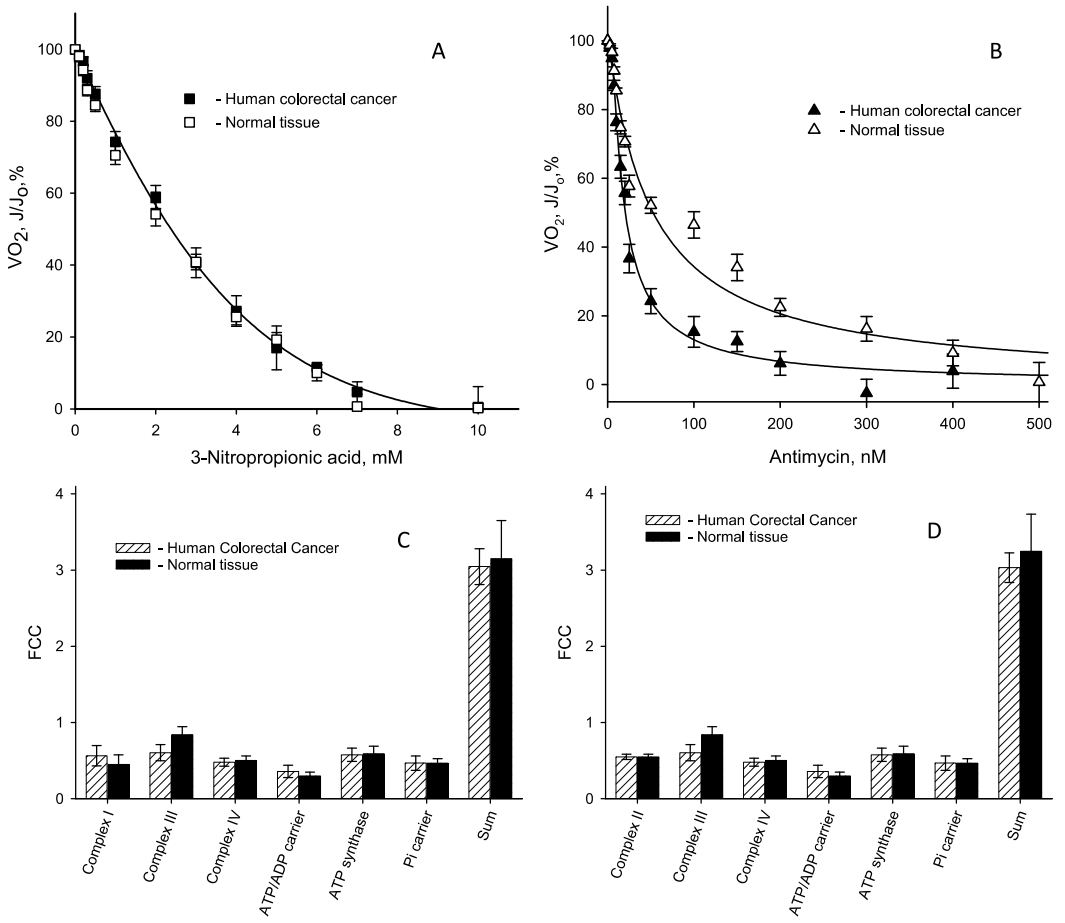

Fig. 12. Titration curves of inhibition of the mitochondrial respiratory chain complexes in permeabilized human colorectal cancer and non-tumorous adjacent tissue fibers with: (A) 3-nitropropionic acid, in the presence of 10 mM succinate as a respiratory substrate (without malate and glutamate); and (B) antimycin-A, respiratory substrates were 2 mM malate and 5 mM glutamate and 10 mM succinate. Similar titration curves were constructed for other mitochondrial respiratory chain and the ATP synthasome complexes; see in Supplementary Fig. 4. On the basis of these graphs, corresponding flux control coefficients were calculated. Two ways of electron transfer were examined: (C) NADH and (D) succinate dependent (FADH) electron transfers. On these figures, every data point was calculated as the mean of 10–15 independent experiments, bars are SEM.

AK isoenzymes and the role of AK system in maintaining energy homeostasis in colorectal carcinomas. Nevertheless, in some malignancies expression of AK2 was found, which had an important role in generation of ATP, providing up to 50% of the total ATP used by mitochondrially-bound HK (Nelson and Kabir, 1985).

Precise coupling of spatially separated intracellular ATP production and ATP-consuming processes is fundamental to the bioenergetics of living organisms, ensuring a fail-safe operation of the energetic system over a broad range of cellular functional activities. For this purpose colonic epithelial cells, like neurons, cardiac and skeletal muscles and many other cells, exert CK and AK systems (Dzeja and Terzic, 2003, 2009; Joseph et al., 1997; Saks et al., 2003). Previous studies on different types of carcinomas, such as human breast carcinoma (Kaambre et al., 2012), neuroblastoma (Klepinin et al., 2014), and sarcoma cells (Bera et al., 2008; Guzun et al., 2011b; Patra et al., 2008, 2012) showed that the suppression of the CK system is associated with loss of uMtCK. In addition, Dzeja et al. (1996) described that suppression of CK-catalyzed phosphotransfer network might result in increased phosphoryl transfer by AK in intact skeletal muscle. Therefore, we propose that the revealed downregulation of CK system may be casually-linked with alterations of the AK phosphotransfer system. In this study we found that the total AK activity in HCC cells exceeds that of adjacent normal tissue cells (Table 1). Also, our respiratory experiments show that HCC cells have higher coupling between the OXPHOS and AK reactions than normal colon cells (Fig. 7). Further studies are needed to determine the profile of AK isoforms expression in HCC cells, as well as their role in tumor energy metabolism.

We evaluated the glycolytic capacity of human colorectal and adjacent normal tissue samples by using hexokinase activities (see Table 1). HCC cells express both HK-1 and HK-2, but levels of these isoenzymes as well as the total glycolytic activity in tumor tissue extracts, do not differ from those in normal tissue samples (Figs. 5 and 9 and Table 1). This obtained result is in a good agreement with some literature data (Chung et al., 1999).

Consequently another mechanism, besides high HK activity in the malignancy, could be responsible for the elevated rates of glucose consumption detected by FDG-PET in colorectal cancers. This could be mediated through increased expression of the plasma membrane glucose transporter GLUT-1, which has already been demonstrated in human colorectal carcinomas (Izuishi et al., 2012). Possible two-compartment energy metabolism also may be the reason of the positive FDG-PET analysis, where the elevated rate of glucose consumption is the feature of the stromal cells.

We established that HK-2 is associated with the mitochondria in HCC cells (Fig. 9). Possible coupling of HK reactions with the OXPHOS system in these malignant cells could explain, to some degree, increased inclination of HCC cells to aerobic glycolysis. The obtained results strongly suggest that in human HCC cells high levels of HK-1 or HK-2 expression could be associated with binding either one or both isoforms to VDAC in the MOM. The interaction of ATP synthase, ANT, VDAC and HK as a single functional complex results in a rapid and very efficient production of glucose-6-phosphate (Mathupala et al., 2006), a precursor for other glycolytic steps and key biosynthetic metabolites *via* the pentose phosphate pathway and Krebs cycle. The mechanism of the Warburgís effect proposed by Pedersen and colleagues (Pedersen, 2008) could only partly explain high rates of aerobic glycolysis in human colorectal carcinomas. Although, the expression levels of HK-1 and HK-2 were similar in tumor and nontumorous tissue (Fig. 5), we did not observed any stimulatory effect of exogenously added glucose on mitochondrial respiration in normal tissue (Fig. 8B). One of the possible explanations for such difference could be that the mitochondria of colorectal carcinomas have an increased binding capacity towards HK-1 and HK-2 in comparison with normal intestinal cells. Indeed, according to some literature data, the increased affinity of tumor mitochondria to HK-2 has already been registered in tumors of another histological type. It was recently hypothesized that some tubulin isotypes (β-tubulin class II) could compete with HK-2 for the binding sites on VDAC and downregulation of the βII-tubulin isotype could promote the HK binding mediating Warburg effect (Guzun et al., 2011a, 2012). Therefore, we studied the levels of βII-tubulin expression (by measurements of its mRNA content) and no substantial differences in expression of βII-tubulin between HCC and normal adjacent tissue cells were found (Figs. 5 and 10). Thus, the proposed model, linked with the competition between HK-2 and βII-tubulin for binding sites on VDAC, does not function in the case of HCC. In cancer cells, other cytoskeletal proteins and mechanisms could control the binding of HK-2 to VDAC, but these mechanisms have not been cleared yet. Furthermore, it is known that various tubulin isotypes can undergo many post-translational modifications, such as detyrosination, acetylation, polyglutamylation and polyglycylation (Janke and Bulinski, 2011). In cancer cells, a spectrum of post-translational modifications of tubulin may differ remarkably from that in normal cells. This could seriously affect the binding capacity of modified tubulins to VDAC, their ability to compete with HK-2 for the binding sites on VDAC as well as regulation of permeability of adenine nucleotides through MOM.

Our results show that although the regulation of mitochondrial respiration in HCC cells differs from the normal oxidative type tissues, the OXPHOS, but not glycolysis, could play a key role in the generation of ATP. Our MCA inhibitor titration studies were carried out on Complex I, II, III, IV, ATP synthase, ANT and PIC upon direct activation of mitochondrial respiration with exogenously added ADP. All these coefficients were found to be with high values. The reason for these high control coefficients is not simply the diffusion restriction, since nearly the same concentration range of inhibitors corresponding FCCs were determined for isolated rat cardiomyocytes and differentiated or undifferentiated neuroblastoma cells (Klepinin et al., 2014). The presence of albumin in the assay medium of detergent-permeabilized cells cannot be the reason for the high FCCs as proposed in the recent work of Moreno-Sanchez et al. (2014). The sum of FCCs for permeabilized cardiomyocytes determined upon direct activation with 2 mM ADP differed, in terms of the sums, by three times despite the use of the same concentrations of albumin (Klepinin et al., 2014). We found that in human HCC cells the control was distributed across several ATP-Synthasome complexes rather evenly, and the key sites of the regulation of respiration were Complex-III and ATP synthase. The same results were achieved for normal tissue. Our MCA studies show that FCCs for Complex-V (ATP synthase) (Fig. 12) do not differ between tumor and control samples. This finding was surprising; it was reported (Sanchez-Cenizo et al., 2010; Willers et al., 2010) that β-F_1-ATPase catalytic subunit of the mitochondrial H^+-ATP synthase is downregulated in human colorectal carcinomas and this was associated with upregulation of the ATPase inhibitory factor 1 expression.

It is most interesting that the sum of FCC(s) for ADP activated respiration in HCC cells and in normal intestinal cells exceeds considerably (>3-times) the theoretic value for linear systems (close to 1) (Kholodenko et al., 1993) (Fig. 12C and D). The value close to 1 was registered for isolated mitochondria and permeabilized cardiac muscle cells during occurrence of ADP activated respiration (Tepp et al., 2011). Previously the large sum of FCC(s) was seen for Cr-activated respiration in permeabilized rat heart cells with ADP recycling inside the ATP synthasome and uMtCK reactions and in permeabilized neuroblastoma cells (Klepinin et al., 2014; Tepp et al., 2011). The theory of MCA claims that in an ideal linear system the sum of FCCs should be 1 (Fell, 1997; Groen et al., 1982; Rossignol et al., 2000; Westerhoff et al., 2009), but may become higher if the system includes enzyme-enzyme interactions, direct substrate channeling and recycling within multi-enzyme complexes (*i.e.*, if system becomes non-linear) (Kholodenko et al., 1993, 1994). In isolated mitochondria or permeabilized highly differentiated cells from normal tissue, the system of OXPHOS works typically as a quasi-linear system with the sum of FCCs close to 1, if respiration is activated with ADP (Moreno-Sanchez et al., 1991; Rossignol et al., 2000; Tepp et al., 2011). The high value (>3) of the sum of FCCs in mitochondrial respiration activated by ADP clearly shows that HCC as well as normal cells of the colon may have an altered structure of the mitochondrial respiratory chain, as compared with non-proliferating highly-differentiated cardiac and some skeletal muscle cells. Our results support the hypothesis that in human colon cells the mitochondrial OXPHOS system may contain supercomplexes with direct substrate (electron carrier) channeling. The veracity of this conclusion is supported by many recent studies (Bianchi et al., 2004; Dudkina et al., 2010, 2011; Genova et al., 2008; Lenaz and Genova, 2010; Quarato et al., 2011; Vonck and Schafer, 2009). Quarato et al. (2011) have examined kinetics of respiration regulation by applying MCA for permeabilized human hepatoma cells and found high FCCs in the de-energized state for three protonmotive Complexes I, III and IV, for which the sum of FCCs exceeded 1. Our results are in accordance with results from a series of studies performed by Lenaz and colleagues (Bianchi et al., 2004; Genova et al., 2008; Lenaz and Genova, 2009, 2010). These researchers, based on flux control analysis, showed that respiratory complexes might kinetically act as a single supramolecular unit, suggesting the existence of substrate channeling within supercomplexes. In this case, the sum of FCCs significantly exceeds 1 (Bianchi et al., 2004; Genova et al., 2008; Lenaz and Genova, 2009, 2010). The presence of supramolecular respiratory complexes (called respirasomes) in mitochondria has been confirmed by electron microscopy, native gel-electrophoresis and single particle image processing (Lenaz et al., 2010; Lenaz and Genova, 2009, 2010).

The formation of respirasomes may be characteristic for cells (both normal and tumorous) which have high proliferative rates. In MCA, high sums of FFC(s) (in the range of 3–5.5, upon direct activation of mitochondrial respiration with exogenously added ADP) have been registered not only for permeabilized HCC and normal colon cells (Fig. 12), but also in the permeabilized tissue of human breast cancer ($\Sigma = \sim 4$ (Kaambre et al., 2012)), undifferentiated (line N2a, $\Sigma = 5.06$), and differentiated neuroblastoma cells ($\Sigma = 3.9$) (Klepinin et al., 2014) where all of them are having high proliferative activity. But for highly differentiated cardiac cells, which have low proliferative activity, the sum of FCCs was found to be substantially lower, close to 1 ($\Sigma = 1.33$ (Tepp et al., 2011)). At present, the mechanism(s) of respirasomes formation is an object of intensive studies. Supercomplex formation and distribution of respiratory complexes between supercomplex and their free forms depend upon the protein/lipid ratio and phospholipid composition (Lenaz and Genova, 2009; Vartak et al., 2013).

Several important functions have been suggested for the formation of respiratory supercomplexes. First – channeling of ubiquinol and cytochrome c, avoiding competition from other enzymes. Second – catalytic enhancement from reduced diffusion times of substrates, and stabilization of the respiratory chain complexes. Third – preventing the generation of superoxide by sequestration of the reactive intermediate ubi-semiquinone (Dudkina et al., 2010; Hoefs et al., 2012). Finally, formation of respirasomes may also protect cells from the mitochondrially-induced apoptosis through suppression of the cytochrome-c release into the cytoplasm.

5. Conclusion

Several new important aspects were uncovered during bioenergetic profiling of HCC. We concluded that the rate of oxygen consumption is higher in colorectal carcinomas as compared to normal adjacent tissues, and that in these tumor cells the OXPHOS system serves as the main provider of ATP. So, immunocytochemical studies have shown that HCC cells contain increased number mitochondria. This is also supported by the hypothesis that the total glucose-phosphorylating capacity of colorectal carcinomas does not differ from surrounding normal tissues. Nevertheless, it was revealed that in HCC cells HK-2 is bound to VDAC, and this could lead to an increased production of lactate by the cells. The kinetics of respiration regulation by ADP is significantly changed during carcinogenesis. Low affinity of mitochondria for ADP in control tissue, in contrast to that of tumor tissue, is obviously a result of diffusion restriction of extracellular ADP and further work is needed to unfold the possible mechanisms of this phenomenon. Thus, during carcinogenesis some specific alterations arise in the regulation of permeability of mitochondrial outer membrane. Our MCA studies have shown that ATP synthasome complexes remain almost unchanged and only small differences were observed in the distribution of control between ETC complexes (small variations in complex III). In the presented work it was shown that there is functional coupling between AK-catalyzed processes and the OXPHOS system in HCC cells, and this could play an important role in maintaining the energy homeostasis in these malignant cells. In HCC cells there is upregulation of AK processes associated with downregulation of the CK system and these events could play an important role in the tumor progression. More extensive studies on HCC are required to identify the precise reasons for this disorganization of cell structure and metabolic compartmentation; remodeling of mitochondria-associated membrane interactions, impairment of intramitochondrial energy conversion and changes in metabolic fluxes.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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References

Agathocleous M, Harris WA. Metabolism in physiological cell proliferation and differentiation. Trends Cell Biol 2013;23:484–92.
Ames A. CNS energy metabolism as related to function. Brain Res Rev 2000;34:42–68.
Anmann T, Guzun R, Beraud N, Pelloux S, Kuznetsov AV, Kogerman L, 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;1757:1597–606.
Appaix F, Kuznetsov AV, Usson Y, Kay L, Andrienko T, Olivares J, et al. Possible role of cytoskeleton in intracellular arrangement and regulation of mitochondria. Exp Physiol 2003;88:175–90.
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;66:763–9.
Bera S, Wallimann T, Ray S, Ray M. Enzymes of creatine biosynthesis, arginine and methionine metabolism in normal and malignant cells. FEBS J 2008;275:5899–909.
Bianchi C, Genova ML, Parenti Castelli G, Lenaz G. The mitochondrial respiratory chain is partially organized in a supercomplex assembly. J Biol Chem 2004;279:36562–9.
Birkenkamp-Demtroder K, Olesen SH, Sorensen FB, Laurberg S, Laiho P, Aaltonen LA, et al. Differential gene expression in colon cancer of the caecum versus the sigmoid and rectosigmoid. Gut 2005;54:374–84.
Bonuccelli G, Tsirigos A, Whitaker-Menezes D, Pavlides S, Pestell RG, Chiavarina B, et al. Ketones and lactate "fuel" tumor growth and metastasis: evidence that epithelial cancer cells use oxidative mitochondrial metabolism. Cell Cycle 2010a;9:3506–14.
Bonuccelli G, Whitaker-Menezes D, Castello-Cros R, Pavlides S, Pestell RG, Fatatis A, et al. The reverse Warburg effect: glycolysis inhibitors prevent the tumor promoting effects of caveolin-1 deficient cancer associated fibroblasts. Cell Cycle 2010b;9:1960–71.
Cesar Mde C, Wilson JE. Further studies on the coupling of mitochondrially bound hexokinase to intramitochondrially compartmented ATP, generated by oxidative phosphorylation. Arch Biochem Biophys 1998;350:109–17.
Chung JK, Lee YJ, Kim C, Choi SR, Kim M, Lee K, et al. Mechanisms related to [^{18}F]fluorodeoxyglucose uptake of human colon cancers transplanted in nude mice. J Nucl Med 1999;40:339–46.
Curry JM, Tuluc M, Whitaker-Menezes D, Ames JA, Anantharaman A, Butera A, et al. Cancer metabolism, stemness and tumor recurrence: MCT1 and MCT4 are functional biomarkers of metabolic symbiosis in head and neck cancer. Cell Cycle 2013;12:1371–84.
Dang CV, Le A, Gao P. MYC-induced cancer cell energy metabolism and therapeutic opportunities. Clin Cancer Res 2009;15:6479–83.
Dias AR, Nahas SC, Camargo EE, Nahas CS. Recent evidences of the use of ^{18}F-fluorodeoxyglucose positron emission tomography in the management of colorectal cancer. J Surg Educ 2007;64:114–9.
Diers AR, Broniowska KA, Chang CF, Hogg N. Pyruvate fuels mitochondrial respiration and proliferation of breast cancer cells: effect of monocarboxylate transporter inhibition. Biochem J 2012;444:561–71.
Donohoe Dallas R, Collins Leonard B, Wali A, Bigler R, Sun W, Bultman Scott J. The Warburg effect dictates the mechanism of butyrate-mediated histone acetylation and cell proliferation. Mol Cell 2012;48:612–26.
Dzeja P, Terzic A. Adenylate kinase and AMP signaling networks: metabolic monitoring, signal communication and body energy sensing. Int J Mol Sci 2009;10:1729–72.
Dzeja PP, Zeleznikar RJ, Goldberg ND. Suppression of creatine kinase-catalyzed phosphotransfer results in increased phosphoryl transfer by adenylate kinase in intact skeletal muscle. J Biol Chem 1996;271:12847–51.
Dzeja PP, Terzic A. Phosphotransfer networks, cellular energetics. J Exp Biol 2003;206:2039–47.
Dzeja PP, Vitkevicius KT, Redfield MM, Burnett JC, Terzic A. Adenylate kinase-catalyzed phosphotransfer in the myocardium: increased contribution in heart failure. Circ Res 1999;84:1137–43.
Dudkina NV, Kouřil R, Peters K, Braun H-P, Boekema EJ. Structure and function of mitochondrial supercomplexes. Biochim Biophys Acta (BBA): Bioenergetics 2010;1797:664–70.

Dudkina NV, Kudryashev M, Stahlberg H, Boekema EJ. Interaction of complexes I, III, and IV within the bovine respirasome by single particle cryoelectron tomography. Proc Natl Acad Sci USA 2011;108:15196–200.

Fell D. Understanding the control of metabolism. London, Miami: Portland Press; 1997.

Fell D. Metabolic control analysis. In: Alberghina L, Westerhoff HV, editors. Systems biology. Berlin: Springer Verlag; 2005. p. 69–80.

Fulda S, Galluzzi L, Kroemer G. Targeting mitochondria for cancer therapy. Nat Rev Drug Discov 2010;9:447–64.

Gao S, Li H, Cai Y, Ye J.-T., Liu Z.-P., Lu J, et al. Mitochondrial binding of α-enolase stabilizes mitochondrial membrane: its role in doxorubicin-induced cardiomyocyte apoptosis. Arch Biochem Biophys 2014;542:46–55.

Gellerich F, Saks VA. Control of heart mitochondrial oxygen consumption by creatine kinase: the importance of enzyme localization. Biochem Biophys Res Commun 1982;105:1473–81.

Gellerich FN, Kunz WS, Bohnensack R. Estimation of flux control coefficients from inhibitor titrations by non-linear regression. FEBS Lett 1990;274:167–70.

Gellerich FN, Laterveer FD, Zierz S, Nicolay K. The quantitation of ADP diffusion gradients across the outer membrane of heart mitochondria in the presence of macromolecules. Biochim Biophys Acta 2002;1554:48–56.

Genova ML, Baracca A, Biondi A, Casalena G, Faccioli M, Falasca AI, et al. Is supercomplex organization of the respiratory chain required for optimal electron transfer activity? Biochim Biophys Acta 2008;1777:740–6.

Geschwind JF, Georgiades CS, Ko YH, Pedersen PL. Recently elucidated energy catabolism pathways provide opportunities for novel treatments in hepatocellular carcinoma. Expert Rev Anticancer Ther 2004;4:449–57.

Gnaiger E. Oxygen solubility in experimental media. Mitochondrial Physiol Netw 2001;6:1–6.

Gogvadze V, Orrenius S, Zhivotovsky B. Mitochondria as targets for cancer chemotherapy. Semin Cancer Biol 2009;19:57–66.

Goldenthal MJ, Marín-García J. Mitochondrial signaling pathways: a receiver/integrator organelle. Mol Cell Biochem 2004;262:1–16.

Gonzalez-Granillo M, Grichine A, Guzun R, Usson Y, Tepp K, Chekulayev V, et al. Studies of the role of tubulin beta II isotype in regulation of mitochondrial respiration in intracellular energetic units in cardiac cells. J Mol Cell Cardiol 2012;52:437–47.

Groen AK, Wanders RJ, Westerhoff HV, van der Meer R, Tager JM. Quantification of the contribution of various steps to the control of mitochondrial respiration. J Biol Chem 1982;257:2754–7.

Gruno M, Peet N, Seppet E, Kadaja L, Paju K, Eimre M, et al. Oxidative phosphorylation and its coupling to mitochondrial creatine and adenylate kinases in human gastric mucosa. Am J Physiol Regul Integr Comp Physiol 2006;291:R936–46.

Guzun R, Gonzalez-Granillo M, Karu-Varikmaa M, Grichine A, Usson Y, Kaambre T, et al. Regulation of respiration in muscle cells in vivo by VDAC through interaction with the cytoskeleton and MtCK within mitochondrial interactosome. Biochim Biophys Acta 2012;1818:1545–54.

Guzun R, Karu-Varikmaa M, Gonzalez-Granillo M, Kuznetsov AV, Michel L, Cottet-Rousselle C, et al. Mitochondria-cytoskeleton interaction: distribution of beta-tubulins in cardiomyocytes and HL-1 cells. Biochim Biophys Acta 2011a;1807:458–69.

Guzun R, Timohhina N, Tepp K, Gonzalez-Granillo M, Shevchuk I, Chekulayev V, et al. Systems bioenergetics of creatine kinase networks: physiological roles of creatine and phosphocreatine in regulation of cardiac cell function. Amino Acids 2011b;40:1333–48.

Guzun R, Timohhina N, Tepp K, Monge C, Kaambre T, Sikk P, et al. Regulation of respiration controlled by mitochondrial creatine kinase in permeabilized cardiac cells in situ, Importance of system level properties. Biochim Biophys Acta 2009;1787:1089–105.

Haber RS, Rathan A, Weiser KR, Pritsker A, Itzkowitz SH, Bodian C, et al. GLUT1 glucose transporter expression in colorectal carcinoma: a marker for poor prognosis. Cancer 1998;83:34–40.

Hoefs SJG, Rodenburg RJ, Smeitink JAM, van den Heuvel LP. Molecular base of biochemical complex I deficiency. Mitochondrion 2012;12:520–32.

Huang LS, Sun G, Cobessi D, Wang AC, Shen JT, Tung EY, et al. 3-Nitropropionic acid is a suicide inhibitor of mitochondrial respiration that, upon oxidation by complex II, forms a covalent adduct with a catalytic base arginine in the active site of the enzyme. J Biol Chem 2006;281:5965–72.

Izuishi K, Yamamoto Y, Sano T, Takebayashi R, Nishiyama Y, Mori H, et al. Molecular mechanism underlying the detection of colorectal cancer by ^{18}F-2-fluoro-2-deoxy-D-glucose positron emission tomography. J Gastrointest Surg 2012;16:394–400.

Janke C, Bulinski JC. Post-translational regulation of the microtubule cytoskeleton: mechanisms and functions. Nat Rev Mol Cell Biol 2011;12:773–86.

Jose C, Rossignol R. Rationale for mitochondria-targeting strategies in cancer bioenergetic therapies. Int J Biochem Cell Biol 2013;45:123–9.

Joseph J, Cardesa A, Carreras J. Creatine kinase activity and isoenzymes in lung, colon and liver carcinomas. Br J Cancer 1997;76:600–5.

Jun YJ, Jang SM, Han HL, Lee KH, Jang KS, Paik SS. Clinicopathologic significance of GLUT1 expression and its correlation with Apaf-1 in colorectal adenocarcinomas. World J Gastroenterol 2011;17:1866–73.

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;44:539–58.

Kasimos JN, Merchant TE, Gierke LW, Glonek T. 31P magnetic resonance spectroscopy of human colon cancer. Cancer Res 1990;50:527–32.

Kholodenko BN, Cascante M, Westerhoff HV. Control theory of metabolic channelling. Mol Cell Biochem 1994;133–134:313–31.

Kholodenko BN, Demin OV, Westerhoff HV. 'Channelled' pathways can be more sensitive to specific regulatory signals. FEBS Lett 1993;320:75–8.

Kholodenko BN, Westerhoff HV. Metabolic channelling and control of the flux. FEBS Lett 1993;320:71–4.

Klepinin A, Chekulayev V, Timohhina N, Shevchuk I, Tepp K, Kaldma A, et al. Comparative analysis of some aspects of mitochondrial metabolism in differentiated and undifferentiated neuroblastoma cells. J Bioenerg Biomembr 2014;46:17–31.

Koukourakis MI, Giatromanolaki A, Simopoulos C, Polychronidis A, Sivridis E. Lactate dehydrogenase 5 (LDH5) relates to up-regulated hypoxia inducible factor pathway and metastasis in colorectal cancer. Clin Exp Metastasis 2005;22:25–30.

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;241:909–15.

Kuznetsov AV, Veksler V, Gellerich FN, Saks V, Margreiter R, Kunz WS. Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. Nat Protoc 2008;3:965–76.

Leandro-Garcia LJ, Leskela S, Landa I, Montero-Conde C, Lopez-Jimenez E, Leton R, et al. Tumoral and tissue-specific expression of the major human beta-tubulin isotypes. Cytoskeleton (Hoboken) 2010;67:214–23.

Lenaz G, Baracca A, Barbero G, Bergamini C, Dalmonte ME, Del Sole M, et al. Mitochondrial respiratory chain super-complex I–III in physiology and pathology. Biochim Biophys Acta (BBA): Bioenergetics 2010;1797:633–40.

Lenaz G, Genova ML. Structural and functional organization of the mitochondrial respiratory chain: a dynamic super-assembly. Int J Biochem Cell Biol 2009;41:1750–72.

Lenaz G, Genova ML. Structure and organization of mitochondrial respiratory complexes: a new understanding of an old subject. Antioxidants Redox Signal 2010;12:961–1008.

Li F, Wang Y, Zeller KI, Potter JJ, Wonsey DR, O'Donnell KA, et al. Myc stimulates nuclearly encoded mitochondrial genes and mitochondrial biogenesis. Mol Cell Biol 2005;25:6225–34.

Li XM, Patel BB, Blagoi EL, Patterson MD, Seeholzer SH, Zhang T, et al. Analyzing alkaline proteins in human colon crypt proteome. J Proteome Res 2004;3:821–33.

Lienhard GE, Secemski II. P1,P5-Di(adenosine-5')pentaphosphate, a potent multisubstrate inhibitor of adenylate kinase. J Biol Chem 1973;248:1121–3.

Lunt SY, Vander Heiden MG. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. Annu Rev Cell Dev Biol 2011;27:441–64.

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;16:819–30.

Marin-Hernandez A, Rodriguez-Enriquez S, Vital-Gonzalez PA, Flores-Rodriguez FL, Macias-Silva M, Sosa-Garrocho M, et al. Determining and understanding the control of glycolysis in fast-growth tumor cells. Flux control by an over-expressed but strongly product-inhibited hexokinase. FEBS J 2006;273:1975–88.

Martinez-Outschoorn UE, Pavlides S, Howell A, Pestell RG, Tanowitz HB, Sotgia F, et al. Stromal-epithelial metabolic coupling in cancer: integrating autophagy and metabolism in the tumor microenvironment. Int J Biochem Cell Biol 2011;43:1045–51.

Martinez-Outschoorn UE, Sotgia F, Lisanti MP. Power surge: supporting cells "fuel" cancer cell mitochondria. Cell Metab 2012;15:4–5.

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;25:4777–86.

Monge C, Beraud N, Tepp K, Pelloux S, Chahboun S, Kaambre T, 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;87:318–26.

Mooney SM, Rajagopalan K, Williams BH, Zeng Y, Christudass CS, Li Y, et al. Creatine kinase brain overexpression protects colorectal cells from various metabolic and non-metabolic stresses. J Cell Biochem 2011;112:1066–75.

Moreno-Sanchez R, Devars S, Lopez-Gomez F, Uribe A, Corona N. Distribution of control of oxidative phosphorylation in mitochondria oxidizing NAD-linked substrates. Biochim Biophys Acta 1991;1060:284–92.

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.

Moreno-Sanchez R, Rodriguez-Enriquez S, Marin-Hernandez A, Saavedra E. Energy metabolism in tumor cells. FEBS J 2007;274:1393–418.

Moreno-Sánchez R, Rodríguez-Enríquez S, Saavedra E, Marín-Hernández A, Gallardo-Pérez JC. The bioenergetics of cancer: is glycolysis the main ATP supplier in all tumor cells? BioFactors 2009;35:209–25.

Moreno-Sanchez R, Saavedra E, Rodriguez-Enriquez S, Gallardo-Perez JC, Quezada H, Westerhoff HV. Metabolic control analysis indicates a change of strategy in the treatment of cancer. Mitochondrion 2010;10:626–39.

Moreno-Sanchez R, Saavedra E, Rodriguez-Enriquez S, Olin-Sandoval V. Metabolic control analysis: a tool for designing strategies to manipulate metabolic pathways. J Biomed Biotechnol 2008;2008:597913.

Mozzetti S, Ferlini C, Concolino P, Filippetti F, Raspaglio G, Prislei S, et al. Class III beta-tubulin overexpression is a prominent mechanism of paclitaxel resistance in ovarian cancer patients. Clin Cancer Res 2005;11:298–305.

Nelson BD, Kabir F. Adenylate kinase is a source of ATP for tumor mitochondrial hexokinase. Biochim Biophys Acta 1985;841:195–200.

Nieman KM, Kenny HA, Penicka CV, Ladanyi A, Buell-Gutbrod R, Zillhardt MR, et al. Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. Nat Med 2011;17:1498–503.
Panayiotou C, Solaroli N, Xu Y, Johansson M, Karlsson A. The characterization of human adenylate kinases 7 and 8 demonstrates differences in kinetic parameters and structural organization among the family of adenylate kinase isoenzymes. Biochem J 2011;433:527–34.
Pastorino JG, Hoek JB. Regulation of hexokinase binding to VDAC. J Bioenerg Biomembr 2008;40:171–82.
Patra S, Bera S, SinhaRoy S, Ghoshal S, Ray S, Basu A, et al. Progressive decrease of phosphocreatine, creatine and creatine kinase in skeletal muscle upon transformation to sarcoma. FEBS J 2008;275:3236–47.
Patra S, Ghosh A, Roy SS, Bera S, Das M, Talukdar D, et al. A short review on creatine-creatine kinase system in relation to cancer and some experimental results on creatine as adjuvant in cancer therapy. Amino Acids 2012;42:2319–30.
Pedersen PL. The cancer cell's "power plants" as promising therapeutic targets: an overview. J Bioenerg Biomembr 2007a;39:1–12.
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 2007b;39:211–22.
Pedersen PL. Voltage dependent anion channels (VDACs): a brief introduction with a focus on the outer mitochondrial compartment's roles together with hexokinase-2 in the "Warburg effect" in cancer. J Bioenerg Biomembr 2008;40:123–6.
Quarato G, Piccoli C, Scrima R, Capitanio N. Variation of flux control coefficient of cytochrome c oxidase and of the other respiratory chain complexes at different values of protonmotive force occurs by a threshold mechanism. Biochim Biophys Acta 2011;1807:1114–24.
Robey RB, Raval BJ, Ma J, Santos AV. Thrombin is a novel regulator of hexokinase activity in mesangial cells. Kidney Int 2000;57:2308–18.
Rodriguez-Enriquez S, Gallardo-Perez JC, Marin-Hernandez A, Aguilar-Ponce JL, Mandujano-Tinoco EA, Meneses A, et al. Oxidative phosphorylation as a target to arrest malignant neoplasias. Curr Med Chem 2011;18:3156–67.
Rodríguez-Enríquez S, Vital-González PA, Flores-Rodríguez FL, Marín-Hernández A, Ruiz-Azuara L, Moreno-Sánchez R. Control of cellular proliferation by modulation of oxidative phosphorylation in human and rodent fast-growing tumor cells. Toxicol Appl Pharmacol 2006;215:208–17.
Rossignol R, Letellier T, Malgat M, Rocher C, Mazat J. Tissue variation in the control of oxidative phosphorylation: implication for mitochondrial diseases. Biochem J 2000;347(Pt 1):45–53.
Rostovtseva TK, Sheldon KL, Hassanzadeh E, Monge C, Saks V, Bezrukov SM, et al. Tubulin binding blocks mitochondrial voltage-dependent anion channel and regulates respiration. Proc Natl Acad Sci USA 2008;105:18746–51.
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;1797:678–97.
Saks V, Kuznetsov A, Andrienko T, Usson Y, Appaix F, Guerrero K, et al. Heterogeneity of ADP diffusion and regulation of respiration in cardiac cells. Biophys J 2003;84:3436–56.
Saks V, Kuznetsov AV, Gonzalez-Granillo M, Tepp K, Timohhina N, Karu-Varikmaa M, et al. Intracellular energetic units regulate metabolism in cardiac cells. J Mol Cell Cardiol 2012;52:419–36.
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;1074:302–11.
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;184:81–100.
Sanchez-Cenizo L, Formentini L, Aldea M, Ortega AD, Garcia-Huerta P, Sanchez-Arago M, et al. Up-regulation of the ATPase inhibitory factor 1 (IF1) of the mitochondrial H^+-ATP synthase in human tumors mediates the metabolic shift of cancer cells to a Warburg phenotype. J Biol Chem 2010;285:25308–13.
Seppet EK, Kaambre T, Sikk P, Tiivel T, Vija H, Tonkonogi M, et al. Functional complexes of mitochondria with Ca, MgATPases of myofibrils and sarcoplasmic reticulum in muscle cells. Biochim Biophys Acta 2001;1504:379–95.
Small JR. Flux control coefficients determined by inhibitor titration: the design and analysis of experiments to minimize errors. Biochem J 1993;296(Pt 2):423–33.
Small JR, Fell DA. Covalent modification and metabolic control analysis. Modification to the theorems and their application to metabolic systems containing covalently modifiable enzymes. Eur J Biochem 1990;191:405–11.
Sotgia F, Martinez-Outschoorn UE, Pavlides S, Howell A, Pestell RG, Lisanti MP. Understanding the Warburg effect and the prognostic value of stromal caveolin-1 as a marker of a lethal tumor microenvironment. Breast Cancer Res 2011;13:213.
Sotgia F, Whitaker-Menezes D, Martinez-Outschoorn UE, Flomenberg N, Birbe RC, Witkiewicz AK, et al. Mitochondrial metabolism in cancer metastasis: visualizing tumor cell mitochondria and the "reverse Warburg effect" in positive lymph node tissue. Cell Cycle 2012;11:1445–54.
Zhdanov AV, Waters AHC, Golubeva AV, Dmitriev RI, Papkovsky DB. Availability of the key metabolic substrates dictates the respiratory response of cancer cells to the mitochondrial uncoupling. Biochim Biophys Acta (BBA): Bioenergetics 2014;1837:51–62.
Zu XL, Guppy M. Cancer metabolism: facts, fantasy, and fiction. Biochem Biophys Res Commun 2004;313:459–65.
Tepp K, Shevchuk I, Chekulayev V, Timohhina N, Kuznetsov AV, Guzun R, et al. High efficiency of energy flux controls within mitochondrial interactosome in cardiac intracellular energetic units. Biochim Biophys Acta 2011;1807:1549–61.
Timohhina N, Guzun R, Tepp K, Monge C, Varikmaa M, Vija H, et al. Direct measurement of energy fluxes from mitochondria into cytoplasm in permeabilized cardiac cells in situ: some evidence for mitochondrial interactosome. J Bioenerg Biomembr 2009;41:259–75.
Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 2009;324:1029–33.
Warburg O, Wind F, Negelein E. The metabolism of tumors in the body. J Gen Physiol 1927;8:519–30.
Vartak R, Porras C-M, Bai Y. Respiratory supercomplexes: structure, function and assembly. Protein Cell 2013;4:582–90.
Vendelin M, Eimre M, Seppet E, Peet N, Andrienko T, Lemba M, et al. Intracellular diffusion of adenosine phosphates is locally restricted in cardiac muscle. Mol Cell Biochem 2004;256–257:229–41.
Westerhoff HV, Winder C, Messiha H, Simeonidis E, Adamczyk M, Verma M, et al. Systems biology: the elements and principles of life. FEBS Lett 2009;583:3882–90.
Whitaker-Menezes D, Martinez-Outschoorn UE, Lin Z, Ertel A, Flomenberg N, Witkiewicz AK, et al. Evidence for a stromal-epithelial "lactate shuttle" in human tumors: MCT4 is a marker of oxidative stress in cancer-associated fibroblasts. Cell Cycle 2011;10:1772–83.
Willers IM, Isidoro A, Ortega AD, Fernandez PL, Cuezva JM. Selective inhibition of beta-F1-ATPase mRNA translation in human tumours. Biochem J 2010;426:319–26.
Wisniewski E, Gellerich FN, Kunz WS. Distribution of flux control among the enzymes of mitochondrial oxidative phosphorylation in calcium-activated saponin-skinned rat musculus soleus fibers. Eur J Biochem 1995;230:549–54.
Witkiewicz AK, Whitaker-Menezes D, Dasgupta A, Philp NJ, Lin Z, Gandara R, et al. Using the "reverse Warburg effect" to identify high-risk breast cancer patients: Stromal MCT4 predicts poor clinical outcome in triple-negative breast cancers. Cell Cycle 2012;11:1108–17.
Vonck J, Schafer E. Supramolecular organization of protein complexes in the mitochondrial inner membrane. Biochim Biophys Acta 2009;1793:117–24.

Publication 3

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Comparative analysis of some aspects of mitochondrial metabolism in differentiated and undifferentiated neuroblastoma cells

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Abstract The aim of the present study is to clarify some aspects of the mechanisms of regulation of mitochondrial metabolism in neuroblastoma (NB) cells. Experiments were performed on murine Neuro-2a (N2a) cell line, and the same cells differentiated by all-trans-retinoic acid (dN2a) served as *in vitro* model of normal neurons. Oxygraphy and Metabolic Control Analysis (MCA) were applied to characterize the function of mitochondrial oxidative phosphorylation (OXPHOS) in NB cells. Flux control coefficients (FCCs) for components of the OXPHOS system were determined using titration studies with specific non-competitive inhibitors in the presence of exogenously added ADP. Respiration rates of undifferentiated Neuro-2a cells (uN2a) and the FCC of Complex-II in these cells were found to be considerably lower than those in dN2a cells. Our results show that NB is not an exclusively glycolytic tumor and could produce a considerable part of ATP via OXPHOS. Two important enzymes - hexokinase-2 and adenylate kinase-2 can play a role in the generation of ATP in NB cells. MCA has shown that in uN2a cells the key sites in the regulation of OXPHOS are complexes I, II and IV, whereas in dN2a cells complexes II and IV. Results obtained for the phosphate and adenine nucleotide carriers showed that in dN2a cells these carriers exerted lower control over the OXPHOS than in undifferentiated cells. The sum of FCCs for both types of NB cells was found to exceed significantly that for normal cells suggesting that in these cells the respiratory chain was somehow reorganized or assembled into large supercomplexes.

Keywords Energy metabolism · Metabolic control analysis · Neuroblastoma · Adenylate kinase · Hexokinase · Warburg effect

Abbreviations

AChE	Acetylcholinesterase
AK	Adenylate kinase
ANT	Adenine nucleotide translocator
CAT	Carboxyatractyloside
CM	Cardiomyocytes
C_{vi}^{J} or FCC	Flux control coefficient
CK	Creatine kinase
ETC	Electron transport chain
G6PDH	Glucose-6-phosphate dehydrogenase
HK	Hexokinase
K_m	Michaelis-Menten constant
MTR-CMXRos	MitoTracker® Red CMXRos
uMtCK	Ubiquitous mitochondrial creatine kinase
MCA	Metabolic Control Analysis
3-NP	3-nitropropionic acid
NB	Neuroblastoma
N2a	Neuro-2a
uN2a	Undifferentiated N2a cells
dN2a	Differentiated N2a cells

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OXPHOS	Oxidative phosphorylation
MOM	Mitochondrial outer membrane
PCr	Phosphocreatine
Pi	Inorganic phosphate
PIC	Inorganic phosphate carrier
PEP	Phosphoenolpyruvate
PK	Pyruvate kinase
RA	All-trans-retinoic acid
SDH	Succinate dehydrogenase
SNS	Sympathetic nervous system
TMPD	N,N,N′,N′-tetramethyl-p-phenylenediamine
VDAC	Voltage dependent anion channel

Introduction

Undifferentiated and poorly differentiated neuroblastomas are very aggressive tumors, which are characterized by high growth rates and strong inclination to metastasis. Studies performed during the past decade suggest that targeting cancer cell energy metabolism may be a new and very effective therapeutic strategy for selective ablation of malignancies (Geschwind et al. 2004; Gogvadze et al. 2009). However, today little is known about the key processes involved in the maintenance of energy homeostasis in NB cells as well as the bioenergetic capacity of their mitochondria.

Documented interest in energy metabolism and mitochondrial function in carcinogenesis started as early as in the 1930s, when Otto Warburg demonstrated (Pedersen 2007a; Warburg 1956) that cancer cells had an increased dependence on glycolysis to meet their energy needs, regardless of whether they were well-oxygenated or not – a phenomenon termed subsequently "aerobic glycolysis" or "Warburg effect". Although, the glycolytic phenotype of many cancer cells and tumors has been demonstrated at the biochemical and molecular levels (Atsumi et al. 2002; Marin-Hernandez et al. 2006; Pedersen 1978; Pedersen 2007b; Xu et al. 2005), the presumed impairment of mitochondrial function as the cause of malignant transformation has unambiguously never been proved in cancer biology (Pedersen 1978). Previous studies have shown that aerobic glycolysis could provide a growth advantage to tumor cells and mediate their increased resistance to apoptotic stimuli (Gatenby & Gillies 2004). A glycolytic phenotype was found in a high percentage of human cancers, and it was suggested that inhibition of glycolysis could be an effective approach for tumor ablation therapy (Ihrlund et al. 2008; Matsushita et al. 2012; Pedersen 2012). Recent studies have shown that the respiratory activity and significance of oxidative phosphorylation (OXPHOS) *versus* glycolysis in bioenergetics of malignancies vary greatly depending on their histological type, growth stage and vascularization (Apte & Sarangarajan 2008; Gatenby & Gillies 2004; Moreno-Sánchez et al. 2009). Cancer cells can utilize lactate, free fatty acids, ketone bodies, butyrate and glutamine as key respiratory substrates and may cause reprogramming of normal surrounding cells towards aerobic glycolysis – "reverse Warburg" effect (Whitaker-Menezes et al. 2011). It has also been demonstrated that targeting mitochondria can be a very effective tool for tumor cell elimination (Gogvadze et al. 2009). Thus, heterogeneity of bioenergetics profiles of tumors requires personalized treatment strategies for choosing effective anticancer treatment, including high-risk NBs. Literature on bioenergetics of human NBs is on the other hand still very limited.

Neurons have a high metabolic rate and use more energy than other cells. They normally derive ATP exclusively through aerobic mechanisms (OXPHOS). Studies performed on post-operational material (Beemer et al. 1984) and data obtained on cultured cells and animal models (Krieger-Hinck et al. 2006) suggest that rapidly proliferating human NBs are highly-glycolytic tumors which display a Warburg phenotype. Even under aerobic conditions, human NB cells can convert remarkable amounts of glucose to lactate instead of using glucose oxidation (Deubzer et al. 2010) and that some inhibitors of glycolysis, such as 3-bromopyruvic acid and 2-deoxyglucose, can suppress NB cell growth *in vitro* (Chuang et al. 2013; Matsushita et al. 2012). Although, cancer cells can produce a significant (~ 50–70 %) part of their ATP via aerobic glycolysis (Pedersen 2007b), the exact molecular mechanisms underlying the Warburg effect are not completely understood. In accordance with the literature data, several aerobic glycolysis key mechanisms, mediating a Warburg phenotype were observed in NB cells, and these could be crucial targets for medical intervention.

Hexokinase (HK) 2 was found to be over-expressed in many human malignancies, including NBs (Beemer et al. 1984; Levy et al. 2012; Pedersen et al. 2002). In this relation, Pedersen and colleagues have formulated the existence of a supercomplex consisting of ATP synthasome, voltage dependent anion channel (VDAC) and HK-2 in cancer cells that helps in explanation of the Warburg effect (Pedersen 2008). It was demonstrated that binding of HK-2 with mitochondria increased almost 5-fold its affinity for ATP (Bustamante & Pedersen 1980). The complex of HK-2 with mitochondria not only decreases product inhibition initiated by glucose-6-phosphate (Bustamante & Pedersen 1977; Nakashima et al. 1988) but also provides rapid access to ATP required by HK-2 to phosphorylate glucose to glucose-6-phosphate and therefore "jump starts" the glycolytic pathway and produces the Warburg effect.

Our recent studies have demonstrated that variations in the expression of some tubulin isotypes in cancer cells could mediate their Warburg phenotype (Guzun et al. 2011; Rostovtseva et al. 2008). We found (Guzun et al. 2011) that the localization and function of β-tubulin isotypes varied in different muscle tissues and tumor cells: in adult rat cardiomyocytes β-tubulin class II is associated with mitochondria, but it is absent in cardiac HL-1

tumor cells having a glycolytic phenotype. The absence of βII-tubulin in cancer cells allows binding of HK-2 to VDAC mediating thereby the initiation of the Warburg effect. It was presumed that HK-2, if directly bound to mitochondria through VDAC and, according to the model proposed by Majewski and coauthors (Majewski et al. 2004), supported VDAC to leave an open state. As a consequence, HK-2 consumes practically all of the ATP produced via OXPHOS and thereby causes elevated glycolysis even in the presence of oxygen. This suggests that increased rates of aerobic glycolysis in cancer cells may occur even without any disturbances in the functional activity of mitochondria.

In NB cells, a metabolic shift from OXPHOS to aerobic glycolysis could be largely mediated by specific alterations in the function of their mitochondria. Defective OXPHOS complexes, linked with mutations of mitochondrial DNA, were found in many other human malignancies. Deficiency of the Complex-I of the mitochondrial respiratory chain, associated with enhanced production of reactive oxygen species (Sharma et al. 2011), has been observed in human gastric cancer tissue (Puurand et al. 2012), renal and thyroid oncocytomas (Bonora et al. 2006; Simonnet et al. 2003). Some literature data suggests that NB cells are deficient in Complex-II activity, since mutations in genes encoding the subunits of the mitochondrial succinate dehydrogenase (SDH) complex have been shown in these malignancies (Cascon et al. 2008; Schimke et al. 2010).

The presented work was, therefore, aimed to clarify the bioenergetic function of mitochondria and main energy fluxes in NB cells. Our experiments were performed on murine NB cells of the Neuro-2a (N2a) line. These cells are well studied and present an excellent model system for clarifying the energetics of human NBs. N2a cells were shown to express mitochondria-bound HK (Krieglstein & Mwasekaga 1987; Krieglstein et al. 1981) and to display the Warburg phenotype (Kaambre et al. 2012a). In our study, the N2a cells differentiated by all-trans-retinoic acid (RA) served as an *in vitro* model of normal neurons. It was shown that RA acid-induced differentiation of human NB cells restored the respiratory activity of mitochondria (Xun et al. 2012).

Understanding the control and regulation of energy metabolism requires analytical tools, which relate the properties of metabolic systems to the kinetic characteristics of the component enzymes and their impact on network function. A powerful experimental approach for this purpose is Metabolic Control Analysis (MCA) (Fell 2005). MCA has been suggested to be beneficial for the description of enzymatic abnormalities in syndromes associated with mitochondrial dysfunction (Kuznetsov et al. 2008). Recently, Moreno-Sanchez and Westerhoff's groups have successfully applied MCA to investigate the control of glycolytic flux and mitochondrial respiration in different types of tumor cells growing in culture. Main conclusion of these studies was that the significance of OXPHOS in bioenergetics of cancer cells should be re-evaluated and experimentally determined for each particular type of neoplasm (Marin-Hernandez et al. 2006; Moreno-Sanchez et al. 2007; Moreno-Sánchez et al. 2009; Moreno-Sanchez et al. 2010).

In the present work MCA along with oxygraphy was applied to characterize the function of OXPHOS in undifferentiated and RA-treated N2a cells. We quantified the control exerted by different components of the respiratory chain and the ATP synthasome complex in both of these cell types. To determine the flux control coefficients, the flux was measured as the rate of O_2 consumption by permeabilized N2a cells when all components of the OXPHOS system were titrated with their specific inhibitors to stepwise decrease separate respiration complex activities. It is important to note that the using of MCA and the permeabilized techniques which enable to estimate OXPHOS function without isolation of mitochondria and thereby avoiding artifacts linked to their isolation procedure and the loss of components involving in the regulation. Furthermore, these methodological approaches preserve intimate interactions between mitochondria in the cell as well as the intactness of their cytoskeletal structures. Additional experiments were also performed in the present work to study the significance of creatine kinase (CK) and adenylate kinase (AK) reactions in the maintenance of energy homeostasis in NB cells.

Materials and methods

Chemicals

Antibodies, chemicals and enzymes were purchased from Abcam®, Sigma-Aldrich, Fluka or Roche, whereas growth media, heat-inactivated fetal bovine serum (FBS) and antibiotics were obtained from PAA Laboratories GmbH, Austria.

Isolation of rat heart cardiomyocytes

Adult cardiomyocytes (CM) were isolated after perfusion of the rat heart with collagenase-A (1.0 mg/ml, Roche) exactly as described in our previous work (Saks et al. 1991). The cells sarcolemma was permeabilized by saponin treatment (25 μg/ml, for 5 min at 25 °C) directly in oxygraphic chambers keeping the mitochondrial membranes intact (Tepp et al. 2011).

Cultivation of N2a cells and their differentiation

Stock culture of N2a cells was obtained from the American Type Culture Collection, Cat. No. CCL-131. Unless otherwise specified, the murine NB cells were grown as a loosely adhering monolayer at 37 °C in 5 % CO_2 in high glucose Dulbecco's modified Eagles medium supplemented with L-

glutamine, 10 % FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (complete growth medium). N2a cells were maintained at 80–90 % confluence at the time of subculture.

Neural differentiation of N2a cells to cholinergic neurons was induced by their treatment with 7 μM all-trans-retinoic acid (RA) in complete growth medium for 5 days (Blanco et al. 2001).

The cells growing in Petri dishes were harvested by centrifugation washed twice with Mitomed-B solution (Kaambre et al. 2012a) supplemented with 10 μM leupeptin and were then resuspended in the medium at a cell density of ~1×10^7 cells/ml.

Determination of cell growth

The effect of RA on the rate of N2a cells growth (seeded in 96-well plates) was estimated by the use of MTT Cell Proliferation Assay Kit (ATCC® 30-1010 K) according to the manufacturer manual. The absorbance of samples at 570 nm (reference wavelength, 620 nm) was measured spectrophotometrically using a microplate reader (Multiscan Spectrum, Thermo Electron Corporation).

Cell permeabilization and oxygraphic measurements

Plasma membrane was permeabilized with saponin treatment (Kuznetsov et al. 2008). Saponin concentration of 40 μg/ml was used as it gave the maximal respiratory response for exogenously added ADP whereas the integrity of the mitochondrial membranes was preserved.

Rates of oxygen consumption by permeabilized N2a cells (at a concentration of ~5×10^5 cells/ml) were measured under magnetic stirring (300 rpm) at 25 °C in 2-ml glass chambers of a two-channel titration-injection respirometer (Oxygraph-2 K, OROBOROS Instruments, Austria) in medium-B supplemented with 5 mM glutamate, 2 mM malate and 10 mM succinate as respiratory substrates. The plasma membrane in the cells was permeabilized by saponin treatment by adding it directly into oxygraphic chambers (incubation time 5 min at 25 °C); solubility of oxygen was taken as 240 nmol/ml.

Effects of exogenously added succinate, creatine, glucose, ADP and Km for AMP were measured in medium-B supplemented with 5 mM glutamate and 2 mM malate and in the presence of pyruvate kinase (PK) –phosphoenolpyruvate (5 mM) ADP trapping system (Kaambre et al. 2012b; Guzun et al. 2009).

Enzymatic activities

HK activity was measured as the total glucose phosphorylating capacity of whole cell extracts, using a standard glucose-6-phosphate dehydrogenase (G6PDH)-coupled spectrophotometric assay (Biswas et al. 1997). One milliunit (mU) of HK activity was calculated as the amount of enzyme activity required to phosphorylate 1 nmol of glucose in 1 min at 25 °C.

The CK activity was assessed spectrophotometrically at 25 °C in the direction of ATP formation in the presence of di(adenosine-5′) pentaphosphate (an adenylate kinase inhibitor (Lienhard & Secemski 1973)), 20 mM phosphocreatine (PCr) and with 2 U/ml G6PDH and 2 U/ml HK as the coupled enzymes (Monge et al. 2009). One mU of CK activity represents the formation of 1 nmole of ATP per minute at 25 °C.

AK activity of whole-cell extracts was measured at 25 °C by a coupled enzyme assay (Dzeja et al. 1999). The reaction was initiated with 2 mM ADP, and the arising changes in absorbance at 340 nm were recorded using a Cary 100 Bio UV-visible spectrophotometer. One mU of AK activity represents the formation of 1 nmole of ATP per minute at 25 °C.

The activity of acetylcholinesterase (AChE) in N2a cells was measured spectrophotometrically applying the technique of Ellman et al. (Ellman et al. 1961). One unit of AChE activity (U) represents the hydrolysis of 1 μmol of the chosen substrate (acetylthiocholine) per minute at 37 °C.

Confocal microscopy imaging of N2a cells

MitoTracker® Red CMXRos (Molecular Probes) was used to label mitochondria in N2a cells. The labeling procedure was carried out according to the manufacturer instructions. MitoTracker Red stained N2a cells were placed over glass microscope slides with ProLong Gold antifade reagent supplemented with DAPI (Molecular Probes®) for visualizing cell nucleus. Cells were imaged by an Olympus FV10i-W inverted laser scanning confocal microscope equipped with a 60× water immersion objective, using 560 nm laser excitation for MTR-CMXRos and light excitation at 405 nm for DAPI.

Metabolic control analysis

MCA quantifies the degree of control exerted by an enzymatic or transport step through the flux control coefficient (FCC). FCC is defined as the ratio of the fractional change in the steady-state flux with respect to an infinitesimal variation in the biochemical activity which may cause the change in flux (Fell 1997). If a small change in an enzyme activity promotes a significant variation in pathway flux, then this enzyme exerts an elevated control over the regulation of pathway. In contrast, if a rather small or negligible change in flux is observed when a complex activity is greatly varied, the enzyme has low FCC and decreased ability to influence on overall flux (Fell 1997; Tepp et al. 2010).

The FCC or C_{vi}^{J} is defined according to the equation (Fell 1997; Moreno-Sanchez et al. 2008):

$$C_{vi}^{J} = \left(\frac{dJ}{dv_i}\right) \Big/ \left(\frac{J}{v_i}\right) = \frac{d\ln J}{d\ln v_i}$$

in which the expression dJ/dv_i describes the variation in flux (J) when an infinitesimal change takes place in the enzyme i concentration or activity. Groen et al. (Groen et al. 1982) have derived a method to determine experimentally the C_{vi}^{J} using titration with specific enzyme inhibitors. The value of the C_{vi}^{J} coefficient is given by Groen (Groen et al. 1982) and Moreno-Sanches (Moreno-Sanchez et al. 2008):

$$C_{E}^{J} = (\Delta J/\Delta I)^{*}(I_{\max}/J_0)$$

where $(\Delta J/\Delta I)$ is the initial slope of the flux/inhibition graph, $I_{\max}$ is the inhibitor concentration giving complete inhibition, and J_0 is the initial steady-state flux value.

Experimentally, the FCCs for permeabilized N2a cells were determined for all mitochondrial respiratory chain and ATP synthasome complexes after their stepwise titration with specific irreversible inhibitors upon direct activation of respiration by exogenously added ADP (at 2 mM). C_{vi}^{J} values were calculated by using graphical method described by Fell (Fell 1997). The following inhibitors were used: rotenone for Complex-I of the mitochondrial electron transport chain (ETC); 3-nitropropionic acid (3-NP) for Complex-II; antimycin for Complex-III; sodium cyanide for Complex-IV; oligomycin for complex-V (ATP synthase); carboxyatractyloside (CAT) for adenine nucleotide translocator (ANT); and mersalyl for inorganic phosphate carrier (PIC).

Statistics

Data in the text, tables and figures are presented as mean± standard error of the mean (SEM). Results were analyzed by Student's t-test. Values of $P<0.05$ were considered statistically significant. To reduce the possibility of random errors, our experiments were repeated at least five times and the fitting technique was used to calculate the C_{vi}^{J} value. Apparent K_m values for ADP and AMP were estimated by fitting experimental data to a non-linear regression (according to a Michaelis-Menten model) equation.

Results

Proliferative activity, the number of mitochondria, morphological and enzymatic changes in N2a cells treated with retinoic acid

Experiments showed that the described protocol for N2a cells differentiation enabled to obtain a practically pure culture of neural cells with morphological and biochemical parameters typical for normal mature neurons; this is in a good agreement with the data from other laboratories (Blanco et al. 2001). We found that treatment of uN2a cells with RA resulted in strong neurite outgrowth that appeared on day 2 and extended on days 4 and 5 (Fig. 1). In addition, RA induced the formation of a population of neural cells with an increased number of mitochondria as illustrated by confocal microscopy (Fig. 1). In both cell types, mitochondria are predominantly localized around the cell nucleus; in dN2a cells a significant part of mitochondria are found to be localized in large neurites possibly attached to microtubule structures.

Moreover, it was established that RA-treated N2a cells lose their proliferative capacity (Fig. 2) and had an elevated AChE activity (Table 1). Similar effects of RA on the activity of AChE in N2a cells were also observed in other laboratories (Sato et al. 2002).

Activities of enzymes, which were involved in the maintenance of cellular energy homeostasis, were also measured in both uN2a and RA-treated cells. Table 1 shows that HK, AK and CK activities in dN2 cells exceed considerably those measured in immature N2a cells, suggesting that dN2a cells have increased metabolic rates and energy demand.

The influence of saponin treatment on the intactness of mitochondrial membranes in N2a cells

For comparison of respiratory activities of mitochondria in undifferentiated and dN2a cells, the technique of permeabilized cell, elaborated by Kuznetsov (Kuznetsov et al. 2008), was used. The technique gives an opportunity to study the mitochondrial function at nearly physiological conditions. The plasma membrane of the studied cells was permeabilized by saponin treatment directly in the oxygraph chamber as described previously (Kuznetsov et al. 2008). To evaluate the intactness of mitochondrial membranes, the cytochrome-c and CAT tests were applied for both undifferentiated N2a and dN2a cells (Saks et al. 1998). Respiration was activated with 2 mM ADP; addition of cytochrome-c (final concentration, 8 μM) did not cause any marked increase in the rate of oxygen consumption, indicating the intactness of the outer mitochondrial membrane. Addition of carboxyatractyloside (1 μM) decreased the respiration rate back to the basal rate (V_o) level showing the intactness of the mitochondrial inner membrane (Fig. 3). Therefore the saponin concentration of 40 μg/ml was considered suitable.

Function of respiratory chain complexes in undifferentiated and differentiated N2a cells

Activities of various segments of the respiratory chain were studied by using a specific step-by-step substrate inhibitor titration oxygraphic protocol. The ADP (at 2 mM) activated

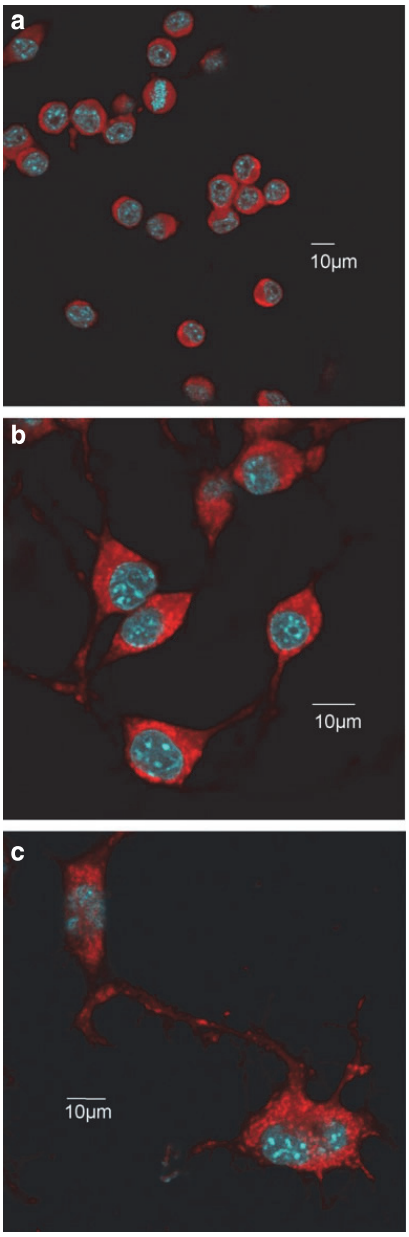

Fig. 1 Confocal microscopy images of N2a cells with and without all-trans-retinoic acid (RA) treatment. **a** -control cells, whereas (**b**) and (**c**) are N2a cells treated with 7 μM RA for 5 days. Mitochondria in the cells were stained with MitoTracker Red CMXRos (red fluorescence) and cell nuclei with DAPI (blue fluorescence). After 5 days of culture, N2a cells treated with RA (see photos **b** and **c**) are characterized by an increased number of mitochondria, display stronger substrate adherent and a flattened morphology, with an increase in cell body size and neurite extension occurring around the soma

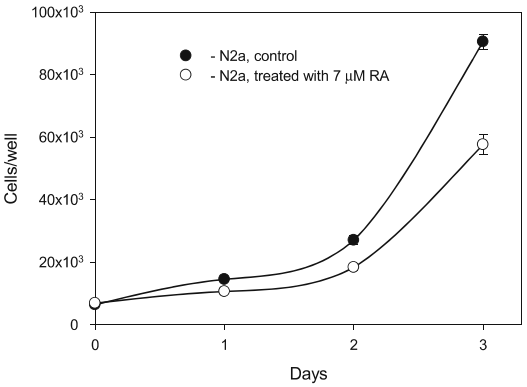

Fig. 2 Effect of all-trans-retinoic acid (RA) on proliferation of N2a cells; cell count was performed by MTT assay at 1, 2 and 3 days of culture. RA-treated N2a cells show decay in their proliferative activity comparing to control N2a cells. Bars are SEM

respiration was found to be inhibited in undifferentiated and dN2a cells by addition of rotenone, this was a characteristic feature of cells with active Complex-I (Fig. 4). The next step was to measure the Complex–II dependent respiration which was achieved by addition of its substrate, succinate (final concentration, 10 mM). The obtained results showed that in normal neurons as well as in the NB cells, the Complex-I was suppressed in comparison with Complex-II. This finding is in good accordance with our previous data on HL-1 tumor cells where the mitochondrial Complex-II is more effective in comparison with Complex-I (Monge et al. 2009). Inhibition of Complex-III with antimycin A (it suppresses the electron flow from Complex-III to cytochrome-c) was followed by measurement of Complex-IV dependent respiration. Experiments with antimycin showed that Complex-III might be functionally active not only in normal neurons, but also in undifferentiated NB cells (Fig. 4). In order to activate Complex-IV, 1 mM TMPD with 5 mM ascorbate was added which resulted in an approximately 3-fold activation of respiration for both cell types (Fig. 4). The cytochrome-c (at 8 μM) exerted only a minor effect on the TMPD-ascorbate activated respiration, suggesting thereby that the applied permeabilization procedure did not injure the intactness of the external mitochondrial membrane in these cells.

Table 1 Enzymatic activities in undifferentiated (uN2a) and all-trans-retinoic acid differentiated (dN2a) N2a cells

Enzyme activities, mU per 1×10^6 cells	uN2a cells, mean±SE, $n=5$	dN2a cells, mean±SE, $n=5$	P-values
Acetylcholinesterase	2.52±0.52	4.62±0.27	< 0.02
Hexokinase	19.57±1.86	32.15±3.73	< 0.05
Creatine kinase	7.59±0.89	11.78±0.59	< 0.001
Adenylate kinase	121.5±9.4	151.1±8.5	< 0.05

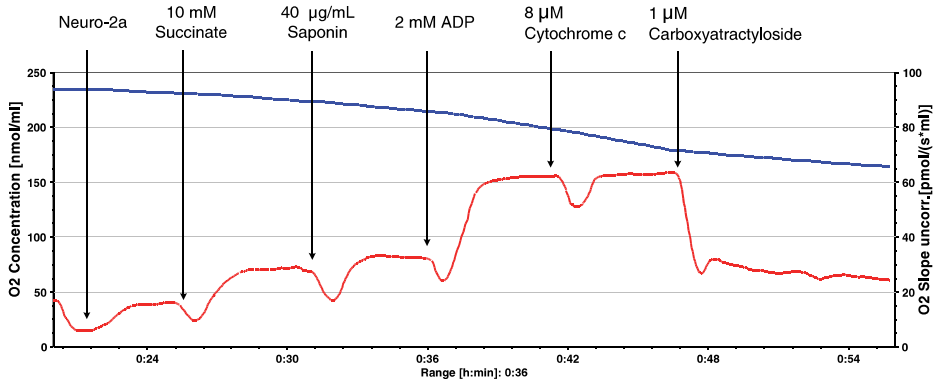

Fig. 3 Quality test for intactness of mitochondrial membranes in permeabilized N2a cells. This experiment was performed in medium-B with 5 mM glutamate, 2 mM malate, and 10 mM succinate as respiratory substrates. Blue traces represent the O_2 concentration and red traces the respiration rates. Respiration was activated with 2 mM ADP; addition of 8 µM cytochrome-c did not cause any marked increase in the rate of oxygen consumption, indicating the intactness of outer mitochondrial membrane. Addition of 1 µM carboxyatractyloside decreased respiration rate back to the basal respiration rate (V_o) level showing the intactness of mitochondrial inner membrane

Coupling of OXPHOS with HK reactions

Our studies confirm, that NB cells have, in comparison with normal differentiated neural cells, decreased activity of OXPHOS. The maximal respiration rate (V_{max}, in the presence of 2 mM ADP) of RA-treated N2a cells exceeds nearly 2 times that measured for undifferentiated cells (Fig. 6a). In cancer cells HK-2 locates predominantly on the outer mitochondrial membrane binding with VDAC. This binding increases HK affinity for ATP which generated via OXPHOS (Pedersen 2008). In this work we investigated the interaction of mitochondrial-associated HK with OXPHOS in NB cells (ADP trapping PK/PEP system was used to exclude involvement of cytoplasmic HK isoforms). We measured the total HK activity in undifferentiated and dN2A cells and performed oxygraphic analysis of the coupling of mitochondria-associated HK with OXPHOS for both types of cells. The total HK activity of dN2a cells was assayed as 32.15± 3.73 nmol glucose/min/10^6 cells; in undifferentiated cells this value was approximately 40 % lower (19.57±1.86 nmol glucose/min/10^6 cells, Table 1). The revealed stimulatory effects of 10 mM glucose on mitochondrial respiration in both undifferentiated and RA-treated N2a cells confirmed the considerable role of HK-2 in energy metabolism of NB cells (Fig. 5). Our results (Fig. 5) are in a good agreement with the previous work (Xun et al. 2012) which reports that RA improves the function of mitochondria in NB cells, but also increases glycolysis (raised HK activity was found in Table 1). In spite of the fact that the aerobic glycolysis in uN2a cells has an important role in the ATP production as compared to that of differentiated cells which have high activity of OXPHOS (Fig. 5).

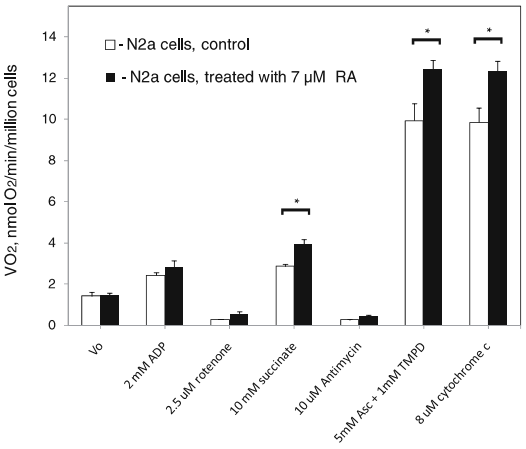

Fig. 4 The activities of the respiratory chain complexes in undifferentiated and all-trans-retinoic acid (RA) differentiated N2a cells. The experiment was performed in medium-B with 5 mM glutamate and 2 mM malate as respiratory substrates. TMPD - N,N,N′,N′-tetramethyl-p-phenylenediamine, Asc - ascorbic acid. Bars are SEM. * indicates a statistical significant difference between the mean values; $p<0.05$

Coupling of OXPHOS with creatine and adenylate kinase systems, K_m values for exogenously added AMP and ADP

Precise coupling of spatially separated intracellular ATP-producing and ATP-consuming processes is fundamental to the bioenergetics of living organisms, ensuring a fail-safe operation of the energetic system over a broad range of cellular functional activities. For this purpose neural cells, like CM, skeletal muscles, and many other cells, exert AK and CK systems (Dzeja & Terzic 2009; Dzeja & Terzic 2003; Saks

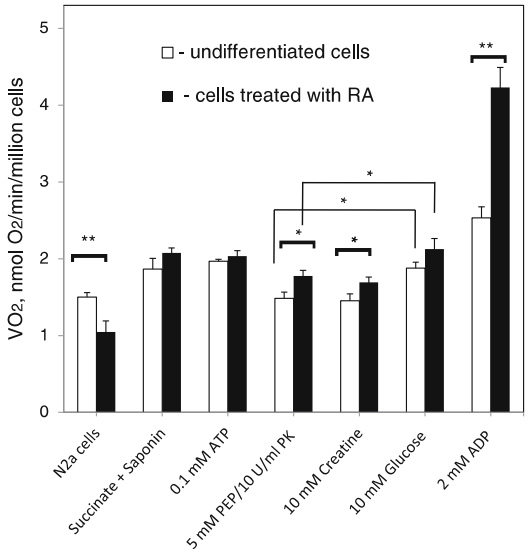

Fig. 5 Effects of exogenously added creatine and glucose on the rate of oxygen consumption by permeabilized RA-treated N2a cells and undifferentiated N2a cells. The creatine (10 mM) and glucose (10 mM) influence on mitochondrial respiration were estimated in the presence of pyruvate kinase/phosphoenolpyruvate ADP trapping system and exogenously added 0.1 mM MgATP. Finally, 2 mM ADP was added for maximal activation of respiration. The used reagents were added to N2a cells successively as shown on the x-axis. Bars are SEM. * and ** indicate a statistical significant difference between the mean values; $p < 0.05$ and $p < 0.01$, respectively

et al. 2003). In cells with high and fluctuating energy requirements, like neurons and muscle cells, ubiquitous mitochondrial creatine kinase (uMtCK) plays a key role in the maintenance of energy homeostasis by coupling of cytoplasmic phosphotransfer reactions with OXPHOS (Dzeja & Terzic 2003; Saks et al. 2003). Recent studies have shown that uMtCK is downregulated in some human cancers (Kaambre et al. 2012a; Patra et al. 2012), but little is known about its expression and functioning in NBs.

Our experiments showed that addition of creatine (up to 10 mM, in the presence of a PK-PEP system and exogenously added 0.1 mM MgATP) had no effect on the rate of O_2 consumption by uN2a as well as RA-treated cells (Fig. 5). These findings indicate that uMtCK plays a minor role in the generation of PCr and energy transfer processes in NB cells. This finding is in a good accordance with our previous results obtained in HL-1 tumor cells (Monge et al. 2009; Seppet et al. 2006). Thus, the measured CK activity in N2a cells can be largely ascribed to CK-BB or perhaps even CK-MM isoform (Table 1).

We found that N2a cells possess AK activity. The total AK activity of uN2a cells is found to be 20 % lower in comparison with RA-treated cells (Table 1). Further studies showed that AMP in the presence of a PK-PEP system could increase the rate of O_2 consumption in permeabilized N2a cells. We measured the apparent K_m value for exogenously added AMP (K_m^{AMP}) for both types of cells. The K_m^{AMP} value for RA-treated N2a cells (175±30 μM) was practically equal to the value found for adult rat cardiomyocytes (175±31 μM) but almost two times less than that for uN2a cells (79±9 μM) (Fig. 6b). These findings confirmed that in undifferentiated NB cells the permeability of the mitochondrial outer membrane (MOM) for exogenously added AMP is increased. Additional studies are needed to uncover possible mechanisms of the phenomenon.

The kinetics of regulation of mitochondrial respiration by exogenously added ADP was also studied in undifferentiated and dN2a cells. Titration curves of the corresponding respiratory experiments are shown in Fig. 6a. In uN2A cells and dN2a cells, the apparent K_m values for ADP were measured as 20.3±1.4 μM and 19.4±3.2 μM, respectively ($n = 5$). The obtained K_m value for exogenously added ADP in uN2a cells was found to be close to the values observed for murine tumor cells of the line HL-1 (47±15 μM) and for isolated mitochondria (in the range of 10–20 μM) (Monge et al. 2008). For permeabilized rat brain synaptosomes the K_m value was remarkably higher - 110±11 μM (Monge et al. 2008).

Metabolic control analysis of respiration regulation in malignant and differentiated N2a cells

The tool of MCA makes it possible to identify the key regulatory complexes of the energy metabolism pathways and to find the best targets for effective antineoplastic treatment (Moreno-Sanchez et al. 2010; Moreno-Sanchez et al. 2008). In undifferentiated and RA-treated N2a cells mitochondrial respiration was activated by exogenously added ADP (final concentration, 2 mM) due to the absence of uMtCK activity.

Figure 7 shows representative traces of O_2 consumption by permeabilized uN2a cells treated with increasing concentrations of 3-NP, an inhibitor of Complex-II (Huang et al. 2006). The measurements were carried out in a steady state conditions. Similar titration curves were obtained for other inhibitors of the mitochondrial ETC and ATP synthasome complexes. They were plotted as relative rates of O_2 consumption (VO_2, J/J_o) *versus* a concentration of used inhibitors (Fig. 8). From these plots, corresponding FCCs, also presented in Table 2, were calculated. The FCCs for undifferentiated and RA-treated N2a cells were determined by the graphical method by Fell (Fell 1997) as described in our previous work (Tepp et al. 2010).

MCA showed that in uN2a cells the key sites of OXPHOS were Complex-I (FCC=1.11), Complex-II (FCC=0.99) and IV (FCC=0.92), whereas FCCs for other mitochondrial complexes were found to be equally and significantly lower (Table 2). FCCs for Complex-I, II and IV have high and close

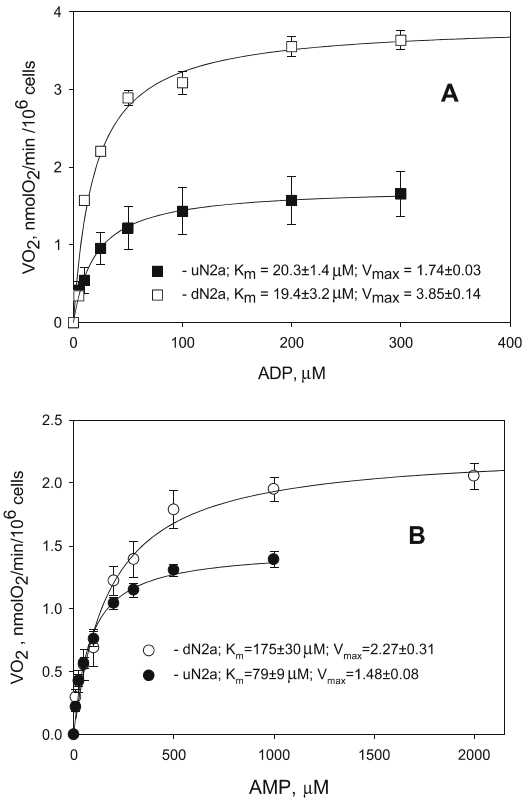

Fig. 6 The dependence of the respiration rate of undifferentiated and RA-differentiated N2a cells (uN2a and dN2a, respectively) on the extracellular ADP (**a**) or AMP (**b**) concentrations. Respiratory substrates served: 5 mM glutamate, 2 mM malate and 10 mM succinate. In the case of AMP activated respiration, rates of O_2 consumption were measured in the presence of 2 mM ATP and pyruvate kinase/phosphoenolpyruvate system. From the presented plots corresponding apparent K_m values for exogenously added ADP and AMP were calculated. Bars are SEM

values in uN2a cells, showing that these complexes may work as one unit in these cells. In dN2a cells, the key points for metabolic control were found to be Complex-II (FCC=1.34) and IV (FCC=0.99); at the same time, the FCC for Complex-I is significantly lower, 0.38 *versus* 1.11 in uN2a cells. Our data indicates that there are considerable differences between uN2a and dN2a cells in FCCs for Complex-II, PIC and ANT (Table 2). In dN2a cells, PIC and ATP/ADP carrier exert lower control over the OXPHOS than in undifferentiated NB cells. It was also shown that the FCC for PIC in uN2a cells exceeded 7 times that of what was measured in oxidative muscle cells (rat cardiomyocytes, FCC=0.064±0.04) (Table 2). Thus, our results confirm that in mitochondria of malignant NB cells these ATP synthasome complexes have more control in regulation of OXPHOS as compared to normal fully differentiated N2a cells or cardiomyocytes.

The sum of FCCs calculated for uN2a cells (5.06) was found to exceed significantly the theoretic value for linear systems (close to 1) (Kholodenko & Westerhoff 1993) and was higher than for RA-treated cells (Σ=3.93) and normal rat CMs (Σ=1.93) (Table 2). It can be concluded that the role of respiratory chain and ATP synthasome complexes in energy metabolism of NB cells may differ considerably from that in normal neuronal cells. The some explanation of MCA theory about the increased sum of the FCCs may be due to the existence of direct channeling of a substrates between the protein complexes or formation of supercomplexes (Kholodenko & Westerhoff 1993). Structurally tight alignment of the complexes of respiratory chain could be one of the ways for malignant cells to avoid apoptosis, as the cytochrome-c, which should be released during the apoptosis process, is tied in the supercomplex.

Discussion

In order to quantitatively characterize some energy fluxes and the functional capacity of mitochondria in NB cells we applied the techniques of permeabilized cell and MCA. In our studies, RA-treated N2a cells served as an *in vitro* model of normal neural cells. A set of experiments demonstrated that the treatment of uN2a cells with 7 μM RA induced the formation of a cell population, where morphological (cell size, neurite organization, substrate attachment, number of mitochondria) and biochemical characteristics (AChE activity) as well as growth rates were very similar to mature fully-differentiated sympathetic neurons (see Figs. 1 and 2, Table 1).

According to established ideas, human high-risk NB is considered to be a highly-glycolytic tumor of the Warburg phenotype (Almeida et al. 2010; Beemer et al. 1984; Feichtinger et al. 2010; Krieger-Hinck et al. 2006; Levy et al. 2012). Our results suggest that in human NB cells high levels of HK-1 or HK-2 expression could be associated with binding either one or both isoforms to VDAC in the MOM. VDAC and ANT move the synthesized ATP to the active sites of HK-2. The interaction of ATP synthase, ANT, VDAC and HK results in a rapid and very efficient production of glucose-6-phosphate (Mathupala et al. 2006), a precursor for other glycolytic steps and key biosynthetic metabolites via the pentose phosphate pathway and Krebs acid cycle. The mechanism of the Warburg´s effect proposed by Pedersen and colleagues (Pedersen 2008) could partly explain high rates of aerobic glycolysis in human NBs. The mitochondrially-bound HK-2 can also provide the elevated resistance of NB cells to apoptotic stimuli. It was discovered that HK-2, a key glycolytic enzyme, played also a role in the regulation of the mitochondria-initiated apoptosis in cancer cells (Chen et al. 2009). Our results support the view (Levy et al. 2012; Matsushita et al. 2012) that in NB cells there are reprogramming of energy metabolism towards aerobic glycolysis.

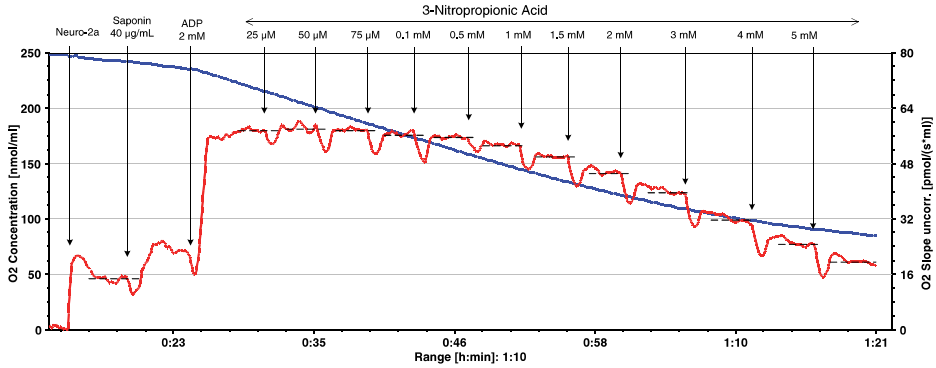

Fig. 7 Representative trace of change in the rate of oxygen consumption by undifferentiated N2a cells during the titration of the reaction medium with increasing concentrations of 3-nitropropionic acid (an inhibitor of complex-II) and in the presence of 10 mM succinate. Steady-state respiration rates are shown by dashed lines

We established that murine N2a cells (Table 1), like human NB cells (Ishiguro et al. 1990), had relatively low CK activity. Our data demonstrates that the MtCK-mediated energy transfer system is not functional in the N2a cells (Fig. 5). The downregulation of uMtCK was also monitored in malignancies of another histological type, such as human breast cancer (Kaambre et al. 2012a), colon adenocarcinoma, and sarcoma cells (Patra et al. 2008; Patra et al. 2012). Since uMtCK is

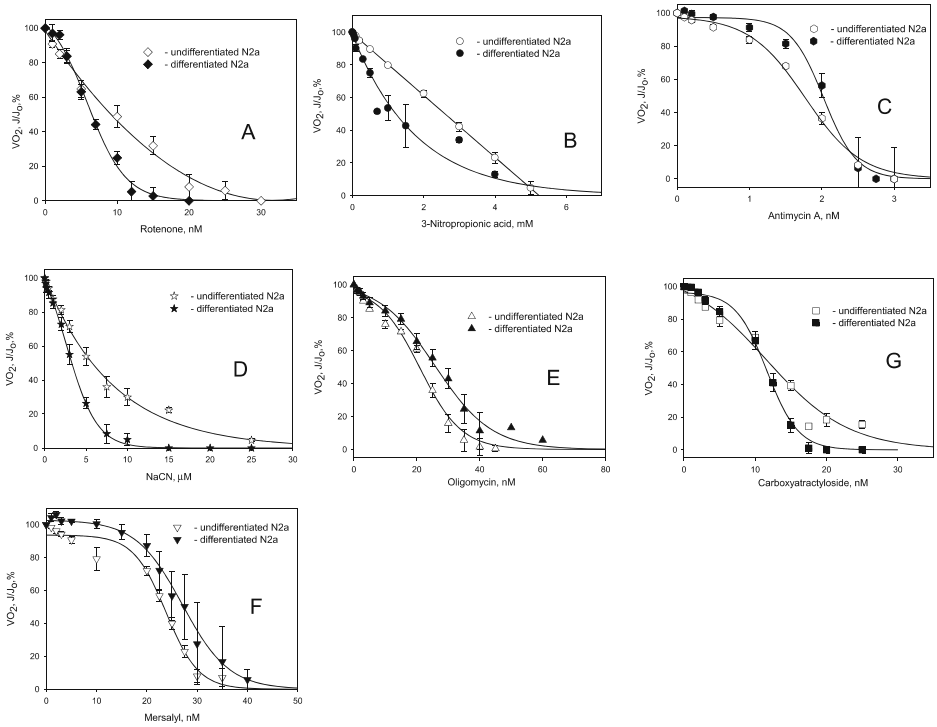

Fig. 8 Titration curves of inhibition of respiratory complexes in permeabilized N2a cells (both undifferentiated and differentiated) with: **a** rotenone, in the absence of succinate; **b** 3-nitropropionic acid, in the presence of 10 mM succinate as a respiratory substrate (without malate and glutamate; **c** antimycin-A; **d** NaCN; **e** oligomycin; **f** mersalyl; and **g** carboxyatractyloside (respiratory substrates 2 mM malate and 5 mM glutamate). Bars are SEM

Table 2 Flux control coefficients (FCC) of respiratory chain complexes for undifferentiated (uN2a) and differentiated (dN2a) by retinoic acid N2a cells as well as isolated rat cardiomyocytes determined upon direct activation of respiration with 2 mM ADP

Complexes	Inhibitors used	uN2a cells, FCC±SEM	dN2a cells, FCC±SEM	Cardiomyocytes, FCC±SEM (Tepp et al. 2011)
Complex-I	Rotenone	1.11±0.32	0.38±0.21	0.20±0.04
Complex-II	3-nitropropionic acid	0.99±0.07	1.34±0.18	0.60±0.06
Complex-III	Antimycin	0.41±0.07	0.36±0.11	0.41±0.08
Complex-IV	NaCN	0.92±0.09	0.99±0.43	0.39±0.09
ATP/ADP carrier	Carboxyatractyloside	0.61±0.17	0.28±0.16	0.20±0.05
ATP synthase	Oligomycin	0.59±0.06	0.55 ±0.26	0.065±0.01
P_i carrier	Mersalyl	0.44±0.08	0.03±0.08	0.064±0.04
Sum	–	5.06	3.93	1.93

downregulated in N2a cells, it could be assumed that the revealed CK activity in the cells can be mediated by CK-BB. Our data suggests (Table 1) that the CK-BB activity in undifferentiated NBs may be about 40 % lower as compared with normal differentiated neurons of the SNS with higher energy needs. Despite the contention that CK-MM is the muscle specific protein, a few research groups have reported that the human brain tissue contains significant amounts of the CK-MM isotype (Hamburg et al. 1990; Tsung 1976). The precise expression of CK isoforms and clarification of the mechanisms of their involvement in energy metabolism of neuronal tumor cells need definitely further clarification.

It is well-known that AK-catalyzed phosphotransfer plays one of the key roles in the maintenance of energy homeostasis in fully differentiated cells with a high-energy demand, particularly, in neural cells (Ames 2000). Currently there is only very limited information about the function of AK system in human NBs. There are some indications in literature that NB cells can express AK1 (de Bruin et al. 2004). In neurons, AK isozymes have a distinct intracellular distribution and preferred substrate. AK1 is a cytosolic enzyme for which ATP is the substrate. AK2 catalyzes the same reaction in the mitochondrial intermembrane space. AK3 is present in the mitochondrial matrix and prefers GTP over ATP as the substrate. Cytoplasmic AK1 participates in energy metabolism when local high energy phosphate levels are low, by sustaining ATP levels at the expense of ADP (Dzeja & Terzic 2003). Previous studies showed that NB cells could express, like normal neurons, the cytoplasmic AK1 and plasma membrane-bound AK1β (de Bruin et al. 2004; Neary et al. 1996). In our experiments, the addition of AMP to permeabilized N2a cells in the presence of a PEP-PK external ADP trapping system and 2 mM ATP led to a strong activation of mitochondrial respiration suggesting the presence of AK2 (Fig. 6b). These results indicate that in NB cells the mitochondrial isoform AK2 may be one of the main ATP producers. But more detailed studies with the use of human material are needed to confirm the assumption. The total AK activity of uN2a cells was slightly (~ 20 %) decreased in comparison with RA-treated cells. In parallel, the apparent K_m value for exogenous AMP in the regulation of respiration in uN2a was much lower (79±9 μM) than for RA treated N2a cells (175±30 μM). These results are taken to show that in RA treated N2a cells the permeability of MOM for AMP is low and possibly controlled by some cytoplasmic proteins. The cellular mechanism of regulation of respiration seems to be similar to CK mediated processes.

In NB cells, the permeability of MOM for adenine nucleotides is significantly higher than in normal tissues of oxidative type. In contrast to K_m values for AMP, the K_m^{app} values of MgADP for undifferentiated and dN2a cells were determined as 20.3 and 19.4 μM, i.e. nearly the same as that for isolated muscle mitochondria (in the range of 10–20 μM) (Monge et al. 2008). In NB cells, the kinetics of regulation of respiration by ADP is changed substantially as compared to normal tissues of oxidative type; for rat cardiac and *m. soleus* the apparent K_m value was found to be in the range of 300–400 μM (Anmann et al. 2006; Seppet et al. 2001). These striking differences in metabolic regulation of respiration could be mediated by a decreased expression of some cytoskeletal proteins. To find out the candidate protein(s) for this regulation in NB cells thorough proteomics studies are needed.

Our studies strongly suggest that NB is not an exclusively glycolytic tumor. Indeed, we found that uN2a cells contain a considerable number of mitochondria (Fig. 1) and displayed the ADP-stimulated respiration suggesting that the OXPHOS system played a notable role in the production of ATP in NB cells. However, we showed that all respiration rates of uN2a as well as the activity of Complex-II in the cells were significantly lower in comparison with RA-treated cells. This suggested that mitochondria in NB cells had a decreased bioenergetic capacity. It is in a good agreement with recent studies performed in other laboratories. Mutations in genes encoding the subunits of the mitochondrial SDH complex were shown in NBs (Cascon et al. 2008; Schimke et al. 2010) as well as in related tumors, such as pheochromocytomas (Astuti et al. 2003; Astuti et al. 2004; Bardella et al. 2011). Moreover, it was reported (Feichtinger et al. 2010) that in human NBs, in comparison with normal adjacent tissue, practically all components of the mitochondrial ETC and the activity of ATP synthase were downregulated; this was attributed to a strong decrease in the number of mitochondrial DNA copies in NB cells.

Our MCA studies were carried out on Complex I, II, III, IV, ATP synthase, ANT and PIC. In uN2a cells, all the flux control coefficients were found to have extremely high values. The reason for these high FCCs is clearly not diffusion restrictions, because the concentration ranges for inhibitors does not differ from those that are used for isolated cardiomyocytes (Table 2). MCA showed that in uN2A cells the key sites in the regulation of OXPHOS were Complex-I (FCC=1.11), Complex-II (FCC=0.99) and IV (FCC=0.92), whereas in dN2a cells these were Complex-II and IV (Table 2). Results obtained for the P_i and adenine nucleotide carriers showed that there were fundamental differences in the control of OXPHOS in undifferentiated and dN2a cells. Indeed, in dN2a cells, these carriers exert much lower control than in undifferentiated cells (Table 2). Our MCA studies suggested that in NB cells the ANT could be upregulated, as compared to normal cells with high OXPHOS rates; for uN2a, dN2a cells and rat CMs the corresponding FCC values were assayed as 0.61, 0.28 and 0.2, respectively (Table 2). One possible explanation for these differences may be the different profiles of ANT isoforms expressions (Dolce et al. 2005). Each ANT isoform possesses a specific expression depending on cell type, nature of tissue, developmental stage and status of cell proliferation. ANT1 is known to be highly expressed in differentiated tissues such as skeletal muscle, heart and brain (Stepien et al. 1992). Unlike the ANT1 and ANT3 isoforms, ANT2 was found to be strongly over-expressed in many types of human cancers (Chevrollier et al. 2005; Chevrollier et al. 2011; Le Bras et al. 2006). In hypoxic conditions, NB cells could maintain the integrity of their mitochondria and effective survival due to over-expression of ANT2. Unlike the ANT1 and ANT3 isoforms, ANT2 is not pro-apoptotic and may therefore contribute to carcinogenesis (Zamora et al. 2004). Since the expression of ANT2 is closely linked to the mitochondrial bioenergetics of tumors, it should be taken into account for individualizing cancer treatments and for the further development of anticancer strategies.

The sum of the FCCs over the entire ATP synthasome and ETC complexes for uN2a was found to be around 5, and this value exceeded remarkably that for RA treated cells (3.93) and some normal tissues of oxidative type – adult rat cardiomyocytes (1.93) (Table 2). Very high FCCs value (with $\Sigma \approx 4$) was also showed in MCA studies performed on human breast cancer fibers (Kaambre et al. 2012a). According to Lenaz and Genova (Lenaz & Genova 2010) a sum of FCCs exceeding one may indicate the presence of supramolecular associations of the respiratory chain complexes that was confirmed by electron microscopy, native gel electrophoresis and single particle image processing (Lenaz & Genova 2009; Lenaz & Genova 2010). We assume that the theory of supercomplexes is one of the possible explanations for high sum of FCCs for NB cells, but more detailed studies are needed to support this hypothesis. It is possible that the presence of respirasomes is the common feature of malignant cells. Several functions for the formation of respiratory supercomplexes have been suggested: channeling of ubiquinol and cytochrome c, avoiding competition from other enzymes; catalytic enhancement from reduced diffusion times of substrates and prevention of the generation of superoxide (Hoefs et al. 2012). The formation of supercomplexes can give an explanation for increased resistance of malignancies to apoptotic stimuli via suppressing the cytochrome-c, a pro-apoptotic factor, release into the cytosol.

Conclusion

Our data supports the opinion that human NB is a tumor with downregulated OXPHOS. But, it should not be regarded as a neoplasm of purely glycolytic type; we found that uN2a cells displayed significant rates of O_2 uptake, and could produce a substantial part of their ATP via OXPHOS. The complex-II of the mitochondrial ETC was found to be downregulated in NB cells as compared to differentiated neural cells. This finding indicates that in NB cells silencing of Complex-II by appropriate agents could provide a selective tumoricidal effect. Our studies suggest that in NB cells, HK, AK, Pi carrier, ANT and Complex-I could play an important role in the regulation of mitochondrial respiration. In NB cells, some of them may be possible targets for chemotherapeutic treatment. Nevertheless, further study is needed to clarify the precise metabolic profile of human NB cells and the regulation of energy fluxes.

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Interest conflicts The authors declare no conflict of interest.

References

Almeida A, Bolaños JP, Moncada S (2010) E3 ubiquitin ligase APC/C-Cdh1 accounts for the Warburg effect by linking glycolysis to cell proliferation. Proc Natl Acad Sci 107(2):738–741. doi:10.1073/pnas.0913668107

Ames A (2000) CNS energy metabolism as related to function. Brain Res Rev 34(1–2):42–68. doi:10.1016/S0165-0173(00)00038-2

Anmann T, Guzun R, Beraud N, Pelloux S, Kuznetsov AV, Kogerman L et al (2006) 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 1757(12):1597–1606. doi:10.1016/j.bbabio.2006.09.008

Apte SP, Sarangarajan R (2008) Cellular respiration and carcinogenesis. Springer, New York

Astuti D, Hart-Holden N, Latif F, Lalloo F, Black GC, Lim C et al (2003) Genetic analysis of mitochondrial complex II subunits SDHD,

SDHB and SDHC in paraganglioma and phaeochromocytoma susceptibility. Clinical Endocrinology 59(6):728–733. doi:10.1046/j.1365-2265.2003.01914.x

Astuti D, Morris M, Krona C, Abel F, Gentle D, Martinsson T et al (2004) Investigation of the role of SDHB inactivation in sporadic phaeochromocytoma and neuroblastoma. Br J Cancer 91(10):1835–1841

Atsumi T, Chesney J, Metz C, Leng L, Donnelly S, Makita Z et al (2002) High expression of inducible 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (iPFK-2; PFKFB3) in human cancers. Cancer Res 62(20):5881–5887

Bardella C, Pollard PJ, Tomlinson I (2011) SDH mutations in cancer. Biochim Biophys Acta (BBA) Bioenerg 1807(11):1432–1443. doi:10.1016/j.bbabio.2011.07.003

Beemer FA, Vlug AM, Rousseau-Merck MF, van Veelen CW, Rijksen G, Staal GE (1984) Glycolytic enzymes from human neuroectodermal tumors of childhood. Eur J Cancer Clin Oncol 20(2):253–259

Biswas S, Ray M, Misra S, Dutta DP, Ray S (1997) Selective inhibition of mitochondrial respiration and glycolysis in human leukaemic leucocytes by methylglyoxal. Biochem J 323(Pt 2):343–348

Blanco V, Lopez Camelo J, Carri NG (2001) Growth inhibition, morphological differentiation and stimulation of survival in neuronal cell type (Neuro-2a) treated with trophic molecules. Cell Biol Int 25(9):909–917

Bonora E, Porcelli AM, Gasparre G, Biondi A, Ghelli A, Carelli V et al (2006) Defective oxidative phosphorylation in thyroid oncocytic carcinoma is associated with pathogenic mitochondrial DNA mutations affecting complexes I and III. Cancer Res 66(12):6087–6096. doi:10.1158/0008-5472.CAN-06-0171

Bustamante E, Pedersen PL (1977) High aerobic glycolysis of rat hepatoma cells in culture: Role of mitochondrial hexokinase. Proc Natl Acad Sci 74(9):3735–3739

Bustamante E, Pedersen PL (1980) Mitochondrial hexokinase of rat hepatoma cells in culture: solubilization and kinetic properties. Biochemistry 19(22):4972–4977

Cascon A, Landa I, Lopez-Jimenez E, Diez-Hernandez A, Buchta M, Montero-Conde C et al (2008) Molecular characterisation of a common SDHB deletion in paraganglioma patients. J Med Genet 45(4):233–238. doi:10.1136/jmg.2007.054965

Chen Z, Zhang H, Lu W, Huang P (2009) Role of mitochondria-associated hexokinase II in cancer cell death induced by 3-bromopyruvate. Biochim Biophys Acta (BBA) Bioenerg 1787(5):553–560. doi:10.1016/j.bbabio.2009.03.003

Chevrollier A, Loiseau D, Chabi B, Renier G, Douay O, Malthiery Y et al (2005) ANT2 isoform required for cancer cell glycolysis. J Bioenerg Biomembr 37(5):307–316. doi:10.1007/s10863-005-8642-5

Chevrollier A, Loiseau D, Reynier P, Stepien G (2011) Adenine nucleotide translocase 2 is a key mitochondrial protein in cancer metabolism. Biochim Biophys Acta (BBA) Bioenerg 1807(6):562–567. doi:10.1016/j.bbabio.2010.10.008

Chuang J-H, Chou M-H, Tai M-H, Lin T-K, Liou C-W, Chen T et al (2013) 2-Deoxyglucose treatment complements the cisplatin- or BH3-only mimetic-induced suppression of neuroblastoma cell growth. Int J Biochem Cell Biol 45(5):944–951. doi:10.1016/j.biocel.2013.01.019

de Bruin W, Oerlemans F, Wieringa B (2004) Adenylate kinase I does not affect cellular growth characteristics under normal and metabolic stress conditions. Experimental Cell Research 297(1):97–107. doi:10.1016/j.yexcr.2004.02.025

Deubzer B, Mayer F, Kuci Z, Niewisch M, Merkel G, Handgretinger R et al (2010) H(2)O(2)-mediated cytotoxicity of pharmacologic ascorbate concentrations to neuroblastoma cells: potential role of lactate and ferritin. Cell Physiol Biochem 25(6):767–774. doi:10.1159/000315098

Dolce V, Scarcia P, Iacopetta D, Palmieri F (2005) A fourth ADP/ATP carrier isoform in man: identification, bacterial expression, functional characterization and tissue distribution. FEBS Lett 579(3):633–637. doi:10.1016/j.febslet.2004.12.034

Dzeja PP, Terzic A (2003) Phosphotransfer networks and cellular energetics. J Exp Biol 206(Pt 12):2039–2047

Dzeja P, Terzic A (2009) Adenylate kinase and AMP signaling networks: Metabolic monitoring, signal communication and body energy sensing. Int J Mol Sci 10(4):1729–1772. doi:10.3390/ijms10041729

Dzeja PP, Vitkevicius KT, Redfield MM, Burnett JC, Terzic A (1999) Adenylate kinase-catalyzed phosphotransfer in the myocardium : increased contribution in heart failure. Circ Res 84(10):1137–1143

Ellman GL, Courtney KD, Andres V Jr, Feather-Stone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95

Feichtinger R, Zimmermann F, Mayr J, Neureiter D, Hauser-Kronberger C, Schilling F et al (2010) Low aerobic mitochondrial energy metabolism in poorly- or undifferentiated neuroblastoma. BMC Cancer 10(1):149

Fell D (1997) Understanding the control of metabolism. Portland Press, London

Fell D (2005) Metabolic Control Analysis. In (pp. 69–80).

Gatenby RA, Gillies RJ (2004) Why do cancers have high aerobic glycolysis? Nat Rev Cancer 4(11):891–899. doi:10.1038/nrc1478

Geschwind JF, Georgiades CS, Ko YH, Pedersen PL (2004) Recently elucidated energy catabolism pathways provide opportunities for novel treatments in hepatocellular carcinoma. Expert Rev Anticancer Ther 4(3):449–457. doi:10.1586/14737140.4.3.449

Gogvadze V, Orrenius S, Zhivotovsky B (2009) Mitochondria as targets for cancer chemotherapy. Seminars in Cancer Biology 19(1):57–66. doi:10.1016/j.semcancer.2008.11.007

Groen AK, Wanders RJ, Westerhoff HV, van der Meer R, Tager JM (1982) Quantification of the contribution of various steps to the control of mitochondrial respiration. J Biol Chem 257(6):2754–2757

Guzun R, Timohhina N, Tepp K, Monge C, Kaambre T, Sikk P et al (2009) Regulation of respiration controlled by mitochondrial creatine kinase in permeabilized cardiac cells in situ. Importance of system level properties. Biochim Biophys Acta 1787(9):1089–1105. doi:10.1016/j.bbabio.2009.03.024

Guzun R, Karu-Varikmaa M, Gonzalez-Granillo M, Kuznetsov AV, Michel L, Cottet-Rousselle C et al (2011) Mitochondria-cytoskeleton interaction: Distribution of beta-tubulins in cardiomyocytes and HL-1 cells. Biochim Biophys Acta 1807(4):458–469. doi:10.1016/j.bbabio.2011.01.010

Hamburg RJ, Friedman DL, Olson EN, Ma TS, Cortez MD, Goodman C et al (1990) Muscle creatine kinase isoenzyme expression in adult human brain. J Biol Chem 265(11):6403–6409

Hoefs SJG, Rodenburg RJ, Smeitink JAM, van den Heuvel LP (2012) Molecular base of biochemical complex I deficiency. Mitochondrion 12(5):520–532. doi:10.1016/j.mito.2012.07.106

Huang LS, Sun G, Cobessi D, Wang AC, Shen JT, Tung EY et al (2006) 3-nitropropionic acid is a suicide inhibitor of mitochondrial respiration that, upon oxidation by complex II, forms a covalent adduct with a catalytic base arginine in the active site of the enzyme. J Biol Chem 281(9):5965–5972. doi:10.1074/jbc.M511270200

Ihrlund LS, Hernlund E, Khan O, Shoshan MC (2008) 3-Bromopyruvate as inhibitor of tumour cell energy metabolism and chemopotentiator of platinum drugs. Mol Oncol 2(1):94–101

Ishiguro Y, Kato K, Akatsuka H, Ito T (1990) The diagnostic and prognostic value of pretreatment serum creatine kinase BB levels in patients with neuroblastoma. Cancer 65(9):2014–2019

Kaambre T, Chekulayev V, Shevchuk I, Karu-Varikmaa M, Timohhina N, Tepp K et al (2012a) Metabolic control analysis of cellular respiration in situ in intraoperational samples of human breast cancer. J Bioenerg Biomembr 44(5):539–558. doi:10.1007/s10863-012-9457-9

Kaambre T, Chekulayev V, Shevchuk I, Karu-Varikmaa M, Timohhina N, Tepp K et al (2012b) Metabolic control analysis of cellular

respiration in situ in intraoperational samples of human breast cancer. J Bioenerg Biomembr. doi:10.1007/s10863-012-9457-9
Kholodenko BN, Westerhoff HV (1993) Metabolic channelling and control of the flux. FEBS Lett 320(1):71–74
Krieger-Hinck N, Gustke H, Valentiner U, Mikecz P, Buchert R, Mester J et al (2006) Visualisation of neuroblastoma growth in a Scid mouse model using [18F]FDG and [18F]FLT-PET. Anticancer Res 26(5A): 3467–3472
Krieglstein J, Mwasekaga S (1987) Effect of methohexital on the relationship between hexokinase distribution and energy metabolism in neuroblastoma cells. Arzneimittelforschung 37(3):291–295
Krieglstein J, Schachtschabel DO, Wever K, Wickop G (1981) Influence of thiopental on intracellular distribution of hexokinase activity in various tumor cells. Arzneimittelforschung 31(1):121–123
Kuznetsov AV, Veksler V, Gellerich FN, Saks V, Margreiter R, Kunz WS (2008) Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. Nat Protoc 3(6):965–976. doi:10.1038/nprot.2008.61
Le Bras M, Borgne-Sanchez A, Touat Z, El Dein OS, Deniaud A, Maillier E et al (2006) Chemosensitization by knockdown of adenine nucleotide translocase-2. Cancer Res 66(18):9143–9152. doi:10.1158/0008-5472.CAN-05-4407
Lenaz G, Genova ML (2009) Structural and functional organization of the mitochondrial respiratory chain: a dynamic super-assembly. Int J Biochem Cell Biol 41(10):1750–1772
Lenaz G, Genova ML (2010) Structure and organization of mitochondrial respiratory complexes: a new understanding of an old subject. Antioxid Redox Signal 12(8):961–1008. doi:10.1089/ars.2009.2704
Levy A, Zage P, Akers L, Ghisoli M, Chen Z, Fang W et al (2012) The combination of the novel glycolysis inhibitor 3-BrOP and rapamycin is effective against neuroblastoma. Investigational New Drugs 30(1):191–199. doi:10.1007/s10637-010-9551-y
Lienhard GE, Secemski II (1973) P 1, P 5 -Di(adenosine-5')pentaphosphate, a potent multisubstrate inhibitor of adenylate kinase. J Biol Chem 248(3):1121–1123
Majewski N, Nogueira V, Bhaskar P, Coy PE, Skeen JE, Gottlob K et al (2004) Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak. Mol Cell 16(5):819–830. doi:10.1016/j.molcel.2004.11.014
Marin-Hernandez A, Rodriguez-Enriquez S, Vital-Gonzalez PA, Flores-Rodriguez FL, Macias-Silva M, Sosa-Garrocho M et al (2006) Determining and understanding the control of glycolysis in fast-growth tumor cells. Flux control by an over-expressed but strongly product-inhibited hexokinase. FEBS J 273(9):1975–1988. doi:10.1111/j.1742-4658.2006.05214.x
Mathupala SP, Ko YH, Pedersen PL (2006) Hexokinase II: cancer's double-edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria. Oncogene 25(34):4777–4786. doi:10.1038/sj.onc.1209603
Matsushita K, Uchida K, Saigusa S, Ide S, Hashimoto K, Koike Y et al (2012) Glycolysis inhibitors as a potential therapeutic option to treat aggressive neuroblastoma expressing GLUT1. Journal of Pediatric Surgery 47(7):1323–1330. doi:10.1016/j.jpedsurg.2011.12.007
Monge C, Beraud N, Kuznetsov A, Rostovtseva T, Sackett D, Schlattner U et al (2008) Regulation of respiration in brain mitochondria and synaptosomes: restrictions of ADP diffusion in situ, roles of tubulin, and mitochondrial creatine kinase. Mol Cell Biochem 318(1–2): 147–165. doi:10.1007/s11010-008-9865-7
Monge C, Beraud N, Tepp K, Pelloux S, Chahboun S, Kaambre T et al (2009) 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 87(4):318–326. doi:10.1139/y09-018
Moreno-Sanchez R, Rodriguez-Enriquez S, Marin-Hernandez A, Saavedra E (2007) Energy metabolism in tumor cells. FEBS J 274(6):1393–1418. doi:10.1111/j.1742-4658.2007.05686.x
Moreno-Sanchez R, Saavedra E, Rodriguez-Enriquez S, Olin-Sandoval V (2008) Metabolic control analysis: a tool for designing strategies to manipulate metabolic pathways. J Biomed Biotechnol 2008: 597913. doi:10.1155/2008/597913
Moreno-Sánchez R, Rodríguez-Enríquez S, Saavedra E, Marín-Hernández A, Gallardo-Pérez JC (2009) The bioenergetics of cancer: Is glycolysis the main ATP supplier in all tumor cells? BioFactors 35(2):209–225. doi:10.1002/biof.31
Moreno-Sanchez R, Saavedra E, Rodriguez-Enriquez S, Gallardo-Perez JC, Quezada H, Westerhoff HV (2010) Metabolic control analysis indicates a change of strategy in the treatment of cancer. Mitochondrion 10(6):626–639. doi:10.1016/j.mito.2010.06.002
Nakashima RA, Paggi MG, Scott LJ, Pedersen PL (1988) Purification and characterization of a bindable form of mitochondrial bound hexokinase from the highly glycolytic AS-30D rat hepatoma cell line. Cancer Res 48(4):913–919
Neary JT, Rathbone MP, Cattabeni F, Abbracchio MP, Burnstock G (1996) Trophic actions of extracellular nucleotides and nucleosides on glial and neuronal cells. Trends Neurosci 19(1):13–18
Patra S, Bera S, SinhaRoy S, Ghoshal S, Ray S, Basu A et al (2008) Progressive decrease of phosphocreatine, creatine and creatine kinase in skeletal muscle upon transformation to sarcoma. FEBS J 275(12):3236–3247. doi:10.1111/j.1742-4658.2008.06475.x
Patra S, Ghosh A, Roy SS, Bera S, Das M, Talukdar D et al (2012) A short review on creatine-creatine kinase system in relation to cancer and some experimental results on creatine as adjuvant in cancer therapy. Amino Acids 42(6):2319–2330. doi:10.1007/s00726-011-0974-3
Pedersen PL (1978) Tumor mitochondria and the bioenergetics of cancer cells. Prog Exp Tumor Res 22:190–274
Pedersen PL (2007a) The cancer cell's "power plants" as promising therapeutic targets: an overview. J Bioenerg Biomembr 39(1):1–12. doi:10.1007/s10863-007-9070-5
Pedersen PL (2007b) 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 39(3):211–222. doi:10.1007/s10863-007-9094-x
Pedersen PL (2008) Voltage dependent anion channels (VDACs): a brief introduction with a focus on the outer mitochondrial compartment's roles together with hexokinase-2 in the "Warburg effect" in cancer. J Bioenerg Biomembr 40(3):123–126. doi:10.1007/s10863-008-9165-7
Pedersen PL (2012) 3-bromopyruvate (3BP) a fast acting, promising, powerful, specific, and effective "small molecule" anti-cancer agent taken from labside to bedside: introduction to a special issue. J Bioenerg Biomembr 44(1):1–6. doi:10.1007/s10863-012-9425-4
Pedersen PL, Mathupala S, Rempel A, Geschwind JF, Ko YH (2002) 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 (BBA) Bioenerg 1555(1–3): 14–20. doi:10.1016/S0005-2728(02)00248-7
Puurand M, Peet N, Piirsoo A, Peetsalu M, Soplepmann J, Sirotkina M et al (2012) Deficiency of the complex I of the mitochondrial respiratory chain but improved adenylate control over succinate-dependent respiration are human gastric cancer-specific phenomena. Mol Cell Biochem 370(1–2):69–78. doi:10.1007/s11010-012-1399-3
Rostovtseva TK, Sheldon KL, Hassanzadeh E, Monge C, Saks V, Bezrukov SM et al (2008) Tubulin binding blocks mitochondrial voltage-dependent anion channel and regulates respiration. Proc Natl Acad Sci USA 105(48):18746–18751. doi:10.1073/pnas.0806303105
Saks VA, Belikova YO, Kuznetsov AV (1991) In vivo regulation of mitochondrial respiration in cardiomyocytes: specific restrictions for intracellular diffusion of ADP. Biochim Biophys Acta 1074(2): 302–311

Saks VA, Veksler VI, Kuznetsov AV, Kay L, Sikk P, Tiivel T et al (1998) Permeabilized cell and skinned fiber techniques in studies of mitochondrial function in vivo. Mol Cell Biochem 184(1–2):81–100

Saks V, Kuznetsov A, Andrienko T, Usson Y, Appaix F, Guerrero K et al (2003) Heterogeneity of ADP diffusion and regulation of respiration in cardiac cells. Biophys J 84(5):3436–3456. doi:10.1016/S0006-3495(03)70065-4

Sato C, Matsuda T, Kitajima K (2002) Neuronal Differentiation-dependent Expression of the Disialic Acid Epitope on CD166 and Its Involvement in Neurite Formation in Neuro2A Cells. J Biol Chem 277(47):45299–45305. doi:10.1074/jbc.M206046200

Schimke RN, Collins DL, Stolle CA (2010) Paraganglioma, neuroblastoma, and a SDHB mutation: Resolution of a 30-year-old mystery. Am J Med Genet A 152A(6):1531–1535. doi:10.1002/ajmg.a.33384

Seppet EK, Kaambre T, Sikk P, Tiivel T, Vija H, Tonkonogi M et al (2001) Functional complexes of mitochondria with Ca, MgATPases of myofibrils and sarcoplasmic reticulum in muscle cells. Biochim Biophys Acta 1504(2–3):379–395

Seppet EK, Eimre M, Anmann T, Seppet E, Piirsoo A, Peet N et al (2006) Structure-function relationships in the regulation of energy transfer between mitochondria and ATPases in cardiac cells. Exp Clin Cardiol 11(3):189–194

Sharma LK, Fang H, Liu J, Vartak R, Deng J, Bai Y (2011) Mitochondrial respiratory complex I dysfunction promotes tumorigenesis through ROS alteration and AKT activation. Hum Mol Genet 20(23):4605–4616. doi:10.1093/hmg/ddr395

Simonnet H, Demont J, Pfeiffer K, Guenaneche L, Bouvier R, Brandt U et al (2003) Mitochondrial complex I is deficient in renal oncocytomas. Carcinogenesis 24(9):1461–1466. doi:10.1093/carcin/bgg109

Stepien G, Torroni A, Chung AB, Hodge JA, Wallace DC (1992) Differential expression of adenine nucleotide translocator isoforms in mammalian tissues and during muscle cell differentiation. J Biol Chem 267(21):14592–14597

Tepp K, Timohhina N, Chekulayev V, Shevchuk I, Kaambre T, Saks V (2010) Metabolic control analysis of integrated energy metabolism in permeabilized cardiomyocytes - experimental study. Acta Biochim Pol 57(4):421–430

Tepp K, Shevchuk I, Chekulayev V, Timohhina N, Kuznetsov AV, Guzun R et al (2011) High efficiency of energy flux controls within mitochondrial interactosome in cardiac intracellular energetic units. Biochim Biophys Acta Bioenerg 1807(12):1549–1561. doi:10.1016/j.bbabio.2011.08.005

Tsung SH (1976) Creatine kinase isoenzyme patterns in human tissue obtained at surgery. Clin Chem 22(2):173–175

Warburg O (1956) On respiratory impairment in cancer cells. Science 124(3215):269–270

Whitaker-Menezes D, Martinez-Outschoorn UE, Lin Z, Ertel A, Flomenberg N, Witkiewicz AK et al (2011) Evidence for a stromal-epithelial "lactate shuttle" in human tumors: MCT4 is a marker of oxidative stress in cancer-associated fibroblasts. Cell Cycle 10(11):1772–1783

Xu R-h, Pelicano H, Zhou Y, Carew JS, Feng L, Bhalla KN et al (2005) Inhibition of Glycolysis in Cancer Cells: A Novel Strategy to Overcome Drug Resistance Associated with Mitochondrial Respiratory Defect and Hypoxia. Cancer Res 65(2):613–621

Xun Z, Lee DY, Lim J, Canaria CA, Barnebey A, Yanonne SM et al (2012) Retinoic acid-induced differentiation increases the rate of oxygen consumption and enhances the spare respiratory capacity of mitochondria in SH-SY5Y cells. Mech Ageing Dev 133(4):176–185. doi:10.1016/j.mad.2012.01.008

Zamora M, Granell M, Mampel T, Vinas O (2004) Adenine nucleotide translocase 3 (ANT3) overexpression induces apoptosis in cultured cells. FEBS Lett 563(1–3):155–160. doi:10.1016/S0014-5793(04)00293-5

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Metabolic control analysis of respiration in human cancer tissue

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Bioenergetic profiling of cancer cells is of great potential because it can bring forward new and effective therapeutic strategies along with early diagnosis. Metabolic Control Analysis (MCA) is a methodology that enables quantification of the flux control exerted by different enzymatic steps in a metabolic network thus assessing their contribution to the system's function. Our main goal is to demonstrate the applicability of MCA for *in situ* studies of energy metabolism in human breast and colorectal cancer cells as well as in normal tissues. We seek to determine the metabolic conditions leading to energy flux redirection in cancer cells. A main result obtained is that the adenine nucleotide translocator exhibits the highest control of respiration in human breast cancer thus becoming a prospective therapeutic target. Additionally, we present evidence suggesting the existence of mitochondrial respiratory supercomplexes that may represent a way by which cancer cells avoid apoptosis. The data obtained show that MCA applied *in situ* can be insightful in cancer cell energetic research.

Keywords: metabolic control analysis, respiratory chain, breast and colorectal cancer, Warburg effect, OXPHOS

INTRODUCTION

Oncologic diseases such as breast and colorectal cancers are still one of the main causes of premature death among people. The low efficiency of contemporary medicine in the treatment of these malignancies is largely mediated by a poor understanding of the processes involved in metastatic dissemination of cancer cells as well as the unique energetic properties of mitochondria from tumors. Current knowledge supports the idea that human breast and colorectal cancer cells exhibit increased rates of glucose consumption displaying a Warburg phenotype, i.e., elevated glycolysis even in the presence of oxygen (Warburg and Dickens, 1930; Warburg, 1956; Izuishi et al., 2012). Notwithstanding, there are some evidences that in these malignancies mitochondrial oxidative phosphorylation (OXPHOS) is the main source of ATP rather than glycolysis. Cancer cells have been classified according to their pattern of metabolic remodeling depending of the relative balance between aerobic glycolysis and OXPHOS (Bellance et al., 2012). The first type of tumor cells is highly glycolytic, the second OXPHOS deficient and the third type of tumors display enhanced OXPHOS. Recent studies strongly suggest that cancer cells can utilize lactate, free fatty acids, ketone bodies, butyrate and glutamine as key respiratory substrates eliciting metabolic remodeling of normal surrounding cells toward aerobic glycolysis—"reverse Warburg" effect (Whitaker-Menezes et al., 2011; Salem et al., 2012; Sotgia et al., 2012; Witkiewicz et al., 2012). In normal cells, the OXPHOS system is usually closely linked to phosphotransfer systems, including various creatine kinase (CK) isotypes, which ensure a safe operation of energetics over a broad functional range of cellular activities (Dzeja and Terzic, 2003). However, our current knowledge about the function of CK/creatine (Cr) system in human breast and colorectal cancer is insufficient. In some malignancies, for example sarcomas the CK/Cr system was shown to be strongly down regulated (Bera et al., 2008; Patra et al., 2008). Our previous studies showed that the mitochondrial-bound CK (MtCK) activity was significantly decreased in HL-1 tumor cells (Monge et al., 2009), as compared to normal parent cardiac cells where the OXPHOS is the main ATP source of and the CK system is a main energy carrier. In the present study, we estimated the role of MtCK in maintaining energy homeostasis in human colorectal cancer cells.

Understanding the control and regulation of energy metabolism requires analytical tools that take into account the existing interactions between individual network components and their impact on systemic network function. Metabolic Control Analysis (MCA) is a theoretical framework relating the properties of metabolic systems to the kinetic characteristics of their individual enzymatic components (Fell, 2005). An experimental approach of MCA has been already successfully applied to the studies of OXPHOS in isolated mitochondria (Tager et al., 1983; Kunz et al., 1999; Rossignol et al., 2000) and in skinned muscle fibers (Kuznetsov et al., 1997; Tepp et al., 2010).

Recent work from Moreno-Sanchez and Westerhoff's groups has applied MCA to investigate the control of glycolytic flux

and mitochondrial respiration in different types of tumor cells growing in culture. A main conclusion of these studies is that the significance of OXPHOS in bioenergetics of cancer cells should be re-evaluated and experimentally determined for each particular type of neoplasm (Marin-Hernandez et al., 2006; Moreno-Sanchez et al., 2007, 2008, 2010). These findings also indicated that MCA may be a very useful approach for studying *in situ* mitochondrial respiration and energy fluxes.

In the present work we applied MCA for *in situ* studies the energy metabolism in human cancer cells. Using oxygraphy and MCA in permeabilized human breast and colorectal cancer cells (Kuznetsov et al., 1997) we quantitatively characterized the control exerted by the different components of the respiratory chain and the ATP synthasome (Tepp et al., 2011).

MATERIALS AND METHODS

PATIENTS AND TISSUE SAMPLING

Bioenergetic proiling was performed on post-operation material derived from patients with human breast (HBC) and colorectal cancers (HCC). Thirty two patients 50–71 year-old were in the HBC group with local or locally advanced disease at pathological stage IA-IIIB (T1-4N0-2M0) and eighteen patients, 63–82 year-old in HCC group with pathological stage (T2-3 N1-M0). Tumor differentiation was into well, moderately, and poorly differentiated adenocarcinoma.

Immediately after the surgery the human samples were placed into pre-cooled Mitomedium-B solution, dissected into small fiber bundles and permeabilized with 50 μg/ml saponin (Kuznetsov et al., 2008). Control experiments showed that this procedure has no effect on the integrity of mitochondrial membranes and that the stimulatory effect on respiration by added cytochrome-c is absent (Kuznetsov et al., 2008; Kaambre et al., 2012).

HIGH-RESOLUTION RESPIROMETRY

Mitochondrial respiration of tissue samples was measured at 25°C under continuous magnetic stirring with an Oxygraph-2 k, (Oroboros Instruments, Innsbruck, Austria) 5 mM glutamate, 2 mM malate and 10 mM succinate were used as respiratory substrates. In permeabilized tumor and muscle fibers, the mitochondrial respiration was activated by exogenously added ADP. The flux control coefficients (FCC) for permeabilized human samples were determined with direct activation of respiration by ADP (state 3 respiration). The presence of MtCK in permeabilized human cancer samples was assayed as described earlier (Monge et al., 2009; Kaambre et al., 2012).

METABOLIC CONTROL ANALYSIS

By applying the principles of MCA, it is possible to quantify the degree of the control, exerted by an enzymatic or transport step through FCC. FCC is defined as the ratio of the fractional change in the steady-state flux with respect to an infinitesimal variation in the biochemical activity that caused the change in flux (Fell, 1997). In the present study FCC was assessed by stepwise titration of the respiratory activity of the system with the specific inhibitors for each step from the respiratory chain and ATP synthasome complexes. Control coefficients are determined from the initial slope of the titration curve and the ratio of inhibitor concentration at maximal flux inhibition over the uninhibited flux.

FCC-values were quantified according to a graphical method (Groen et al., 1982; Fell, 2005) modified by Small (Small and Fell, 1990) and results obtained were compared with the computer estimated coefficients (Gellerich et al., 1990; Small and Fell, 1990). Previous studies indicated that similar values can be obtained with either methods, but special attention should be paid to systems with branched pathways or direct channeling due to possible unreliable estimates (Kholodenko et al., 1993; Kholodenko and Westerhoff, 1993; Tepp et al., 2011).

RESULTS AND DISCUSSION

BIOENERGETIC PROFILING OF HUMAN CANCER AND MCA

First, we evaluated the impact of Cr, ADP, mitochondrial-bound hexokinase (HK) and CK reactions on OXPHOS in permeabilized human tumor samples. It has been proposed that in cancer cells the binding of HK-2 to the mitochondrial voltage-dependent anion channel (VDAC) mediates their Warburg behavior further suggesting that this enzyme could be used as a target for antineoplastic therapy (Pedersen, 2008). We found that affecting mitochondria-bound CK and HK only produces minor effects on mitochondrial respiration in HCC cells. Indeed, the addition of 10 mM Cr and 5 mM glucose with 0.2 mM ATP (in the presence of phosphoenolpyruvate-pyruvate kinase ADP trapping system) had no effect on the rates of oxygen consumption by these cells. Similar effects were also registered in HBC cells (Kaambre et al., 2012). From these results, it appears that MtCK does not play a significant role in HBC and HCC cells *in situ*. The role of another CK isoforms in maintaining of energy homeostasis in these cancer cells will be examined in future work. The data obtained suggest that mitochondrial, but not glycolytic ATP, plays a key role in maintaining life processes in HCC and HBC cells. In contrast to HCC cells, a marked stimulatory effect on mitochondrial respiration by glucose addition (in the presence of exogenous MgATP) was observed in saponized HL-1 tumor cells that display a glycolytic phenotype (Eimre et al., 2008; Monge et al., 2009). Furthermore, we found that adding respiratory substrates and 2 mM ADP to HCC and HBC fibers resulted in a notable increase in O2 consumption rate (**Table 1**).

Due to the absence of MtCK in HCC cells, we further analyzed OXPHOS in these cells upon direct activation of respiration with exogenous ADP. In order to evaluate the functionality of individual respiratory complexes of the electron transport chain (ETC) in HCC and HBC cells, the rates of O_2 consumption were measured after sequential addition of specific substrates and inhibitors in the following order: 2 mM ADP, 10 μM rotenone, 10 mM succinate, 10 μM antimycin A, 5 mM ascorbate with 1 mM tetramethyl-p-phenylenediamine (TMPD). We found that the addition of 2 mM ADP activates mitochondrial respiration by ∼3.2 and 3.3 times in HCC and HBC samples, respectively (**Table 1**). Our studies showed that these human malignancies have a functionally active Krebs cycle as well as the ETC. Accordingly ADP stimulated respiration of human breast and colorectal tumors was found to be strongly depressed

Table 1 | Values of basal (V_o) and maximal respiration rate (V_{max}, in the presence of 2 mM ADP) and apparent Michaelis Menten constant (K_m) for ADP in permeabilized human breast and colorectal cancer samples as well as health tissue.

Tissues	$V_o^{\#}$	V_o(succ)	K_m^{app} ADP, μM	V_{max}	RCI	Source
Colorectal cancer	1.4 ± 0.21	2.62 ± 0.34*	34.2 ± 11.1	4.51 ± 0.47*	3.2 ± 0.8	Our data
Control tissue	1.19 ± 0.17	1.61 ± 0.24	46.3 ± 15.5	2.56 ± 0.32	2.2 ± 0.6	Our data
Breast cancer	0.33 ± 0.03	0.56 ± 0.04	114.8 ± 13.6	1.09 ± 0.04	3.3 ± 0.4	Kaambre et al., 2012
Control breast tissue	0.02 ± 0.01	0.10 ± 0.02	–	–	–	Kaambre et al., 2012
Rat soleus	2.19 ± 0.30	–	354 ± 46	12.2 ± 0.5	5.6 ± 1.0	Kuznetsov et al., 1996; Monge et al., 2009
Rat gastrocnemius white	1.23 ± 0.13	–	14.4 ± 2.6	4.10 ± 0.25	3.3 ± 0.6	Kuznetsov et al., 1996; Monge et al., 2009

*# Respiration rate is expressed in nmol O_2/min/mg dry weight; V_o—in the presence of 2 mM malate and 5 mM glutamate as respiratory substrates; V_o(succ)—in the presence of 2 mM malate, 5 mM glutamate, and 10 mM succinate; RCI—respiratory control index is the ratio of V_{max} value to V_o; *$p < 0.05$ as compared to control tissue; data are expressed as mean ± standard error of the mean(SEM).*

upon addition of 10 μM rotenone (an inhibitor of Complex-I), antimycin (an inhibitor of Complex-III), 1 mM NaCN (an inhibitor of Complex-IV) and, on the contrary, it was strongly (>5 times) activated in the presence of ascorbate with TMPD, indicating the presence of active cytochrome-c-oxidase (data not shown). Apparently, the activity of Complex-II in HCC exceeds that in normal tissue, as the addition of succinate to permeabilized fibers led to a stronger stimulation of respiration than in control tissue, although it has been reported that SDHD gene expression is reduced in ∼80% colorectal cancers (Habano et al., 2003).

The results shown in **Table 1** demonstrate that the respiratory activity of breast and colorectal cancers differ significantly that of normal adjacent tissues. Both tumors exhibited respiratory rates close to tissue from rat skeletal muscles (**Table 1**). These data may indicate the presence of a "reverse Warburg" effect, which depends on the properties of the tumor microenvironment. The microenvironment (e.g., substrate availability) is a strong determinant of mitochondrial content and activity in tumors, which could play an important role in the definition of tumors bioenergetic profile (Bellance et al., 2012; Jose and Rossignol, 2013).

When we analyzed respiration as a function of exogenously added ADP, we found that mitochondria from human breast and colorectal cancer cells exhibit an increased affinity toward exogenously added ADP compared with normal oxidative type tissues. The apparent Michaelis Menten constants (K_m) for MgADP were determined as 114.8 ± 13.6 μM and 34.2 ± 11.1 μM for breast and colorectal cancer, respectively (**Table 1**). These values are significantly lower as compared to rat soleus (K_m = 354 ± 46 μM) or isolated cardiomyocytes [K_m = 360 μM (Anmann et al., 2006)], but this value is still higher than the apparent K_m for isolated mitochondria (10–20 μM). The observed difference in the metabolic regulation of respiration could be linked to a decreased expression or absence of some cytoskeletal proteins (Appaix et al., 2003; Saks et al., 2010; Guzun et al., 2012). It has been shown that in normal oxidative muscle, βII-tubulin can bind to VDAC and thereby strongly limit the permeability of mitochondrial outer membrane to adenine nucleotides (Rostovtseva et al., 2008; Guzun et al., 2011). In addition to βII-tubulin, candidate proteins are desmin, microtubule-associated proteins, other isoforms of tubulin and plectin (Appaix et al., 2003; Guzun et al., 2012).

We used MCA to quantitate the control exerted by the different ETC complexes and the ATP synthasome on the respiratory flux in human colorectal and breast carcinomas. FCCs were determined in permeabilized human fibers using the inhibitor titration method in ADP-stimulated respiration.

In HBC cells, the main rate-controlling steps of respiration were Complex IV ($FCC = 0.74$), ATP synthase ($FCC = 0.61$), and phosphate carrier ($FCC = 0.60$). The highest control was exerted by adenine nucleotide translocase (ANT), $FCC = 1.02$ (Kaambre et al., 2012). Our preliminary data for HCC showed high FCCs for: Complex-I ($FCC = 0.62$), Complex-III ($FCC = 0.73$), and Complex-IV ($FCC = 0.58$).

The FCC was calculated graphically as shown in **Figure 1** and as explained in Methods. In the case of HCC, the FCC-values for PIC was calculated as 0.52 (graphic method) (Groen et al., 1982) and 0.47 [according to the Small equation (Small, 1993)]. Although both these methods gave similar values, the use of the Small equation for calculating of FCC(s) was preferred over the graphical one because it is more robust and less subjective. Further investigations are needed to determine the FCC-values for other respiratory chain complexes in HCC and in healthy colon tissue.

In the case of HBC, the summation of the determined FCC(s) for all steps evaluated in the ATP synthasome and ETC complexes was found to be around 4. This value significantly exceeds the theoretically expected summation for linear pathways (value 1). According to Lenaz et al. (2010), a sum of FCC(s) exceeding 1 indicates the existence of supramolecular association of the respiratory complexes that was confirmed by electron microscopy, native gel electrophoresis and single particle image processing (Lenaz and Genova, 2009, 2010). Although more studies are needed to elucidate this important matter, supercomplex formation would allow to explain, at least in part, the high intrinsic resistance to apoptotic stimuli that tumor cells exhibit, namely via suppression of cytochrome-c release. The formation of respiratory supercomplexes could occur not only in HBC but also in HCC cells.

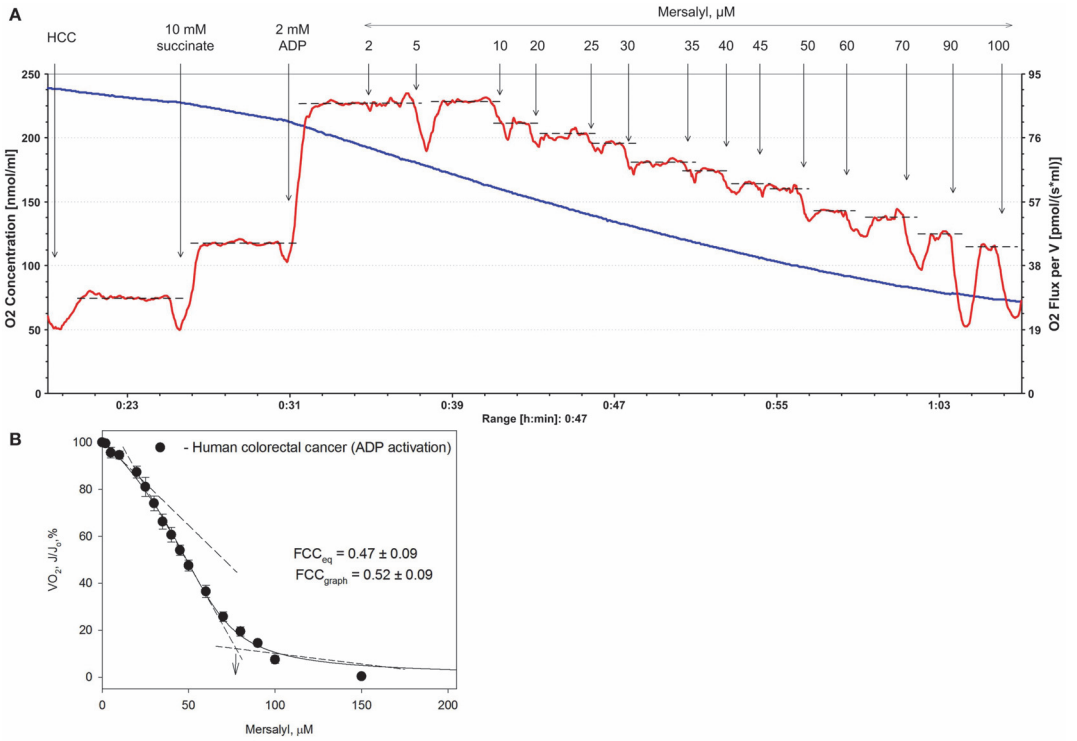

FIGURE 1 | Representative traces of change in the rate of oxygen consumption by permeabilized human colorectal cancer (HCC) fibers after their titration with increasing concentrations of mersalyl, an inhibitor of inorganic phosphate carrier (panel A). The values of respiration rate obtained were plotted vs. mersalyl concentration (panel **B**) and from the plot the corresponding flux control coefficient was calculated. Bars are ±SEM.

CONCLUSION

In this work we show that MCA can be applied to *in situ* quantitative analysis of respiration in cancerous and normal tissues obtained from small amounts of biopsy material. *In situ* studies have the advantage of preserving the cellular ultrastructure such as the cytoskeleton thus enabling the study of their role in controlling energetics in cancer cells. It is important to emphasize that the use of MCA for studying mitochondrial function *in situ* allows us to avoid changes in microenvironment that happen during the isolation procedure. Our studies were performed on cells from tissue samples isolated from patients. This may represent a limitation because recently it has been emphasized that the bioenergetic profile of tumors cells depends largely, among other factors, on the stage of tumor growth and its degree of vascularization (Moreno-Sanchez et al., 2007). Large number of studies were performed on tumor cell cultures, which exhibit a strong dependency on glycolysis, but there might be a strong impact of the artificial culture conditions on energy metabolism (Jose and Rossignol, 2013). One example of the impact of cell culture, the so-called "culture shock," modulates the activity of some genes, which possibly upregulate glycolysis (Gnaiger and Kemp, 1990; Gstraunthaler et al., 1999). This specificity underscores the importance of examining tumor cell behavior in their natural environment.

We quantified the control of respiration in two different types of human cancer cells. The main result obtained is that the ANT exerts a high flux control, implicating the role of adenine nucleotide exchange between mitochondrial and cytoplasmic compartments as a key energetic trait in cancer cells. This result may be important for cancer therapy. Possible suppression of ANT2 and/or overexpression of ANT1 and ANT3 isoforms in cancer cells may induce their death via apoptosis (Jang et al., 2008). We also show that HCC cells exhibit increased respiratory rates as compared to adjacent normal cells suggesting the presence of "reverse Warburg" effect (Whitaker-Menezes et al., 2011). The novel concept of reverse Warburg in cancer metabolism denotes that tumor cells provoke aerobic glycolysis in the tumor stroma thus lactate secretion from cancer-associated fibroblasts. Secreted lactate then fuels OXPHOS in epithelial cancer cells, by acting as a paracrine onco-metabolite. Our data suggest a new strategy for HCC treatment; namely, by

inhibitors of some monocarboxylate transporters (Queiros et al., 2012).

From our MCA studies it can be inferred the presence of respiratory supercomplexes in mitochondria from cancer cells. Recent investigations have shown that respiratory chain complexes I, III and IV can interact to form supercomplexes (respirasomes) (Acín-Pérez et al., 2008; Lenaz and Genova, 2010; Dudkina et al., 2011). Future studies using MCA should unravel how the FCC-value depend upon structural organization of the respirasomes and how exactly the respiratory chain is organized in tumor cells.

Further development in the area of metabolic flux analysis and cellular bioenergetics is important to link future studies on tumor metabolomics to clinical research.

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REFERENCES

Acín-Pérez, R., Fernández-Silva, P., Peleato, M. L., Perez-Martos, A., and Enriquez, J. A. (2008). Respiratory active mitochondrial supercomplexes. *Mol. Cell.* 32, 529–539. doi: 10.1016/j.molcel.2008.10.021

Anmann, T., Guzun, R., Beraud, N., Pelloux, S., Kuznetsov, A. V., Kogerman, L., et al. (2006). 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* 1757, 1597–1606. doi: 10.1016/j.bbabio.2006.09.008

Appaix, F., Kuznetsov, A. V., Usson, Y., Kay, L., Andrienko, T., Olivares, J., et al. (2003). Possible role of cytoskeleton in intracellular arrangement and regulation of mitochondria. *Exp. Physiol.* 88, 175–190. doi: 10.1113/eph8802511

Bellance, N., Pabst, L., Allen, G., Rossignol, R., and Nagrath, D. (2012). Oncosecretomics coupled to bioenergetics identifies alpha-amino adipic acid, isoleucine and GABA as potential biomarkers of cancer: differential expression of c-Myc, Oct1 and KLF4 coordinates metabolic changes. *Biochim. Biophys. Acta* 1817, 2060–2071. doi: 10.1016/j.bbabio.2012.07.004

Bera, S., Wallimann, T., Ray, S., and Ray, M. (2008). Enzymes of creatine biosynthesis, arginine and methionine metabolism in normal and malignant cells. *FEBS J.* 275, 5899–5909. doi: 10.1111/j.1742-4658.2008.06718.x

Dzeja, P. P., and Terzic, A. (2003). Phosphotransfer networks and cellular energetics. *J. Exp. Biol.* 206, 2039–2047. doi: 10.1242/jeb.00426

Dudkina, N. V., Kudryashev, M., Stahlberg, H., and Boekema, E. J. (2011). Interaction of complexes I, III, and IV within the bovine respirasome by single particle cryoelectron tomography. *Proc. Natl. Acad. Sci. U.S.A.* 108, 15196–15200. doi: 10.1073/pnas.1107819108

Eimre, M., Paju, K., Pelloux, S., Beraud, N., Roosimaa, M., Kadaja, L., et al. (2008). Distinct organization of energy metabolism in HL-1 cardiac cell line and cardiomyocytes. *Biochim. Biophys. Acta* 1777, 514–524. doi: 10.1016/j.bbabio.2008.03.019

Fell, D. (1997). *Understanding the Control of Metabolism.* London; Miami: Portland Press.

Fell, D. (2005). "Metabolic control analysis" in *Systems Biology*, eds L. Alberghina and H. V. Westerhoff (Berlin: Springer-Verlag), 69–80.

Gellerich, F. N., Kunz, W. S., and Bohnensack, R. (1990). Estimation of flux control coefficients from inhibitor titrations by non-linear regression. *FEBS Lett.* 274, 167–170. doi: 10.1016/0014-5793(90)81355-R

Gnaiger, E., and Kemp, R. B. (1990). Anaerobic metabolism in aerobic mammalian cells: information from the ratio of calorimetric heat flux and respirometric oxygen flux. *Biochim. Biophys. Acta* 1016, 328–332. doi: 10.1016/0005-2728(90)90164-Y

Groen, A. K., Wanders, R. J., Westerhoff, H. V., Van Der Meer, R., and Tager, J. M. (1982). Quantification of the contribution of various steps to the control of mitochondrial respiration. *J. Biol. Chem.* 257, 2754–2757.

Gstraunthaler, G., Seppi, T., and Pfaller, W. (1999). 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.* 9, 150–172. doi: 10.1159/000016312

Guzun, R., Gonzalez-Granillo, M., Karu-Varikmaa, M., Grichine, A., Usson, Y., Kaambre, T., et al. (2012). Regulation of respiration in muscle cells *in vivo* by VDAC through interaction with the cytoskeleton and MtCK within mitochondrial interactosome. *Biochim. Biophys. Acta* 1818, 1545–1554. doi: 10.1016/j.bbamem.2011.12.034

Guzun, R., Karu-Varikmaa, M., Gonzalez-Granillo, M., Kuznetsov, A. V., Michel, L., Cottet-Rousselle, C., et al. (2011). Mitochondria-cytoskeleton interaction: distribution of beta-tubulins in cardiomyocytes and HL-1 cells. *Biochim. Biophys. Acta* 1807, 458–469. doi: 10.1016/j.bbabio.2011.01.010

Habano, W., Sugai, T., Nakamura, S., Uesugi, N., Higuchi, T., Terashima, M., et al. (2003). Reduced expression and loss of heterozygosity of the SDHD gene in colorectal and gastric cancer. *Oncol. Rep.* 10, 1375–1380.

Izuishi, K., Yamamoto, Y., Sano, T., Takebayashi, R., Nishiyama, Y., Mori, H., et al. (2012). Molecular mechanism underlying the detection of colorectal cancer by 18F-2-fluoro-2-deoxy-D-glucose positron emission tomography. *J. Gastrointest. Surg.* 16, 394–400. doi: 10.1007/s11605-011-1727-z

Jang, J. Y., Choi, Y., Jeon, Y. K., and Kim, C. W. (2008). Suppression of adenine nucleotide translocase-2 by vector-based siRNA in human breast cancer cells induces apoptosis and inhibits tumor growth *in vitro* and *in vivo*. *Breast Cancer Res.* 10:R11. doi: 10.1186/bcr1857

Jose, C., and Rossignol, R. (2013). Rationale for mitochondria-targeting strategies in cancer bioenergetic therapies. *Int. J. Biochem. Cell. Biol.* 45, 123–129. doi: 10.1016/j.biocel.2012.07.005

Kaambre, T., Chekulayev, V., Shevchuk, I., Karu-Varikmaa, M., Timohhina, N., Tepp, K., et al. (2012). Metabolic control analysis of cellular respiration *in situ* in intraoperational samples of human breast cancer. *J. Bioenerg. Biomembr.* 44, 539–558. doi: 10.1007/s10863-012-9457-9

Kholodenko, B. N., Demin, O. V., and Westerhoff, H. V. (1993). 'Channelled' pathways can be more sensitive to specific regulatory signals. *FEBS Lett.* 320, 75–78. doi: 10.1016/0014-5793(93)81661-I

Kholodenko, B. N., and Westerhoff, H. V. (1993). Metabolic channelling and control of the flux. *FEBS Lett.* 320, 71–74. doi: 10.1016/0014-5793(93)81660-R

Kunz, W. S., Kuznetsov, A. V., Clark, J. F., Tracey, I., and Elger, C. E. (1999). Metabolic consequences of the cytochrome c oxidase deficiency in brain of copper-deficient Mo(vbr) mice. *J. Neurochem.* 72, 1580–1585. doi: 10.1046/j.1471-4159.1999.721580.x

Kuznetsov, A. V., Tiivel, T., Sikk, P., Kaambre, T., Kay, L., Daneshrad, Z., et al. (1996). Striking differences between the kinetics of regulation of respiration by ADP in slow-twitch and fast-twitch muscles *in vivo*. *Eur. J. Biochem.* 241, 909–915. doi: 10.1111/j.1432-1033.1996.00909.x

Kuznetsov, A. V., Veksler, V., Gellerich, F. N., Saks, V., Margreiter, R., and Kunz, W. S. (2008). Analysis of mitochondrial function *in situ* in permeabilized muscle fibers, tissues and cells. *Nat. Protoc.* 3, 965–976. doi: 10.1038/nprot.2008.61

Kuznetsov, A. V., Winkler, K., Kirches, E., Lins, H., Feistner, H., and Kunz, W. S. (1997). Application of inhibitor titrations for the detection of oxidative phosphorylation defects in saponin-skinned muscle fibers of patients with mitochondrial diseases. *Biochim. Biophys. Acta* 1360, 142–150. doi: 10.1016/S0925-4439(96)00072-5

Lenaz, G., and Genova, M. L. (2009). Structural and functional organization of the mitochondrial respiratory chain: a dynamic super-assembly. *Int. J. Biochem. Cell. Biol.* 41, 1750–1772. doi: 10.1016/j.biocel.2009.04.003

Lenaz, G., and Genova, M. L. (2010). Structure and organization of mitochondrial respiratory complexes: a new understanding of an old subject. *Antioxid. Redox. Signal.* 12, 961–1008. doi: 10.1089/ars.2009.2704

Lenaz, G., Baracca, A., Barbero, G., Bergamini, C., Dalmonte, M. E., Del Sole, M., et al. (2010). Mitochondrial respiratory chain

super-complex I-III in physiology and pathology. *Biochim. Biophys. Acta* 1797, 633–640. doi: 10.1016/j.bbabio.2010.01.025

Marin-Hernandez, A., Rodriguez-Enriquez, S., Vital-Gonzalez, P. A., Flores-Rodriguez, F. L., Macias-Silva, M., Sosa-Garrocho, M., et al. (2006). Determining and understanding the control of glycolysis in fast-growth tumor cells. Flux control by an over-expressed but strongly product-inhibited hexokinase. *FEBS J.* 273, 1975–1988. doi: 10.1111/j.1742-4658.2006.05214.x

Monge, C., Beraud, N., Tepp, K., Pelloux, S., Chahboun, S., Kaambre, T., et al. (2009). Comparative analysis of the bioenergetics of adult cardiomyocytes and non-beating HL-1 cells: respiratory chain activities, glycolytic enzyme profiles, and metabolic fluxes. *Can. J. Physiol. Pharmacol.* 87, 318–326. doi: 10.1139/Y09-018

Moreno-Sanchez, R., Rodriguez-Enriquez, S., Marin-Hernandez, A., and Saavedra, E. (2007). Energy metabolism in tumor cells. *FEBS J.* 274, 1393–1418. doi: 10.1111/j.1742-4658.2007.05686.x

Moreno-Sanchez, R., Saavedra, E., Rodriguez-Enriquez, S., Gallardo-Perez, J. C., Quezada, H., and Westerhoff, H. V. (2010). Metabolic control analysis indicates a change of strategy in the treatment of cancer. *Mitochondrion* 10, 626–639. doi: 10.1016/j.mito.2010.06.002

Moreno-Sanchez, R., Saavedra, E., Rodriguez-Enriquez, S., and Olin-Sandoval, V. (2008). Metabolic control analysis: a tool for designing strategies to manipulate metabolic pathways. *J. Biomed. Biotechnol.* 2008, 597913. doi: 10.1155/2008/597913

Patra, S., Bera, S., Sinharoy, S., Ghoshal, S., Ray, S., Basu, A., et al. (2008). Progressive decrease of phosphocreatine, creatine and creatine kinase in skeletal muscle upon transformation to sarcoma. *FEBS J.* 275, 3236–3247. doi: 10.1111/j.1742-4658.2008.06475.x

Pedersen, P. L. (2008). Voltage dependent anion channels (VDACs): a brief introduction with a focus on the outer mitochondrial compartment's roles together with hexokinase-2 in the "Warburg effect" in cancer. *J. Bioenerg. Biomembr.* 40, 123–126. doi: 10.1007/s10863-008-9165-7

Queiros, O., Preto, A., Pacheco, A., Pinheiro, C., Azevedo-Silva, J., Moreira, R., et al. (2012). Butyrate activates the monocarboxylate transporter MCT4 expression in breast cancer cells and enhances the antitumor activity of 3-bromopyruvate. *J. Bioenerg. Biomembr.* 44, 141–153. doi: 10.1007/s10863-012-9418-3

Rossignol, R., Letellier, T., Malgat, M., Rocher, C., and Mazat, J. P. (2000). Tissue variation in the control of oxidative phosphorylation: implication for mitochondrial diseases. *Biochem. J.* 347(Pt 1), 45–53. doi: 10.1042/0264-6021:3470045

Rostovtseva, T. K., Sheldon, K. L., Hassanzadeh, E., Monge, C., Saks, V., Bezrukov, S. M., et al. (2008). Tubulin binding blocks mitochondrial voltage-dependent anion channel and regulates respiration. *Proc. Natl. Acad. Sci. U.S.A.* 105, 18746–18751. doi: 10.1073/pnas.0806303105

Saks, V., Guzun, R., Timohhina, N., Tepp, K., Varikmaa, M., Monge, C., et al. (2010). Structure-function relationships in feedback regulation of energy fluxes *in vivo* in health and disease: mitochondrial interactosome. *Biochim. Biophys. Acta* 1797, 678–697. doi: 10.1016/j.bbabio.2010.01.011

Salem, A. F., Whitaker-Menezes, D., Lin, Z., Martinez-Outschoorn, U. E., Tanowitz, H. B., Al-Zoubi, M. S., et al. (2012). Two-compartment tumor metabolism: autophagy in the tumor microenvironment and oxidative mitochondrial metabolism (OXPHOS) in cancer cells. *Cell Cycle* 11, 2545–2556. doi: 10.4161/cc.20920

Small, J. R. (1993). Flux control coefficients determined by inhibitor titration: the design and analysis of experiments to minimize errors. *Biochem. J.* 296(Pt 2), 423–433.

Small, J. R., and Fell, D. A. (1990). Covalent modification and metabolic control analysis. Modification to the theorems and their application to metabolic systems containing covalently modifiable enzymes. *Eur. J. Biochem.* 191, 405–411. doi: 10.1111/j.1432-1033.1990.tb19136.x

Sotgia, F., Whitaker-Menezes, D., Martinez-Outschoorn, U. E., Flomenberg, N., Birbe, R. C., Witkiewicz, A. K., et al. (2012). Mitochondrial metabolism in cancer metastasis: visualizing tumor cell mitochondria and the "reverse Warburg effect" in positive lymph node tissue. *Cell Cycle* 11, 1445–1454. doi: 10.4161/cc.19841

Tager, J. M., Wanders, R. J., Groen, A. K., Kunz, W., Bohnensack, R., Kuster, U., et al. (1983). Control of mitochondrial respiration. *FEBS Lett.* 151, 1–9. doi: 10.1016/0014-5793(83)80330-5

Tepp, K., Shevchuk, I., Chekulayev, V., Timohhina, N., Kuznetsov, A. V., Guzun, R., et al. (2011). High efficiency of energy flux controls within mitochondrial interactosome in cardiac intracellular energetic units. *Biochim. Biophys. Acta* 1807, 1549–1561. doi: 10.1016/j.bbabio.2011.08.005

Tepp, K., Timohhina, N., Chekulayev, V., Shevchuk, I., Kaambre, T., and Saks, V. (2010). Metabolic control analysis of integrated energy metabolism in permeabilized cardiomyocytes - experimental study. *Acta Biochim. Pol.* 57, 421–430.

Warburg, O. (1956). On the origin of cancer cells. *Science* 123, 309–314. doi: 10.1126/science.123.3191.309

Warburg, O. H., and Dickens, F. (1930). *The Metabolism of Tumours.* London: Constable.

Whitaker-Menezes, D., Martinez-Outschoorn, U. E., Lin, Z., Ertel, A., Flomenberg, N., Witkiewicz, A. K., et al. (2011). Evidence for a stromal-epithelial "lactate shuttle" in human tumors: MCT4 is a marker of oxidative stress in cancer-associated fibroblasts. *Cell Cycle* 10, 1772–1783. doi: 10.4161/cc.10.11.15659

Witkiewicz, A. K., Whitaker-Menezes, D., Dasgupta, A., Philp, N. J., Lin, Z., Gandara, R., et al. (2012). Using the "reverse Warburg effect" to identify high-risk breast cancer patients: stromal MCT4 predicts poor clinical outcome in triple-negative breast cancers. *Cell Cycle* 11, 1108–1117. doi: 10.4161/cc.11.6.19530.

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Klepinin, Aleksandr; Chekulayev, Vladimir; Timohhina, Natalja; Shevchuk, Igor; Tepp, Kersti; Kaldma, Andrus; Koit, Andre; Saks, Valdur; Kaambre, Tuuli (2014). Comparative analysis of some aspects of mitochondrial metabolism in differentiated and undifferentiated neuroblastoma cells. Journal of Bioenergetics and Biomembranes, 46 (1), 17−31, s10863-013-9529-5.

Kaldma, Andrus; Shevchuk, Igor; Koit, Andre; Chekulayev, Vladimir; Timohhina, Natalja; Tepp, Kersti; Mado, Kati; Khandashvili, Manana; Heck, Karoliina; Truu, Laura; Planken, Anu; Kaambre, Tuuli (2014). Bioenergetic profiling of human colorectal cancer clinical material. *Cell Symposia, Hallmarks of Cancer: Asia.* Beijing, China,.

Kaambre, Tuuli; Chekulayev, Vladimir; Shevchuk, Igor; Tepp, Kersti; Timohhina, Natalja; Varikmaa, Minna; Bagur, Rafaela; Klepinin, Aleksandr; Anmann, Tiia; Koit, Andre; Kaldma, Andrus; Guzun, Rita; Valvere, Vahur; Saks, Valdur (2013). Metabolic Control Analysis of Respiration in Human Cancer Tissue. Frontiers in Physiology, 4, 1−6, fphys.2013.00151.

Kaldma, Andrus; Koit, Andre; Shevchuk, Igor; Chekulayev, Vladimir; Tepp, Kersti; Timohhina, Natalja; Bogovskaja, Jelena; Valvere, Vahur; Saks, Valdur; Kaambre, Tuuli (2013). Regulation of respiration of human colorectal cancer: application of Metabolic Control Analysis. *Mitochondrion, 13(6): Mitochondrial Medicine 2013, Newport Beach, CA, USA.* Newport Beach, CA, USA, 900.

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Kaldma, Andrus; Klepinin, Aleksandr; Chekulayev, Vladimir; Mado, Kati; Shevchuk, Igor; Timohhina, Natalja; Tepp, Kersti; Kandashvili, Manana; Varikmaa, Minna; Koit, Andre; Planken, Margus; Heck, Karoliina; Truu, Laura; Planken, Anu; Valvere, Vahur; Rebane, Egle; Kaambre, Tuuli. (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, j.biocel.2014.09.004.

Klepinin, Aleksandr; Chekulayev, Vladimir; Timohhina, Natalja; Shevchuk, Igor; Tepp, Kersti; Kaldma, Andrus; Koit, Andre; Saks, Valdur; Kaambre, Tuuli (2014). Comparative analysis of some aspects of mitochondrial metabolism in differentiated and undifferentiated neuroblastoma cells. Journal of Bioenergetics and Biomembranes, 46 (1), 17−31, s10863-013-9529-5.

Kaldma, Andrus; Shevchuk, Igor; Koit, Andre; Chekulayev, Vladimir; Timohhina, Natalja; Tepp, Kersti; Mado, Kati; Khandashvili, Manana; Heck, Karoliina; Truu, Laura; Planken, Anu; Kaambre, Tuuli (2014). Bioenergetic profiling of human colorectal cancer clinical material. *Cell Symposia, Hallmarks of Cancer: Asia.* Beijing, China,.

Kaambre, Tuuli; Chekulayev, Vladimir; Shevchuk, Igor; Tepp, Kersti; Timohhina, Natalja; Varikmaa, Minna; Bagur, Rafaela; Klepinin, Aleksandr; Anmann, Tiia; Koit, Andre; Kaldma, Andrus; Guzun, Rita; Valvere, Vahur; Saks, Valdur (2013). Metabolic Control Analysis of Respiration in Human Cancer Tissue. Frontiers in Physiology, 4, 1−6, fphys.2013.00151.

Kaldma, Andrus; Koit, Andre; Shevchuk, Igor; Chekulayev, Vladimir; Tepp, Kersti; Timohhina, Natalja; Bogovskaja, Jelena; Valvere, Vahur; Saks, Valdur; Kaambre, Tuuli (2013). Regulation of respiration of human colorectal cancer: application of Metabolic Control Analysis. *Mitochondrion, 13(6): Mitochondrial Medicine 2013, Newport Beach, CA, USA.* Newport Beach, CA, USA, 900.

Shevchuk, Igor; Koit, Andre; Kaldma, Andrus; Timohhina, Natalja; Chekulayev, Vladimir; Tepp, Kersti; Bogovskaja, Jelena; Valvere, Vahur; Saks, Valdur; Kaambre, Tuuli (2013). Comparative aspects of bioenergetic profiling of tumor clinical material and cell cultures. *Mitochondrion, 13(6): Mitochondrial Medicine 2013, Newport Beach, CA, USA.* Newport Beach, CA, USA, 907.

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