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Mitochondrial dysregulation: early Warburg effect as a means of risk stratification in colon cancer

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MITOCHONDRIAL DYSREGULATION: EARLY WARBURG EFFECT AS A
MEANS OF RISK STRATIFICATION IN COLON CANCER

by

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MITOCHONDRIAL DYSREGULATION: EARLY WARBURG EFFECT AS A MEANS OF RISK STRATIFICATION IN COLON CANCER

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ABSTRACT

There exists a profound need for biomarkers that will allow for better screening and risk stratification for colorectal cancer (CRC). With the advent of the newly termed “metabolic syndrome”, CRC prevalence is trending upwards even with much improved screening protocols and remains the second leading cause of cancer related morbidity. The “metabolic syndrome” refers to a range of environmental risk factors, including diabetes and obesity, thought to be increasing the prevalence of CRC. An altered metabolism is seen in metabolic syndrome, which affects cancer through changes in the relationship between glycolysis, the Krebs cycle, and mitochondrial oxidative phosphorylation (OXPHOS). Specifically, it has been observed that highly proliferative tumorigenic cells are undergoing a shift away from the energy efficient OXPHOS and toward aerobic glycolysis even under normoxic conditions. This effect has been termed, the Warburg Effect. As a consequence of endogenous (e.g. genetic, diabetes etc.) and exogenous (e.g. diet, smoking etc.) factors, alterations in cell proliferation/death have been shown to occur throughout the colon reflecting the diffuse “field of injury” (field carcinogenesis). Also due to high energy demands it is recognized that the hyper-proliferative mucosa contiguous to colonic tumors may be hyper-metabolic. Our
group has been interested in elucidating the biological nature of field carcinogenesis and assesses expression of key metabolic markers in the rectal biopsies from patients who harbor neoplasia elsewhere in their colon. We found key indications of a glycolytic shift toward aerobic glycolysis with upregulation of glucose transporter (GLUT1) as well as pyruvate shunting away from OXPHOS via pyruvate kinase muscle 2 (PKM2). These changes were further corroborated by an increase in hypoxia inducible factor 1 alpha (HIF1α), which is normally seen to increase glycolytic function in hypoxic conditions. Along with these glycolytic changes we also found mitochondrial dysfunction in patients with adenomas. Specifically, mitochondrial mass was found to be increased, with increases in mtDNA as well as upregulation of mitochondrial fusion via optic atrophy 1 (OPA1). Uncoupling protein 2, which decouples OXPHOS from ATP synthesis in the mitochondria, was also found to be upregulated. These findings represent a novel panel of biomarkers for assessing CRC risk via analysis of metabolic dysfunction in the easily accessible rectal epithelium.
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LIST OF ABBREVIATIONS

AOM ........................................................................Azoxymethane
APC ........................................................................Adenomatous Polyposis Coli
ATP ........................................................................Adenosine tri-phosphate
BMI ........................................................................Body Mass Index
CRC ........................................................................Colorectal Cancer
DNA .........................................................................Deoxyribonucleic Acid
ETC .........................................................................Electron Transport Chain
GLUT1 .......................................................................Glucose Transporter 1
HIF1α .................................................................Hypoxia Inducible Factor 1 alpha
mtDNA .................................................................Mitochondrial Deoxyribonucleic Acid
mRNA ......................................................................Messenger Ribonucleic Acid
OPA1 ........................................................................Optic Atrophy 1
OXPHOS ..................................................................Oxidative Phosphorylation
Pirc ........................................................................Polyposis in Rat Colon
PDK1 .......................................................................Pyruvate Dehydrogenase Kinase 1
PKM2 .......................................................................Pyruvate Kinase Muscle 2
ROS .........................................................................Reactive Oxygen Species
TCA .........................................................................Tricarboxylic Acid Cycle
UCP2 .......................................................................Uncoupling Protein 2
VEGFR .....................................................................Vascular Epithelial Growth Factor Receptor

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INTRODUCTION

Despite many recent innovations in testing procedures and practices, colorectal cancer (CRC) remains the second leading cause of cancer related mortality in the United States. This year alone there are estimated to be 93,090 new cases as well as 49,700 deaths due to colon cancer (Siegel, Miller, & Jemal, 2015). Yet if detected early, CRC is highly curable; but because of the nature of screening recommendations and underutilization of screening by at risk populations, CRC remains under diagnosed at the localized stage. The at-risk population for CRC is generally seen as patients over the age of 50. However, because this screening recommendation is very broad with a low yield of CRC incidence in the general population (under 10%), it remains under utilized by those who need it and perhaps over utilized by those who may not (Roy, Backman, & Goldberg, 2006). A better and more personalized approach to screening guidelines will be needed to accurately identify patients in need of earlier or more frequent screening intervals rather than just advising everyone over the age of 50 to have a colonoscopy.

Presently, numerous genetic and exogenous factors have been identified for risk stratification but the utilization of these factors together to come up with a predictive model for colon cancer remains difficult because the interactions between these factors are complex and unpredictable (H. Roy et al., 2013). It is posited that utilization of a field effect approach, whereupon micro-environmental changes along the entire colonic mucosal tract are detected, in combination with the use of an accurate biomarker may be a more robust method of early identification of patients at a higher risk of developing
CRC (H. K. Roy, Backman, & Goldberg, 2006). This topic remains the crux of my thesis and will be revisited later in the paper.

**Colorectal Cancer Pathway**

Colon cancer has various progression pathways determined by differing molecular and genetic events. The main overarching pathway is the transformation from a benign growth or polyp (adenomatous polyp/adenoma) in the colonic mucosa to an adenocarcinoma with varying degrees of invasion and lymph node metastases. The adenomatous polyp is characterized by its cellular dysplasia but is still benign. However, as the neoplastic process continues, mutations in critical genes and other genetic disturbances begin to occur. In the APC/β-catenin pathway (shown in Figure 1), which accounts for the majority of sporadic colon cancers (>80%), the tumor suppressor adenomatous polyposis coli (APC) gene is initially mutated at a relatively early stage. Loss of APC promotes cellular proliferation through the disruption of Wnt-signaling and leads to further mutations such as in proto-oncogene KRAS (Jänne & Mayer, 2000). Activating mutations in KRAS are seen in advanced stages and promote further growth and inhibition of apoptosis. In advanced stages of the adenoma-adenocarcinoma paradigm of CRC, many tumor suppressor genes including p53 will have accumulated mutations and growth occurs uninhibited (Raskov, Pommergaard, Burchardt, & Rosenberg, 2014).

Another major pathway for sporadic colorectal cancers is the microsatellite instability (MSI) pathway. This pathway results from mutations in DNA mismatch repair genes (hMLH1, hMSH2, hMSH6). Microsatellite repeats in DNA accrue as a result of
these mutations and lead to deficiencies in proteins regulating cell growth such as the pro-apoptotic Bax protein. MSI associated CRC is typically characterized by mutations in the BRAF oncogene, silencing of tumor suppressor genes through CpG island hypermethylation, as well as the presence of microsatellite instability. In contrast to the adenomas seen in the traditional pathway, MSI results in sessile serrated adenomas (SSAs) which typically have no cellular dysplasia and must be distinguished from the nonmalignant hyperplastic polyps (HPs) especially if found in the proximal or transverse colon (Birgisson et al., 2015).

There are also well-described hereditary forms of colon cancer that have provided great insight into the progression of sporadic colon cancers. About 3-6% of all colorectal cancers are hereditary with a lifetime CRC risk of 70-90% for individuals possessing these syndromes. Lynch syndrome, or hereditary non-polyposis colorectal cancer (HNPCC), is characterized as an autosomal dominant disease. A germ line mutation in DNA mismatch repair genes exists in patients with HNPCC. This causes extensive DNA mismatches especially in microsatellite regions leading to microsatellite instability. Familial adenomatous polyposis (FAP) is also an autosomal dominant disease but results from germ line mutations in APC. It is diagnosed clinically with a high frequency of large numbers of polyps. In contrast to FAP and HNPCC, MUTYH associated polyposis (MAP) is an autosomal recessive disorder characterized by bi-allelic mutations in the base excision repair gene, MUTYH. MAP presents in a similar fashion to FAP but generally has significantly fewer adenomas (less than 100) (Samadder, Jasperson, & Burt,
2015). The pathways for these hereditary gene mutations help us understand how the same gene mutations in sporadic colon cancer work to potentiate the cancer.

Catching colon cancer at its earliest stage when it is still characterized by the adenomatous polyp is crucial to preventing CRC related morbidity. At this stage, removal of the polyps by endoscopic polypectomy will greatly decrease chances of CRC incidence and death (Levin et al., 2008). This fact puts into perspective the extreme importance of accurate and efficient colorectal cancer screening.

**Figure 1. Colon Cancer APC/β-catenin pathway:** This figure illustrates the morphological changes as well mutational changes that occur as a hyperplastic epithelium progresses to colon adenocarcinoma. The APC mutation occurs early and is considered the gateway into the process. KRAS mutations lead to progression into the larger or more advanced adenomas (Jänne & Mayer, 2000).
Colorectal Cancer Screening

Currently there are multiple recommended CRC screening tests: colonoscopy, flexible sigmoidoscopy, and fecal occult blood tests (FOBTs) (H. K. Roy et al., 2006). Flexible sigmoidoscopy (FS) is a structural examination of the distal colon and rectum using a colonoscope. FS’s efficacy is based around the theory that most colorectal adenomas and cancers are located in the distal or sigmoid section of the colon however because only this small section is observed, adenomas arising in the proximal or transverse sections will be missed. Figure 2 shows a comparison between FS and general colonoscopy. Fecal occult blood tests, on the other hand, rely upon the observation that colorectal tumors emit small amounts of blood or other tumor products that can then be analyzed from stool samples (Bretthauer, 2010).

These tests have a range of invasiveness, patient discomfort, accuracy, and cost. FOBT is the least invasive and the least costly but also has the lowest sensitivity due to the test having to find small traces of neoplastic occult blood in large samples of stool (Levin et al., 2008). FS is similar in accuracy and design to screening colonoscopy but suffers the major flaw of only screening a small portion of the colon. Recently, the use of FOBTs and flexible sigmoidoscopy is dropping while there has been a sharp increase in the use of screening colonoscopy, which is attributable to Medicaid beginning to reimburse screening colonoscopy in 2001 (Holden, Jonas, Porterfield, Reuland, & Harris, 2010). However, it should be also noted that currently only FOBTs and flexible sigmoidoscopy have been shown via long term randomized control trials (RCTs) to reduce CRC incidence and mortality. It is believed that colonoscopy will show better
results in RCTs that have been recently started because of its higher sensitivity for adenomas than FOBTs and its more thorough screening of the colon in comparison to flexible sigmoidoscopy (Bretthauer, 2010; Zauber, 2015).

Colonoscopy screening is widely held to be the gold standard by which to assess other screening tests for cancer (Lieberman et al., 2012). In the United States, it is almost always the recommended choice of screening by gastroenterologists. It allows for complete structural investigation of the colon and prompt removal of the precancerous lesion, the adenomatous polyp (Zauber, 2015). However, various issues with screening

Figure 2. Flexible Sigmoidoscopy vs. Colonoscopy: Illustration A demonstrates the capacity of a flexible sigmoidoscope for monitoring the colon. This illustration is even generous with regards to how far up the distal colon the scope can reach. Generally only the sigmoid colon and rectum are surveyed. Illustration B displays the reach of a general colonoscopy endoscope. This method monitors the entire colon including the length of the proximal and transverse colon.


colonoscopy are preventing CRC screening rates from rising to the levels necessary to significantly reduce CRC incidence and mortality.

The underuse of screening colonoscopies on at risk patients is perhaps the largest problem. One issue is that the cost of colonoscopies inhibits its use by the underinsured, particularly those in lower socioeconomic groups (Meester et al., 2015). Another major factor for underuse of colonoscopy remains the fact that less than 10% of patients yield screen relevant neoplasms during screening (Lieberman et al., 2012). These two issues combined with its high invasiveness lead to vast under utilization. Yet even if everyone decides to submit to a colonoscopy at age 50, the healthcare system cannot support screening the entire population (H. K. Roy et al., 2006). In addition to underuse, there is also overuse whereby patients who are not likely to benefit are screened or when patients are having screening procedures too frequently. For instance, patients with irritable bowel Disease (IBD, ex. Ulcerative Colitis, Chron’s Disease, etc) symptoms are frequently recommended for full screening colonoscopies while the prevalence of adenomas remains significantly lower for this group than “average risk” patients (Lieberman et al., 2014). Improvements must be made to the system of performing early risk stratification so as to be able to efficiently utilize the limited endoscopic capacity for CRC prevention in the real at risk population.

**Increased Incidence of CRC in Younger Patients**

Surprisingly, there has been increased incidence of CRC and CRC related mortality in younger patients under the age of 50. As shown in Figure 3, colon and rectal
cancer incidence, while declining steadily for patients over the age of 50, has seen a sharp rise in individuals under 50 years of age (Ahnen et al., 2014). This group of patients is generally not given screening colonoscopy early enough and CRC often progresses to advanced stages before diagnosis. An improved risk stratification method that can be utilized early for colonoscopy recommendation would greatly benefit patients likely to develop early onset CRC.

Currently, earlier screening is only recommended to patients with familial patterns of colon cancers and genetic predispositions such as familial adenomatous polyposis (FAP), Lynch syndrome, and MUTYH associated polyposis. These conditions only account for 15-20% of early onset CRC while the majority of cases remain etiologically undetermined (Jones et al., 2015). In these cases, there are many factors that would lead to late diagnosis, such as younger patients deciding not to seek care when symptoms present, primary care physician misdiagnosis, and lack of insurance (Ahnen et al., 2014). Assessing risk factors earlier in a new novel way will be necessary to decrease rate of early sporadic CRC incidence.

Lifestyle and environmental factors are currently thought to have a profound impact on the development of colon cancer (Anand et al., 2008). These include tobacco use, alcohol consumption, diet, exercise, diabetes mellitus, and obesity. And it is these factors in addition to underuse of screening that may explain the rise in early onset CRC. These factors also are the same or increased in the older age groups but because of higher colonoscopy screening rates the incidence rates trend downward (Ahnen et al., 2014).
Obesity’s Impact on Cancer

Obesity has become an epidemic worldwide, particularly in the United States with an estimated 20% of men and 25% of women being considered obese. Obesity is defined by a body mass index (BMI) of 30 kg/m² or higher. Prevalence of obesity is on the rise with more and more individuals falling under its classification (Kopelman, 2000). This trend is sobering, as obesity has been linked as a major risk factor in numerous diseases. In particular, a recent meta-analysis of 70,000 cases of CRC found that patients with a
BMI greater than 30 had a 20% greater risk of developing CRC when compared with patients in the normal weight range (defined by BMI <25) (Moghaddam, Woodward, & Huxley, 2007). Obesity has also been seen to accelerate the onset of early colorectal cancer, as shown in Figure 4 (Umar & Greenwald, 2009).

Figure 4. Impact of Obesity on Early Onset CRC: This graph illustrates the increase in colon cancer incidence associated with obesity along with poor diet and sedentary lifestyle. It also points out that healthy diet, active lifestyles, and especially early detection will decrease risk of CRC incidence. Figure taken from Umar A and Greenwald P Cancer Epidemiol Biomarkers Prev 2009; 18:1672-1673

Although increasing obesity in the population is certainly having an impact on CRC incidence trends, the exact mechanism by which obesity increases the risk of CRC is unknown (Tandon, Imam, Ismail, & Castro, 2015). Adipose tissue functions as an
endocrine organ and produces various factors that are collectively called adipokines. Adipokines can be proteins, cytokines, or hormones. These factors have many functions but in particular, have a profound role in modulating the inflammatory response. High levels of these inflammatory adipokines may be the triggers for CRC initiation (Comstock et al., 2014).

A recent study of serum adipokine levels in an adult white male cohort by Comstock et al. suggests that the following adipokines have the most positive correlation with colorectal adenoma formation: leptin, interferon-γ-inducible protein (IP-10), and tumor necrosis factor-α (TNFα). All three of these factors promote cellular growth and inhibit apoptosis through the phosphoinositol-3-kinase/Akt signaling pathway. It is clear then that high amounts of these adipokines may give rise to a suitable environment for cancer to potentiate in. However, a definitive correlation still may not exist between these adipokines and increased incidence of CRC since the studies did not address locally produced adipokines (only serum adipokines were measured) and the patient cohort was small and only included adult white males (Comstock et al., 2014).

Dysfunctional or dysbiotic gut flora compositions have also been noted in patients harboring CRC adenomas. Normally, bacteria in the gut exist in a symbiotic manner with intestinal epithelial cells. The mutually beneficial relationship allows the colon to process dietary polysaccharides and the bacteria benefit through the use of carbohydrates for energy. These bacteria also affect inflammatory response, insulin sensitivity, and adipokine release (Zhu, Michelle Luo, Jobin, & Young, 2011). Thus, when the microbiome is dysfunctional as seen in obese patients, negative changes in apoptotic
signaling as well as activation of cellular growth/proliferation signaling can occur providing a proliferative environment for cancer. This relationship is extremely complex because of the multiple signaling pathways that are affected as shown in Figure 5 (Bardou, Barkun, & Martel, 2013).

Evidence also shows that obesity, as part of a range of environmental factors termed the metabolic syndrome, is heavily linked to type II diabetes. This linkage occurs through altered insulin sensitivity, deficient glucose metabolism, and other cardiovascular risk factors (Després & Lemieux, 2006). The altered metabolism seen in metabolic syndrome also affects cancer through changes in the relationship between glycolysis, tricarboxylic acid (TCA) cycle, and mitochondrial oxidative phosphorylation (OXPHOS) pathways. First discovered by Otto Warburg over 90 years ago, these changes are now considered one of the hallmarks of cancer (Yadava, Schneider, Jerry, & Kim, 2013). This effect is called the Warburg effect and is described in the next section.

It is important to note that changes mediated through obesity and the metabolic syndrome can be reversed through lifestyle changes and reduction in body mass index (BMI). In particular, increased exercise and improvement in diet have been shown to decrease risk of CRC and other diseases linked to metabolic syndrome (Boutron-Ruault, Senesse, Méance, Belghiti, & Faivre, 2001; Després & Lemieux, 2006).
Figure 5. The yellow arrows indicate how dysbiotic microbiota can affect obesity associated colon cancer through various pathways. This illustrates the complexity of the interactions of colon epithelial cells with not only gut microbiota but also with other obesity related conditions such as adipokine release. Also shown is how the obesity associated inflammation as well as metabolic syndrome can influence the progression of CRC. Image modified from Bardou M., Barkun A., and Martel M. Gut 2013; 62:933-947
Warburg Effect and Cancer

Over 90 years ago, Otto von Warburg discovered what has been termed the Warburg Effect. The Warburg Effect describes the phenomena seen in tumorigenic cells whereby aerobic glycolysis is utilized over mitochondrial OXPHOS even in oxygen rich or normoxic conditions. This is strange considering that cancer cells, which are proliferating at high rates, need a large amount of energy in the form of adenosine triphosphate (ATP). Mitochondrial OXPHOS yields much more ATP than aerobic glycolysis. Yet, this preferential use of aerobic glycolysis along with mitochondrial dysfunction is evident in many cancers (Boland et al., 2013).

To understand this phenomenon lets first take a look at normal energy metabolism in cells. Normally, glucose undergoes glycolysis in the cytosol to generate pyruvate as well as a small amount of ATP. Pyruvate can then enter the mitochondria to promote OXPHOS as well as other mitochondrial pathways or stay in the cytosol to undergo the lactic acid pathway via lactate dehydrogenase. In normoxic conditions (in which O2 presence is abundant), the mitochondrial pathway predominates. Once in the mitochondria, pyruvate is converted into acetyl CoA through oxidative decarboxylation by pyruvate dehydrogenase. This rate limiting step is regulated by pyruvate dehydrogenase kinase, which functions to down-regulate pyruvate dehydrogenase through phosphorylation. If this phosphorylation does not occur, acetyl CoA can enter the Krebs Cycle to generate NADH and FADH$_2$ which donate electrons to the electron transport chain (ETC). These electrons are passed down the ETC to eventually reduce respiratory oxygen into water and concurrently generate ATP through ATP synthase. The
ATP synthase is powered by the proton gradient formed as the electrons move down the chain (Longo & Archer, 2013). This is a highly efficient process when compared to aerobic glycolysis, with OXPHOS producing around 30 ATP per molecule glucose and glycolysis only producing 2 net ATP per molecule glucose (Yadava et al., 2013). See Figure 6 for an overview of this process.

Why then are tumorigenic cells seen to shift the flux of glucose away from the OXPHOS pathway and instead into various biosynthetic pathways? This question has yet to be answered in a satisfying manner although various hypotheses on why cancer cells exhibit this behavior have been suggested such as evasion of apoptosis via regulation of mitochondrial cytochrome c release or in response to reactive oxygen species (ROS) release. It is also recognized that in normal embryogenesis, hyper proliferative embryonic cells also utilize the Warburg effect perhaps due to biosynthetic needs for nucleosides, amino acids, and macromolecules. This may explain aerobic glycolysis’ role in tumor cell potentiation as well (Boland et al., 2013). Although it’s purpose has not fully been elucidated, it is clear through many studies of different cancers that mitochondrial dysregulation does exist in a manner consistent with the Warburg effect. In fact, the Warburg effect is now considered one of the eight hallmarks of cancer in Hanahan and Weinberg’s latest review article (Hanahan & Weinberg, 2011).

There are three areas of interest in Warburg: the glycolytic alterations, pyruvate shunting, and mitochondrial dysregulation. In regards to glycolysis, it has been seen that glucose transporter 1 (GLUT1) is up-regulated to increase glucose uptake by the cancerous cells. High glucose flux into the tumor cell is necessary to support energy
demands without the use of OXPHOS. Glucose is converted to pyruvate through glycolysis with the last step occurring via pyruvate kinase. A different spliced variant of pyruvate kinase, pyruvate kinase muscle 2 (PKM2) is seen expressed in both embryonic development and in the Warburg Effect. PKM2 is seen as a marker for aerobic glycolysis for Warburg. Pyruvate’s conversion to acetyl CoA via pyruvate dehydrogenase is regulated by pyruvate dehydrogenase kinase 1 (PDK1) which deactivates pyruvate dehydrogenase. Both GLUT1 and PDK1 are upregulated via hypoxia-inducible factor 1 α (HIF1α) (H. Jones et al., 2015). HIF1α is transcriptional factor, which normally seeks to shunt pathways away from OXPHOS under low oxygen conditions. It is also important in promoting angiogenesis via regulation of vascular endothelial growth factor receptor (VEGFR). However, in normal conditions with plentiful oxygen, HIF1α is post-translationally modified through prolyl-hydroxylation. In this state, HIF1α associates with the tumor suppressor protein, von Hippel-Lindau (VHL) and is subsequently targeted for degradation. On the flip side, in normal low oxygen conditions, mitochondria release reactive oxygen species (ROS) and cause the activation of HIF1α. Up regulation of HIF1α also occurs in normoxic conditions in tumor cells through mutations in tumor suppressor genes such as VHL, which contributes to the potentiation of Warburg Effect (DeBerardinis, Lum, Hatzivassiliou, & Thompson, 2008). It is clear then that HIF1α, glucose transporters, PKM2 and PDK1 are important in Warburg mediated metabolic dysfunction.

The other characteristic of Warburg is its mitochondrial dysregulation. Mitochondria are dynamic organelles undergoing various cycles of growth and division.
in response to cellular demands and extracellular factors such as oxygen availability. Mitochondrial mass increases via replication of mitochondrial DNA (mtDNA) as well as increases in protein mass. Conversely mitochondria can also undergo degradation or mitophagy by an autophagosome. Biogenesis of mitochondria occurs via two main processes: fusion and fission.

Fusion is the process by which neighboring mitochondria fuse together to form a continuous mitochondrial reticulum. This process is mediated by a couple factors, namely optic atrophy 1 GTPase protein (OPA1) in the inner mitochondrial membrane mitofusin-1 (MFN1) and mitofusin-2 (MFN2) in the outer mitochondrial membrane as shown in Figure 6. MFN1 and MFN2 mediate fusion of the outer membrane while OPA1 mediates fusion of the inner membrane (Longo & Archer, 2013).

Fission on the other hand is the process in which mitochondria split into smaller more fragmented mitochondria. A different GTPase mediates this process, dynamein related protein 1 (DRP1). DRP1 is recruited from the cytosol to the outer mitochondrial membrane where it works to pinch off the membrane in order to split the mitochondria. It is an important process in biogenesis of daughter mitochondrial as well as in mitophagy and cellular apoptosis (Boland et al., 2013).

Biogenesis is seen to be upregulated in response to cellular demands, as well as many other factors such as mutations in tumor suppressor genes like p53 and activated oncogenes such as c-MYC. Currently, peroxisome proliferator activator receptor gamma coactivator 1 alpha (PGC1α) is thought to be the major integrator of transcriptional control on mitochondrial biogenesis (Boland et al., 2013).
In tumorigenic cells there also exists an upregulation in uncoupling protein 2 (UCP2). UCP2 is anion carrier protein in the inner mitochondrial membrane that decouples ATP generation from the proton gradient, thus preventing OXPHOS. Derdak et. al found overexpression of UCP2 in human colon cancer cells which was postulated to be due to protection of cancer cells from apoptosis via suppression of mitochondrial ROS release. UCP2 also provides a mechanism by which a shift toward aerobic glycolysis can be promoted (Derdak et al., 2008).

**Early Warburg Effect as a Biomarker**

Getting back to the topic of finding a method to improve early colon cancer risk stratification, it is clear that biomarkers need to be investigated. Successful biomarkers have been found for other cancers such as breast cancer and prostate cancer but there is a profound need for finding biomarkers for early identification of sporadic cancer risk (Hudson, 2013). Utilization of a field effect approach may be necessary to utilize biomarkers through less invasive measures. The field effect model of colon carcinogenesis posits that the microscopic alterations in the distal colon mucosa will show changes indicative of an existing adenoma or increased risk of developing an adenoma. Previous work by Wali et al. has shown that microvascular blood in particular was altered in endoscopically normal mucosa (Wali et al., 2005). This suggests that field carcinogenesis rather than adenomas and tumors themselves may be the key to locating useful biomarkers (Roy et al., 2013). In particular, it may be possible to assess mitochondrial and metabolic dysfunction through the use of a field carcinogenesis model.
to perform risk stratification.

Figure 6. This illustration details the various relevant biochemical pathways as well as mitochondrial biogenesis control. The Warburg Effect has effects on many points in this diagram: pyruvate shunting via PDK and HIF1, upregulation of glucose transport into the cell, and mitochondrial fission and fusion. Figure modified from Archer, S. New England Journal of Medicine 2013;369:2236-51
Aims and Objectives

The purpose of this thesis is to assess if a Warburg/Warburg-like effect is evident through select molecular markers that may possess implications for risk stratification of colon carcinogenesis. We hypothesized that patients with adenomas would have significant changes in mitochondrial mass and upregulation of certain key metabolic and mitochondrial genes (GLUT1, UCP2, PKM2, HIF1α, and OPA1). Furthermore, based on the field carcinogenesis effect, these changes could be assessed in the easily accessible distal colon (rectum) irrespective of the location of the colonic lesion.

For this study we used two rodent models for CRC as well as a patient cohort of approximately 80 individuals with around 40 harboring adenomas. Specifically we hope to achieve the following goals:

1) Quantify changes in gene expression for glycolytic and mitochondrial biogenesis markers to establish a Warburg Effect in patients and rodent models of CRC.

2) Quantify a change in mitochondrial mass due to Warburg effect in rectal colon epithelial cells for patients and rodent models of CRC.

3) Explore the utilization of these changes as a means of early risk stratification for CRC.
METHODS

Animal Studies

The animals protocols/animal samples used for this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of NorthShore University HealthSystem (Evanston, IL). Animal housing was climate-controlled (ambient temperature at 25°C) and at a 12 hr light/dark cycle.

Azoxymethane Rat (AOM) Model

Twelve male rats of Fisher F-344 background were procured from Harlan Teklad (Madison, Wisconsin) and maintained on AIN76-A rodent chow (Harlan Teklad) with ad libitum access to water. These rats were randomized to either 2 weekly intraperitoneal injections of azoxymethane (administered at a concentration of 15mg/kg of body weight) or saline. Serial colonoscopies were performed on the rodents for adenoma detection/frequency. Rats were housed for 40 weeks and euthanized. Colons were excised and cleansed with ice cold phosphate saline buffer (PBS). Colonic epithelial mucosa was collected for genetic analysis through Real Time PCR.

Polypopsis In Rat Colon Rat Model

Six Polypopsis in Rat Colon (Pirc) Rats were obtained from Taconic Laboratories (Hudson, NY) at an age of roughly 10wks. These rodents have been genetically modified APCΔ1137 and this rodent model (partially) recapitulates the human disease FAP, however
can be seen as a useful model for CRC as ~80% of sporadic CRCs are attributed to APC truncation (Amos-Landgraf et al., 2007). Six age-matched controls (Fisher F-344 rats consistent with Pirc rat background) were also obtained (Taconic Laboratories, Hudson, NY). Serial colonoscopies were performed on the rodents for adenoma detection/frequency. These animals were housed for 24 weeks and euthanized. Colons were excised and flushed with PBS. Colonic epithelial mucosa was obtained for Real Time PCR analysis.

**Human/Patient Studies**

Human samples were obtained with an approved Institutional Review Board (IRB) protocol from NorthShore University HealthSystem with informed consent. Patients undergoing either screening or surveillance colonoscopies were included in this study. Patients who received incomplete colonoscopies (failure to intubate the cecum), taking anti-coagulants, or other confounding factors were excluded from this study. Briefly, rectal biopsies were obtained during colonoscopy. Samples were collected in PBS and frozen for preservation and later analysis. Forty patients with no adenomas and thirty nine patients harboring adenomas were used for the following study.

**RNA and DNA Isolation**

Rat epithelial mucosa was homogenized through mechanical disruption. RNA and gDNA were stabilized in Trizol (Life Tech, Foster City, CA). The nucleic acids were isolated from these samples with RiboPure RNA Isolation Kit (Life Tech, Foster City,
CA). Human rectal biopsies were also homogenized by mechanical disruption and stored in Trizol for RNA stabilization. RNA and gDNA from human samples were isolated with Ribopure RNA isolation Kit for isolation. All RNA samples (animal and human) were quantified with NanoDrop (Fisher Scientific, Hanover Park, IL).

**Polymerase Chain Reaction (PCR)**

Quantified RNA was synthesized using the High Capacity cDNA Synthesis Kit (Life Tech, Foster City, CA). cDNA synthesis was facilitated with Step-One Plus Thermocycler (Life Tech, Foster City, CA). cDNA and gDNA was prepared for Real Time PCR using Taqman Gene Expression Assay primers (for each respected marker) and Real Time Universal Master Mix. For quantification of mt-NADH Dehydrogenase (mtND1), 18s gDNA was used as control. For all other markers, beta actin was used as a loading control. Concentrations of these markers were assessed with comparative \(2^{-\Delta\Delta C_t}\) and quantitative Real Time PCR data analysis was performed using RQ Manager 1.2.1 (Life Tech, Foster City, CA).
RESULTS

Mitochondrial Mass in AOM Rats and Patients

Mitochondrial mass was evaluated via quantification of the expression of a common mitochondrial DNA fragment, mtND1. This was normalized with the expression of nuclear DNA, 18s gDNA. In the AOM rat model, we found a significant ~2.4-fold (p≤0.024) increase in expression in AOM rats versus the control rats as shown in Figure 7. For this study, 10 AOM rats and 6 control rats were used. This trend was also seen in human rectal biopsies, with a ~1.76-fold (p≤0.05) increase in patients harboring adenomas as shown in Figure 8.

Figure 7. AOM rats show ~2.4-fold increase in expression of mtND1 (p≤0.024) over expression in control rats injected with only saline. Std. error bars are also shown.
OPA1 Expression in Pirc Rats and Patients

Expression of OPA1 was quantified in both the Pirc rat model as well in human rectal biopsies. In Pirc rats, a ~1.67-fold change in expression of OPA1 was observed (p ≤ 0.027) as shown in Figure 9. Patients with adenomas showed a ~1.52-fold change in expression when compared to patients with no adenomas (p ≤ 0.045) as shown in Figure 10.

Figure 8. Patients with adenomas showed a ~1.76-fold increase in expression of mtND1 (p ≤ 0.024) indicating increased mitochondrial mass. Std. error bars are also shown.
Figure 9. PIRC rats show ~1.67-fold increase in expression of OPA1 ($p \leq 0.027$) over expression in control rats. Std. error bars are also shown.

Figure 10. Patients with adenomas showed a ~1.52-fold increase in expression of OPA1 ($p \leq 0.045$) indicating increased mitochondrial fusion. Std. error bars are also shown.
**UCP2 Expression in Pirc Rats and Patients**

Expression of UCP2 was quantified in both the Pirc rat model as well in human rectal biopsies. In Pirc rats, a ~2.01-fold change in expression of UCP2 was observed (p≤0.05) as shown in Figure 11. Patients with adenomas showed a ~2.65-fold change in expression when compared to patients with no adenomas (p≤0.01) as shown in Figure 12.

![Figure 11](image_url)

**Figure 11.** PIRC rats show ~2.01-fold increase in expression of UCP2 (p≤0.05) over expression in control rats. Std. error bars are also shown.
Expression of HIF1α was measured in the patient rectal biopsies. Patients with adenomas showed a ~1.97-fold increase in HIF1α expression (p ≤ 0.01) as shown in Figure 13.

**Figure 12.** Patients with adenomas showed a ~2.65-fold increase in expression of UCP2 (p ≤ 0.01) indicating uncoupling of ATP production in the mitochondria. Std. error bars are also shown.

**HIF1α Expression in Patients**

Expression of HIF1α was measured in the patient rectal biopsies. Patients with adenomas showed a ~1.97-fold increase in HIF1α expression (p ≤ 0.01) as shown in Figure 13.
Expression of GLUT1 was measured in the patient rectal biopsies. Patients with adenomas showed ~3.46-fold increase in GLUT1 expression ($p \leq 0.01$) as shown in Figure 14.

**Figure 14.**

Expression of GLUT1 was measured in the patient rectal biopsies. Patients with adenomas showed ~3.46-fold increase in GLUT1 expression ($p \leq 0.01$) as shown in Figure 14.
PKM2 Expression in Patients

Expression of PKM2 was measured in the patient rectal biopsies. Patients with adenomas showed ~1.94-fold increase in PKM2 expression (p≤0.01) as shown in Figure 15.
Figure 15. Patients with adenomas showed a ~1.94-fold increase in expression of PKM2 ($p \leq 0.01$) indicating increased aerobic glycolysis. Std. error bars are also shown.
DISCUSSION

The search for biomarkers for cancer phenotypes and in high risk patients is strongly sought out for translational detective measures and personalized chemoprevention as well as chemotherapies. With credit to innovations and findings for early CRC detection, prognosis has markedly improved over the past 20 years with the use of better screening procedures and administration of non-steroidal anti-inflammatory drugs (NSAIDs). CRC risk has been reduced by a significant 30-50%. However, the incidence of CRC related mortalities in the United States is only preceded by that of lung cancer in American men and women combined (Siegel, Miller, & Jemal, 2015). This underscores the need for more definitive/effective measures for screening and risk stratification. The prevalence of cancer risk in our society has focused much attention on metabolic disease, where diabetes and obesity status have been the attributing platforms. The changes in metabolic demand as well as its pathway are criteria for carcinogenesis. One of the hallmarks of cancer, the Warburg Effect, was discovered in the first half of last century and provides potential insight in the understanding of progression and malignant features in colon cancer. To cover the first aims of this thesis, we chose to explore the expression of select molecular markers well established in the Warburg Effect in patient rectal biopsy samples. We compared patients possessing detectable adenomas versus control patients with no significant colonoscopic findings. Patients harboring lesions (depending on factors such as size, histological features and location) are presented as the at risk population for CRC.
To establish that early Warburg/Warburg-like effects exist in the field of carcinogenesis model of CRC we first looked at changes in expression of glycolytic genes. For aerobic glycolysis to achieve enough glucose flux to generate sufficient energy in the absence of OXPHOS, glucose uptake via glucose transporters must be upregulated. We found a significant ~3.53-fold change in GLUT1 in patients with adenomas compared to patients without. GLUT1 regulation is intrinsically tied with HIF1α expression, which we looked at next.

HIF1α is extremely important for cellular response to hypoxic conditions, mediating angiogenesis through vascular endogenic growth factor (VEGF) as well as other functions to fulfill metabolic demands. In the case of Warburg effect, effects normally associated with hypoxic conditions are seen in normoxic conditions. In our studies, we found that HIF1α was significantly overexpressed in the patient cohort (~1.98-fold change) suggesting presence of the Warburg effect. HIF1α upregulates PKM2 and GLUT1, which are both suggestive of aerobic glycolysis (Semenza, 2011). To add to this we found that PKM2 was also overexpressed in the patient cohort (~1.94-fold change). PKM2 is recognized through many studies as a marker of the Warburg effect through its role in shunting pyruvate away from the oxidative phosphorylation pathway. These findings are strongly suggestive of a Warburg or Warburg-like phenomena in our precancerous at risk patients.

To further explore the Warburg effect in our patient studies, we choose to examine mitochondrial changes in patient rectal biopsies. It is understood the there is reduced OXPHOS in malignant cells, however the cancer implications in mitochondrial
changes (response to ROS, shunting from OXPHOS to aerobic glycolysis and other metabolic pathways) remain to be fully elucidated. We therefore coupled our human studies with the well-established rodent model of CRC, the AOM-treated rat.

Mitochondrial mass was seen in our studies to be increased via both increased levels of mitochondrial DNA and increased levels of the major mitochondrial fusion gene, OPA1. The measurement of mtND1 gene fragment in mtDNA is commonly used to establish a mitochondrial copy number and give relative mitochondrial mass (Lin et al., 2012). In our studies, we found marked overexpression in both the AOM rat model (~2.01-fold change) as well as in our patient population (~1.76-fold change). However, the relationship between a mitochondrial mass increase and the increased aerobic glycolysis in Warburg effect seems counterintuitive at first glance. If mitochondrial OXPHOS is downregulated, why then would mitochondrial mass be increasing? But it must also be noted that mitochondrial mass increase and mitophagy exist in a balance in normal cells (Chourasia, Boland, & Macleod, 2015). In a tumorigenic state, cancer cells must evade apoptosis and it is possible that inhibiting degradation of mitochondria through mitophagy might prevent this apoptosis. This evasion of apoptosis could also be the result of increased mass of mitochondria attenuating the apoptotic qualities of cancer induced mtDNA mutations via complementation (Youle & van der Bliek, 2012).

Another important factor is the presence of high cellular levels of reactive oxygen species (ROS) in tumor driven cancer. ROS is primarily produced in the mitochondria and is believed to be critical in tumor cell proliferation. Importantly, ROS is necessary for HIF1α stabilization, which allows for GLUT1 and PKM2 upregulation (Hamanaka &
Chandel, 2010). Increased mass may generate the ROS necessary to potentiate the Warburg/Warburg-like effects.

Along with mitochondrial mass, expression of the mitochondrial fusion GTPase, OPA1 was studied. OPA1 showed a modest increase in both patients (~1.52-fold change) and the Pirc rat model (~1.67-fold change). In hypoxic states, mitochondria increase their OXPHOS capacity through increased fusion. However, in the normoxic conditions of Warburg phenomena, increased OPA1 may be suggestive of cells trying to recover OXPHOS in the face of increased aerobic glycolysis and mitochondrial dysfunction (Tondera et al., 2009). Secondly, Frezza et. al found that OPA1 protected cells from apoptosis by preventing release of cytochrome c from the mitochondria (Frezza et al., 2006). This may be another anti-apoptotic factor related to the Warburg effect.

Pyruvate utilization by the mitochondria is not only for use in oxidative phosphorylation but also necessary for other biosynthetic pathways such as the production of acetyl CoA for use in fatty acid biosynthesis. Acetyl CoA normally can condense with oxaloacetate to form citrate, allowing it to be transported out of the mitochondria via a shuttle. Once in the cytosol, citrate is converted back to acetyl CoA via ATP citrate lyase. Inhibition of this cytosolic enzyme was found to prevent cellular proliferation as well as fatty acid biosynthesis via pyruvate derived acetyl CoA (R. Jones & Thompson, 2009). Thus, cancer cells may need these mitochondrial biosynthetic pathways for proliferation even when OXPHOS is not needed.

Lastly, UCP2 expression was analyzed to show that the electron transport chain was indeed dysfunctional. We found overexpression in both the Pirc rat model (~2.01-
fold change) as well as in the patient cohort (~2.65-fold change). UCP2 promotes glycolysis as it uncouples the electron transport chain from ATP production via proton leak as acting as a pyruvate transporter thereby shunting pyruvate out of the mitochondria. It also regulates high levels of ROS preventing apoptosis from high oxidative stress (Baffy, 2010).

These findings in patients with neoplastic lesions speculate changes in mitochondrial mass, morphology and dynamics. However these patients are classified as at risk, where not all adenomas will undergo malignant transformation. Additionally, cosegregating factors (smoking, diabetes, family history) were not accounted for. We also did not analyze the actual activity of the proteins and only looked at the relative quantities of their mRNA transcripts.

In summary we have shown an early Warburg-like effect in the rectal mucosa of patients possessing adenomas through established metabolic markers. We propose an increased mitochondrial mass in both human and animal studies and changes in mitochondrial dysfunction associated genes, which may support increase in mass as well as fusion. The metabolic implications of colon cancer risk have been of great interest with diabetes and BMI status being shown as risk factors. Our results of a Warburg-like phenomena in the premalignant colon can start to reveal the mitochondrial changes in colon field carcinogenesis, inducing further studies in the potential of these changes as CRC biomarkers, mitochondria-mediated Warburg effects, and in targeting these markers for chemoprevention.
REFERENCES


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X-ray Crystallography of MutYH base repair enzyme implicated in colon cancer
Boston University Medical Center, Department of Gastroenterology, Roy Lab, September 2014-Current
Principle Investigator: Dr. Hemant K. Roy M.D.
Metabolic Alterations in Early Colon Cancer

Presentations
2012 Richard Larock Undergraduate Research Conference, Davis Ca.
Bilal Latif, Ryan Woods, Sheila David. *Analysis of trajectory of nucleophilic attack of water during adenine glycosylase activity catalyzed by MutY.*

Academic Achievements
UC Davis Deans List
Winter 2011, Spring 2011, Fall 2011, Winter 2012

Volunteer
Endoscopy, UC Davis Medical Center, Sacramento CA, Jan 2012 – April 2012 (50 hours)
- Stocked and cleaned endoscopy operating rooms
- Comforted patients before, during, and after operations
- Helped with simple tasks during operations

Surgery, Oncology, and Quality Department, Round Rock Medical Center, Round Rock TX, Oct 2012 – March 2013 (100 hours)
- Stock Nurse Stations
- Transport Patients
- Clerical Work in quality department