Integrating Biomarkers into Translational Research on Diet and Cancer

BY

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THESIS

Submitted as partial fulfillment of requirements
for the degree of Doctor of Philosophy in Pathology
in the Graduate College of the
University of Illinois at Chicago, 2015

Chicago, Illinois

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This thesis is dedicated to my mom, Manju Kataria. Without your continuous support, dedication, hard work, sacrifices, and love this would not have been possible. Love you mom!

ACKNOWLEDGEMENTS

First and foremost I want to thank my advisor, my academic father, Dr. Peter Gann for fostering my scholarship and intellectual growth. Furthermore, for instilling principals of being a good scientist and always making me think of the bigger picture. You have challenged me scientifically in countless ways that I didn't think were possible. I am a better scientist because of you. You have allowed me to pursue my own ideas and more importantly to learn from my own mistakes. One of my fondest memories with you as when the CV's for my PCR experiments were unexpectedly high. These were my precious LCM and I had thought of everything possible. Upon approaching you, you had me to walk you through my protocol; from picking up the pipette to running the plate. While explaining each exact step, I had quickly realized my mistake and was then able to fix it. I had an "Aha" moment – a moment that I still remember on my bad days. Thank you for always making time for me, even when you were busy. You've not only let me grow as a scientist but also as a person. It has been a great honor to be your first PhD student.

I would also like to thank rest of the Gann lab, Ryan Deaton, Peter Nugyen, and Erika Enk – my second family. You have been there on my worst and my best days. I cannot ask for a better support group. Ryan you have been my second advisor in many ways. Thank you for the countless times you've helped me sculpt and refine my projects. Also for always putting up with me, especially with my impatience. Peter has always been a helping hand and has always gone out of his way to help with all of my projects. I don't know what I would do with you. Erika, you have taught me so much about clinical research. Your optimism and generosity is contagious.

Dr. Bosland, thank you for always going above and beyond to help me achieve my goals. Also for always helping me figure out alternatives when I faced roadblocks – there were many of those. Your dedication to help others is always going to motivate me to do the same.

I would also like to thank the rest of my committee, Drs. Balla, Walden and Fantuzzi, for their generous support and guidance. Dr. Balla you have been a pleasure to work with. You always offer a different perspective and ask instrumental questions. Dr. Walden and Fantuzzi thank you for assisting in the development of scientific ideas presented in my thesis.

ACKNOWLEDGEMENTS (continued)

CONTRIBUTIONS OF AUTHORS

Chapter 1 is a broad introduction about diet and cancer and sets the stage for subsequent chapters.

Chapter 2 represents a published manuscript (**Kataria, Y**., Wright, M., Deaton, R.J., Rueter, E.E., Rybicki, B.A., Moser, A.B., Ananthanrayanan, V., and Gann, P.H. (2015). Dietary influences on tissue concentrations of phytanic acid and AMACR expression in the benign human prostate. *The Prostate.*) for which I am the primary author and completed the main components of the study. Drs. Gann, Wright, and Dr. Anantharyanan asked the original questions. Dr. Gann also assisted in shaping the manuscript. Dr. Wright helped conduct serum phytanic acid measurements, ensure subject enrollment, and find collaborators in Detroit. Ryan Deaton assisted with data analysis. Erika Enk, the study coordinator was in charge of patient recruitment and sample processing. Dr. Rybicki provided samples from Henry Ford Health systems. **Chapter 3** represents a cross-sectional study studying the retinoid and carotenoid in patients at high risk for liver cancer. This study is currently under review for publication. Ryan Deaton assisted with data analysis. Dr. Jin helped with the serum clinical chemistry assays. Drs. Dong and van Breemen quantitated retinoid and carotenoid concentrations in the biospecimens. Dr. Jensen and Cotler were the study hepatologist at UIC and University of Chicago, respectively. Joseph Goldenberg quantitated serum RBP4 measurements. Erika Enk, the study coordinator was in charge of patient recruitment and sample processing.   **Chapter 4** is a extension of chapter three and assessed the joint relationship between retinoids and iron in patients at high risk for liver cancer. I anticipate this study will continue to be further investigated after my departure.

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LIST OF ABBREVIATIONS

PCa	Prostate Cancer
AMACR	Alpha-methylacyl-CoA Racemase
SCP2	Sterol Carrier protein 2
CAT	Catalase
DBP	D-bifunctional Protein
RXRα	Retinoid X Receptor-alpha
PPARα	Peroxisome Proliferator-activated receptor-alpha
UIC	University of Illinois Hospital at Chicago
JBVAMC	Jesse Brown Veterans Affairs Medical Center
FFPE	Formalin-fixed paraffin-embedded
HFHS	Henry Ford Health Systems
LCM	Laser Capture Microdissection
FFQ	Food Frequency Questionnaire
CV	Coefficients of Variation
DAB	Diaminobenzidine
HCV	Hepatitis C
HCC	Hepatocellular carcinoma
ROS	Reactive oxygen species
DHQ	Diet History Questionnaire
NAFLD	Non-alcoholic fatty liver disease
CLD	Chronic liver disease
RBP4	Serum retinol binding protein 4
αSMA	Alpha-Smooth muscle actin
HSCs	Hepatic stellate cells
RAE	Retinol activity equivalents
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase

LIST OF ABBREVIATIONS (continued)

hs-CRP   High sensitivity C-reactive protein

APRI   Aminotransferase to platelet ratio index

FIB-4   Fibrosis-4

UHPLC-MS/MS Ultrahigh pressure liquid chromatography-tandem mass spectrometry

NASH   Non-alcoholic steatohepatitis

SUMMARY

This thesis integrates data from bench and bedside to help clarify some aspect of the complex relationship between diet and cancer. We elucidated dietary factors that contribute to or protect against prostate and liver cancer, and their relationship with underlying pathophysiologic mechanisms.

The first project evaluated the possible link between AMACR intake from dairy or red meat and its expression in the prostate. Alpha-methylacyl-CoA racemase (AMACR) is an enzyme involved in fatty acid metabolism that is markedly over-expressed in virtually all prostate cancers (PCa), relative to benign tissue. One of AMACR's primary substrates, phytanic acid, is obtained predominately from red meat and dairy product consumption. Epidemiological evidence suggests links between dairy/red meat intake, as well as phytanic acid levels, and elevated PCa risk. Specifically, this study investigated the relationships between dietary intake and serum and tissue concentrations of phytanic acid and AMACR expression (mRNA and protein) in the histologically benign human prostate. Our data could not support the hypothesis that excess levels of dietary phytanic acid are responsible for both the overexpression of AMACR in prostate cancer and the potential association between PCa risk and intake of dairy foods and red meat.

The second project evaluated retinoid and carotenoid status in serum and liver tissue in patients at high-risk for liver cancer. Increased oxidative stress is regarded as a major mechanism of hepatitis C virus (HCV) -related liver disease progression. Deficiencies in retinoid and carotenoid antioxidants may represent a major modifiable risk factor for liver cancer progression. The aims of this study were to identify key predictors of serum antioxidant levels in patients with HCV, to examine the relationship between retinoid/carotenoid concentrations in serum and hepatic tissue, to quantify the association between systemic measures of oxidative stress and antioxidant status, and to examine the relationship between retinoids and stellate cell activation. We observed a decrease in serum retinol, β-carotene, and retinol binding protein 4 which is associated with early stage HCV infection. Furthermore, retinoid and carotenoid levels declined as disease progressed and our data suggest that this decline occurs early in the disease process, even before fibrosis is apparent. Measures of oxidative stress were associated with fibrosis stage and concurrent with antioxidant depletion. Vitamin A loss was accompanied by stellate cell activation in hepatic tissue.

SUMMARY (continued)

The last project examined the joint relationship between iron and vitamin A in patients at high-risk for liver cancer. This study aimed to quantify the correlation between iron and retinoid concentrations in CLD and to examine the joint effects of retinoids and iron in CLD. We observed an increase in serum iron biomarkers in CLD patients compared to controls. Serum retinoids were inversely associated with serum iron biomarkers. Overall, HCV infection was generally associated with serum antioxidant levels but also higher ferritin concentrations; however, we did not observe a relationship with fibrosis stage.

Chapter 1: Introduction

In 1950, Richard Doll and Bradford Hill published a groundbreaking paper, which indisputably linked smoking to lung cancer.(1) This suggested that individuals could alter their cancer risk through lifestyle modification. Over the next several decades the findings of this study have translated into changes in the tobacco industry and health policy to revolutionize public health. The role of diet in carcinogenesis was only investigated decades later. In 1981, Doll and Peto published a landmark report on the causes of cancer; estimating that 30 – 35% of cancer mortalities in the United States were attributable to diet.(2) This report resulted in efforts to elucidate the roles of diet, nutrition, anthropometry, and physical activity in cancer risk. Lifestyle and diet, in principle, offer an opportunity to prevent cancer in high-risk populations. More recently, the World Cancer Research Fund estimated that diet, nutrition and physical activity still account for 24% of all cancers in the United States.(3)

Global differences in cancer rates and migration studies suggest the importance of lifestyle and environment in cancer pathogenesis. Cancer rates among populations migrating from low to high incident countries change significantly, adopting the risk ratios of the new host country within the first few generations. For example, prostate cancer incidence increased almost four fold in Japanese men who migrated to the United States.(4)

Dietary constituents, additives, toxin contamination, processing and preparation of food have all been implicated to modify the risk of many cancer types including prostate and liver cancer. Multiple lines of evidence suggest that diet can influence any of the following mechanisms: proliferation, hormonal regulation, all differentiation, inflammation, immunity, apoptosis, cell cycle, carcinogen metabolism, and DNA repair. Mechanistic links between dietary exposure and cancer risks have rarely been deciphered. Aflatoxin is an exception to this that provides an example of a link between diet and cancer.(5) Aflatoxin is a naturally occurring toxin produced by mold, and is a source of contamination in grains. Aflatoxin exposure occurs primarily through the ingestion of contaminated foods and is a known cause of liver cancer.(5) Contamination largely occurs in hot and humid countries with inadequate storage facilities.

Aflatoxin metabolism leads to reactive metabolites that covalently bind to DNA, which causes mutations in the p53 gene, a tumor suppressor gene. This mutation affects cell cycle progression and apoptosis.(6)

The study of the relationship between diet and cancer is challenging, as diet is a highly complex mixture with highly correlated elements. Determination of a critical window of exposure is also important, as adolescent and pre-natal exposure can be crucial for cancer pathogenesis. Moreover, research conducted in a laboratory setting is not always translatable to human clinical research. Often individual dietary constituents are examined *in vitro*; isolating those constituents for human consumption. These isolated compounds may be toxic at high doses and comparing these studies to complex whole foods is of limited value. Moreover, micronutrients that are given as isolated supplements as opposed to components of a whole diet may have unexpected outcomes. Both the CARET trial and Alpha-Tocopherol, Beta-Carotene cancer prevention trial reported increased incidence of lung cancer for high dose beta-carotene supplementation. Additionally, an individual's past dietary exposures cannot be accurately measured.(7, 8) Diet is commonly measured by 24-hour recalls, food frequency questionnaires (FFQ), and by maintaining a food diary. In a 24-hour dietary recall, detailed reports are generated about dietary consumption over the previous 24 hours. Most epidemiological studies utilize the FFQ for its ease of use. In the FFQ, participants report their frequency of intake and portion size typically over the past year. Food diaries require participants to record what they eat over a certain period of time. The 24 hour recall is a major alternative, and when well conducted is detailed and rich in data about nutrients, cooking practices, and eating frequency. However, all dietary assessments are prone to measurement error as they rely on self-report. This leads to misclassification of exposure and reduces the power to detect possible effects in research studies. These shortcomings emphasize the need to identify subgroups of individuals with elevated risk using alternative methods other than reported diet alone.

Observational epidemiological research aims to explain the magnitude of exposure (i.e., diet) with health and disease in human populations. Different types of epidemiological studies such as case-control, cohort studies, and randomized control let trials (RCT) each have strengths and weakness. Case-control studies are retrospective in nature generally utilizing tools such as FFQ and single time point 24-hour

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recall, whereas cohort studies and RCTs are prospective and allow researchers to gather dietary consumption data and follow subjects forward in time for disease occurrence. However, cohort studies are expensive and need to be large for adequate statistical power. Lastly, RCTs are ideal as they avoid bias, but are often financially and ethically unfeasible and frequently have no appropriate placebo control. Thus, there are limited RCT data available for evaluating the relationship between diet and cancer.

Studies supported by biomarkers of exposure and outcomes allowing smaller studies of shorter duration are needed to better understand the relationship between diet and cancer. Molecular epidemiology is of great utility as it aims to open up the black box by examining the events between exposure and disease, occurrence and progression. Biomarkers improve exposure assessment compared to an FFQ, while providing insight about disease pathogenesis. For example, selenium levels vary widely in individual foods and food composition tables are inaccurate. Serum selenium levels provide a more accurate measurement of intake levels than self-report. Appropriate quality control of biomarkers increases the validity of a study by minimizing systemic bias. We have gained a lot of insight from data gathered by many lines of evidence however, many questions remain unresolved about the role of diet in cancer. Here we elucidate dietary factors that contribute to or protect against liver and prostate cancer, and their relationship with underlying pathophysiologic mechanisms.

This thesis consists of two different studies. The first study investigated the relationships between dietary intake and serum and tissue concentrations of phytanic acid and AMACR expression (mRNA and protein) in the histologically benign human prostate. Alpha-methylacyl-CoA racemase (AMACR) is an enzyme that is markedly over-expressed in virtually all prostate cancers (PCa), relative to benign tissue. One of AMACR's primary substrates, phytanic acid, is almost exclusively obtained from red meat and dairy product consumption. Epidemiological evidence suggests links between dairy/red meat intake, as well as phytanic acid levels, and elevated PCa risk. The aim of this study was to quantify the relationships between dietary intake, serum and prostatic phytanic acid concentrations, AMACR expression (mRNA and protein) in benign prostate tissue.

The second project investigated vitamin A status in patients at high-risk for liver cancer. Deficiencies in retinoid and carotenoid antioxidants may be a modifiable risk factor for CLD progression as increased

oxidative stress is a major mechanism in progression of CLD. The aims of this study were to identify key predictors of serum antioxidant levels in patients with HCV, to examine the relationship between retinoid/carotenoid concentrations in serum and hepatic tissue, to quantify the association between systemic measures of oxidative stress and antioxidant status, and to examine the relationship between retinoid and stellate cell activation. In an extension of the second project we explored the joint relationship between iron and vitamin A in the same CLD patients. The first aim of this study was to measure the correlation between iron and vitamin A concentrations in the CLD patients. The second aim was to examine the join effect of retinoid and iron status in CLD.

With these three projects our goal is to further integrate data from bench and bedside to help clarify the complex relationships between diet and cancer. Our overarching goal was to collect supporting evidence for future chemoprevention studies in diet and cancer.

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Chapter 2 - Dietary Influences on Tissue Concentrations of Phytanic Acid and AMACR Expression in the Benign Human Prostate

ABSTRACT

Alpha-methylacyl-CoA racemase (AMACR) is an enzyme involved in fatty acid metabolism that is markedly over-expressed in virtually all prostate cancers (PCa), relative to benign tissue. One of AMACR's primary substrates, phytanic acid, is derived predominately from red meat and dairy product consumption. Epidemiological evidence suggests links between dairy/red meat intake, as well as phytanic acid levels, and elevated PCa risk. This study investigates the relationships among dietary intake, serum and tissue concentrations of phytanic acid, and AMACR expression (mRNA and protein) in the histologically benign human prostate.

Men undergoing radical prostatectomy for the treatment of localized disease provided a food frequency questionnaire (n = 68), fasting blood (n = 35), benign fresh frozen prostate tissue (n = 26), and formalin-fixed paraffin-embedded (FFPE) sections (n = 67). Serum and tissue phytanic acid concentrations were obtained by gas chromatography–mass spectrometry. We extracted RNA from epithelial cells using laser capture microdissection and quantified mRNA expression of AMACR and other genes involved in the peroxisomal phytanic acid metabolism pathway via qRT-PCR. Immunohistochemistry for AMACR was performed on FFPE sections and subsequently quantified via digital image analysis. Associations between diet, serum, and tissue phytanic acid levels, as well as AMACR and other gene expression levels were assessed by partial Spearman correlation coefficients.

High-fat dairy intake was the strongest predictor of circulating phytanic acid concentrations (r = 0.35, P = 0.04). Tissue phytanic acid concentrations were not associated with any dietary sources and were only weakly correlated with serum levels (r = 0.29, P = 0.15). AMACR gene expression was not associated with serum phytanic acid (r = 0.13, P = 0.47), prostatic phytanic acid concentrations (r = 0.03, P = 0.88), or AMACR protein expression (r = −0.16, P = 0.20).

Our data underscore the complexity of the relationship between AMACR and its substrates and do not support the unifying hypothesis that excess levels of dietary phytanic acid are responsible for both the overexpression of AMACR in prostate cancer and the potential association between PCa risk and intake of dairy foods and red meat.

INTRODUCTION

Studies of migrants and a large body of laboratory and epidemiological evidence suggest that a Westernized diet rich in animal fat and protein plays an important role in prostate carcinogenesis (1-8). The responsible dietary factors are not well established, but both case-control and cohort studies have shown associations between prostate cancer (PCa) risk and intake of red meat and dairy foods (4, 6, 7). Alpha-methylacyl-CoA racemase (AMACR) is an enzyme that is strongly overexpressed in virtually all PCa cases, with low levels present in normal prostatic tissues (9, 10). AMACR is essential in the metabolism of phytanic acid – a 20-carbon saturated branched chain fatty acid that humans obtain by consuming meat and dairy products from ruminant animals, whose gut fermentation is able to release the phytanic acid precursor phytol from chlorophyll (11). Our group has reported that in radical prostatectomy specimens, benign glands near a focus of cancer had a higher expression of AMACR than distant glands, indicating the existence of a field effect for AMACR in prostate carcinogenesis (12). These observations suggest that alterations in AMACR expression occur very early in cancer development and that they constitute a characteristic of high-risk but morphologically benign tissue (13). Addition of phytanic acid to cultured prostate cancer cells increases AMACR expression(14); therefore, we and others have hypothesized that excess levels of phytanic acid could explain the overexpression of AMACR in PCa while at the same time strengthening support for the epidemiological association of dairy and red meat intake with risk.

Dietary branched-chain fatty acids contribute to several processes that may have great relevance to prostate carcinogenesis, including oxidative stress and nuclear receptor signaling. After ingestion, phytanic acid is transported into peroxisomes via sterol carrier protein 2 (SCP2) (**Figure 1**) (15). Unlike most fatty acids, phytanic acid cannot be metabolized by β-oxidation; thus, it first undergoes removal of

one carbon by α-oxidation in peroxisomes to form pristanic acid, which is isomerized by AMACR, rapidly β-oxidized, and eventually fully broken down to CO_2 and water in mitochondria. During multiple rounds of β-oxidation, this metabolic process generates reactive oxygen species with the potential to create molecular damage (15). An inherited defect in this pathway causes Refsum disease, which results from the accumulation of branched chain fatty acids (16). Catalase (CAT), an antioxidant enzyme, is present in peroxisomes to counteract free radicals. Downstream proteins include D-bifunctional protein (DBP), an enzyme involved in peroxisomal β-oxidation. Finally, both phytanic and pristanic acid are ligands for retinoid X receptor-alpha (RXRα) and peroxisome proliferator-activated receptor-alpha (PPARα), which are transcription factors with a host of downstream effects on cellular metabolism, proliferation and apoptosis.
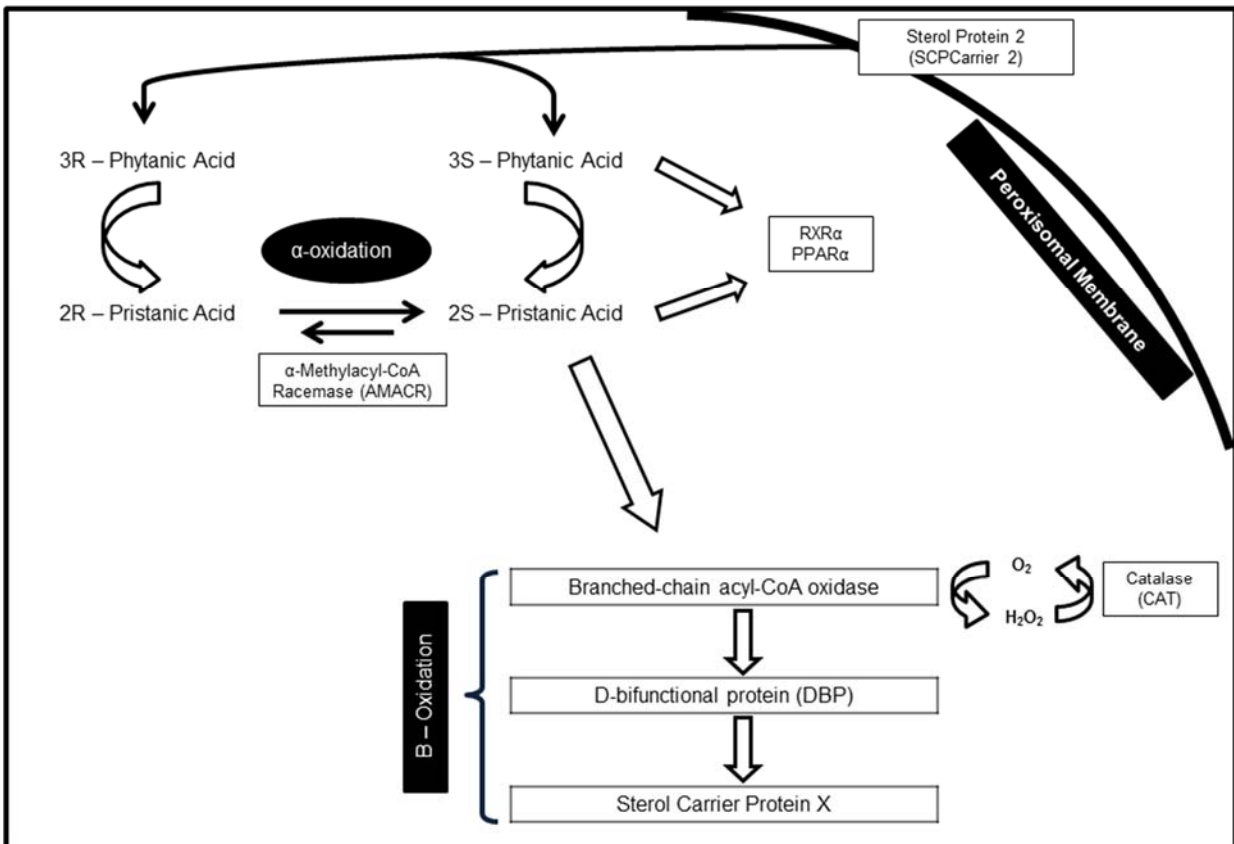
Figure 1. Role of AMACR in the metabolism of phytanic acid

It is not yet clear whether AMACR is an "innocent bystander" or whether it or its dietary substrates in red meat and dairy food lie within the causal pathway leading to the development of PCa. The primary aim of this study was to quantify relationships between dietary intake, serum and prostatic phytanic acid concentrations, mRNA and protein expression of AMACR and other genes in the phytanic acid metabolic pathway in benign prostate tissue. We postulated that men with a higher intake of ruminant animal products would have higher serum and prostatic levels of phytanic acid and consequently higher AMACR expression levels in benign prostate tissue. Pentadecanoic and heptadecanoic saturated fatty acids, which are found in ruminant animal products and have been suggested as biomarkers for dairy intake in serum and adipose tissue, were also tested as alternate measures of dairy intake and thus phytanic acid levels in the blood and tissue (17-19).

MATERIALS AND METHODS

Study population. A total of 81 men who underwent radical prostatectomy for the treatment of localized PCa were included in this study. Thirty-nine men were recruited from the University of Illinois Hospital at Chicago (UIC) or the Jesse Brown Veterans Affairs Medical Center (JBVAMC) in Chicago. A pre-surgical research visit was completed to obtain dietary and lifestyle data, medical history and a fasting blood sample. Immediately after surgery, a pathologist obtained fresh samples of benign tissue distant from tumor foci during gross examination of the prostatectomy specimens. Complete diet history, formalin-fixed paraffin-embedded (FFPE) blocks were obtained from 39 Chicago subjects, and fresh frozen benign prostate tissue was available for 26 Chicago subjects.

Existing fresh frozen prostate tissue, FFPE sections, and medical, lifestyle, and diet data from 42 men enrolled in a previous study at Henry Ford Health Systems (HFHS) in Detroit were also included. These men provided fresh frozen prostate tissue and medical/lifestyle and diet history questionnaires; however, fasting blood samples were not available. Specimens and data were combined across all three study sites. Patients receiving exogenous hormones, or neoadjuvant anti-hormone therapy were excluded. The UIC, JBVAMC, and HFHS institutional review boards approved the study.

Laser capture microdissection (LCM) and RNA isolation. Laser capture microdissection (LCM) was utilized to quantify mRNA expression in frozen prostate tissue as described by Nonn et al (20). Hematoxylin and eosin stained slides were reviewed by a study pathologist to determine areas of benign glandular tissue from the peripheral zone of the prostate. Four 8μ frozen sections from each patient were cut and placed on RNAse-free polyethylene naphthalate slides (Leica, Buffalo Grove, IL, USA). Slides were fixed in 100% ethanol for 15 minutes one day prior to LCM collection and stored at -80°C before staining with 0.5% toludine blue. A Leica LMD-ASLMD instrument was used to collect 150-200 microdissected acini of benign epithelium into Eppendorf caps containing 50μl digestion buffer (Life Technologies, Carlsbad, CA, USA) that were stored overnight at -80°C prior to RNA isolation. RNA was extracted with the RecoverAll® kit (Life Technologies) using the protocol provided by the manufacturer. RNA quality and quantity were evaluated 260 to 280 nm ration using the NanoDrop® spectrophotometer.

qRT-PCR. RNA (50ng) was reverse-transcribed using the VilocDNA kit (Life Technologies). cDNA was preamplified according to manufacturer's protocol using TaqMan® Pre-AMP master mix and TaqMan® assays. Assays included six genes of interest: AMACR, DBP, SCP2, PPARα, RXRα, and CAT. Three housekeeping genes were also measured: beta-actin (ACTB), tata-box binding protein (TBP), and hypoxanthine phosphoribosyltransferase 1 (HPRT-1) (**Figure 19 – Appendix A**).

The resulting pre-amplified product was diluted 1:20 and served as a template for the individual TaqMan® qPCR reactions, which were performed on an HT7900 instrument. Each reaction was completed in triplicate and genes for each subject were run on the same plate. No-template control reactions were included on every plate to evaluate contamination. Expression was normalized to the housekeeping genes, and the ΔCT method was utilized to quantify gene expression. Sample raw data and calculation of AMACR mRNA expression in the normal region is shown in **Figure 20 – Appendix A.**

Dietary assessment. A 100-item Block 98.2 or Block Brief Food Frequency Questionnaire (FFQ) was completed by Chicago participants (NutritionQuest, Berkeley, CA). HFHS participants completed an FFQ developed by the Nutrition Assessment Shared Resources of the Fred Hutchinson Cancer Research Center (21). All three questionnaires captured information on the frequency of consumption and portion sizes of foods consumed during the previous year. The FFQs provided estimates of total daily energy,

macronutrient and micronutrient intakes, as well as consumption of specific foods and food groups, including red meat, dairy foods and fish, in grams per day. Branched chain fatty acids have been identified in some oil-rich fish, presumably as a result of phytoplankton in the food chain (13). Meat and dairy items were indexed as either high- or low-fat, since only the former contain significant amounts of branched chain fatty acid.

Serum and tissue phytanic acid assays. Singlicate measurement of fatty acids in fasting serum samples was performed by capillary gas chromatography, mass spectrometry following derivatization of total lipid fatty acids at the Peroxisomal Diseases Laboratory of the Kennedy Krieger Institute, as described in detail by Lagerstedt, et al (22). In brief, fatty acids were quantified by selective ion monitoring in ratio to stable isotope-labeled internal standards. A four deuterium labeled standard was used for phytanic acid quantitation. Each fatty acid was treated as a percentage of the total lipid levels. Mean intra- and inter-batch coefficients of variation (CV) for phytanic acid in serum samples were 1.3% and 12.7%, respectively, based on anonymous replicates from a quality control serum pool.

The protocol for tissue fatty acid analysis was similar; however, due to the limited volume of tissue available, lipids were extracted from samples before analysis. The glycerol linked fraction was measured because phytanic acid is mainly contained in glycerides and glycerophospholipids (23). An average of 339 mg of prostate tissue per subject was provided by the Chicago subjects, and a minimum of 100 mg was considered desirable for phytanic acid quantification. Samples from Detroit subjects were too small to be analyzed. Results for individual fatty acids were expressed as their percentage of total tissue lipids. Mean intra-batch CV for phytanic acid in tissue was 18.6%, based on non-identical but adjacent samples from a random subject within each batch.

Immunohistochemistry. A tumor and normal FFPE block was selected for each subject. Slides were stained with AMACR (Dako, 1:100, Clone 13H4) and the assay was titrated to detect variation of expression in normal and tumor regions. Briefly, IHC was carried out as follows: 5 µm sections were cut on to charged slides and deparaffinized. After rehydration, antigen retrieval was carried out using a pressure cooker for 15 minutes. Slides were incubated for 60 minutes with the primary antibody, followed by incubation in a ready-to-use anti-rabbit secondary antibody from BioCare®. Color reaction was

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developed using diaminobenzidine (DAB) as the chromagen and slides were counterstained with hematoxylin. Positive and negative controls were run in each batch.

Slides were scanned at 20x on an Aperio (Leica) ScanScope® CS whole-slide digital microscope. A digital draw tool was used to demarcate normal, PIN, and tumor regions. The Genie® module in the Aperio system is a machine learning program that classifies each pixel in an image according to a set of hand-drawn, pre-classified training images provided by a trained individual. We created three classes of training images: epithelium, stroma, and blank slide. The resulting classes were determined to be highly accurate in the training set images, and were then applied to the study slides. The positive pixel algorithm within ImageScope® was utilized for digital image analysis (**Figure 21 – Appendix A).** We determined the percent positive pixels and pixel stain intensity in the epithelium of the normal, PIN, and tumor compartments.

Statistical Analysis. Frequency distributions of dietary intake, mRNA levels, and serum and tissue fatty acid concentrations were examined for normality and outlier values. A difference in distribution amongst the three types of FFQs was noted, thus results are adjusted for FFQ type. Scatterplots and Spearman rank correlation coefficients were used to examine relationships among the variables of interest. A *P*-value of ≤ .05 (two-sided) was considered statistically significant. Analyses were performed using SAS Version 9.2, (SAS, Inc., Cary, NC, USA).

RESULTS

Table I shows selected demographics and clinical characteristics of the study participants in Chicago and Detroit. Over three-quarters of the participants were overweight or obese (78%). The majority (58%) were African American and had a history of hypertension (64%) as reported by medical records. The average pre-surgical PSA was 9 ng/mL. About half of the men (46%) had a Gleason score ≤6. Serum and tissue phytanic acid were not associated with Gleason grade.

TABLE I. Selected characteristics of the study population[a]

	Chicago (n=39)	Detroit (n=42)	All (n=81)
	mean (SD)	mean (SD)	mean (SD)
Age	62.5 (5.8)	61.7 (6.2)	62.1 (6.0)
PSA level, mean, ng/ml	8.2 (5.6)	9.6 (7.5)	9.0 (6.7)
	n (%)	n (%)	n (%)
BMI, kg/m^2			
<25	8 (20)	9 (21)	17 (21)
25-30	15 (40)	22 (52)	37 (46)
≥30	15 (40)	11 (26)	26 (33)
Race			
White	11 (28)	23 (55)	34 (42)
Black	28 (72)	19 (45)	47 (58)
Hypertension			
Yes	28 (72)	24 (57)	52 (64)
No	11 (28)	18 (43)	29 (36)
BPH			
Yes	5 (13)	9 (21)	14 (17)
No	34 (87)	33 (79)	67 (83)
Diabetes			
Yes	13 (33)	4 (10)	17 (21)
No	26 (67)	38 (90)	64 (79)
Gleason score			
≤ 6	23 (59)	14 (33)	37 (46)
3+4	11 (28)	8 (19)	19 (23)
4+3	3 (8)	6 (14)	9 (11)
8-10	2 (5)	14 (33)	16 (20)
Pathologic Stage			
T2a	0 (0)	1 (2)	1 (1)
T2b	4 (10)	27 (64)	31 (38)
T2c	21 (54)	1 (2)	22 (27)
T3	2 (5)	0 (0)	2 (2)
T3a	5 (13)	9 (21)	14 (17)
T3b	2 (5)	4 (10)	6 (7)
Missing	5 (13)	0 (0)	5 (6)

[a] Not all Chicago participants had a value for BMI (n=38), Age (n=38) & PSA (n=35)

Table II shows that no correlation was observed between total dairy intake and serum phytanic acid levels; however, when we restricted analysis to high-fat dairy foods, we observed a positive and significant correlation with serum phytanic concentrations (r = 0.36, $P = 0.04$). High-fat ruminant meat intake by itself was not associated with serum phytanic acid levels (r = 0.07, $P = 0.68$), and adding this meat intake to high-fat dairy intake did not materially change the aforementioned significant correlation with serum. Fish intake did not show any association with phytanic concentrations in serum. Concentrations of pristanic acid, the direct substrate of AMACR, were correlated with phytanic levels in serum and tissue (r = 0.64 and r = 0.43, respectively) and gave similar results; thus they are not discussed further.

TABLE II. Partial Spearman correlation coefficients between dietary intake and tissue concentrations of phytanic acid in serum and prostatic tissue[a]

Intake (grams/day)	Serum Phytanic (% FA) (n=35)		Serum Heptadecanoic (% FA) (n=35)		Tissue Phytanic (% FA) (n=24)	
	r	P-value	r	P-value	r	P-value
Dairy						
Total	0.03	0.85	0.02	0.93	0.02	0.91
High-fat	0.35	0.04	-0.02	0.88	0.07	0.74
Whole milk	-0.17	0.33	-0.06	0.70	0.06	0.79
Butter	-0.03	0.87	-0.11	0.53	-0.32	0.14
Meat (Ruminant)						
Total	0.06	0.75	0.15	0.38	0.15	0.50
High-fat	0.07	0.68	0.00	0.99	0.15	0.50
Combined High-fat						
Dairy and meat	0.36	0.04	0.006	0.97	0.11	0.59

[a] The data have been adjusted for the three different types of FFQ.

Tissue phytanic acid concentrations did not correlate with combined high-fat dairy and meat intake, nor with any other dietary measure or serum biomarker. Furthermore, **Figure 2** shows that serum and tissue phytanic concentrations were only weakly correlated (r= 0.29, P = 0.15).

Pentadecanoic and heptadecanoic acids were strongly correlated with each other in both serum and prostatic tissue (r = 0.69, P < 0.01 and r = 0.95, P < 0.01); therefore, we only show results for heptadecanoic acid. No association was seen between high-fat dairy intake and serum (r= 0.02, P = 0.93) or tissue (r= 0.07, P = 0.76) heptadecanoic concentration in this population. However, phytanic acid concentrations were strongly correlated with heptadecanoic acid concentration in both the serum and tissue (**Figure 3a & 3b**). Furthermore, **Figure 3c** shows that serum and tissue levels of heptadecanoic acid were positively correlated (r = 0.67, P < 0.01). Phytanic acid concentrations in the prostate range from 0.05 – 0.30%, whereas heptadecanoic acid concentrations range from 5.49 - 38.00% - approximately a hundred-fold difference.
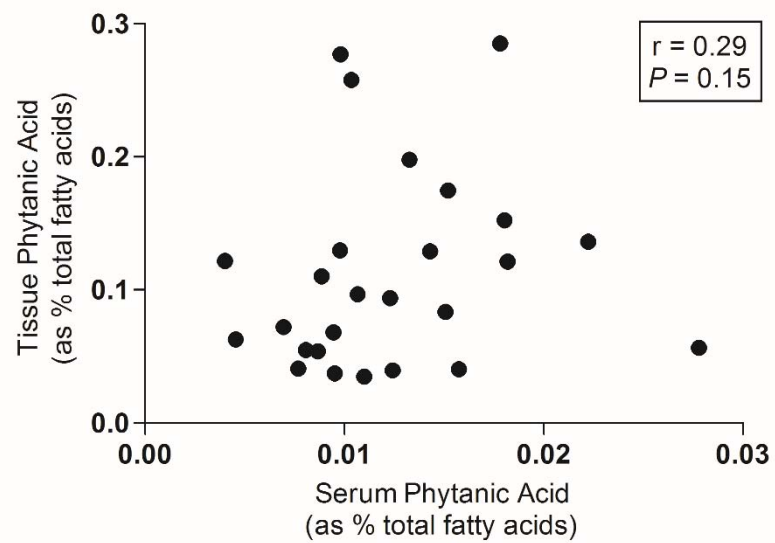
Figure 2. Scatterplot showing correlation (Spearman r) between serum and tissue phytanic acid
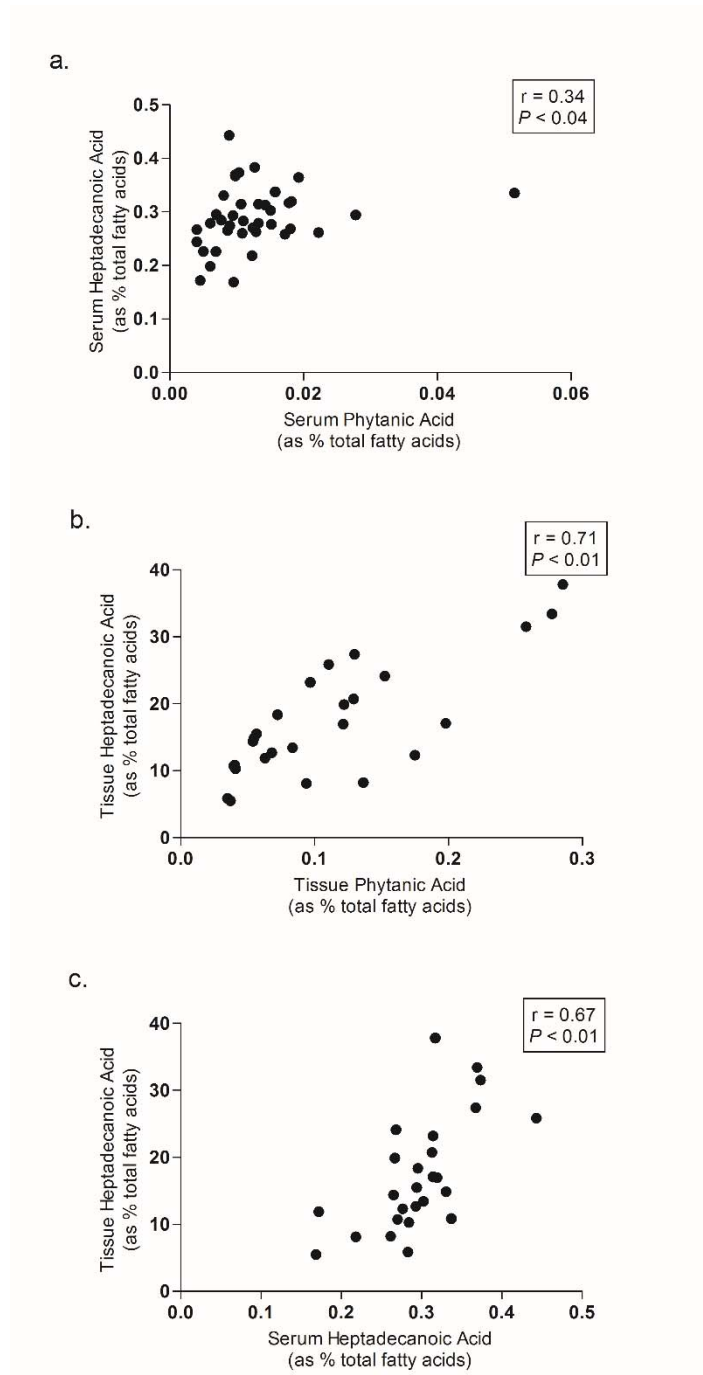
Figure 3. Scatterplots showing correlations (Spearman r) between a. Serum phytanic and pentadecanoic acid, b. Tissue phytanic and pentadecanoic acid, and c. Serum pentadecanoic acid and tissue pentadecanoic acid

AMACR expression at the mRNA level was positively correlated with expression of other genes in the

peroxisomal phytanic acid metabolism pathway. Overall, there was strong evidence for inter-correlated

expression among genes in the pathway, as shown in **Table III**. As was the case for tissue phytanic acid

concentration, we found that AMACR mRNA expression was not associated with combined high-fat dairy

and meat intake. **Table IV** shows that ruminant meat intake alone was marginally correlated with AMACR

mRNA (r = 0.23, $P = 0.06$). However, we did not observe a stronger relationship with high-fat ruminant

meat – a better estimate of phytanic acid intake – and thus this observation is probably due to chance.

Overall, **Table IV** indicates no clear patterns of association between dietary determinants of phytanic acid

and expression of genes associated with peroxisomal phytanic acid metabolism and function.

TABLE III. Spearman correlations between expression levels of genes involved in peroxisomal phytanic acid metabolism or function (n=70)

	AMACR	SCP2	CAT	DBP	PPARα
AMACR					
SCP2	0.58				
	<.01				
CAT	0.65	0.71			
	<.01	<.01			
DBP	0.57	0.42	0.75		
	<.01	<.01	<.01		
PPARα	0.43	0.52	0.64	0.59	
	<.01	<.01	<.01	<.01	
RXRα	0.18	0.65	0.4	0.11	0.3
	0.14	<.01	<.01	0.37	0.01

TABLE IV. Partial Spearman correlation coefficients between dietary intake and mRNA expression levels in benign prostate tissue (n=68)[a]

	AMACR		SCP2		DBP		CAT		RXRα		PPARα	
	r	P-value	r	P-value	r	P-value	r	P-value	r	P-value	r	P-value
Dairy												
Total	-0.07	0.55	0.03	0.80	-0.09	0.46	-0.02	0.85	0.20	0.11	-0.14	0.26
High-fat	0.03	0.84	0.07	0.58	-0.02	0.87	0.05	0.67	0.16	0.20	-0.17	0.17
Whole milk	-0.15	0.24	-0.15	0.24	-0.21	0.09	-0.24	0.05	0.05	0.71	0.25	0.04
Butter	0.14	0.25	-0.01	0.91	-0.08	0.50	-0.01	0.92	0.01	0.93	-0.06	0.59
Meat (Ruminant)												
Total	0.23	0.06	0.07	0.55	0.22	0.07	0.09	0.49	0.08	0.53	0.07	0.58
High-fat	0.21	0.09	0.02	0.90	0.15	0.23	0.02	0.90	-0.03	0.81	-0.01	0.95
Combined High-fat												
Dairy and meat	0.13	0.31	0.11	0.35	0.10	0.42	0.13	0.33	0.16	0.19	-0.13	0.30

[a]The data have been adjusted for the three different types of FFQ.

Specifically, whole milk intake was inversely associated with catalase mRNA expression (r = -0.24, $P =$ 0.05), however it was positively associated with PPARα mRNA expression (r = 0.25, $P = 0.04$). Serum phytanic acid concentration was not associated with AMACR, SCP2, DBP, CAT, and PPARα mRNA expression in benign prostate, but was positively correlated with mRNA expression of RXRα (r = 0.41, P $= 0.02$) (**Table V**). Tissue phytanic acid concentrations were not correlated with mRNA expression for AMACR; similar findings were observed for tissue heptadecanoic acid (r = 0.03, $P = 0.89$).

AMACR protein expression significantly increased between normal (mean % positivity = 0.07; 95% CI, 0.06 – 0.08), PIN (mean % positivity = 0.12; 95% CI, 0.10-0.13), and tumor (mean % positivity = 0.17; 95% CI, 0.16-0.18) compartments. Overall there was no clear pattern of association between AMACR protein expression and dietary determinants of phytanic acid. AMACR protein expression in the normal compartment was not correlated with mRNA expression (r = -0.16, $P = 0.20$). However, AMACR protein expression in the normal compartment was inversely and significantly correlated with SCP2 (r = -0.25, $P =$ 0.05) and RXRα (r = -0.37, $P = 0.01$) mRNA levels. AMACR protein expression did not correlate with tissue phytanic levels (r = -0.09, $P = 0.46$).

TABLE V. Spearman correlation coefficients between serum and tissue phytanic acid levels and mRNA expression in benign prostate tissue

mRNA	Serum phytanic n = 31		Tissue phytanic n = 26	
	r	*P*-value	r	*P*-value
AMACR	0.13	0.47	0.03	0.88
SCP2	0.22	0.23	0.09	0.66
DBP	0.26	0.16	0.24	0.23
CAT	0.12	0.51	0.14	0.48
RXRα	0.41	0.02	0.11	0.61
PPARα	0.00	0.97	0.21	0.31

DISCUSSION

This is the first study, to our knowledge, to investigate the interrelationships among intake of ruminant dairy and meat products, serum and tissue phytanic acid concentrations, and AMACR expression in the benign prostate. Our results show that among the dietary sources of branched chain fatty acids, only high-fat dairy intake was correlated with serum phytanic acid concentration. However, tissue concentrations of phytanic acid were not discernibly associated with any dietary source and were only weakly correlated with serum levels. Moreover, we observed no significant association of diet, serum or tissue phytanic acid levels, with AMACR gene or protein expression in the benign prostate. We found, as expected, that AMACR gene expression was highly correlated with expression of other genes in related pathways, and thus no overall patterns suggesting a relationship between these genes and food intake or fatty acid levels emerged. Taken together, the present results do not provide support for the hypothesis that excess levels of phytanic acid in the diet could explain both the overexpression of AMACR in prostate cancer and the epidemiological association between prostate cancer risk and intake of dairy foods and red meat.

The present data confirm previous findings in which serum phytanic acid levels were correlated with dietary intake of high-fat dairy food (2, 24, 25). Allen et al., found butter intake to be significantly correlated to serum phytanic acid (r = 0.44, $P < .01$) in the EPIC subcohort from the UK (2). The association we observed was not attributable to butter intake, but most likely due to other dairy components such as cheese, yogurt or ice cream. This discrepancy may be explained by differences in patterns of dairy fat consumption between countries, and variation in the branched chain fatty acid concentration across and even within food categories.

The lack of correlation between dietary intake and tissue phytanic acid, supported by the weak correlation between serum and tissue phytanic acid, suggests that metabolic activity leading to a high rate of turnover within prostate tissue might be an important factor. The tissues evaluated in this study were histologically normal but from cancerous prostates; therefore it is conceivable that the samples reflect a microenvironment affected by field cancerization. Proliferating cells may have important metabolic requirements such that fatty acids undergo accelerated oxidation to produce acetyl-CoA, which could

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serve as a precursor for glycolytic intermediates, amino acid synthesis and ribose for nucleotides (26).

Measurement error, resulting from the FFQ approach to measuring usual intake, and the technical difficulty of assaying low abundance fatty acids in tissue samples, also could have attenuated true relationships. However, we observed strong correlations between prostate and serum concentrations of relatively low abundance long chain omega-3 fatty acids, which are determined largely by diet and supplement use. For example, serum-tissue correlations were r = 0.75 ($P < 0.01$) for docosahexaenoic acid, and r = 0.52 ($P = 0.01$) for docosapentaenoic acid.

We observed that levels of heptadecanoic acid, an alternative biomarker for dairy intake, were correlated with phytanic acid in serum (as previously reported by Allen, et al.) and in prostate tissue (2). Since heptadecanoic levels were not correlated with other fatty acids such as linoleic and eicosapentanoic acid, we assume these relationships are due to common food sources as opposed to sample artifacts. Serum and tissue levels of heptadecanoic were also highly correlated with each other. However, we found no discernible relationship between dairy intake and heptadecanoic acid in either serum or tissue. Once again, this raises the possibility that true relationships were attenuated by measurement error involving dietary intake. However, recently reported correlations for dairy intake and pentadecanoic acid, such as those from the Multi-Ethnic Study of Atherosclerosis (MESA), are rather small (r = 0.13-0.22) and thus could have been missed in a small study such as ours (27). More importantly, our results indicate that heptadecanoic acid, although approximately 100-times more abundant than phytanic acid, is not a superior biomarker to phytanic for identifying a relationship between dairy intake and AMACR expression in the prostate.

Phytanic acid has been shown to bind and activate the nuclear receptors PPARα and RXRα (28-32). These receptors play a role in a variety of cellular processes, including adipogenesis, lipid homeostasis, fatty acid, and glucose metabolism (30). As there was no relationship observed between RXRα mRNA expression and tissue phytanic acid, the association seen with serum phytanic concentrations could be explained by chance or by local factors within the prostate.

Overall there was no association found between dietary intake and mRNA gene expression of AMACR or its pathway partners. Whole milk consumption was inversely associated with catalase and positively

associated with PPARα mRNA expression. These findings could be due to chance because of multiple hypothesis testing; however, Suhara et al., reported that high fat cow's milk products increased the activity of PPARα and RXRα in a reporter gene assay (33). Activation of PPARα promotes fatty acid catabolism and insulin sensitivity, which could favor tumor growth in the prostate microenvironment, although PPAR activation could also be invoked to explain epidemiological findings that milk consumption is inversely related to risk of type 2 diabetes (34).

AMACR protein expression was significantly increased between normal, PIN, and tumor compartments as seen in previous studies (12). However, we observed no association between AMACR mRNA and protein expression in the benign prostate. The *in vitro* study by Mobley et al. demonstrated that treatment with phytanic acid markedly increased AMACR protein levels in androgen-sensitive LNCaP cells but had little effect on mRNA expression, indicating that post-transcriptional modifications or effects on protein half-life might be operative (14). Our quantitative IHC results are consistent with this, and further studies are needed to explore possible post translational processes and mechanisms.

The present study, which to our knowledge is the first to measure phytanic acid concentrations in prostate tissue, benefitted from laser capture microdissection to collect a homogeneous histologically normal epithelial cell population for gene expression analysis. Additionally, quantitative image analysis is an accurate and reproducible way to evaluate IHC in these RP specimens. However, certain limitations of this study are acknowledged, including a relatively small sample size that limits power and the ability to control for potential confounders. Additionally, the FFQs utilized did not allow us to discriminate between fatty and non-fatty fish while only the former is a source of phytanic acid (13). Of further concern is the high CV (18%) for tissue phytanic acid concentration, which suggests poor reproducibility. However, this could be attributed to the heterogeneity of the prostate tissue, because repeat samples were not taken from precisely the same tissue location and hence were not strictly identical.

In conclusion, we found that there is no simple chain of association linking dairy intake to phytanic acid concentrations in the prostate and to AMACR expression in benign tissue, despite evidence that dairy intake and serum levels are linked and *in vitro* data indicating upregulation of AMACR expression when phytanic acid is added to cultured PCa cells (14). These results do not support a direct relationship

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between local prostatic phytanic acid concentration and AMACR expression. Studies that examine

temporality and distribution of phytanic acid concentration in the prostate are warranted. The reason for

AMACR overexpression in prostate cancer, and its potential link to the etiology of this disease, remains

unresolved.

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Chapter 3 - Retinoid and Carotenoid Depletion in Serum and Liver Tissue Concentrations in Patients at High-Risk for Liver Cancer

ABSTRACT

Approximately 2.3 million Americans are chronically infected with hepatitis C virus (HCV). HCV patients with cirrhosis form the largest group of persons at high risk for hepatocellular carcinoma (HCC). Increased oxidative stress is regarded as a major mechanism of HCV-related liver disease progression. Deficiencies in retinoid and carotenoid antioxidants may represent a major modifiable risk factor for HCC progression. This study aims to identify key predictors of serum antioxidant levels in patients with HCV, to examine the relationship between retinoid/carotenoid concentrations in serum and hepatic tissue, to quantify the association between systemic measures of oxidative stress and antioxidant status, and to examine the relationship between retinoids and stellate cell activation. There was a significant inverse association between serum retinol, lycopene, and RBP4 concentrations with fibrosis stage. Serum β-carotene and lycopene were strongly associated with their respective tissue concentrations. There was a weak downward trend of tissue retinyl palmitate with increasing fibrosis stage. Tissue retinyl palmitate was inversely and significantly correlated with hepatic αSMA expression, a marker for hepatic stellate cell activation (r = -0.31, P < 0.02). Urinary isoprostanes levels were inversely correlated with serum retinol, β-carotene, and RBP4. A decrease in serum retinol, β-carotene, and RBP4 is associated with early stage HCV. Retinoid and carotenoid levels decline as disease progresses, and our data suggest that this decline occurs early in the disease process, even before fibrosis is apparent. Measures of oxidative stress are associated with fibrosis stage and concurrent antioxidant depletion. Vitamin A loss is accompanied by stellate cell activation in hepatic tissue.

INTRODUCTION

The progression of hepatitis C virus (HCV) infection can lead to cirrhosis and in some cases to hepatocellular carcinoma (HCC), which has limited treatment options and poor prognosis. Approximately

2.3 million Americans are chronically infected with HCV. HCV patients with cirrhosis form the largest group of persons at high risk for HCC.(1) (2-5) Oxidative stress, resulting from chronic inflammation, is purported to be a major mechanism for hepatic fibrosis and cirrhosis.(6) An imbalance between production of reactive oxygen species and antioxidant defense induces a number of pathophysiological changes in the liver, including activation of hepatic stellate cells (HSCs), oxidative damage to lipids, nucleotides and proteins, and initiation of proliferative processes associated with regeneration. Vitamin A and its carotenoid precursors are an important part of the body's antioxidant defense, due to their ability to scavenge and directly neutralize free radicals in the tissue. In normal liver, quiescent HSCs are responsible for the storage of more than 90% of the body's vitamin A reserves as retinyl esters. However, when activated, HSCs lose their capacity to store vitamin A while acquiring contractile, proliferative and pro-inflammatory properties that are believed to play a major role in fibrogenesis.(7)

In this context, deficiencies in dietary antioxidants, such as retinoids and carotenoids, could represent a major modifiable risk factor for chronic liver disease (CLD) progression. Two prospective epidemiological studies evaluating the relationship between serum retinoids and liver cancer among subjects with chronic hepatitis B found that higher pre-diagnostic serum retinol was strongly associated with a subsequent reduced risk of liver cancer.(8, 9) More recently, a large cohort study in Finland observed that higher baseline serum retinol and β-carotene were inversely associated, many years later, with the incidence of liver cancer and death from CLD.(10) Additionally, a randomized trial has reported compelling evidence that polyprenoic acid, a synthetic retinoid, reduced the incidence of second primary liver tumors and prolonged survival in HCC patients.(11) A small number of studies to date have found that these serum micronutrients are depleted in cirrhotic patients and only three studies to date have assessed hepatic antioxidant levels in small and generally heterogeneous populations of pre-cirrhotic individuals. (12-15) (16) (17-19)

Despite vast improvements in HCV treatment in recent years, there are potential opportunities for therapeutic or preventive strategies involving antioxidant repletion, particularly in patients who have progressed to cirrhosis and are at risk for HCC even if they achieve virological cure.(20) (21, 22) Thus there is a crucial need to develop adjuvant strategies to prevent cirrhosis and HCC in high-risk

individuals.(23) However, supplementation with vitamin A itself must be approached cautiously in individuals with liver disease, as hypervitaminosis A causes accelerated liver fibrosis and may also promote cancer development.(24) Therefore, there is a need for more information on the spectrum and causes of retinoid and carotenoid depletion in the HCV-infected population, so that optimal strategies for clinical trials can be identified.

Among HCV-infected persons, antioxidant depletion could be explained by a combination of dietary, lifestyle, and physiological factors. Apart from processes directly linked to HCV infection, these factors include inadequate dietary antioxidant intake, smoking, alcohol intake or diabetes, all of which have been reported to diminish defense against oxidative stress. The present study aims to: a) determine the prevalence and predictors of retinoid and carotenoid depletion in a well-defined patient population, b) examine the relationships between retinoid and carotenoid concentrations in serum and hepatic tissue, c) quantify the association between systemic measures of oxidative stress and antioxidant status, and d) examine the relationship between antioxidant levels and stellate cell activation. We postulated that lower retinoid and carotenoid concentrations and higher levels of oxidative stress would be associated with fibrosis stage among HCV-infected patients.

MATERIALS AND METHODS

Study population. We conducted a cross-sectional study among patients with HCV infection at the University of Illinois at Chicago (UIC) and University of Chicago (UC). A total of 91 subjects were included in this study. We consented and enrolled patients with confirmed HCV infection undergoing percutaneous liver biopsy (n = 69) who provided fasting blood, fresh tissue, urine, and completed a diet history questionnaire. Liver histology was staged into F0-4 according to the Batts-Ludwig scoring system. Subjects with F0 were categorized as having no fibrosis. Subjects with fibrosis stage 1-2 and fibrosis 3-4 were categorized as mild/moderate and severe fibrosis, respectively. The liver histology staging criteria was utilized to define fibrosis stage in our analysis.

All biopsies were performed with 16 or 18-gauge needles; an average of 7.7 mm^3 fresh tissue was taken from the end of each core and snap frozen for research purposes. We collected serum, urine, and questionnaire data from healthy volunteers (n=11). We obtained post-mortem normal liver tissue from the Cooperative Human Tissue Network (CHTN) repository (n = 8). The remaining controls included patients with no chronic liver disease (n = 3) who provided serum, tissue, urine, and questionnaire data.   The UIC and UC Institutional Review Boards approved the study.

Dietary assessment. The National Cancer Institute Diet History Questionnaire I (DHQ) was completed by all participants who provided serum. The DHQ was subsequently analyzed by the Diet*Calc Analysis Program (Version 1.4.3. National Cancer Institute, Applied Research Program). This program generated nutrient estimates based on frequency and portion sizes over the past year. Approximate retinoid and carotenoid intake was calculated from the DHQ. Pre-formed vitamin A is primarily found in animal sources such as eggs, dairy produce, fish, and meat. Carotenoids, which are found primarily in plant foods, include α-carotene, β-carotene, β-cryptoxanthin, lutein, and zeaxanthin; of those only α-carotene, and β-carotene are pro-vitamin A carotenoids; i.e., vitamin A precursors. To account for both pre-formed and pro-vitamin A dietary sources, the recommended dietary allowance for vitamin A is expressed in retinol activity equivalents (RAE).

Serological measurements. Serum analyses were performed by the clinical pathology laboratories at UIC. The serum chemistry panel included aspartate aminotransferase (AST), alanine aminotransferase (ALT), international normalized ratio (INR), albumin, total bilirubin, total protein, BUN, hemoglobin, creatinine, and platelet count. Fasting serum insulin, glucose, and high sensitivity C-reactive protein (hs-CRP) were measured in single batches on SYNCHRON® Systems from Beckman Coulter (Pasadena, California). Based on blinded duplicates from a quality control pool of serum, intra-batch coefficients of variation (CV) aliquots for fasting serum insulin, glucose, and hs-CRP were 2.4%, 0.65%, and 0.98%, respectively. Insulin resistance was measured using the homeostatic model assessment of insulin resistance (HOMA-IR). Aminotransferase to platelet ratio index (APRI) and FIB4 (Fibrosis 4) were calculated using published formulas.(25) (26)

Serum retinoid/carotenoid assays. Singlicate measurements of retinoids and carotenoids in fasting serum samples were performed by using atmospheric pressure chemical ionization mass spectrometry as described previously, with the following changes.(27) Ultrahigh pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) was carried out using a Shimadzu (Kyoto, Japan) LCMS-8040 triple quadrupole mass spectrometer equipped with a Shimadzu Shim-pack XR-ODSIII column (2.0 x 50 mm, 1.6 µm) at 35°C. After holding at 5:95 (v/v) methyl-*tert*-butyl ether/methanol for 0.3 minutes, a 0.45-minute linear gradient was used from 5% to 30% methyl-*tert*-butyl ether at a flow rate of 0.6 mL/minute. Carotenoids and retinoids were detected by MS/MS during the same analysis using polarity switching and the following selected reaction monitoring transitions: lycopene *m/z* 536 to 467 (-), $[^{13}C_{10}]$-lycopene (internal standard) *m/z* 546 to 477 (-), β-carotene *m/z* 536 to 536 (-), lutein *m/z* 551 to 135 (+), retinol and retinyl palmitate *m/z* 269 to 93 (+).

Based on blinded replicates from a quality control pool of serum, mean intra- and inter- batch CVs for retinol in serum samples were 9.3% and 13.9%, respectively. Average intra- and inter- batch CV for all serum retinoids and carotenoids were 13.9%, and 9.7%, respectively.

The Relative Dose Response (RDR) test has been proposed as a better alternative for determining vitamin A deficiency compared to serum retinol. A retinol increase of greater than 20% following a challenge dose of retinyl palmitate is considered a positive test indicating deficient liver reserves. Serum delta RDR values were calculated according to the following formula: $100 \; x \; \frac{A_5 - A_0}{A_5}$. A_0 is the serum retinol concentration at baseline, and A_5 is the serum retinol concentration at 5 hours post-retinol dose. Test participants provided serum for baseline retinol levels after an overnight fast, and then ingested 1000 RAE of retinyl palmitate dissolved in corn oil on a cracker. Serum retinol levels were measured again five hours later. Of the 24 subjects who completed the RDR, eleven had tissue available.

RBP4 measurement. Serum RBP4 was measured in duplicate using an enzyme-linked immunosorbent assay (ALPCO Diagnostics, Salem, NH) according to the manufacturer's instructions. The kit utilized a polyclonal rabbit anti-RBP antibody.

Tissue retinoid/carotenoid assays. Hepatic tissue retinoid and carotenoid measurements were performed using UHPLC-MS/MS as described above, except that the tissue (.5 to 5 mg) was homogenized in 200 µl water and extracted twice using 600 µl portions of ethanol/hexane (20:80; v/v) containing 0.1% butylated hydroxytoluene. Based on adjacent samples from a random subject within each batch, mean intra-batch CV for hepatic tissue retinyl palmitate, β-carotene, and lycopene were 6.9%, 10.0%, and 4.1%, respectively.

Urinary isoprostanes. Urine concentrations of 8-iso-PGF2α were measured using a rapid UHPLC-MS/MS assay as described previously.(28) Based on anonymous replicates from a quality control pool of urine, mean intra- and inter-batch CV for the urinary isoprostanes measurements were 6.4% and 7.8%, respectively.

Immunohistochemistry. Tissue sections of 4 µm each were placed on charged slides, dehydrated, and then deparaffinized. Immunostaining was carried out using a BondRX (Lecia Biosystems, Buffalo Grove, IL) autostainer with a mouse monoclonal antibody for αSMA (DAKO, Clone 1A4) in a 1:2000 dilution for 15 minutes at room temperature to identify HSC. The sections were then incubated in rabbit anti-mouse IgG (Bond Polymer Refine Detection, Leica Biosystems) for 20 minutes. No antigen retrieval was used for this stain. Color reaction was developed using diaminobenzidine (DAB) as the chromagen, followed by counterstaining with hematoxylin. Positive and negative controls were included in each batch.

Slides were scanned at 20x on an Aperio ScanScope® CS whole-slide digital microscope (Leica Biosystems). A digital draw tool was used to identify hepatic parenchymal areas. Large vessels, inflammation, and artifacts *(e.g.,* folds, debris, etc.) were excluded from analysis. αSMA quantitation was restricted to the hepatic parenchymal region to exclude αSMA positive cells (i.e. portal fibroblasts and bone marrow derived collagen-producing cells) in the portal region.(29) Definiens Tissue Studio® 3.6.1 (Definiens, Munich, Germany), a digital image analysis platform, was used to measure the percent positivity of αSMA stain area within the hepatic parenchyma (**Figure 22 – Appendix A**).

Statistical analysis. Frequency distributions of dietary intake, urinary isoprostanes, retinoid, and carotenoid concentrations were examined for normality. Scatterplots and Spearman rank correlation

coefficients were used to examine relationships among the variables of interest. A *P*-value of < 0.05 was considered statistically significant, and all tests were two-sided. Analyses were performed using SAS Version 9.2 (SAS, Inc., Cary, NC).

RESULTS

Table VI shows selected demographics and clinical characteristics of the study participants at both institutions by fibrosis stage. Participants in the control group were more likely to be white, overweight, and non-smokers. There were no differences in race, BMI, smoking status, or diabetes among the disease groups. The no fibrosis group was more likely to be overweight or obese compared to the mild/moderate fibrosis group (94% vs 59%). A majority of the HCV-positive subjects had either genotype 1a (52%) or 1b (38%) as reported by medical records. The median AST, APRI, and FIB-4 levels in disease subjects were positively associated with the fibrosis stage (**Table VII**). However, hs-CRP concentrations were inversely and significantly associated with fibrosis stage (*P* < 0.05) (**Table VII**). BMI was positively and significantly correlated with hs-CRP concentrations (r = 0.46, *P* < 0.01).

TABLE VI. Selected characteristics of the study population by fibrosis stage

Characteristics	Control	No Fibrosis	Mild/Moderate Fibrosis	Severe Fibrosis	Total	P - value[1]	P - value[2]
	n=22	n=18	n=34	n=17	n=91		
Age - Median (IQR)	45 (42-54)	55 (51-62)	53 (48-58)	56 (53-57)	53 (46-58)	0.17	0.17
Gender							
Male	12	6	18	10	46	0.42	0.27
Female	10	12	16	7	45		
Race							
Asian	0	0	1	0	1	**<0.01**	0.68
Black	2	12	17	7	38		
White	18	6	15	9	48		
Unknown	2	0	1	1	4		
BMI							
<25	0	1	13	4	18	**0.02**	0.11
25-30	5	9	12	7	33		
>30	9	8	8	6	31		
Unknown	8	0	1	1	10		
Smoking Status							
Never	17	7	10	7	41	**0.02**	0.60
Former	2	7	11	7	27		
Current	3	4	13	3	23		
Diabetes							
Yes	3	2	3	4	12	0.56	0.33
No	19	16	31	13	79		
HCV Genotype							
1	-	11	29	13	53	0.15	0.15
2	-	1	0	0	1		
3	-	0	0	1	1		
Unknown	-	6	5	3	14		

[1] P-values are Fisher exact (2-tailed) for comparison of proportions and Kruskal-Wallis test for comparison of medians amongst all groups
[2] P-values are Fisher exact (2-tailed) for comparison of proportions and Kruskal-Wallis test for comparison of medians amongst diseased groups only

TABLE VII. Clinical assay results for the study population by fibrosis stage - median (interquartile range)

| Laboratory Data	Control	No Fibrosis	Mild/Moderate Fibrosis	Severe Fibrosis	*P* - value[1]	*P* - value[2]
	n=14	n=17	n=34	n=17		
AST (IU/L)	23.5 (20.0-29.0)	42.0 (30.0-60.0)	46.5 (35.0-68.0)	94.0 (43.0-127.0)	**<0.01**	**<0.02**
ALT (IU/L)	22.5 (18.0-29.0)	57.0 (31.0-67.0)	56.5 (32.0-76.0)	89.0 (42.0-115.0)	**<0.01**	0.27
INR	1.10 (1.07-1.12)	1.00 (0.95-1.1)	1.02 (1.0-1.1)	1.05 (1.0-1.1)	0.08	0.08
Albumin (g/dL)	4.0 (3.9-4.3)	4.1 (3.8-4.2)	4.0 (3.8-4.2)	4.0 (3.7-4.2)	0.83	0.70
Total Bilirubin (mg/dL)	0.7 (0.5-0.9)	0.6 (0.3-0.9)	0.6 (0.5-0.8)	0.8 (0.5-1.0)	0.35	0.21
Total Protein (g/dL)	6.9 (6.6-7.2)	7.6 (7.0-8.1)	7.4 (7-8.1)	7.2 (6.9-7.7)	**0.04**	0.52
BUN (mg/dL)	12.5 (10.0-16.0)	13.5 (11.0-16.0)	12.0 (9.0-14.0)	14.0 (11.0-15.0)	0.26	0.14
Hemoglobin (g/dL)	14.2 (13.8-14.6)	14.2 (12.8-14.8)	14.4 (11.9-14.9)	14.0 (13.5-15.2)	0.78	0.99
Platelet Count (10^9/L)	288 (238-292)	207 (141-251)	212 (181-258)	190 (153-228)	0.10	0.35
APRI[3]	0.18 (0.16-0.29)	0.50 (0.42-0.83)	0.51 (0.39-0.87)	1.01 (0.48-2.03)	**<0.01**	**<0.05**
FIB-4[4]	0.7 (0.5-0.7)	1.7 (1.2-2.2)	1.5 (1.1-2.2)	2.7 (1.7-3.6)	**<0.01**	**<0.02**
Fasting Serum Assays	n=14	n=18	n=32	n=13		
HS-CRP (mg/L)	2.3 (1.1-9.5)	2.3 (0.6-2.1)	0.7 (0.2-1.7)	0.6 (0.5-2.1)	**0.04**	0.39
Insulin (µIU/mL)	6.5 (5.3-14.5)	9.4 (6.4-16.2)	8.4 (6.4-15.9)	11.8 (8.8-16.4)	0.40	0.41
Glucose (mg/dL)	96 (81-120)	89.5 (82-97)	93 (182-98)	94 (87-105)	0.60	0.71
HOMA-IR Score	1.6 (1.1-3.6)	2.3 (1.4-3.4)	2.0 (1.2-4.0)	2.7 (1.8-4.5)	0.49	0.42

[1] Kruskal-Wallis test for comparison of medians amongst all groups

[2] Kruskal-Wallis test for comparison of medians amongst diseased groups only

[3] AST to Platelet Ratio Index (APRI)

[4] Fibrosis 4 (FIB-4) is based on age, aspartate aminotransferase, alanine aminotransferase levels, and platelet counts

Dietary intake of individual retinoids and carotenoids did not differ between controls and HCV subjects. There was evidence of a downward trend for total vitamin A intake (expressed as retinol activity equivalents, RAE) with increasing fibrosis stage, however this trend was weak and not significant or distinguishable from chance, as shown in **Figure 4**. Total vitamin A, or individual retinoid and carotenoid intake also did not differ by fibrosis stage or predictive markers of fibrosis (APRI and FIB-4). Mean total vitamin A intake was 1182 and 1295 mcg RAE for men and women, respectively; values well above the general population based on data from NHANES III (682 and 606 mcg RAE, for men and women, respectively). Total vitamin A or individual retinoid and carotenoid intake levels were not associated with BMI, smoking status, alcohol consumption, or insulin resistance.
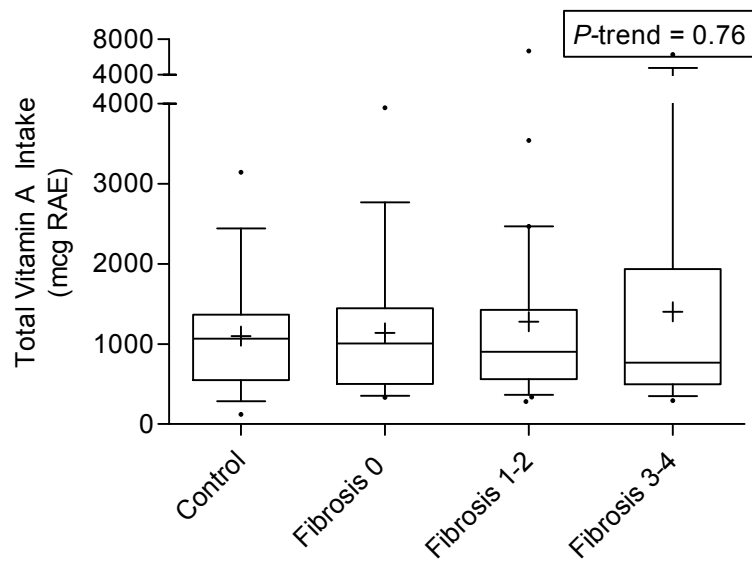
Figure 4. Median (mean = +, whiskers = 25th and 75th percentile) total vitamin A intake (mcg retinol activity equivalents) by fibrosis stage

Dietary β-carotene and lutein intake were positively and significantly correlated with their respective serum concentrations (β-carotene: r = 0.24, $P = 0.05$; lutein: r = 0.33, $P < 0.01$). However, total vitamin A intake did not correlate with serum retinol concentrations (r = 0.07, $P = 0.53$). Similarly, no relationships between intake and serum were observed for other dietary carotenoids. Tissue retinoid and carotenoid levels were not associated with dietary intake (**Table XII – Appendix A**). **Figure 5** shows that serum retinol and hepatic retinyl palmitate concentrations, the respective dominant forms of vitamin A in serum and liver, were not correlated (r < 0.01, $P = 0.99$). However, serum and tissue concentrations of β-carotene (r = 0.56, $P < 0.01$) and lycopene (r = 0.77, $P < 0.01$) were moderately correlated in this study population.
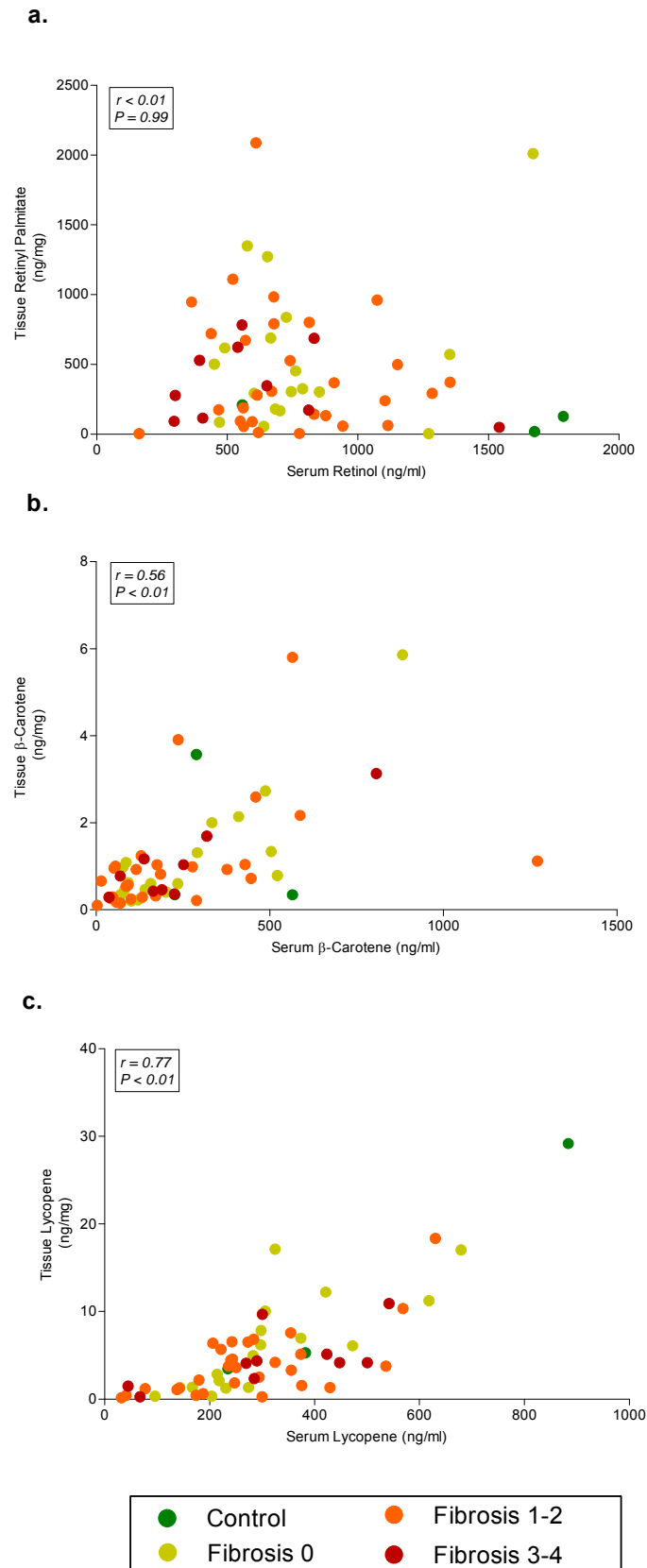
Figure 5. Scatterplots showing correlations (Spearman r) between (a) Serum retinol and tissue retinyl palmitate (n=60), (b) Serum and tissue β-carotene (n=58), and (c) Serum and tissue lycopene (n=60)

Serum retinol and RBP4 concentrations were significantly lower in HCV subjects compared to controls, although no HCV subjects met the standard serum criteria for vitamin A deficiency (<200 ng/mL). **Figure 6** shows a significant downward trend of serum retinol and RBP4 concentrations with increasing hepatic fibrosis. Similar relationships were also observed for serum β-carotene, lycopene, and lutein concentrations (**Figure 7**).

a.
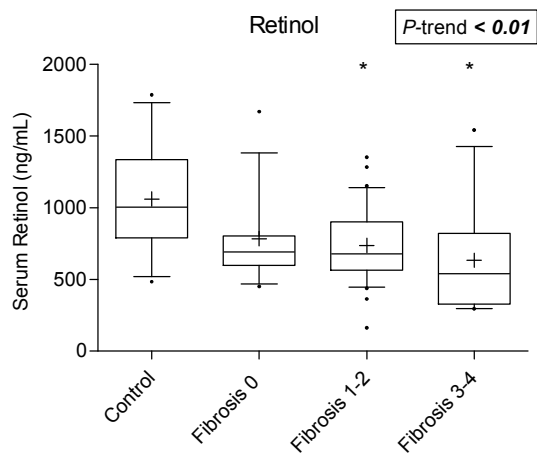
b.
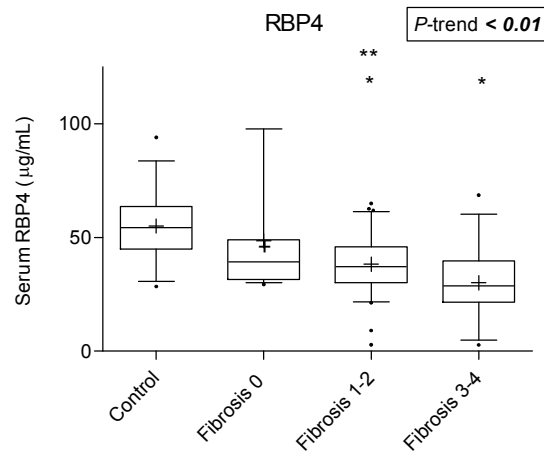
Figure 6. Boxplots (Whiskers = 10th and 90th percentile) for serum concentrations of (a) retinol and (b) RBP4 by fibrosis stage

* P < 0.05 compared to Control ** P < 0.05 compared to Fibrosis 0 group

Figure 7. Boxplots (Whiskers = 10th and 90th percentile) for serum concentrations of (a) b-carotene, (b) lycopene, and (c) lutein by fibrosis stage

* P < 0.05 compared to control group
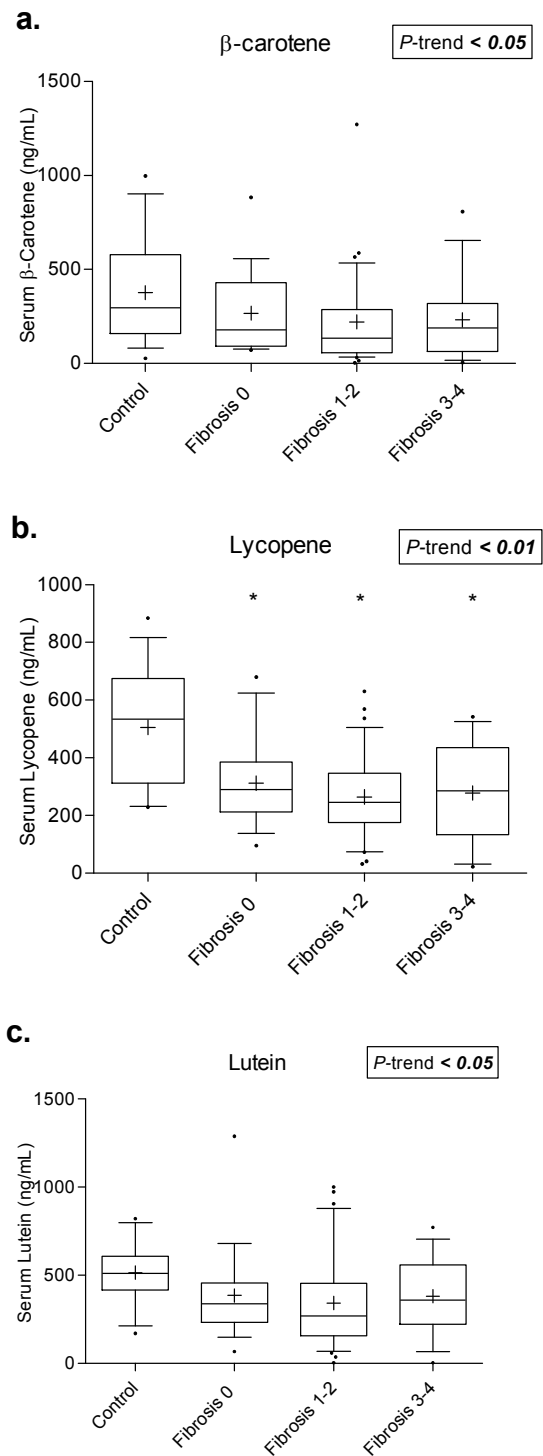
No relationships were observed for serum retinoids and carotenoids with either APRI or FIB-4 scores (**Table XIII – Apendix A**). However, RBP4 levels were inversely and significantly correlated with AST and ALT levels (r = -0.34, $P < 0.01$; r = -0.28, $P = 0.015$, respectively). Individual retinoid and carotenoid concentrations did not vary according to sex, BMI, smoking status, alcohol consumption, or insulin resistance. Of the 24 HCV participants who completed the RDR test, two of them had RDR values >20%, suggestive of a vitamin A deficiency. There was no apparent relationship between delta RDR retinol concentrations and retinyl palmitate concentrations in hepatic tissue (Spearman r = -0.03, $P = 0.92$) or fibrosis stage (P-trend = 0.55).

Hepatic retinyl palmitate levels were lower in control tissue samples, which were mostly obtained post-mortem, compared to diseased subjects (Wilcoxon $P = 0.04$). In contrast, hepatic lycopene and β-carotene concentrations were higher in controls compared to diseased subjects (Wilcoxon $P = 0.07$, $P = 0.34$, respectively). There was a very weak inverse relationship between hepatic retinyl palmitate concentration and fibrosis stage among all subjects ($P = 0.36$) **(Figure 8)**. Furthermore, hepatic β-carotene and lycopene concentrations showed a weak downward trend with increasing fibrosis stage. Hepatic retinyl palmitate was positively and significantly correlated with APRI, FIB-4, ALT, and AST (r = 0.27, $P = 0.03$; r = 0.29 $P = 0.02$; r = 0.30, $P = 0.015$; and r = 0.24, $P = 0.05$, respectively). These relationships were not observed for tissue carotenoids.
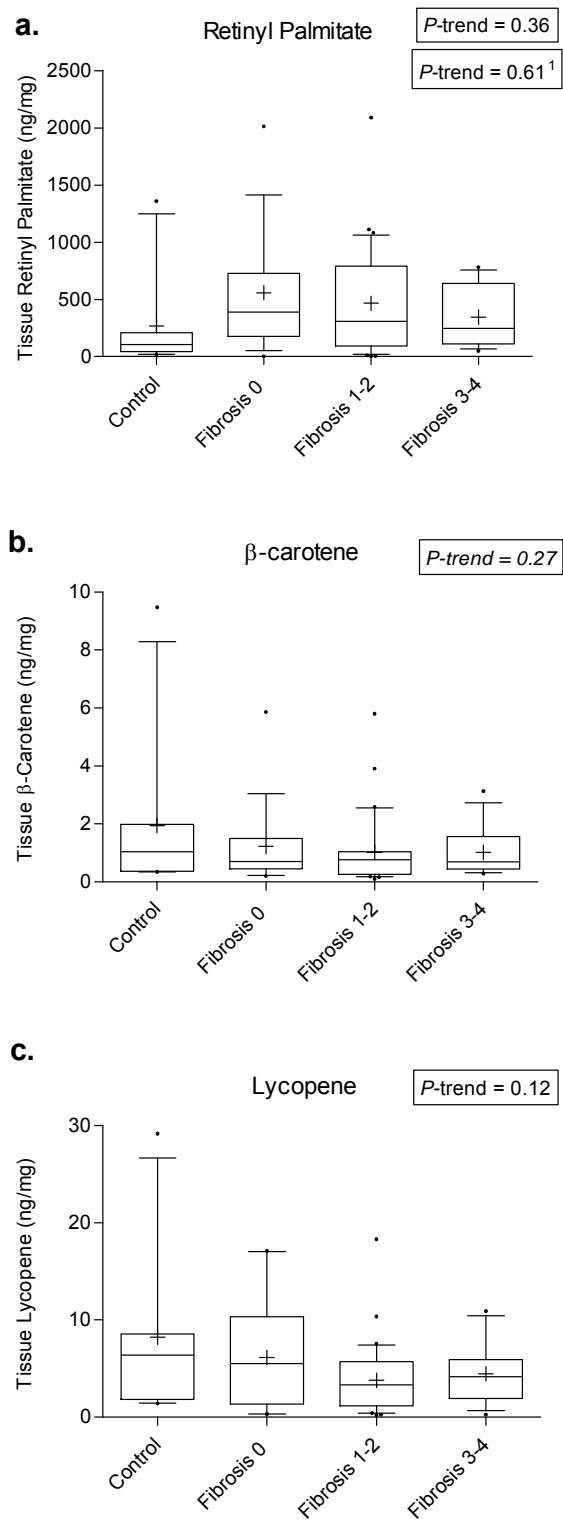
Figure 8. Boxplots (Whiskers = 10th and 90th percentile) for tissue concentrations of (a) retinyl palmitate, (b) β-carotene, and (c) lycopene by fibrosis stage. [1] Trend test including HCV subjects only

Parenchymal αSMA expression in hepatic tissue appeared to increase only among subjects with fibrosis 3-4 (**Figure 9**) (Wilcoxon $P = 0.12$).   αSMA expression was not associated with serum retinol concentrations (r = -0.03, $P = 0.79$). However, αSMA expression was inversely and significantly correlated with tissue retinyl palmitate concentrations (r = -0.31, $P = 0.013$) (**Figure 10**). This relationship was not observed for any other tissue carotenoids (**Table XIV – Appendix A**). In particular, hepatic lycopene levels were not correlated with αSMA expression (r = -0.03, $P = 0.81$).
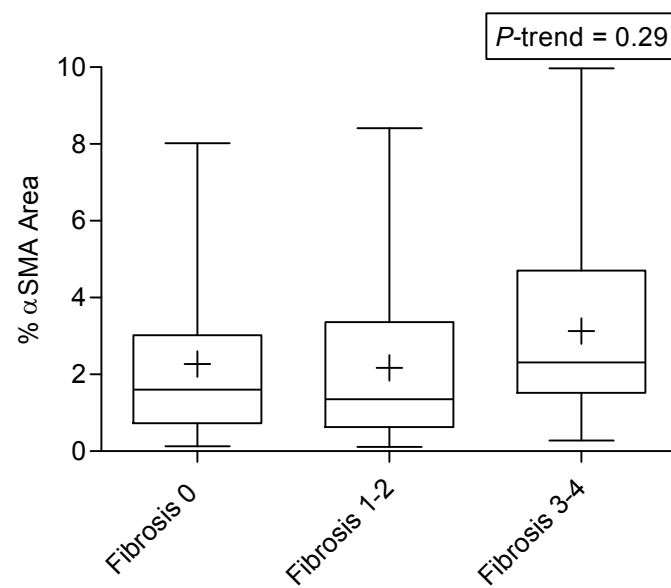
Figure 9. Boxplots (Whiskers = 10th and 90th Percentile) of percent protein expression of αSMA area by fibrosis group
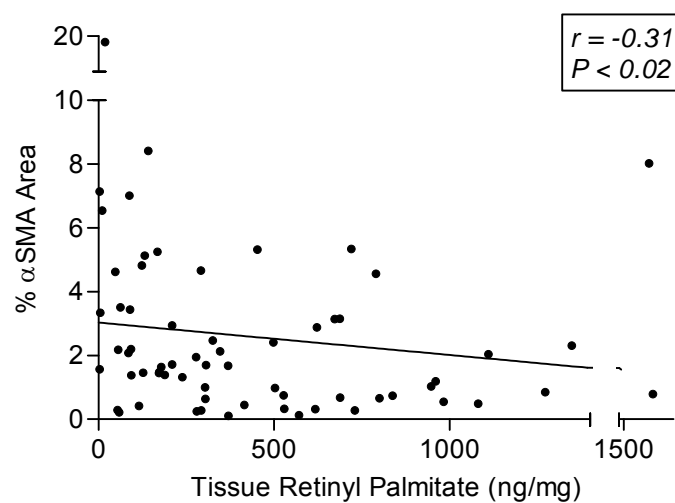
Figure 10. Scatterplot showing correlation (Spearman r) between hepatic retinyl palmitate and % marker area of αSMA protein expression (n=65)

Urinary isoprostane levels were positively and significantly associated with fibrosis stage (**Figure 11**). Serum retinol, β-carotene, and RBP4 concentrations were all inversely and significantly associated with urinary isoprostane concentrations. Tissue retinoid concentrations were not correlated with urinary isoprostane levels (**Table VIII**). However, both serum and hepatic lycopene were suggestively correlated (r = -0.18, $P = 0.12$; r = -0.22, $P = 0.09$, respectively).
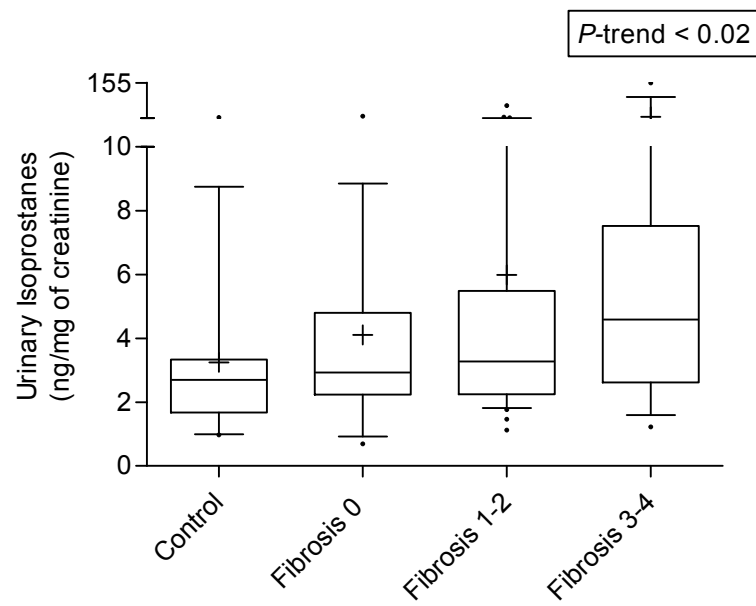
Figure 11. Boxplots (Whiskers = 10th and 90th Percentile) of urinary isoprostanes by fibrosis stage

TABLE VIII. Spearman correlations between urinary isoprostanes, serum and tissue retinoids/carotenoids

	Urinary Isoprostanes (ng/mg creatinine)	
Serum Retinoids/Carotenoids (ng/mL) (n=77)	r	*P*-value
Retinol	**-0.23**	**0.05**
Lycopene	-0.18	0.12
β-Carotene	**-0.22**	**0.05**
Lutein	-0.12	0.31
RBP4 (ug/L)	**-0.25**	**0.03**
Tissue Retinoids/Carotenoids (ng/mg) (n=60)		
Retinol	-0.05	0.72
Lycopene	-0.22	0.09
β-Carotene	-0.08	0.53
Retinyl Palmitate	-0.03	0.79

DISCUSSION

Depletion of vitamin A, lycopene, and β-carotene is widespread among patients with chronic HCV infection. This phenomenon appeared to occur early in the disease process, even before fibrosis is apparent, and cannot be explained, based on our results, by diet, obesity, alcohol intake, smoking, or insulin resistance. Inverse associations with fibrosis progression were more apparent for serum retinoids and carotenoids as opposed to hepatic levels, and were especially clear for serum retinol and RBP4. While we found relatively strong correlations between serum and liver tissue for lycopene and β-carotene, hepatic retinyl palmitate was poorly correlated with serum retinol, suggesting differential factors modulating these levels.(17, 18) It is also possible that declines in serum retinoids appear earlier in the disease process than declines in hepatic stores. We further observed that depletion of serum antioxidants is linked to increasing levels of urinary isoprostanes, which are reflective of systemic oxidative stress due to lipid peroxidation. An important finding was that hepatic retinyl palmitate levels were significantly and inversely associated with stellate cell activation, as measured by αSMA expression in liver biopsy specimens. Taken together, results from this cross-sectional analysis support the hypotheses that depletion of retinoid and carotenoid antioxidants occurs early in the disease process and that this depletion parallels an increase in oxidative stress and evidence of hepatic stellate cell activation.

In the present study, the reduced serum retinol levels associated with CLD progression were well above the widely accepted WHO cut-off point of 200 ng/mL for vitamin A deficiency. Moreover, only two of the HCV participants had a positive RDR test, indicating inadequate liver vitamin A reserve. Serum retinol and β-carotene levels in our HCV participants were also generally comparable to the NHANES III participants, a nationally representative sample of the US population.(30) Reduced dietary intake of retinoids and carotenoids do not appear to be responsible for the observed associations with fibrosis stage, which could be the result of diminished storage capacity, increased metabolism or defective mobilization of retinol due to impaired RBP4 synthesis.(15, 31, 32) In any event, a cross-sectional study such as this is unable to determine whether antioxidant depletion is a causal factor in fibrosis progression, or simply an epiphenomenon that accompanies progression.

The current study supports prior evidence that serum RBP4 concentrations are inversely related to disease severity in HCV patients.(33) (34) We observed a high correlation between serum RBP4 and retinol (r = 0.78, P < 0.001). Serum RBP4 measured by an enzyme immunoassay might be a feasible and cost-effective alternative for assessing vitamin A status. No association was detected between RBP4 levels and albumin, suggesting that generally decreased hepatic protein synthesis did not contribute to the reduction in RBP4. However, impaired mobilization of hepatic stores could explain the decrease of RBP4 seen in CLD. Increased serum RBP4 has been reported to contribute to insulin resistance associated with type 2 diabetes and obesity, which are possible risk factors for CLD progression.(35) However, we observed no correlation between RBP4 concentrations and BMI, glucose, or insulin levels. The possible effects of reduced RBP4 levels on insulin resistance among CLD patients might warrant further study.

A few studies have evaluated serum and tissue concentrations of dietary antioxidants in patients with CLD, although these have focused on more severe, later stage disease. The lack of correlation between serum retinol and tissue retinyl palmitate could be explained by impaired release of retinol from damaged hepatocytes or by the presence of homeostatic mechanisms that maintain hepatic retinol until vitamin A stores are severely depleted. We did not observe a definite decline in hepatic retinyl palmitate concentrations with increasing disease severity in earlier stages of CLD. Yadav et al. reported lower levels of retinyl esters in 20 HCV patients compared to controls.(17) In our study, however, the lower concentration in controls could have been caused by the degradation of retinyl palmitate into retinol in the cadaver tissue by endogenous esterase. Indeed, we observed higher levels of hepatic retinol in the control subjects. The reason for the positive relationship between hepatic retinyl palmitate and liver enzymes is unclear, but this relationship would also be consistent with mechanisms that favor retention of vitamin A in the presence of early but ongoing liver damage.

Lycopene cannot be converted to vitamin A and thus it presents an attractive potential alternative for antioxidant supplementation in CLD. Adverse effects have not been reported with consuming lycopene supplements or high amounts of lycopene-rich foods.(36, 37) We observed an inverse relationship of serum lycopene levels with fibrosis stage. Moreover, unlike vitamin A, there was also a strong correlation

between serum and hepatic lycopene levels, thus suggesting that dietary supplementation could result in higher hepatic lycopene concentrations. Yuan et al. demonstrated an inverse association between lycopene concentration in baseline serum and risk of developing HCC in China.(8) Lycopene is a potent carotenoid antioxidant that is also thought to affect processes related to mutagenesis, carcinogenesis, cell differentiation, and proliferation.(38, 39) Epidemiological data suggest that lycopene may act as a chemopreventive agent for many cancer types such as prostate, breast, and lung.(40) *In vitro* studies have observed that lycopene can inhibit proliferation mouse and human hepatocytes by inducing cell cycle arrest.(41) (42) However, dietary lycopene did not prevent liver cancer in a rat model of spontaneous hepatocarcinogenesis.(43) These discrepant results warrant further investigation.

Serum hs-CRP is synthesized by hepatocytes in response to inflammation and is regulated by pro-inflammatory cytokines such as IL-6. In a recent cohort analysis in China, higher serum CRP levels at baseline were associated with liver cancer incidence and death from CLD.(44) As such, we hypothesized that higher levels of hs-CRP would occur with increasing fibrosis stage. However, in this study, hs-CRP concentrations decreased with increasing fibrosis stage. While these findings are counterintuitive, a few smaller studies have suggested similar results.(45) (46) (47) Nasciemento, et al. observed lower hs-CRP to IL-6 ratio compared to controls, suggesting that IL-6 stimulation of CRP production in the liver might be mitigated by HCV.(45) Our data suggest that CRP production is affected early during the natural history of HCV infection, thus negating its utility as a biomarker of inflammation in this population.

F_2-isoprostanes are a sensitive and validated urinary marker of systemic oxidative stress due to lipid peroxidation. In our study, smokers had higher isoprostane levels compared to non-smokers. Our data indicate a positive association between urinary isoprostane concentrations and fibrosis stage. To our knowledge, this extends beyond previous data that have shown patients with CLD and cirrhosis have increased isoprostane levels compared to controls.(16) We further validate the validity of urinary isoprostanes as an indicator of systemic antioxidant status. As hypothesized, we observed a significant inverse association of F_2-isoprostanes with serum retinol, β-carotene, and RBP4 levels. However, tissue retinoid and carotenoid levels were not associated with urinary isoprostane levels with the possible

exception of lycopene. Urinary isoprostanes are a measure of total body lipid peroxidation, and therefore do not reliably reflect oxidative stress in the target organ

As we expected, subjects with moderate to severe fibrosis had higher levels of αSMA expression in hepatic tissue compared to subjects with mild fibrosis or none at all. Thus, it appears that HSC activation might not be apparent, at least by immunohistochemical assessment, until substantial fibrosis is present. Our data indicate that vitamin A depletion could occur prior to development of significant fibrosis and evidence of HSC activation. The hepatic vitamin A depletion might be attributable to either loss of storage capacity or increased consumption via autophagy. Increased metabolism of vitamin A droplets through an autophagic mechanism may generate substrates needed for energy intensive pathways to meet the metabolic demands of proliferation, fibrogenesis, and contractility.(48)

This is the first comprehensive analysis to examine the interrelationships among dietary, serum, and hepatic levels of retinoids and carotenoids with early progression of disease in HCV patients. The present study, to our knowledge, is the largest to date to quantitate hepatic retinoids and carotenoids. In addition, the study benefitted from quantitative image analysis that provided objective and reproducible measures of αSMA expression in liver biopsy specimens. However, certain limitations are acknowledged including a limited sample size for specific subgroups and a cross sectional design that cannot be used to infer causal direction. Unfortunately, we were able to administer the RDR to only few patients due to difficulties to obtain the additional blood sample required five hours after the initial retinyl ester dose. Additionally, the post-mortem tissue samples of normal liver were not ideal as they could have undergone artifactual changes in retinoid and carotenoid concentrations.

Findings from this study could have implications regarding the emerging epidemic of obesity-related non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH).(49) Oxidative stress is believed to play a key role in these disease processes, similar to HCV-related liver disease. Currently, there are no specific therapies for NAFLD beyond dietary modifications and exercise. Lower levels of retinoids have been implicated in NAFLD. Transgenic mice with impaired retinoid signaling develop steatohepatitis and eventually HCC.(23) It has also been found that NAFLD patients have higher levels of oxidative stress and lower vitamin A intake, independent of metabolic syndrome status, suggesting that

adequate vitamin A intake is important in protecting against oxidative stress in NAFLD patients.(50) In conclusion, our data highlight the potential importance of dietary retinoids and carotenoids as modifiers of progression in early chronic liver disease and support the need for further research leading to well-designed intervention studies.

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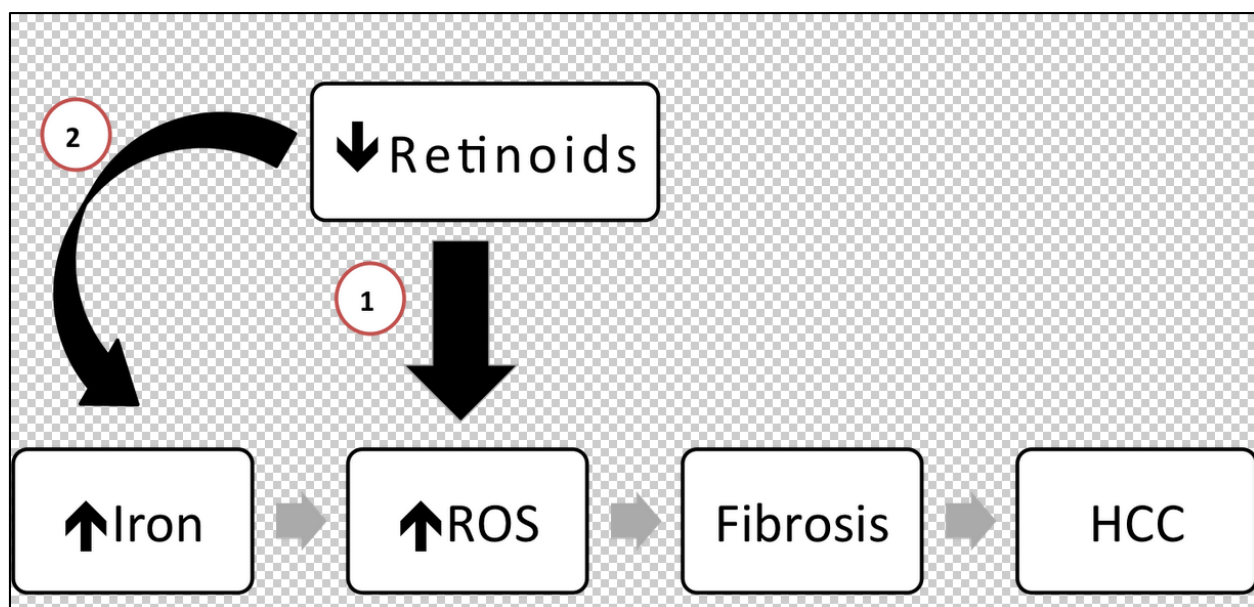# Chapter 4 – Joint Relationship between Iron and Retinoids

ABSTRACT

Approximately 2.3 million Americans are chronically infected with hepatitis C virus (HCV). HCV patients

with cirrhosis form the largest group of persons at high risk for hepatocellular carcinoma (HCC).

Increased oxidative stress is regarded as a major mechanism of HCV-related liver disease progression.

We demonstrated depletion of retinoids and carotenoids occurs early in HCV related chronic liver

disease, even before fibrosis is apparent. Elevated serum and hepatic iron are also relatively common in

HCV patients. It is plausible that free radicals generated from excess iron may further exacerbate

damage by depleting antioxidants. This study investigates the relationship between iron and retinoids in

CLD patients. We hypothesized that patients with more advanced fibrosis would be more likely have

lower retinoids and higher iron concentrations. Our results do not support the hypothesis that individuals

with relatively advanced fibrosis are more likely to have low retinol and high ferritin. Subjects with low

retinol and high iron concentrations did not have higher levels of oxidative DNA damage biomarker than

persons with one or neither factor. Overall, HCV infection is generally associated with lower serum

retinoids/carotenoids levels but also higher ferritin concentrations; however, we do not observe a

relationship with fibrosis stage.

INTRODUCTION

Approximately 2.3 million Americans are chronically infected with HCV, forming the largest group of

persons at high risk for HCC. Increased oxidative stress is regarded as a major mechanism of

progression of chronic liver disease (CLD) and subsequent HCC. Deficiencies of retinoids and carotenoid

antioxidants have been reported in patients with advanced CLD.(1) In chapter 3, we demonstrated that

depletion of Vitamin A, lycopene, and β-carotene is widespread among patients with early chronic HCV

infection early in the disease process, even before fibrosis is apparent. Additionally, serum retinol and β-

carotene were inversely associated with liver cancer incidence and subsequent death from CLD in a large

cohort study.(2) Elevated serum and hepatic iron are also relatively common in HCV patients.(3) It is plausible that free radicals generated from excess iron may further exacerbate damage by depleting antioxidants **(Figure 12)**. However, the relationship between iron and retinoids in CLD remains to be clarified in human populations.

Figure 12. Proposed joint relationship between iron and retinoids/carotenoids

Liver is the predominant site for iron storage, and excess iron facilitates the production of reactive oxygen species (ROS) thus making the liver vulnerable to iron induced damage. Iron reacts with hydrogen peroxide to generate hydroxyl radicals (i.e., Fenton reaction) which then cause oxidative damage to lipids, proteins, and nucleic acids. Modest increases in serum and hepatic iron levels are known to exacerbate liver injury and hepatic fibrosis progression.(4, 5) (6, 7) (8-10) Excess iron may contribute to the inflammatory microenvironment thereby disrupting normal liver function. Furutani et al. have shown that transgenic mice expressing the HCV polyprotein fed an excess iron diet had greater levels of hepatic lipid peroxidation, DNA damage and increased risk for HCC compared to controls.(11)

Iron metabolism is tightly regulated and slight changes in iron metabolism can have severe consequences. Iron in circulation is bound to transferrin, which delivers iron to extra-hepatic tissue. Transferrin saturation reflects the percentage of transferrin protein bound by iron. In normal individuals, transferrin is approximately 15-50% saturated with iron, corresponding to serum iron levels of 49-181 mcg/dL and 37-170 mcg/dL in men and women, respectively. Iron is stored in hepatocytes and reticuloendothelial macrophages as ferritin. Lower levels of ferritin are present in the serum and is an indicator of total body iron stores. Normal serum ferritin concentrations are 10-259 ng/mL and 5-116 ng/mL for men and women, respectively. Serum iron biomarkers (iron, ferritin, and transferrin saturation) are elevated above the clinically normal range in approximately 40% of HCV patients.(4, 12) (13)

Iron depletion therapy is utilized in iron overload diseases, such as hereditary hemochromatosis. Hemochromatosis patients regularly treated with iron depletion (maintaining a serum ferritin of at most 10ng/ml) had less hepatic inflammation and slower fibrosis progression.(14) Moreover, several groups have demonstrated that iron depletion in HCV patients reduces liver enzymes, lipid peroxidation, and oxidative stress.(15-17) Iron depletion combined with a low iron diet has also been found to decrease hepatic oxidative damage to DNA, decrease hepatic fibrosis grade, and decrease HCC risk compared to subjects who declined treatment.(8) Together these findings indicate the importance of maintaining normal iron levels in HCV patients.

Only one study to date has examined the combined relationship between retinoids and iron in fibrosis progression. Tsuchiya et al. fed wild type mice three different types of diet: normal, retinoid deficient

(diet devoid of vitamin A), and retinoid excess (diet + vitamin A), followed by daily intraperitoneal administration of 1 mg of dextran iron for five days.(18) Iron content was significantly lower in the liver of the high retinoid diet group compared to the retinoid deficient mice. While this study has inherent limitations such as IP administration of iron that will first enter the liver the results suggest that retinoid may decrease iron content in the liver and therefore could suppress iron induced oxidative stress. These findings emphasize the importance of elucidating the effects of retinoid depletion on iron metabolism in CLD patients. Correction of retinoid or carotenoid deficiency via altered diets and/or iron reduction in high-risk patients may be safe and attractive options for preventing the progression of CLD due to HCV or non-alcoholic fatty liver disease (NAFLD).

To address the joint relationship of iron and retinoids in early CLD progression, we conducted a cross-sectional study in HCV patients. This study aimed to: a) quantify the correlation between serum iron and retinoid concentrations in CLD and b) examine the joint effects of retinoids and iron on CLD. We hypothesized that patients with more advanced fibrosis would be more likely have lower retinoids and higher iron concentrations.

MATERIALS AND METHODS

Study population. We conducted a cross-sectional study among patients with HCV at the University of Illinois at Chicago (UIC) and University of Chicago (UC). A total of 91 subjects were included in this study. We consented and enrolled patients with confirmed HCV undergoing percutaneous liver biopsy (n = 69) who provided fasting blood, fresh tissue, urine, and completed a diet history questionnaire. Liver histology was staged into F0-4 according to the Batts-Ludwig scoring system. Portal inflammation, periportal inflammation and lobular inflammation grading were also recorded. Subjects with Fibrosis 0 were categorized as having no fibrosis. Subjects with Fibrosis stage 1-2 and Fibrosis stage 3-4 were categorized as mild/moderate and severe fibrosis, respectively.

We collected serum, urine, and questionnaire data from healthy volunteers (n=11). We obtained post-mortem normal liver tissue from the Cooperative Human Tissue Network (CHTN) repository (n = 8). The

remaining controls included patients with no chronic liver disease (n = 3) who provided serum, tissue, urine, and questionnaire data. The UIC and UC Institutional Review Boards approved the study.

Dietary assessment. The National Cancer Institute Diet History Questionnaire I (DHQ) was completed by all participants who provided serum. Participants reported their frequency of intake and portion size of 124 food items over the past year. The DHQ was subsequently analyzed by the Diet*Calc Analysis Program (Version 1.4.3. National Cancer Institute, Applied Research Program). The program generated nutrient and food group intake estimates based on frequency and portion sizes. The approximate iron intake was calculated from the DHQ.

Serum iron panel measurements. The clinical pathology laboratories at UIC measure serum iron, transferrin, and ferritin levels. Serum iron and transferrin were measured on the SYNCHRON LX System® (California, USA) by a timed-endpoint method and a turbidimetric method, respectively. Serum ferritin was measured as a two-site immunoenyzmatic assay. Based on blinded duplicates from a quality control pool of serum, intra-batch coefficients of variation (CVs) aliquots for fasting serum iron, transferrin, and ferritin were < 0.1%, 1.47%, and < 0.1%, respectively.

Tissue iron measurements. Two pilot studies were conducted to determine the reproducibility of iron levels in 4-5mm from 18-gauge biopsy samples. The first pilot study included the following samples: replicates FFPE biopsy tissue from two different patients with HCV, 18 meg-ohm water, paraffin, nitric acid, nitric acid treated eppendorf vials, and National Institute of Standard Technology (NIST) bovine liver. The NIST bovine liver is considered a gold standard as it has a known quantity of iron (184 µg/g). CVs for the HCV patient replicates were 13% and 131%. NIST bovine liver iron concentration values were precise and reproducible (mean = 184.02 µg/g, CV = < 0.1%). The high CVs for the small biopsy samples indicate that tissue volume normalization to mass on a small volume of tissue is a concern. A second pilot study was designed to test normalization of amino acid concentration via a ninhydrin assay (i.e. amino acid concentration) and to cross-validate iron content in the same FFPE biopsy from the same patient with a CLIA certified lab. Results from the second pilot study revealed that iron content varied tremendously in small tissue volumes (CV range: 17%-100%). Furthermore, ranking also varied among normalization methods. It is likely that the tissue heterogeneity contributed to the variance observed.

Ultimately, due to the small tissue sizes available to us, we were unable to overcome sample volume and tissue heterogeneity for hepatic iron quantitation.

Immunohistochemistry. Tissue sections of 4 µm each were placed on charged slides, dehydrated, and then deparaffinized. Heat-induced epitope retrieval was done with low pH buffer for 10 minutes. Immunostaining was carried out using a BondRX (Lecia Biosystems, Buffalo Grove, IL) autostainer with a primary mouse monoclonal antibody for 8OHdG (JAICA, Clone N45.1) in a 1:600 dilution for 30 minutes at room temperature. The sections were then incubated in rabbit anti-mouse IgG (Bond Polymer Refine Detection, Leica Biosystems) for 20 minutes. Color reaction was developed using diaminobenzidine (DAB) as the chromagen followed by counterstaining with hematoxylin. Positive and negative controls were included in each batch.

Slides were scanned at 200x on an Aperio ScanScope® CS, a whole-slide digital microscope (Leica Biosystems, Elk Grove, IL). A digital draw tool was used to distinguish hepatic parenchymal areas from large vessels, inflammation, and artifacts (e.g. folds, debris, etc.) which were excluded from the analysis. Definiens Tissue Studio® 3.6.1 (Definiens, Munich, Germany), a digital image analysis platform, was used to measure the nuclear staining of 8OHdG within the hepatic parenchyma (**Figure 23 – Appendix A**).

Statistical analysis. Frequency distributions of dietary intake, 8OHdG levels, and iron biomarker concentrations were examined for normality. Scatterplots and Spearman rank correlation coefficients were used to examine the relationships among the variables of interest. A *P*-value of < 0.05 was considered statistically significant, and all tests were two-sided. Analyses were performed using SAS Version 9.4 (SAS, Inc., Cary, NC).

RESULTS

Demographics of the patient population are shown in **Table VI** (Chapter 3). Dietary iron intake did not differ between controls and HCV subjects and it remained similar with increasing fibrosis stage with no

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apparent trend (**Figure 13**). Iron intake also did not differ by predictive markers of fibrosis (APRI and FIB-

4). Mean dietary iron intake was 26.3 and 16.9 mg for men and women, respectively. In comparison,

these values were above the mean iron intake from the NHANES III data (18.1 and 13.5 mg, for men and

women, respectively). Iron intake levels were not associated with age or insulin levels (r = -0.09, $P =$

0.48; r = 0.41 $P < 0.02$; r = 0.14, $P = 0.29$; respectively).
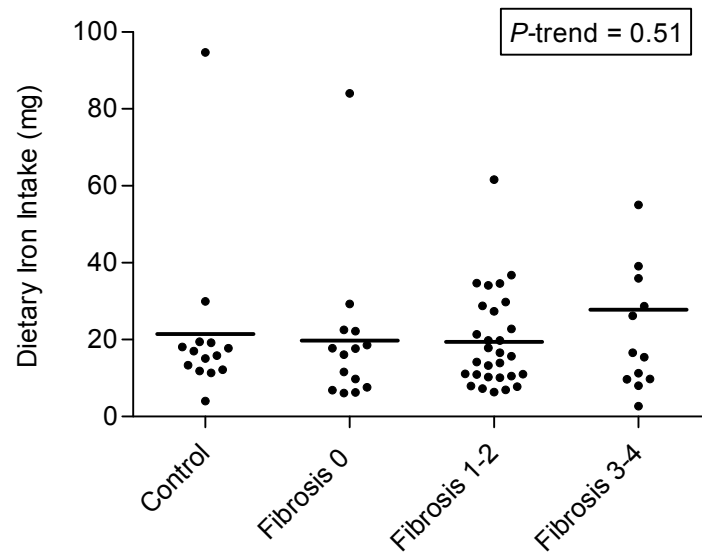
Figure 13. Plot of total iron intake (mg) by fibrosis stage (line = mean)

Dietary iron intake did not correlate with serum iron, transferrin saturation or ferritin concentrations (r = 0.03, $P = 0.83$; r = 0.01 $P = 0.92$; and r < 0.01, $P = 0.95$, respectively) in this well-nourished population. Similar to the NHANES III population, serum iron and transferrin saturation were modestly higher in HCV subjects compared to control subjects.(3) However, serum ferritin levels were significantly higher in all HCV subjects compared to controls (Wilcoxon: $P < 0.02$), and a subset of the patients had levels above the clinically normal range (43%). However, no significant linear trends were observed with fibrosis stage for any of the serum iron biomarkers **(Figure 14)**. We measured three serum iron biomarkers, but observed no relationship between any variables of interest with serum iron and transferrin saturation. Therefore, results presented from here on will focus on serum ferritin.
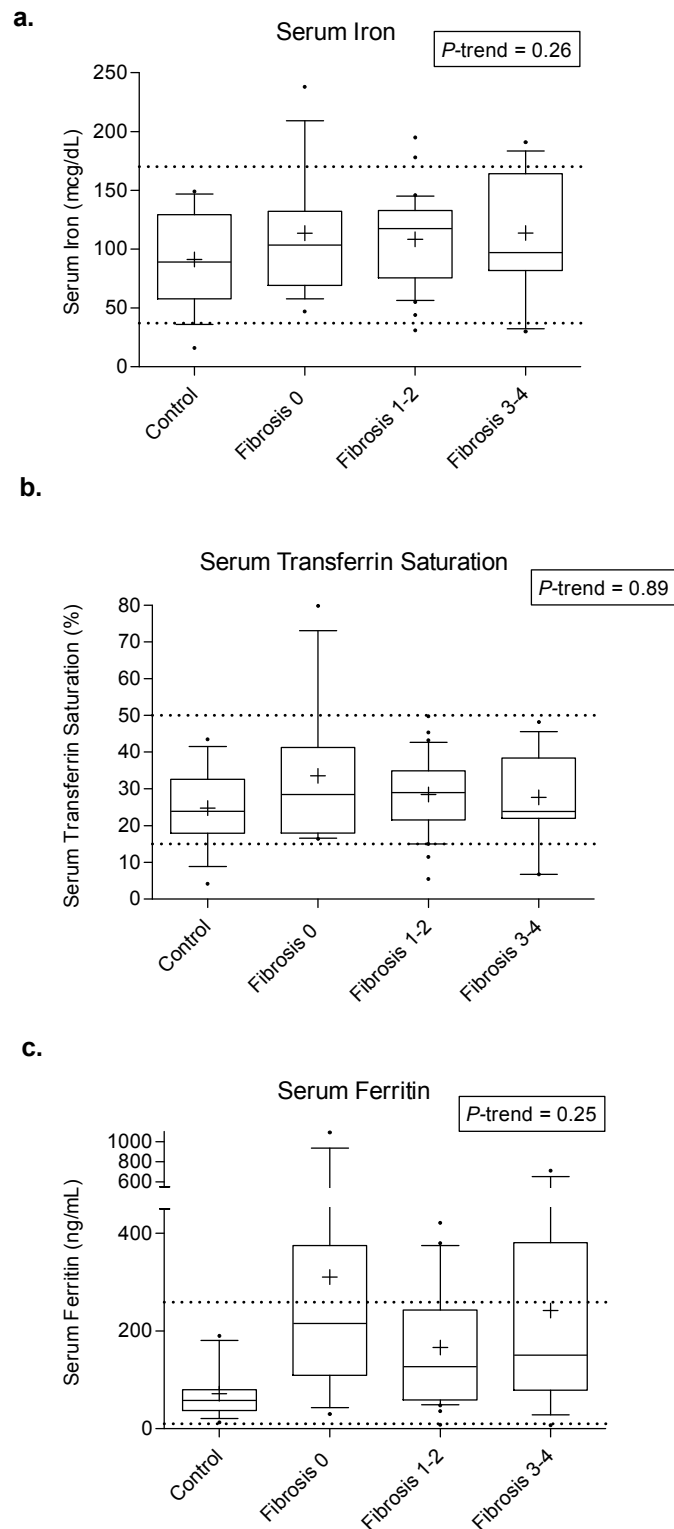
Figure 14. Boxplots (Whiskers = 10th and 90th percentile) for serum concentrations of (a) iron, (b) transferrin saturation, and (c) ferritin by fibrosis stage. Dotted lines represent clinical normal ranges

Serum ferritin was positively and significantly correlated with AST, ALT, and APRI (r = 0.35, *P* < 0.01; r = 0.41 *P* < 0.02; r = 0.24, *P* = 0.05; respectively). Serum ferritin did not significantly vary by age, smoking history, or BMI (r = 0.15, *P* = 0.20; P = 0.47 Wilcoxon; r = 0.08, *P* = 0.50; respectively). Mean female and male serum ferritin concentrations were 169 and 224 ng/mL, respectively; however there was no significant difference (Wilcoxon *P* = 0.64).

Serum ferritin was inversely associated with both serum lycopene and β-carotene levels **(Table IX & Figure 15).** No relationships were observed between serum ferritin, hepatic tissue retinoid, and carotenoids with the possible exception of an inverse association between serum ferritin and hepatic lycopene levels (r = -0.23, *P* = 0.07) **(Table X)**. **Figure 16** shows serum ferritin levels by fibrosis stage in low and high lycopene groups. Generally, serum ferritin levels were lower in the high lycopene group compared to the low lycopene group, especially in the Fibrosis 3-4 group. A contingency table analysis revealed that in the low lycopene and high ferritin group the proportion of subjects with Fibrosis 3-4 was 17%, whereas, in the high lycopene and low ferritin group the proportion of subjects with Fibrosis3-4 was 28%, a difference that was not statistically significant (Fishers Exact, *P = 0.26).*

**TABLE IX. Spearman correlation coefficients between biomarkers of iron, serum retinoid and carotenoid (n=77)**

	Serum iron (mcg/dL)		Serum ferritin (ng/mL)		Serum transferrin saturation (%)	
	r	p-value	r	p-value	r	p-value
Retinol, ng/ml	0.04	0.76	0	0.98	0.01	0.94
Lycopene, ng/ml	-0.11	0.34	**-0.29**	**0.01**	-0.19	0.09
β-carotene, ng/ml	-0.06	0.58	**-0.29**	**0.01**	-0.11	0.35
Lutein, ng/ml	0.01	0.9	-0.11	0.34	-0.07	0.52
RBP4, ug/ml	-0.13	0.26	-0.11	0.41	-0.13	0.24
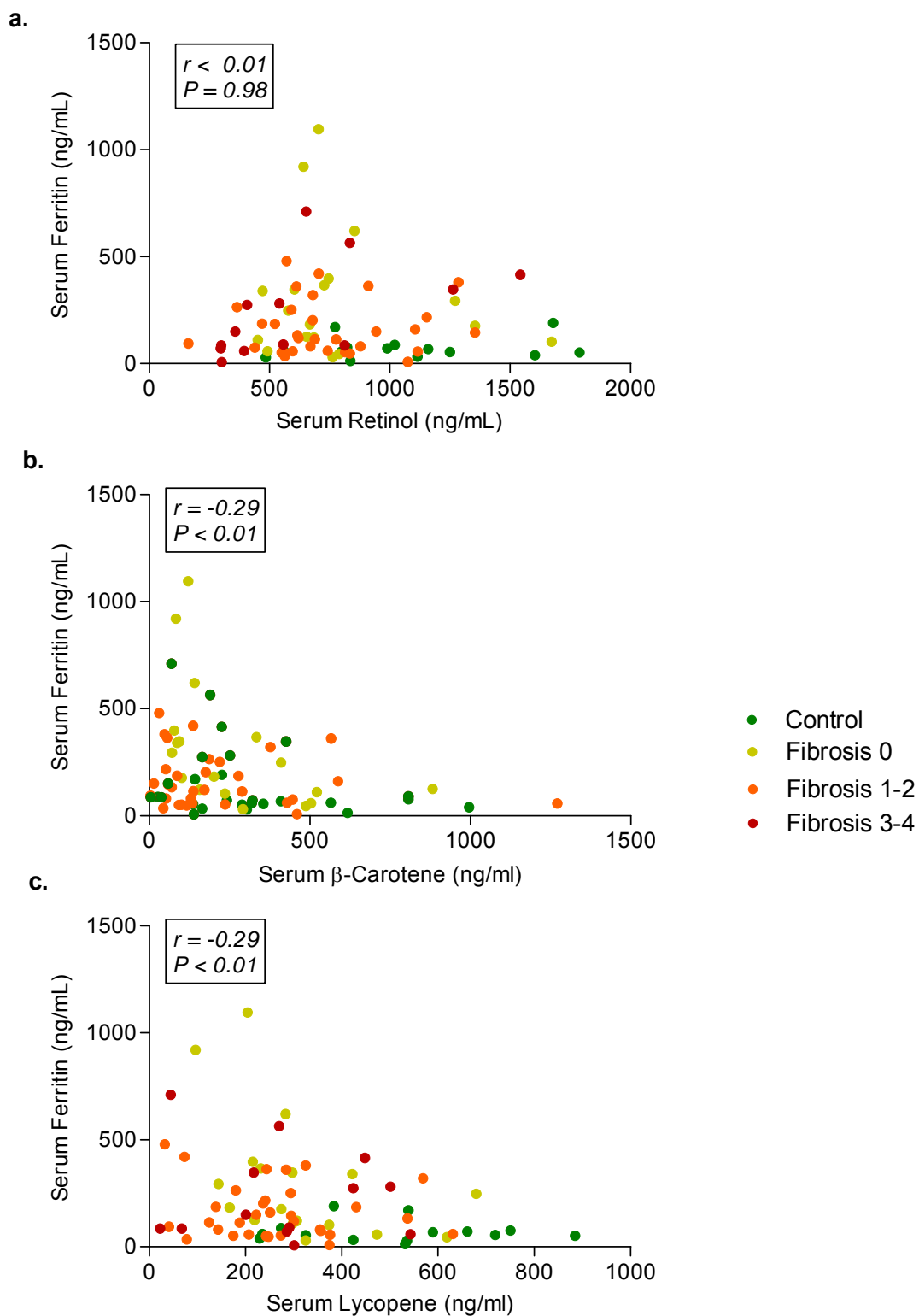
Figure 15. Scatterplots showing correlations (Spearman r) between serum ferritin and (a) serum retinol (n=77), (b) serum b-carotene (n=77), and (c) serum lycopene (n=77)

TABLE X. Spearman correlation coefficients between biomarkers of iron, tissue retinoid and carotenoid (n=60)

	Serum Iron		Serum Ferritin		Transferrin Saturation	
	(mcg/dL)		(ng/mL)		(%)	
	r	p-value	r	p-value	r	p-value
Retinyl Palmitate, g/mg	0.18	0.17	0.10	0.46	0.12	0.34
Lycopene, ng/mg	-0.09	0.51	**-0.23**	**0.07**	-0.15	0.24
β-carotene, ng/mg	-0.04	0.73	-0.19	0.16	-0.07	0.57
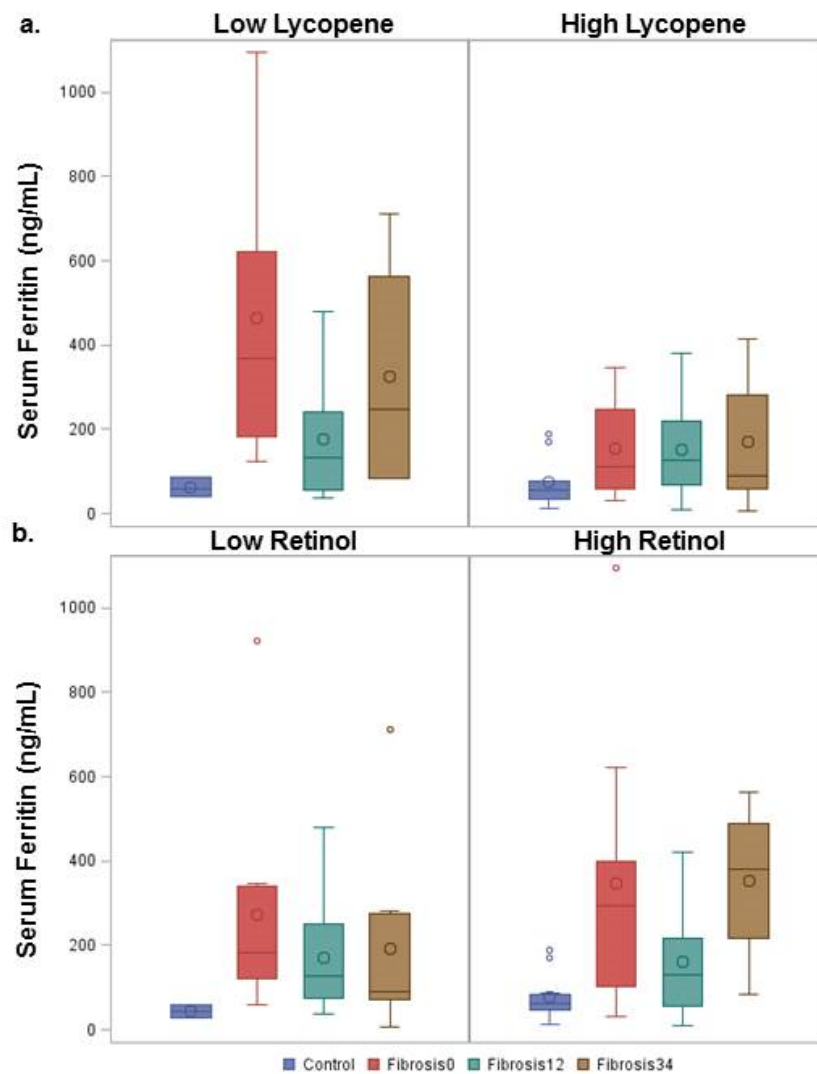
Figure 16. Boxplots of serum ferritin by median split a. lycopene b. retinol concentrations

Figure 16 shows ferritin levels by fibrosis stage in low and high retinol groups. Serum ferritin levels were similar in the low and high retinol groups with the possible exception of Fibrosis 3-4. Fibrosis 3-4 subjects in the low retinol group had lower serum ferritin levels compared to Fibrosis 1-2. However, Fibrosis 3-4 subjects in the high retinol group had higher serum ferritin levels compared to Fibrosis 1-2. A contingency table analysis revealed that in the low retinol and high ferritin group the proportion of subjects with Fibrosis 3-4 was 19%. In contrast, in the high retinol and low ferritin group the proportion of subjects with Fibrosis 3-4 was 9% **(Table XI),** a difference that was not statistically significant (Fishers Exact, *P = 0.30).*

Ferritin							
	Low				**High**		
	17				21		
	Control	HCV (n =15)			Control	HCV (n =21)		
Low		Fib 0	Fib1-2	Fib3-4		Fib 0	Fib1-2	Fib3-4
	2	2	8	5	0	7	10	4
	21				8		
	Control	HCV (n =11)			Control	HCV (n =16)		
High		Fib 0	Fib1-2	Fib3-4		Fib 0	Fib1-2	Fib3-4
	10	3	7	1	2	6	7	3

Retinol

**TABLE XI.** Frequency of subjects in median split retinol and ferritin by fibrosis stage

There was no difference in hepatic 8OHdG levels between HCV and control subjects (Wilcoxon $P = 0.72$).

However, 8OHdG levels based on strongly positive nuclei was increased only among subjects with

Fibrosis 3-4 (**Figure 17**). 8OHdG levels were not associated with serum or tissue retinoid and carotenoid

concentrations and did not correlate with any of the serum iron biomarkers (**Table XV – Appendix A**).

However, as shown in **Figure 18,** 8OHdG levels were positively and significantly associated with αSMA
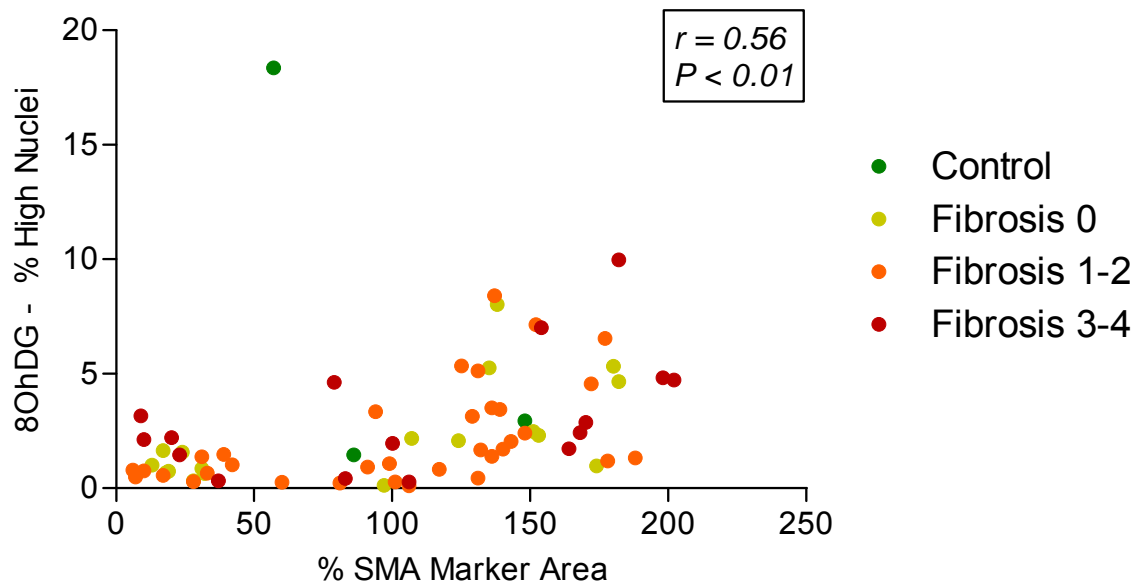
expression (r = 0.55, $P < 0.01$).

a.
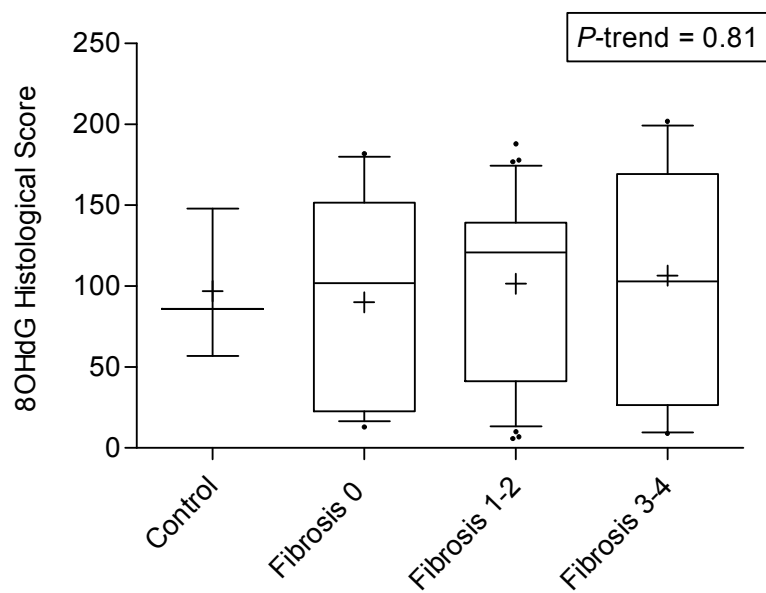
b.
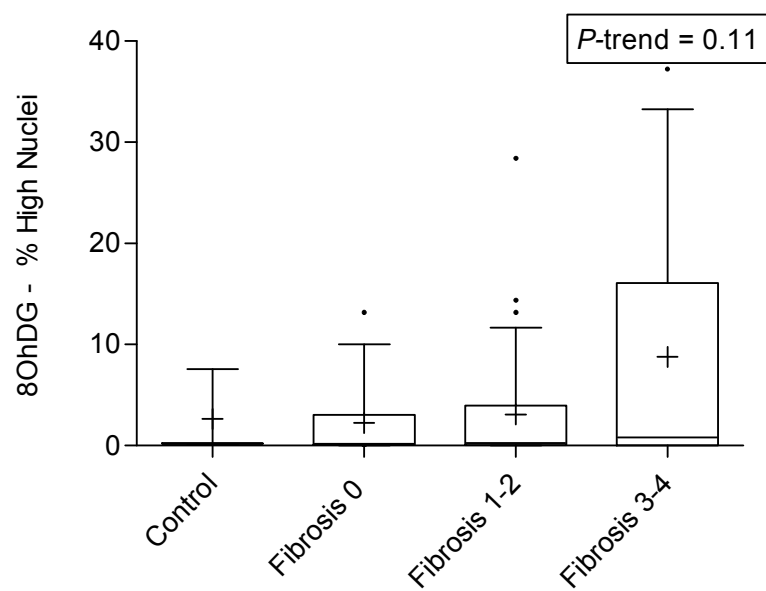
Figure 17. Boxplots (10th and 90th Percentile) of (a) histological score of 8OHdG levels by fibrosis severity (b) strong 8OHdG positive nuclei by fibrosis stage

Figure 18. Scatterplots showing correlations (Spearman r) between 8OHdg- % High Nuclei and % SMA Marker Area

DISCUSSION

This is the first study, to our knowledge, to investigate the joint relationship of retinoids and iron in HCV subjects. Our results do not support the hypothesis that individuals with relatively advanced fibrosis are more likely to have low retinol and high ferritin. Subjects with low retinol and high iron concentrations did not have higher levels of oxidative DNA damage biomarker than persons with one or neither factor. Hepatic 8OHdG levels of strongly positive nuclei was higher in Fibrosis 3-4 subjects compared to other HCV subjects; this indicates a threshold effect. 8OHdG levels were positively and significantly correlated with αSMA expression. Overall, HCV infection is generally associated with lower serum retinoids/carotenoids levels but also higher serum ferritin concentrations; however, we do not observe a relationship with fibrosis stage.

We hypothesized that the relationship of ferritin concentration to fibrosis stage was dependent on retinol concentrations in that, specifically, ferritin levels would increase with fibrosis stage when retinol concentrations were low. However, we observed that subjects with low retinol and high ferritin levels are not statistically more likely to have advanced fibrosis. In fact, Fibrosis 3-4 subjects with low retinol tend to have lower serum ferritin levels compared to Fibrosis1-2, whereas, Fibrosis 3-4 subjects with high retinol had higher serum ferritin levels compared to Fibrosis 1-2. This observation is the opposite of the hypothesized relationship suggested by the experimental data from Tsuchiya et al. The absence of a biological interaction between retinol and ferritin may be attributable to the fact that our population consisted of patients with mostly mild disease as opposed to severe liver disease.

We further examined the joint relationship of iron with other antioxidants specifically, β-carotene and the non pro-vitamin A carotenoid lycopene. The joint relationship between serum ferritin and β-carotene was similar to the relationship observed between serum ferritin and retinol. However, we observed that subjects in Fibrosis 3-4 with high lycopene had lower serum ferritin levels compared to Fibrosis 3-4 subjects with low lycopene. Additionally, we observed an inverse correlation of serum ferritin with serum lycopene and β-carotene concentrations. Hepatic lycopene levels were also inversely associated with serum ferritin levels. This relationship is possibly due to the ability of retinoids and carotenoids to scavenge free radicals and quench ROS.(19-21) Furthermore, lycopene has shown to be protective

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against DNA oxidation, lipid peroxidation and histopathological changes in liver damage induced by ferric nitrilotriacetate (Fe-NTA).(22) Although there is no direct evidence of lycopene lowering serum ferritin concentrations, hemochromatosis patients who underwent iron depletion therapy had significantly increased serum vitamin A concentrations compared to pretreatment levels (1360 ng/mL vs. 3000 ng/mL, pre-treatment, post-treatment respectively).(23) (24) Thus, lycopene continues to be an attractive option for chemoprevention in CLD.

Our findings confirmed a previously reported modest elevation of serum iron biomarkers in HCV subjects compared to healthy controls.(8) The observed elevation was not attributable to confounding factors such as diet, age, gender, BMI, insulin resistance or alcohol consumption. The positive association observed between serum ferritin and liver enzymes suggested that ferritin might be a marker HCV induced disease. It is possible that iron facilitates viral replication in hepatocytes or that cells with the virus tend to accumulate more iron.

It is plausible that retinoid depletion and iron deposition in CLD are linked via hepcidin. The liver regulates iron homeostasis largely by modulating expression of hepcidin in hepatocytes. Hepcidin binds to ferroportin, an iron efflux protein and facilitates its degradation, thus preventing the release of iron from cells. It has been reported that serum hepcidin and hepatic hepcidin expression are significantly lower in HCV patients compared to controls.(25-28) This suggests that low hepcidin expression is involved in iron overload in HCV. Additionally, Tsuchiya et al. found evidence in animal models to suggest that retinoids suppress hepcidin expression, accompanied by lower hepatic iron concentrations.(29) Moreover, vitamin A deficiency has been shown to increase liver hepcidin mRNA expression in rats, resulting in an increase in splenic and hepatic iron concentrations. It appears that vitamin A helps maintain iron homeostasis by regulation of hepatic hepcidin expression.(30)

Hepatic 8OHdG levels have been shown to be a risk factor for HCC development in CLD.(31, 32) Similar to Fujita et al. but contrary to Tanaka et al., we observe no linear trend of 8OHdG levels with fibrosis stage.(5, 33) Also contrary to our results, Tanaka et al. and others have observed a positive association between serum iron levels and 8OHdG levels. Tanaka et al. had a relatively higher proportion of severe fibrotic patients compared to our study population (53% vs. 25%), this could influence the association

between 8OhdG and serum ferritin levels.(31) Additionally, the difference in disease severity may explain why we found marked 8OHdG levels markedly increased only in the more advanced fibrosis group. A threshold effect may exist whereby a prerequisite amount of damage must occur before a change in 8OHdG levels is observable. Quantitation of 8OHdG levels is a function of arbitrary thresholds set by the observers, thus this signifies the importance of examining the intensity of the 8OHdG levels in the nuclei. The positive association observed between αSMA and 8OHdG levels suggests involvement of oxidative damage in the pathogenesis and progression of HCV. However, the underlying mechanism orchestrating hepatic stellate cell activation and nuclear damage is not clear.

The present study is, to our knowledge, is the first to assess the joint effect of retinoid and iron on CLD patients. We utilized a monoclonal antibody (N45.1) against 8OHdG that has been widely used to evaluate oxidative DNA damage in animal and human tissues. Our study also benefitted from quantitative image analysis. However, certain limitations are acknowledged including the limited sample size within subgroups, and a cross sectional design that cannot identify reverse causality. The number of patients available to evaluate iron and retinoid interaction in the Fibrosis 3-4 group was small (n=14). Although, statistical modeling was also conducted to assess the interaction between retinoid and ferritin on CLD, the data were too sparse to draw any conclusions. Lastly, we were not able to successfully quantitate hepatic iron levels via ICP-MS due to the small tissue sample volume available.

Further studies are needed to provide insight into the apparent reciprocal interrelationship between iron and retinoids in CLD. These studies should consider investigating the retinoid/carotenoids status in individuals heterozygous for the hemochromatosis (HFE) gene who are known to have markedly elevated serum ferritin levels. Findings from our study could also have implications for the emerging epidemic of NAFLD. Tsuchiya et al., in a cross-sectional study, reported that dysregulation of retinoid metabolism genes in NAFLD patients with iron accumulation.(29) In conclusion, levels of retinoid/carotenoids were generally lower in HCV subjects and serum ferritin levels were higher, but they were not associated with fibrosis stage.

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Chapter 5 - DISCUSSION

This work has concentrated on understanding aspects of the relationship between diet and cancer risk by investigating the underlying pathophysiological mechanisms. The projects in this thesis have focused on intermediate biomarkers of disease specifically AMACR in normal prostate and levels of retinoid/carotenoids in liver. Use of these biomarkers comes with a specific set of challenges. (1-3)

The first among these challenges involves patient recruitment and obtaining biological samples, especially human tissue for biomarker quantitation. The amount of effort and coordination required by referring physicians, research staff and the patient is substantial. Patients and healthcare providers are often concerned about providing tissue samples above the minimum required for diagnosis. Additional coordination between the provider and research staff is often required to ensure that the necessary biological samples have been collected and examined for relevant clinical outcomes. Difficulties in doing so may limits the material available for research.

Second, obtaining "normal" tissue is also proven to be difficult. Studying normal tissue biology is crucial as it provides insight into the etiology and progression of disease. AMACR overexpression is well established in prostate cancer, but, it is not well characterized in normal prostate tissue. Likewise, retinol content of the normal hepatic tissue is not well established making it harder to interpret levels observed in the liver of HCV patients for establishing future intervention guidelines. Additionally, while serum ferritin levels in HCV patients are frequently elevated, but within the clinical normal range they are reported to be an independent predictor of fibrosis progression.(4) Thus, better definition of "normal" helps identify high-risk populations for intervention studies.

In our liver studies, the quality of the hepatic iron and retinoid measures were directly affected by limited tissue availability and quality of the cadaver tissue used. Obtaining cadaver tissue is not always ideal as it is prone to unknown post-mortem and processing artifacts or underlying medical conditions that may affect the results. We observed that hepatic vitamin A stores were far lower in control tissue compared to

the HCV infected patients. It is likely that endogenous esterases cleaved the retinyl palmitate to retinol prohibiting us from accurately quantitating hepatic vitamin A stores in the tissue available for study.

A third challenge involved in biomarker studies involves selecting appropriate quality control strategies, especially when the availability of specimens is limited. Before completion of any of the assays on valuable biospecimens, conducting pilot studies are vital to increase the validity of the study. This minimizes systemic bias in the results, as each assay will be optimized specifically for the study samples and removes potential technical difficulties. The carefully designed pilot study for hepatic iron measurement revealed that measurement was impossible with the small volume of tissue available from valuable study biopsy FFPE blocks.

Additionally, including appropriate quality control measures beyond a positive and negative control is also crucial for biomarker measurements. These controls provide insight into possible technical and methodological variability that could bias the measure of association between the outcome and exposure. For example, blinded aliquots from pooled urine in the urinary 8OHdG assay revealed extremely high intra- and inter-batch CVs; in some instances, calculating a CV was impossible as one of the replicates had undetermined values. Blinded duplicate quality control data indicated that the assay was not reproducible or reliable and the results for the entire assay were subsequently omitted from the study. Lastly, tissue heterogeneity can limit how much sample is needed for accurate measurements. We were unable to measure phytanic acid in prostate tissue obtained from HFHS. Even though, mass spectrometry is a sensitive assay, and can handle a small amount of tissue input, the spatial heterogeneity or uneven distribution of the analyte in the tissue can limit accuracy.

The studies conducted as a part of this thesis were all cross-sectional in design. While cross-sectional studies are cost effective and can be informative, they are limited in the conclusions that can be derived since reverse causality cannot be eliminated. These studies are also relatively small and prohibit us from doing convincing regression and sub-group analyses, as data tend to get sparse in subgroups and thus become difficult to interpret. Diet involves a complex behavior that is clearly related to other lifestyle factors that can influence disease (i.e. age, smoking, exercise, occupation). These lifestyle factors can easily confound observed relationships. The subjects of all three studies presented here could largely

benefit from investing in more *in vitro* experiments and possibly animal studies. Potential studies could include manipulating AMACR expression in cultured benign and malignant cell lines or using radioisotope tracers to determine the fate of phytanic acid within cells under various conditions. Post-translational modifications and turnover mechanisms of AMACR as discussed in Chapter 2 are also interesting topics to explore *in vitro* to help understand the results of our study.

Further *in vivo* and *in vitro* studies are also needed to examine the mechanisms of the biological interaction between retinoids and iron. For example, vitamin A deficiency often coexists iron deficiency. It is plausible that vitamin A deficiency may exacerbate iron deficiency anemia by altering iron metabolism.(5) Rat studies have shown that iron deficiency alters vitamin A levels in serum and hepatic tissue.(6, 7) It is also important to measure serum hepcidin to explore its role in regulating both retinol and iron in the liver. Immediate future directions should also include exploration of alternative techniques for hepatic iron measurements that allow to use smaller tissue volume. Ultimately, such experimental studies can provide future direction for epidemiologists and yield information that can aid in the interpretation of our findings in Chapter 4.

The overarching goal in conducting these studies was to evaluate substances, especially antioxidants, for use in disease prevention intervention studies. Further work is needed before such intervention studies can be optimally designed and conducted, especially in the context of HCV and retinoids/carotenoids. We need to identify the best agent for supplementation to prevent liver fibrosis. Our data suggest that lycopene is possibly a better candidate than dietary vitamin A. With the creation of new anti-viral medications the HCV patients who could benefit from antioxidants supplementation need to be carefully selected to include those who either do not respond to anti-viral treatment or those who continue to have increasing liver fibrosis despite viral clearance.

Dietary factors are thought to account for about 30% of cancers in Western countries, but the mechanisms involved in this relationship are unclear.(8)   The studies presented here do not provide support for the hypothesis that AMACR's role in prostate cancer is mediated by phytanic acid consumption. However, we found that retinoid loss occurs early in CLD progression and that lycopene may be an attractive choice for dietary supplementation for liver cancer prevention. Iron accumulation in

the liver may be a significant source of oxidative stress in CLD, but how this exposure relates to hepatic antioxidant status remains unclear. Future biomarker development and dietary intervention studies for cancer and CLD prevention can benefit from investigating normal tissues, collecting adequate quality control measures and conducting *in vivo* and *in vitro* laboratory studies.

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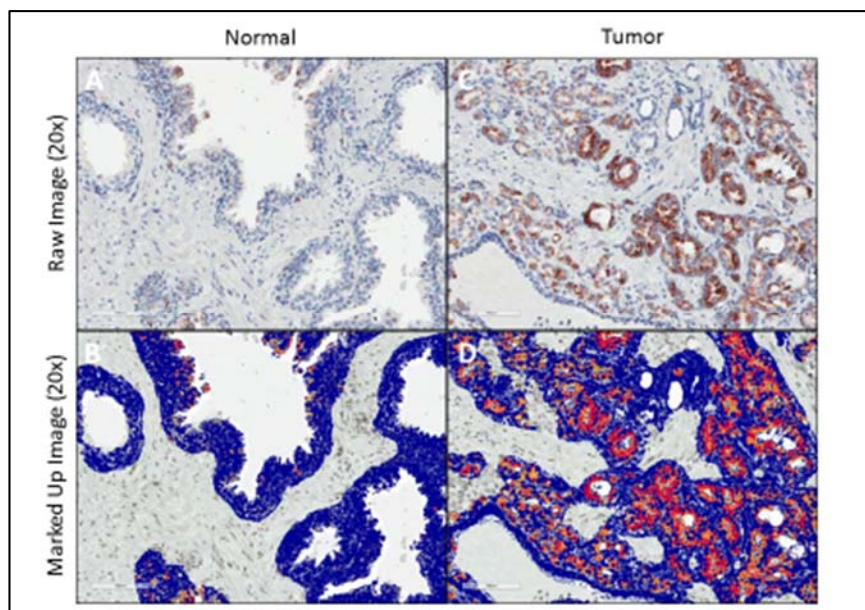
APPENDICES

APPENDIX A
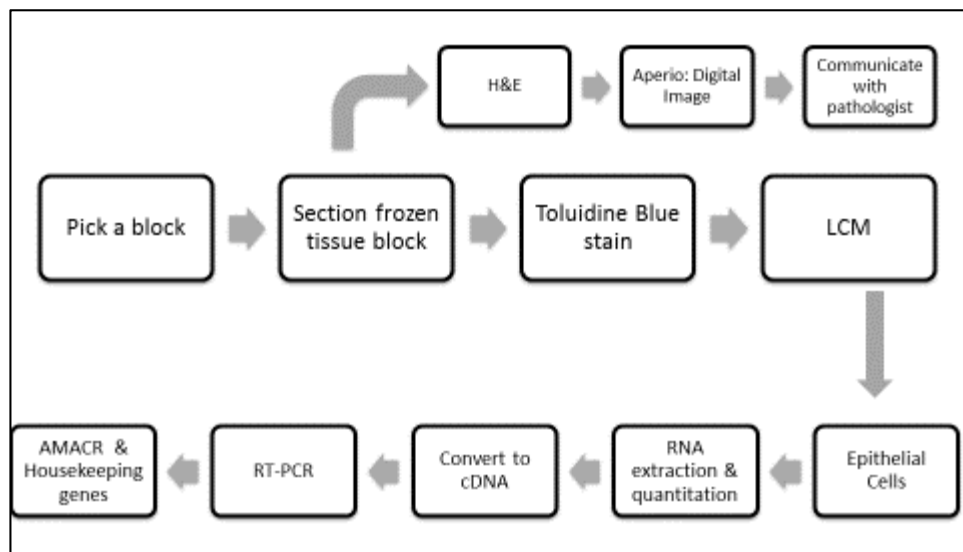
**Figure 19.** Workflow of steps taken to measure AMACR mRNA in normal tissue
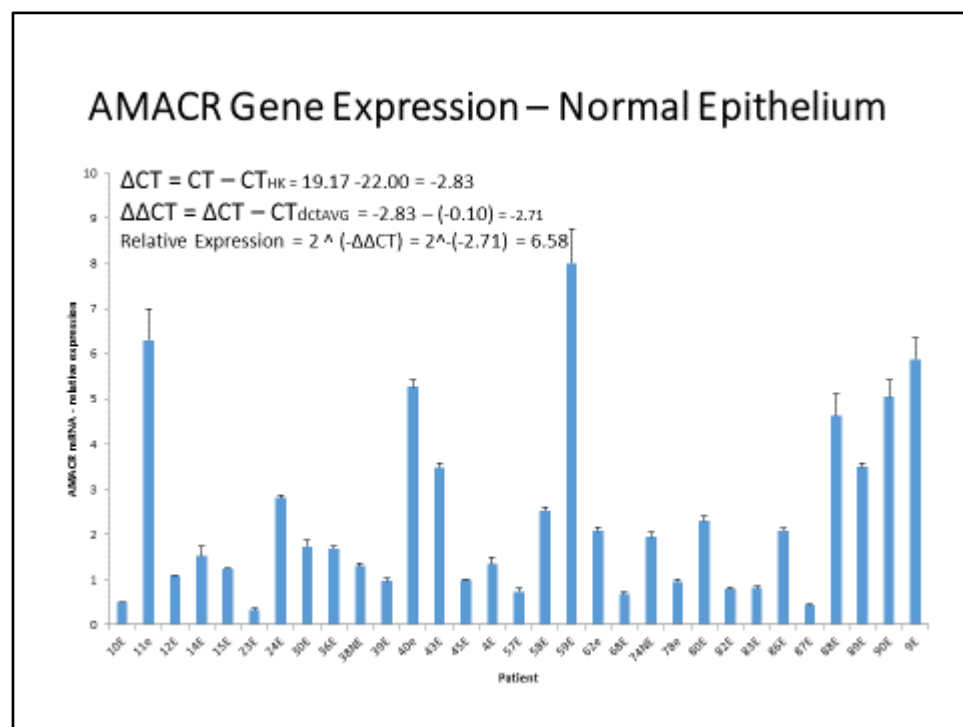
Figure 20. Relative AMACR mRNA expression. Data is normalized to three housekeeper genes utilizing the equations shown.

Figure 21. A-B. Example of AMACR expression in normal region of interest with its corresponding digital marked up image C-D. Example of AMACR expression in a tumor region of interest with its corresponding digital marked up image.
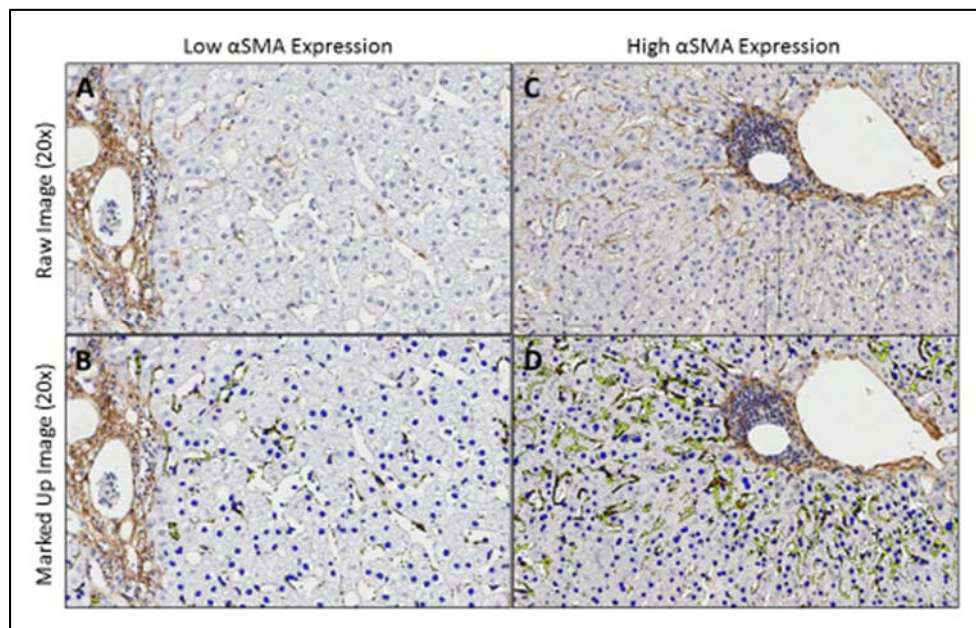
Figure 22. A-B. Example of low SMA expression of SMA with corresponding digital marked up image. C-D. Example of high SMA expression of SMA with corresponding digital marked up image.

Table XII. Spearman correlation coefficients between vitamin A intake and hepatic retinoid and carotenoid

	Tissue Retinyl Palmitate (ng/mg)	Tissue β-Carotene (ng/mg)	Tissue Lycopene (ng/mg)
Total vitamin A (mcg RAE)	r = 0.00 P = 0.99	r = 0.17 P = 0.22	r = 0.11 P = 0.43

Table XIII. Spearman correlations between serum retinoids/carotenoids with disease activity markers

	APRI		FIB-4	
Serum Retinoids/Carotenoids (ng/mL) (n=77)	r	*P*-value	r	*P*-value
Retinol	-0.04	0.72	-0.07	0.59
Lycopene	0.14	0.26	0.05	0.67
β-Carotene	0.16	0.20	0.15	0.23
Lutein	0.26	0.06	0.22	0.07
RBP4 (ug/L)	-0.20	0.12	-0.14	0.28

Table XIV. Spearman correlations between αSMA expression and tissue retinoid and carotenoid

	Tissue Retinyl Palmitate (ng/mg)	Tissue β-Carotene (ng/mg)	Tissue Lycopene (ng/mg)
% αSMA Area	r = -0.31 P = 0.01	r = 0.03 P = 0.83	r = -0.03 P = 0.80
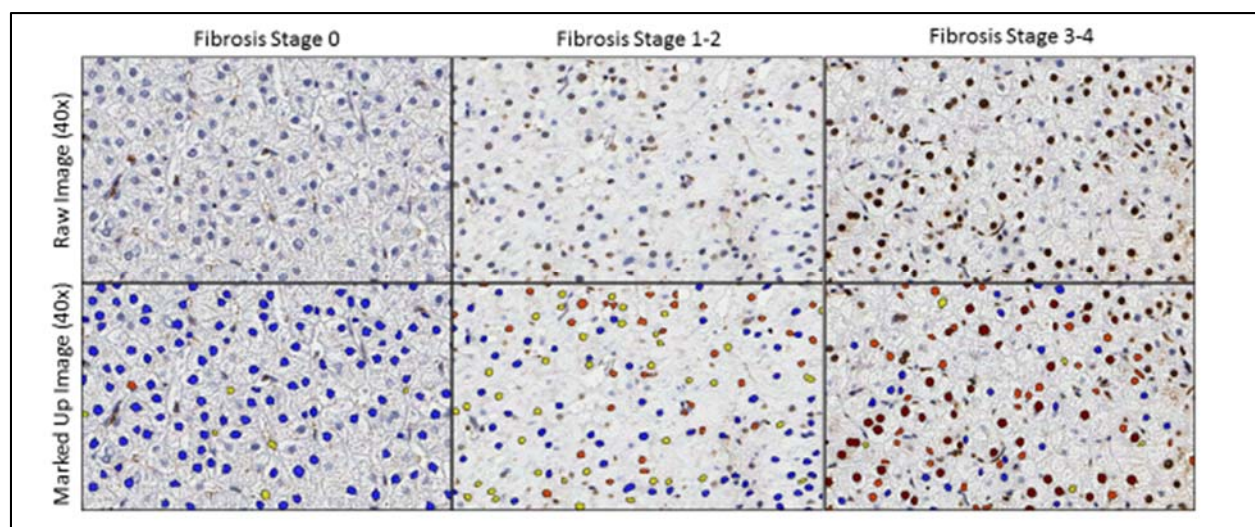
Figure 23. Examples of 8OHdG stained slides with the corresponding digital marked-up image by fibrosis stage

Table XV. Spearman correlations between 8OHdG levels, serum and tissue retinoid/carotenoid

	8OHdG - % High Nuclei	
Serum Retinoids/Carotenoids (ng/mL) (n=77)	r	*P*-value
Retinol	-0.04	0.70
Lycopene	0.11	0.35
β-Carotene	0.08	0.51
Lutein	-0.03	0.80
RBP4 (ug/L)	-0.02	0.84
Tissue Retinoids/Carotenoids (ng/mg) (n=77)	r	*P*-value
Retinyl Palmitate	-0.06	0.62
Lycopene	0.11	0.40
β-Carotene	0.20	0.09
Iron Biomarkers (n=65)		
Ferritin (ng/mL)	-0.26	0.60
Iron (mcg/dL)	-0.11	0.38
Transferrin Saturation (%)	-0.12	0.27

APPENDIX B

Approval Notice

Continuing Review

September 17, 2014

Peter Gann, MD, ScD

Pathology

840 South Wood Street

CSN 130, M/C 847

Chicago, IL 60612

Phone: (312) 355-3723 / Fax: (312) 996-4812

RE: Protocol # 2006-0132

"HSRRB No. A-13486 (Protocol No. PC050393) Dietary Influences on Alpha-Menthylacyl-CoA Racemase (AMACR) Expression in the Prostate"

Dear Dr. Gann:

Your Continuing Review was reviewed and approved by the Expedited review process on September 9, 2014. You may now continue your research.

Please note the following information about your approved research protocol:

Notice: Both Wancai Yang and Ryan Deaton have lapsed investigator training, and require 2 credit hours of continuing education before they can resume work on this protocol.

Protocol Approval Period: September 10, 2014 - September 9, 2015

Approved Subject Enrollment #:   110

Additional Determinations for Research Involving Minors: These determinations have not been made for this study since it has not been approved for enrollment of minors.

Performance Sites: UIC, Henry Ford Health System

Sponsor: Department of Defense Prostate Research
Program

PAF#:   2006-04083

Grant/Contract No: PC050393

Grant/Contract Title: Dietary Influences on Alpha-Methylacyl-CoA
Racemase (AMACR) Expression in the Prostate

Research Protocol:

    a) "HSRB No. A1348 (Protocol No. PC050393) Dietary Influences on Alpha-ethylacyl-CoA Racemase (AMACR) expression in the Prostate," v.12, 8-7-2014"

Your research meets the criteria for expedited review as defined in 45 CFR 46.110(b)(1) under the following specific categories:

(2) Collection of blood samples by finger stick, heel stick, ear stick, or venipuncture as follows:

(a) from healthy, nonpregnant adults who weigh at least 110 pounds. For these subjects, the amounts drawn may not exceed 550 ml in an 8 week period and collection may not occur more frequently than 2 times per week; or

(b) from other adults and children, considering the age, weight, and health of the subjects, the collection procedure, the amount of blood to be collected, and the frequency with which it will be collected. For these subjects, the amount drawn may not exceed the lesser of 50 ml or 3 ml per kg in an 8 week period and collection may not occur more frequently than 2 times per week.

(5) Research involving materials (data, documents, records, or specimens) that have been collected, or will be collected solely for nonresearch purposes (such as medical treatment or diagnosis).

Please note the Review History of this submission:

Receipt Date	Submission Type	Review Process	Review Date	Review Action
09/04/2014	Continuing Review	Expedited	09/09/2014	Approved

Please remember to:

→ Use your **research protocol number** (2006-0132) on any documents or correspondence with the IRB concerning your research protocol.
→ Review and comply with all requirements on the enclosure,

"**UIC Investigator Responsibilities, Protection of Human Research Subjects**"
(*http://tigger.uic.edu/depts/ovcr/research/protocolreview/irb/policies/0924.pdf*)

Please note that the UIC IRB has the right to seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

Please be aware that if the scope of work in the grant/project changes, the protocol must be amended and approved by the UIC IRB before the initiation of the change.

We wish you the best as you conduct your research. If you have any questions or need further help, please contact OPRS at (312) 996-1711 or me at (312) 413-3788. Please send any correspondence about this protocol to OPRS at 203 AOB, M/C 672.

Sincerely,

Fachel Olech, B.A., CIP

Assistant Director, IRB # 3

Office for the Protection of Research Subjects

cc: Meha Singh, Cancer Center, 318 MCA, MC 700
       Frederick G. Behm, Pathology, M/C 847
       OVCR Administration, M/C 672
       Privacy Office, Health Information Management Department, M/C 772

APPENDIX C

UNIVERSITY OF ILLINOIS
AT CHICAGO

Office for the Protection of Research Subjects (OPRS)
Office of the Vice Chancellor for Research (MC 672)
203 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

Approval Notice

Continuing Review

September 17, 2014

Peter Gann, MD, ScD

Pathology

840 South Wood Street

CSN 130, M/C 847

Chicago, IL 60612

Phone: (312) 355-3723 / Fax: (312) 996-4812

RE: Protocol # 2006-0132

"HSRRB No. A-13486 (Protocol No. PC050393) Dietary Influences on Alpha-Menthylacyl-CoA Racemase (AMACR) Expression in the Prostate"

Dear Dr. Gann:

Your Continuing Review was reviewed and approved by the Expedited review process on September 9, 2014. You may now continue your research.

Please note the following information about your approved research protocol:

Notice: Both Wancai Yang and Ryan Deaton have lapsed investigator training, and require 2 credit hours of continuing education before they can resume work on this protocol.

Protocol Approval Period: September 10, 2014 - September 9, 2015

Approved Subject Enrollment #:   110

Additional Determinations for Research Involving Minors: These determinations have not been made for this study since it has not been approved for enrollment of minors.

Performance Sites: UIC, Henry Ford Health System

Sponsor: Department of Defense Prostate Research
Program

PAF#:   2006-04083

Grant/Contract No: PC050393

Grant/Contract Title: Dietary Influences on Alpha-Methylacyl-CoA
Racemase (AMACR) Expression in the Prostate

Research Protocol:

       a) "HSRB No. A1348 (Protocol No. PC050393) Dietary Influences on Alpha-ethylacyl-CoA Racemase (AMACR) expression in the Prostate," v.12, 8-7-2014"

Your research meets the criteria for expedited review as defined in 45 CFR 46.110(b)(1) under the following specific categories:

(2) Collection of blood samples by finger stick, heel stick, ear stick, or venipuncture as follows:

(a) from healthy, nonpregnant adults who weigh at least 110 pounds. For these subjects, the amounts drawn may not exceed 550 ml in an 8 week period and collection may not occur more frequently than 2 times per week; or

(b) from other adults and children, considering the age, weight, and health of the subjects, the collection procedure, the amount of blood to be collected, and the frequency with which it will be collected. For these subjects, the amount drawn may not exceed the lesser of 50 ml or 3 ml per kg in an 8 week period and collection may not occur more frequently than 2 times per week.

(5) Research involving materials (data, documents, records, or specimens) that have been collected, or will be collected solely for nonresearch purposes (such as medical treatment or diagnosis).

Please note the Review History of this submission:

Receipt Date	Submission Type	Review Process	Review Date	Review Action
09/04/2014	Continuing Review	Expedited	09/09/2014	Approved

Please remember to:

→ Use your **research protocol number** (2006-0132) on any documents or correspondence with the IRB concerning your research protocol.
→ Review and comply with all requirements on the enclosure,

"**UIC Investigator Responsibilities, Protection of Human Research Subjects**"
(*http://tigger.uic.edu/depts/ovcr/research/protocolreview/irb/policies/0924.pdf*)

Please note that the UIC IRB has the right to seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

Please be aware that if the scope of work in the grant/project changes, the protocol must be amended and approved by the UIC IRB before the initiation of the change.

We wish you the best as you conduct your research. If you have any questions or need further help, please contact OPRS at (312) 996-1711 or me at (312) 413-3788. Please send any correspondence about this protocol to OPRS at 203 AOB, M/C 672.

Sincerely,

Rachel Olech, B.A., CIP

Assistant Director, IRB # 3

Office for the Protection of Research Subjects

cc: Meha Singh, Cancer Center, 318 MCA, MC 700
       Frederick G. Behm, Pathology, M/C 847
       OVCR Administration, M/C 672
       Privacy Office, Health Information Management Department, M/C 772

APPENDIX D

COMRB 6120

CHICAGO, IL 60607
United States
Attn: Yachana Kataria

Billing Type	Invoice
Billing Address	Yachana Kataria 909 S. Wolcott COMRB 6120 CHICAGO, IL 60607 United States Attn: Yachana Kataria
Total	0.00 USD

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VITA
Yachana Kataria, PhD

University of Illinois at Chicago
Department of Pathology
840 S. Wood Street, Suite 130 CSN
Chicago, IL 60612

EDUCATION

PhD – Department of Pathology, *University of Illinois at Chicago (UIC)*, Fall 2010 - July 2015
    Advisor – Peter Gann, MD, ScD
    GPA: 3.74

BA – Department of Chemistry, *University of Illinois at Chicago (UIC)*, Fall 2006 - December 2009
    Honors, Magna Cum Laude
    GPA: 3.79

Certificate – HarvardX Health in Numbers: Quantitative Methods in Clinical & Public Health Research

PROFESSIONAL SKILLS

Statistical Software: SAS, STATA, SPSS

Molecular biology techniques: RT-PCR, laser capture microdissection (LCM) of human tissue, RNA & DNA extractions, western blots, protein extraction and quantitation

Histology: immunohistochemistry (IHC) on human tissue, cryosection of human tissue, basic histology stains

Digital Pathology: whole slide imaging, machine learning, digital image analysis (Aperio, & Tissue Studio - Definiens)

Clinical Research: Institutional Review Boards (IRB) applications for clinical/epidemiological research, processing human sera, urine, and tissue, running pilot studies for ICP-MS and GC-MS, analyzing nutrition/dietary data, questionnaire design, coordinating and collecting human specimen retrieval in bio-repository and tissue banks, initiate and manage collaborations for research, working with clinical pathology lab personnel, managing databases, patient recruitment for clinical studies

RESEARCH EXPERIENCES

Research Assistant, *Department of Pathology, UIC*
August 2010 to Present
- Developed expertise in pathology, molecular epidemiology, strengthened skills in clinical and translational cancer research which incorporate serological and tissue biomarkers of exposure and disease
- Independently designed tissue based studies that incorporate sophisticated molecular epidemiological approaches to the study of dietary factors in the etiology and prevention of prostate and liver cancer.
- Completed two cross-sectional clinical studies.
- Manage undergraduate students (>2 years) for senior capstone thesis projects.

Research Assistant, *Department of Psychiatry, UIC*

January 2009 to August 2010

- Evaluated and analyzed the quality of patient care amongst patients seen in inpatient and outpatient settings, and in the metabolic screening clinic in patients with serious mental illness and concurrent antipsychotic therapy

Research Specialist, *Department of Pathology, UIC*

May 2009 to August 2010

- Assisted with patient recruitment in ongoing molecular epidemiology studies of prostate and liver cancer and completed IRB applications
- Processed blood samples, collected and analyzed human tissue specimens, extracted and analyzed RNA, performed basic cell culture

Research Assistant, *Department of Pharmacology, UIC*

September 2008 to January 2009

- Applied molecular biochemistry research methods and techniques (i.e. gel electrophoresis, making buffers, polymerase chain reaction (PCR), making stacking and running gels)

Research Assistant, *Department of Psychiatry, UIC*

May 2008 to September 2008

- Assessed a metabolic screening checklist model and quantified baseline metabolic risk of outpatients treated with antipsychotics
- Interacted with and attained data from patients who were diagnosed with psychosis for the metabolic screening clinic

TEACHING EXPERIENCE

Teaching Assistant, Molecular Epidemiology and Biomarkers of Disease, *Department of Pathology, UIC*

Spring Semester 2012 to May 2015

- Helped design the syllabus
- Assisted graduate students in difficult concepts in molecular epidemiology
- Graded and created exam questions
- Solo taught a week of classes on the topic of epidemiological study designs

Student Mentor, *Department of Pathology, UIC*

August 2010 to Present

- Mentored undergraduates and rotating graduate students in the lab
- Taught molecular epidemiology and molecular biology concepts and applications
- Assisted with completion of senior capstone thesis projects
- Assisted with presenting at research forums

Academic Tutor, *Various Clients*

September 2006 to August 2010

- Taught various courses (n=25), with a primary focus on mathematics and science among a wide range of students (grade school to college level)

Teaching Assistant, Introduction to Honors College, *Honors College, UIC*

August 2008 to December 2009

- Acquainted honors college students as an undergraduates with the UIC community

PUBLICATIONS

Kataria, Y., Deaton, R.J., Rueter, E.E., and Gann, P.H. (2015). Retinoid and Carotenoid in Serum and Liver Tissue Concentrations in Patients at High-Risk for Liver Cancer – *Under Review*

Lugli, G., **Kataria, Y**., Gann, P.H., Xhou, X., and Nonn, L. (2015). Laser-capture microdissection of human prostatic epithelium for RNA analysis. *Journal of Visualized Experiments (JOVE)*

Kataria, Y., Wright, M., Deaton, R.J., Rueter, E.E., Rybicki, B.A., Moser, A.B., Ananthanrayanan, V., and Gann, P.H. (2015). Dietary influences on tissue concentrations of phytanic acid and AMACR expression in the benign human prostate. *The Prostate*.

Batscha, C., Schneiderhan, M.E., **Kataria, Y**., Rosen, C., and Marvin, R.W. (2010). Treatment settings and metabolic monitoring for people experiencing first-episode psychosis. *Journal of psychosocial nursing and mental health services.*

GRANTS AND FELLOWSHIPS

2014 to 2015 – Center for Clinical and Translational Science Pre-doctoral Education for Clinical and Translational Scientists (CCTS - PECTS) Fellowship, *College of Medicine, UIC*

2012 to 2014 – UIC Chancellor's Graduate Research Multidisciplinary Fellowship, *Graduate College, UIC (*campus wide competition)

AWARDS AND HONORS

2015 – UIC Graduate Student Council Travel Award

2015 – UIC Graduate College Student Travel Presenter's Award

2014 – UIC Graduate Student Council Travel Award

2014 – UIC Graduate College Student Travel Presenter's Award

2007 to 2009 – Chancellors Student Service Awards

2008 – Outstanding Volunteer Award, UIC Medical Center

2006 to 2009 – Dean's List UIC

ORAL PRESENTATIONS

February 2015 – "Integrating Biomarkers into Translational Research on Diet and Cancer"
UIC – Department of Pathology Research Seminar Series

November 2013 – "Preliminary Results - Retinoid and Carotenoid Depletion in Patients at High-Risk for Liver Cancer"
UIC – Department of Pathology Research Seminar Series

December 2012 – "Second Look at Phytanic Acid"
UIC – Department of Pathology Research Seminar Series

December 2011 – "New Results from the "Dietary Influences on AMACR Expression in the Prostate Study""
UIC – Department of Pathology Research Seminar Series

PANELS

January 2009 – **Panelist for Blended Learning**
E-Teaching Symposium of Blended Learning at UIC, Chicago, Illinois, USA

April 2008 – **Panelist for Multiple Perspectives Plenary Panel**
Sloan-C Workshop on Blended Learning and Higher Education, Chicago, Illinois, USA

CONFERENCE ACTIVITY / POSTERS PRESENTED

April 2015 – "Joint Relationship Between Iron and Retinoid in Patients at High-Risk for Liver Cancer"
American Association for Cancer Research (AACR) – Annual Meeting, Philadelphia, Pennsylvania, USA

April 2014 – "Retinoid and Carotenoid Depletion in Patients at High-Risk for Liver Cancer"
UIC – Cancer Center Research Forum, Chicago, Illinois, USA

April 2014 – "Retinoid and Carotenoid Depletion in Patients at High-Risk for Liver Cancer"
American Association for Cancer Research (AACR) – Annual Meeting, San Diego, California, USA

April 2012 – "Associations between Phytanic Acid and Alpha-methyl acyl-CoA Racemase (AMACR) Expression in the Normal Human Prostate"
American Association for Cancer Research (AACR) – Annual Meeting, Chicago, Illinois, USA

March 2012 – "Associations between Phytanic Acid and Alpha-methylacyl-CoA Racemase (AMACR) Expression in the Normal Human Prostate"
UIC – Cancer Center Research Forum, Chicago, Illinois, USA

November 2011 – "Dietary Influences of Alpha-methylacyl-CoA Racemase (AMACR) Expression in the Normal Human Prostate"
UIC – College of Medicine Research Forum, Chicago, Illinois, USA

Other Posters
March 2014 – "Retinoid/Carotenoid Depletion in Hepatitis C: Fibrosis Stage in Relation to Serum Biomarkers for Antioxidant Status, Insulin Resistance, and Oxidative Stress"
BM Chung, **Y Kataria,** EE Rueter, S Cotler, D Jensen, G Guzman, M Jin, R van Breemen, P Gann
United States & Canadian Academy of Pathology (USCAP) – Annual Meeting, San Diego, California, USA

April 2014 – "The Relationship Between Oxidative Stress and Antioxidants in Hepatitis C Virus-infected Patients"
Christine Solans, **Y Kataria**, P Nguyen, Ryan Deaton, EE Rueter, S Cotler, D Jensen, P Gann
UIC – Student Research Forum, Chicago, Illinois, USA

UNIVERSITY SERVICE

Lead Consultant, *Enterprise Work, UIC*
June 2014 to September 2014
- Assessed commercialization potential of inventions and discoveries in the field of molecular diagnostics
- Composed marketing summaries and understand technology highly regarded projects
- Managed a team of 4 consultants

Project Leader - Undergraduate Research Experience Program, *Office of the Provost & Dean of Student Affairs, UIC*
June 2007 to August 2010
- Worked with Provost Lon Kaufman to develop undergraduate courses at UIC that functioned as research electives in order to increase undergraduate research opportunities
- Chaired & managed a committee of Deans and Associate Deans of the undergraduate colleges
- Attended and presented at various meetings with administration on campus to spearhead this project, and to coordinate its logistics
- Performed a campus-wide survey to measure student research interest

Project Coordinator, Interactions of UIC & Community, *Office of the Vice-Provost for Programming and Planning, UIC*
May 2010 to August 2010
- Integrated UIC with its surrounding community utilizing geographical information systems (GIS) as a platform
- Created a database within Google Maps as an interactive web based resource for the project

LANGUAGES

English, Hindi, Urdu (fluent in all three)

PROFESSIONAL MEMBERSHIPS/AFFILIATIONS

2011 to Present – American Association for Cancer Research (AACR)

2014 to Present – Graduate Women in Science

COMMUNITY INVOLVEMENT

2013 to Present – Graduate Education in Medical Sciences Student Association (GEMMSA)

2010 to Present – Volunteer for the Bank of America Chicago Marathon and other running events

2010 to Present – Volunteer at homeless shelters, judge high-school science fairs