DIFFERENTIAL GENE EXPRESSION IN RESPONSE TO CONSUMPTION OF VARYING 2-AMINO-1-METHYL-6-PHENYLIMIDAZO [4,5-B]PYRIDINE (PHIP) LEVELS OF WELL-DONE RED MEAT

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ABSTRACT

Background: Heart disease and cancer are the top two causes of mortality in the United States. Diet is recognized to play a significant role in the prevention of both. Limitation of red meat in the diet has been recommended to decrease chances of developing these maladies. Components in red meat suspected to contribute to disease development include heterocyclic amines formed from well-done preparation. Elucidating the genes/pathways that are affected by the consumption of well-done red meat may aid in the prevention and treatment of these diseases.

Methods: 30 participants were asked to maintain a low heterocyclic amine diet for three weeks and then fed one meal of well-done meat of varying PhIP levels five days a week for four weeks. Peripheral blood samples were obtained at the end of the low heterocyclic amine phase and the conclusion of the well-done meat feeding phase. mRNA was isolated and transformed into single stranded cDNA before hybridization to Affymetrix Human Gene 1.0 Sense Target Arrays. Differential gene expression between PhIP group levels and pre-feeding and post-feeding samples was tested using the ANCOVA model in Partek analysis software.

Results: Differential gene expression was found in ten genes with the consumption of different PhIP levels. All ten may be associated with the acquisition of the ability of the cell to become malignant. One gene associated with heart disease, diabetes, and angiogenesis was found to be differentially up-regulated in the Asian group.

Conclusions: Consumption of well-done meat of varying PhIP levels may lead to the up-regulation of genes associated with the progression of normal cells to malignant cells.

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INTRODUCTION

Cancer is the second leading cause of death in the United States(American Cancer Society 2012). Intake of red meat, in particular well-done red meat, has been associated with higher risk of developing cancer. Heterocyclic amines are compounds in well-done red meat that are suspected carcinogens and hypothesized to contribute to the development of cancer. The association between the compounds in well-done red meat and cancer makes it important to study the genes and pathways that may be associated with intake of well-done red meat. In order to study genes and pathways that may be affected, the present study employed a nutrigenomic approach by studying gene expression changes in the peripheral whole blood of participants who were fed varying concentrations of PhIP in well-done meat. Differential gene expression was found in genes that may be affected in cells as they progress to malignancy as defined by the "hallmarks of cancer"(Hanahan and Weinberg 2011).

BACKGROUND

Introduction

Heart disease and cancer are such prevalent causes of mortality and morbidity in the United States. Research to pinpoint factors that may contribute to the development of these diseases is critical. Both of these maladies have been associated with dietary intake, in particular the consumption of red meat. These associations have led to the exploration of the components in red meat that may influence disease development. A group of preparation-dependent components known as heterocyclic amines have been identified as mutagens, and their intake has been positively associated with cancer risk, in particular. Three of these mutagens have been named as suspected carcinogens, so the study of their metabolism, to include foods/substances that may alter their metabolism with co-consumption, may provide important insight into how exposure may contribute to the development of cancer.

A recent technology being employed to study the effects of diet or compounds is "omics" technology. This technology allows for a study of effects on the whole genome at one time. Transcriptomics is a branch of "omics" that specifically looks at what mRNA is being produced at a particular point in time. This allows for examination of the effect on gene expression of the whole genome following an intervention. In order to study transcriptomics, microarray technology is utilized where tens of thousands of genes can be explored simultaneously. Microarray technology is being utilized to explore the effects of diet on gene expression, explore the effects of disease on gene expression and in the present study how diet may be related to disease by studying gene expression.

Diet and Disease

The leading causes of death in the United States are heart disease followed very closely by cancer, with stroke and diabetes placing in the top ten (Murphy, Xu et al. 2012). According to the American Cancer Society and the American Heart Association, dietary choices, weight control and physical activity are the most important modifiable risk factors for cancer, heart disease, stroke and diabetes (American Heart Association 2012; Kushi, Doyle et al. 2012) A recent study from McCullough et al concluded that nonsmokers who adhered to the American Cancer Society guidelines for nutrition, weight, physical activity and alcohol consumption were less likely to die from cancer, cardiovascular disease and all causes (McCullough, Patel et al. 2011). Each year in the United States over 190,000 cancer deaths may be linked to diet and physical inactivity, and thus, may be preventable(American Cancer Society 2012)(American Cancer Society 2012).

Red Meat and Disease

The association between diet and cardiovascular disease, cancer, and diabetes has spurred numerous recommendations concerning modification of diet to prevent these diseases (Kushi, Doyle et al. 2012). One recommendation is the avoidance or limitation of red meat intake. Red meat is defined as pork, beef, veal, goat, and lamb. Red meat consumption has been associated with increased risk for all-cause mortality, in addition to mortality from cardiovascular disease and cancer (Sinha, Cross et al. 2009; Pan, Sun et al. 2012). Several studies have shown increased risk of cardiovascular disease including coronary heart disease, stroke and myocardial infarction with red meat consumption(McAfee, McSorley et al. 2010; Bernstein, Pan et al. 2012). However,

many of these studies did not adjust for important factors, such as age, BMI, caloric intake, smoking, alcohol, physical activity, or family history. After adjusting for these factors, the association between red meat consumption and increased disease risk became insignificant (McAfee, McSorley et al. 2010). These methodological limitations make the evidence connecting red meat intake with cardiovascular disease inconclusive.

Red meat consumption has also been associated with diabetes and metabolic syndrome. A study of three large prospective cohorts in the United States found a positive association with the risk of diabetes for both processed and unprocessed red meat, while controlling for potential confounders, such as age, family history, smoking status, alcohol, and energy intake (Pan, Sun et al. 2011). However, other studies of red meat intake found associations with diabetes only with processed red meat but not with unprocessed red meat (Fretts, Howard et al. 2012). Processed meat includes meats such as bacon, SPAM, and deli meats that have been processed using salt or other preservatives such as nitrates in order to prepare for long term storage.

One of the most studied associations of red meat consumption and disease is that of red meat intake and its association with cancer. Intake of red meat has been associated with an increased risk of several types of cancer to include breast, pancreatic, and prostate cancer, in addition to several digestive tract cancers, such as oral, pharyngeal, and stomach cancers (Aune, Stefani et al. 2009). The evidence for some of these cancer sites is contradictory or less than convincing. However, the World Cancer

Research Fund has designated the evidence linking red meat to colorectal cancer as convincing (World Cancer Research Fund/ American Institute for Cancer Research 2011).

Meat Intake & Cancer

Several components of red meat have been thought to contribute to the link between red meat consumption and cancer risk including fat, ammonia, and heme iron (Cross and Sinha 2004). Dietary fat, in particular saturated fat, has been suspected to be the link between red meat and cancer and cardiovascular disease. The increase in fecal secondary bile that has been identified as a result of a high red meat and fat intake may be hyperproliferative and irritating to the colon leading to an increase in colorectal cancer risk (Cross and Sinha 2004; Bouwens, Grootte Bromhaar et al. 2010). In addition, high intakes of dietary fat may lead to elevated plasma androgen and estrogen levels, a decrease in immune function, and an increase in overweight and obesity (Aune, Stefani et al. 2009). These effects of fat may lead to increased risk for both cardiovascular disease and cancer. Many studies, however, have failed to show an association between dietary fat and many types of cancer, including colorectal cancer, prostate cancer, and breast cancer (Cross and Sinha 2004; Bouwens, Grootte Bromhaar et al. 2010; Park, Murphy et al. 2012). Associations between dietary fat and cancers have mainly been linked to dietary fat from meat which may indicate that the association is more due to meat intake than the dietary fat.

Ammonia produced endogenously from the consumption of red meat has also been proposed for the link between red meat and cancer, especially colon cancer. Red meat is a rich source of protein. Most of the protein is digested and subsequently

absorbed in the small intestine (Cross and Sinha 2004). However, small amounts are able to continue to the large intestine where intestinal bacteria break down proteins via deamination, decarboxylation, and fermentation, which produces amines, amides and ammonia, respectively. High protein diets have been shown to be associated with high levels of fecal ammonia (Cross and Sinha 2004). Ammonia has been shown to alter the morphology and intermediary metabolism of intestinal cells, DNA synthesis, and reduce cell life span. However, there is little conclusive evidence linking the production of ammonia with occurrence of cancers (Cross and Sinha 2004).

Another suspected component underlying the association between red meat and cancer is heme iron. Iron is a metal that is thought to be carcinogenic because of its pro-oxidant nature. Endogenously, iron together with hydrogen peroxide may potentially lead to the creation of hydroxyl radicals (Choi, Neuhouser et al. 2008) which subsequently may lead to increases in the Fenton and Haber-Weiss reactions, themselves responsible for the production of more free iron (Choi, Neuhouser et al. 2008). Cumulative damage from reactive oxygen species is thought to be linked to DNA damage and subsequently cancer. Suggested mechanisms for the carcinogenic activity of heme iron are its effects on cell proliferation of intestinal mucosa, oxidative breakdown of lipids and/or toxicity of fecal water to the intestinal cells (Ferguson 2010). However, there is limited evidence connecting heme iron from meat sources directly to cancer.

Meat Mutagens/Carcinogens

Preparation-dependent mutagenic and/or potentially carcinogenic compounds have also been identified in red meat, such as polycyclic aromatic hydrocarbons (PAHs), heterocyclic aromatic amines (HCAs) and N-nitroso compounds (NOCs). Polycyclic aromatic hydrocarbons (PAHs) are produced at high temperatures from the degradation of organic materials into free radicals that later recombine to form the polynuclear aromatic structure (Augustsson, Skog et al. 1999). There is a linear relationship between the temperature of the heat source and the amount of PAHs formed at temperature ranging from 400°C - 1000°C (Jägerstad and Skog 2005). When meat is grilled over an open flame, meat juices and fat may drip onto the fire which produces flames containing PAHs (Jägerstad and Skog 2005). Some of the PAHs subsequently adhere to the surface of the meat. One of the main studied PAHs is benzo[a]pyrene (BaP) (Jägerstad and Skog 2005).

N-nitroso compounds (NOC) are suspected mutagens to which meat eaters are exposed. N-nitroso compounds can be formed exogenously from specific meat preparation techniques or endogenously from reactions within the body. Exogenous N-nitroso compounds are associated with red meat that has been processed by smoking or fire-drying (Cross and Sinha 2004). Fecal concentrations have been shown to exceed ten times the dietary intake which indicates formation of NOCs by endogenous means (Holtrop, Johnstone et al. 2012). Intake of nitrates, nitrites and heme iron have all been suspected to contribute to NOC formation endogenously in the colon (Holtrop, Johnstone et al. 2012). Tumors arising from NOCs have been found in 39 different

species(Cross and Sinha 2004). Because NOCs are alkylating agents, they have the potential of reacting with DNA which may lead to carcinogenesis (Cross and Sinha 2004).

Heterocyclic aromatic amines (HCAs) are another group of suspected carcinogens/mutagens that are created during high-temperature cooking of red meat. HCAs consist of a group of greater than twenty compounds that consist of an exocyclic amino group attached to one of two or three aromatic rings; therefore, are referred to as amino-imidazoazaarenes (Jägerstad and Skog 2005). HCAs are produced by the Malliard reaction of creatine or creatinine, amino acids and sugar in cooked meats. The uncooked muscle protein and stored glycogen provide the source of free amino acids and sugar respectively (Jägerstad and Skog 2005). The quantity of HCAs produced depends on the cooking method, temperature of the cooking device and duration of exposure to heat (Cross and Sinha 2004). There is a direct relationship between the pan surface temperature and quantity of HCA's produced even when internal temperatures where constant (Knize and Felton 2005). In addition, the amount of time the meat was in contact with the heat source also varied directly with the amount of HCA's created. For example, more HCA's are created by allowing meat to remain in contact with a pan for five minutes rather than flipping every minute (Salmon, Knize et al. 2000).HCAs are typically present in parts per billion (Turesky and Le Marchand 2011). Marinating meat in barbecue, teriyaki and turmeric-garlic sauce has been shown to decrease the formation of heterocyclic amines (Salmon, Knize et al. 1997; Nerurkar, Le Marchand et al. 2009). The average daily intake of HCAs range from less than 2 to greater than 25 ng/kg per day (Turesky and Le Marchand 2011).

The 11th Report on Carcinogens from the National Toxicology Program of the Department of Health and Human Service named three heterocyclic amines as "reasonably anticipated to be human carcinogens."(National Toxicology Program 2004) Those HCAs selected are 2-Amino-3,4-dimethoylimidazo,[4,5-f]quinolone (MeIQ), 2-Amino-3,8,-dimethylimidazo[4,5-f]quinoxaline (MeIQx), and 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (National Toxicology Program 2004). The "reasonably anticipated" category includes those "substances for which there is limited evidence of carcinogenicity in humans and/or sufficient evidence of carcinogenicity in experimental animals indicating a cause and effect relationship between exposure to the substance and cancer." (National Toxicology Program 2004) Several animal studies have shown that HCAs increase the incidence of tumors in mammary glands, lung, colon, stomach and prostate (Zheng and Lee 2009). HCA-DNA adducts have been identified in breast, colorectum, pancreas and prostate tissues of humans (Turesky and Vouros 2004; Malfatti, Dingley et al. 2006; Zhu, Rashid et al. 2006; Tang, Liu et al. 2007; Tang, Liu et al. 2007).

The most abundant heterocyclic amine by mass in the human diet has been identified as 2-amino-1-methyl-6-phenylimidazo (4,5-b) pyridine or PhIP comprising approximately 70% of the intake of HCAs ingested by humans (Zheng and Lee 2009; Turesky and Le Marchand 2011; Gu, Neuman et al. 2012). Concentrations of PhIP up to 500 parts per billion (ppb) can be found in well-done meat, as well as poultry (Turesky and Le Marchand 2011). Using a model system, Knize and Felton determined PhIP could be produced from phenylalanine, creatinine and glucose (Knize and Felton 2005).

However, PhIP has also been shown to form in the absence of glucose (Turesky and Le Marchand 2011). Animal studies have shown that PhIP induces tumors of the colorectum, pancreas, prostate, and mammary gland (Gu, Neuman et al. 2012). Several studies have found a specific positive association, some as a dose response relationship, between cancers and intake of PhIP in humans (Zheng and Lee 2009). PhIP and MeIQx are the most abundant HCAs detected in well done meat (Cross and Sinha 2004; Knize and Felton 2005)

2-Amino-3,8,-dimethylimidazo[4,5-f]quinoxaline, also known as MeIQx, together with PhIP, is the most detected heterocyclic amine in well done meat. MeIQx was produced from a mixture of glycine creatinine and glucose in aqueous buffers in a model system (Knize and Felton 2005). The average daily intake of MeIQx is estimated to be between 14 and 47 ng/day (Cross 2004). Human studies have found positive associations between MeIQx intake and breast, lung and colorectal cancers. In animal studies, MeIQx was shown to induce lung, liver, gastrointestinal tract, Zymbal gland, and skin cancers as well as lymphomas and leukemia (U.S. Department of Health and Human Services: National Toxicology Program 2002).

Another heterocyclic aromatic amine created is 2-amino-3-methylimidaazo [4,5-f] quinolone or MeIQ. MeIQ is found at much reduced levels when compared to PhIP and MeIQx (ROC Background). In animal studies, MeIQ was shown to induce colon, liver, forestomach, skin, mammary, Zymbal gland and oral cancer (U.S. Department of Health and Human Services: National Toxicology Program 2002).

Heterocyclic Aromatic Amine Metabolism

Heterocyclic amines may be referred to as a xenobiotic substance. A xenobiotic substance is one that is "foreign to a living organism" (Merriam Webster). Most xenobiotic substances are lipophilic which means they need to be transformed into water soluble substances in order to be excreted from the body. Therefore when a xenobiotic substance is ingested, metabolic pathways are activated to detoxify the substance so it can subsequently be removed. The first group of reactions is referred to as Phase I reactions and functions to modify the substance by reactions, such as oxidation, reduction, hydrolysis, and cyclization or decyclization. One group of the more studied of the Phase I enzymes that help catalyze these reactions is that of the cytochrome p450 enzymes. The second phase of reactions include conjugation reactions that conjugate the activated products from Phase I. Examples of Phase II enzymes that catalyze these reactions are N-acetyltransferases (NATs), sulfotransferases (SULTs), glutathione s-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs). Though the main function of the metabolism of xenobiotics is detoxification/excretion, the reactions may lead to genotoxic metabolites.

Heterocyclic amines become mutagenic only after bioactivation through specific enzymes and pathways of xenobiotic metabolism (Donghak and Guengerich 2005). The genotoxicity of the heterocyclic amines may depend on the pathway taken, enzymes utilized, and the phenotype of the enzyme utilized. If the aromatic or heterocyclic aromatic ring systems are oxidized, the metabolites are detoxified. However, if the exocyclic amine group is oxidized, genotoxic N-hydroxy-HAA and arylhydroxylamine

metabolites result (Turesky and Le Marchand 2011). Enzymes within these pathways can serve to activate the HCAs while others detoxify them. Some of the main enzymes involved in the metabolism of HCAs are cytochrome P450 enzymes, N-acetlytransferases (NAT1 and NAT2), sulfotransferases (SULTs), glutathione sulfur-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs).

When heterocyclic aromatic amines enter the body they may bind the intracellular aromatic hydrocarbon receptor (AHR) causing an increased rate of synthesis of several Phase I cytochrome p450 enzymes (Turesky and Le Marchand 2011). One of these enzymes, CYP1A2, is hypothesized to perform the first step of bioactivation of HAAs by N-oxidation producing the arylhydroxylamine and N-hydroxy-HAA metabolites (Coles, Nowell et al. 2001; Turesky and Le Marchand 2011). After undergoing primary activation by CYP1A2 in the liver, the metabolites may be further modified through conjugation reaction catalyzed by Phase II enzymes in the target organs (Coles, Nowell et al. 2001). NAT, SULT, L-seryltRNA and L-prolyl-tRNA synthetases catalyze the esterification of the activated arylhydroxylamine and N-hydroxy-HCA metabolites (Turesky and Le Marchand 2011). These unstable esterification products may be susceptible to heterolytic cleavage which may produce a nitrenium ion that subsequently may bind to DNA through the formation of N-C bonds at the guanine bases (Jägerstad and Skog 2005).

Inhibition of Heterocyclic Amine Formation, Digestion, Absorption by Food Products

Certain foods have been investigated that may decrease the formation of heterocyclic amines when preparing meats. Specifically, a decrease in the formation of

HCA has been shown when meat has been prepared with garlic, onions, citrus juices as well as spices such as rosemary, thyme and sage (Shin, Rodgers et al. 2002; Cheng, Chen et al. 2007; Gibis 2007). In addition, active compounds in these foods, such as quercetin, found in onions, apples, broccoli, green leafy vegetables and berries, and naringenin, a flavonoid found in many citrus fruits, have been found to inhibit the formation of HCAs in model systems (Cheng, Chen et al. 2007). Also, red wine and tea polyphenols have been shown to decrease HCA formation (Busquets, Puignou et al. 2006; Gibis 2007). Fiber has been shown to adhere to heterocyclic aromatic amines, which may decrease their digestion, absorption, and subsequently its ability to be bioactivated to a carcinogen/mutagen (Funk, Braune et al. 2007).

Genes, Metabolism, and Cancer Susceptibility

Variation in the gene structure that alters the function of the gene may influence individual cancer susceptibility. Single nucleotide polymorphisms and other genetic variants (insertions, deletions), and DNA methylation may alter the expression of RNA transcripts, post translational modifications and modify the proteins. Differences in these genes may alter the metabolism of carcinogens and mutagens, such as heterocyclic aromatic amines, and subsequently the risk of disease among exposed individuals.

One of the main enzymes for the bioactivation of heterocyclic amines is CYP1A2. mRNA expression of CYP1A2 may vary as much as 70 fold (Turesky and Le Marchand 2011). This variation may be attributed to genetic polymorphisms in the regulatory region as well as CpG-island methylation. With CYP1A2 being the main bioactivator

involved in the metabolism of heterocyclic amines, individuals with higher CYP1A2 activity may bioactivate more HAAs leading to a higher risk for DNA damage. Measurement of CYP1A2 activity may be accomplished indirectly through the consumption of caffeine. CYP1A2 also catalyzes the 3-N-demethylation of caffeine which produces metabolites that can be measured in the urine (Turesky and Le Marchand 2011).

Sulfurotransferases (SULTs) are another group of Phase II enzymes, which are expressed in all parts of the gastrointestinal tract and catalyze the transfer of sulfur from a donor molecule to an alcohol or amine group. SULTs may function as detoxifiers or bioactivators. Heterocyclic amines may be N-sulfonated by SULTs to detoxify the compound leaving it ready for excretion. However following N-hydroxylation, HAAs may be bioactivated by SULTs via O-sulfation which may result in the heterolytic cleavage of sulfate anions and neutrophils that may subsequently bind to DNA (Suzuki, Morris et al. 2008). The SULT1A1 and 2 isoforms have been shown to catalyze the binding of metabolites of PhIP activation to DNA.

N- acetyl transferases (NATs) catalyze the transfer of an acetyl group from acetyl CoA to hydrazamine and arylamine chemicals. These Phase II enzymes can either detoxify or bioactivate aromatic amines. There are two isoenzymes of NAT1 and NAT2. NAT1 is found in all human tissue while most NAT2 is of hepatic or intestinal origin (Suzuki, Morris et al. 2008). N-hydroxylated HAA metabolites from CYP1A2 are substrates for O-actelyation by NAT1 and NAT2 producing an N-acetoxy intermediate

which may bind to DNA forming adducts. If people are both rapid N-oxidizers (from CYP1A2) and rapid O acetylators from NAT2, cancer risk may be greatly increased (Turesky and Le Marchand 2011)

Glutathione s-transferases catalyze the nucleophilic conjugation of a reduced glutathione via a sulfhydryl group to the electrophilic center containing a carbon, nitrogen, or sulphur atom. GSTs serve as detoxifying enzymes with the highest amounts produced in the liver and testis and very little in the colon. GST isoenzyme GST1A conjugates the activated PhIP metabolite, N-acetoxy-PhIP, to detoxify

Another Phase II detoxification enzyme is the UDP-glucuronosyltransferases. These enzymes are found in the endoplasmic reticulum of hepatic cells as well as other body tissues and catalyze the conjugation with glucuronic acid. Glucuronidation is a major factor in the elimination of lipophilic compounds from the body (Radominska-Pandya, Czernik et al. 1999). The UGT1A isoenzyme has been shown to participate more in the detoxification of HCA than UGT2B (Turesky and Le Marchand 2011).

Genomics Technology

The completion of the Human Genome Project, i.e. the sequencing of the entire human genome in April of 2003 opened up a new world of opportunity for scientists to elucidate the code of nucleotide bases. This led to the desire to not only examine each gene individually, but to also examine what is going on with the whole genome at a single snapshot in time. In other words, how the genes may be interacting with one another. This gave birth to the field of genomics which focuses on examining the entire genome. Within the field of omics technology, there are several subspecialties including

transcriptomics, proteomics, and metabolomics which examine all of the mRNA, proteins, and metabolites, respectively, being produced at one time.

Transcriptomics, in particular, is the examination of what genes are being transcribed, or expressed, at a single point in time. Unlike the genome which is fairly static, the transcriptome is constantly changing based on environmental conditions. These environmental conditions may be affected by diet, pharmacological agents, toxins, and disease. Studying the transcripts may allow for elucidation of biological pathways.

In order to study the effect on the transcriptome based on environmental conditions, the gene expression microarray was developed. There are different types of microarrays including cDNA and oligonucleotide arrays. cDNA microarrays use cloned DNA libraries spotted on glass slide to mRNAs or protein coding RNAs while oligonucleotide arrays use short oligonucleotide gene probes. Tens of thousands of genes can be analyzed simultaneously generating large amount of data on the up-regulation and down-regulation of genes when comparing two types of tissue (e.g., normal and diseased) or same tissue type before and after an intervention. The field of bioinformatics was developed in response to the need to analyze all of the generated information. Analysis of microarray data includes the challenge of separating background noise from the actual signal representing differential gene expression.

Current Research in Transcriptomics

Transcriptomic technology has allowed for the testing of differential gene expression in the hopes of identifying genes and pathways differentially expressed in

response to disease, diet and exposure to potential carcinogens. Recently, a number of studies utilizing this technology have been reported, most of them investigating disease states. For example, Rotunno, et al. (Rotunno, Hu et al. 2011) recently described a study of differential gene expression between cases and paired controls for early lung adenomacarcinoma. Fifty differentially expressed genes were identified which may provide insight into early changes that occur in lung cancer (Rotunno, Hu et al. 2011). Similarly, Landi et al. (Landi, Dracheva et al. 2008 examined the gene expression signature of malignant and benign lung tissue of former, current and never smokers and found similar gene expression patterns involving disregulation of mitotic spindle formation in former and current smokers (Landi, Dracheva et al. 2008). In addition, Field et al was able to identify differentially expressed genes that play a role in cellular growth and differentiation, invasion, metastasis and immune response comparing malignant breast tissue in matched African American and Caucasian cases (Field, Love et al. 2012). This approach may allow for the elucidation of the causes for the higher mortality rates of younger African American women diagnosed with breast cancer (Field, Love et al. 2012).

Gene Expression in Peripheral Blood in Response to Diet

Most studies have analyzed gene expression patterns specifically in diseased organ tissue. This approach may be expensive, invasive and impractical for some research. Examining differential gene expression in peripheral whole blood allows for a minimally invasive specimen collection. Several recent studies have shown that peripheral blood is capable of showing differential expression patterns in response to

dietary interventions in the field of nutritional genomics. In 2011, Ghosh et al demonstrated change in gene expression in whole-blood in response to caloric restriction among obese patients (Ghosh, Dent et al. 2011). A study from Bouwens et al showed that peripheral blood mononuclear cells can reveal fatty acid specific gene expression profiles in young healthy men after the consumption of different fatty acids, as evidenced by the opposite effects of PUFA and SFA intakes on the expression of genes involved in liver X receptor signaling (Bouwens, Grootte Bromhaar et al. 2010). Another study by Bohn et al described the up-regulation of genes in the DNA repair, apoptosis, and hypoxia pathways in the blood of smokers in response to antioxidant rich, plant-based diet (Bohn, Myhrstad et al. 2010).

AIMS AND HYPOTHESIS

Because red meat and well-done red meat consumption has been associated with prevalent mortalities and morbidities, elucidation of the molecular basis for these associations could provide critical information leading to the prevention and/or treatment of associated maladies. The purpose of this MS thesis was to determine the effects of consumption of well-done meat of varying PhIP levels on genome-wide mRNA expression in peripheral whole blood. It was expected that metabolic genes and genes involved in the progression of a normal cells to cancerous cells would be differentially expressed in the blood of the subjects before consuming well-done meat when compared to that of the blood after consuming well-done meat for four weeks. In addition, it was hypothesized there would also be differential gene expression when comparing different levels of PhIP. This was accomplished by comparing gene expression in peripheral whole blood before and after four weeks of consumption well-done red meat of different levels of PhIP as part of semi-controlled feeding study.

METHODS

Participant Selection

Recruitment

In order to recruit participants for the present study, advertisements were posted throughout the campus of the University of Hawaii, as well as in the University newspaper. All potential participants were required to attend an initial screening visit. At the beginning of the eligibility screening, informed consent was obtained. During screening, potential participants were required to answer a short eligibility questionnaire and a comprehensive lifestyle questionnaire, including a detailed food frequency questionnaire, and to maintain a 3-day food record. They were also required to complete a medical history questionnaire, and to provide a blood sample for obtainment of a biochemical blood profile and a complete blood count.

Inclusion Criteria

In order to be included in the study, participants were required to be non-smokers over the age of 18 years, not use any hair dyes and have a weight not less than 90% or greater than 130% of the 1983 Metropolitan Life Insurance criteria, with no unexplained weight change of more than 10 pounds within the past twelve months (Metropolitan Life Foundation 1983). The 1983 Metropolitan Life Insurance height and weight tables are a published reference table that outline ideal body weight based on sex, height and body frame size (Metropolitan Life Foundation 1983). In addition, the diet questionnaire was used to determine that all participants were regular beef eaters, did not follow any special diet such as a vegetarian, macrobiotic, weight loss or diabetic diet, consumed less than seven servings of fruits and vegetables per day, consumed less

than 22 grams of fiber per day, drank no more than two alcoholic drinks per day and consumed no more than two caffeinated beverages each day. Participants also had to be in good general health with no history of previous gastrointestinal tract disorders, such as inflammatory bowel disease, or cancer which was determined based on the medical history questionnaire, as well as the biochemical blood profile and the blood count obtained at screening. Any subjects found to have clinically abnormal values in their blood test results were advised to consult their physician.

Meat Preparation

To estimate the amount of heterocyclic amines, specifically PhIP and MeIQx, that would be produced, a meat sample preparation pilot was conducted. Three pounds of fresh ground beef with 15% fat was purchased from a local grocery store. Six ½ pound patties were formed each with an approximate diameter of 4.5 inches and thickness 0.5 inches. A flat top gas griddle was used to cook the patties with surface temperatures varying between 400°F to < 500°F throughout the duration of the cooking. Three of the patties were cooked for 12 minutes total cooking time and rotating half-way through. The remaining three patties were cooked for 24 minutes total, rotating half-way through. The three patties cooked for a total of 12 minutes were homogenized into a single batch using a food processer. The three patties cooked for 24 minutes were also homogenized into a single batch. Samples from each of the batches were subsequently sent to Dr. Robert Turesky's laboratory at the New York State Department of Health to quantify the amount of HCAs. The amount of HCAs in the meat was determined using tandem solid phase extraction methods of LC/ESI-MS/MS (liquid chromatography/

electrospray ionization-mass spec/mass spec.) and GC/MS respectively (Turesky, Taylor et al. 2005) (Mottier, Parisod et al. 2000). Results from the pilot meat sample preparation can be found in Table 1.

Table 1. MeIQx & PhIP levels in parts per billion from the Meat Preparation Pilot

	MeIQx (ppb)	**PhIP(ppb)**
6 minutes/side Batch 1	7.0	15.2
6 minutes/side Batch 2	5.3	16.9
6 minutes/side Batch 3	4.8	14.3
6 min/side Average ±SD	**5.7 ±1.1**	**15.5±1.3**
12 min/side Batch 1	7.7	32.1
12 min/side Batch 2	8.3	33.2
12 min/side Batch 3	7.2	38.2
12 min/side Batch 4	7.4	35.5
12 min/ side Average ± SD	**7.9 ± 0.5**	**35.7 ± 2.5**

Using the data obtained from the meat preparation pilot, a standardized protocol for meat preparation was developed. Not more than 15% fat ground beef was cooked on a flat metal grill surface in pre-formed ½ pound patty portions with a starting grill temperature of 527°F. Patties were to be cooked for 9-12 minutes per side flipping only

once. After cooking, the patties were homogenized by thoroughly mincing in a food processor in two to four batches. Subsequently, samples from each batch were sent for quantification of HCAs as described previously for the pilot. After quantification of the heterocyclic amines in parts per billion (ppb), PhIP/serving amount was determined for each group taking into account the amount of PhIP that would be consumed along with a reasonable portion size. Actual cooking times, HCA amount, and portion sizes for each study group can be found in Table 2.

Participant Feeding

For the first three weeks of participation in the study, referred to as the pre-feeding phase, the participants were asked to avoid consumption of any well done meat, poultry or fish products and, more generally, any of the same products cooked using pan-frying grilling or oven broiling methods.

Table 2. Amounts of Well Done Meat Fed and Doses of PhIP and MeIQx per Study Day

Group #	PhIP (µg/ serving)	MeIQx (µg/serving)	Portion Size (ounces)	Cooking Time (minutes/side)	# of Participants
1	2.689	0.945	7	6-9	10
2	11.568	0.981	5	9-14	14
3	7.966	1.296	5	9-12	6

For the next four weeks, the participants were fed the prepared well done meat for dinner. The participants were required to eat dinner 5 days a week for a total of 19

feeding days at Paradise Palms cafeteria on the University of Hawaii at Manoa campus. The meal included a specific portion of meat (refer to Table 2), as well as a starch, a vegetable, a fruit, dessert and a drink. For the remainder of their daily meals and on Saturdays and Sundays, the participants followed their normal diet with the exception that they were asked to avoid eating any well done meat/fish. In addition, participants were instructed to avoid certain foods 2 hours prior to the meal and until the next morning. A list of the foods they were asked to avoid can be found in Table 3. The chefs in charge of preparation of the meals for serving were also asked to avoid the use of the same foods in meal prep. These foods are rich in fiber and antioxidants which may block the formation, digestion and/or absorption of the heterocyclic amines or other active compounds in the well-done meat as described previously.

Table 3. Foods to Avoid for Participants/Chefs in Consumption/Preparation of Study Meals

Component	Foods to Avoid
Meats/Protein	Meats cooked well-done, tofu, soybeans, all types of beans
Starch	High fiber starches: whole grain breads and pastas, oatmeal/oats, bran, buckwheat, brown rice
Fruit	High fiber fruits (prunes, raisins, pears, raspberries), citrus fruits (grapefruit, oranges, lemons, limes), Other fruits (apples, peaches, strawberries, mangoes, cherries, nectarines, grapes)
Vegetable	High fiber vegetables (Brussels sprouts, canned pumpkin, green peas, green beans), beans/legumes (all kinds), dark green leafy veg (broccoli, spinach, turnip greens, anything that is dark green), Other vegetables (bean sprouts, mushrooms, onions, celery)
Dessert	Chocolate and coffee flavored desserts, high fiber fruits (prunes, raisins, pears, raspberries), citrus fruits (grapefruit, lemons, limes)
Beverage	Orange juice, grape juice, grapefruit juice, lemon/lime flavored beverages (Fresca, Sprite, lemonade), caffeinated beverages (coffee, tea, colas, Barqs root beer, energy drinks), alcohol
Condiments/Sauces/ Spices	Gravies and sauces made from actual meat drippings; Garlic, garlic powder, onion powder

CYP1A2 Levels

In order to assess CYP1A2 activity levels, participants were required to complete a caffeine urine test. Twenty-four hours prior to completion of the test, participants were instructed to avoid all use of caffeine including caffeinated beverages and chocolate. After fasting for at least six hours, participants were instructed to consume 2 cups Maxwell House brand of freeze dried instant coffee (provided to them) with only sugar or sugar alternative and no cream or dairy of any kind. From the start of drinking the coffee, they were given thirty minutes to finish consumption. Two hours after drinking the coffee, they were allowed to eat. Four hours after drinking the coffee, they were instructed to empty their bladder and to discard any urine. For the next hour, i.e., between the fourth and fifth hour after consumption of coffee, participants collected all urine and stored the sample refrigerated in a cooler with frozen blue ice until given to the study staff. Participants were instructed to avoid any caffeine throughout the whole testing period, except for the study dose. When obtained by the study staff the urine volumes were measured and the samples were kept at -80°C until analysis. Urine was subsequently sent to Dr. Adrian Franke's laboratory at the University of Hawaii Cancer Center for analysis with HPLC as previously described (Le Marchand, Franke et al. 1997) Quality control was conducted by including blind duplicates analyzed simultaneously with the study samples. Results from the quality control analysis are included in Table 4. After receiving the results the median of the group was found for the entire data set. All participants whose values fell below the median were classified as having a "low CYP1A2 activity" whiles all who were above were classified as having a "high CYP1A2 activity".

Table 4. Results from the Quality Control of CYP1A2 Activity.

Participant #	Ratio (17U + 17X)/ caf	Blind QC Duplicate Ratio (17U + 17X)/ caf
43	6.64	6.65
45	10.05	9.35
49	11	10.4
59	7.24	7.15
64	4.55	4.96

Specimen collection

Blood specimens for this study were collected from each participant on Days 21 and 49 of the study. Day 21 was after following the three weeks pre-feeding basal diet avoiding well done meats. Day 49 was at the conclusion of the consumption of the four-week intervention with well-done meat.

2.5 ml of whole blood was collected via antecubital fossa venipuncture directly into PAXgene tubes (PreAnalytiX , Switzerland) from each participant according to the manufacturer's instructions. The PAXgene tubes (PreAnalytiX , Switzerland) that came prefilled with proprietary solution that enables immediate stabilization of RNA when collecting blood. After collection, the tubes were inverted 10 times to mix the blood with the solution, stored upright at room temperature for two hours, transferred to a -20°C freezer for 24 hours and then moved to a -80°C freezer for storage until processing.

Prior to processing, the PAXgene tubes were thawed overnight in a rack and once again inverted ten times to assure that the solution was adequately mixed.

RNA Extraction

In the PAXgene blood collection tube, the nucleic acids and proteins were pelletized by centrifugation. After being washed and resuspended in order to digest the proteins, the pellet was incubated in optimized buffers and proteinase K. To homogenize the cell lysate and remove any remaining cell matter, the solution was then put through the PAXgene shredder spin column. The lysate was then applied to the PAXgene RNA spin column that selectively binds RNA while allowing contaminants to pass through. In order to remove any remaining impurities, several wash steps were performed, as well as the removal of DNA using DNase. RNA was then eluted and heat denatured before quality and quantity were assessed using the Bioanalyzer and Nanodrop, respectively (PreAnalytiX 2009).

Removal of Globin mRNA

Following extraction, alpha and beta globin mRNA was removed from the total RNA using Ambion's GLOBINclear Kit (Life Technologies, Carlsbad). Whole blood may contain a large percentage of hemoglobin mRNA (Liu, Walter et al. 2006). Hemoglobin mRNA from PAXgene whole blood has been shown to decrease the sensitivity of transcript detection using microarrays (Liu, Walter et al. 2006). Therefore, 14 µl of ≥ 70 ng/µl of total RNA was combined with a biotinylated Capture Oligo mixture specific for binding alpha and beta globin mRNA . Magnetized streptavidin beads were then incubated with the mixture which allowed them to bind the biotinylated oligonucleotides. When

applied to the magnet, the GLOBIN mRNA was captured by the magnet, and the globin mRNA-depleted RNA was purified with a RNA binding bead mixture. Assessment of quality and quantity was performed on the globincleared RNA using the Bioanalyzer and Nanodrop, respectively.

Single stranded cDNA, Labeling and Hybridization

A total of 100 ng globin-cleared mRNA at a concentration of 33 ng/µl was then used to generate single strand complementary DNA using Ambion's WT Expression Kit . When beginning the generation of cDNA , diluted PolyA controls were added to each sample in order to "provide exogenous positive controls to monitor the entire target labeling process." Reverse transcription was then utilized to produce single stranded cDNA sequence that contained a T7 promoter sequence. A second strand of cDNA was then synthesized followed by the in vitro transcription to create antisense cRNA. After the purification of the cRNA, quantity and quality were again assessed using Bioanalyzer and Nanodrop, respectively. Reverse transcription was then utilized to synthesisize sense strand cDNA with incorporated dUTP from at least 10 ug of the cRNA product using random primers designed specifically to exclude rRNA sequences. After hydrolyzing the cRNA template using RNaseH, the sense strand cDNA was purified and the quantity and size distribution were assessed using Nanodrop and Bioanalyzer , respectively. Uracil DNA glycolsylase (UDG) and apurinic/ apyrimidinic endonuclease 1 (APE1) was then added to 2.75 ug of the single stranded cDNA. The UDG and APE1 recognize the added dUTP from the second strand synthesis completed previously and fragment the DNA strand at that point. After assessing the size of the fragmented DNA

with the Bioanlayzer, the DNA was labeled using terminal deoxynucleotidyl transferase enzyme in addition to Affymetrix's proprietary biotin linked DNA labeling solution. After fragmentation and labeling, the samples were combined with a hybridization cocktail and controls, then injected into the Affymetrix Human Gene 1.0 Sense Target (ST) Array and incubated overnight at 45°C for 16 hours. After the incubation, each chip was washed and stained according to the Affymetrix GeneChip Expression Wash, Stain and Scan User Manual using the Affymetrix GeneChip Fluidics Station 450/250. Each chip was scanned immediately following the wash using the Affymetrix GeneChip Scanner 3000.

Affymetrix Gene Expression Console

All CEL files were imported into the Affymetrix Gene Expression Console vs 1.1.2800.2681 in order to evaluate the hybridization quality of all chips. The software allowed for visualization of quality control metrics through histogram and box and whisker plots of intensities for each chip. A signal histogram for all 60 chips was created, in addition to relative probe cell intensity, in order to identify divergent probe cell intensities.

Differential Gene Expression Analysis

All statistical analysis was performed using Partek Genomics Suite Version 6.6. CEL files were imported from Affymetrix Gene Console using default import settings including background correction, quantile normalization and summarization using Robust Multichip Analysis. The data was also log2 transformed. The contrast method used was the Fisher's least significant difference test (Fisher 1925) . Multiple test

correction was accomplished using Benjamini-Hochberg methods of False Discovery Rate (Benjaminin and Hochberg 1995). Confounding variables, or sources of variation, were visualized using F-means for signal to noise ratio.

A subsequent analysis was conducted on only the pre-feeding study samples to verify that the genes differentially expressed between post-feeding PhIP group levels were not the same genes differentially expressed between pre-feeding PhIP group levels. The same variables of scan date, sex, ethnicity, age, BMI and CYP1A2 activity were used in the model.

RESULTS

Participant Characteristics

A summary of the participant characteristics can be found in Table 5. A total of 60 samples from 30 individuals were analyzed in the present study. There were 25 males and 5 females with eleven Caucasians, nine Asians, and ten individuals who were of other or mixed ethnicity. The mean age was 25.6 years and the range: 19 to 40 years. Ten, six and fourteen people were fed 2.69, 7.97, and 11.57 µg of PhIP per serving, respectively (Table 2). Participants had a mean BMI of 24.60 ± 4.67 at the start of feeding with a mean BMI of 25.06 ± 4.68 at the conclusion of the feeding. A paired t-test performed using Excel showed that the weight gain was significant with a p-value of 0.007.

Table 5. Characteristics of participants who participated in the well done meat feeding study.

Age (Mean ± SD)	25.6 ± 8.12
BMI (Mean ± SD)	
BMI_start	24.60 ± 4.67
BMI_end	25.06 ± 4.67
Sex [n (%)]	
Male	25 (83.3)
Female	5 (16.6)
Ethnicity [n (%)]	
Caucasian	11 (36.7)
Asian	9 (30)
Mixed/other	10 (33.3)

Affymetrix Gene Expression Console

Analysis with Affymetrix Gene Expression Console allowed for visualization of the hybridization quality of all sixty microarray chips. Figure 1 is a signal histogram showing relative signal values of all sixty microarrays. There does not appear to be any extreme outliers. Figure 2 shows a box plot of relative probe cell intensity for all sixty chips. This plot was utilized to compare the distribution of the intensities on each microarray using the median probe intensity as a reference value for the entire set of microarray chips tested in the study. If there were a large divergence from the median probe intensity, it could have been identified using this plot.

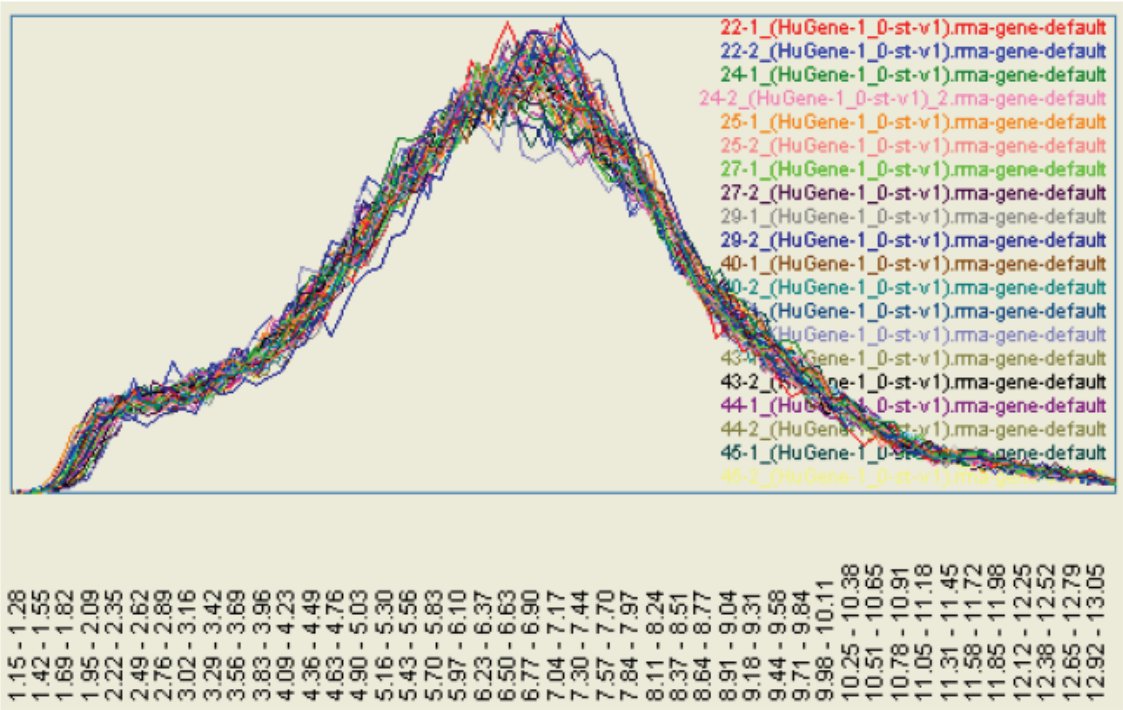

Figure 1. Affymetrix Gene Expression Console QC Metrics Signal Histogram illustrates relative signal values of all 60 microarrays.

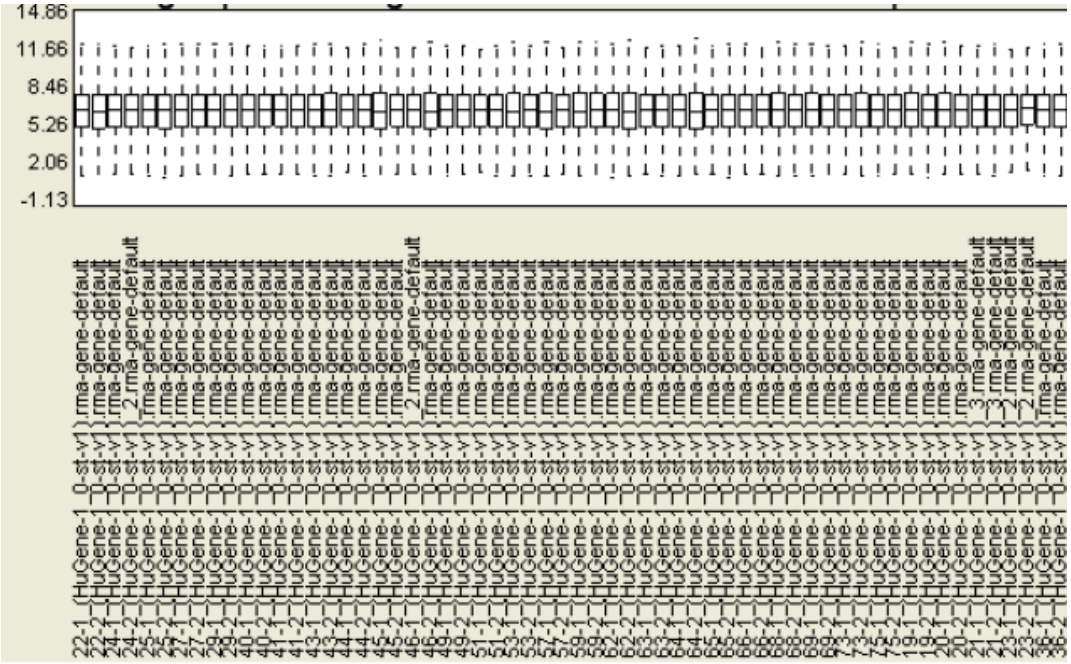

Figure 2. Box Plot of Relative Probe Cell Intensity from Affymetrix Gene Expression Console shows the distribution of intensities for each microarray utilizing median probe intensity as the reference value.

Partek Analysis

For the first analysis, all sixty samples were analyzed in Partek using a six-way analysis of covariance (ANCOVA) with pre-feeding vs. post-feeding, scan date, PhIP dose

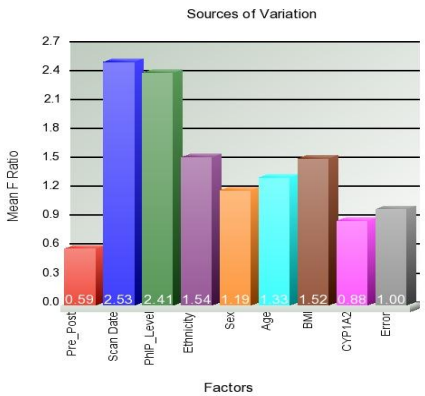

Figure 3. Sources of Variation in mean F ratio demonstrating signal to noise ratio before any batch removal using 6-way ANCOVA (n=60 samples) with pre-feeding vs. post-feeding, scan date, PhIP dose level, sex, ethnicity, age, BMI and CYP1A2 activity as response variables. This plot allows for the identification of variation in the model.

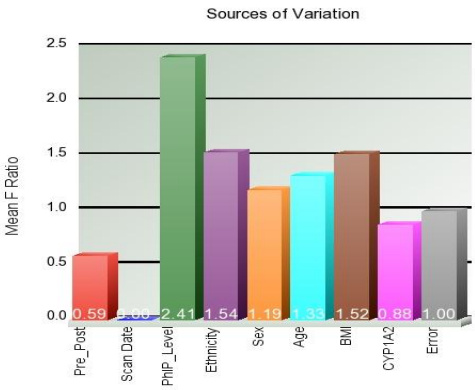

Figure 4. Sources of Variation in the mean F ratio from 6-way ANCOVA (n=60 samples) with pre-feeding vs. post-feeding, scan date, PhIP dose level, sex, ethnicity, age, BMI and CYP1A2 activity as response variables after the batch removal tool has been applied using a mixed model ANOVA.

level, sex, ethnicity, age, body mass index (BMI) and CYP1A2 activity as response variables. This analysis served as an exploratory analysis which allowed for the visualization of the sources of variation using F-means (see Figure 3). As is apparent in the figure, scan date is the largest source of variation in the model followed closely by PhIP dose level. Ethnicity, BMI, sex and age also came in with F ratio higher than error. Thus, each of these response variables played a significant role in the variation in gene expression which indicated the need to include them in future models. Because scan date was such a large source of variation, the effect of scan date was removed in all future analysis using Partek's batch removal tool. The batch removal tool used the mixed-model ANOVA method which allowed for visualization of the other response variables without the batch effect. Figure 4 shows the sources of variation in gene expression after the batch removal tool has been utilized. No genes were found to be differentially expressed between the pre-feeding and post-feeding samples and CYP1A2 activity when multiple test correction was applied. However, 3,243, 262, 28, 2 and 1 genes were differentially expressed between the different PhIP level groups, ethnicities, sex, age, and BMI, respectively, when multiple testing parameters were applied.

Next, since each subject had pre-feeding and post-feeding samples, the ANOVA method was used with subject (pairing), pre-feeding vs. post-feeding, and PhIP level response variables. This analysis allowed for the subjects to each serve as their own control reducing the effect of interindividual variability. The largest source of variation remaining was the PhIP level, with a mean F ratio of 3.81 (Figure 5). The variation in gene expression due to the intervention was not above the error in this analysis. When applying the multiple test correction parameters, no genes were differentially expressed between pre-feeding and post-feeding, but there was a change in the expression of 6186 genes when comparing PhIP levels.

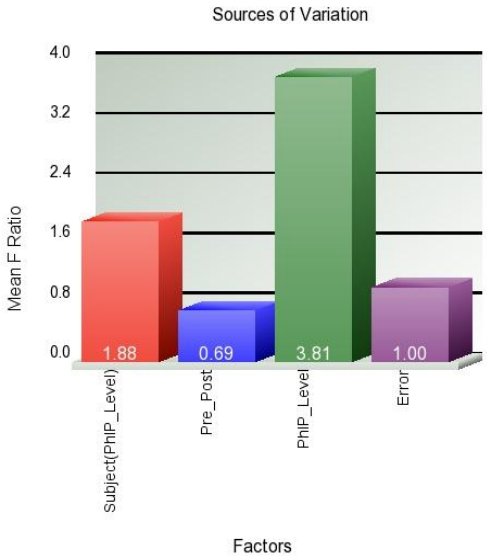

Figure 5. Sources of Variation in the mean F ratio demonstrating signal to noise in the 3-way ANOVA (n=60 samples) with subject (pairing), pre-posting feeding, and PhIP level as response variables. PhIP level is the largest source of variation in the model.

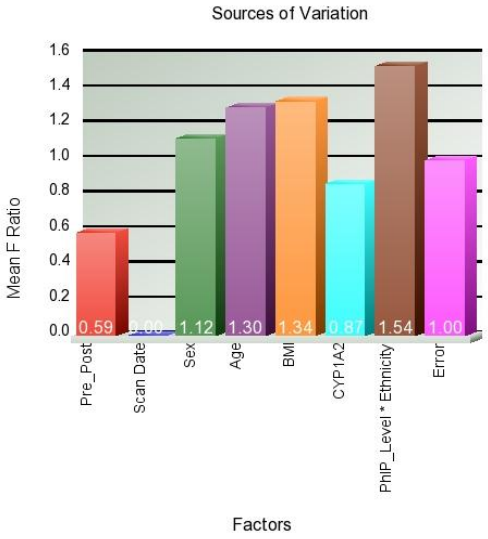

Figure 6. Sources of Variation in the mean F ratio demonstrating signal to noise ratio using 6-way ANCOVA (n=60 samples) with pre-feeding vs. post-feeding, scan date, PhIP dose level*ethnicity interaction term, sex, age, BMI and CYP1A2 activity as response variables.

In order to test if there was an effect of PhIP simultaneously interacting with ethnicity a six-way ANCOVA was used with PhIP*ethnicity interaction term, pre-feeding vs. post-feeding, scan date, age, BMI and CYP1A2 activity as response variables. The greatest source of variation was the PhIP*ethnicity

interaction as can be seen in Figure 6. When applying the multiple test correction parameters, 2031, 22, 11, and 1 gene(s) were differentially expressed between PhIP*Ethnicity levels, sexes, ages, and BMI levels, respectively. No genes were found to be significantly differentially expressed after the multiple test correction between pre-feeding and post-feeding samples or CYP1A2 activity groups.

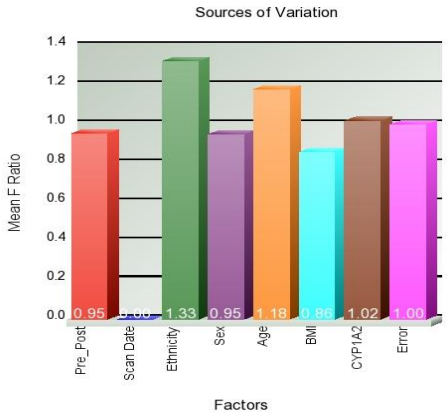

Figure 7. Sources of Variation in the mean F ratio for all of the pre-feeding samples vs post-feeding PhIP 11.568 µg/d PhIP level group using 5-way ANCOVA (n=44) with pre-feeding vs post-feeding, scan date, sex, ethnicity, age, BMI and CYP1A2 activity as response variables.

In order to test if there was an effect of pre-feeding versus post-feeding when looking at all of the pre-feeding samples versus the highest dose of PhIP post-feeding samples, all of the pre-feeding samples (N=30) were compared to the post-feeding samples of the 11.568 µg/d PhIP level group (N=14) using five-way ANCOVA with pre-feeding vs post-feeding, scan date, sex, ethnicity, age, BMI and CYP1A2 activity as response variables. The largest source of variation was ethnicity followed closely by age (see Figure 7). In this analysis, only differential expression in one and 16 genes were found that passed multiple correction when examining ethnicity and sex respectively. No genes passed multiple correction when analyzing pre-feeding versus post-feeding, age, BMI or CYP1A2.

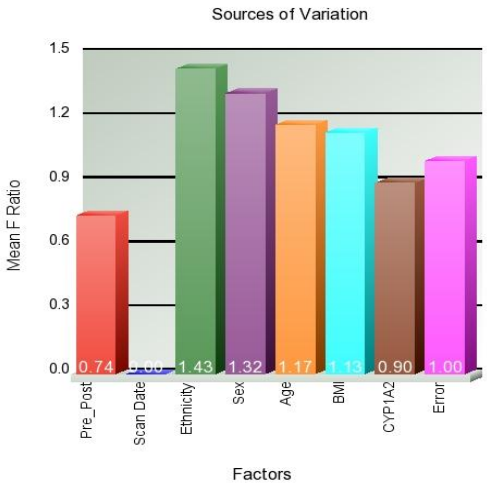

Figure 8. Sources of Variation in mean F ratio demonstrating signal to noise ratio using 6-way ANCOVA (n=50 samples) with pre-feeding vs. post-feeding, scan date, sex, ethnicity, age, BMI and CYP1A2 activity as response variables.

Then in order to increase the sample size from the previous analysis, the gene expression in the pre-feeding samples (N=30) were compared to that in the two highest PhIP level groups (post-feeding samples of the 7.966 µg/d and 11.568 µg/d PhIP level groups combined) (N=20) using a five-way ANCOVA with pre-feeding vs post-feeding, scan date, sex, ethnicity, age, BMI and CYP1A2 activity as response variables. Once again ethnicity was the largest source of variation with sex, age, and BMI following closely (Figure 8). Differential expression which passed multiple correction parameters was seen between ethnicities and sex with 49 and 22 genes varying respectively. No differential expression that passed the testing parameters was observed when comparing pre-feeding versus post-feeding, age, BMI and CYP1A2 groups.

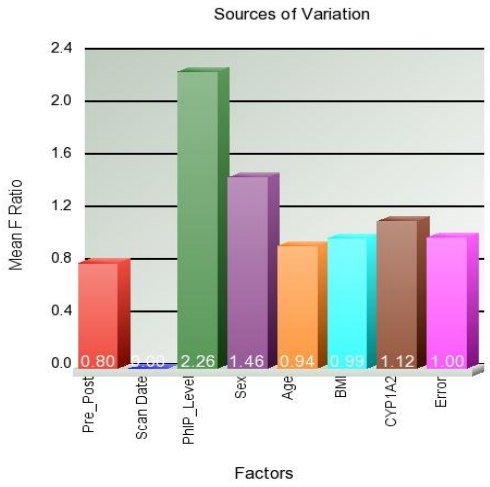

Figure 9. Sources of Variation in mean F ratio demonstrating signal to noise ratio using 6-way ANCOVA (n=22 samples) with pre-feeding vs. post-feeding, scan date, sex, age, BMI and CYP1A2 activity as response variables when testing only the Caucasian samples.

Because each of the previous analysis where ethnicity was a response variable found genes significantly expressed between ethnic groups when applying even the stringent multiple correction tests and ethnicity was consistently one of the largest sources of variation, analyses were conducted separately on only the Caucasian samples and, subsequently, on only the Asian samples. The Caucasian analysis included only the eleven subjects (twenty-two samples) included in the Caucasian ethnicity grouping ethnicity group. The samples were analyzed using a five-way ANCOVA with pre-feeding vs post-feeding, scan date, PhIP level, sex, age, BMI and CYP1A2 activity as response variables. PhIP level was the largest source of variation (Figure 9). When examining genes that were differentially expressed and passed the multiple testing parameters, PhIP, sex, age, BMI and CYP1A2 experienced a change in gene expression among 439, 19, 1, 1, and 2 genes respectively.

In the same way, an analysis was conducted including only the eighteen samples from the Asian ethnicity group. A five-way ANCOVA was utilized to compare the pre-feeding and post-feeding samples of only the Asian ethnicity group with pre-feeding vs post-feeding, scan date, PhIP level, sex, age, BMI and CYP1A2 activity as response variables. When examining the Sources of Variation graph (Figure 10), this was the only analysis conducted where the variation that could be attributed to the pre-feeding samples versus post-feeding samples was greater than the error. In addition, BMI and CYP1A2 soared above the other response variables as the greatest sources of variation. When looking at differentially expressed genes between CYP1A2 groups, 33 genes were found to be differentially expressed when applying multiple testing parameters. In addition, 17, 12, 10 and 1 gene(s) were differentially expressed when analyzing BMI, age, sex and PhIP level respectively.

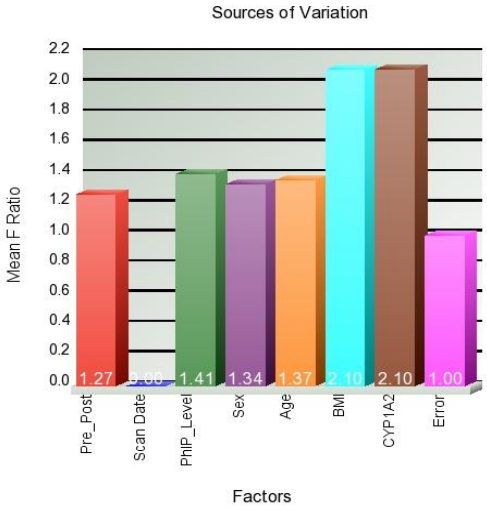

Figure 10: Sources of Variation in mean F ratio demonstrating signal to noise ratio using 6-way ANCOVA (n=18 samples) with pre-feeding vs. post-feeding, scan date, sex, age, BMI and CYP1A2 activity as response variables when testing only the Asian samples.

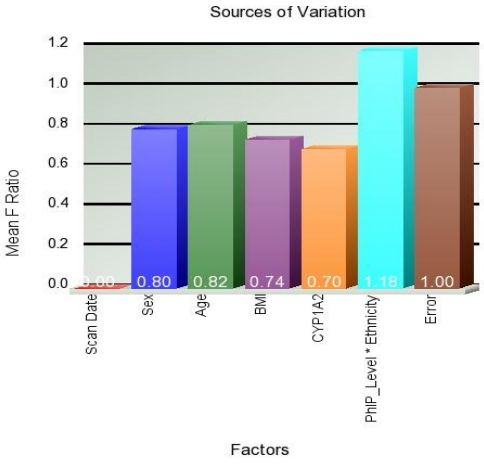

Figure 11: Sources of Variation in mean F ratio demonstrating signal to noise ratio using 5-way ANCOVA (n=30 samples) with PhIP level*ethnicity, scan date, sex, age, BMI and CYP1A2 activity as response variables when testing only the post-feeding samples.

After looking at all the data from the analysis conducted thus far, it appeared that the

variation in gene expression in the pre-samples was too large for the study to detect a difference when the intervention was applied. Therefore, analyses were subsequently performed utilizing only the thirty post-feeding samples with the PhIP level as the main treatment effect. The first analysis conducted with only the post-feeding samples was a five-way ANCOVA comparing the interaction of PhIP*ethnicity of all of the post-feeding samples with scan date, sex, age, BMI and CYP1A2 activity as response variables. When examining the sources of variation from this analysis (Figure 11), the PhIP * ethnicity interaction was the only response variable with a mean F ratio that was greater than error. However, no gene was found to be differentially expressed when applying the multiple test correction parameters for any of the response variables.

In order to examine if there was an effect only with PhIP levels, without ethnicity, the interaction term was removed for the next analysis utilizing only the post-feeding samples. A six-way ANCOVA was used to compare differences in gene expression between PhIP levels with scan date, sex, ethnicity, age, BMI and CYP1A2 activity as response variables. Ethnicity and PhIP level were the only two variables that surpassed the error among the sources of variation (Figure 12). When applying the multiple test correction, differential expression was found in 21, 13 and 2 genes between the different PhIP levels, sex and ethnicity groups respectively. Out of the 21, 13, and 2 genes

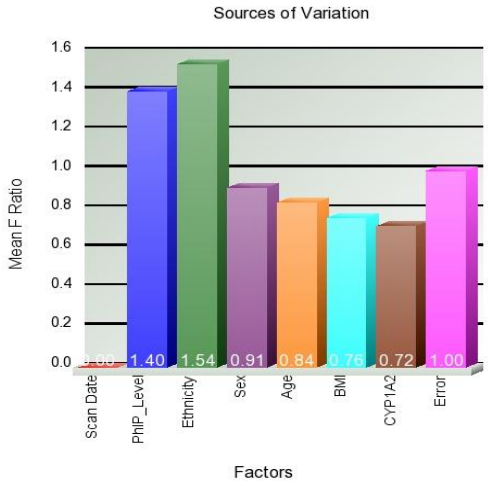

Figure 12: Sources of Variation in mean F ratio demonstrating signal to noise ratio using 6-way ANCOVA (n=30 samples) with PhIP level, ethnicity, scan date, sex, age, BMI and CYP1A2 activity as response variables when testing only the post-feeding samples.

differentially expressed between PhIP levels, sex , and ethnicity groups only 7, 12, and 1 gene(s), respectively, were annotated.

Because there are more than two groups being compared in the ANCOVA directionality of the differential expression is not given by PARTEK. In order to calculate directionality of the expression changes, contrasts comparing one group against another group must be built into the model. Therefore after reviewing the results, the ANCOVA with the same response variables was performed in addition to adding multiple contrasts which allowed for contrasting of gene expression between the different PhIP level groups (11.568 vs. 2.69; 7.97 vs 2.69; 11.568 vs 7.97; 11.568 and 7.97 vs 2.69 μg/d) and different ethnicities (Asian vs Caucasian; Asian vs Mixed/Other; Caucasian vs Mixed/Other; Asian vs Caucasian and Mixed/Other; Caucasian vs Asian and Mixed/Other). The contrast method allows for not only multiple test correction parameters but also fold-change parameters. The fold-change parameters applied were ±1.5 which means that between the two comparison groups the genes were up-regulated or down-regulated by a factor of no less than 1.5. The results of the contrast analysis can be found in Table 6. When comparing the highest and lowest PhIP groups (11.568 vs. 2.69 μg/d) and the middle group (7.97 μg/d) with the lowest group, 19 and 5 genes were found to be up-regulated, respectively. No genes were found to be down-regulated. Of those 19 and 5 genes, 8 and 1 have been annotated. No genes passed the parameters when contrasting the two highest PhIP grouping levels against each other (11.568 vs 7.97 μg/d). However, when contrasting the two highest PhIP levels (11.568 vs 7.97 μg/d) together as one group against the lowest PhIP level (2.69), 54 genes were

found to be up-regulated with 18 of those being annotated. When analyzing the contrasts performed between the ethnicity groups, 4, 2 and 1 gene(s) were found to be differentially expressed when contrasting the Caucasian vs Asian, Asian vs Caucasian and Mixed/Other groups and Caucasian vs Asian and Mixed/Other groups respectively. However, only 1 gene was annotated in each group. No genes were found to be - differentially expressed when contrasting the Caucasian vs Mixed/Other and Asian vs Mixed/Other.

Table 6. Number of Genes Differentially Expressed/Annotated Genes When Contrasting PhIP Levels & Ethnicities

Contrasting Analyses	Number of Genes Differentially Expressed	Number of Annotated Genes Differentially Expressed
PhIP Level Contrasts		
11.568 vs. 2.69 µg/d	19	8
7.97 vs 2.69 µg/d	5	1
11.568 vs 7.97 µg/d	0	0
11.568 and 7.97 vs 2.69 µg/d	54	7
Ethnicity Contrasts		
Asian vs Caucasian	4	1
Asian vs Mixed/Other	0	0
Caucasian vs Mixed/Other	0	0
Asian vs Caucasian and Mixed/Other	2	1
Caucasian vs Asian and Mixed/Other	1	1

A subsequent analysis was conducted on only the pre-feeding study samples to verify that the genes differentially expressed between post-feeding PhIP group levels

were not the same genes differentially expressed between pre-feeding PhIP group levels. The same response variables of scan date, sex, ethnicity, age, BMI and CYP1A2 activity were used in the five-way ANCOVA model. When performing the subsequent analysis on only the pre-feeding study samples, no genes were found to differ between PhIP group levels that passed multiple test corrections and/or fold change parameters. Age and BMI were the largest sources of variation (Figure 13).

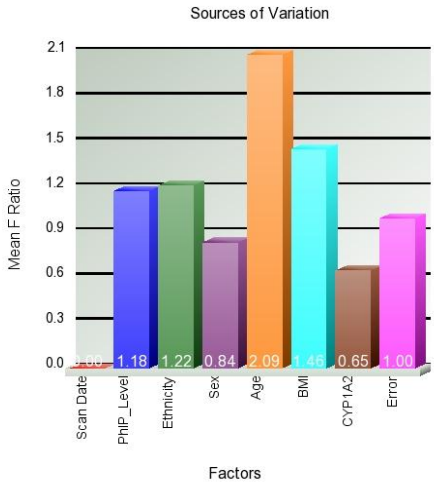

Figure 13. Sources of Variation in mean F ratio demonstrating signal to noise ratio using 6-way ANCOVA (n=30 samples) with PhIP level, ethnicity, scan date, sex, age, BMI and CYP1A2 activity as response variables when testing only the pre-feeding samples.

Genes Up-regulated in Response to PhIP

As mentioned previously, the ANCOVA model was able to identify seven annotated genes that were up-regulated across all PhIP levels with a false discovery rate (FDR) of <0.05 (*ATP6V0B, MPZ, DRAP1, ZNF552, HCG27, HN1,* and *UBE2Q1)*. A list of these genes official symbols and official names and their p-values can be found in Table 7. A visualization of the distribution of the genes across each of the PhIP levels can be seen in each gene's dot plot in Figure 14.

Table 7. Genes Up-regulated across all PhIP levels with FDR <0.05.

Gene Symbol	Name	p-value
ATP6V0B	ATPase, H+ transporting, lysosomal 21kDa, V0 subunit b	5.81×10^{-7}
MPZ	Myelin Protein Zero	3.63×10^{-6}
DRAP1	Dr1-associated protein (negative cofactor 2 alpha)	3.91×10^{-6}
ZNF552	Zinc Finger 552	7.34×10^{-6}
HCG27	HLA complex group 27 (non-protein coding)	1.48×10^{-5}
HN1	hematological and neurological expressed 1	2.19×10^{-5}
UBE2Q1	ubiquitin-conjugating enzyme E2Q family member 1	2.36×10^{-5}

When examining the results of the contrasts performed with the ANCOVA model with a false discovery rate of <0.05 and fold change parameter of ± 1.5, the contrasts between the 11.568 vs 2.689 µg/d groups found eight annotated genes (*ATP6VDB, DRAP1, ZNF552, HCG27, MPZ, HN1,* and *VAC 14)* that had been up-regulated with a fold-change greater than 1.5. No genes were found to have been down-regulated. A list of these genes official symbols and official names and their p-values and fold changes can be found in Table 8. The corresponding dot plots can be found in Figure 14.

Table 8. Genes Up-regulated when comparing 11.568 to 2.689 µg/d PhIP groups with FDR <0.05 and fold change >1.5.

Gene Symbol	Gene name	p-value	Fold Change
ATP6V0B	ATPase, H+ transporting, lysosomal 21kDa, V0 subunit b	2.35×10^{-7}	1.53
DRAP1	Dr1-associated protein (negative cofactor 2)	8.39×10^{-7}	3.19
ZNF552	Zinc finger protein 552	2.51×10^{-6}	1.72
HCG27	HLA complex group 27 (non-protein coding)	4.04×10^{-6}	1.51
MPZ	Myelin protein zero	4.05×10^{-6}	1.66
HN1	hematological and neurological expressed 1	4.99×10^{-6}	1.79
VAC14	Vac14 homolog	2.35×10^{-5}	1.57

A single annotated gene (*MPZ*) was found to be differentially expressed when analyzing the contrasts from the ANCOVA model contrasting the 7.97 vs. 2.689 µg/d with a FDR of <0.05 and a fold change of ≥1.5. No genes were found to be down regulated. This gene was found to be up-regulated with a fold change of 2.02 and a p-value of 5.44×10^{-7}. A dot plot illustrating the expression values for the different PhIP levels can be found in Figure 14.

The contrasts conducted on the 11.568 and 7.97 µg/d vs 2.689 µg/day groups determined that seven genes (*MPZ, ZNF552, DRAP1, HCG27, HN1, LY6G5B,* and *FBX09)* were up-regulated with a FDR of < 0.05 and a fold change of ≥1.5. No genes were found to be down-regulated. Table 9 lists the gene names, identifiers, p-values and fold

change values for each gene. In addition, Figure 14 shows dot plots of each of the genes.

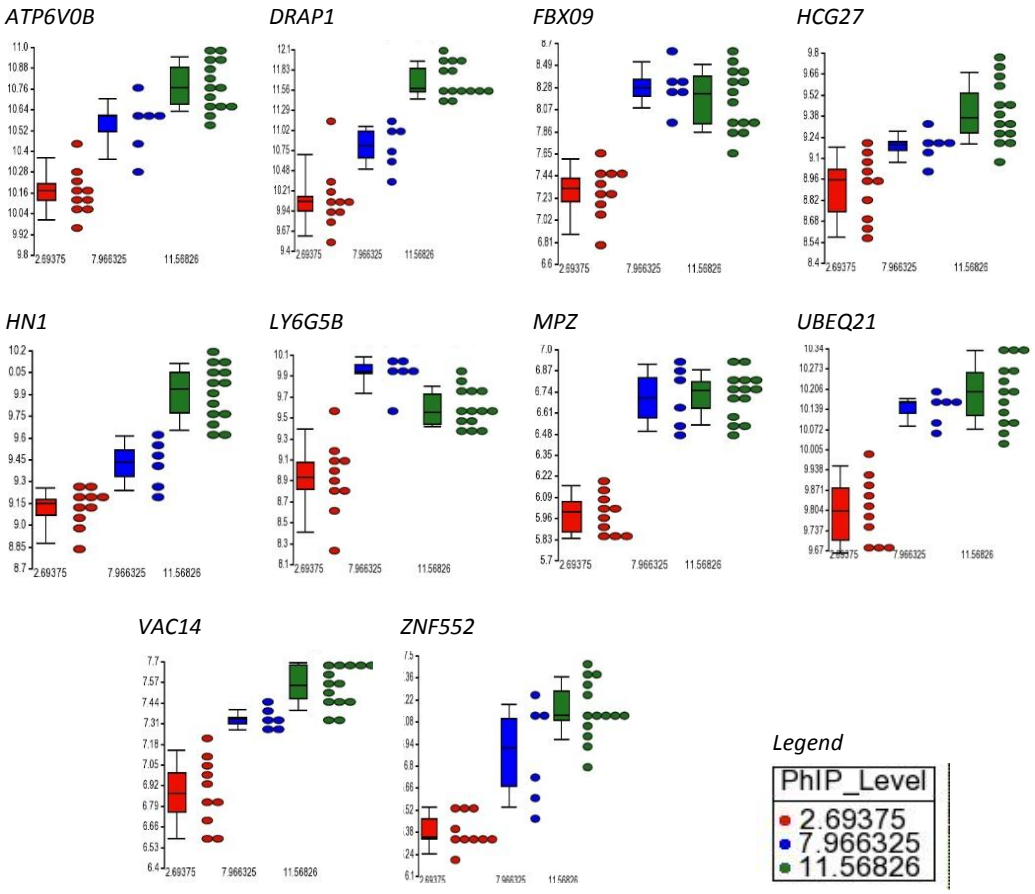

Figure 14. Dot plots for each gene differentially expressed when examining PhIP levels in the analysis. Each dot represents one sample. The x- axis represents the different PhIP levels. The y-axis represents the log2 normalized intensity of the gene.

Table 9. Genes Up-regulated when comparing 11.568 & 7.97 to 2.689 µg/d PhIP groups with FDR <0.05 and fold change >1.5.

Gene Official Symbol	Gene Official name	p-value	FC
MPZ	Myelin protein zero	7.83×10^{-7}	1.67
ZNF552	Zinc finger protein 552	2.51×10^{-6}	1.62
DRAP1	Dr1-associated protein (negative cofactor 2)	6.53×10^{-6}	2.42
HCG27	HLA complex group 27 (non-protein coding)	1.94×10^{-5}	1.68
HN1	hematological and neurological expressed 1	4.69×10^{-5}	1.52
LY6G5B	lymphocyte antigen 6 complex	8.91×10^{-5}	1.88
FBX09	F-box protein 9	9.13×10^{-5}	1.90

Genes differentially expressed When Examining Ethnicity

Upon further examination of the data, a single annotated gene (*UTS2)* was found to be differentially expressed in the ANCOVA model in the contrasts between Asian vs Caucasian, Asian vs Caucasian and Mixed/Other groups and Caucasian vs Asian and Mixed/Other groups with a FDR of <0.05 and a fold change of ±1.5. Urotensin 2 (*UTS2*) was up-regulated in the Asian population for all of the contrasts. A list of

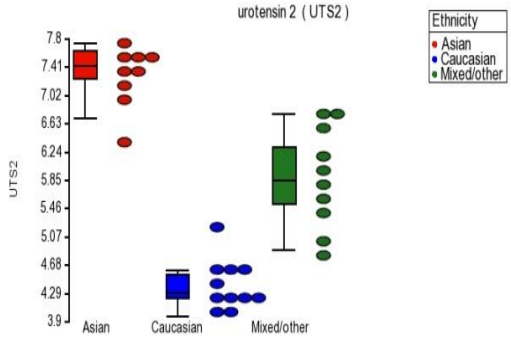

Figure 15. Dot plot illustrating the differential expression of Urotensin2 (*UTS2*) when comparing ethnicities. Each dot respresents a single sample. The Y-axis represents the log2normalized intensity of the *UTS2* gene. The X-axis represents the different ethnicities.

p-values and fold change values for each of the contrasts can be found in Table 10. A list of the analyses with the corresponding p-values for *UTS2* can be found in Table 11. In the pre-feeding only analysis *UTS2* did not pass multiple test correction. However, there was a trend towards differential expression with an unadjusted p-value of 3.17×10^{-6} and a fold change of 7.81.

Table 10. p-values and fold changes for UTS2 when contrasting different ethnicities in response to varying PhIP levels in the post-feeding only sample analysis

Contrast	p-value	Fold Change
Asian vs Caucasian	6.56938e-008	+ 8.40259
Asian vs Caucasian and Mixed/Other	3.15464e-007	+ 5.37226
Caucasian vs Asian and Mixed/Other	6.48005e-007	-4.5338

Table 11. p-values for differential expression of UTS2 in each of the analyses where ethnicity was included in the ANOVA/ANCOVA model *Pre-feeding only sample is unadjusted p-value before multiple testing parameter.

Analysis	p-value
All Samples	2.7366e-014
All Samples with PhIP*Ethnicity interaction	6.36788e-013
All Pre-feeding Samples vs 2.69 µg/d Post-feeding Samples	6.69651e-008
All Pre-feeding Samples vs 11.568 µg/d Post-feeding Samples	1.53893e-010
All Pre-feeding Samples vs 7.97 and 11.568 µg/d Post-feeding Samples	1.55599e-012
All Pre-feeding Samples and 2.69 µg/d vs 7.97 and 11.568 µg/d Post-feeding Samples	2.12104e-015
Post-feeding Only Samples	3.15464e-007
Pre-feeding Only Samples	3.17 e -006*

DISCUSSION

The present study investigated the effects of well-done meat of varying PhIP concentrations on peripheral whole blood gene expression. PhIP has been identified as a suspected carcinogen and mutagen, so elucidating genes/pathways affected by its consumption may contribute to the understanding of its pathogenic behavior. It was expected that there would be differential gene expression between the pre-feeding and post-feeding samples, and also between the different PhIP group levels. No genes were found to be differentially expressed between the pre-feeding and post-feeding samples. Seven genes (*HCG27, UBE2Q1, HN1, ZNF552, DRAP1, MPZ, and ATP6V0B*) were discovered to be upregulated when comparing the three PhIP levels at one time. Seven genes (*HCG27, VAC14, HN1, ZNF552, DRAP1, MPZ, and ATP6V0B*) were upregulated when contrasting the 11.568 µg/d to the 2.689 µg/d groups. Seven genes (*LY6G5B, HCG27, FBX09, HN1, ZNF552, DRAP1,* and *MPZ*) were up-regulated when contrasting the 11.568 and 7.97 µg/day groups to the 2.689 µg/d groups. In addition, one gene (*UTS2*) was found to be expressed 8.45 fold more, on the average, in Asian subjects than in Caucasian subjects both prior to and after the intervention.

Table 12. Genes Up-regulated in response to the varying levels of PhIP.

Gene Symbol	Name	All PhIP levels p-value	11.568 vs 2.689 µg/d p-value [Fold change]	11.568 & 7.97 vs 2.689 µg/d p-value [Fold change]
ATP6V0B	ATPase, H+ transporting, lysosomal 21kDa, V0 subunit b	5.81×10^{-7}	2.35×10^{-7} [1.53]	N/A
MPZ	Myelin Protein Zero	3.63×10^{-6}	4.05×10^{-6} [1.66]	7.83×10^{-7} [1.67]
DRAP1	Dr1-associated protein (negative cofactor 2 alpha)	3.91×10^{-6}	8.39×10^{-7} [3.19]	6.53×10^{-6} [2.42]
ZNF552	Zinc Finger 552	7.34×10^{-6}	2.51×10^{-6} [1.72]	2.51×10^{-6} [1.62]
HCG27	HLA complex group 27 (non-protein coding)	1.48×10^{-5}	4.04×10^{-6} [1.51]	1.94×10^{-5}[1.68]
HN1	hematological and neurological expressed 1	2.19×10^{-5}	4.99×10^{-6} [1.79]	4.69×10^{-5} [1.52]
UBE2Q1	ubiquitin-conjugating enzyme E2Q family member 1	2.36×10^{-5}	N/A	N/A
VAC14	Vac14 homolog	N/A	2.35×10^{-5} [1.57]	N/A
LY6G5B	lymphocyte antigen 6 complex	N/A	N/A	8.91×10^{-5} [1.88]
FBX09	F-box protein 9	N/A	N/A	9.13×10^{-5} [1.90]

It was hypothesized that the main effect of feeding well-done meat would be to produce differential gene expression in the metabolic genes previously associated with PhIP and heterocyclic amines and genes involved in the progression of normal cells to malignancy. However, when the stringent multiple testing correction parameters were applied to the ANOVA, no genes were found to be differentially expressed. Changes in metabolic genes may occur as a result of post-transcriptional or post-translational modifications; therefore, these changes may not be detected in a transcriptomic analysis. In addition, I believe that the heterogeneity of the diets at baseline and differences in gene expression among the participants may have overshadowed any subtle changes that may have occurred as a result of feeding well-done meat. At the conclusion of the feeding, the diet of all participants had been partially standardized, since all ate well-done meat added to a standardized meal. This may have resulted in reduced background variation of gene

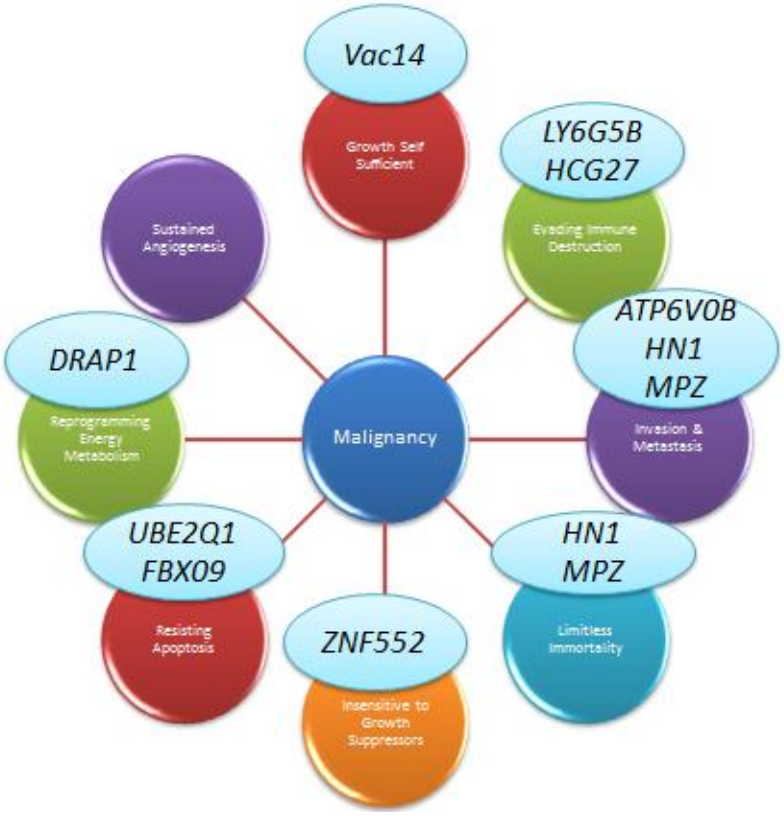

Figure 16. Illustration depicting the Hallmarks of Cancer as described by Hannahan & Weinberg in the circles with the genes differentially expressed between PhIP levels in the ovals attached to their respective hallmarks.

expression, and may have helped detect statistically significant differential gene expression observed across PhIP dose groups in the present study, even after the multiple testing correction parameters were applied.

Consumption of and preference for well-done red meat and heterocyclic amines has been associated with several types of cancers (Le Marchand, Hankin et al. 2002; Cross, Peters et al. 2005). The present study may have identified genes differentially expressed that may be linked to early alterations in homeostasis that may occur as a result of consumption of well-done meat. Acquisition of cancer has been hypothesized to be a multistep process requiring the malfunction of several different regulatory mechanisms. Hanahan and Weinberg have recently identified eight "Hallmarks of Cancer" acquired by normal functioning cells on their path to tumorigenicity and subsequently malignancy (Hanahan and Weinberg 2011). All ten of the genes differentially expressed between varying PhIP levels in the present study may be associated with seven out of eight of these hallmarks: altered growth signals, altered immune function, invasion and metastasis, , evading cell death, evading growth suppressors and reprogramming energy metabolism.

One hallmark of malignant cells is altered growth signals leading to self-sufficiency in growth. In the present study *Vac14* expression was found to be altered between varying PhIP levels. *Vac14* produces the protein VAC14 homolog which is a HEAT repeat protein that functions as a scaffold in the multi-protein complex with PIKfyve/FAB1/PIP5K3 and FIG4/SAC3 (PIKfyve/VAC14/FIG4) (Zhang, McCartney et al.

2012). This complex regulates the biosynthesis and turnover of the phosphoinositide (PI) PI(3,5)P2 (Zhang, McCartney et al. 2012). Decreases and increases in the amount of VAC14 protein results in a decreases and increases in PI (3,5)P2 respectively (Bonangelino, Nau et al. 2002; Zhang, McCartney et al. 2012). Jones et al hypothesized that PI (3,5)P2 may be an intracellular signaling molecule involved in controlling responses to stress (Jones, Gonzalez-Garcıa et al. 1999). Levels of PI (3,5)P2 increased via an IL-2 mitogenic pathway in response to stress in lymphocytes (Jones, Gonzalez-Garcıa et al. 1999). Therefore, *Vac14* up-regulation may lead to an increase in mitogenic signaling.

Immune function may be altered with consumption of well-done meat by the differential gene expression of *LY6G5B* and *HCG27. LY6G5B* and *HCG27* are clustered together on the 21st region of the short arm of chromosome 6. Lymphocyte antigen 6 complex, locus G5B (*LY6G5B*) and Human leukocyte antigen complex group 27 (*HCG27*) are part of the major histocompatibility complex (MHC) class region of chromosome 6. *HCG27* is identified as a non-protein coding gene. However, SNP variants in its chromosomal location (6p21.33) have been associated with increased risk of lung cancer in two genome wide association studies (Broderick, Wang et al. 2009), as well as with hepatitis C virus induced hepatocellular carcinoma(Kumar, Kato et al. 2011), and susceptibility to follicular lymphoma (Skibola, Bracci et al. 2009) .

LY6G5B is a member of the lymphocyte antigen-6 (Ly-6) family of cysteine rich proteins located at 6p21.3 within the MHC class III region (Mallya, Campbell et al.).

MHC class III Ly-6 proteins are suspected to play a role in immune function. *LY6G5B* , in particular, is a secreted protein suspected to be involved in cell-cell interactions as well as in cell signaling (Mallya, Campbell et al.). Though *LY6G5B* has not been characterized, other Ly-6 genes have been identified as markers for T-cell differentiation and breast cancer as well as increased expression in prostate and stomach cancers (Horie, Okutomi et al. 1998; Lee, Lee et al. 2006; Lu, Chen et al. 2010).

ATP6VOB and *HN1* may be associated with invasion and metastasis. The *ATP6V0B* gene was up-regulated in the expression analysis 1.53 fold ($p=2.35 \times 10^{-7}$). *ATP6V0B* encodes the B subunit, V0 sub-complex of a vacuolar ATPase (v-ATPase) that serves as a proton pump to mediate the acidification of various intracellular organelles (Ma, Xiang et al. 2011). This enzyme has two major subunits, A and B. The B subunit is suspected to serve as the regulatory unit while the A subunit is thought to have catalytic function (Ma, Xiang et al. 2011) . The V0 sub-complex is embedded in the membrane and includes the proton translocation pore that transports protons while the V1 sub-complex binds and hydrolyzes ATP. It has been suggested that v-ATPases play a role in the acidic microenvironment of tumors leading to the development and metastasis of cancer (Ma, Xiang et al. 2011).

Similarly, Hematological and neurological expressed 1 (*HN1*) gene produces the HN1 protein. Increased expression of *HN1* has been associated with metastatic cancer progression (Varisli, Gonen-Korkmaz et al. 2011). In addition when comparing normal tissues to lung adenocarcinoma in two independent studies, bladder urothelial

carcinoma, ductal breast carcinoma, pancreatic carcinoma, skin squamous cell carcinoma, prostatic intraepithelial neoplasia, and ovarian serous and endometroid adenocarcinoma, *HN1* expression was up-regulated in the malignant tissues (Varisli, Gonen-Korkmaz et al. 2011). Increased expression of *HN1* has been associated with decreased AKT signaling which in turn increases the stabilization of β-catenin (Varisli, Gonen-Korkmaz et al. 2011). β-catenin has been associated with invasion and metastasis (Hiendlmeyer, Regus et al. 2004).

In the same way, the altered gene expression of *MPZ* may be associated with invasion and metastasis. The *myelin protein zero (MPZ)* gene encodes the myelin protein zero protein and was up-regulated 2.2 fold ($p= 5.44 \times 10^{-7}$) in the present study. Myelin protein zero, considered an adhesion molecule, is the most abundant protein in myelin. Recently it has been shown that the Wnt/ β-catenin pathway is not only essential but is also a direct driver in the increase in the gene expression of the *MPZ* gene (Tawk, Makoukji et al. 2011). Because β-catenin is associated with invasion and metastasis, up-regulated expression of *MPZ* may be associated with up-regulation of β-catenin leading to invasion and metastasis as well.

HN1 and *MPZ* up-regulation may lead to an increase in β-catenin. Jaitner et al has recently demonstrated that β-catenin may regulate the expression of *c-myc* (Jaitner, Reiche et al. 2012). *c-myc* acts as a transcription factor regulating the expression of *hTERT (human telomerase reverse transcriptase)* gene (Jaitner, Reiche et al. 2012). This gene product plays a critical role in lengthening of the telomeres that may result in

replicative immortality which is another hallmark of cancer acquisition (Hanahan and Weinberg 2011). Therefore, *HN1* and *MPZ* up-regulation may also play a role in replicative immortality.

Evading growth suppressors may be occurring in the present study with the up-regulation of the transcriptional regulator, *Zinc finger protein 552* (*ZNF552*). Zinc finger proteins, in general, function during cell differentiation and development with the binding of transcription factors to their DNA recognition site where they can either up-regulate or suppress gene expression (Deng, Liu et al. 2012). *ZNF552* is a member of the Kruppel-like protein family that has two or more C2H2-type zinc-fingers with a Kruppel-associated box (KRAB) which functions as a regulatory domain (Deng, Liu et al. 2012). Upregulation of expression of *ZNF552* has been associated with the repression of the transcriptional activities of AP1 and SRE. This has led to the suggestion that *ZNF552* may function as a negative regulator of the MAPK signaling pathway (Deng, Liu et al. 2012). MAPK signaling pathways function in times of stress in the cell in regulation of cell survival and repopulation. PhIP has been associated, in a previous study, with decreased expression of TNF-α and down regulation of the MAPK/ERK1 signaling pathway which are both associated with tumor suppression (Im, Choi et al. 2009).

Genes that alter normal ubiquitination functioning have been associated with altering normal apoptosis. Ubiquitination is the process of attaching ubiquitin molecules to a protein. Under many circumstances, this attachment tags the protein for degradation. The ubiquitination system has been associated with the regulation of

apoptosis, cell cycle progression, signal transduction and DNA repair mechanisms. Two genes in the present study are associated with ubiquitination: *UBE2Q1* and *FBOX09.* Ubiquitin-conjugating enzyme E2Q family member 1 encodes for an E2 family ubiquitin-conjugating enzyme with the ubiquitin conjugate/RWD-like domain located at the N-terminal and at the C terminal a ubiquitin conjugating E2 domain (Seghatoleslam, Nikseresht et al. 2012). The RWD domain is a RING finger containing domain, a WD-repeat containing proteins and yeast DEAD (DEXD)-like helicases which has been suggested to be a recognition domain for protein-protein interactions (Seghatoleslam, Nikseresht et al. 2012). *UBE2Q1* mRNA was found to be overexpressed in approximately 61.5 % of human breast tissue when compared to normal tissue while protein was overexpressed in 61.9% of malignant breast tissue (Seghatoleslam, Nikseresht et al. 2012). *UBE2Q1* frameshift mutations and microsatellite instability has also recently been associated with gastric and colorectal adenomas (Yoo, Park et al. 2011).

FBOX09 is an uncharacterized member of the F-box family of proteins which is characterized by the "F-box" or a 40 amino acid motif. Characterized F-box proteins make up one of the proteins in an ubiquitin protein ligase complex known as SCFs (SKP1-Cullin-F-box). The SCF complex functions in phosphorylation-dependent ubiquitination. Proper formation and function of this complex is critical in cell cycle regulation providing unidirectional control of cell cycle progression (Burrows, Prokop et al. 2012).

Reprogramming the cell's energy metabolism has been identified as a hallmark of cancer (Hanahan and Weinberg 2011). This alteration becomes critical for the survival

of the tumor under hypoxic conditions (Hanahan and Weinberg 2011). *DR1-associated protein 1* (*DRAP1*), also known as negative cofactor 2 alpha, produces the DR1 associated protein that works to enhance the transcriptional repression ability of DR1. The DR1-DRAP1 complex, also referred to as the NC2 complex, inhibit the entry of transcription factors into the preinitiation complex by interacting with the TATA binding protein which results in repression of RNA polymerase II transcription initiation (Yeung, Kim et al. 1997). Barton, et al found DR1-DRAP1 complex was up-regulated in a hypoxic state allowing hepatoma cells to conserve energy (Denko, Wernke-Dollries et al. 2003). Therefore, the up-regulation of *DRAP-1* may be associated with reprogramming the cell's energy metabolism.

A summary of the genes, literature, hypothesized functions and associations with the hallmarks of cancer found to be up-regulated differentially between well-done meat of varying PhIP concentration is located in Table 13. Each gene up-regulated may be associated with the small changes that may occur on the pathway from normal cells to malignancy. It is also important to note, however, that these hallmarks are found downstream of the differentially expressed genes. A normal functioning cell may be able to repair the effects of differential expression. However, over time and with chronic exposure the changes may lead to the acquisition of the varying hallmarks resulting in cancer.

In addition to genes differentially expressed between varying PhIP levels, there was also a gene up-regulated in the Asian group when compared to Caucasian up to

8.45 fold. Increased expression of *Urotensin 2 (UTS2)* has been associated with cardiovascular disease, as well as diabetes (Suguro, Watanabe et al. 2008; Sáez, Smani et al. 2011). In addition, it has been identified as an angiogenic factor (Guidolin, Albertin et al. 2010). Sustained angiogenesis is a hallmark of cancer. No studies to date have been identified that have found this differential expression between ethnicities. This up-regulation may have important implications for Asian population in regards to the susceptibility of development of disease and should be further explored.

Table 13. Summary of genes up-regulated in response to varying PhIP levels, cited literature and hypothesized function and hallmark of cancer associations

Gene Symbol	Literature	Hypothesized Function	Hallmark Association
VAC14	(Jones, Gonzalez-Garcıa et al. 1999; Zhang, McCartney et al. 2012)	Increased production of PI (3,5) P2 via up-regulation of mitogenic pathway	Altered growth signals
HCG27	(Broderick, Wang et al. 2009; Skibola, Bracci et al. 2009; Kumar, Kato et al. 2011)	Non-coding gene in the MHC region with SNP cancer associations	Altered immune function
LY6G5B	(Horie, Okutomi et al. 1998; Mallya, Campbell et al. 2006)	Codes for Ly-6 cell surface molecule involved in cell signaling and T-cell differentiation	Altered immune function
ATP6V0B	(Ma, Xiang et al. 2011)	Creation of an acidic microenvironment leading to metastatic environment	Invasion & metastasis
HN1	(Hiendlmeyer, Regus et al. 2004; Varisli, Gonen-Korkmaz et al. 2011)	Decreases AKT signaling leading to increased β-catenin which results increased invasion and metastasis as well as *hTERT* expression	Invasion & metastasis; replicative immortality
MPZ	(Tawk, Makoukji et al. 2011)	Direct product of Wnt/ β-catenin pathway indicating increase in β-catenin	Invasion & metastasis; replicative immortality
UBE2Q1	(Yoo, Park et al. 2011; Seghatoleslam, Nikseresht et al. 2012)	Alters the ubiquitination pathway	Evasion of apoptosis
FBX09	(Burrows, Prokop et al. 2012)	Involved in phosphorylation dependent ubiquitination through the SCF complex	Evasion of apoptosis
ZNF552	(Im, Choi et al. 2009; Deng, Liu et al. 2012)	Transcriptional repression; Decreases AP-1 & SRE leading to negative regulation of MAPK pathway	Altered growth suppression
DRAP1	(Yeung, Kim et al. 1997; Denko, Wernke-Dollries et al. 2003)	Transcriptional repression allowing the cell to reprogram metabolism in hypoxic conditions	Reprogramming the cell's metabolism

Limitations

Microarray gene expression analysis presents with challenges and limitations. One of the major challenges is the statistical analysis of the plethora of data generated from analysis of 22,000 genes concurrently. When performing statistical operations of microarray analyses, many hypotheses are being tested simultaneously when attempting to identify which genes may be associated with the intervention. This multiplicity introduces a greater possibility of Type I errors. Benjamini -Hochberg correction method attempts to limit the amount of false positives which occur as a result of the multiplicity problems. In addition, microarray experiments often have the problem in the inability to replicate results. Another independent study with identical interventions needs to be performed in order to remedy the replication problem. Technical validation of the results using qPCR should also be performed. Another limitation of gene expression analysis is that evaluation is based on mRNA transcripts. Post-trancriptional and post-translational modifications and events may further influence the actual protein products and the occurring cellular processes. A proteomic analysis is suggested for future studies.

Though peripheral whole blood is readily available and minimally invasive to obtain, it also presents with certain limitations when examining global gene expression through transcript analysis. mRNA transcripts may vary greatly in proportion to various blood cell components. Isolation of the different types of lymphocytes and measuring gene expression only on the same types of lymphocytes may help eliminate this bias.

Further limitations are present in the current study. First, the short duration of the study may not have been long enough to adequately show changes that occur as a result of chronic exposure. Also, the sample size was relatively small. In addition, changes occurring in the blood may have been better assessed post-prandially. Some changes may have already been occurring in the tissue since blood was not drawn until the day after the last feeding of well-done meat.

Conclusions & Future studies

In conclusion, differential gene expression was observed in the peripheral whole blood of subjects who had been fed 2.689 µg/d, 7.966 µg/d, and 11.568 µg/day of PhIP. These genes may be the connection between well-done red meat consumption and the early alterations in cell function that occurs in normal cells in their progression to cancerous cells. Chronic consumption of well-done meat may lead to further dysregulation of these pathways and eventually result in carcinogenesis.

Future studies need to be conducted to further explore the connection between these genes and their relationship with disease development. In addition, a similar feeding study where proteomic methods are employed to detect what proteins are being produced may further elucidate pathways affected by the consumption of well-done meat. Lastly, a study where blood is collected at specific intervals within hours following the consumption of well-done meat may be helpful in measuring more genes affected by consumption. In addition, more studies should be conducted to further explore the up-regulation of expression of *UTS2* in Asians when compared to Caucasians since *UTS2* expressions may have important health consequences.

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