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Malignant Transformation Causes Rearrangement of Energy Metabolism in Colorectal and Breast Cancers

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Defence of the thesis: 31st of January 2018.

Declaration:

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

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Kartsinogenees toob kaasa energiametabolismi ümberkorralduse jämesoole- ja rinnakasvajates

ANDRE KOIT

TTÜ KIRJASTUS

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

III. 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.

IV. 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.

V. Kaambre, Tuuli; **Koit, Andre**; Shevchuk, Igor; Chekulayev, Vladimir; Tepp, Kersti; Timohhina, Natalja; Bogovskaja, Jelena; Saks, Valdur; Valvere, Vahur (2013) Uutest suundadest kasvajate energiametabolismi uuringutes. Eesti Arst, 92(5): 261–267.

Author's Contribution to the Publications

I – Author participated in preparing the study design with major emphasis on the comparison of cell cultures and human samples, but also on clinical data interpretation. Additionally, carried out oxygraphic measurements, participated in data analysis and had leading role in writing the paper.

II-IV – Author, together with colleagues, processed human samples, conducted oxygraphic studies, participated in manuscript writing and data analysis.

V – Author prepared the part regarding truncated citric acid cycle and contributed to preparation of the manuscript.

Abbreviations

HBC	human breast cancer
HCC	human colorectal cancer
EU	European Union
EC	European Council
DCIS	ductal carcinoma in situ
HER2	human epidermal growth factor receptor 2
PDAC	pancreatic ductal adenocarcinoma
ADP	adenosine diphosphate
ATP	adenosine triphosphate
OXPHOS	oxidative phosphorylation
PPP	phosphate pentose pathway
ECAR	extracellular acidification rate
OCR	oxygen consumption rate
PEP	phosphoenolpyruvate
ANT	adenine nucleotide transporter
TCA	tricarboxylic acid cycle
SDH	succinate dehydrogenase
ROS	reactive oxygen species
HIF-1α	hypoxia-inducible factor 1 alpha
FCC	flux control coefficient
NADH	nicotinamide adenine nucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
$FADH_2$	flavin adenine dinucleotide
PET	positron emission tomography
^{18}FDG	^{18}F-fluorodeoxyglucose
LDH	lactate dehydrogenase
Δp	proton motive force
ΔpH	chemical component of proton motive force
Δψ	mitochondrial membrane potential
FH	fumarate hydratase
CI	Complex I of the respiratory chain
CII	Complex II of the respiratory chain
CIII	Complex III of the respiratory chain
CIV	Complex IV of the respiratory chain
SCAF	supercomplex assembly factor
MOM	mitochondrial outer membrane
CAT	carboxyatractyloside
WB	Western blot

Review of literature

1. Breast and colorectal cancer

1.1 Incidence, mortality and survival rates

Cancer is the second leading cause of death globally. 8.8 million deaths were caused by cancer in 2015, therefore accounting for 1 death out of every 6. In addition, incidence of cancer is on the rise. In 2012, a total of 14 million new cases were registered and by 2020 the number of new cases is projected to rise up to 17 million globally. Partly, the rise is ascribed to demographic effect, but it nevertheless forecasts elevated need to address this complex malady [1].

The biggest number of new cases is caused by cancer of the lung. In 2012, lung cancer accounted for 1.82 million new cases globally, and it was followed by breast and colorectal cancers, which accounted for 1.67 million and 1.36 million new cases, respectively. For cancer deaths, lung cancer remains as the leading cause (1.59 million deaths globally), but deaths caused by breast and colorectal cancers are falling to the fourth and fifth place with 0.69 million and 0.52 million deaths, respectively, indicating to improved outcomes for those latter types [2].

In this thesis, breast and colorectal cancers are the study objects and for that reason the main emphasis is given to those two cancer types on the following pages.

According to Estonian National Institute for Health Development, a total of 8558 new cancer incidences were registered in 2014 for Estonia with prostate cancer being the leading diagnosis (1083 new diagnoses; ICD-10-CM diagnose code C61). The second leading cause is cancer of the colon, rectum and anus/anal canal (combined codes C18-C21) with 914 new cases and breast cancer was the fourth most common cancer diagnosis with 728 new cases [3].

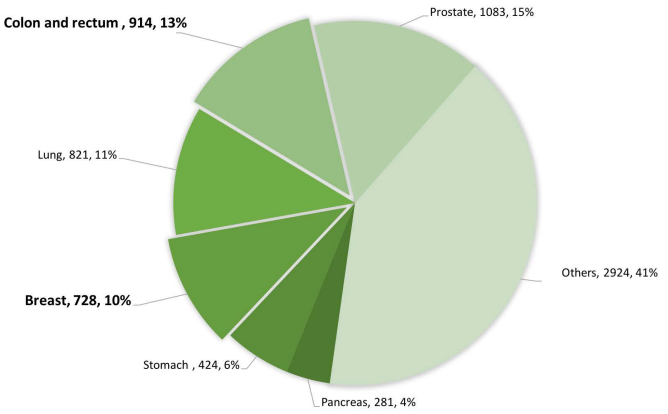

Figure 1. Cancer incidences in Estonia in 2014, shown as location, number of cases and percentage of all cases [3].

During the same year, 2014, 15 467 deaths were registered in Estonia with cardiovascular reasons being the leading cause (8258 deaths) and malignancy related deaths as the second most common cause (3812 deaths). Similarly to global data, the leading cause of cancer-related death was lung cancer (715 deaths; C33-C34); colorectal cancer (C18, C19) and breast cancer (C50) were the second and the fifth cause with 334 and 244 deaths, respectively [3].

Despite the rise in cancer incidences, survival rates in Europe have improved, especially in the Easter-European countries, including Estonia, which previously had more lower rates compared to rest of the Europe than they have according to recent studies [4]. Based on data from 2000 to 2009, five-year relative survival in Estonia for colon/rectal cancer is 49/50% and for breast cancer, 76% [5]. Both results are lower than the European average found in the EUROCARE-5 study which covers similar time period. European average 5-year relative survival for colon/rectal cancers is 57.0/55.8% and 81.8% for breast cancer. Survival rates in Eastern-European countries were found to be systematically lower than that in rest of the Europe. To an extent, it can indicate problems with data quality, but mainly it is explained by limited or delayed access to better treatments and reduced efficacy of existing treatments due to late diagnosis. But additionally, other factors like amount of diagnostic testing, cancer biology, comorbidities, and socioeconomic status can directly or indirectly affect trends in survival and EUROCARE-5 study did not have access to major prognostic factors like stage at diagnosis and therefore comparison of survival differences in different regions is difficult. Additionally, the authors of the EUROCARE-5 study conclude that survival and mortality, as basis for this study, are too limited for sufficient analysis of different regions as survival can be inflated by overdiagnosis and lead-time bias and mortality on its own – a consequence of past incidence and survival trends – is insufficient [6]. Therefore, analysis of survival or morbidity can be useful and relevant within a single region which allows to estimate effects of different region-wide initiatives like cancer screening.

1.2 Population screening

WHO has estimated that 30-50% of all cancers are preventable and prevention itself offers the most cost-effective long-term strategy for the control of cancer. Main risk factors that should be avoided, reduced or managed, are tobacco use, physical inactivity, obesity, alcohol use, infections, environmental pollution, occupational carcinogens and ionizing radiation [7]. However, whether the named risk factors are managed or not, early detection of any cancer significantly increases the likelihood of improved prognosis or successful curative treatment and therefore many countries have screening programs to find those early stage patients.

European Council recommends screening for three types of cancer - breast, cervical and colorectal cancers - to reduce the burden of the common cancers in the member states. In 2012, 2.6 million new cancer cases were recorded in the European Union and 1.26 million deaths caused by the same disease. From the recommended screening targets, colorectal cancer is the second cause of cancer-

related death for men and the third for women; breast cancer is the leading cause of cancer-related death in women, but incidence and hence, deaths, from cervical cancer are comparatively low in the EU. Low incidence of cervical cancer is explained by effective implementation of the screening program [8].

95% of nearly 67.5 million women residing in the EU who belong to chosen target age for breast cancer screening, 50-69 years, reside in the 25 member states who have implemented nationwide population-based screening programs. In case of cervical cancer, target age is 30-59 years and 72% of women residing in EU are in member states that have screening programs for it. In case of colorectal cancer, target age is 50-74 years and out of the total 152 million EU residents meeting that target, 72% are residing in member states that have initiated some type of screening program for the disease [8].

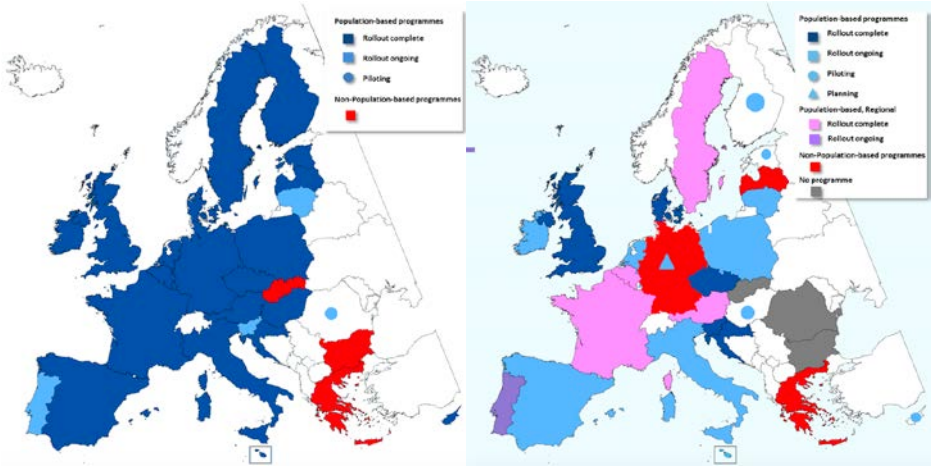

Figure 2. Implementation of breast cancer screening (on the left) and colorectal cancer screening (on the right) in the EU member states as of 2016 [8].

In USA, similarly to EU, American Cancer Society recommends screening for breast, cervical and colorectal cancer, but additionally, for lung and prostate cancer [9]. Controversially, US Preventive Task Force, what also issues recommendations, agrees with screening for breast, colorectal, cervical and lung cancers, but recommends against screening for prostate cancer (using PSA), but also against screening for ovarian, testicular and thyroid cancers [10].

Recommending against some screening programs is driven by non-balanced outcome of the programme or by insufficient methodologies used for the screening. Despite positive effects of different recommended screening programs have been confirmed with randomized controlled trials, some negative aspects are also involved. Mainly the negative aspects are false positive and false negative results, but also sample collection method or sample quality can cause problems. Additionally, in case of colorectal cancer screening, endoscopists should reach adenoma-detection-rate above 25% (≥30% for men, ≥20% for women), because most colorectal cancers that develop between

screenings are believed to arise from lesions that were missed at the time of previous colonoscopy [9].

For breast cancer, mortality benefit from screening is estimated to be around 20% (95% CI 11-27%). It is also acknowledged that such benefit assessment is directly affected by the study design and comes with uncertainties, but 20% was found to be the most reasonable estimate [11]. For colorectal cancer, up to 50% decline was concluded for both incidence and mortality in US, which was attributed to widespread use of colonoscopy in that region for screening purposes [12]. Such result for colorectal cancer screening, however, seems to be biased. Meta-analysis on almost 745 000 individuals showed that if faecal guaiac-based occult blood testing was used for screening, it led to 14% reduction in mortality and use of flexible sigmoidoscopy led to 28% reduction in colorectal cancer mortality compared to unscreened individuals [13]. But as general background, colorectal cancer incidence has dropped almost 40% since 1975 and by more than 45% since its peak in mid-1980s and importantly, associated mortality has fallen by more than half. Screening alone cannot explain such decline as uptake of screening has been too slow when the timeframe is considered. Some authors explain reduced mortality with improved treatment options, earlier detection of symptomatic disease and with fewer cases of colorectal cancer occurring in the first place. Role of screening cannot and should not be excluded from these declining numbers, but it should be noted that changes in diet and microbiome can have a significant role in reduced incidence of colorectal cancer seen in those studies [14].

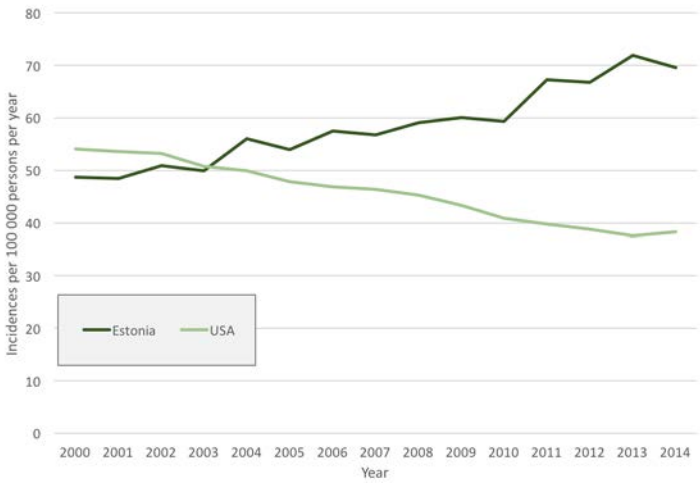

Figure 3. Colorectal cancer incidences in Estonia [3] and USA [15] between 2000 and 2014. Data presented as new cases per 100 000 persons per year.

Described decline in colorectal cancer incidence in some western societies is, however, not characteristic to Estonian population during the past decades as shown by the increasing incidence trend (Figure 3). Colorectal cancer

incidences peaked in the US in the middle of 1980s and have declined since [14] and when compared to Estonian data, the opposite trends might be explained by the difference in socioeconomic development stage of those two societies.

1.3 Overdiagnosed cancers

Widespread screening programs have introduced a new and unexpected phenomenon – overdiagnosis. Overdiagnosis refers to the detection of cancers on screening, which would not have become clinically apparent in the patients' lifetime if the patient would not have been screened [11].

Using search string "cancer overdiagnosis" in PubMed, gives a total of 2656 results (Figure 4). Earliest use of the string was in 1948 which was followed by 14 years of silence until the string surfaced in 1962 and then another 15 years of silence until 1976. After 1980, there has not been a year without a use of this string in an article. Surprisingly, use of this string declined in 2016 compared to 2015, despite the elevated interest in the society.

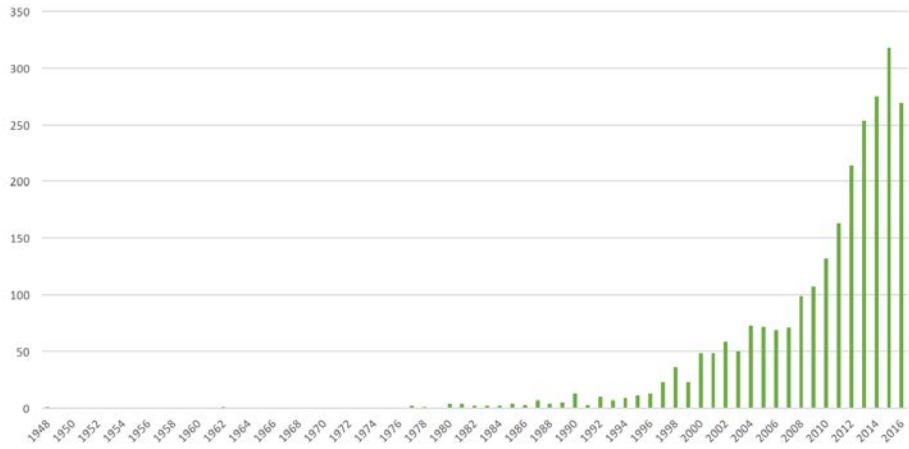

Figure 4. Use of string "cancer overdiagnosis" in scientific papers per year based on PubMed "All fields" search.

Main harm associated with overdiagnosis, in case of breast cancer, is overtreatment, such as lumpectomy, mastectomy, chemotherapy, and radiation therapy followed by additional unnecessary diagnostic procedures. Additionally, women would experience anxiety of knowing that they have breast cancer [16].

Mixed results have been published on this subject for screening mammography and the overdiagnosis rate has been reported anywhere between 0% and upwards of 30% of diagnosed cases. This striking difference indicates that such rate estimates are not straightforward calculations, but rather are based on different sets of assumptions and are often biased by methodological flaws [17]. American Cancer Society concluded in their 2017 review that higher estimates of overdiagnosis were based on studies that did not adequately adjust

for lead time or contemporaneous trends in incidence and had inadequate follow-ups. As a result, if those methodological criteria were met, overdiagnosis of invasive disease should be uncommon [9]. Therefore, some authors consider overdiagnosis an epidemiological rather than a pathological concept [18].

Overdiagnosis of invasive cancers is less likely, largely because these cancers tend to grow more quickly and the authors of [17] support this notion with and absence of documented cases of an invasive cancer regressing without treatment. Despite the authors notion, there are several case reports for spontaneous breast cancer remission [19,20] and reviews indicating that almost all human cancers, including metastatic, can spontaneously regress [21]. These spontaneous regression cases, however, are very rare. In case of breast cancer screening, the likely subtype to be associated with overdiagnosis, is ductal carcinoma in situ (DCIS). In the pre-mammography era, DCIS was extremely uncommon, representing around 5% of all breast cancer diagnoses. By now, strikingly, DCIS represents 30% of all breast cancers detected at screening and 20% of all newly diagnosed breast cancer cases [16]. Despite the increasing number of DCIS diagnoses, controversy still exists, whether these are overdiagnosed patients or is the screening program already picking out patients who will develop invasive breast cancer from the same lesion in 5-20 years.

One possibility to reduce or postpone treatment in DCIS patients should be active surveillance, similarly to that established for low-risk prostate cancer. Such programs are already in development, but there are still many open questions, as to what constitutes appropriate active surveillance, how long should it last for and when exactly further intervention is needed [22].

Altogether, overdiagnosis, based on current knowledge, represents disparity between improved efficacy of radiological methods and limitations in pathological methods, so that a cancer, once discovered, is difficult to segregate into high risk (immediate attention needed) or low risk (no immediate attention needed) categories. Therefore, despite some exceptions with active surveillance in prostate cancer, all cancers discovered on screening, for now, are considered to be high risk. To a large extent, however, even greater problem is caused by people who decide not to participate in the screening programs even if these are made readily available for them.

2. Changed metabolism in malignant cells

2.1 Dissonance between models and human biology

Researchers prefer simple models to describe the complexity of human biology, and additionally, many experiments would not be possible with a living human being. Cultured human cells, as the most widely used model system in research laboratories today, were first established in 1952 when HeLa cells were isolated from a patient with cervical cancer and immortalised. The same cell culture, shortly after its introduction, was used to develop vaccine against the polio virus [23] and has been used in various trials ever since together with many other cell cultures. Cells in culture are commonly used to study metabolism and despite the well-known culture medias used to cultivate those cells were developed shortly after introduction of the HeLa cells (DMEM in 1959 [24] and RPMI-1640 in 1967 [25]), it has only recently come under wider attention, that those culture medias were not developed to mimic human plasma, but, rather, to address the need for large amounts of media with more homogenous composition than biological fluids or tissue extracts could offer [26]. Therefore, use of these medias, due to nonphysiological composition, can affect properties of cells and render the outcome uncharacteristic to the human body. Indeed, it has been shown that severe genetic and epigenetic changes emerge once cells from mammalian tissues are taken into culture. It is explained through adaption to culture conditions and alternatively referred to as “culture shock”, but it drastically undermines the very foundation of cultured cells, that these *in vitro* models should represent the original tissue the cells were isolated from – it has become more and more evident, that to a large extent, they don’t. [27,28]. In addition to culture cells, mice are widely used in biological research, but composition of mouse plasma is also considerably different from that of humans. For example, uric acid, missing in culture medias and minimally present in mice, was confirmed to have profound effect on intracellular metabolite levels once its level in media was taken to that in humans. In addition, presence of uric acid antagonized cytotoxicity of 5-fluorouracil, a widely used agent in cancer treatment – a find that could have not been possible to make if only cell cultures or mice were used [26].

In case of breast cancer, cell cultures follow the molecular subtypes (discussed in more detail in paragraph 2.2). Metastatic breast cancer cells, when compared to non-metastatic cells, have shown to have elevated oxidative phosphorylation (OXPHOS) together with elevated glycolysis, but importantly, the metastatic cells in distal metastases have gone through metabolic reprogramming when compared to the initial lesion. This adaption is flexible as the metastatic cells with changed metabolic profile resume to initial program once taken back to growth media. It can have severe implications on defining cancer types, but also on drug development. 4T1 breast cancer cells (aggressive triple negative subtype) display sensitivity to glutamine deprivation, but once metastasized to the lung, become glutamine independent. Glutamine sensitivity is resumed once the cells from lung metastases are recultivated, but systemic

treatment with glutaminase inhibitors would leave the lung metastases unaffected. Similarly, liver metastases of the same model system display 40-50% reduction in total respiration when compared to the parental cells, but other studies have shown, that breast cancer cells that metastasize preferably to the lungs, have increased respiration compared to the parent cells [29-32].

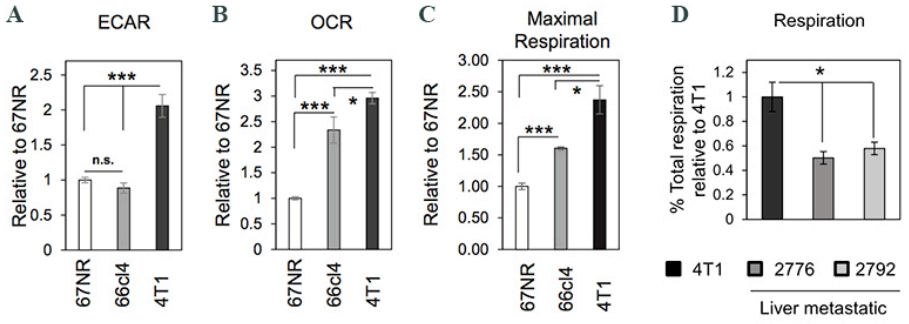

Figure 5. (A-C) in culture, 4T1 cells display elevated extracellular acidification rate (ECAR), oxygen consumption rate (OCR) and maximal respiration compared to 67NR and 66cl4 cell lines; 67NR is non-metastatic, 66cl4 metastasizes preferably to the lung and 4T1 to various sites. **(D)** Cultured cells with preference for forming liver metastases display reduced respiration when compared to parental 4T1 cells. From [29] with permission.

Interestingly, once 4T1 cells have metastasised to the lung and display changed metabolic program together with elevated pyruvate uptake and resistance to specific metabolic drugs, it does not correlate with changes in DNA expression pattern [29]. It renders many of the standard IHC-based biomarkers useless and proves growing need for new generation of biomarker technologies.

2.2 Critical aspects of tumor formation and progression

Each person is unique and tumors in different patients, even if the disease originates from the same site, tend to behave differently. Historically, tumor types have been segregated based on organs they arise from, but advances in science have and continue to find possibilities to fractionate malignancies based on their intrinsic properties, or their counteraction with the host. Genetic testing has led to many molecular treatment targets that, once treated against, spare the patient, but facilitate cytotoxic or cytostatic effect on the tumor tissue (like human epidermal growth factor receptor 2 (HER2) in breast [33] and isocitrate dehydrogenase (IDH) mutations [34] in certain solid and blood cancers). Trials have shown, however, that this picture has to be widened and science needs to consider the tissue of origin (e.g. breast, brain or liver) together with additional aspects like inclusion of immune cells in the tumor mass, perfusion status and possibility of stromal symbiosis. The picture is even more complicated, if cancer is considered a systemic disease from the onset where all those aspects

must be taken into account starting from very early diseases and treatment adjusted accordingly [35].

Breast tumors are among the most studied malignancies and patients are treated based on multiple tumor properties lacking for most of the other tumor types. Molecular subtypes are defined based on expression patterns of receptors for oestrogen (ER), progesterone (PR), and HER2 together with levels of proliferation marker Ki-67. Presence, absence and abundance of each marker are basis for segregating tumors into specific molecular subtypes and for making prognostic and predictive assessments (Table 1).

Table 1. Breast cancer most common molecular subtypes. From [36] with permission.

Subtype	**IHC status**	**Prevalence**	**Outcome**
Luminal A	ER+, PR+, HER2-, low Ki-67	35-45%	Best
Luminal B	ER+, PR+, HER2-, high Ki-67	10-20%	Intermediate
HER2 overexpressing	ER-, PR-, HER2+	10-15%	Poor
Triple negative	ER-, PR-, HER2-	15-20%	Poor

Each subtype indicates to differences in tumor biology that will lead to segregation in recurrence and treatment outcomes [37]. There are many different genetic tests for subgrouping different cancer types even further, but the value of these is more evident if the test outcome can be coupled to specific treatment options with olaparib from AstraZeneca being one of the latest addition for breast cancer patients in metastatic setting (BRCA mutation as prerequisite for treatment) [38].

Cancers are highly heterogeneous [39] from very early onset, but often the clonal development will sustain certain molecular mechanisms characteristic to the tissue of origin. In wider view, metastatic breast or prostate cancer cells can retain hormone receptors or melanoma cells remain dark due to elevated melanin production in the metastatic cells and despite some of these specific inherited properties can be used for prognostic or predictive purposes, metabolic phenotype might have already changed during disease spatial progression. For example some authors have found that malignant cells require increased OXPHOS and mitochondrial activity to acquire metastatic phenotype [40], others have shown that tumor biology in distal sites has to adapt even further to maintain viability and growth. Breast cancer most commonly spreads to bone, lung and liver, but in addition to elevated OXPHOS and glycolysis in metastatic cells, site-specific differences emerge [29] indicating, from one hand, to intrinsic flexibility in those cells, but on the other, increased difficulties in treating all different lesions with a single agent.

Circulatory system is used to introduce anti-cancer treatments to patients, but the same system delivers nutrients to the malignant cells and also facilitates migration of the tumor cells to distal sites within the body. Many vasculatory

abnormalities in the tumor are contributing to both tumor progression and treatment outcome: heterogeneous permeability, irregular blood flow, high interstitial pressure and endothelial cell anergy [41]. The named abnormalities can limit nutrient availability for malignant cells, but equally, to tumor infiltrating immune cells. Cancer cells can adapt to limited nutrient availability by manipulating its stromal cells via two compartment metabolism. It is particularly evident in pancreatic ductal carcinomas (PDAC) that commonly present with intense fibrotic stroma, which impairs the vasculature and leads to highly hypoxic and nutrient poor environment.

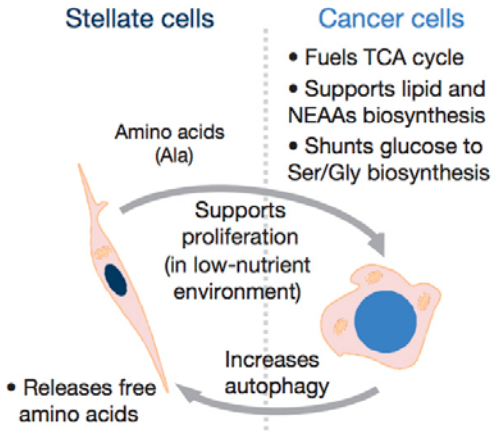

Figure 6. Model of tumour–stroma metabolic cross-talk in PDAC. From [42] with permission.

PDAC stimulate autophagy in stroma-associated pancreatic stellate cells which in turn secrete alanine and the tumor takes it up to support its elevated OXPHOS and lipid biosynthesis [42]. Similar cross-talk between tumor cells and fibroblasts has been shown in breast tumors [43], possibly indicating to wider presence of two compartment metabolism in different cancers.

2.3 Changes in glucose metabolism

Metabolic profiles of different tumor types or even metastases from the same initial lesion, together with mitochondrial function, can have significant variability and plasticity as described above. Albeit, cancer cells have shown to share certain commonalities.

Two principal nutrients that support survival and biosynthesis in mammalian cells are glucose and glutamine. Through catabolism of both of these nutrients, various anabolic pathways are maintained to synthesise different macromolecules and, via controlled oxidation of carbon skeletons, their reducing power is captured in the form of nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide ($FADH_2$) to feed the electron transport chain and regenerate adenosine triphosphate (ATP). Related cofactor nicotinamide adenine dinucleotide phosphate (NADPH) provides reducing power to a wide variety of biosynthetic reactions and helps maintain cellular

redox capacity [44]. Increased glucose uptake by malignant cells in comparison to non-proliferating cells was first described almost a century ago by the German physiologist Otto Warburg [45]. Many years later, based on the same discovery, positron emission tomography (PET)-based imaging of the uptake of a radioactive fluorine-based glucose analogue, ^{18}F-fluorodeoxyglucose (^{18}FDG-PET), was developed and widely distributed to clinics for tumor diagnosis and staging, as well as for monitoring responsiveness to treatment [46,47].

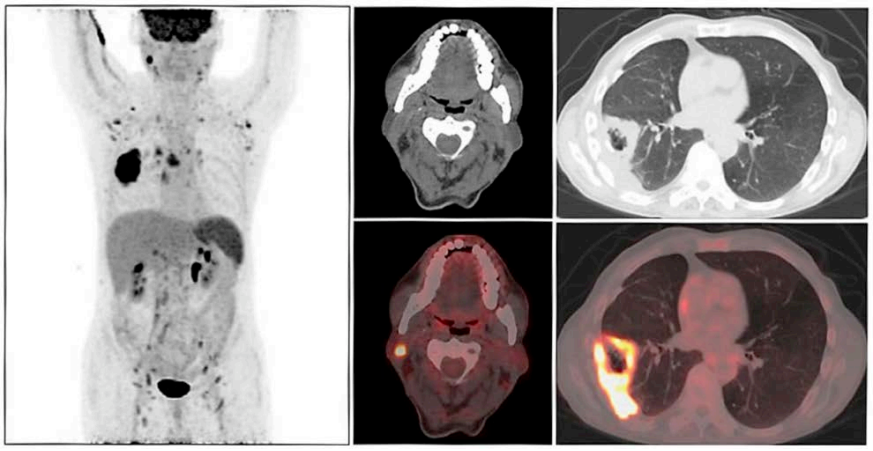

Figure 7. Combined analysis of ^{18}FDG-PET with computer tomography to improve anatomic localization. Lung cancer patient with extensive metastases; focal uptake of ^{18}FDG in neck muscle. From [47] with permission.

Warburg initially speculated that cancers cells, due to defective mitochondria, switch to glycolysis for ATP synthesis even in the presence of abundant oxygen and hence the term, “aerobic glycolysis”. Interestingly, he initially noted, but later discarded, that respiration alone could maintain tumor viability and therefore it was concluded that both glucose and oxygen need to be eliminated to kill tumor cell. Indeed, research in recent decades has shown that while glycolysis is dramatically upregulated in almost all cancer cells, mitochondria continue to operate normally [48,49]. Glycolysis, in addition to ATP regeneration, serves as a source for synthesis of biomass and reducing equivalents. Via pentose phosphate pathway (PPP) oxitative branch NADPH is produced and ribose-5-phosphate is produced via nonoxidative branch of PPP. Non-oxidative branch of PPP has been suggested to be the main source for ribose-5-phosphate synthesis in tumor cells which in turn is needed to produce nucleotides for rapid proliferation. Therefore, tumor cells need to upregulate glycose consumption to keep the fructose-6-phosphate and/or glyceraldehyde-3-phospate levels elevated to push the nonoxidative PPP pathway towards products as the reactions in this pathway are all reversible [50].

Glycolysis converts one molecule of glucose into two molecules of pyruvate describable with the following chemical equation:

$$C_6H_{12}O_6 + 2P_i + 2ADP + 2NAD^+ \rightarrow 2C_3H_3O_3 + 2H_2O + 2ATP + 2NADH$$

In addition to the generation of pyruvate, two molecules of ATP and two molecules of NADH are regenerated. Thus, for glycolysis to be maintained, a flux balance that consumes its products (ATP and NADH) must be achieved and supply of glucose and inorganic phosphate must be continuous. In case of terminally differentiated cells, cytosolic NADH/NAD^+ imbalance is eliminated using mitochondrial shuttles like malate-aspartate or glycerol phosphate shuttle and therefore NADH (or $FADH_2$ in case of the less studied glycerol phosphate shuttle) is consumed by OXPHOS to regenerate more ATP. These shuttles can balance the amount of NADH generated by small rates of glycolysis, but in case of rapid glycose uptake, the production of NADH is increased many orders of magnitude and the named shuttles quickly become saturated. As a result, additional mechanisms are necessary to regenerate cytosolic NAD^+. The major mechanism for that involves the reduction of pyruvate to lactate to regenerate NAD^+ from NADH via lactate dehydrogenase (LDH), which is frequently overexpressed in many cancers (Figure 8). As a result, high rates of glycolysis needed for rapid proliferation can induce the Warburg phenotype via concomitant need for NAD^+ regeneration to sustain intracellular redox balance [49,51].

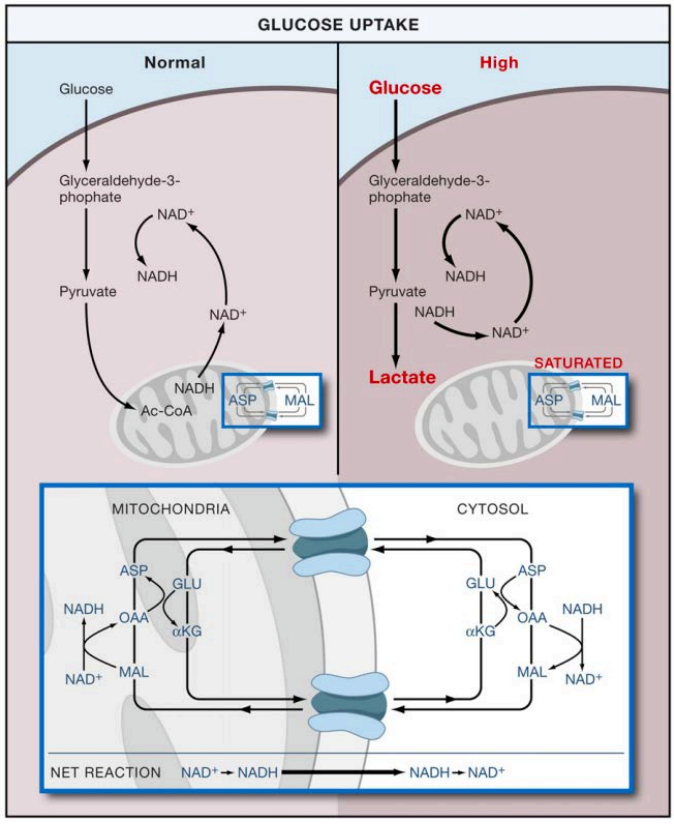

Figure 8. Glucose metabolism during high or low glycose uptake. From [51] with permission.

Glycolysis turns a molecule of glycose into lactate 10-100 times faster than it would take to oxidise the same molecule of glycose to CO_2 in mitochondria [49] and therefore it has been suggested that that rapid ATP production can be the biggest benefit of aerobic glycolysis [51]. At the same time, glycolysis in the cytosol is almost 20-fold less effective in generating ATP from a molecule of glycose than complete oxidation of glycose would give in the mitochondria, but it still confers a proliferative advantage for tumor cells [52]. Surprisingly, calculations suggest that the amount of ATP needed for mammalian cell growth and division may be far less than that required for basal cellular maintenance [53], and an excess amount of ATP can have inhibitory effect on proliferation [52]. Therefore, cells must adapt mechanisms to cope with increasing concentration of ATP to sustain elevated glycolysis. One clue in resolving this situation is the fact that growing cells express a single isoform of pyruvate kinase, PKM2, which is biochemically two times less active than its other isoform PKM1, which is primarily expressed in differentiated cells. As pyruvate kinase catalyzes the final step of glycolysis by converting phosphoenolpyruvate (PEP) to pyruvate and transferring a phosphate to ADP to regenerate ATP, its less active isoform PKM2 should be also less active in regenerating ATP and should hence be limiting the amount of free ATP. Additionally, an alternate enzymatic activity has been discovered for the pyruvate kinase substrate PEP which can act as a phosphate donor in mammalian cells expressing PKM2 by participating in phosphorylation of the glycolytic enzyme phosphoglycerate mutase. Therefore, pyruvate can be generated without inclusion of PKM2 and also without concomitant ATP production, again, lowering the amount of ATP and allowing increased glycolysis. Additionally, at least theoretically, pyruvate kinase, though thermodynamically unfavourable, can function in reverse and consume ATP together with pyruvate to generate PEP [52,54]. Intriguingly, large studies have shown that if ATP contribution from aerobic glycolysis and OXPHOS are compared, then based on 31 different cell lines, average ATP contribution from aerobic glycolysis is a mere 17%. This data does not support the hypothesis that cancer cells exhibit aerobic glycolysis to generate ATP faster [51].

Warburg effect has also been associated to elevated need for biomass and that metabolic pathways branching from glycolysis are contributing carbon to synthesis of nucleotides, lipids and proteins. During aerobic glycolysis most of the glucose carbon is not retained, but excreted as lactate. Less than 10% of glucose carbon is directed into anabolic pathways in different proliferating cell types, and if even as little as 0.1-1% of the dramatically increased glucose uptake enters biosynthetic pathways, it can more than double the basal flux into the same pathways in non-transformed cells. Therefore, these rapidly proliferating cells are benefiting from excreting more than 90% of glucose taken up by the cell. One suggestion for explaining this is the notion that if the glycolytic pathway from glucose to lactate is kinetically very active, but biosynthetic pathways stemming from it are less active, the less active pathways require increased substrate pools for sufficient throughput and hence, the

glycolysis must be enormously elevated to sustain the less active branched pathways. Additionally, tumors might benefit from excreting large amounts of lactate as it can condition the microenvironment, promote invasion and suppress immune reaction and through that facilitate tumor survival, growth and invasion. But inversely, many normal proliferating cells utilize aerobic glycolysis without evident benefit from excreted lactate and hence it indicates that high glycolysis is unlikely chosen for in cancer cells because of high lactate excretion [49,51,52]. Therefore, though Warburg effect is evident in many types of cancer, benefits of it for the cancer cells are unclear. At the same time OXPHOS in the same cancer cells is still functional and seems to provide most of the intracellular ATP, but there are many aspects that are affecting this system, whether arising from within the tumor cell or from environmental restrictions.

2.4 Functionality of OXPHOS

In differentiated physiological cells the mitochondrial respiratory chain (Figure 9) is functioning to pump protons across inner mitochondrial membrane (by complexes I, III and IV) with concomitant transfer of electrons through the chain to the final electron acceptor, molecular oxygen, to generate water. Pumping of the proteins generates proton motive force (Δp), composing of a small chemical component (ΔpH) and a large electrical component (membrane potential $\Delta\psi$), which is then used by complex V (ATP-synthase) to regenerate ATP from ADP and inorganic phosphor P_i. Chemiosmotic nature of this process was first suggested by Peter D. Mitchell [55] in 1961, who later received the Noble Price for his remarkable work in this field.

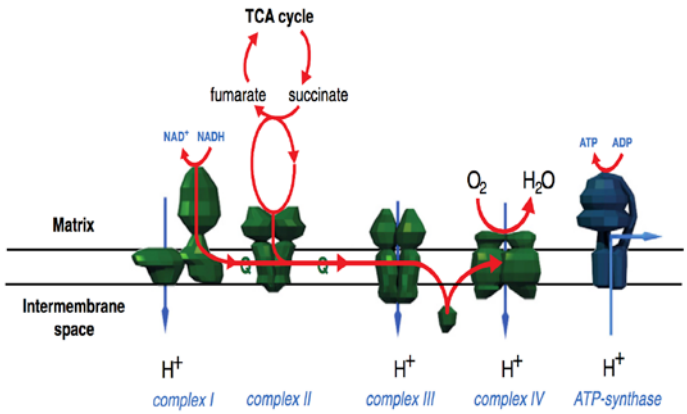

Figure 9. Unassembled model of mitochondrial respiratory chain. From [56] with permission.

For the ATP regeneration to remain functional, ATP and ADP are shuttled between cytosol and mitochondrial matrix by the adenine nucleotide transporter ANT. Respiratory chain is feed by tricarboxylic acid cycle (TCA) which generates NADH and $FADH_2$. Mitochondrial respiratory chain disorders,

however, are the most common forms of mitochondrial disease and have direct effects on ATP regeneration and metabolism. There can be underlying genetic mutations to it, but it is rarely clear how different mutations cause specific clinical phenotypes and affect enzyme activity [56], and additionally, severe conditions in tumor microenvironment, like limited oxygen supply, drive non-genetic changes caused by exogenous effectors.

Most known oncogenic mutations in the respiratory chain are found in Complex II (succinate dehydrogenase, SDH), an enzyme which is also part of the TCA cycle. Out of other TCA cycle enzymes, mutations in fumarate hydratase (FH) and isocitrate dehydrogenase (IDH) are also well described. Mutations in SDH subunits have been described in pheochromocytoma, neuroblastoma, thyroid and ovarian cancers, whilst FH mutations have been reported in kidney, bladder, breast and testicular cancers [57]. SDH dysfunction results in accumulation of its substrate, succinate, which acts as a competitive inhibitor of the 2-oxoglutarate-dependent HIF-prolyl-hydroxylases and as a result stabilizes hypoxia-inducible factor 1 alpha (HIF-1α), which in turn activates genetic programs that facilitate angiogenesis, anaerobic metabolism, and suppress both mitochondrial pyruvate catabolism and oxygen consumption. HIF-1α is one of the key regulators of metabolism and is often highly expressed in many tumors [50,58]. Unlike SDH or FH, mutations in IDH1 and IDH2 change the function of those enzymes and cause these to produce an oncometabolite, 2-hydroxyglutarate [34], which in turn acts as a competitive inhibitor on alpha-ketoglutarate dependent dioxygenases and leads to genome wide histone and DNA alterations [59].

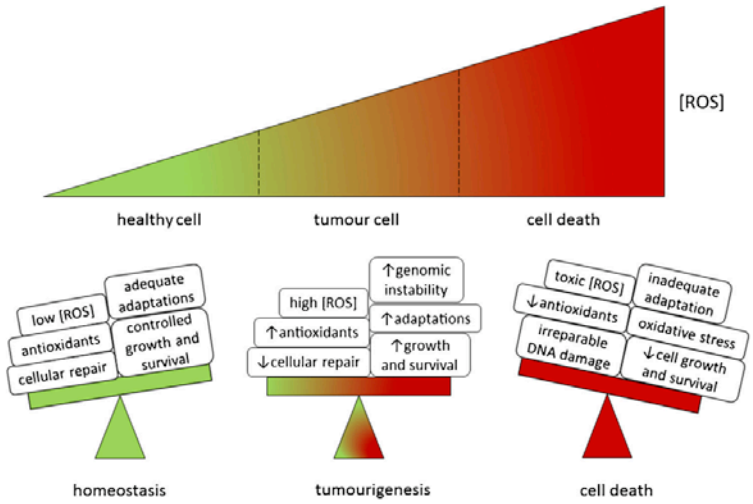

Figure 10. Role of ROS at different concentrations. From [60] with permission.

Oxygen is the final acceptor of electrons in the respiratory system as described before, but if for some reason this reduction is incomplete, it leads to formation of reactive oxygen species (ROS) [61]. ROS are short-lived and highly unstable oxygen free radicals, for example, superoxide ($O_2^{\bullet -}$) and

hydroxyl ($OH^{\bullet}$), which are usually converted into freely diffusible non-radicals like peroxide (H_2O_2) [60]. ROS formation is present in all respiring cells, and despite these were initially thought to exclusively cause damage and to lack physiological function, recent years have shown that ROS act as a signalling molecules to regulate wide variety of intracellular processes like adaption to hypoxia, regulation of autophagy, regulation of immunity, regulation of differentiation, and regulation of longevity [62]. Although ROS can originate from various endogenous processes or arise from interactions with exogenous sources, most of ROS are coming from the mitochondria with estimation that 1% of O_2 consumed by mitochondria is converted to $O_2^{\bullet-}$. ROS levels in physiological setting are relatively low, but if cellular antioxidant defence system is overwhelmed, whether by increased ROS or decrease in cellular antioxidant capacity, oxidative stress occurs (Figure 10). Oxidative stress results in direct or indirect ROS-mediated damages to nucleic-acids, proteins, and lipids, and has been implicated in neurodegeneration, atherosclerosis, diabetes, aging, and carcinogenesis. In malignant cells, overproduction of ROS has been shown to induce variety of biological effects including enhanced cell proliferation, DNA damage and genetic instability, cellular injury and cell death, autophagy and resistance to therapies [60,63].

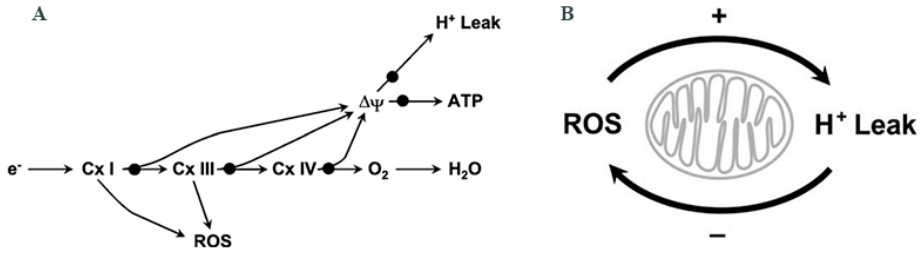

Figure 11. (A) Network of energy flow in mitochondria starting from electrons e^- (e.g. from NADH) to generate H_2O as a result of canonical TCA together with $\Delta\psi$. The latter is then used to generate ATP or dissipated as H^+ leak. **(B)** Model of feedback loop, where increased formation of ROS increases proton leak, which in turn reduces the amount of ROS formation. From [64] with permission.

Interestingly, elevated ROS formation induces proton leak through the mitochondrial inner membrane to decrease Δp and that in return reduces ROS formation. Therefore, a feedback loop is formed (Figure 11). Proton leak in mitochondria is a highly important evolutionary phenomenon as it has been reported in every organism studied to date, but there are still many controversies regarding its mechanism, regulation and exact physiological relevance in health and disease [64].

2.5 OXPHOS suppression and supercomplexes

Mitochondria are the main source of ROS and Complex I (CI) of the respiratory system is the main source of mitochondrial ROS production [65].

CI, with 45 subunits, is the first and largest complex of the respiratory chain. It is under bigenomic control (7 units from mitochondrial plus 38 units from nuclear DNA) and therefore tightly regulated interaction between the mitochondrial and nuclear genomes is required for successful biogenesis and functioning of the complex. Isolated CI deficiency is most frequently diagnosed form of mitochondrial OXPHOS disorders and it has wide clinical variety. Interestingly, in most of the patients, underlying cause of the CI deficiency remains unknown. Hoefs *et al* [66] studied 34 patients with known CI deficiency (non-malignant diseases) and concluded that genetic cause was identifiable only in 14 patients (i.e. 59% of the patients had CI deficiency of unknown origin). Therefore, in patients with unknown aetiology, the observable deficiency in CI might be caused by mutations in unknown assembly factors, by posttranslational modifications or possibly by misaligned supercomplex formation [66,67]. In malignancies, mutations in CI are found most frequently out of all respiratory chain complexes and these changes have been linked to development of colon, thyroid, pancreas, breast, and bladder cancers [68].

Role of CI dysfunction in cancers, however, is controversial and despite increased ROS formation due to mutations in CI have been linked to metastatic phenotype in some lung and breast tumors, functionality of CI is still necessary under low glucose conditions – a condition frequently found in cancer microenvironment [68,69]. In addition, it has been shown that severe loss-of-function in CI increases NAD+/NADH ratio, increases alpha-ketoglutarate concentration and via that, contributes to destabilization of HIF-1α and hence, to inability to adapt aerobic glycolysis which in turn results in hampered cell growth. However, certain degree of CI dysfunction may be advantageous under different selective pressures, but complete impairment of OXPHOS can be fatal for tumor development [70]. As CI in cancers is not only important in energy generation, but also in maintaining redox homeostasis, production of ROS and regulation of biosynthetic pathways, it is not unexpected that CI has become a target for drug development [71]. CI, however, should not be considered as a single entity in mitochondrial inner membrane.

Up to 30% of the cytosolic volume consists of proteins and this volume occupation is even higher in mitochondria, where it takes up about 60-70% of the mitochondrial volume. Additionally, mitochondrial inner membrane is ~50% protein by mass and ~70% by area. Together, it inevitably creates spatially restrictive environment that in turn supports formation of protein-protein interactions, micro compartmentalization, metabolic channelling, and functional coupling [64,72]. One possible example of it is the respiratory chain. The respiratory chain complexes have historically been considered as separate entities under a simple “fluid model”, where the complexes freely float in the inner mitochondrial membrane and for example electrons from Complex I transfer to Complex III via random diffusion of Coenzyme Q molecules in the lipid bilayer. Starting from year 2000, however, increasing number of studies have shown that respiratory chain complexes are forming higher assemblies (supercomplexes) and if all complexes are assembled, a “solid model” is

formed. Based on a third interpretation, termed “plasticity model”, supercomplexes in the solid model are not fixed and (some of the) supercomplexes can dissociate back to separate entities under certain conditions and function based on a shared Coenzyme Q pool in parallel to supercomplexes (Figure 12). Additionally, a specific type of proteins, termed supercomplex assembly factors or SCAFs, are required for supercomplex formation, but exact mechanistic role of SCAFs has not been described in sufficient terms and role of cardiolipin has also been proposed in some models [73-75].

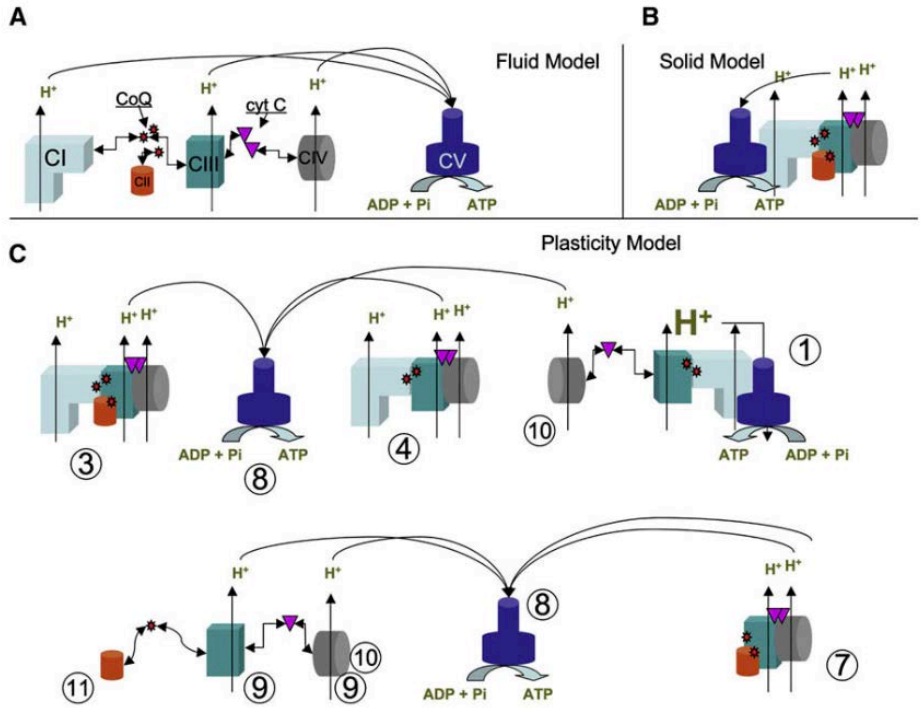

Figure 12. Three models of supercomplex formation in the respiratory chain. **(A)** Fluid model, where all complexes are separate entities and coenzyme Q/cytochrome c are freely moving in the inner mitochondrial membrane. **(B)** Solid model, where all respiratory chain complexes are incorporated into supercomplexes with inclusion of coenzyme Q/cytochrome c. **(C)** Plasticity model, where supercomplexes with different stoichiometry exist together with freely moving complexes. Numbers indicate to different assemblies or co-assemblies of the complexes. From [75] with permission.

Stoichiometry of respiratory supercomplexes remains debatable and it can be largely attributed to shortcomings in understanding exact drivers and mechanisms behind formation and dissociation. However, studies have demonstrated that CI is an integral part of all respirosomes and inclusion of Complex II (CII), as a second entry point to the respiratory chain, is unclear. CI forms higher assemblies with Complex III (CIII) and Complex IV (CIV) and therefore such supercomplex can easily transfer electrons to the final acceptor,

molecular oxygen. One explanation to benefits of supercomplexes is that they confer catalytic and kinetic advantages, as catalytic centres of different complexes are in very close proximity and reducing equivalents do not leave the supercomplex structure and transfer of these is kinetically favoured. Stable structures of $CI_1III_2IV_1$ have been proposed in porcine heart mitochondria, but despite complexes I, III, and IV are found in these, they are also found as separate entities, again supporting the plasticity model. Recently, even higher assembly has been proposed, termed mitochondrial respiratory megacomplex, having stoichiometry of $MCI_2III_2IV_2$. This megacomplex was extended even further and hypothesis was proposed that 2 copies of Complex II fit into caps between complexes I and IV, conclusively forming electron transport chain supercomplex which includes all electron transport chain components (Figure 13) [73,76].

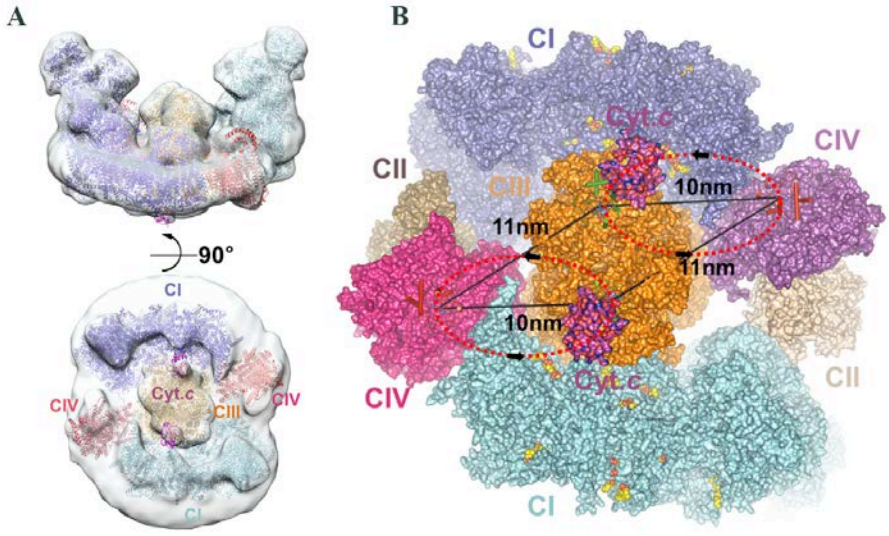

Figure 13. (A) Structure of megacomplex $MCI_2III_2IV_2$. **(B)** Structure of electron transfer chain megacomplex $MCI_2II_2III_2IV_2$. Different colours represent different respiratory chain complexes and these coincide with colours of the respective abbreviations. From [73] with permission.

Structure, composition and stoichiometry of respiratory chain supercomplexes remains to be an issue of debate and thorough investigation, as better understanding of how and why these are formed, which additional cellular components are necessary for the formation, and what leads to dynamic dissociation of the supercomplexes, can improve our understanding of cellular respiration in physiological setting, but also in disease.

3. Adaption of Metabolic Control Analysis

During a large part of last century, there was a general agreement among biochemists that regulation and control of metabolism is centered around key rate-limiting enzymes. In this case, varying activity of this single rate-limiting enzyme in a pathway would change the flux of the pathway, but varying activity of any other (non-rate limiting) enzyme in the same pathway would have no effect and hence a simple binary system is defined. Different methods were developed to identify those single entities having a central role, but the concept of rate-limiting step proved to be unsatisfactory both theoretically and experimentally. An alternative approach was introduced in the framework of Metabolic Control Analysis (MCA), where not just one but several enzymes in a pathway can affect the metabolic flux and in that way regulatory mechanism in complex metabolic systems can be determined. Term Flux Control Coefficient (FCC) is used to describe the rate of effect each enzyme in a pathway has, specifically, FCC shows fractional change in the overall metabolic flux of a pathway that is caused by 1% change in the rate of an enzyme in this pathway. In terms of mathematical representation, FCC, or C_{vi}^{J}, is the degree of control that the rate (*V*) of a given enzyme (*i*) exerts on flux *J*, or:

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

where the expression dJ/dV_i describes the variation in the flux (*J*) when infinitesimal change is done in the concentration or activity of enzyme *i*. In practice, however, infinitesimal changes in V_i are undetectable and only measurable changes are introduced. If a small change in V_i promotes a significant variation in *J*, then this enzyme exerts an elevated flux control and the opposite is true, if even significant change in V_i produces only very small or negligible change in *J*. [77-80].

In case of OXPHOS in functional, unperturbed mitochondria, changing the amount of specific enzyme is possible through genetic means, but it is hugely more beneficial to use varying amounts of specific inhibitors to change the activity of a desired enzyme. There are inhibitors for all enzymes in OXPHOS system and the MCA theory can be rearranged accordingly. The exact adaptation is dependent on the nature of an inhibitor (irreversible, noncompetitive, and competitive), but in case of irreversible inhibition the mathematical result would be:

$$C_{Vi}^{J} = \frac{dJ/J}{dI/J_{max}} \qquad \text{(Equation 2)}$$

where *I* represents used inhibitor concentration and I_{max} the inhibitor concentration necessary for total inhibition of the enzyme [80,81].

Once a metabolic pathway of interest has been defined and analyzed, whether via manipulating enzyme quantity or altering enzyme activities with inhibitors, the sum of all FCC in the given pathways should be 1 (termed summation theorem). Interestingly, this proves that FCC of an enzyme is not specific to that enzyme in isolation, but it is a property of a whole system – once an amount of an enzyme is increased, its FCC decreases, but it requires that the FCC of some other enzyme in the same pathway increases to meet the requirement that the sum of all FCCs must equal to 1. But it also shows that as FCCs of a pathway can be redistributed among different enzymes based on alternating circumstances and every measured value specifically represents the metabolic state it was measured in [79-81]. However, there are limitations to the summation theorem, because it has been shown that the sum of 1 is true only for enzymes in linear, unbranched pathways. If enzyme-enzyme interactions are present, the sum of FCCs will exceed that of 1 [82] and it can be concluded that higher organisatory levels between proteins are indicated by increased sum of FCCs.

Aims of the thesis

The main aim of this PhD thesis was to quantitate changes in energy metabolism caused by malignant transformation in human breast and colon epithelial tissues. For the given aim, both malignant and healthy tissue samples were used from patients undergoing routine breast cancer or colorectal cancer surgery.

The objectives of the current study were the following:

1. Analyse properties of respiratory chain complexes comparatively in HBC, HCC and in their respective healthy epithelial counterparts.
2. Quantify flux control coefficients for components of the ATP synthasome in order to understand similarities and differences between different tissues, but simultaneously, assess suitability of MCA for detecting presence of supercomplexes.
3. Measure ADP-dependent respiration in four different tissue sample types under different metabolic conditions and compare the results with equal *in vitro* models, and preferably, with results from pathology reports and longitudinal data.
4. Measure and calculate apparent K_m (ADP) values for the different human sample types to position the results based on mitochondrial affinity for ADP, but also to detect uniformity of mitochondrial populations.

Materials and methods

4. Materials

4.1 Human samples

The tissue samples were provided by the Oncology and Hematology Clinic at the North Estonian Medical Centre (Tallinn). All the samples were analyzed immediately after surgery. Only primary tumors were examined and information from respective pathology reports was provided by the North Estonian Medical Centre for all the analyzed samples. Informed consent was obtained from all the patients and coded identity protection was applied. All investigations were approved by the Tallinn Medical Research Ethics Committee and were in accordance with Helsinki Declaration and Convention of the Council of Europe on Human Rights and Biomedicine. The entire group consisted of 34 patients with breast cancer and 55 with colorectal cancer.

4.2 Cell cultures

MDA-MB-231 and MCF-7 cells were grown as adherent monolayers in low glucose (1.0 g/L) Dulbecco's Modified Eagle's Medium (DMEM) with stable L-glutamine and sodium pyruvate (from CAPRICORN scientific) supplemented with 10% heat-inactivated fetal bovine serum, 10 μg/mL human recombinant Zn-insulin, and antibiotics: penicillin (100 U/ml), streptomycin (100 μg/mL) and gentamicin at a final concentration of 50 μg/mL. Cells were grown at 37 °C in humidified incubator containing 5% CO_2 in air and were sub-cultured at 2-3 day intervals.

4.3 Chemicals

All chemicals were purchased from Sigma-Aldrich Chemical Com. (USA) and were of the highest purity available (>98%).

5. Methods

5.1 Mitochondrial respiration in permeabilized tissue samples

Numerous studies have demonstrated that isolated mitochondria behave differently from mitochondria *in situ* [83-86]. We therefore have investigated respiratory activity of tumor and control tissues *in situ* using the skinned sample technique [83,87-89]. This method allows to analyze the function of mitochondria in their natural environment and leaves links between cytoskeletal structures and mitochondrial outer membrane intact. The preservation of this link is crucial, as these intracellular structures have pivotal role in regulating permeability of the outer mitochondrial membrane [90-93]. Cytochrome c test was used to confirm integrity of the mitochondrial outer membrane (MOM) [87-89,94]; mitochondrial inner membrane quality was checked using carboxyatractyloside (CAT) test as the last procedure in every experiment [87-89,94]. Rates of O_2 consumption were assayed at 25 °C using Oxygraph-2k

high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria) loaded with pre-equilibrated respiration buffer - medium-B [89]. Activity of the respiratory chain was measured by substrate-inhibitor titration as described earlier [89,95]. The solubility of oxygen at 25 °C was taken as 240 nmol/ml [96]. The solubility of oxygen is much lower at 37 than at 25 °C, but also the skinned samples from malignant clinical material are more stable at 25 °C. All rates of respiration (V) are expressed as nmol O_2/min per mg dry tissue weight for solid tumors and as nmol O_2/min per million cells for cell cultures.

5.2 Metabolic control analysis

The method is described in more detail in paragraph 3.

We applied MCA to human breast and colorectal cancer skinned samples to determine the FCCs for respiratory chain complexes. The flux was measured as the rate of O_2 consumption in permeabilized tissues derived from HCC and HBC patients when all components of the OXPHOS system were titrated with specific irreversible or pseudo irreversible inhibitors to stepwise decrease selected respiratory chain complex activities according to a previously published method. 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 [89,97-99].

5.3 Western blot analysis

For describing expression levels of mitochondrial RC complexes, postoperative human tissue samples (70-100 mg) were crushed in liquid nitrogen and homogenized in 20 volumes of RIPA lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 0.1% Triton X-100 and complete protease inhibitor cocktail (Roche)) by Retsch Mixer Mill at 25 Hz for 2 min. After homogenization, samples were incubated for 30 min on ice and centrifuged at 12,000 rpm for 20 min at 4 °C. The proteins in the supernatants were precipitated using acetone/TCA to remove non-protein contaminants. Briefly, supernatants were mixed with 8 volumes of ice-cold acetone and 1 volume of 100% TCA, kept at -20 °C for 1h and then pelleted at 11500 rpm for 15 min at 4 °C. The pellets were washed twice with acetone and resuspended in 1x Laemmli sample buffer.

Proteins were separated by polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride (PVDF) membrane and subjected to immunoblotting with the total OXPHOS antibody cocktail (ab110411). Then, the membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibody and visualized using an enhanced chemiluminescence system (ECL; Pierce, Thermo Scientific). After chemiluminescence reaction, the PVDF membranes were stained with Coomassie Brilliant Blue R250 to measure the total protein amount. Signal intensities of complexes I-V were calculated using ImageJ software and results normalized to total protein intensities.

Expression levels of Complex I in HCC and normal tissues were additionally estimated using anti-NDUFA9 antibody that corresponds to NADH

dehydrogenase 1α subcomplex 9 (SAB1100073). The samples were incubated and visualized as described above. Levels of NDUFA9 encoding protein were normalized to total protein content.

5.4 Citrate synthase activity

Activity of citrate synthase in tissue homogenates was measured as described by Srere [100]. Reactions were performed in 96-well plates containing 100 mM Tris-HCl pH 8.1, 0.3 mM AcCoA, 0.5mM oxaloacetate and 0.1 mM DTNB using FLUOstar Omega plate reader spectrophotometer (BMG Labtech).

5.5 Confocal microscopy

The mitochondrial content was quantified in paraffin embedded neoplastic and normal colon or breast tissue samples via selective marking of mitochondrial outer membrane translocase Tom20 (Santa Cruz Biotechnology, sc17764). The Tom20 fluorescence intensity was normalized against whole -tubulin (Abcam®, ab6046) fluorescence. Conforal images were collected using Olympos FV10i-W inverted laser scanning confocal microscope.

5.6 Data analysis

Data in the text, tables and figures are presented as mean ± standard error (SEM). Results were analyzed by Student-test, p-values <0.05 were considered statistically significant.

Results

6. Properties of mitochondrial respiratory chain

6.1 Quantitative changes in number of mitochondria

Quantitative change in relative number of mitochondria in both HBC and HCC were measured to describe the general changes caused by tumor formation. In case of HCC, analysis with confocal microscopy showed, that once normalized against total level of tubulin in both malignant and healthy colon tissue, malignant tissue harbours ~70% more mitochondria than the surrounding normal tissue (Figure 14).

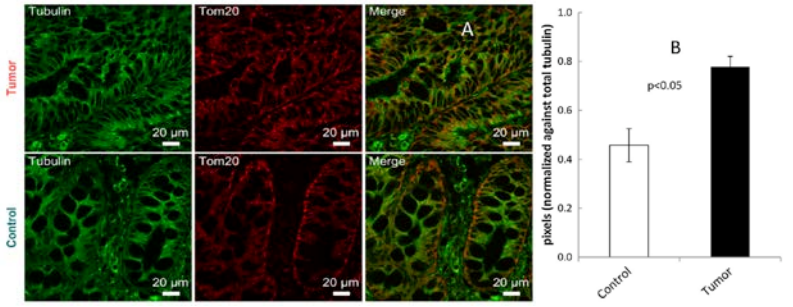

Figure 14. Relative number of mitochondria in HCC and in respective healthy colon tissue. Displayed as confocal image **(A)** and as quantified results **(B)**.

Similar tests have previously been conducted in our lab also on HBC to describe the changes in respective tissues and it was found that the number of mitochondria increase up to 100% (Figure 15) [89].

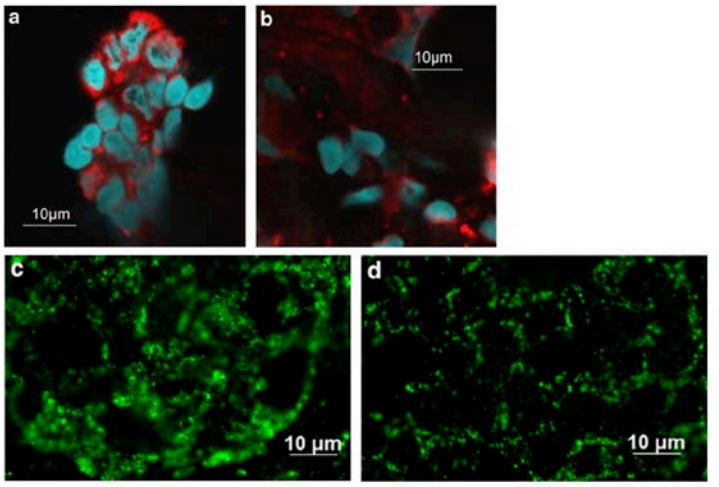

Figure 15. Confocal microscopy images of mitochondria in HBC (**a, c**) and healthy control tissues (**b, d**). Mitotracker Red® together with DAPI staining (**a, b**); VDAC staining (**c, d**). From [89] with permission.

Additionally, activity of citrate synthase was measured in all four tissue types to estimate mitochondrial mass. Enzymatic activity was measured as mU/mg protein (Table 2). Citrate synthase activity confirms that in case of HCC, mitochondrial number increase in range of 80%, but in case of HBC the increase was even larger, as it rose up to 250% when compared to healthy breast tissue taken from the same patients.

Table 2. Activity of citrate synthase as marker of mitochondrial mass, in human breast and colorectal cancers as well as in corresponding normal tissues. Enzymatic activity measured as mU/mg protein, mean ± SE.

Samples	**Enzymatic activity**	***p*-values***
HBC	14.55 ± 2.59; n = 9	< 0.005
Healthy breast tissue	4.09 ± 1.15; n = 9	
HCC	83 ± 19; n = 6	< 0.05
Healthy colon tissue	46 ± 6; n = 6	

Changes in increases between those two locations can be explained by proliferative activity in their respective control samples, as colon tissue renews much more rapidly than ductal epithelium in mostly post-menopausal women in our studies.

6.2 Quality of permeabilized samples

Quality of samples is paramount for assuring adequacy of results and conclusions thereof. Over or under-treatment with saponin, or mechanical damage in device chamber can lead to false results when working with skinned tumor fibres. These damages can rupture mitochondrial outer or inner membrane and render measurements misleading, but quality control measures are applied to exclude such shortcomings.

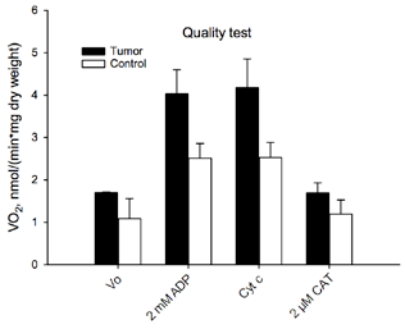

Figure 16. Quality control tests on HCC and respective control tissues to confirm intactness of both mitochondrial outer and inner membranes in saponized samples.

When cytochrome c is added to samples with ADP-activated respiration, no changes are expected in respiratory rate if mitochondrial outer membrane is intact. Similarly, if CAT (ANT inhibitor) is added to the same test thereafter,

the respiratory rate should decrease to its initial basal rate to conform simultaneous intactness of the mitochondrial inner membrane. Quality control results for HCC samples are depicted on Figure 16 and a typical illustration of HBC quality test on Figure 17.

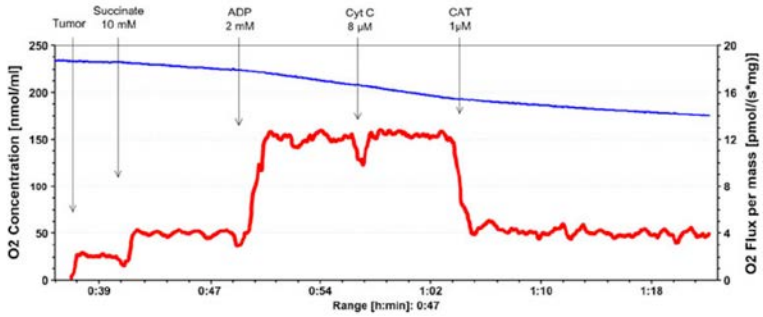

Figure 17. Quality control test on HBC sample successfully confirming intact outer and inner mitochondrial membranes. From [89] with permission.

The described quality tests were performed on all analysed tissue samples in medium-B solution [89] loaded with 5mM glutamate, 2mM malate and 10mM succinate as respiratory substrates. 8µM cytochrome c and 2 µM CAT were used.

6.3 Respiratory chain analysis

Basal (state 2) respiration rates were compared in all tissue types of interest. Measurements were conducted in medium-B in presence of 5mM glutamate, 2mM malate and 10mM succinate as respiratory substrates, equally to that described for the quality control. Control samples were separated from malignant material by hospital pathologists and therefore the same patients also donated control samples for the measurements (Figure 18).

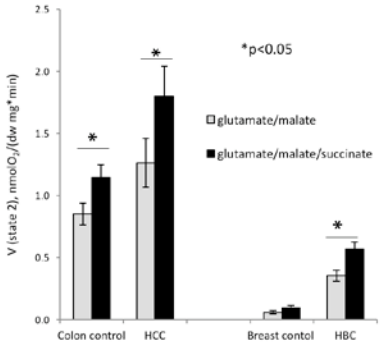

Figure 18. Comparison of basal respiration rates in permeabilized HBC, HCC and their respective control samples. Bars are mean ± SEM, n = 8 for colon samples, n = 12 for breast samples, *p<0.05.

Basal respiration in both malignant sample types is higher than that in their respective healthy tissues. Interestingly, addition of succinate to glutamate/malate increased basal respiration in all sample types.

To assess respiratory capacities of different respiratory chain segments in ADP-dependent manner, multiple substrate-inhibitor titration protocols were used. Addition of 2mM ADP caused increased respiration in all sample types. For assessing CI activity, only glutamate/malate were used as substrates when respiration was activated with ADP. Subsequent addition of rotenone, a CI-specific inhibitor, caused the respiration rates to decrease back to the basal levels. For assessing CII activity, CI was inhibited with rotenone, glutamate/malate was not added and only succinate was used as a respiratory substrate – under these conditions CII is the only entry point for electrons into the respiratory chain. To assess CIII, both CI and CII were activated with their respective substrates and exogenous ADP, but CIII was thereafter inhibited with its specific inhibitor antimycin-A (ANT). To assess the maximal capacity of CIV, mixture of 5mM ascorbate and 1mM tetramethyl-p-phenylenediamine was added to the reaction chamber, which feeds electrons directly to cytochrome c and therefore keeps the function of CIV at its maximum. Respective results of all the described experiments are indicated in Table 3.

Table 3. Multiple parameters of the respiratory chain in permeabilized samples.

Parameter	HBC, n = 7 [89]		HCC, n = 7 [88]	
	tumor	control	tumor	control
V_o	0.294 ± 0.024	0.004 ± 0.007	1.06 ± 0.14	0.82 ± 0.15
V_{ADP}	0.71 ± 0.06	0.055 ± 0.004	2.02 ± 0.21	1.39 ± 0.21
V_{rot}	0.34 ± 0.04	0.070 ± 0.015	0.91 ± 0.11	0.85 ± 0.14
V_{succ}	0.74 ± 0.10	0.076 ± 0.008	2.22 ± 0.26	1.33 ± 0.18
V_{ANM}	0.38 ± 0.04	0.071 ± 0.018	1.04 ± 0.09	0.69 ± 0.07
V_{cox}	2.36 ± 0.33	1.23 ± 0.18	6.59 ± 0.71	3.84 ± 0.58

As is evident from Figure 18 and Table 3, ADP-dependent oxygen consumption in HBC control tissue is extremely small and reliable results are difficult to be obtained using Oxygraph-2k high-resolution respirometers. To avoid artifacts and misleading calculations, results for this sample type have been left out in most of the following data.

Results in Table 3 can be used to calculate relative contribution of different respiratory complexes. V_{glut}/V_{succ} is indicative of CI activity and the experiments suggested that CI deficiency is evident in HCC on functional level. No significant differences were registered for V_{succ}/V_{COX}, which serves as a surrogate marker for assessing CII activity (Figure 19).

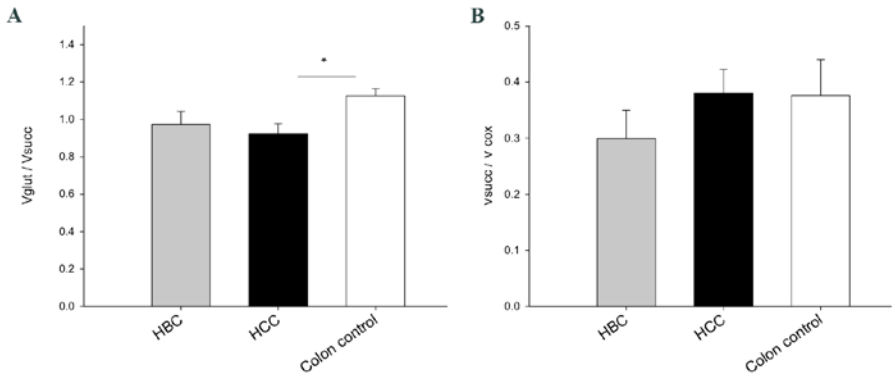

Figure 19. Assessment of functionality of CI (**A**) and CII (**B**) in HBC and HCC samples as described by V_{glut}/V_{succ} for CI and by V_{succ}/V_{COX} for CII. Bars are mean ± SEM, n = 7 for HBC [89], n = 7 for HCC, **p*<0.05. Partially from [89] with permission.

To further evaluate the possible suppression of CI in HCC, expression levels of all respiratory chain complexes were quantified in this sample type using total OXPHOS antibody cocktail. Surprisingly, no suppression was confirmed on expression level once the data was normalised to total protein content (Figure 20).

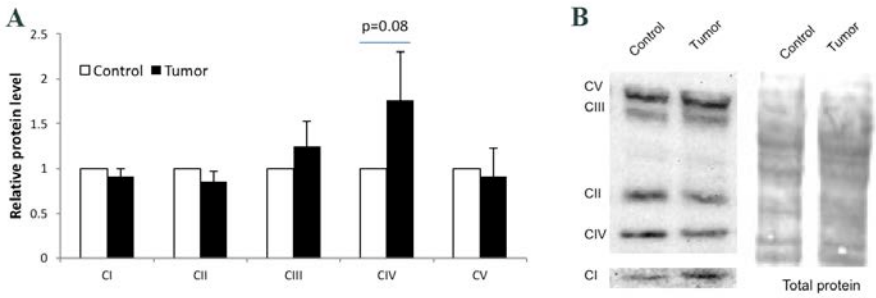

Figure 20. Quantitative analysis of expression levels of the respiratory chain complexes in HCC and respective normal tissue samples (**A**) along with a representative Western blot image (**B**). Protein levels were normalized to total protein staining by Coomassie Brilliant Blue; data shown as mean ± SEM of 5 independent experiments.

As expression level of respiratory chain complexes did not confirm the suppression of CI as was previously evident on functional level, expression of CI component NDUFA9 was measured to confirm the initial Western blot result. Surprisingly, the second approach confirmed the suppression of CI on the expression level (Figure 21).

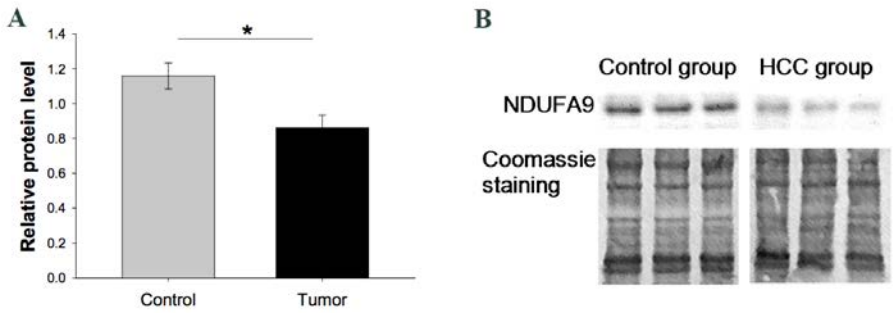

Figure 21. Quantification of Complex I subunit NDUFA9 expression levels using Western blot (WB) and anti-NDUFA9 antibody in HCC and healthy colon tissue samples (**A**); respective WB image of NDUFA9 protein levels (**B**). Levels of NDUFA9 were normalized to total protein content quantified by Coomassie blue staining. Data are represented as mean values ± SEM from 4 independent experiments; * $p<0.05$.

Conflicting results between two approaches of Western blot are indicating that those results are directly dependent on which respiratory chain complex, or specific subunits, are targeted and possibly, which normalization method is applied. Therefore, multiple methodologies or approaches should be applied when studying functionality of respiratory chain complexes.

To understand whether respiratory chain complexes have formed supercomplexes in the studied tissue types, metabolic control analysis was applied to calculate FCCs for all the respiratory chain complexes (I, II, III, IV) and for components of the ATP synthasome (ANT, ATP synthase, P_i transporter). For that purpose, the selected targets in different sample types were specifically inhibited by stepwise dose increase and the FCCs were calculated using three different models (graphical [77,101,102], Small [103], Gellerich [104]). The results were consistent regardless of which calculation method was applied to the dataset (Figure 22, Table 4).

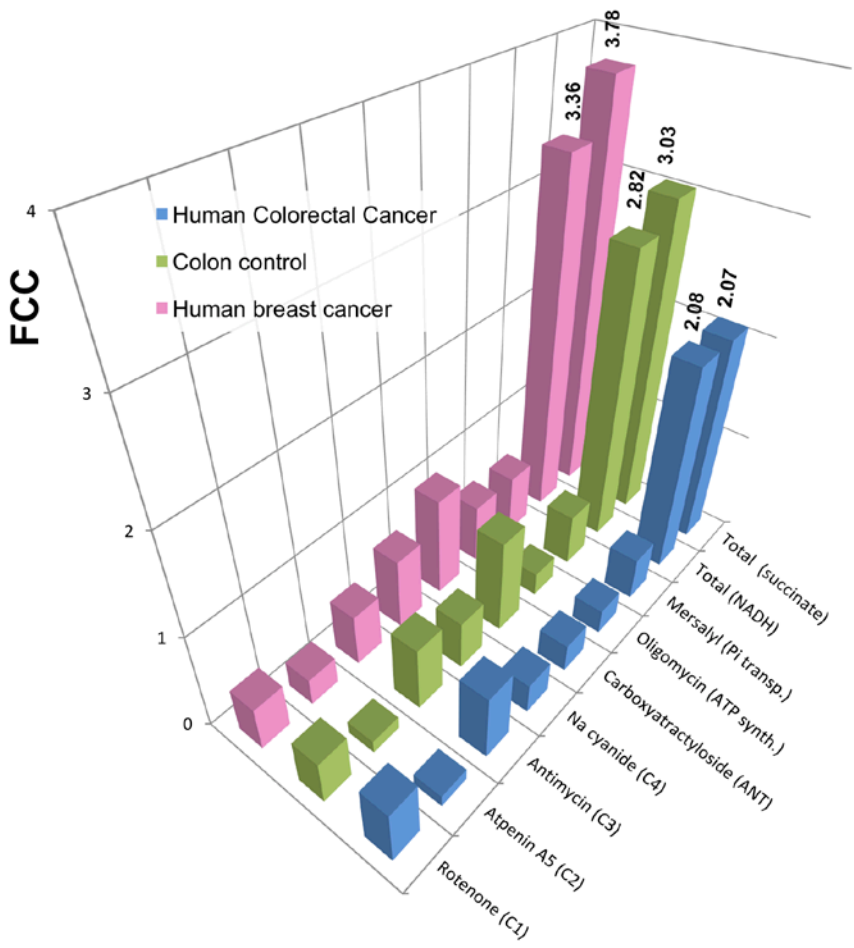

Figure 22. FCCs for ATP synthasome and RC complexes as determined by MCA. Sums of FCCs are calculated as the last bars. Data for HBC is published before in [89], except for Complex II with atpenin A5. Isolated mucosal tissue was used for colon control. From [89] with permission.

For the MCA experiments, 1-100nM rotenone was used as CI inhibitor, 0.1-6μM atpenin A5 as CII inhibitor, 1-200nM antimycin-A as CIII inhibitor, 0.1-40μM Na-cyanide as CIV inhibitor, 1-200nM CAT as ANT inhibitor, 1-600nM oligomycin as ATP synthase inhibitor, and 1-200μM mersalyl as Pi inhibitor. Additionally, sum of FCCs was calculated for two different respiratory chain pathways – NADH-based electron flow through complexes I, III, IV and succinate-based electron flow through complexes II, III, IV (Table 4). FCCs were summarised in the named order to analyse functional coupling between complexes that mediate the electron flow from different sources (NADH or succinate).

Table 4. FCCs for different components of both mitochondrial respiratory chain and ATP synthasome together with used inhibitors and inhibitor concentration ranges. * from [89] with permission.

Component	Inhibitor		FCC		
	Name	**Range**	**HCC**	**Colon control**	**HBC**
CI	Rotenone	1-100nM	0.56	0.45	0.46*
CII	Atpenin A5	0.1-6µM	0.12	0.13	0.28
CIII	Antimycin	1-200nM	0.68	0.66	0.54*
CIV	Na cyanide	0.1-40µM	0.31	0.50	0.74*
ANT	CAT	1-200nM	0.28	0.97	1.02*
ATP synthase	Oligomycin	1-600nM	0.25	0.24	0.61*
Pi transporter	Mersalyl	1-200µM	0.43	0.53	0.60*
Sum 1, 3-7	**Total (NADH)**		**2.08**	**2.82**	**3.36**
Sum 2-7	**Total (succinate)**		**2.07**	**3.03**	**3.78**

Calculation of FCCs is complicated due to high heterogeneity of human tumor samples, which can originate from different tumor subtypes (e.g. Luminal A/B, HER2 or triple negative in HBC; unknown subtypes in HCC), but also from heterogeneity within each tumor and hence, from exact region of the tumor under analysis, or from irregular stromal burden.

6.4 ADP-regulated respiration

For analysing respiratory activity on a higher mitochondrial level, maximal ADP-activated respiration was measured in different human samples and comparison was made with that in respective cell lines. As previously indicated, ADP-activated respiration in breast control tissue is not sufficiently measurable with Oroboros instruments. Healthy breast tissue contains high amount of fat tissue, but extremely low respiration rates were present even if clearly lobular/ductal structures were isolated and measured and hence these data have been left out. In contrast, ADP-activated respiration in colon control tissue is relatively high. To isolate mucosal and smooth muscle contributions to overall colon control respiration results, those two layers were separated and measured in isolation. Results confirmed that mucosal layer is respiring more actively than the underlying smooth muscle layer (V_{max} 1.41 ±0.02 vs 0.65 ±0.03, respectively). Maximal respiration rate in both malignant sample types is remarkably higher than that in the tissue these tumors arose from (Table 5).

Table 5. Apparent K_m for ADP [K_m(ADP)] and maximal rate of respiration (V_{max}) values for ADP dependent respiration calculated for HBC, HCC and their adjacent healthy tissue samples.

Tissues	**V_{max}** ± SEM	**K_m** (ADP), µM ± SEM
HBC	1.09 ± 0.04*	114.8 ± 13.6*
Breast control tissue	0.02 ± 0.01*	-
HCC	2.41 ± 0.32	93.6 ± 7.7**
Colon control tissue	0.71±0.23	256** ± 34

Notes: *from [89] and **[95] with permission; V_{max} values are presented as nmolO_2/min/mg dry tissue weight without proton leak rates. 35 patients used for analysis of HBC and 35 for HCC.

HBC samples can be divided into clinically significant molecular subtypes (Table 1), with Luminal A being least and triple negative the most aggressive. To establish link between human samples and respective cell cultures, maximal respiration in MCF-7 and MDA-MB-231 cell lines was measured as these represent most studied Luminal A and triple negative subtype cell lines, respectively. Based on pathology reports, equal division was made between human samples. The results revealed striking difference as cell cultures and human material showed opposite correlation. In cell lines, triple negative subtype consumed oxygen less actively than the Luminal A cell line, but in human samples, the maximal respiration rate was much higher for the most aggressive triple negative subtype than for Luminal A subgroup (Figure 23).

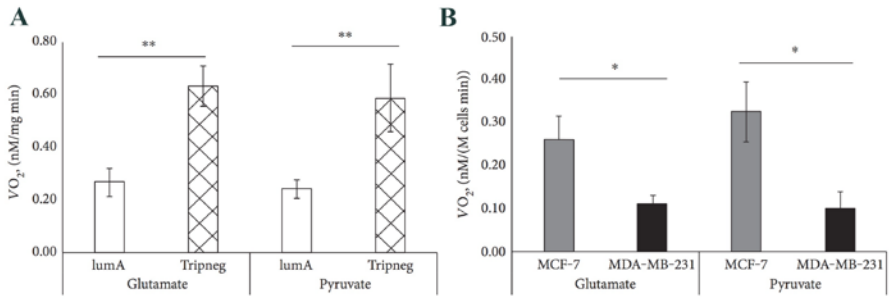

Figure 23. (**A**) Respiration rates for human samples of Luminal A (lumA) and triple negative (Tripneg) HBC subtypes in the presence of 5 mM glutamate or 5 mM pyruvate; n=13/12 for Luminal A and n=7/8 for triple negative subtypes, respectively. **(B)** Respiration rates for Luminal A type MCF-7 and triple negative MDA-MB-231 cells in the presence of 5 mM glutamate or 5 mM pyruvate; n=3 for each measurement; *p<0.05, **p<0.005.

Respiratory parameters for HCC are more difficult to subdivide as there are no standard clinically relevant subtypes for that tumor type. However, all HCC samples were grouped based on disease stage and compared against respective average V_{max} values for each subgroup. Even though increase in V_{max} can be

calculated for early stages, the values for stage IIIC and IV do not fit the expected dynamics (Figure 24). It must be emphasized that disease stage at diagnosis is not a valid biomarker of aggressiveness and therefore such plotting can be debated. Longitudinal data for HCC patients in the study cohort was thereafter analysed and it was found that 7 out of 32 eligible patients had deceased (median follow-up time 47.3 ±4.9 months). When longitudinal data was plotted against V_{max} values in the deceased and alive group, it became apparent that respiratory activity was higher in HCC patients who were more likely to die during the follow-up time (Figure 24).

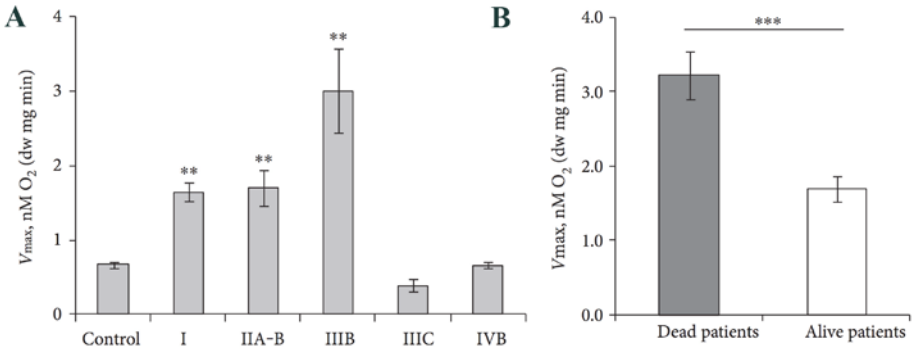

Figure 24. (**A)** Dependence of maximal mitochondrial respiration rate (V_{max}) and HCC stage at diagnosis; compared to V_{max} in colon control. Stage I was calculated as the mean of 13 patients, IIA, IIB - 13 patients, IIIB-4 patients, IIIC-3 patients and IVB-1 patient. Control colon tissue is obtained from 34 patients. Bars are SEM; **p<0.005. **(B)** V_{max} in HCC patients based on disease state in follow-up setting. 7 patients out of 32 are confirmed to have succumbed to HCC (V_{max} = 3.19 ± 0.34), 25 patients out of 32 have stayed in remission (V_{max} = 1.70 ± 0.17), ***p<0.001.

To quantify mitochondrial affinity for exogenous ADP (i.e. permeability of mitochondrial outer membrane), we measured respective apparent Michaelis-Menten constants (K_m) from titration experiments. The data was plotted as rates of O_2 consumption versus ADP concentration and apparent K_m values were calculated thereafter by nonlinear regression equation. Healthy colon tissue exerts low affinity for exogenous ADP (K_m=256 ±3μM), whereas that in HCC is significantly higher (K_m=93,6 ±7.7μM). For HBC, the K_m value (K_m=114.8 ±13.6μM) was close to that of HCC (Table 5). However, when the experimental data was fitted into double reciprocal Lineweaver-Burk plots and K_m/V_{max} values calculated from linearization approach, additional aspects emerged. K_m for HCC was recalculated to be close to that from previous calculation (69±10μM), but biphasic respiration regulation emerged for both colon control and HBC (Figure 25(a)). It has been previously shown that biphasic regulation indicates to two distinct mitochondrial populations within the same sample [83]. In case of colon control, the samples were provided as cross-sections of entire colon wall, and therefore these included both mucosal and smooth muscle phases. To separate the effects of those two physiological layers, the layers were

isolated and measured separately. Measurements confirmed that both layers have distinct values for both K_m and V_{max} and those differences can explain the biphasic result on Lineweaver-Burk plots for colon control (Figure 25 (b)). But aside from colon control, biphasic regulation appeared also for HBC and was evident in samples from 32 patients out of 34.

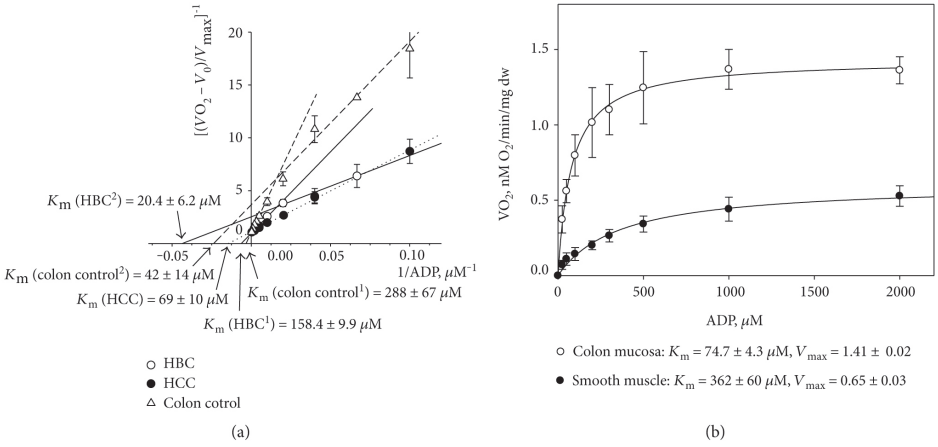

Figure 25. (A) Dependences of normalized respiration rate values for HCC (dotted line), HBC (solid line) and healthy colon tissue samples (dashed line); double reciprocal Lineweaver–Burk plots. Samples from 34 patients with breast cancer and 10 patients with colorectal cancer were examined. **(B)** ADP-dependent respiration in healthy colon mucosa and smooth muscle tissue samples (Michaelis-Menten curve, n=8). Here, V_o and V_{max} are rates of basal and maximal ADP-activated respiration, respectively.

Taken together, there are several distinct changes when HBC or HCC are compared to their respective healthy samples, but interestingly, distinct changes are also present when HBC is compared to HCC (Figure 26). The changes are discussed in more detail in the following Discussion section.

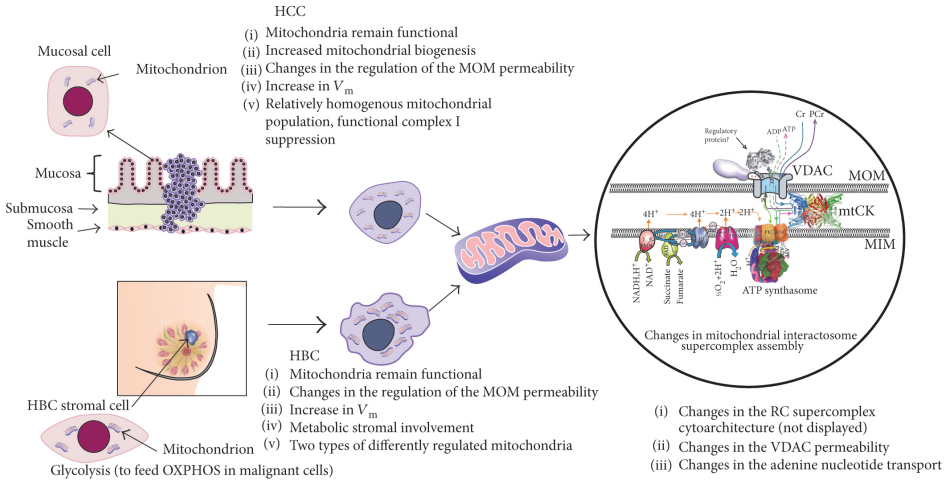

Figure 26. Mitochondrial alterations in HCC and HBC.

Discussion

Under physiological setting and in differentiated cells, mitochondria have central role in regeneration of ATP, maintaining redox homeostasis and in serving certain metabolic substrates for anabolic processes in the cytosol. In cancerous cells, however, mitochondrial significance is maintained, but its exact role seems to have gone through noticeable changes. Pseudohypoxic state, brought on by stabilization of HIF-1α, deregulates normal glucose metabolism, facilitates the Warburg phenotype, and alters microenvironment for the respiratory chain in the mitochondria. Whether caused by changed glycolytic nature, or by intrinsic requirements to maintain cell homeostasis, malignant cells turn their mitochondria into metabolic hubs for breaking down substrates taken up from the environment [29,105]. Simultaneously, however, mitochondrial role in regenerating ATP is also maintained. Therefore, the role of this organelle is central for successful development of malignant cells, and it is often accompanied by cells increasing its number, but looking at mitochondrial number alone does not differentiate the mitochondrial phenotypic changes or allow making any prediction on metabolic phenotype of the entire cell.

In the current study, we confirmed that based on enzymatic activity of citrate synthase, HBC has 3.5 times more mitochondria and HCC has 1.8 times more mitochondria than their healthy epithelial counterparts. It indicates that increased number of mitochondria is necessary for homeostasis in malignant cells. Contradictory, however, is the fact that based on the same results, HBC has 5.7 times less mitochondria than HCC has, but it would be false to conclude that breast cancer, therefore, develops slower or is less life-threatening than colorectal cancer is just because a known source of ATP or hub for metabolic intermediates is not presented in equal numbers. As both of these cancers can and will progress fairly quickly, if left untreated, then it would be logical to conclude that whether mitochondria are actually not that important for malignant cells (because their numbers in similarly aggressive tumors is ~6 times different), or that the number of mitochondria does not define its functional capacity or exact role as discussed above. Interestingly, although out of the scope of this work, glucose metabolism in malignant cells is changed, tricarboxylic acid cycle is not functioning as is known from the transformed cells and role of the citrate synthase has changed. For example, upregulated glutamine metabolism in many cancers is sufficient to replenish TCA cycle intermediates downstream of alpha-ketoglutarate, but more importantly, citrate can be synthesised also from glutamine via reductive carboxylation and this is more prominent under hypoxic conditions [106], a situation common for most cancer cells. Citrate in turn, is exported to cytosol to feed many metabolic pathways that maintain redox homeostasis in the cytosol and mitochondria, provide carbons for fatty acid synthesis, or acetyl groups for protein acetylation, among others [44]. Therefore, the significance of citrate synthase activity as an indicator for mitochondrial number in malignant tumors, should be revisited.

In the light of previous considerations, we analysed function of mitochondrial respiratory chain complexes as separate entities or together with ATP synthasome (includes ATP synthase, ANT and inorganic phosphate carrier) to understand the functional changes that have developed in breast and colorectal cancers in comparison to their healthy counterparts.

To quantify respiratory capacities of different respiratory chain segments, different substrate-inhibitor sets were used to isolate the contribution of each enzyme of interest. All respiratory parameters were elevated for both HBC and HCC (Table 3) and interestingly, basal respiration was also elevated for both malignancies (more pronounced if succinate was added to the media), which might be caused by alternative oxidases or can be related to elevated proton leak across mitochondrial inner membrane (Figure 18) that in turn contributes to maintenance of redox potential under hypoxic conditions [64]. Additional comparisons of different respiratory parameters for different respiratory chain complexes allowed to make additional conclusions. For example, ratio of V_{succ}/V_{COX} is used to quantify functional capacity of CII, but no significant differences were present in the analysed tissue samples. V_{glut}/V_{succ} ratio is used for the same in CI and the results indicated that relative suppression is present in the activity of CI in HCC (but not in other tissue types). CI deficiency is common in most mitochondrial pathologies [66] and has also been confirmed to be present in different cancers, but its exact functional significance has remained controversial. Attempts to confirm the suppression of CI on protein expression level gave conflicting results as whole-OXPHOS antibody cocktail did not indicate reduced CI expression, but when additional antibody against one component of CI (NDUFA9) was used in another attempt to clarify the expression results, the suppression was evident (Figure 20 versus Figure 21). The semi-quantitative Western blot method used in both expression studies can therefore be strongly dependent on exact conditions used for the analysis, but also, these results can be driven by altered conformation of respiratory chain components due to supercomplex formation, what might render some sites of these enzymes incompatible for different antibodies.

Formation of supercomplexes in the respiratory chain is widely accepted understanding, but there are disputes to its benefits or lack thereof. It has been proposed that supercomplex offers kinetic advantages due to substrate channelling between enzymes in the complex, but other studies have concluded that such structures are simply caused by spatial restrictions and confer no functional or catalytic benefits [107]. We concentrated on using oxygraphic measurements to confirm the presence of these entities and possibly, their composition. First, FCCs for each respiratory chain complex and components in the ATP synthasome were measured. FCCs help to define which steps exert the biggest control over the flux through the given pathway. Previous results from our lab have shown that in HBC, CIV (FCC = 0.74) and ANT (FCC = 1.02) are the main respiratory rate controlling steps [89], but this is not the case in HCC. ANT in HCC (FCC = 0.28), in controversy to HBC, has lost its regulatory function when compared to healthy colon mucosa (FCC = 0.97). As ANT-

exerted flux control between HBC and HCC is regulated to opposing extremes (Table 4), then again, it indicates that mitochondria do not undergo simple and uniform modifications as a result of tumor formation, but metabolic interplay between nuclear programs and mitochondria are more complex and can have many different homeostatic outcomes. However, FCCs can also be used to predict functional assemblies between the measured respiratory chain complexes. In a linear system, the sum of FCCs for a pathway should be equal to 1, but the sum increases if the system includes enzyme-enzyme interactions, direct channelling, and/or recycling within multi-enzyme complexes, or in other words, the system has become nonlinear [108-111]. Electron can have two different entry points to the respiratory chain (from CI to CIII/CIV or from CII to CIII/CIV) and both of these points are equally linked to the function of ATP synthasome to close the ATP regeneration loop. Sum of FCCs for those two pathways are in the range from 2.07 to 3.78 in HBC, HCC and colon control (breast control is left out as discussed before; Table 4, Figure 22), and hence, presence of supercomplexes can be expected. More detailed look, however, shows that, similarly to previous findings, CII does not seem to be included to widespread enzyme-enzyme interactions as FCCs for CII are low in all three sample types. In HBC, FCCs for CI and CIII display close values (0.46 vs. 0.54, respectively) and similar situation was registered also for CI and CIII in HCC (0.56 vs 0.68, respectively). In colon control, too, CI and CIII share close values (0.45 vs 0.66, respectively), but equally high FCC for CIV in colon control (0.50) has lowered in HCC (0.31). Such results for the colon samples allow to conclude that under physiological setting, CI is attached to CIII (possibly with multiple copies of CIV), but inclusion of CIV to this supercomplex becomes uncertain as a result of HCC formation. As breast control tissue is not suitable for oxygraphic tests as discusses before, then assessment of dynamics is complicated, but CIV in HBC displays high FCC (0.74), and therefore, unlike in HCC, CIV has maintained a role in supercomplexes in HBC.

The organization of respiratory chain complexes in the mitochondrial inner membrane has been an object of intense debate and it is not studied systematically in human normal or cancerous tissues. Given the known theoretical framework, our results confirm the plasticity model and agree with the data from Bianchi et al. [108], but the distribution of Complex IV remains unclear - both random distribution and association into CI-III-IV supercomplex can be possible.

Despite the uncertain nature of supercomplexes and different distribution of flux control along the respiratory chain and ATP synthasome, whole-mitochondria functional properties, like maximal respiration, can be measured and compared for each sample type. Maximal respiration, V_{max}, is measured in the presence of different substrates together with exogenous 2mM ADP so that the respiratory chain should be saturate to the maximum and ATP regeneration is supported with continuous free ADP. In addition to four clinical sample types, we included two breast cancer cell lines to this study to understand the

links between human samples and *in vitro* models. To our surprise, breast cancer taken freshly from humans displays exact opposite respiratory profile than cultured cell lines. The comparison was made between matching molecular subtypes so that the clinically least aggressive Luminal-A was compared to the clinically most aggressive triple negative subgroup (Table 1, Figure 23). Luminal-A cell line (MCF-7) displayed more than 2-times higher respiration (with both pyruvate and glutamate) than the respective triple negative MDA-MB-231 cell line. In humans, however, under equal test conditions, the opposite is true, and triple negative tumor sample respiratory rates exceed that in Luminal-A samples more than 2 times. In both cases the difference is significant (Figure 23). There are no clinically relevant subgroups for HCC and therefore similar comparison cannot be made, however, we compared disease stage with the respiratory rate in the given stage group (Figure 24). Initial dynamics from stage I to IIIB discontinues thereafter in stages IIIc and IV. Disease stage at diagnosis is not a valid biomarker for disease aggressiveness and hence the approached correlation could not be considered relevant in clinical setting, but, interestingly, respiratory capacity in HCC, irrespective of stage at diagnosis, does seem to have correlation with life expectancy. Longitudinal data for 32 patients was gathered (median follow-up time 47.3 ±4.9 months) and 7 patients were confirmed to be deceased. The results established significant difference between dead and alive patients (Figure 24). It can be argued, based on similarity to HBC subgroup results, that higher respiratory capacity in the deceased HCC patients was already initially indicating to more aggressive disease. In the same framework, it can be suggested that triple negative samples with lower than expected respiratory capacity, could be considered as less aggressive disease, but larger patient cohorts should be analysed to confirm either of these hypotheses.

To characterize mitochondrial affinity for ADP, Michaelis-Menten constants (K_m) were measured next. Initial results gained from nonlinear regression equation (Table 5) were reconsidered once the data was plotted into Lineweaver-Burk plots and K_m calculated through linearization approach. Despite K_m for HCC stayed similar, additional conclusions had to be made for other two sample types. In HBC and colon control, biphasic respiration control was evident on the graph (Figure 25), which indicates to presence of two populations of mitochondria with different affinities for ADP [83]. In colon control, the two populations had very different K_m values (42 ±24 vs 288 ±76) and by separating mucosal and the smooth muscle phases in the colon samples, these results were assigned to the named two layers with additional tests, respectively. In HBC, however, presence of two populations of mitochondria could indicate to several possibilities: a) high stromal burden, but in this case similar results should be present in HCC, but were not; b) metabolic heterogeneity between tumor cells, where some are well oxygenated and others are not, but again, similar results should have been seen in HCC, but were not; c) presence of two-compartment metabolism, where tumor has altered metabolism in the stromal cells. The two-compartment hypothesis has been

described in breast tumors, and in current case the K_m with high value (158.4 ±9.9) represents the tumor cells having high mitochondrial mass, elevated OXPHOS together with β-oxidation, while low K_m (20.4 ±6.2) would represent stromal compartment with high level of glycolysis. Low K_m values (10-30) are usually measured in cultured cells or isolated mitochondria, but in the present work, K_m values in HBC and HCC were up to 7 times higher than that. From one hand, it indicates that isolated mitochondria have lost a layer of regulatory mechanisms, but from the other, that cell cultures, too, even if permeabilized similarly to human samples, have lost some regulatory restrictions. In *in vivo* tumor samples the regulation of mitochondrial outer membrane permeability is more complicated and probably related to interplay between energy transfer pathways or changes in the phosphorylation state of VDAC channels [86,112-115] and also associated with modulation of cytoskeleton or membrane potential as a result of tumor formation.

Taken together, physiological function of epithelial tissue in the breast and in the colon, are vastly different, and there are differences also in malignancies arising from the named tissue types, but both of these cancers share changes that require functional analysis for precise and correct understanding as fixed-state studies alone can yield conflicting or misleading results.

Conclusions

This work offers new insight into how regulation of energy conversion systems has changed as a result of malignant transformation in both colorectal and breast cancers. Main conclusions of the study are summarized as follows:

1. Mitochondrial number in HCC and HBC increases when compared to that in their healthy counterparts, but number of mitochondria is not indicative of their function or functional capacity.
2. Relative CI suppression in evident in HCC, but definite result is present only on functional level as Western blot is giving conflicting results. CI is not supressed in HBC.
3. Metabolic Control Analysis can be used as a kinetic method for confirming presence of respiratory chain supercomplexes.
4. Functional level MCA data regarding supercomplexes in HBC, HCC and colon control tissues, are confirming previous steady-state studies where CII is not included in these assemblies, but CI and CIII, together with varying copy number of CIV, are forming respiratory chain supercomplexes.
5. Respiratory rates in human breast cancer samples do not comply with data from equal *in vitro* cell lines (MCF-7, MDA-MB-231), indicating to severe biological differences between these two sample types.
6. Apparent K_m for ADP confirms presence of two differently regulated mitochondrial populations in HBC that can be associated with two-compartment metabolism in the named disease, but no such phenomenon was registered for HCC nor for either of the control tissues.

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Abstract

Cancer poses an increasing burden for the Western world healthcare systems in particular due to aging society, lifestyle choices and environmental problems. Similar situation is also true for Estonia where deaths due to malignancies are on the second place after cardiovascular reasons. Cancer can be curable if diagnosed in early stages and for that reason many screening programs have been initiated in a number of countries primarily for breast and colorectal cancers – both of these maladies are the object of study in the current thesis.

Despite cancer has been long considered to be a purely genetic disease, it has become increasingly evident that there are numerous changes to metabolism and bioenergetic programs that cannot be attributed to specific mutations. Even though changes in glucose metabolism and cellular respiration were first described more than a century ago, still many controversies exist. To describe functional changes in oxidative phosphorylation and compare the results of equal analysis in both breast and colorectal cancers, we used human cancer samples and certain breast cancer cell lines to generate a comparable structure. Metabolic Control Analysis (MCA) together with accompanying static methods were used to detect that relative suppression of Complex I in the human colorectal cancer samples is evident on functional level, but Western blot analysis can, under standard settings, offer contradicting results to the functional studies, which in turn elevates the use of methods like MCA. But further, results from MCA also suggested that respiratory chain complexes I and III (together with varying copies of Complex IV) are forming supercomplexes in both malignancies, Complex II, however, does not assemble with others and both of these outcomes are in line with previous studies where static methods were used. When respiratory properties of breast cancer two opposing molecular subtypes (luminal A vs triple negative) were compared between human samples and cell cultures, striking difference emerged as opposing relation between respiratory capacity and aggressiveness was concluded. Therefore, underlying biology of cell cultures and human samples might often come to conflicting results and hinder translational research. When further analysis was conducted on permeability of mitochondrial outer membrane for ADP, K_m values suggested presence of two populations of differently regulated mitochondria in human breast cancer samples – a result which was not found for other sample types and therefore illustrates that different metabolic programs exist that lead to homeostasis in malignant diseases.

Results of the current thesis highlight the elevated need to run functional studies on biological samples as static methods widely used today, might not be sufficient to describe the underlying biology in both physiological or diseased states.

Kokkuvõte

Vähktõvest on saamas üks suurimatest probleemidest läänemaailma tervishoiusüsteemidele, peamiselt tänu vananevale populatsioonile, käitumisharjumistele ja saastunud keskkonnale. Eestiski on olukord sarnane ning seda kinnitab ilmekalt tõsiasi, et vähist tingitud surmad on südameveresoonkonna haigustest tingitud surmade järel teisel kohal. Varakult avastatud pahaloomulised kasvajad on enamasti ravitavad ning selle eesmärgiga on ellu kutsutud mitmed skriiningprogrammid näiteks varases faasis rinnavähi, soolekasvajate või emakakaelavähi avastamiseks. Kuigi skriining-programmidega seostatakse teatud probleeme, nagu näiteks ülediagnoosimine, on Eesti näitel suuremaks kitsaskohaks inimesed, kes on otsustanud taolistel uuringutel mitte osaleda. Rinna- ja soolevähk on ka antud doktoritöö uurimisobjektiks.

Vähki on tihti peetud puhtalt geneetiliseks haiguseks, kuid järjest selgemalt on esile tõusnud, et energiametabolismiga seotud süsteemides on tekkinud mitmed ulatuslikud muudatused, mida enamasti ei ole võimalik seostada geneetiliste põhjustega. Kuigi muutunud glükoositarvet ja rakuhingamist kirjeldati esimest korda juba enam kui sajand tagasi, on selles osas tänaseni säilinud mitmed vasturääkivused, mis omakorda viitavad vajadusele teostada täiendavaid teadusuuringuid. Käesolevas doktoritöös kasutati nii inimese vähimaterjali kui ka rakukultuure, et lähemalt kirjeldada ja võrrelda muudatusi oksüdatiivses fosforüülimises kolorektaal- ja rinnavähi näitel. Metaboolse Kontrolli Analüüsi (MKA) tulemused viitasid hingamisahela Kompleks I mahasurutusele ja seda üksnes kolorektaalvähis, kuid see oli selgelt eristatav üksnes funktsionaalsel uuringul, kuna staatilise iseloomuga Western blot analüüs andis kahe erineva lähenemise tulemusena üksnes vastuolulisi tulemusi. Samuti kasutati MKA analüüsi hindamaks superkomplekside olemasolu ja nende võimalikku koostist. Tulemused viitasid, et kompleksid I ja III (teatud ulatuses koos Kompleks IVga) on seostunud superkompleksideks, kuid Kompleks II neis ei osale. Taoline järeldus kinnitas varasemaid uuringuid, kus samaväärsele seisukohale jõuti erinevaid staatilisi meetodeid kasutades. Täiendavalt võrdlesime rinnavähi korral rakuhingamise seoseid haiguse oodatava agressiivsusega ja kasutasime selleks nii inimese materjali kui ka vastavaid rakuliine. Võrdlesime kahte vastandlikku molekulaarset alatüüpi (luminal A ja kolmiknegatiivne), millest esimene on vähim ning teine kõige agressiivsema kliinilise kuluga. Saadud tulemused olid üllatavad, kuna selgus, et laboratoorsetes uuringutes laialt kasutatavad rakuliinid ja vastavad kliinilised proovid näitasid pöördvõrdelist seost ning see omakorda võib viidata ka põhjustele, miks laboriuuringute põhjal saadud tulemused enamasti inimesele üle kandes läbi kukuvad. Kasutades Michaelis-Menteni võrrandeid (K_m), uurisime mitokondri välismembraani afiinsust eksogeense ADP suhtes ning selgus, et inimese rinnavähi proovides on kaks erinevalt reguleeritud mitokondrite populatsiooni, kuid kolorektaalvähiproovides taolist jaotumist ei esinenud. Tegemist on pelgalt ühe viitega, et kartsinogeneesil võib olla mitmeid erinevaid väljundeid, mis siiski tagavad vähirakkude homeostaasi.

Käesoleva töö tulemused viitavad vajadusele teostada bioloogilisel materjalil funktsionaalseid uuringuid, kuna täna laialdaselt kasutust leidvad staatilised (sh genoomsed) meetodid ei pruugi olla piisavad, et adekvaatselt kirjeldada erinevates kudedes toimivaid bioloogilisi protsesse, nende aktiivsust või ulatust.

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Research Article

Mitochondrial Respiration in Human Colorectal and Breast Cancer Clinical Material Is Regulated Differently

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We conducted quantitative cellular respiration analysis on samples taken from human breast cancer (HBC) and human colorectal cancer (HCC) patients. Respiratory capacity is not lost as a result of tumor formation and even though, functionally, complex I in HCC was found to be suppressed, it was not evident on the protein level. Additionally, metabolic control analysis was used to quantify the role of components of mitochondrial interactosome. The main rate-controlling steps in HBC are complex IV and adenine nucleotide transporter, but in HCC, complexes I and III. Our kinetic measurements confirmed previous studies that respiratory chain complexes I and III in HBC and HCC can be assembled into supercomplexes with a possible partial addition from the complex IV pool. Therefore, the kinetic method can be a useful addition in studying supercomplexes in cell lines or human samples. In addition, when results from culture cells were compared to those from clinical samples, clear differences were present, but we also detected two different types of mitochondria within clinical HBC samples, possibly linked to two-compartment metabolism. Taken together, our data show that mitochondrial respiration and regulation of mitochondrial membrane permeability have substantial differences between these two cancer types when compared to each other to their adjacent healthy tissue or to respective cell cultures.

1. Introduction

The field of cellular bioenergetics is gaining increased attention and studies performed during the last years have shown that targeting cancer cell energy metabolism may be a new and promising area for selective tumor treatment [1]. The literature describing changes in energy metabolism and mitochondrial function during carcinogenesis is, unfortunately, full of contradictions. Majority of previous studies about the bioenergetics of malignant tumors were performed in vitro on different cell models with the conclusion that cancer cells have increased glucose uptake and, due to mitochondrial damage, it is not metabolized via oxidative phosphorylation (OXPHOS) [2–4]. It is clear that for many tumors, glycolysis is the main energy provider, but in others, OXPHOS is still crucial for survival and progression and produces necessary ATP [1, 5, 6]. Recently, a new concept for tumor metabolism was proposed—metabolic coupling between mitochondria in cancer cells and catabolism in stromal cells—which promotes tumor growth and development of metastases. In other words, tumor cells induce reprogramming in surrounding nontumor cells so that the latter acquire the Warburg phenotype [7] and start producing and exporting the necessary fuels for the anabolic cancer cells ("reverse Warburg"). The cancer cells will then metabolize these fuels via their tricarboxylic acid cycle and OXPHOS [8–10]. Complex interplay between developing cancer cells and host physiology, possibly mediated by "waves" of gene expression

in the tumor [11, 12], can only develop in vivo and therefore in vitro studies cannot give conclusive information about the functional activity and capacity of OXPHOS in human samples. In vitro models ignore many factors arising from the tumor microenvironment (TME), which can and will exert significant effects in vivo. TME consists of nonmalignant cells, soluble growth factors, signaling molecules, and extracellular matrix that support tumor progression [13], but high heterogeneity within cancers cell population on top of it contributes to even further complexity in clinical samples [14]. At the same time, the metabolic profiles of tumor cells that are grown in culture have significant variations primarily due to the culture conditions, such as concentrations of glucose, glutamine, and/or fetal serum. Cells grown in glucose-free medium display relatively high rates of oxygen consumption, but cultivation in high-glucose medium increases their glycolytic capacity together with reduced respiratory flux [15–19].

In addition to intercellular differences, there are also intracellular rearrangements resulting from tumor formation. The functional units within cells are often macromolecular complexes rather than single species [20]. In case of OXPHOS, it has been shown that complexes of the respiratory chain can form assemblies—supercomplexes—that lead to kinetic and possibly homeostatic advantages [21]. Therefore, pure genome or transcriptome data are not sufficient for describing the final in situ modifications and the final outcomes of a pathway or cellular processes are defined by actual activities of their separate proteins—or their assemblies—together with the respective regulatory mechanisms. More specifically, previous studies have shown that in cardiac and yeast cells, a large protein supercomplex is centrally positioned in regulation of mitochondrial respiration and mitochondrial energy fluxes. The supercomplex consists of ATP synthasome, mitochondrial creatine kinase (MtCK) or hexokinase (HK), voltage-dependent anion channel (VDAC), and some regulatory proteins expectedly coordinate the selective permeability of it. This complex is known as mitochondrial interactosome (MI) [22], and it is located in the contact sites of outer and inner mitochondrial membranes. This unit also includes supercomplexes formed by the respiratory chain [23, 24]. Changes in the content of ATP synthasome and respiratory chain supercomplexes in pathological conditions are still poorly studied. Inhibiting key respiratory enzymes or avoiding restructuring of mitochondrial supercomplexes in tumors has potential to disrupt disease progression without affecting normal cells, thus, providing a powerful new approach for developing novel therapeutic targets. Specifically, Rohlenova et al. recently demonstrated that breast cancer cells expressing HER2 oncogene develop specific RC supercomplexes which make complex I in these susceptible to treatment with chemically altered tamoxifen called MitoTam [25]. MitoTam is taken to a phase I clinical study [25], and there are other clinical studies undergoing that target OXPHOS in different cancer types (e.g., trial numbers NCT01957735 and NCT02650804). Therefore, despite the assumed glycolytic nature of human tumors, inhibition of oxidative respiration is proving to be a viable therapeutic strategy and further studies are needed to define differences between cancer types but also individual patients in regard to such treatment.

We have previously shown on clinical samples that both human breast cancer (HBC) and human colorectal cancer (HCC) are not purely glycolytic, but these tumors have sustained OXPHOS as a substantial provider of ATP [26–28]. Here, we extend our studies by comparing bioenergetics of HBC and HCC using kinetic methods.

2. Materials and Methods

2.1. Chemicals. All chemicals were purchased from Sigma-Aldrich (USA) and were of the highest purity available (>98%).

2.2. Clinical Materials. The tissue samples were provided by the Oncology and Haematology Clinic at the North Estonia Medical Centre (Tallinn). All the samples were analyzed immediately after surgery. Only primary tumors were examined and information from respective pathology reports was provided by the North Estonia Medical Centre for all the analyzed samples. Informed consent was obtained from all the patients and coded identity protection was applied. All investigations were approved by the Tallinn Medical Research Ethics Committee and were in accordance with the Helsinki Declaration and Convention of the Council of Europe on Human Rights and Biomedicine. The entire group consisted of 34 patients with breast cancer and 55 with colorectal cancer.

2.3. Cell Cultures. MDA-MB-231 and MCF-7 cells were grown as adherent monolayers in low glucose (1.0 g/L) Dulbecco's modified Eagle's medium (DMEM) with stable L-glutamine and sodium pyruvate (from Capricorn Scientific GmbH) supplemented with 10% heat-inactivated fetal bovine serum, 10 μg/mL human recombinant Zn insulin, and antibiotics: penicillin (100 U/mL), streptomycin (100 μg/mL), and gentamicin at a final concentration of 50 μg/mL. Cells were grown at 37°C in a humidified incubator containing 5% CO_2 in air and were subcultured at 2-3-day intervals.

2.4. Mitochondrial Respiration in Saponin-Permeabilized Tissue Samples. Numerous studies have demonstrated that isolated mitochondria behave differently from mitochondria in situ [29–32]. We therefore have investigated respiratory activity of tumor and control tissues in situ using the skinned sample technique [26, 28, 29, 33]. This method allows analysis of the function of mitochondria in cells in their natural environment and leaves links between cytoskeletal structures and mitochondrial outer membranes intact [34–37]. Cytochrome c test was used to confirm integrity of the mitochondrial outer membrane (MOM) [22, 26, 28, 33]; mitochondrial inner membrane quality was checked using a carboxyatractyloside (CAT) test as the last procedure in every experiment [22, 26, 28, 33]. Rates of O_2 consumption were assayed at 25°C using Oxygraph-2k high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria) loaded with pre-equilibrated respiration buffer medium B [26]. Activity of the respiratory chain was measured by substrate-inhibitor

titration as described earlier [26, 38]. The solubility of oxygen at 25°C was taken as 240 nM/mL [39]. The solubility of oxygen is much lower at 37 than at 25°C, but also, the skinned samples from malignant clinical material are more stable at 25°C. All rates of respiration (V) are expressed in nM O_2/min per mg dry tissue weight for solid tumors and in nM O_2/min per million cells for cell cultures.

2.5. Metabolic Control Analysis. Metabolic control analysis (MCA) is a method for studying regulatory mechanisms in complex metabolic systems [40–42]. Flux control coefficient (FCC) is defined as the ratio of fractional change in a system variable to fractional change in a biochemical activity that caused the change in the given system [42]. FCC or C^J_{vi} is the extent to which an enzyme in a pathway controls the flux (J); it corresponds to the percentage decrease in flux caused by a 1% decrease in the activity (v_i) of that enzyme [41, 43]:

$$C^J_{vi} = \frac{(dJ/dv_i)}{(J/v_i)} = \frac{d\ln J}{d\ln v_i}. \quad (1)$$

This method shows how the control is shared between the enzymes and the transporters of the pathway and enables to identify the steps that could be modified to achieve successful alteration of the flux or metabolite concentration in the pathway. But it also permits the identification of system components that are crucial in the regulation of energy transfer and regulatory networks [40–42, 44–46].

MCA has previously been applied in our lab to human breast and colorectal cancer skinned samples to determine the FCCs for respiratory chain complexes. The flux was measured as the rate of O_2 consumption in permeabilized tissues derived from HCC patients when all components of the OXPHOS system were titrated with specific irreversible or pseudoirreversible inhibitors to stepwise decrease selected respiratory chain complex activities according to a previously published method [26, 27, 47, 48].

2.6. Western Blot Analysis of the Level of Mitochondrial RC Complexes Expression. Postoperative human tissue samples (70–100 mg) were crushed in liquid nitrogen and homogenized in 20 volumes of RIPA lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 0.1% Triton X-100, and complete protease inhibitor cocktail (Roche)) by Retsch Mixer Mill at 25 Hz for 2 min. After homogenization, samples were incubated for 30 min on ice and centrifuged at 12,000 rpm for 20 min at 4°C. The proteins in the supernatants were precipitated using acetone/TCA to remove nonprotein contaminants. Briefly, supernatants were mixed with 8 volumes of ice-cold acetone and 1 volume of 100% TCA, kept at −20°C for 1 h and then pelleted at 11500 rpm for 15 min at 4°C. The pellets were washed twice with acetone and resuspended in 1x Laemmli sample buffer.

Proteins were separated by polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride (PVDF) membrane, and subjected to immunoblotting with the total OXPHOS antibody cocktail (ab110411). Then, the membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibody and visualized using an enhanced chemiluminescence system (ECL; Pierce, Thermo Fisher Scientific). After chemiluminescence reaction, the PVDF membranes were stained with Coomassie brilliant blue R250 to measure the total protein amount. The complexes I–V signal intensities were calculated by ImageJ software and normalized to total protein intensities.

Expression levels of complex I in HCC and normal tissues were additionally estimated using anti-NDUFA9 antibody that corresponds to NADH dehydrogenase 1α subcomplex 9 (SAB1100073). The samples were incubated and visualized as described above. Levels of NDUFA9 encoding protein were normalized to total protein content.

2.7. Citrate Synthase Activity. Activity of citrate synthase in tissue homogenates was measured as described by Srere [49]. Reactions were performed in 96-well plates containing 100 mM Tris-HCl pH 8.1, 0.3 mM AcCoA, 0.5 mM oxaloacetate, and 0.1 mM DTNB using FLUOstar Omega plate reader spectrophotometer (BMG Labtech).

2.8. Data Analysis. Data in the text, tables, and figures are presented as mean ± standard error (SEM). Results were analyzed by the Student t-test; p values <0.05 were considered statistically significant.

3. Results and Discussion

3.1. Respiratory Chain Analysis and Presence of Supercomplexes. Suppression of mitochondrial electron transport chain function is widespread in cancer, and this is closely connected to apoptosis resistance [50–54]. However, studies are often conducted on cell cultures and therefore little is known about respiratory chain (RC) function in clinical human breast and colorectal carcinomas in situ. To reveal possible disturbances, we conducted comparative quantitative analysis on the respiration rates for different RC complexes in permeabilized HBC and HCC and their adjacent normal tissue samples. Data for healthy breast tissue has been left out from most of the following calculations due to very low ADP-dependent oxygen consumption in this tissue type as it is not sufficient to assess inhibitory effects of antimycin A or rotenone or compare these results to other studied samples.

Multiple substrate-inhibitor titration protocol was used for measuring respiratory capacities of different respiratory chain segments (Table 1) [30, 55]. All respiration rates corresponding to the activities of different RC complexes are increased in both investigated human cancers when compared to their adjacent normal tissue. The mean value of basal respiration (state 2, Vo) in skinned HCC samples is higher than that in normal tissue and depends on the used respiratory substrates. Specifically, in the presence of glutamate and malate, HCC and its control tissue fibers exhibit lower state 2 respiration rates than in the presence of glutamate, malate, and succinate; similar dependence was observed for the breast cancer samples (Figure 1). One possible reason for this difference can be succinate-dependent proton leak in tumor tissue [56–58]. Addition of 2 mM

MgADP for studying complex I-based state 3 (in the presence of glutamate and malate without succinate) increased mitochondrial respiration rates in all tissue samples and following addition of complex I-specific inhibitor (rotenone) inhibited the respiration back to the initial state 2 levels (Table 1). Similarly, the function of complex II was quantified upon ADP-stimulated respiration in the presence of rotenone and succinate; at these conditions, the complex I activity is inhibited and apparent respiration rate originates from complex II. Complex III in both HBC and HCC was confirmed to be fully functional as an addition of antimycin A inhibited the electron flow from complex III to mitochondrial complex IV (COX) (Table 1). The activation of mitochondrial complex IV (addition of 5 mM ascorbate and 1 mM tetramethyl-p-phenylenediamine) resulted in a remarkable increase in the rate of O_2 consumption in all examined samples, both cancerous and normal, but the increase was nearly two times higher in cancer tissue.

Complex I deficiency is the hallmark of multiple mitochondrial diseases and is generally considered to be an intrinsic property of some cancers [58–63]. Indeed, our experiments confirm that development of HCC results in reduced V_{Glut}/V_{Succ} ratio which indicates relative suppression of the complex I-dependent respiration [58]. Similar results have previously been described for gastric and ovarian cancer tissues but also in some cancer cell cultures [58, 63–66]. Deficiency of complex I in some tumors might be an early event causing an increase in mitochondrial biogenesis in an attempt to compensate for the reduction in OXPHOS function [63]. Computer modeling predicts that the mechanisms of this compensation can use multiple pathways like β-oxidation of fatty acids, mitochondrial folate metabolism, and others [67]. Our results showed that this suppression is pronounced on the functional level in HCC (Figure 2(a)), but to identify the changes on the protein expression level, we analyzed the RC complexes with total OXPHOS antibody cocktail (Figure 3). Based on this type of approach, the suppression of RC complex I was found to be absent if the results were normalized to total protein. The suppression of complex I in HCC was additionally studied by Western blot analysis with antibodies against only one complex I subunit—NDUFA9 (see Supplementary Fig 1 available online at https://doi.org/10.1155/2017/1372640). This result, however, confirmed the suppression of complex I. As seen from those experiments, analysis of RC, using the semi-quantitative WB method, can be strongly dependent on experimental conditions: against what complex I subunit the antibodies were used and which normalization conditions are applied. Additionally, complex II in colon samples did not indicate possible suppression in that alternative pathway as differences in V_{Succ}/V_{COX} ratios were not significant (Figure 2(b)) [68].

In contrast to HCC, mitochondrial respiration in HBC samples is not accompanied with suppression of complex I-dependent respiration (Figures 2(a) and 2(b)). Altogether, the relative complex I deficiency on the functional level in our oxygen consumption measurements is characteristic for HCC but not for HBC tissue.

Table 1: Characterization of respiratory parameters of permeabilized tissue samples derived from patients with breast or colorectal cancer.

Parameters	HBC patients, $n = 7$ [26]		HCC patients, $n = 7$ [28]	
	Tumor	Control	Tumor	Control
Vo	0.294 ± 0.024	0.004 ± 0.007	1.06 ± 0.14	0.82 ± 0.15
V_{ADP}	0.71 ± 0.06	0.055 ± 0.004	2.02 ± 0.21	1.39 ± 0.21
Vrot	0.34 ± 0.04	0.070 ± 0.015	0.91 ± 0.11	0.85 ± 0.14
V_{Succ}	0.74 ± 0.10	0.076 ± 0.008	2.22 ± 0.26	1.33 ± 0.18
VANM	0.38 ± 0.04	0.071 ± 0.018	1.04 ± 0.09	0.69 ± 0.07
V_{COX}	2.36 ± 0.33	1.23 ± 0.18	6.59 ± 0.71	3.84 ± 0.58

Note: here, each data point is the mean ± SEM of respiratory values. Vo: basal respiration without ADP or ATP; V_{ADP}: ADP-stimulated respiration (final concentration 2 mM) in the presence of 5 mM glutamate and 2 mM malate (indicating the function of the respiratory chain complex I); Vrot: rates of respiration after addition of 50 μM rotenone (an inhibitor of complex I); V_{Succ}: ADP-stimulated respiration in the presence of rotenone and 10 mM succinate (to estimate the function of complex II); VANM: rates of respiration after addition 10 μM antimycin-A (an inhibitor of complex III); V_{COX}: rates of O_2 consumption in the presence of complex IV substrates (5 mM ascorbate jointly with 1 mM tetramethyl-p-phenylenediamine).

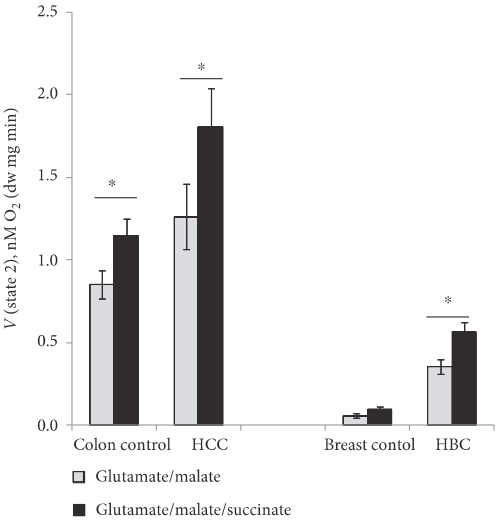

Figure 1: Assessment of state 2 respiration rates of the permeabilized HCC, HBC, and normal adjacent tissue samples in the presence of different combinations of respiratory substrates (5 mM glutamate, 2 mM malate, and 10 mM succinate). Bars are SEM, $n = 8$ for colon samples, and $n = 12$ for breast tissue samples, $^*p < 0.05$.

Remarkable numbers of studies have shown that RC complexes can form protein assemblies (supercomplexes). These supramolecular structures provide kinetic advantage such as substrate channeling, increased efficiency in electron transport, prevention of destabilization, and degradation of respiratory enzyme complexes [21] and means to regulate

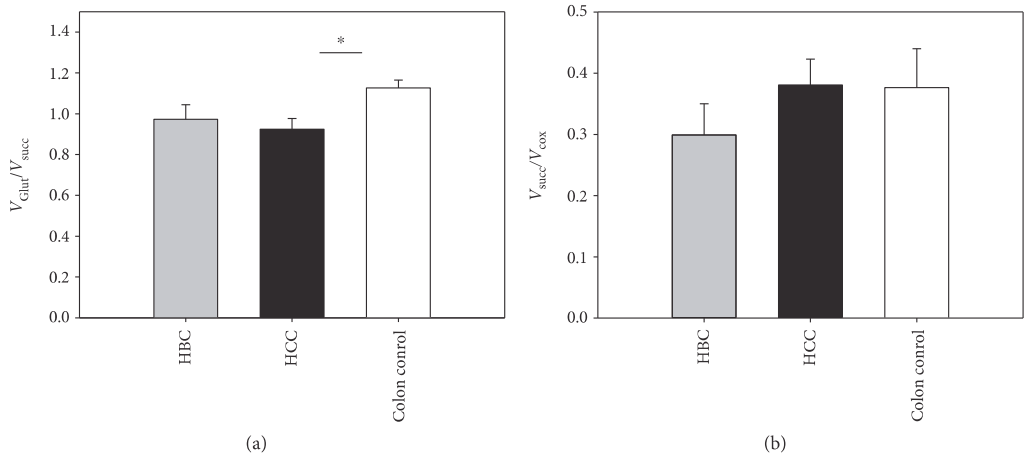

Figure 2: (a) Oxygraphic analysis of the functioning of complex I in skinned tissues from patients with HBC or HCC; here, V_{Glut}/V_{Succ} is the ratio of ADP-stimulated respiration rate in the presence of 5 mM glutamate and 2 mM malate (activity of complex I) to ADP-stimulated respiration rate in the presence of 50 μM rotenone and 10 mM succinate (activity of complex II). (b) V_{Succ}/V_{COX} is the ratio of complex II respiration rate to complex IV respiration rate. Data shown as mean ± SEM; $n = 7$ for colon [28] and breast tissue samples [26], $^*p < 0.05$.

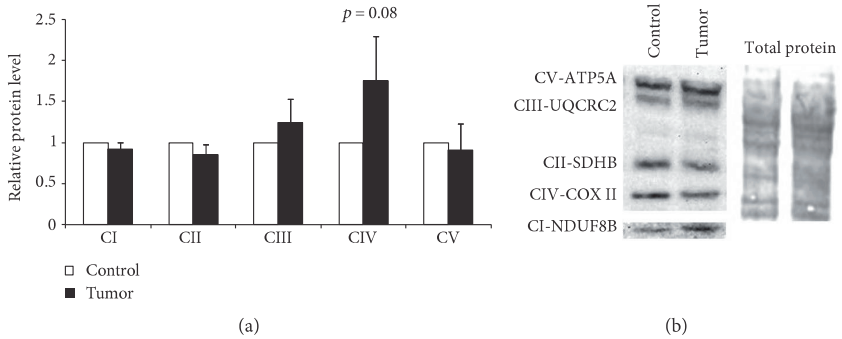

Figure 3: Quantitative analysis of the expression levels of the respiratory chain complexes in HCC and normal tissue samples (a) along with a representative Western blot image (b). Protein levels were normalized to total protein staining by Coomassie blue; data shown as mean ± SEM of 5 independent experiments.

ROS levels in the cell (most of the mitochondrial ROS originates from complexes I and III) [69] and hence, homeostasis. The RC complex I is considered to be the most important component in these assemblies, and it is a member of almost all known respirasomes [70–74]. In previous studies, complexes I, III, and IV were found to be assembled into supercomplexes in different configurations, but complex II was not confirmed to be a component of these RC supercomplexes and was assumed to move freely in the mitochondrial inner membrane [70, 75, 76]. Relative deficiency of the RC complex I on the functional level (as shown above) may be a result of changes in supercomplex composition as a part of malignant transformation.

In addition to RC supercomplexes, along with respirasomes, one more molecular transmembrane protein supercomplex (which is known as ATP synthasome; [77]) was identified as the component of the OXPHOS system. The ATP synthasome complex consists of ATP synthase, inorganic phosphate carrier, and adenine nucleotide translocator (ANT) [48]. The current model of mitochondrial interactosome (MI) considers the ATP synthasome and RC complexes together with voltage-dependent anion channel (VDAC) and mitochondrial creatine kinase (MtCK) as components of intracellular energetic units [22]. Even though MI is proven in striated muscles, the functional role of it, together with MtCK, in malignant samples remains controversial [78], but it indicates that both ATP synthasome and RC complexes can form even more complex functional structures.

In addition to steady-state proteome studies, kinetic testing of metabolic fluxes using MCA can provide preliminary information about supramolecular organization in the

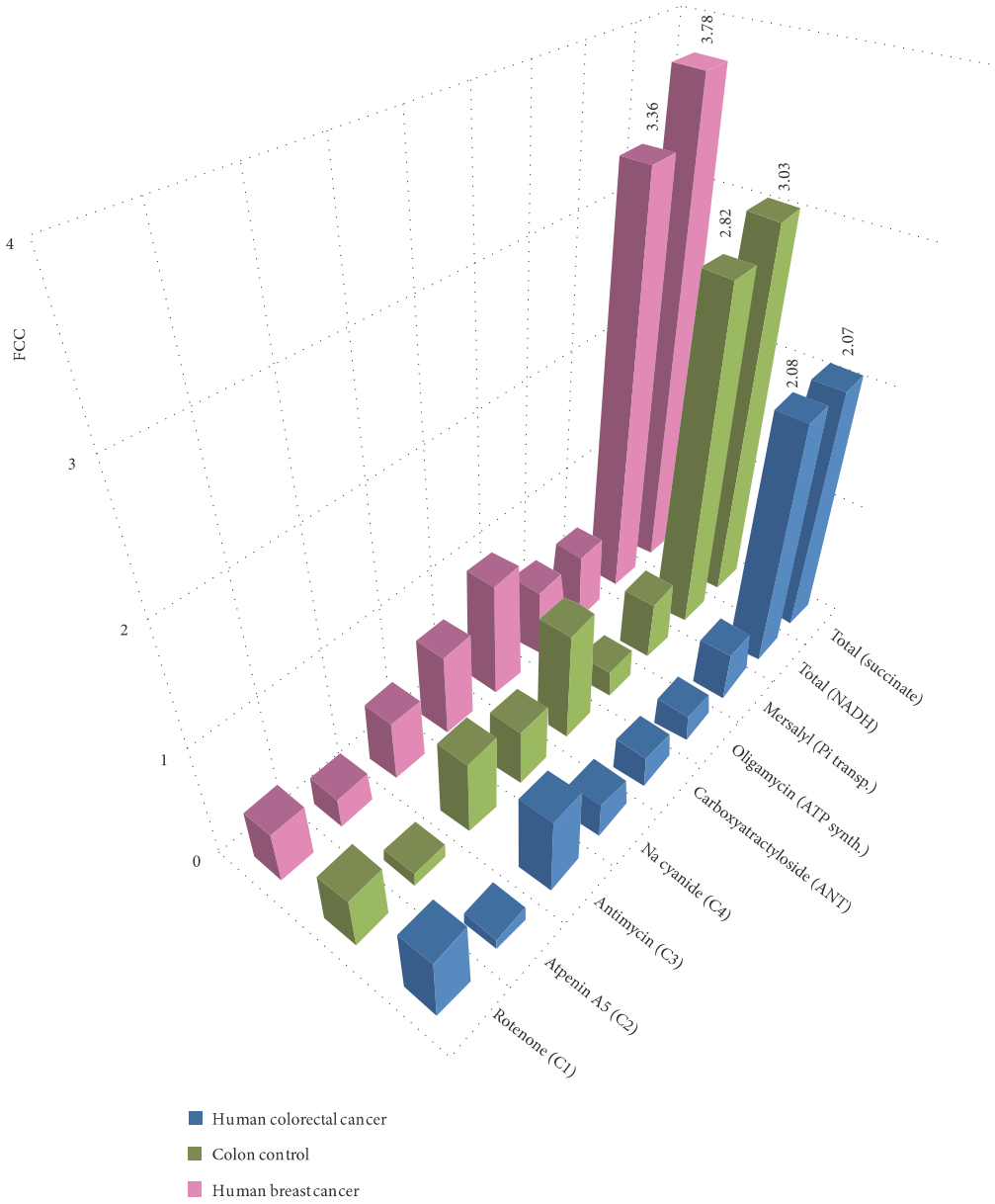

Figure 4: FCCs for ATP synthasome and RC complexes as determined by MCA. Two ways of electron transfer were examined: NADH-dependent and succinate-dependent electron transfers, and respective sums of FCCs are calculated as the last bars. Data for HBC is published before in [26], except for complex II with atpenin A5. Isolated mucosal tissue was used for colon control.

energy transfer system and enables to quantify the flux exerted by the different RC and the ATP synthasome complexes [27, 28, 79]. MCA can discriminate between two prevailing models: the former model, based on the assumption that each enzyme can be rate controlling to a different extent, and a subsequent model, where whole metabolic pathway can behave as a single channel and inhibition of any of its components would give the same flux control [80]. Bianchi et al. proposed that both complexes I and III are highly rate controlling in NADH oxidation, suggesting the existence of

Table 2: FCCs for different components in mitochondrial Interactosome and ranges of the concentrations of inhibitors.

MI component	Inhibitor	Range of inhibitor concentration	FCC		
			HCC	Control colon tissue (mucosa)	HBC
Complex I	Rotenone	1–100 nM	0.56	0.45	0.46*
Complex II	Atpenin A5	0.1–6 μM	0.12	0.13	0.28
Complex III	Antimycin	1–200 nM	0.68	0.66	0.54*
Complex IV	Na cyanide	0.1–40 μM	0.31	0.50	0.74*
ANT	Carboxyatractyloside	1–200 nM	0.28	0.97	1.02*
ATP synthase	Oligomycin	1–600 nM	0.25	0.24	0.61*
Pi transporter	Mersalyl	1–200 μM	0.43	0.53	0.60*
Sum 1, 3–7	**Total (NADH)**		**2.08**	**2.82**	**3.36**
Sum 2–7	**Total (succinate)**		**2.07**	**3.03**	**3.78**

Note: *from [26].

functional association between these two complexes [80]. To confirm the formation of supercomplexes in HCC- and HBC-skinned samples using MCA, we investigated the flux control coefficients (FCCs) for the complexes involved in aerobic NADH oxidation (I, III, and IV), in succinate oxidation (II, III, and IV), and for components of the ATP synthasome. For this purpose, cancerous and normal tissue samples were titrated with increasing concentrations of specific inhibitors against all of the ATP synthasome and RC complexes. Figure 4 summarizes the data analyzed in three different ways: by a graphical model [40, 41, 81], according to Small [82], and the Gellerich model [44]. The obtained FCC values did not depend on which exact method was used for calculations. The main problem in these calculations is high heterogeneity of the clinical material, which from the one hand originates from cancer molecular subtypes (e.g., Lumianal A/B, HER2 or triple negative in HBC; unknown subtypes in HCC) but on the other hand originates from heterogeneity of tumor cells within each tumor [14] or irregular stromal burden. Therefore, the obtained coefficient values do not only depend on which patients were included to the study, but the results may also depend on which particular tumor region was used from each patient sample. This can be considered as an inevitable part in analyzing clinical samples.

Previous work has shown that the main respiratory rate-controlling steps in HBC cells are complex IV (FCC = 0.74) and adenine nucleotide transporter (ANT, FCC = 1.02) [26]. Similar control distribution was not observed within HCC ATP synthasome complex as FCCs for ANT were found to be significantly lower when HCC was compared to the results of healthy colon mucosa (FCC = 0.284 for HCC and FCC = 0.970 for healthy colon). These results show that ANT exerts high flux control in healthy colon tissue (and in HBC), but ANT seems to lose its limiting role in HCC. Ramsay et al. believe that hexokinase-voltage-dependent anion channel-ANT complex, which spans across the outer and inner mitochondrial membranes, is critical in cancer cells as this complex is the link between glycolysis, oxidative phosphorylation, and mitochondrial-mediated apoptosis [83]. Therefore, the difference between HBC and HCC, in regard to ANT-exerted flux control, indicates to distinct difference in energy metabolism between these two tumor types (Table 2; Figure 4). In addition, HBC is showing equal FCCs for ATP synthase and inorganic phosphate transporter (Pi) in ATP synthasome, but this phenomenon is not characteristic neither for healthy colon mucosa nor for colorectal cancer. These alterations could be related to mitochondrial permeability transition pore (mtPTP) and apoptosis. Bernardi et al. studied the key regulatory features of the mtPTP [84–87], and the same group of authors has pointed to the fact that ANT can modulate the mtPTP, possibly through its effects on the surface potential, but it is not a mandatory component of this channel.

FCCs within the RC system in HBC do not differ significantly and the flux control is distributed almost uniformly throughout the different complexes (Table 2, Figure 4). Such condition is an indication of possible presence of protein supercomplexes (approximately equal values of FCCs for RC complexes I and III—0.46 versus 0.54, resp.). On the other hand, the flux distribution for normal colon tissue, when compared to HCC, showed slight difference for that for complex IV (FCC 0.50 versus 0.31), but flux control coefficients with close values were calculated for complex I (FCC 0.45 versus 0.56) and complex III (FCC 0.66 versus 0.68). Similarity in FCCs for complex I and complex III for both HCC and healthy colon tissue enables to propose that in healthy conditions, complex III is attached to complex I (possibly together with multiple copies of complex IV), but during carcinogenesis, the supercomplex assembly changes and even though complex I and complex III seem to stay linked, the participation of complex IV in this assembly becomes uncertain. Functional assembly of complexes I and III together with their rate-limiting roles will lead to sum of FCCs being greater than 1 [73] (see below).

Role of complex IV is multifaceted as three populations of it have previously been suggested: population assembled with complex I and complex III, population assembled with complex III alone and a non-interacting population [74]. Several data show that the absence of functional supercomplex assembly factor I (SCAF1) may be involved in distribution of complex IV [74, 75, 88]. As outlined in the review article by Enrìquez, total cell respiration (glucose, pyruvate, and glutamine as substrates) was significantly higher in cells lacking functional SCAF1

[74]. High total cell respiration was registered also for both cancer types described in this paper, but presence or absence of functional SCAF1 was not investigated.

The sums of the determined FCCs within cancerous and healthy sample groups were calculated to be in the range from 2.07 to 3.78. In theory, sum of FCCs in a linear system is 1 [5, 40, 42–44, 89, 90], but the value of it can increase if the system includes enzyme–enzyme interactions, direct channeling, and/or recycling within multienzyme complexes (i.e., system becomes nonlinear) [79, 80, 91, 92]. The higher sum of FCCs from our tests is not a result of diffusion restrictions because the concentration ranges for all of the inhibitors in various samples were similar and did not depend on the nature of the samples [26, 28, 47, 48].

The organization of RC complexes in the mitochondrial inner membrane has been an object of intense debate and it is not studied systematically in human normal or cancerous tissues. Given the known theoretical framework, our results confirm the plasticity model and agree with the data from Bianchi et al. [80], but the distribution of complex IV remains unclear—both random distribution and association into I-III-IV supercomplex can be possible. Large FCC for complex II is not characteristic neither for HBC, for HCC, nor for healthy colon tissue, and therefore, our kinetic studies confirmed previous findings that this complex is not a part of RC supercomplexes.

The question about the changes in the composition and stoichiometry of protein supercomplexes, which result from carcinogenesis, needs further studies, and in addition, as mitochondria have other additional roles in cellular metabolism, it can be presumed that changes in RC are also affecting cataplerotic processes sprouting from the mitochondria, but such link has not yet been studied yet.

3.2. ADP-Regulated Mitochondrial Respiration in HBC and HCC Fibers. Table 3 summarizes ADP-regulated mitochondrial respiration parameters determined for skinned tissue samples taken from both patient groups. Differences in the rates of maximal ADP-activated respiration (V_{max}) in colon tissue samples are corresponding to the differences in the content of mitochondria in these cells (the amount of mitochondria in HCC is 50% higher than that in healthy control tissue [28] (supplementary Table 1)). Our previous experiments have shown that HBC tissue, too, contains an increased number of mitochondria in comparison to its adjacent normal tissue [27, 93] (supplementary Table 1). As indicated above, ADP-dependent respiration in healthy human breast tissue is absent. Breast samples contain lot of fat tissue, but low V_{max} values were evident even if clearly lobular/ductal structures were separated and tested. Low respiratory capacity can also be indicating to lowered metabolic activity in normal ductal/lobular tissue in older women (average age of HBC patients in this study was 63.4 years). In contrast to normal breast tissue, the colon control tissue samples have significantly higher respiration rates (Table 3). Specifically, respiratory capacity is higher in apparent mucosal/submucosal section of the normal colon tissue samples compared to that of the underlying smooth muscle part as we manually separated and tested these two layers in a selection of colon tissue samples (Figure 5(b)).

Table 3: Apparent K_m ($^{app}K_m$) and maximal rate of respiration (V_{max}) values for ADP-dependent respiration calculated for HBC, HCC and their adjacent healthy tissue samples.

Tissues	$^{app}K_m$, μM ± SEM	V_{max} ± SEM
Human breast cancer tissue	114.8 ± 13.6*	1.09 ± 0.04*
Healthy adjacent breast control tissue	—	0.02 ± 0.01*
Human colorectal cancer tissue	93.6 ± 7.7**	2.41 ± 0.32
Healthy adjacent colon control tissue	256** ± 34	0.71 ± 0.23

Note: *from [26] and ** [38]; V_{max} values are presented as nM O_2/min/mg dry tissue weight without proton leak rates. These K_m and V_{max} values for ADP were determined from corresponding titration curves by fitting experimental data to non-linear regression equation according to a Michaelis–Menten model. 35 patients used for analysis of HBC and 35 for HCC.

HBC arises from tissue with almost absent ADP-related respiration, but once formed, the mechanism of energy conversion seems to acquire a more complicated form and it can be associated with both increased mitochondrial biogenesis and interplay between cancer and stromal cells [26]. HBC can be classified into four clinically distinct and significant molecular subtypes: luminal-A, luminal-B, HER2 expressing, and triple negative. Clinically, luminal-A is considered the least and triple negative as the most aggressive subtype. Therefore, we expected to see clear differences when respiratory parameters of those two extreme subtypes were measured. Initially, respiration rates were analyzed in Luminal-A type MCF7 and triple negative MDA-MB-231 cell lines. When compared, respiration rates in presence of glutamate or pyruvate clearly showed that oxygen consumption in luminal-A subtype cells is remarkably higher (Figure 5(b)). But in contrast, the exact opposite was registered for the same parameters in clinical samples (Figure 5(a)) as the highest respiratory rates were registered for the most aggressive triple negative subtype. From the one hand, this contradicting result shows that cell cultures are not directly comparable to respective clinical counterparts and can lead to misguiding expectations. On the other hand, it proves that the role of OXPHOS becomes increasingly important in clinical samples as aggressiveness of the tumor increases, but it is not evident in the respective culture cells. In the present case, it is not a result of increased glucose availability in the growth medium, which could lead the cells to acquire glycolytic phenotype and explain the difference with clinical samples, because low-glucose media was used.

For HCC, which is without distinct clinical subtypes, we compared disease stage to average V_{max} value for that stage (Figure 6(a)). Even though increase in V_{max} in initial stages can be calculated in comparison to control sample, the decrease in V_{max} for stages IIIC and IVB does not fit this increase in dependence. The disease stage at diagnosis itself is not a valid marker of aggressiveness and therefore such plotting can be debated. Therefore, we gathered initial longitudinal data on patient progression in our HCC cohort and confirmed that 7 out of 32 eligible patients had died (median

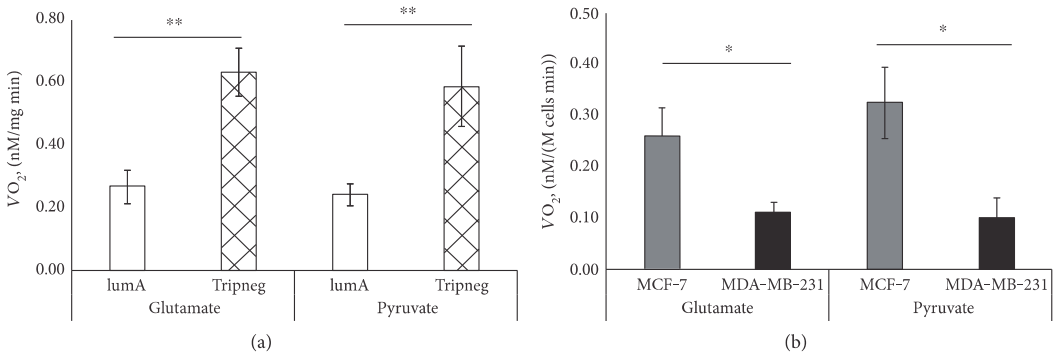

FIGURE 5: (a) Respiration rates for clinical samples of luminal-A and triple negative HBC subtypes in the presence of 5 mM glutamate or 5 mM pyruvate; $n = 13/12$ for luminal-A and $n = 7/8$ for triple negative subtypes, respectively. (b) Respiratory rates for luminal-A type MCF-7 and triple negative MDA-MB-231 cells in the presence of 5 mM glutamate or 5 mM pyruvate; $n = 3$ for each measurement; $^{*}p < 0.05$, $^{**}p < 0.005$.

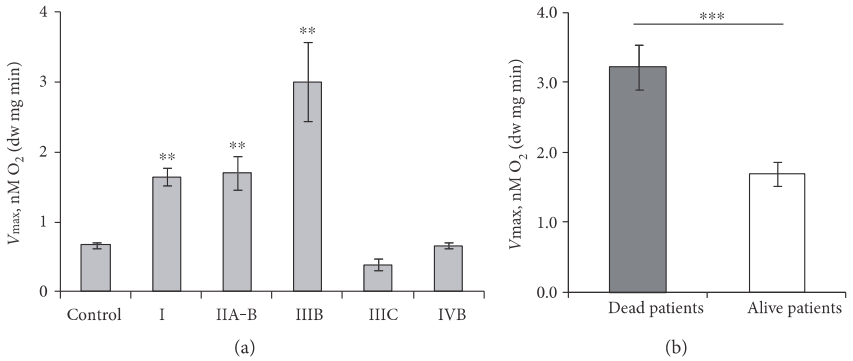

FIGURE 6: (a) Dependence of maximal rate of mitochondrial respiration (V_{max}) compared with the HCC at different stages. Stage I was calculated as the mean of 13 patients, IIA, IIB - 13 patients, IIIB-4 patients, IIIC-3 patients and IVB-1 patient. Control colon tissue is obtained from 34 patients. Maximal respiration rate V_{max} is compared with that in control tissue. Bars are SEM; $^{**}p < 0.005$. (b) V_{max} in HCC patients based on disease state in follow-up setting. Seven patients out of 32 are confirmed to have succumbed to HCC ($V_{max} = 3.19 \pm 0.34$); 25 patients out of 32 stay in remission ($V_{max} = 1.70 \pm 0.17$), $^{***}p < 0.001$.

follow-up time 47.3 ± 4.9 months). V_{max} values in patients that succumbed to the disease were significantly higher than that in the currently not progressed group (Figure 6(b)). As was shown for HBC above, higher respiratory capacity was registered for the most aggressive triple negative subgroup. Therefore, it can be argued based on similarity that higher tumor respiratory parameters in the dead HCC patients were indicating to more aggressive disease. In addition, lower than expected respiratory rate in some triple negative tumors can therefore indicate that given patient, when compared to the average in the triple negative subgroup, has less aggressive disease than could be expected. To confirm this in larger cohorts and relate aggressiveness in HCC and HBC to V_{max} value, additional longitudinal studies are necessary.

We next measured apparent Michaelis–Menten constants (K_m) for ADP to characterize the affinity of mitochondria for exogenous ADP (i.e., permeability of mitochondrial outer membrane). Corresponding K_m values for permeabilized tumor and nontumorous tissues were determined from titration experiments using exogenously added ADP. The obtained data were plotted as rates of O_2 consumption versus ADP concentration and apparent K_m values were calculated from these plots by nonlinear regression equation. Healthy colon tissue displayed low affinity for ADP ($K_m = 256 \pm 3\,\mu$M), whereas that in HCC is significantly higher ($K_m = 93.6 \pm 7.7\,\mu$M) [38]. The K_m (ADP) value for HBC tissue samples ($K_m = 114.8 \pm 13.6\,\mu$M) was similar to that for HCC [26].

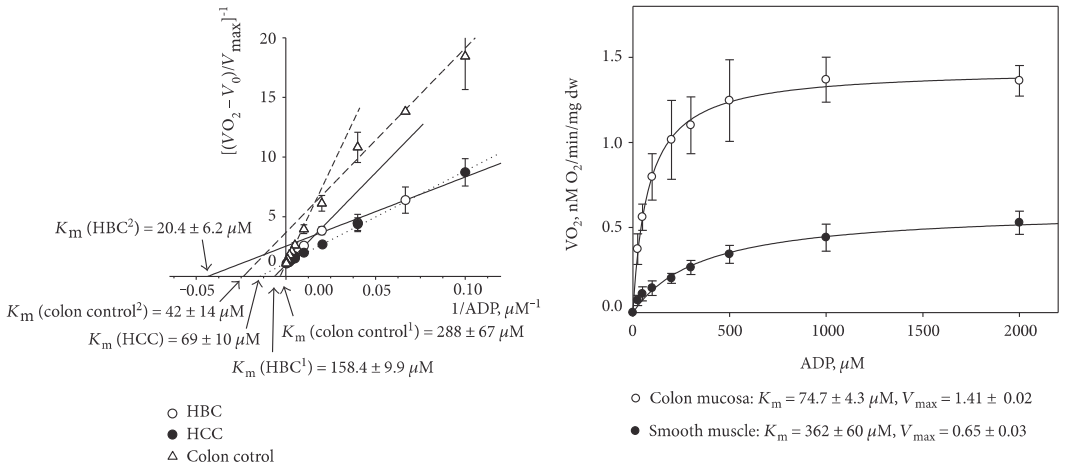

Figure 7: (a) Dependences of normalized respiration rate values for HCC (dotted line), HBC (solid line), and healthy colon tissue samples (dashed line); double reciprocal Lineweaver–Burk plots. Samples from 32 patients with breast cancer and 10 patients with colorectal cancer were examined. (b) ADP-dependent respiration in healthy colon mucosa and smooth muscle tissue samples (Michaelis–Menten curve, $n = 8$). Here, Vo and V_{max} are rates of basal and maximal ADP-activated respiration, respectively.

According to the classical studies by Chance and Williams [94, 95] and the data of many other investigators [29, 30, 37], the apparent K_m value for ADP for isolated mitochondria is low, about 15 μM, but the observed apparent K_m values in our study for permeabilized clinical HBC and HCC samples were 6–8 times higher than this value (Table 3). Our previous studies have shown that sensitivity of the mitochondrial respiration for exogenous ADP for permeabilized NB HL-1 cells is also high as the apparent K_m equaled to 25 ± 4 μM and was similar to that of isolated heart mitochondria [34, 96]. The similar low apparent K_m values were also registered for undifferentiated and differentiated neuroblastoma culture cells, where the corresponding K_m for ADP were measured as 20.3 ± 1.4 μM and 19.4 ± 3.2 μM, respectively [97]. The registered difference between culture cells and clinical samples, despite the used preparation method, again indicates to differences present in these two sample groups.

We treated permeabilized samples with incremental concentrations of ADP and the measured O_2 consumption rates (normalized to V_{max}) were analyzed against respective ADP concentration values as double reciprocal Lineweaver–Burk plots (Figures 7(a) and 7(b)) [29]. Figure 7(a) shows the results of the Lineweaver–Burk treatment of the experimental data linked with ADP-regulated mitochondrial respiration in skinned fibers of HCC, healthy colon, and HBC. Corresponding V_{max} and K_m values were calculated from the linearization approach. Saks and colleagues have previously shown that the presence of biphasic respiration regulation on the graph curve indicates the existence of two populations of mitochondria with different affinities for ADP [29]. Our results indicated such differences in colon control and HBC samples. Specifically, monophasic regulation of mitochondrial respiration is apparent in HCC tissue, but in healthy colon tissue, two populations of mitochondria with very different properties were found (Figure 7(a)). One population of mitochondria is characterized with lower K_m (42 ± 14 μM), whereas the apparent K_m (ADP) value for the second mitochondrial population is nearly seven times higher (288 ± 67 μM). We thereafter again separated mucosal and smooth muscle parts from the colon samples before additional K_m measurements to characterize their isolated contributions. Apparent K_m value for mucosal part was measured to be 74.7 ± 4.3 μM and the same value for colon smooth muscle tissues was found to be 362 ± 60 μM (Figure 7(b)). Therefore, results after separation explain the results from the initial experiment where the entire colon wall was analyzed and two separate groups of mitochondria were discovered. Additionally, we could also distinguish two differently regulated types of mitochondria in HBC samples: one with apparent K_m value for MgADP of 20.4 ± 6.2 μM, but the same for the second mitochondrial population was nearly ten times higher, 158.5 ± 9.9 μM (Figure 4(a)). The phenomenon shown in Figure 7(a) can be associated, on the one hand, simply with elevated stromal content (in such case, similar results should have been also registered for HCC), but on the other hand, with possible two-compartment tumor metabolism in HBC, what states that tumor cells function as metabolic parasites and extract energy from supporting host cells such as fibroblasts [98–103]. In such case, the stromal part of the HBC samples can be characterized with glycolytic metabolism representing the low K_m value due to high levels of autophagy, mitophagy, glycolysis, and lipolysis, while cancer cells

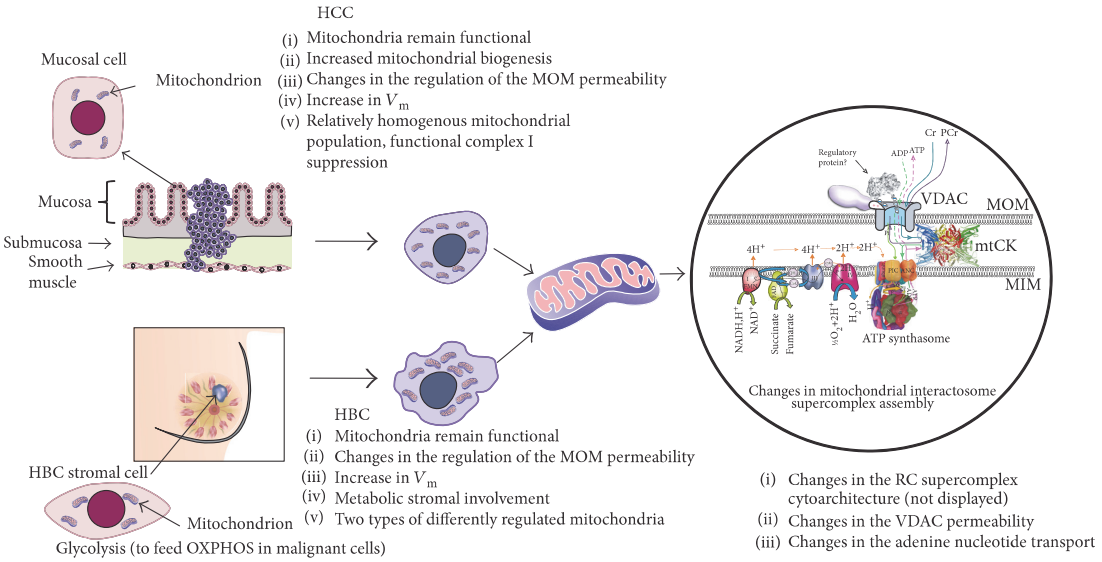

FIGURE 8: Mitochondrial alterations in HCC and HBC tissue cells. Mitochondrial interactosome is a large supercomplex consisting of ATP synthasome, VDAC, mitochondrial kinases like adenylate kinase, hexokinase or mitochondrial creatine kinase (MtCK), and respiratory chain (super)complexes. Here, the octameric MtCK characteristic is shown for the striated muscles and also as the possible component of the MI in the healthy colon [108–110]. The complex of VDAC together with other proteins controls the exchange of adenine nucleotides and regulates energy fluxes between mitochondrial and cytosolic compartments. Changes in the structure and function of MI are the important parts of cancer mitochondrial metabolism.

have high mitochondrial mass, OXPHOS, and β-oxidation activity, which is represented by the mitochondrial population with the high K_m (ADP) values. From the given comparison between HBC and HCC, the two subpopulations of mitochondria are specific only to HBC samples (confirmed in 32 cases out of the total 34) but not to HCC samples, and it indicates that tumor formation leads to distinct changes, which is related to the tissue type the tumor originates from.

Altogether, these results indicate the remarkable differences in the regulation of mitochondrial outer membrane (MOM) permeability between cultured tumor cells and clinical material (including between different tumor types and even between patients). Even further, the results can be contradictory as registered for respiration parameters. It can be estimated, based on the results from our lab, that low K_m value for ADP can be a common characteristic for cancer cells grown in culture, but in in vivo tumor samples, the regulation of MOM permeability is more complicated and probably related to interplay between energy transfer pathways and changes in the phosphorylation state of VDAC channels [32, 104–107] and also with modulation of cytoskeleton or membrane potential as a result of tumor formation.

4. Concluding Remarks

To understand the energy metabolism of tumors, it is necessary to detect bioenergetic fingerprints of each individual tumor type. Our results confirmed that respiratory capacity is preserved in both HBC and HCC as these both demonstrated substantial rates of oxidative phosphorylation, which contradicts with earlier widespread understanding that the metabolism of human breast and colorectal carcinomas is prevalently glycolytic. Studies on cell lines up to now have led to many lifesaving technologies and treatments in humans, but the scientific level might be nearing the end of readily transferrable results between the cell model and human physiology. Our results indicated that apparent glycolytic nature of some breast cancer types could be expected based on cell cultures, but this presumption was in sharp conflict when culture cell results were compared with these from respective clinical samples. In addition, when compared to their healthy adjacent tissue, both clinical cancer types showed increased respiratory capacity. Despite the increased respiratory capacity in HCC, relative deficiency of complex I was registered for it on the functional level Western blot analysis was not sufficient to confirm this deficiency on the protein level as two different antibody approaches gave conflicting results, but this result proves the necessity to measure pathways also on the functional level whenever possible to compare the function to steady-state markers like presence or abundance of certain enzymes. Our experiments indicate that the respiratory chain and ATP synthasome can form macromolecular assemblies (supercomplexes) with reorganized composition and/or stoichiometry while the changes are specific for different tumor types. This is in good agreement with recent studies from other laboratories [25] and

the current work shows that equal results can be obtained using kinetic methods, but additional studies are warranted to include results from protein level studies using the blue native gel electrophoresis (BNGE) technique. Our K_m measurements confirmed that two populations of mitochondria registered in healthy colon tissue can be categorized as different layers of the colon wall, but in HBC, the subgroups can be linked to two-compartment metabolism where tumor acts as a metabolic parasite on normal stromal cells. Mitochondria of HCC are homogenous in terms of regulation of the mitochondrial outer membrane permeability and MCA (Figure 8).

Mitochondria are not only the centers of cellular energy conversion but are also the important part in biosynthetic metabolism and apoptosis. Therefore, direct detection of profound changes in the ATP synthasome components and in the architecture of the respiratory chain complexes, as shown in the current work, can support development of new predictive models or therapies.

Abbreviations

ANT: Adenine nucleotide translocator
CAT: Carboxyatractyloside
COX: Cytochrome c oxidase
FCC: Flux control coefficient
HBC: Human breast cancer
HCC: Human colorectal cancer
HK: Hexokinase
MCA: Metabolic control analysis
MI: Mitochondrial interactosome
MOM: Mitochondrial outer membrane
MtCK: Mitochondrial creatine kinase
mtPTP: Mitochondrial permeability transition pore
OXPHOS: Oxidative phosphorylation
RC: Respiratory chain
SCAF1: Supercomplex assembly factor I
TME: Tumor microenvironment
VDAC: Voltage-dependent anion channel.

Additional Points

Highlights. (1) Relative complex I functional deficiency is characteristic for HCC but not for HBC. (2) HBC respiratory capacity severely higher than in adjacent normal breast tissue. (3) Complexes I and III expectedly assembled in both tumorous and normal tissues. (4) K_m for ADP shows distinct differences between cell cultures and clinical samples. (5) Two distinct mitochondrial populations present in HBC but not in HCC.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Publication II

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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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OXPHOS

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

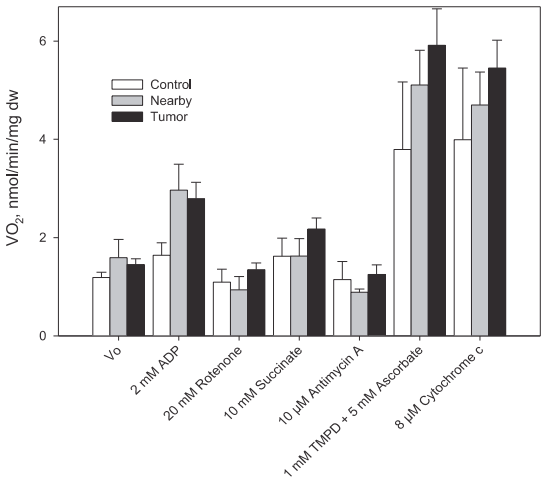

Fig. 1. Analysis of the mitochondrial respiratory chain function in permeabilized human colorectal cancer, junction area between cancer and normal mucosa (nearby) and healthy tissue samples. These studies were carried out in medium-B with 5 mM glutamate and 2 mM malate as respiratory substrates. TMPD 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-

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.

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

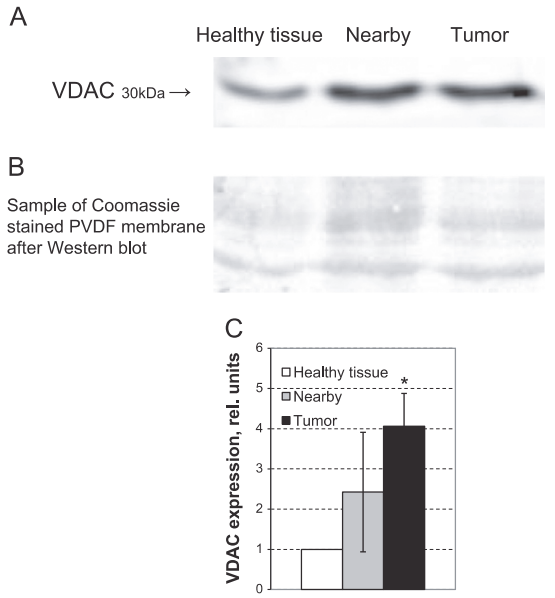

Fig. 2. (A) Western blot analysis of the level of voltage-dependent anion channel (VDAC) porin protein expression in human CRC, nearby and unaffected non-tumorous tissue. The VDAC bands in reference samples correspond to the molecular mass of 30 kDa. (B) Sample of Coomassie-stained PVDF membrane (cutoff 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

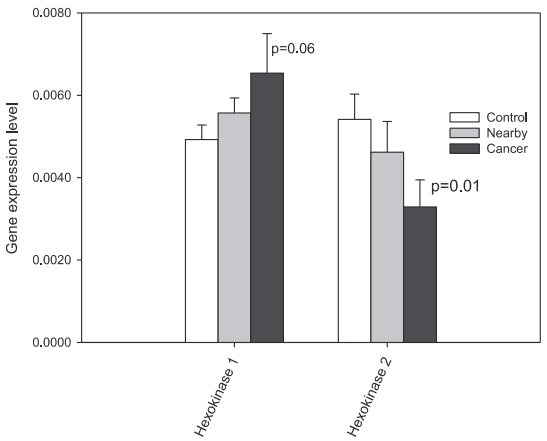

Fig. 3. Q-PCR analysis of the levels of mRNA expression for hexokinase 1 and 2 in human CRC and surroundings tissues; bars are SEM; $p=0.06$ for HK 1 and $p=0.01$ for HK 2. Average results for 29 CRC patients.

$I_{GLU} \approx 11.7\%$. 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 ($\sim$ 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

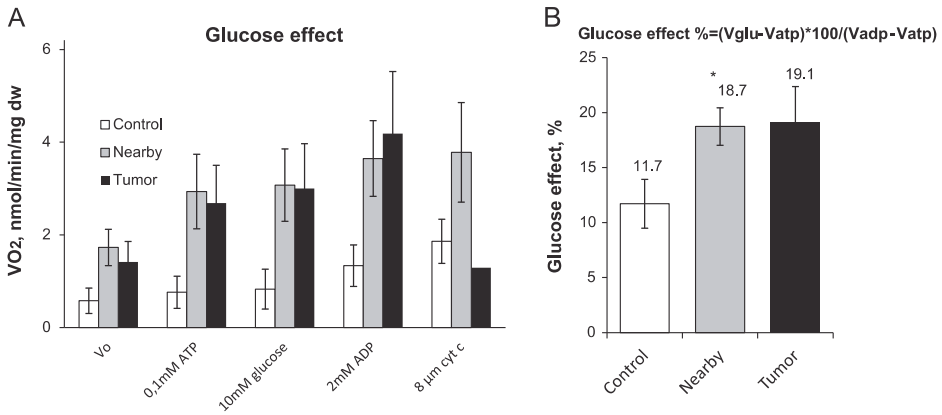

Fig. 4. (A) Oxygraphic analysis of coupling of HK to OXPHOS in permeabilized CRC, nearby and control (healthy large intestine) tissue samples. The addition of 10 mM glucose to CRC fibers in the presence of 0.1 mM Mg-ATP caused a stimulatory effect on mitochondrial respiration in nearby and tumor tissue.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

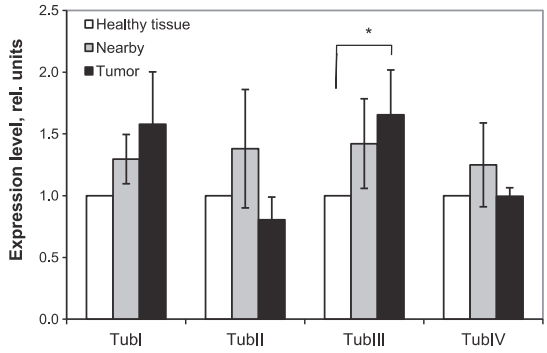

Fig. 5. Western blot analysis of the level of various β-tubulin isotypes: relative expression compared to control. Bars are SEM, $n=7$, * $p<0.05$. Additional data on Supplementary Fig. 2.

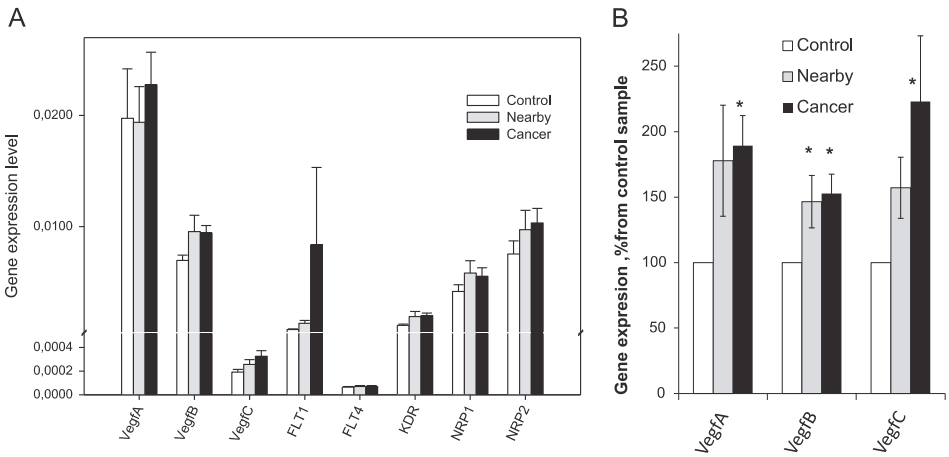

Fig. 6. (A) Q-PCR analysis of the mRNA expression for some vascular endothelial growth factors and their receptors in human CRC and surrounding tissue samples. (B) VEGF (A-C) gene expressions normalized to the unaffected tissue. Bars are SEM, $n=29$, * $p<0.05$.

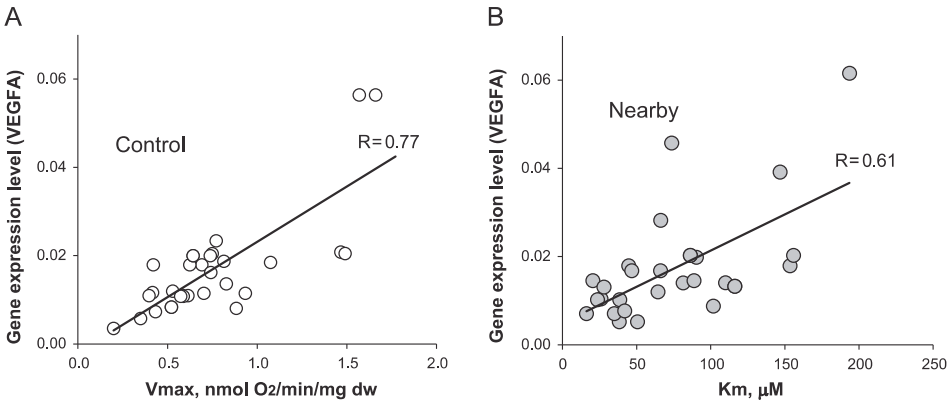

Fig. 7. Interrelationships between the level of VEGF-A expression in tissue samples of CRC patients and their respiratory parameters; rates of maximal ADP-activated respiration (V_m) and K_m values for ADP. (A) The relationship between the level of VEGF-A expression and V_m values for healthy (control) 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.

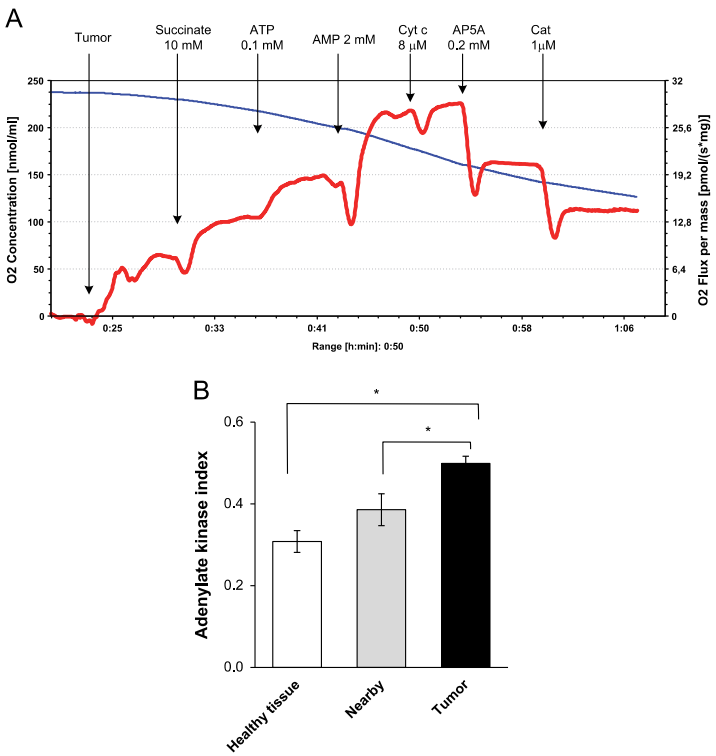

Fig. 8. Oxygraphic analysis of the functional coupling between adenylate kinase (AK) catalyzed processes and OXPHOS in permeabilized CRC, nearby and adjacent healthy intestine tissue samples. (A) Representative tracing of rates of O_2 consumption by CRC fibers; addition of 2 mM AMP in the presence of 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, $n=10$; $^{*}\ p<0.05$.

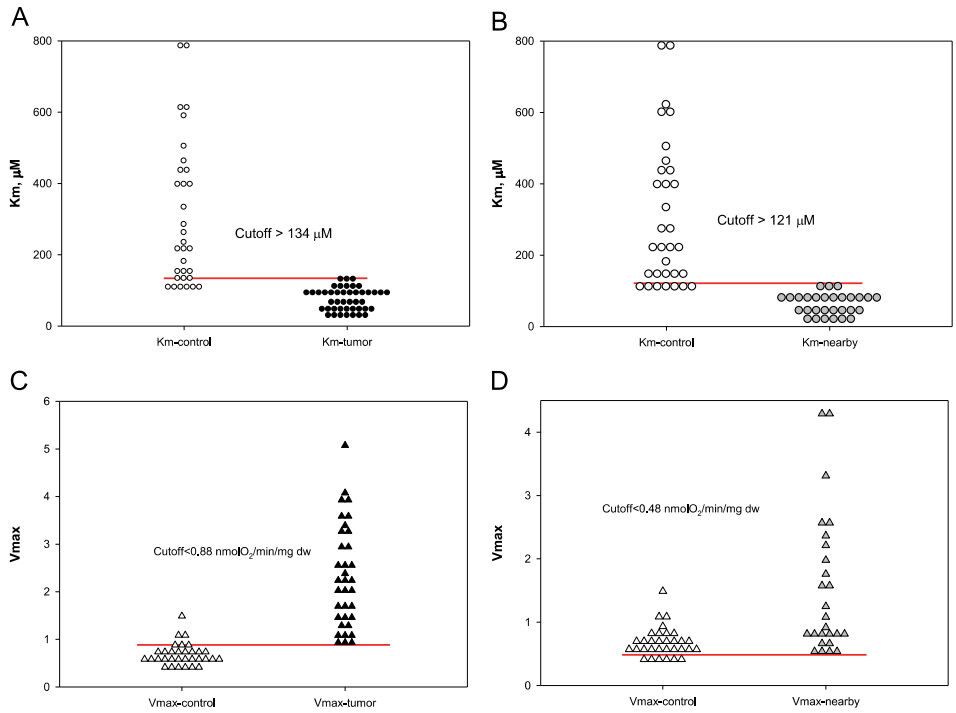

Fig. 9. Cutoff analysis of apparent K_m (ADP) for control-tumor (A), and control-nearby (B) permeabilized samples. V_m values cutoff analysis was also performed for these two datasets: (C) control-tumor and (D) control-nearby. For these calculations samples were taken from 46 patients.

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

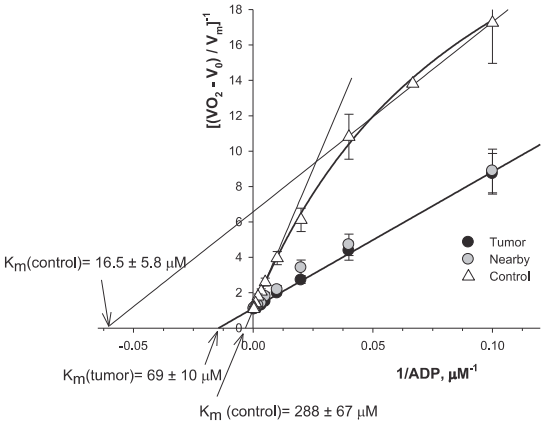

Fig. 10. The dependence of the normalized values of respiration rates of CRC, nearby and unaffected colon tissue permeabilized samples: double reciprocal Lineweaver–Burk plots ($n=9$).

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.

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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,

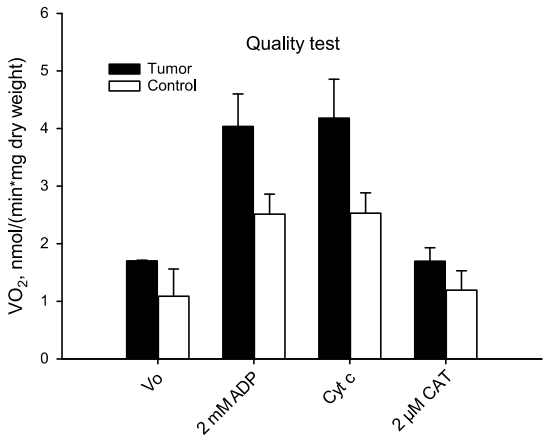

Fig. 1. Quality tests for the intactness of mitochondrial membranes in permeabilized human colorectal cancer and surrounding normal tissue fibers. These experiments were performed in medium-B with 5 mM glutamate, 2 mM malate, and 10 mM succinate as respiratory substrates. Respiration of permeabilized 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.

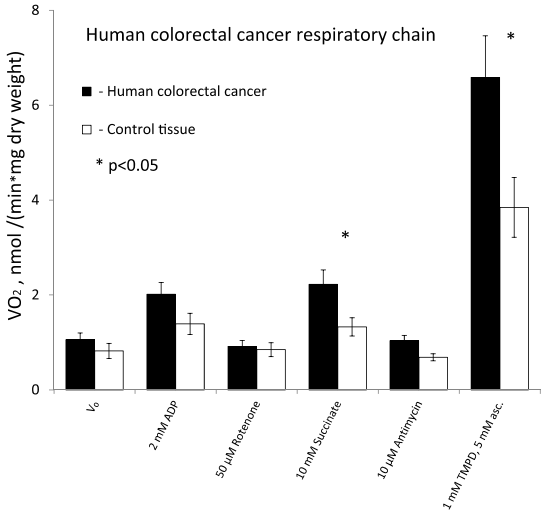

Fig. 2. Evaluation of the respiratory chain functions in permeabilized human colorectal cancer fibers and normal adjacent tissues. The experiment was performed in medium-B with 5 mM glutamate and 2 mM malate as respiratory substrates. TMPD is N,N,N′,N′-tetramethyl-phenylenediamine, and asc – ascorbate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

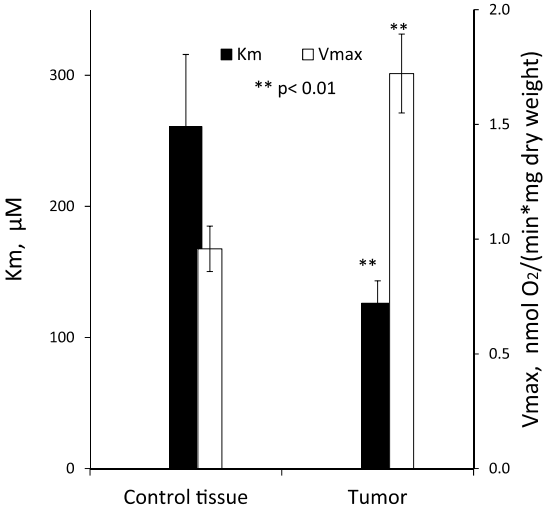

Fig. 3. Comparative analysis of the apparent K_m values for ADP and the maximal respiration rate (V_{max}) for permeabilized skinned HCC and normal colon samples. Bars are SEM, $n = 35$, $p < 0.01$.

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.

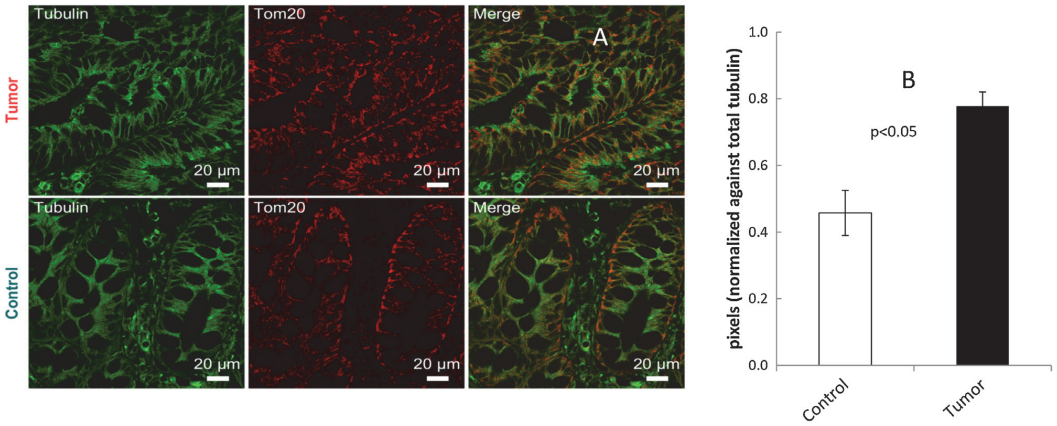

Fig. 4. Immunohistochemistry quantification of the total mitochondrial content in postoperative tissue samples; this was performed *via* measurements of Tom20 expression levels. (A) Representative confocal images of paraffin-embedded sections of HCC and normal colorectal tissue preloaded with anti-tubulin 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

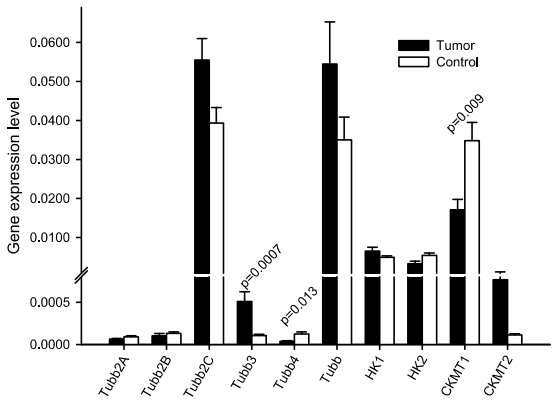

Fig. 5. Expression profile of various β-tubulin isotypes, some creatine kinase and hexokinase isoforms in human colorectal cancer and normal tissue; mRNA levels were assayed by real-time quantitative RT-PCR (bars are SEM, $n = 29$). Here: HK-1 and HK-2 are hexokinase-1 and -2; CKMT1 and CKMT2 are ubiquitous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

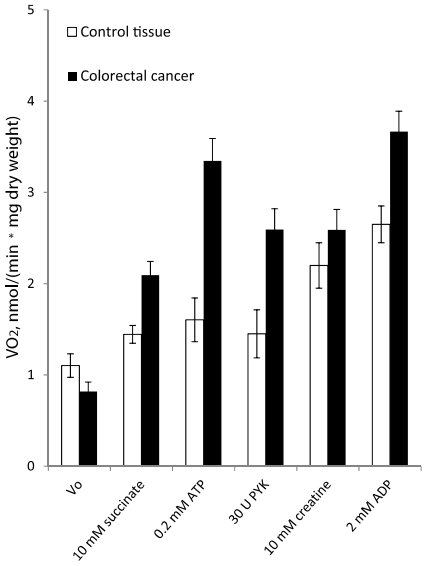

Fig. 6. Efficiency of creatine in the control of OXPHOS by mitochondrial creatine kinase (uMtCK) *via* local ADP production in permeabilized HCC and normal tissue fibers. The experiment was performed in medium-B with 5 mM glutamate and 2 mM malate and 10 mM succinate as respiratory substrates. Creatine 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, $n = 8$.

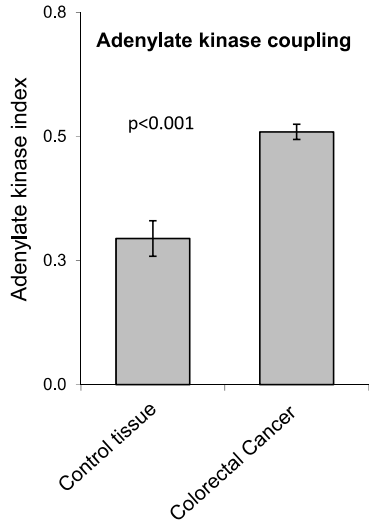

Fig. 7. Adenylate kinase (AK) functional coupling in permeabilized control and HCC tissues. Adenylate kinase coupling with mitochondria was expressed using AK index. Outer mitochondrial intactness was controlled by effect of exogenously-added cytochrome *c* (cytochrome *c* effect <20%). Bars are SEM,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

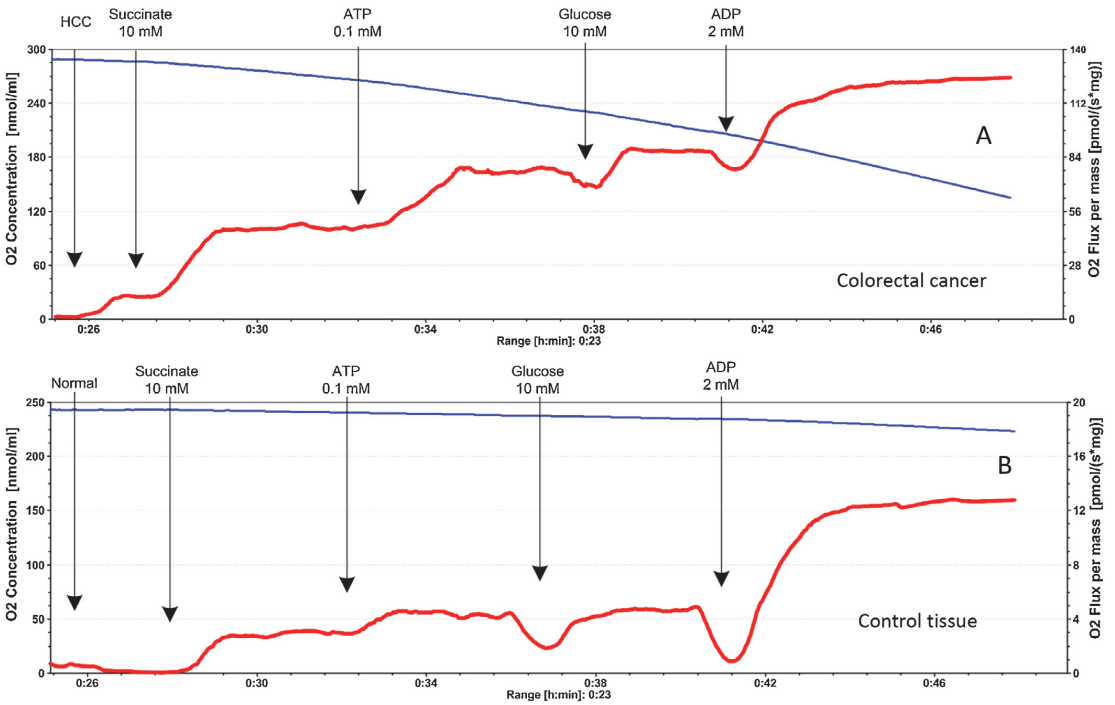

Fig. 8. Oxygraphic analysis of coupling of HK to OXPHOS in permeabilized HCC (A) and normal tissue fibers (B); recording of original traces of O_2 consumption. The addition of 10 mM glucose to HCC fibers in the presence of 0.1 mM Mg-ATP caused a stimulatory effect on mitochondrial respiration, but this 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)

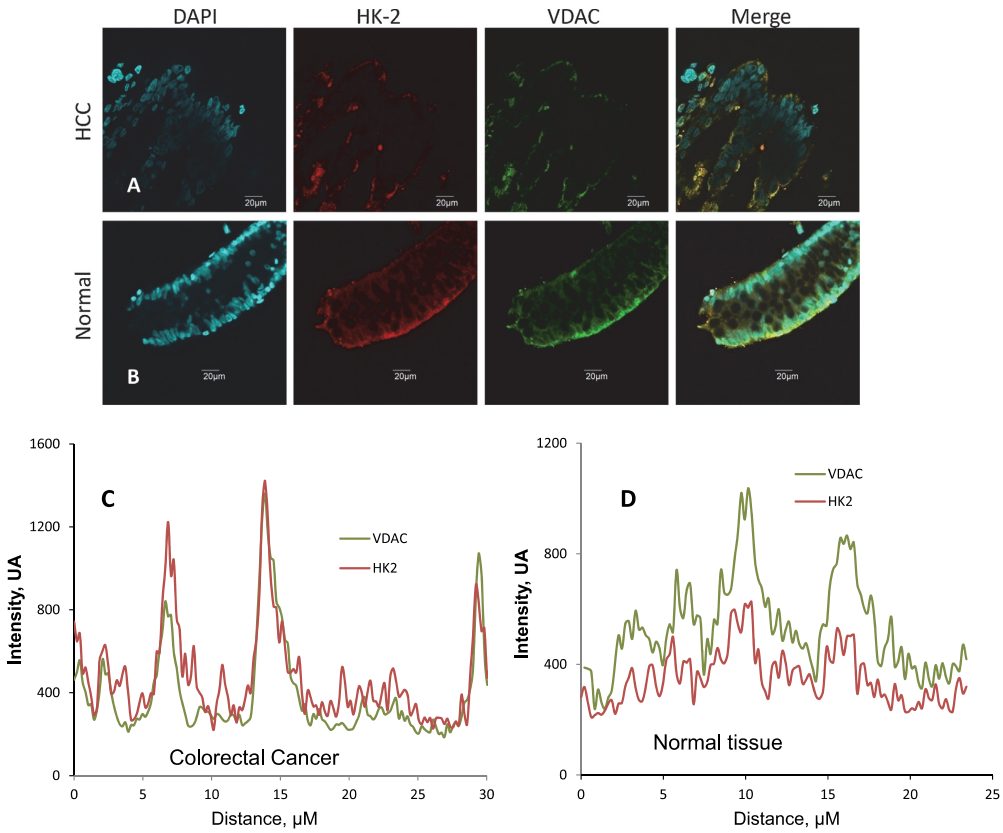

Fig. 9. Confocal imaging immunofluorescence of hexokinase-2 (HK-2), mitochondrial VDAC and their colocalization in saponin-skinned human colorectal cancer (HCC) fibers (A) and adjacent normal tissue (B) obtained by immunocytochemistry: blue fluorescence (nucleus, DAPI), red color indicates the presence 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

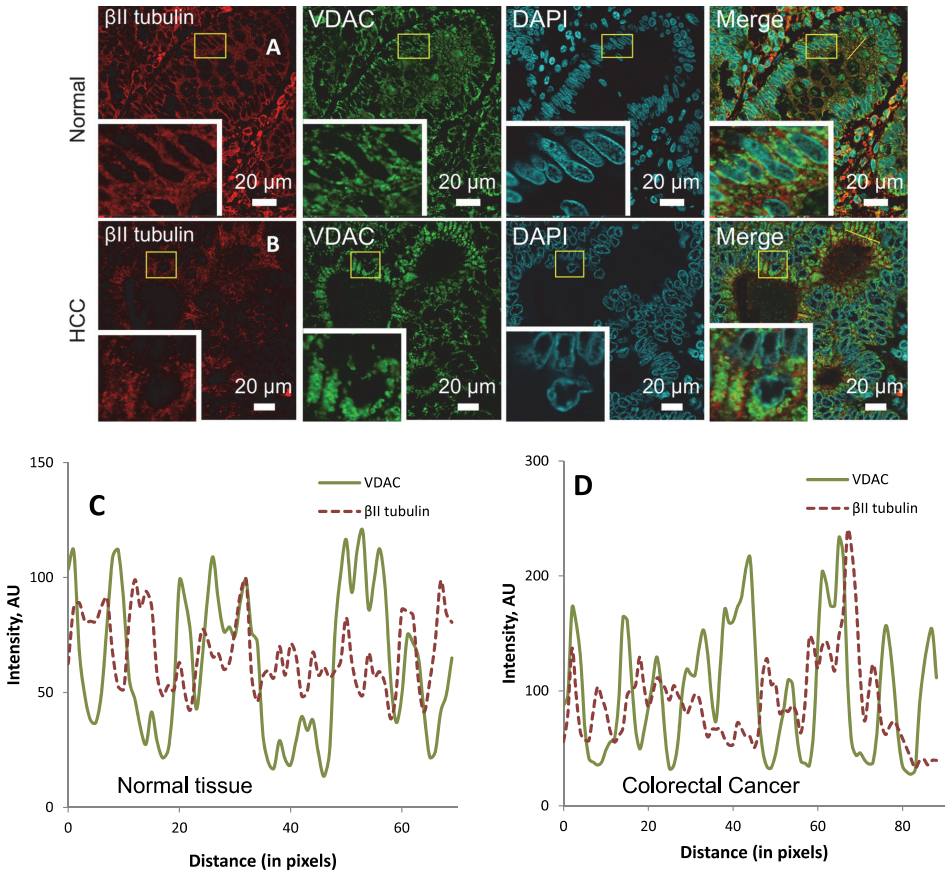

Fig. 10. Confocal imaging immunofluorescence of mitochondrial VDAC, cytoskeletal βII-tubulin, and their colocalization in normal (A) and human colorectal cancer (HCC) (B) tissue slices. Here: blue fluorescence (nucleus, DAPI); red color indicates the presence of βII-tubulin (Dylight-550 labeled Ig, ab96880), 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

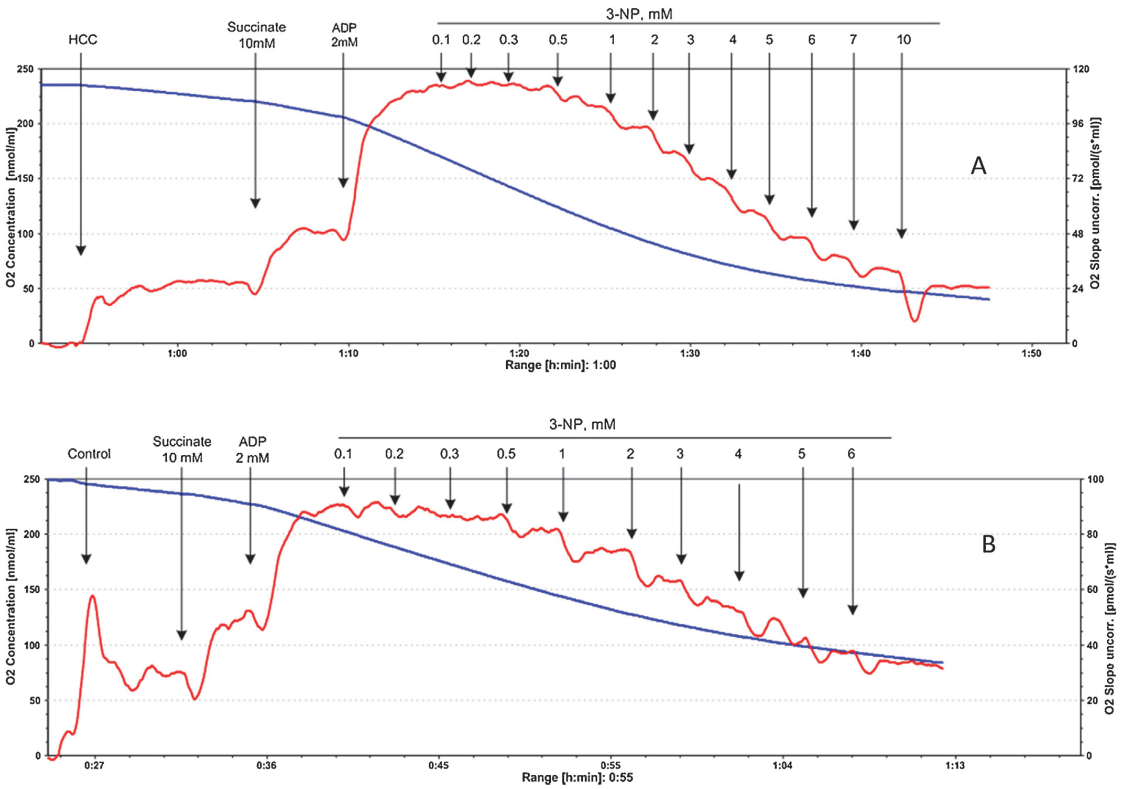

Fig. 11. Representative tracing of change in the rate of O_2 consumption (red lines) by permeabilized human colorectal cancer (A) and adjacent normal tissue fibers (B) during their titration with increasing concentrations of 3-nitropropionic acid (3-NP, a specific inhibitor of Complex-II) in the presence 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

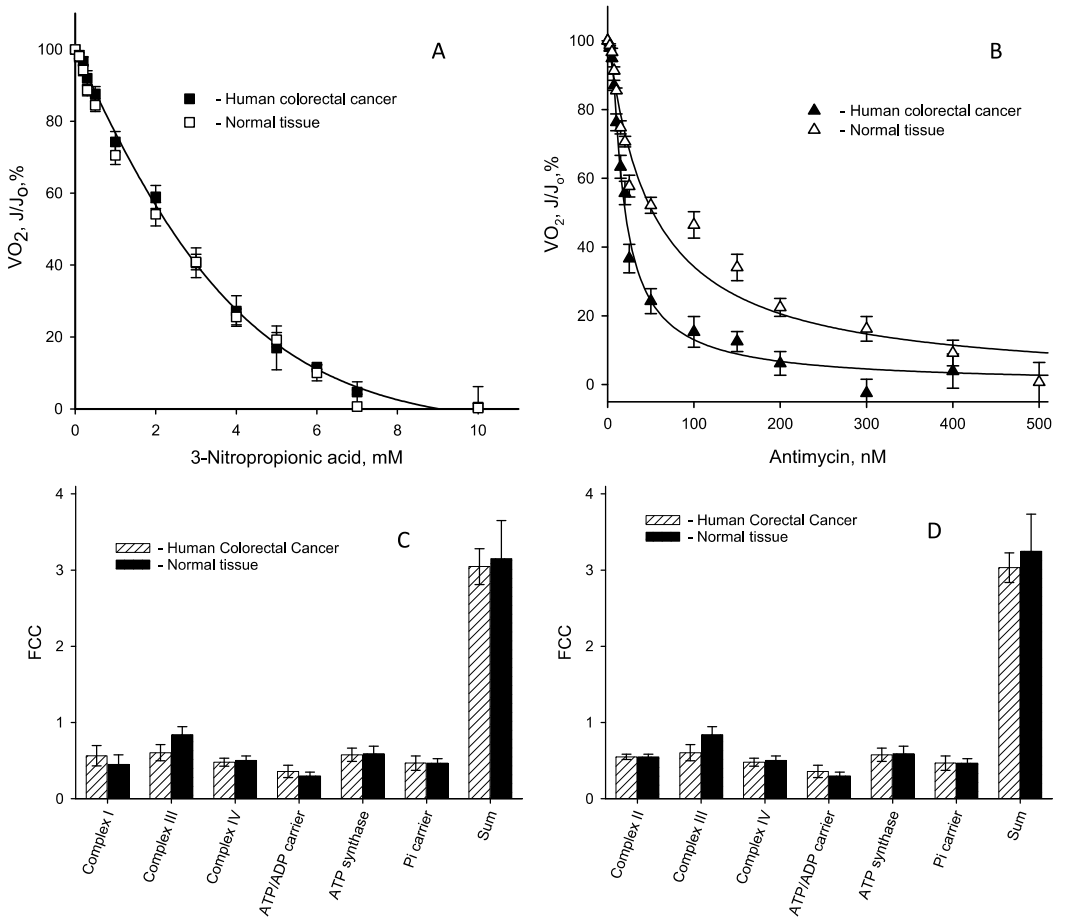

Fig. 12. Titration curves of inhibition of the mitochondrial respiratory chain complexes in permeabilized human colorectal cancer and non-tumorous adjacent tissue fibers with: (A) 3-nitropropionic acid, in the presence of 10 mM succinate as a respiratory substrate (without malate and glutamate); and (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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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.

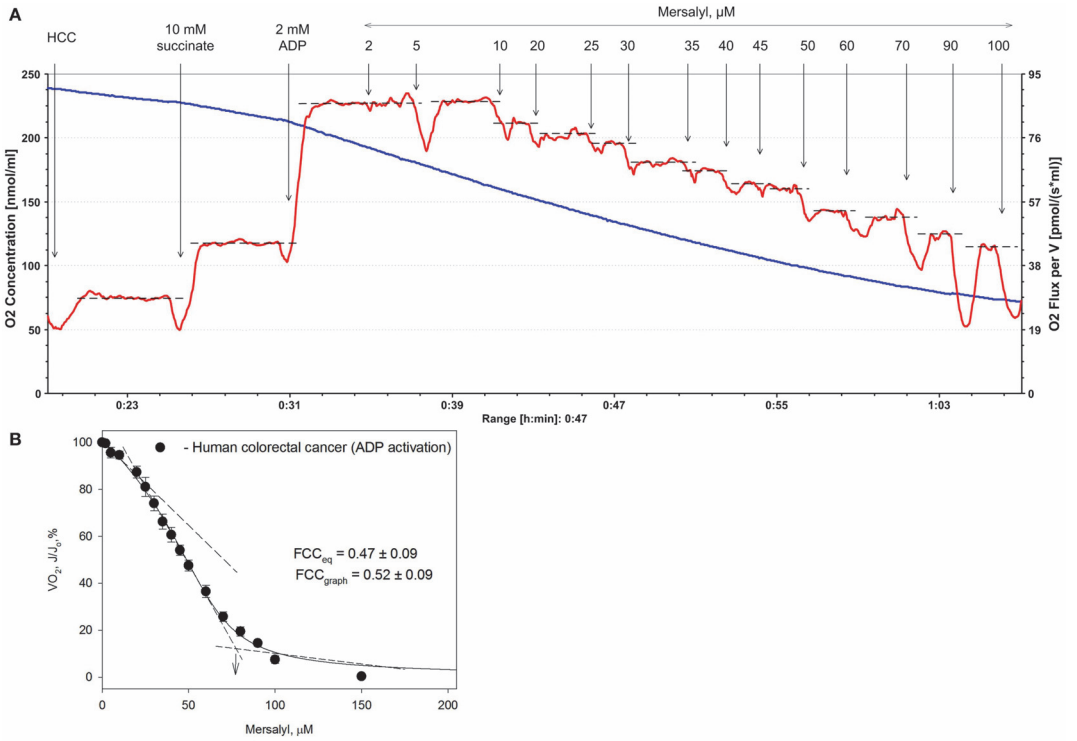

FIGURE 1 | Representative traces of change in the rate of oxygen consumption by permeabilized human colorectal cancer (HCC) fibers after their titration with increasing concentrations of mersalyl, an inhibitor of inorganic phosphate carrier (panel A). The values of respiration rate obtained were plotted 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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Uusi suundi kasvajate energiametabolismi uuringutes

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Genoomika kiire arengu käigus on selgunud, et selle valdkonna meetoditega ei ole võimalik erinevaid metabolismihäireid terviklikult kirjeldada ning täiendavalt on vaja kasutusele võtta teisi meetodeid rakuenergeetikast ning proteoomikast. Äärmiselt huvitavaks kujuneb selline süsteemsem käsitlus ulatuslike patoloogiliste muutustega maliigses koes. Eelmise sajandi alguses kirjeldas Otto Warburg efekti, kus tuumorirakkudes toimus eelistatult glükolüüs isegi normoksiatingimustes. Tema esmane arvamus, et just see asjaolu ongi raku maliigsuse allikas, lükati järgnevatel aastatel uute avastuste valguses ümber. Lisaks ulatuslikele rakuenergeetilistele ümberkorraldustele maliigse raku sees (nt kärbitud Krebsi-tsükkel, hingamisahela superkompleksid) on viimastel aastatel erinevate vähipaikmete juures korduvalt tõestatud ka kahe kompartmendi olemasolu, kus maliigne rakk allutab ümbritseva strooma enda jaoks vajalikke metaboliite tootma. Maliigsuse täpsem olemus, paremad ravimisihtmärgid ning -strateegiad võivad peituda just kasvajate süsteemsemate uuringute tulemustes.

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Võtmesõnad:
metaboolse kontrolli analüüs, mitokonder, hingamisahel, rinnavähk, kolorektaalvähk, Warburgi efekt

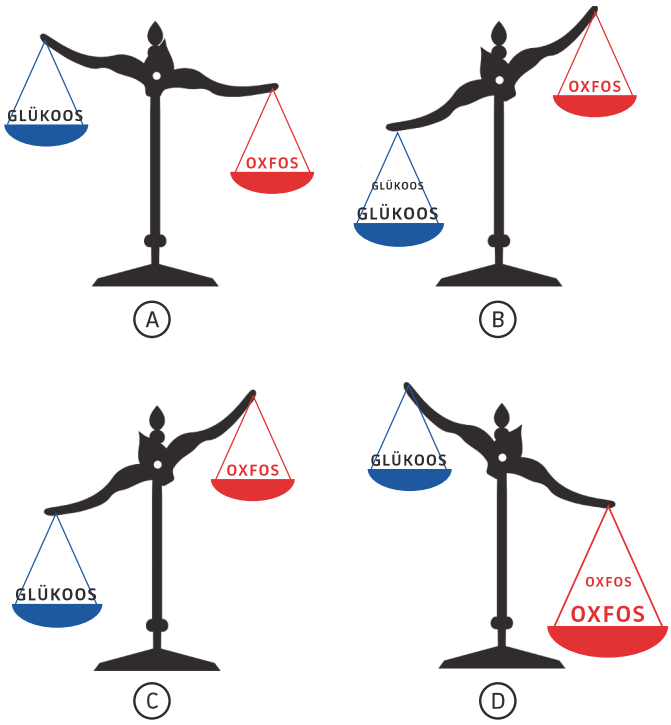
Kuigi tänapäeva nn postgenoomsel ajastul on paljude organismide geenijärjestused juba kindlaks tehtud, on see omakorda tõstatanud uusi ja senisest tunduvalt keerulisemaid probleeme. Hoolimata sellest et me oskame kirjeldada raku üksikuid struktuurseid komponente, ei ole me veel ikkagi võimelised mõistma ja kvantitatiivselt kirjeldama nii üksikut rakku kui ka tervet organismi ning ka mitmete haiguste patogeneesi mehhanisme. Praegu on paljusid patoloogilisi seisundeid uuritud genoomi tasemel, kuid see ei võimalda mõista patoloogiliste muutuste korral kõiki sündmusi raku metabolismis. Edasiarendamist vajavad siin raku energeetika ja proteoomika valdkonnad. Üheks põhimõtteliseks kitsaskohaks on, et puudub süsteemne käsitlus, mille käigus peaksid tihedat koostööd tegema füsioloogid, bioloogid, biofüüsikud, meedikud ja matemaatikud. Pahaloomulised kasvajad on äärmiselt heterogeensed ja keerulised bioloogilised süsteemid ning seetõttu on kompleksne käsitlus nende mõistmiseks kriitilise tähtsusega. Bioenergeetilised muutused maliigsetes rakkudes on viimastel aastatel maailmas väga aktuaalne ja kiiresti arenev teema. Ammu on teada, et selliseid rakke iseloomustab ka nende ebanormaalne energiametabolism.

Kasvajate energiametabolismi tüübid

Paljude vähitüüpide korral toimub rakkudes metaboolne nihe oksüdatiivselt fosforüülimiselt glükolüüsi suunas ning seda nimetatakse Warburgi efektiks ning aeroobsetes tingimustes toodetakse neis rakkudes suurel hulgal laktaati. Brandi (1) andmetel kaitseb suure glükolüütilise aktiivsusega fenotüüp prolifereeruvaid rakke oksüdatiivse stressi eest. Pedersen koos kaasautoritega (2–5) on oletanud, et heksokinaas (HK) II seostumine pingest sõltuva anioonkanaliga (*voltage dependent anion channel*, VDAC) on võtmesündmuseks, et terve, oksüdatiivse energiametabolismiga rakk lülituks ümber kantserogeenesi rajale. Meie eelnevad uuringud näitavad, et südame fenotüübiga hiire HL-1 tüüpi rakkudes, mis on saadud hiire südame koja kasvajarakkudest ning milles mitokondrid moodustavad ebaregulaarse, dünaamilise, filamendilaadse võrgustiku ning esineb HK II suurenenud avaldumus, lülitub oksüdatiivse fosforüülimise kontroll mitokondriaalse kreatiinkinaasi (MtCK) toodetud ADP pealt

ümber tsütosooli ADP-le (6, 7). Kasvajate metaboolne fenotüüp, mida iseloomustab lahtisidestatud mitokondriaalne hingamine ja suurenenud oksüdatiivne stress, muudab selle ravimiarenduse jaoks atraktiivseks mudeliks (8).

On andmeid, et rakkude oksüdatiivse fosforüülimise staatust on võimalik kasutada kasvaja pahaloomulisuse hindamisel, s.t väga agressiivsete kasvajate korral võib rakkudes mitokondrite aktiivsus olla maha surutud. Pikka aega arvati, et glükolüüs on maliigsete rakkude jaoks universaalne mehhanism, mis seletab kogu nende energeetilise metabolismi olemuse. Juba pool sajandit tagasi tekkisid kahtlused, et see ei ole nii (9), ja mitmed hiljutised uuringud on seda ka kinnitanud (10, 11). Vaatamata viimastel aastatel toimunud intensiivsele teadustööle pole siiani selge, kuidas muutub kasvajarakkude energeetiline metabolism kantserogeneesi eri staadiumites. Erineva paikmega kasvajaid iseloomustab äärmiselt erinev energiavahetuse viis (vt joonis 1).



Joonis 1. Erinevalt normaalsest rakust (A) võib vähirakkudes metaboolse remodelleerimise tõttu esineda mitmesuguseid energeetiliste protsesside muutusi (B–D). Maliigne rakk võib olla ülimalt glükolüütiline (B), puuduliku oksüdatiivse fosforüülimisega (C) või hoopis suurenenud oksüdatiivse fosforüülimisega (D). Joonis tehtud 31. allika alusel.

Järjest enam leiab kinnitust hüpotees, et kasvajates esineb mitu kompartmenti, mis on omavahel seotud ja tõenäoliselt muudavad maliigse raku eriti hästi oludega kohanevaks. Järjest enam on kirjanduses ilmunud viiteid, et maliigsete rakkude kõrval asuvad makrofaagide aktiveeritud stroomarakud, milles toimub aktiivne glükolüüs ja need n-ö toidavad kasvajarakke substraatidega, mida tuumor kasutab oksüdatiivse fosforüülimise jaoks. Seda nähtust nimetatakse Warburgi pöördefektiks (10, 12–14). Sellise ainevahetusega kasvajate hulka kuulub ka rinnavähk ning arvatavasti on Warburgi pöördefektiga iseloomustatav energiametabolism rinna pahaloomuliste kasvajate üldine omadus.

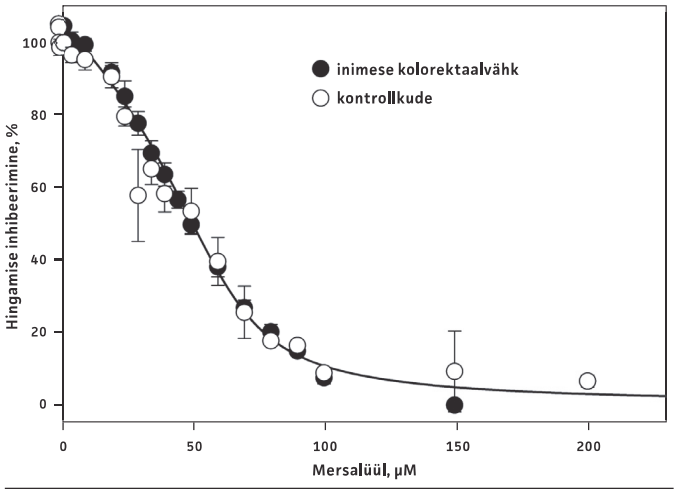
Metaboolse kontrolli analüüsi meetodi kasutamine kasvajate energiametabolismi uurimisel

Tänapäeval on oluline välja selgitada, millised on need mehhanismid, mis kontrollivad kasvajate energiametabolismi ja kuidas need on omavahel seotud. Reaalselt on võimalik rakkude metaboolsete protsesside kineetikat iseloomustada matemaatilise modelleerimise abil ning konstrueerida mudeleid, mis ensüümkineetika ja metaboliitide transpordi kohta saadud eksperimentaalsete andmete alusel võimaldavad kirjeldada kogu süsteemi käitumist molekulaarsel tasemel. Kokkuvõttes sisaldavad saadud mudelid kümneid kiirus-, tasakaalu- ja difusioonikonstante, mille õigsus ei tarvitse olla täismahus täielikult kontrollitav. Sellekohased töid ilmus juba üle kümne aastat tagasi (15, 16) ning praeguseks on see käsitlusviis teinud läbi märkimisväärse arengu (17).

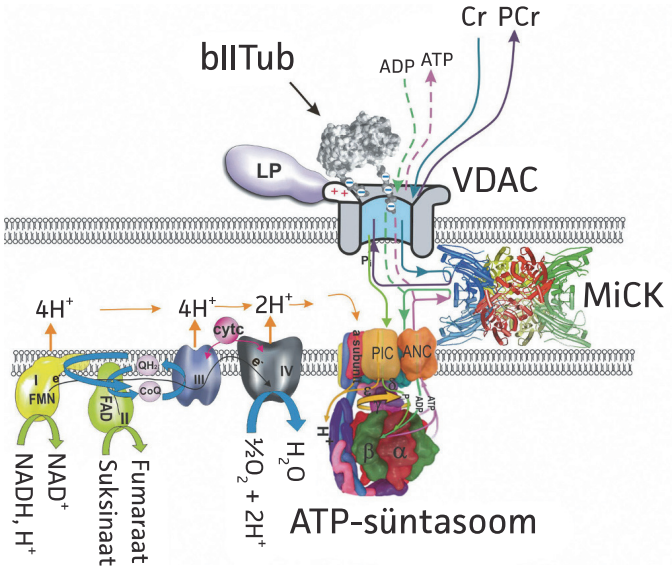
Sisendparameetrite suure hulga tõttu on võimalik konstrueerida erinevaid, sisuliselt alternatiivsetest kontseptsioonidest lähtuvaid mudeleid, mis võrdväärselt kirjeldavad süsteemi käitumist. Nende mudelite sisuline kontroll on võimalik, kui nende alusel saab kavandada uusi reaalselt teostatavaid mudelite sobivust hindavaid katseid, mis paraku ei osutu alati siiski võimalikuks. Sedalaadi piirangutest mudelite hindamisel on võimalik üle saada, kui kasutada metaboolse kontrolli analüüsi meetodit (MKA). Tegemist on eksperimentaalse meetodiga, mistõttu selle abil saadud info on objektiivne ning seda rakendades on võimalik reaalsetes ja sobivalt piiritletud tingimustes saada usal-

dusväärseid empiirilisi andmeid. See matemaatilise modelleerimise meetod ennetas nn sisulist modelleerimist ning mõlemad on olnud pikka aega rööbiti kasutusel (18, 19). Meetodi abil käsitletakse keerukat süsteemi koosnevana sõltumatute elementide jadast, mida sisuliselt käsitatakse mustade kastidena, mille sisemist struktuuri ignoreeritakse. Selle meetodi puhul määratakse eksperimentaalselt teatud summaarset parameetrit, kõne all oleval juhul rakkude hapnikutarbimise kiirust, ning hinnatakse iga sõltumatu muutuja osakaalu selles protsessis (20, 21). Eksperimentaalselt kasutatakse vastava struktuuriüksuse (muutuja) järkjärgulist, osalist, soovitatavalt pöördumatut, kuid selektiivset inhibeerimist mingi keemilise ühendi poolt (vt joonis 2) ning määratakse mõõdetava parameetri (hapnikutarbimise kiiruse) tundlikkus selle konkreetse elemendi blokeerimise suhtes (metaboolse kontrolli koefitsient). Seega määratakse reaktsioonide jada üldist aktiivsust piiravad staadiumid ning elementide suhteline n-ö osakaal protsessis. Selline sisuliselt formaalne käsitlusviis võib osutuda väga efektiivseks näiteks uute ravimite otsingul, et leida patoloogiliste protsesside "tundlikke" punkte.

Varem on selliselt kasvajaid uurinud dr R. Rossignol kaastöötajatega, määrates oksüdatiivse fosforüülimise kontrollikoefitsiente roti isoleeritud mitokondrites, nii et selle tulemusel on võimalik identifitseerida vähiteraapia jaoks olulisi märklaud-ensüüme rakkude metabolismi radades (22, 23). Selle meetodi puhul tiitritakse uuritava metabolismiraja komplekse astmeliselt, kasutades nende komplekside jaoks spetsiifilisi pöördumatuid inhibiitoreid. Samuti on seda meetodit kasutatud muutuste väljaselgitamiseks rakkude energiametabolismis lihaste patoloogiliste seisundite puhul (24, 25). Meie kasutasime metaboolse kontrolli analüüsi meetodit rinnavähi energiametabolismi uurimiseks. See uuring on esimeseks omalaadseks, kus kasutatakse skineeritud kiudude meetodit rinnavähi kliinilise materjali uurimiseks. Selgus, et kuigi rinnavähki peetakse puhtalt glükolüütilise metabolismiga kasvajaks, on selline arusaamine tulnud peamiselt sellest, et uuringuteks kasutatakse rakukultuure, mis ei pruugi peegeldada vähirakkude omadusi koes endas. Seda nähtust on tabavalt nimetatud kultuurišokiks (26)

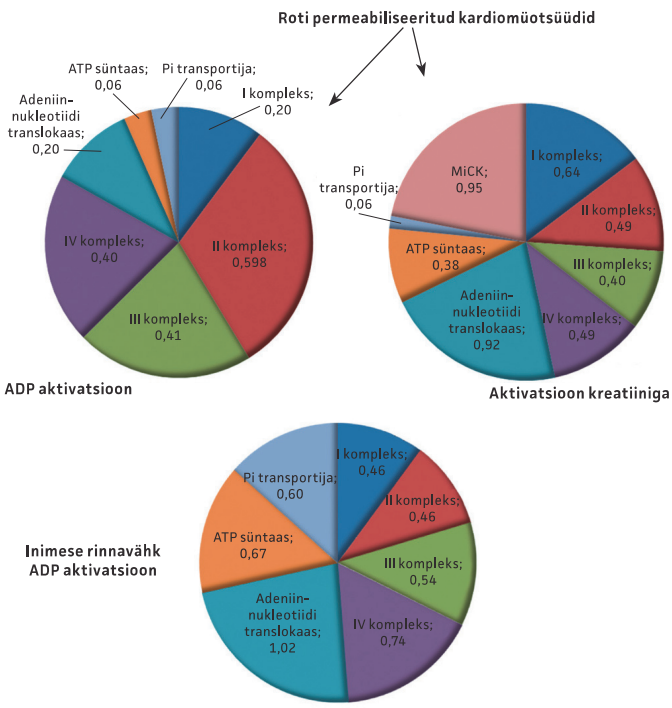


Joonis 2. Anorgaanilise fosfaadi kanali mersalüüliga inhibeerimise kõver. Kontrollikoefitsiendid arvutatakse kõverast Smalli võrrandi abil (32) või graafilise meetodiga, kasutades kõvera algtõusu ja inhibiitori kontsentratsiooni, kus reaktsioon on täielikult pidurdunud. Siin erinevust kasvaja ja terve jämesoole (kontroll) koe vahel pole, seega tõenäoliselt ei mängi fosfori transportija rolli kolorektaalvähi tekkes.



Joonis 3. Hapnikutarbimise regulatsioon superkompleksis. Mitokondriaalne interaktosoom (MI), millesse kuuluvad ATP süntasoom (koosneb ATP süntaasist, fosfori kandjast (PIC) ning adeniinnukleotiidi trasnslokaasist (ANC)), mitokondriaalne kreatiinkinaas (MiCK), pingest sõltuv anioonkanal (VDAC) ning selle läbitavuse võimalikud regulaatorvalgud tubuliin (bIITub) ja võimalik *linker*-valk (LP). Südamerakkudes asub oktameerne MiCK mitokondri membraanide vahelises ruumis ning seostub kontaktaladel nii sise- kui ka välismembraaniga. Ei ole selge, kas teda esineb ka maliigsetes rakkudes. VDAC läbitavust erinevatele metaboliitidele (ATP, ADP, AMP, fosfokreatiin, kreatiin (Cr)) reguleerib tubuliini heterodimeer, võimalik, et selles osaleb seni identifitseerimata LP. MI üheks võimalikuks komponendiks on ka hingamisahel.

ning sellest tulenevalt on oluline uurida vähirakke nende naturaalses ümbruses, kus on säilinud koele iseloomulikud omadused, kontaktid rakuorganellide ja tsütoskeleti vahel. Võimaluse selleks annab skineeritud permeabiliseeritud kiudude meetod, kus bioloogilist preparaati töödeldakse saponiiniga, mis muudab rakkude välismembraani läbitavaks rakust väljastpoolt sisseviidavatele ainetele. Keemilise ja Bioloogilise Füüsika Instituudi bioenergeetika laboratooriumis esitatud kontseptsiooni kohaselt toimub energiaproduktsioon ja selle regulatsioon südamerakkudes mitokondriaalse interaktosoomi (MI) raamides, mis ühendab hingamisahela komponendid, ATP süntaasist, adeniinnukleotiidi transportijast (ANT) ja anorgaanilise fosfaadi transportijast (PIC) koosneva ATP süntasoomi ning mitokondri välismembraanis asuva ja selle läbitavust reguleeriva poriinkanali (VDAC) (vt joonis 3). Sarnase bioloogilise struktuuri olemasolu on tõenäoline ka kasvajarakkudes, kus energiatootmiseks kasutatakse oksüdatiivset fosforüülimist. Reaalselt tähendab metaboolse kontrolli analüüs siin rakkude bioenergeetika jaoks oluliste komponentide osalist inhibeerimist koos kontrollikoefitsientide määramisega hingamisahela I (inhibiitoriks rotenoon), II (antimütsiin), IV (NaCN) kompleksi, ANT (karboksüatraktülosiid), PIC (mersalüül) ja ATP süntaasi (oligomütsiin) jaoks. Selline komplekt osutub esialgu piisavaks maliigsete rakkude metaboolse remodelleerimise hindamiseks ning võimaldab täpsustada edaspidiseid uurimissuundi. Seepärast kasutasimegi sama strateegiat rinnavähi kliinilise materjali analüüsil, kusjuures selgus, et rinna kontrollkoes hapnikutarbimist peaaegu ei esinenud, küll aga võis seda sedastada tuumori koes (12). Selgus, et võrdluses normaalse oksüdatiivse koega (nagu näiteks südamelihas) ning samuti koest isoleeritud mitokondritega on rinnavähi energiaülekande süsteem väga tundlik ning saadud voo kontrollikoefitsiendid väga suured (vt joonis 4). Suured voo kontrollikoefitsiendid esinevad südamelihases, kus energiametabolism toimub kreatiinkinaasi raja kaudu. Mittetöötava kreatiinkinaasi puhul on MI kontrollikoefitsientide summa südamerakkudes ligikaudu 1, mis tähendab, et seal on tegemist lineaarse energeetilise süsteemiga. Kui mingi mõõdetud metabolismiraja vookontrolli koefitsientide summa on rohkem kui 1, on tegemist keerulisema ülesehitusega energiaülekande süsteemiga. Tõenäoliselt on südamerakkudes füsioloogilistes tingimustes, kus kreatiin aktiveerib MI, raku energiaülekanne reguleeritud väga efektiivselt. Selles regulatsioonis on oluline osa täita mitokondriaalsel kreatiinkinaasil, adeniinnukleotiidi translokaasil ja tsütokroom c oksüdaasil. Seda näitab ka tehtud analüüs, mille käigus leiti, et südamerakkudes on voo kontrollikoefitsientide summa 4,31. Voo suurema kontrollikoefitsiendiga süsteemides esineb nn kanaliseerimist, erinevate ensüümide funktsionaalset sidestatust, protsesside hargnemisi või suuri valgukomplekse, mida nimetatakse superkompleksideks.



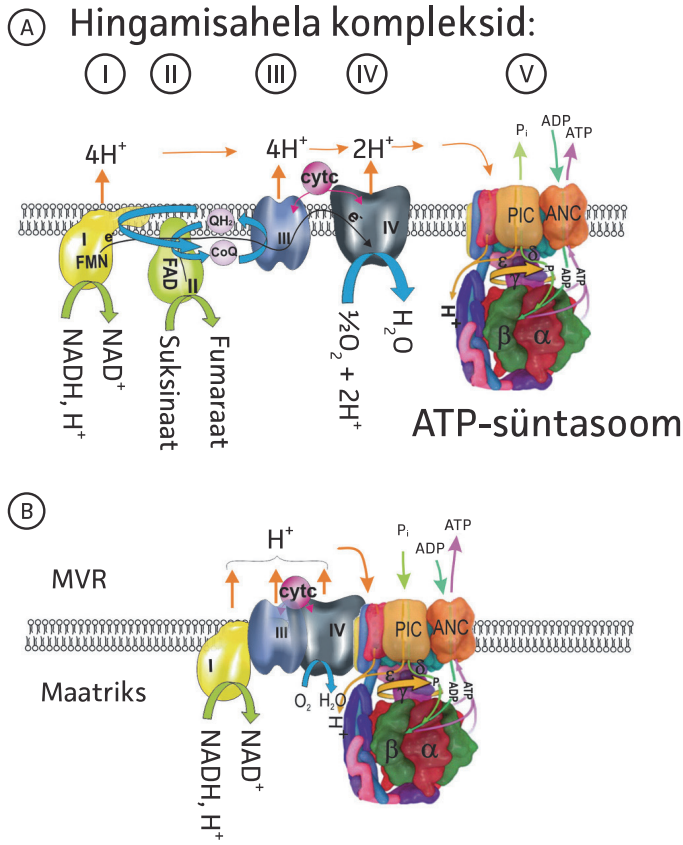
Joonis 4. Metaboolse kontrolli analüüsi tulemused südamelihase ja rinnavähi rakkudes. Südamelihases on voo kontrollikoefitsiendid mõõdetud hingamist aktiveerides kas ADP lisamisega või füsioloogilistes tingimustes, kus kreatiini mõjul hakkab tööle kreatiinkinaas. Rinnavähipreparaatide puhul on kasutatud ADP aktivatsiooni, kuna seal tõenäoliselt puudub mitokondriaalne kreatiinkinaas või pole see aktiivne (12, 33). Ringi pindala moodustab süsteemi koefitsientide summa (100%), erinevad sektorid näitavad süsteemi tundlikkust erinevate komponentide mõjukuse suhtes.

Hingamisahel maliigsetes rakkudes

Rinnavähi bioloogilise materjali uurimisel selgus, et selle rakkude hingamisahela

(hingamisahela kohta vt joonis 5a) kõiki komplekse iseloomustavad suured kontrolli-koefitsiendid. Samuti on nende metaboolse kontrolli koefitsientide summa 4,49. See viib otsesele järeldusele, et maliigsetes rakkudes on hingamisahel teisiti organiseeritud, kui me oleme seda harjunud vaatlema tervete rakkude puhul ning tegemist ei ole lihtsa lineaarse süsteemiga. Sellise süsteemi tekkimiseks on mitu võimalust, millest üks on valguliste superkomplekside moodustumine hingamisahelas ja mida on täheldatud näiteks skeletilihastes. Selle protsessi käigus moodustavad suure valgukompleksi hingamisahela I, III ja IV kompleks (vt joonis 5b). Sellise superkompleksi teke võib olla üheks põhjuseks, miks vähirakud ei ole võimelised apoptoosiks. Rakusurmaks vajalik valk tsütokroom c on seotud selle superkompleksi sisemusse ning ta ei saa moodustada apoptoosi teostumiseks vajalikku kompleksi – apoptosoomi. Teiseks võimalikuks apoptoosi puudumise põhjuseks on ebanormaalse ehitusega hingamisahel vähirakkudes.

Klassikalise arusaama kohaselt seisnevad mitokondriaalsed protsessid peamiselt püruvaadi või rasvhapete oksüdatsioonis tsitraaditsükli ning hingamisahelasse kuuluva nelja kompleksi poolt, mille tulemusena vabanevad keskkonda CO_2 ja H_2O ning suurel hulgal ATPd. Need kaks süsteemi on omavahel tihedalt ning mõnevõrra ka harmooniliselt seotud, kuid kasvajakoes võib vaadelda nende harjumuspäraste protsesside kulgemises olulisi muutusi. Eelkirjeldatud Warburgi mehhanismi tõttu muudetakse suur osa püruvaadist enne mitokondrisse jõudmist juba tsütosoolis laktaadiks (selle käigus toodetakse ligi 16 korda vähem ATPd kui mitokondrist oleks võimalik saada). Siiski siseneb mitokondrisse jõudev püruvaat (või rasvhappejääk) atsetüül-CoA-na tsitraaditsüklisse, kuid ei läbi seda enamasti täies ulatuses, vaid väljub sellest juba esimese kolmandiku läbimisel ehk pärast tsitraadiks muundamist (tegemist on nn kärbitud Krebsi-tsükliga, *truncated Krebs cycle*) (27). Ühest küljest põhjendatakse seda oksüdatiivse stressi mõjul vähenenud akonitaasi (katalüüsib tsitraadi muundamist edasi isotsitraadiks) aktiivsusega (28), kuid teisalt võib seda seletada maliigse raku suurenenud vajadusega rasvhapete järele (nt membraanideks prolifereerumise protsessis) (27). Rasvhapete neogeneesiks



Joonis 5. A. Hingamisahela kompleksid. I kompleks: NADH – koensüüm Q reduktaas; II kompleks: suktsinaat – koensüüm Q reduktaas; III kompleks: tsütokroom c reduktaas; IV kompleks: tsütokroom c oksüdaas, mõnikord peetakse hingamisahela V kompleksiks ATP-süntaasi.
B. I, III ja IV kompleks on moodustanud superkompleksi, kuhu on seotud ka tsütokroom c (cyt c). MVR – mitokondri membraanide vaheline ruum.

suunatakse mitokondrisse kuhjuv tsitraat vastava antiporteri kaudu tsütosooli, kus vastav süntees ka aset leiab.

Kuna tsitraaditsükkel on maliigsest protsessist kahjustunud, siis mõjutab see eelduslikult ka hingamisahela tööd. Hingamisahelas toimub elektroni ülekanne ning see elektron võib ahelasse siseneda kas I (substraadiks NADH) või II (substraadiks suktsinaat) kompleksi kaudu. Mõnede vähipaikmete korral on näidatud, et tavapäraselt hingamisahela moodustavast viiest kompleksist võivad nii I, II, III või ka IV ja V olla funktsionaalselt kahjustunud, kusjuures kahjustuste eripära on seostatav kasvaja maliigsusega (29). Kui hingamisahela kas I või II kompleksi aktiivsus on allareguleeritud ja tsitraaditsükkel minetanud oma klassikalise toimimismustri, aga mitokonder on

siiski säilitanud rolli maliigse raku tinglikus homeöstaasis, siis võib see üheselt viidata laiaulatuslikele ümberkorraldustele pahaloomuliste rakkude mitokondrite harjumuspärases töös. Selliste ümberkorralduste parem kirjeldamine, süstematiseerimine ning valideerimine võib oluliselt aidata kaasa muu hulgas kantserogeneesi, ravimiefektide (sealhulgas kõrvalmõjude) ning konkreetse patsiendi maliigse haiguse eripära paremale mõistmisele.

Rakuenergeetikast lähtuvad uued võimalused kliinilises onkoloogias

Suurimaks rakuenergeetikaga seotud väljakutseks onkoloogias on leida farmakoloogiliselt aktiivseid aineid, mis ründavad spetsiifiliselt vähirakke. Perspektiivikaks peetakse võimalust muuta mitokondri välismembraani regulatsiooni maliigsetes rakkudes, kus on ülekaalus glükolüüs, samuti glükolüüsi otsest inhibeerimist. Universaalsemaid viise kasvajarakkude vastu toimimiseks pakuvad raku metaboolse reprogrammeerimise inhibeerimine, samuti glutaminolüüsi, oksüdatiivse fosforüülimise või Krebsi tsükli ensüümide inhibeerimine. Teiseks perspektiivikaks võimaluseks on vähirakkude suunamine apoptoosi, kasutades selleks võimaluseks apoptoosi aktiveerumise esilekutsumist või reaktiivsete hapnikuosakeste hulga suurendamist. Rakuenergeetika võib olla võimaluseks leida uusi biomarkereid pahaloomuliste kasvajate diagnostika jaoks (30). Osa neist ainetest võib olla seotud selliste onkogeenidega nagu c-Myc, KLF4 ja Oct1. Näiteks c-Myc defitsiit võib viidata mitokondriaalse hingamise vähenemisele ning selgelt välja kujunenud Warburgi efekti olemasolule maliigsetes rakkudes. KLF4 puudulikkus stimuleerib samuti glükolüüsi, kuid sealjuures ei muutu rakkude hapnikutarbimine. Erinevalt kahest eelmisest suurendab Oct1 puudulikkus oksüdatiivse fosforüülimise efektiivsust ning vähenenud või peaaegu puuduvat glükolüütilist aktiivsust maliigsetes rakkudes. Seda laadi muutused mõjutavad aminohapete ja rasvhapete metabolismi ning tänu sellele võivad osutuda onkoloogilisteks biomarkeriteks isoleutsiin, α-aminoadipiinhape ja γ-aminoisovõihape. Peale nende on üheks paljulubavaks biomarkeri kandidaadiks monokarboksülaadi transportija MCT-4, mis võimaldab hinnata rinnavähipatsientide prognoosi (10).

Kasvajate bioenergeetiline iseloomustamine annab kindlasti uut infot kantserogeneesi kohta fundamentaalteaduslikust aspektist, kuid võib tulevikus luua eeldused edukama ravitulemuse saamiseks, võimaldades oluliselt täpsemalt hinnata haiguse prognoosi ning uutest prognostilistest markeritest lähtudes individualiseerida ravi. Süsteemibioloogiline käsitlus võib anda ootamatuid, kuid vähiravi seisukohalt suure praktilise väärtusega tulemusi.

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SUMMARY

New developments in studies of tumor energy metabolism

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During the development of genomic methods it has become evident that they are not able to yield sufficient results for completely describing metabolic pathologies and therefore additional methods from cellular energetics and proteomics have to be applied. Such systematic approach has led to quite surprising results in samples from the tumorous tissue. Otto Warburg was the first to describe high glycolytic rate in malignant cells even under normoxia, however, his hypothesis about this being the main cause of malignancy was discredited with the emergence of new discoveries in following years. In addition to widespread reorganization in the cellular energetics of cancerous cells (e.g. truncated Krebs cycle, respiratory chain supercomplexes), it has been shown that the malignant tissue can make the surrounding stroma to produce metabolites needed by tumour cells. It is believed that an intrinsic understanding of malignancy, as well as of possible drug targets and better treatment strategies can be gained from such systematic approach in tumour biology.

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2005 – 2008 University of Tartu, master course in applied chemistry where I concentrated on studying industrial processes; Master thesis: „Renewing furcellaran production. A study for industrial application", **MA**;

2002 – 2005 University of Tartu, chemistry, Bachelor thesis: „A study on Maillard-like browning reaction using G4S- DA/glycine model system", **BA**;

Work Experience:

2015 - ... **Mitogro OÜ** – co-founder. Research-phase company concentrated on stratifying tumor metabolism in clinical samples to facilitate improved treatment decisions.

2010 – 2016 **AS Elcogen** – *Sales Manager*. Responsible for creating sales structure and customer base on global fuel cell market. We started from zero, but ended up with the leader role in that specific industry (customers mainly in Japan, Korea, West-Europe, USA).

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