

ABSTRACT

ERSOY, EZGI. Effects of Carotenoids in Carrots on Obesity Induced Non-Alcoholic Fatty Liver Disease and Inflammation in a High-Fat Diet Mouse Model (Under the direction of Dr. Abdulkerim Eroglu)

Obesity development through high-fat diets increases hepatic lipid accumulation, oxidative stress and inflammation that advance non-alcoholic fatty liver disease (NAFLD). Plant-based foods can help mitigate liver inflammation and fibrosis due to their bioactive compounds, such as carotenoids. These tetraterpenoid pigments give the yellow, orange, and red colors to plants and include both provitamin A (α -carotene, β -carotene, and β -cryptoxanthin) and non-provitamin A forms (lycopene, lutein, and zeaxanthin). Carotenoids show antioxidant and free radical defense capabilities due to the double-bonded conformation in their extended structures. After absorption in the small intestine, carotenoids can be metabolized and stored in the liver, transferred to adipose tissue, or circulated to peripheral tissues.

The research in this thesis investigates how whole food interventions containing carotenoids affect the liver during obesity caused by diets with poor nutrition. The project aimed to close a gap in existing research regarding how most studies use supplemented doses of isolated carotenoids, rather than whole foods, together with high-fat diets above normal human eating patterns. The study evaluated how doses of lycopene, α -carotene, β -carotene, and lutein from colored carrots would affect liver-specific lipid metabolism and inflammation markers in mice consuming a 45% fat diet. The fundamental hypothesis behind this project states that whole food sourced carotenoid intervention methods trigger different hepatic responses linked to NAFLD while the effect depends on tissue-based action mechanisms of different carotenoids.

In the diet pellets, the orange carrot group contained 102.64 nmol/g β -carotene (0.276 mg/g OC powder), 76.76 nmol/g α -carotene (0.206 mg/g OC), and 1.65 nmol/g lutein (0.005 mg/g OC), with

no detectable lycopene. Red carrot pellets had 22.78 nmol/g β -carotene (0.061 mg/g RC), 0.96 nmol/g α -carotene (0.003 mg/g RC), 95.19 nmol/g lycopene (0.255 mg/g RC), and 0.96 nmol/g lutein (0.003 mg/g RC). Yellow carrot pellets provided 1.16 nmol/g β -carotene (0.003 mg/g YC) and 2.06 nmol/g lutein (0.006 mg/g YC), with no detectable α -carotene or lycopene. White carrot powder contained none of the four quantified carotenoids.

Serum analyses confirmed carotenoid absorption, with detectable levels of α -carotene (32.09 ± 4.45 nM) and β -carotene (7.76 ± 0.01 nM) in the orange carrot group, and lycopene (33.89 ± 5.80 nM) in the red carrot group. While several trends to mitigate the experimental conditions were observed including reduced hepatic fat accumulation, inflammatory markers, and expression levels of acyl-CoA oxidase 1 (ACOX1), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1- α), carnitine palmitoyltransferase 2 (CPT2), stearoyl-CoA-desaturase 1 (SCD-1), and peroxisome proliferator- activated receptor alpha (PPAR- α), most of the trends did not reach statistical significance. Red carrot intake consistently showed steady positive effects in reducing fat mass and hepatic steatosis, as well as modulating lipid transport and antioxidant defense mechanisms, though changes in gene expression were modest. In contrast, the orange carrot stimulated higher expression of lipid oxidation proteins, but this was offset by a concurrent increase in lipid uptake markers, which diminished its overall metabolic benefits.

Quantitative proteomic analysis affirmed these metabolic tissue changes although multiple groups lacked significant statistical interpretation. The whole food-based approach brought in added biological factors which possibly contributed to study outcomes. The study period of 20 weeks and 45% dietary fat content upheld standards of a high-fat diet study design but likely failed to create adequate metabolic stress needed to see differences in NAFLD progression among intervention groups.

This research points out the difficulties of studying NAFLD using whole food dietary interventions within moderate fat dietary models that involve multifactorial diseases. Carotenoid-containing carrot varieties directly impacted liver pathways related to lipid metabolism and oxidative stress, however comparison between intervention arms were mostly insignificant and the metabolic changes were not consistently validated with laboratory findings.

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Effects of Carotenoids in Carrots on Obesity Induced Non-Alcoholic Fatty Liver Disease and
Inflammation in a High-Fat Diet Mouse Model

by
Ezgi Ersoy

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APPROVED BY:

Dr. Abdulkerim Eroglu
Committee Chair

Dr. Mary Ann Lila

Dr. Slavko Komarnytsky

DEDICATION

I dedicate my thesis to my mother and father, for all the support they provided me to be successful in my journey, to my teachers for the path they paved, and to all my friends for being there for me.

BIOGRAPHY

Ezgi Ersoy was born and raised in the capital city of Turkey, Ankara. Earlier in her academic journey, she studied Biotechnology at Kent State University and Molecular Biology & Genetics at Baskent University. She finished her undergraduate education of Food Engineering at Izmir Institute of Technology. As an undergraduate student, she worked in Gulec Lab with a funded project on the effects of molasses on iron deficiency anemia. After graduation, she joined MEY DIAGEO, Turkey's largest beverage production company, as a Senior Production Specialist. There, she worked on the production of Rakı, gaining experience in fermentation, distillation, and process optimization. She later attended NC State University and moved to Kannapolis, North Carolina, to work as a research assistant in the Eroglu Lab and was enrolled in the Master of Science program in Nutrition. With the lead of Dr. Eroglu she started her research into the effects of carotenoids on liver health, where she took the steps to learn the skillsets needed to perform laboratory analysis of organic compounds and animal tissues. Making contributions to research and science was a great opportunity and she aims to further improve herself to make a change in the science and health community.

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CHAPTER 1

1.Introduction

1.1. Non-Alcoholic Fatty Liver Disease: Overview and Epidemiology

Non-alcoholic fatty liver disease (NAFLD) is a condition identified as excess fat accumulation in the liver, specifically triglycerides, without any overconsumption of alcohol (daily intake ≤ 25 g) by the individual.^{1,2} In the United States, NAFLD is the most common liver disease with more than 100 million diagnosed patients and is the second main cause of liver transplants overall, as well as the most common reason for liver transplantation in individuals under 50.³ The excess fat deposits in the liver can cause steatosis ($\geq 5\%$ fatty liver cells) and the condition can worsen into non-alcoholic steatohepatitis when inflammation and scarring of tissue occurs.⁴ It is also observed that the disease may eventually transform to a more severe form known as cirrhosis as the scar tissue spreads in hepatocytes. Around 24% of adults in the United States are estimated to have NAFLD, while 1.5% to 6.5% are impacted by the more chronic form called non-alcoholic steatohepatitis (NASH), according to researchers.⁵ Of NAFLD patients with simple steatosis, around 20–30% are likely to develop NASH; of those with NASH, roughly 7–25% may advance to fibrosis and cirrhosis⁶, leaving approximately 4.5 million undiagnosed cases which more than any other known liver disease causing cirrhosis.⁷ According to data NAFLD has surpassed hepatitis B and C as the leading risk factor for primary liver cancer and the first stages of NAFLD are harder to diagnose compared to other liver diseases.⁸ In a healthy liver, fat builds up without inflammation or hepatocyte ballooning.⁹ However, in nonalcoholic fatty liver disease, steatosis can worsen with persistent inflammation, resulting in cirrhosis, fibrosis, and an elevated risk of hepatocellular carcinoma.⁹ This process can start early, even in children and teenagers, and can cause serious illness by the ages of 30 to 40.⁹ Fatty liver is most commonly seen in people aged

45–64 and slightly more in men than women, but it also affects a significant number of those aged 20–44.¹⁰ This shows that NAFLD isn't just a disease of older adults, but affects younger people as well, which explains why it's the leading cause of liver transplants for those under 50.

1.2. Diagnosis and Biomarkers of Non-Alcoholic Fatty Liver Disease

NAFLD usually shows itself in asymptomatic conditions and is mainly discovered by chance as studies show that NAFLD identification is often an incidental medical finding, seen in abnormal liver tests, or bright liver on screening and hepatomegaly.³ Vague symptoms such as malaise, fatigue or a dull discomfort in the right abdominal area can also be observed.¹¹ The amount of scarring in liver, also known as the fibrosis stage, is shown to be one of the most important predictive factors in mortality, either from liver related disease or all-cause mortality.¹² A systematic review published in 2017, showed that the probability of death increases at each stage of NAFLD, exceeding six times higher for all-cause mortality and more than forty times higher for liver related mortality at the most advanced stage.¹² After diagnosis, disease severity can be seen by serum biomarkers or imaging tools. Main parameters that are looked at include age, body mass index (BMI)(kg/m²), insulin-like growth factor (IGF) or diabetes, aspartate transferase (AST), alanine aminotransferase (ALT),platelets count (x10⁹/l) and albumin concentration (g/l).¹³ When identifying fatty liver, increased ALT in blood is the first indication of the disease. ^{14,15} Although 40 U/L is thought to be the top limit of normal for these enzymes, more precise thresholds indicate that ideal ALT levels should be roughly 25 U/L for women and 35 U/L for men. ^{14,15} However, higher results could still be considered normal by current laboratory reference ranges, and may be then followed by a liver ultrasound, which in fact may not show unhealthy symptoms during examination. For fibrosis, elevated liver fibrosis (ELF) is checked during blood tests where a result in 10.51 or above indicates advanced liver fibrosis.¹⁶ If the ELF test results in

high-degree liver scarring, stiffness of the liver can be analyzed using high-frequency sound waves by a technique called transient elastography to diagnose cirrhosis.¹⁷ NAFLD fibrosis score (NFS) can also be calculated to observe the severity of the disease, which takes into account similar factors such as age, BMI, liver enzyme ratios (ALT/AST > 0.8), albumin, and diabetes.¹⁸ Fibrosis-4 (FIB-4) score is also used to assess the condition of the liver, which is a similar algorithm to NFS.¹⁹ FIB-4 and NFS are the most commonly utilized tests that are done with bloodwork as well as transient elastography, FibroScan-AST (FAST) score are noninvasive ways to perform imaging to observe NAFLD biomarkers.²⁰ These tests are great indicators of fat and scarring levels in liver however, none of them apply for NASH, as in order to examine liver inflammation, a biopsy is needed.²¹ As such procedures are not easy, medical professionals make presumptive diagnoses and provide recommendations on diet and lifestyle of the individual. The goals of NASH treatment are to improve metabolic abnormalities, decrease inflammation on the liver, and prevent and reverse fibrosis. AASLD (American Association for the Study of Liver Diseases) suggests that pharmacological treatments should mainly be recommended to those who have NASH diagnosis with biopsy results.^{22,23} Screening for NAFLD is challenging because many people appear healthy, feel fine, and have normal liver tests. Different organizations have varying recommendations: AASLD advises against routine screening of the liver unless the patient has type 2 diabetes, while EASL (European Association for the Study of the Liver) recommends screening obese individuals and those with metabolic syndrome using liver enzymes and possibly an ultrasound, with more advanced testing for high-risk groups like those over 50, diabetics, and people with cardiovascular disease.²⁴ Interestingly, the most aggressive approach comes from the ADA (American Diabetes Association) endocrinologists, who recommend screening for NASH and fibrosis in type 2

diabetics and even pre-diabetics with elevated ALT, which makes sense given the strong link between NAFLD and insulin resistance.²⁵

1.3. Pathophysiology and Risk Factors of NAFLD

As mentioned, NAFLD progresses in three pathways where fat builds up in the liver (hepatic steatosis), accumulated fat causes inflammation (steatohepatitis) and finally scarring develops (fibrosis) due to severe damage.²⁶ The free fatty acids in the bloodstream can directly reach the liver. Since fat in the body is constantly broken down and released, the more fat circulating in the blood, the more the liver absorbs.²⁶ Through de novo lipogenesis (DNL), the liver by itself may turn excess amounts of nutrients, such as dietary sugars, into fat.²⁷ Although a lot of studies have been done, the basic molecular mechanisms connected to hepatic DNL remain not completely understood as it occurs by a set of enzymes controlled by liver transcription factors. Stearoyl-CoA desaturase (SCD-1) is involved in transforming saturated fatty acids into monounsaturated fatty acids which eventually forms triglycerides.²⁸ Peroxisome proliferator-activated receptor alpha (PPAR- α) is a major transcription factor which activates oxidation genes such as carnitine palmitoyltransferase-2 (CPT-2), which is essential for transporting long-chain fatty acids into mitochondria for β -oxidation.²⁹ Peroxisome proliferator-activated receptor gamma coactivator 1 (PGC1) is involved in the breakdown of fatty acids in the mitochondria of hepatocytes for energy use.^{30,31} On the other hand, fatty acid synthase (FAS) which sterol regulatory element-binding protein-1c (SREBP-1c) regulates, produces fatty acids that help maintain the balance of fat concentration.³² Another important transporter protein of fatty acids is cluster of differentiation 36 (CD36) and it functions in moving fatty acids into the liver cells and helps with signaling of cell metabolism pathways.^{33,34} Acyl-CoA oxidase 1 (ACOX1) has peroxisomal fatty acid oxidation properties and breaks down fatty acids.³⁵ This system is maintained in a healthy liver to balance

between fatty acid synthesis, oxidation, and uptake; however with NAFLD, abnormal levels of gene and protein expressions are present.^{27,36-38} With the accumulation of fat, lipotoxicity is seen in the liver where major oxidative stress and inflammation are caused by the release of harmful cytokines, such as interleukin-1 beta (IL-1 β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF- α), and transforming growth factor-beta (TGF- β), which leads to hepatocyte apoptosis.³⁹ Immune cells, including macrophages, Kupffer cells and regulatory T cells (Treg) increase in concentration which leads to elevated inflammation.⁴⁰ To balance the excess fat, the liver burns fat for energy through mitochondrial β -oxidation, but fat builds up and damages the liver when this process fails.⁴¹ Ongoing inflammation triggers hepatic stellate cells to create scar tissue, leading to fibrosis.⁴¹ Factors like cholesterol, uric acid, low oxygen, and gut bacteria causes the damage to worsen.⁴² Diabetes improvement, in addition to better heart health and kidney health are some of the systematic outcomes that are seen with prevention efforts to prevent further liver diseases such as liver failure and cancer.⁴³ There are medical procedures that improve a patient's overall condition, such as gastric band (LAGB), gastric bypass (RYGB), sleeve gastrectomy (LVSG) and biliopancreatic diversion with duodenal switch (BPD-DS) as they cause weight loss.⁴⁴⁻⁴⁷ Targeting pathophysiological processes such as insulin resistance and lipid metabolism has been worked on by pharmaceutical industries, where pathways related to lipotoxicity, oxidative stress, inflammation, immune activation, apoptosis, fibrogenesis and collagen turnover are targeted. Drugs such as Lanifibranor decreases hepatic fat by targeting multiple PPARs which are nuclear receptors that are key regulators in lipid metabolism, liver inflammation, and fibrosis.^{48,49} Semaglutide is another drug that targets glucagon-like peptide-1 receptor (GLP-1), which is involved in cell signaling mechanisms that have effects on metabolic syndrome characteristics which are closely linked to non-alcoholic fatty liver disease.⁵⁰ Firsocostat is

a medication in development for the treatment of fatty liver by inhibiting acetyl-CoA carboxylase (ACC) enzyme that regulates hepatic fat, as ACC together with diacylglycerol O-acyltransferase 2 (DGAT2) inhibitors are demonstrated to mitigate liver fat in patients.⁵¹ Aramchol functions to block the enzyme stearoyl coenzyme A desaturase (SCD1), which is implicated in hepatic fatty acid metabolism and with inhibition results can be seen in less fat synthesis, enhanced fat oxidation, and reduced hepatic fat accumulation.⁵² However, emerging therapies for NAFLD treatment have to be more applicable and widespread. The endpoint of taking these drugs as far as their long-term use raises questions, as no one medicine using one mechanism will likely be sufficient to treat fatty liver. Therefore, basic tenants of treatment comes down to first line of therapy for NAFLD which is comprehensive lifestyle changes. Reduction of calorie intake ($\geq 30\%$ of their baseline or ~ 750 - $1,000$ kcal/day) helps to improve insulin resistance in obese patients as well as improvements in hepatic steatosis, and is especially effective when high fructose beverages are eliminated in the diet.⁵³ Fructose defeats the purpose of caloric restriction as it leads to a higher chance of the occurrence of nonalcoholic steatohepatitis in high risk patients and chronic liver fibrosis risk in those who already have NAFLD.⁵⁴ Weight loss is recommended when it results in 3-5% improvement of steatosis, however it is known that NASH and liver fibrosis outcomes dramatically lessen with 6-10% body fat loss.⁵³⁻⁵⁵ Exercise itself may reduce steatosis but doesn't effectively reduce inflammation without proper nutrition.⁵³⁻⁵⁵ Decreasing heavy alcohol consumption (one drink per day) protects against adverse health effects and drinking two or more cups of caffeinated coffee is also proven to reduce the risk of liver fibrosis in several liver diseases.⁵⁶⁻⁵⁹ The reasons behind NAFLD are a set of issues seen in the body, including genetic factors, older age (≥ 50 years), raised cholesterol and blood pressure, smoking, low activity levels, poor diet and other underlying diseases such as Weber-Christian disease, type 1 diabetic

hepatopathy and type 2 diabetes¹¹. The top two risk factors for NAFLD are being diabetic, particularly with type 2 diabetes due to insulin resistance, and being obese, even if the obesity is mild. To demonstrate this point, a prospective 7-year cohort study analyzed predictors and remission of NAFLD with 213 participants that did not have NAFLD at the beginning.⁶⁰ Each subject was followed over time in order to observe what factors were important for leading to development of NAFLD, and for the disease to improve later on. The study found that weight gain of around 6 kilograms caused the development of NAFLD and those who lost weight around 5% or more of their baseline weight were more likely to have remission of the disease compared to those who didn't lose any. Additionally, the baseline homeostasis model of insulin resistance (HOMA) score which is a marker of insulin resistance was higher in those who developed NAFLD. Other markers such as leptin, cholesterol and baseline weight were not predictive factors of fatty liver. Other studies show there are additional risk factors including genetic predispositions like PNPLA3 (Patatin Like Domain 3, 1-Acylglycerol-3-Phosphate O-Acyltransferase), as well as conditions such as dyslipidaemia, hypertension, and PCOS (Polycystic Ovary Syndrome).⁶¹ Global data shows that 50% of people with type 2 diabetes also have NAFLD.⁶¹ Compared to people without diabetes, diabetics have a far higher chance of developing cirrhosis within ten years.⁶² Over half of the people who have diabetes are predicted to have NASH and detrimental effects can be seen on outcomes from liver transplantation.⁶³ Diabetes is linked to many other complications including cardiovascular diseases which can make transplantation more difficult. The outcome of these concerns is significant because more than 70% of obese individuals have fatty liver disease, and about 30% develop NASH with the risk of cirrhosis and its consequences.⁶⁴ The frequency of these related metabolic and liver illnesses is predicted to increase dramatically by 2030, when half of American people are predicted to be medically obese.⁶⁵

1.4. Prevention Measures for NAFLD: Dietary and Lifestyle Interventions

Unhealthy eating habits contribute greatly to progression of NAFLD, especially those that are high in saturated fatty acids.⁶⁶ High fat processed meat products and high fat dairy, as well as consumption of highly processed carbohydrates have been shown to increase the risk of NAFLD.^{67,68} In a study in which a healthy group of healthy men and women (normal liver enzyme levels, ALT below 30 U/L for men and 19 U/L for women) had at least two meals from fast food chains per day for a period of four weeks, ALT levels after the first week increased sharply to the point of significant liver damage.⁶⁹ By the end of the examination, 11 of 18 subjects had ALT levels that were consistently exceeding the normal range. Eating fast food has been associated with consuming more soft drinks and meat, which can substantially influence the occurrence of NAFLD.

In the last decades, changes in the food system have also impacted population health. Food insecurity is a problem that leads to NAFLD, which is defined as the state of not having access to nutritious and healthy food that leads to high consumption of more affordable, less nutritious, high calorie foods.⁷⁰ This societal concern is especially seen in places that have food deserts, where there is a lack of health insurance, mostly in populations that have lower levels of education, and it is seen in younger people with health problems such as obesity.⁷¹ The impact of food insecurity shows itself as an increase in metabolic syndrome and risk of diabetes, as well as a higher chance of developing hepatic fibrosis.⁷² Another observation can be made about meal sizes and increases in calories in processed foods. As food manufacturing developed in technology and fast produce took place it became easier to bulk produce foods with a lot of additives to improve taste and increase consumerism, however the health of the customer was neglected. Fat content, sugar amount, sodium and quality of the ingredients in packaged and fast foods are considered unhealthy

when consumed regularly, which is not optimal when looked at the portion sizes of such addictive foods.⁷³ Sugars such as sucrose and fructose in processed forms have been studied in relation to their effects on liver health. Strong evidence backed with human data predominantly focused on sugar sweetened beverages showed increased hepatic triglyceride synthesis, steatosis⁷⁴, intestinal composition alterations in gut permeability and endotoxemia⁷⁵, hepatic uric acid production⁷⁶ and weight gain.⁷⁷ The substantial quantities of fructose, cholesterol, saturated fat, and sugar found in fast food have been shown to cause metabolic diseases, heart disease, and obesity concerns. Fast food consumption is associated with higher risk of diabetes caused by elevated levels of triglycerides, and cholesterol⁷⁸ and excessive saturated and trans fats found in these foods can disrupt metabolism.⁷⁹ Most high fat processed foods exceed daily recommended intakes of fat, sugar, and sodium.⁸⁰ Other studies have shown that consumption of foods high in fructose and cholesterol causes liver disease and obesity in animal models⁸¹, and the standard American diet high in processed foods is not suitable for health recommendations in terms of fat, sodium, and sugar.⁸² Research shows that individuals who consume more processed meat are approximately three times more likely to develop liver problems than those who consume less, and those who drink high sugar soft drinks on a regular basis were 45% more likely to get NAFLD.⁸³ Simple sugars like fructose, without any intake of fiber, cause NAFLD by activating PPAR γ which leads to de novo lipogenesis and increased triglyceride buildup in the liver.⁸⁴ Fructose affects the gut flora which leads to lipopolysaccharides (LPS) translocation and toll-like receptor-4 (TLR-4) activation in Kupffer cells in liver, resulting in hepatic inflammation.⁸⁴ Dietary saturated fat intake also further interacts with these pathways and as it facilitates the growth of commensal bacteria in the gut, increases intestinal permeability, releases and translocates inflammatory molecules, such as LPS, which increase the leakiness of the gut. LPS activates TLRs in liver and contributes to

fatty liver disease as well as increases lipid storage and inflammation.⁸⁵ A study on NAFLD patients with different body weights found that, regardless of obesity status, visceral fat accumulation was present in both groups and patients had similar liver inflammation according to their TNF- α levels.⁸⁶ It was observed in the same study that non-obese individuals had dietary cholesterol that was superabundant and dietary polyunsaturated fatty acids (PUFAs) that were significantly lower.⁸⁶ Particularly in non-obese participants, the study discovered that too high cholesterol levels raise oxysterol levels, which activates liver X receptor alpha (LXR α) and upregulates its expression. Since LXR α controls SREBP-1c, this activation stimulates fatty acid production in hepatocytes. Therefore, it can be stated that the characteristics of the fat that is consumed matters and overconsumption of cholesterol can also worsen NAFLD symptoms. Research shows that a high-fat, high-cholesterol diet triggers an increased immune response and raises inflammatory markers like interleukin 1 beta (IL-1 β).⁸⁷ This leads the transition from simple steatosis to NASH as cholesterol accumulates in liver cells. In both NASH patients and C57BL/6J mice on high fat diets, cholesterol was found to accumulate in liver cells as crystals, which then caused immune cells (Kupffer cells) to cluster around the liver cells, leading to fatty liver inflammation.⁸⁷

As written above, pathophysiology of fatty liver stems from three major factors; excess sugar intake causes de novo lipogenesis increase in liver; high fatty acid intake causes abnormal levels of fat accumulation in hepatocytes and an individual's own excess body fat which increases likelihood of fatty acid release (lipolysis) from adipose tissue.⁸⁸ The disease is associated with lower insulin sensitivity, lower insulin suppression of glucose production and free fatty acids (FFAs), and lower hepatic fatty acid oxidation. On the other hand, adipose and hepatic tissue insulin resistance increases with NAFLD conditions, as well as de novo lipogenesis in the liver.⁸⁹

This can be closely associated with excess calorie intake and obesity as it causes hyperinsulinemia and lower levels of adiponectin.⁸⁹ A randomized clinical research including 40 adolescent males with nonalcoholic fatty liver disease indicated that over the course of eight weeks, those adhering to a low added sugar diet ($\leq 3\%$ of daily calories) showed significant recovery from hepatic steatosis relative to those who were on their typical diet.⁹⁰ In another randomized study, significant differences in hepatic fat buildup were seen within two weeks when subjects were assigned to a low-calorie diet with either low-fat or high-fat content.⁹¹ The liver fat of the low-fat diet group decreased by 20%, while the liver fat of those following the same calorie intake but with a higher fat content saw an increase by 35%. The same study showed that insulin levels increased by a comparable amount in the high-fat group but decreased by about 15% in the low-fat group. According to research, just one meal high in saturated fat increased hepatic triglycerides by 35%, boosted gluconeogenesis by 70%, and decreased whole-body, hepatic, and adipose insulin sensitivity by 25%, 15%, and 34%, respectively.⁹² These findings suggested a rapid metabolic shift in insulin sensitivity as short as within four hours. Another experiment was done with rodents where important regulators such as PPARs and LPS increased with dietary saturated fat.⁹³ The supporting notion is that excessive fat consumption is directly related to intrahepatic triglycerides (IHTG) pathways and hepatic insulin resistance mediation by the activation of many intrahepatic mechanisms. In another study that examined how 38 overweight participants' liver fat buildup and metabolic health were affected by eating 1,000 extra calories per day from various sources, including sugar (soda and candy), unsaturated fat (nuts and olive oil), and saturated fat (cheese and butter).⁹⁴ The study results showed that saturated fat increased lipolysis, harmful ceramides, triggered insulin resistance and increased IHTG level by 55% which was higher than the 15% increase caused by unsaturated fat intake and the 33% increase caused by sugar intake. This data

illustrates that excess fat intake is overall not helpful for liver health and additionally the type of fat consumed matters. This brings the question of how popular weight loss diets that are effective in body fat loss may still have a contradictory effect on human health, such as low carbohydrate high fat diets like the Atkins diet, having adverse impacts on liver health, caused by high saturated fat content in the main foods of such diets like red meat, high fat dairy and animal fat.⁹⁵ A study evaluating data from the 2005-2010 National Health and Nutrition Examination Survey (NHANES) indicated that higher adherence to plant-based foods improved liver function, as shown by lower ALT, AST, and fatty liver index (FLI). In particular, individuals who included more whole food plant sources in their diets had 21% reduction in developing NAFLD.⁹⁶ In a study done in the United Kingdom among 124,546 participants, those who replaced 80 g/week of red and processed meat with legumes had a 4% lower rate of developing NAFLD,⁹⁷ and another study confirmed that increased consumption of legumes, lentils, and beans were associated with considerably decreased ratios for NAFLD up to 65%.⁹⁸

Another research study with 293 NAFLD patients that adopted healthy diet habits and physical activity, showed that with 10% weight loss, there was 90% improvement with NASH resolution, meaning that inflammation stopped, 81% improvement with fibrosis regression, and 100% improvement with steatosis improvement with 52 weeks of intervention. However, it was also observed that only 10% of the participants (29 subjects) could achieve 10% body weight loss during the experiment.⁹⁹ There is insufficient evidence with specific diet plans in order to reverse and protect against liver disease. A randomized controlled trial reported in the Journal of American Medical Association grouped patients into different diets for one year, such as the Atkins, Zone, Weight Watchers and Ornish.¹⁰⁰ Amount of weight loss was around 10 pounds for each of the groups and adherence dropped for all participants over time. Therefore, it can be stated that rather

than short term diets, lifestyle modifications where sustainability is the main goal is the most important treatment. A case control study was administered with NAFLD patients where individuals' sugar intake was tracked and adjusted.¹⁰¹ Fatty liver was positively correlated with added sugar consumption and was found to be more frequently present in those who consumed more sugar-sweetened beverages or total fructose. More glucose consumption increases liver glycogen or passes to other tissue and may get stored in liver in fat form, however fructose mainly contributes to significantly more de novo lipogenesis which generates uric acid and causes liver inflammation. AASLD does not provide specific recommendations for sugar restriction in the diet however studies indicate that limiting hepatic exposure to fructose and insulin would result in beneficial health outcomes.¹⁰² Popular diets and nutrition plans follow various techniques such as altering macronutrient intake, feeding time restrictions and eliminating food groups in which the most successful ones in weight loss are the individuals that have high adherence where patients can apply them for long term, negative energy balance implications and nutritious ingredients.¹⁰³ An example of effective popular diets includes the dietary approached to stop hypertension (DASH) diet that has been linked to a lower prevalence of NAFLD where lots of plant based foods were consumed with low fat dairy and moderate amounts of grains and meat.¹⁰⁴ Newer intermittent fasting approaches or more strict commercial diets showed short term weight loss, yet did not produce sufficient data to show consistent superiority in correlation with NAFLD improvements.^{105,106} However, it was found that frequency of the meals matters in terms of liver health. Dietary behaviors of 37 healthy men were examined in a six-week randomized controlled trial, where participants consumed 40% more calories than their normal intake.¹⁰⁷ The excess calories were provided either as high sugar and/or high fat foods that were incorporated into meals or consumed as snacks. Those who had their calories in snack form had the highest level of fat

accumulation in liver, just by increased frequency of calorie intake. The Mediterranean diet has the most evidence behind it for a healthy dietary pattern linked to NAFLD and its related conditions. This particular diet is rich in fruits, vegetables, nuts, legumes, fish, and olive oil while being low in processed meats and sugars. The Mediterranean diet has been shown to reduce hepatic steatosis and aminotransferase levels related to inflammation¹⁰⁸, lower the risk of metabolic and cardiovascular diseases¹⁰⁹, helps with weight loss¹¹⁰ and is recommended by EASL guidelines for NAFLD.^{111,112} A 6-month adherence to the Mediterranean diet showed positive results with NAFLD patients by a steatosis grade decreased in 80% of the participants with resolution in 20% of them.¹¹³ In comparison a low-fat diet showed a significant decrease in hepatic fat content within 18 months of intervention, from 24% to 16% and had positive effects on cardiovascular health.¹¹⁴ The Mediterranean diet is also known for consumption of fish for protein choice, which is rich in omega-3 fatty acids. Omega-3 polyunsaturated fatty acids, especially in the forms of eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA), are proven to improve insulin sensitivity,^{115,116} reduce intrahepatic triglyceride (IHTG) content,¹¹⁷ and improve steatohepatitis.¹¹⁸ The Mediterranean diet is also low in saturated fatty acids which are the type of fats known to cause weight gain, increase liver triglyceride concentration and visceral fat.¹¹⁹ Consumption of various fresh produce is essential for liver health as it is also observed that patients with NAFLD generally have low levels of zinc, copper and vitamins A, D and E.¹²⁰ The liver is known to be a major organ that functions in transportation and storage of these micronutrients but the correlation of fatty liver severity and lower blood levels of these components are not well understood, with an exception of vitamin E.¹²⁰⁻¹²² AASLD suggests that Vitamin E at 800 IU per day improved liver fibrosis in nondiabetic individuals however supplementation can have adverse effects on diabetic NASH patients or NAFLD diagnosis without biopsy.¹²³

1.5. The Role of Carotenoids in Liver Health

Fruit and vegetable intake has been shown to be a great source of vitamins and minerals, as well as protective compounds necessary for health.¹²⁴ Plants produce beneficial compounds as a defense against environmental stressors such as ultraviolet light, however they are also useful for human health in immunity, fighting against reactive oxygen species and anti-cancerous effects.¹²⁵ “Eat the rainbow” is a method of getting sufficient amounts of phytonutrients, as some of these chemical compounds provide color to plants.¹²⁶ Flavonoids, tannins, anthocyanins and carotenoids are the main categories of these chemical compounds.¹²⁷ Carotenoids are pigments that are biosynthesized by not only plants, but also by other photosynthetic organisms such as fungi and some bacteria.¹²⁸ Most animals cannot produce carotenoids themselves, therefore they have to be incorporated through diet and structurally modified in order to be used in active forms.¹²⁸ More than a thousand types of carotenoids are studied, which can be divided into two subgroups.¹²⁹ Carotenes are linear hydrocarbons that include lycopene, β -carotene and α -carotene and xanthophylls are oxygenated derivatives of carotenes which include β -cryptoxanthin, lutein and zeaxanthin.¹²⁹ The biosynthetic pathway of carotenoids begins with lycopene as a precursor to β -carotene and α -carotene, as its open chain structure is cyclized.¹³⁰ β -carotene is then hydroxylated into β -cryptoxanthin, which is further transformed into zeaxanthin¹³¹, while lutein is derived from α -carotene; therefore these derivatives contain hydroxyl (-OH) groups within their structure.¹³¹ Furthermore, carotenoids are known to be precursors of vitamin A. Some animal-based foods such as dairy, liver and seafood contain a significant amount of retinol and retinyl palmitate which are called preformed vitamin A or retinyl esters and can be readily used just by absorption, however carotenoids such as β -carotene, α -carotene, and β -cryptoxanthin are considered provitamin A.¹³²

Provitamin A carotenoids need to be enzymatically converted by small intestine enzymes, which cleaves them into shorter molecules called apocarotenoids which are transformed into retinal, retinol and retinoic acid.¹³³ Uptake of β -carotene into the mucosal cells is highly controlled by β -carotene dioxygenase 1 (BCO1) and scavenger receptor B-1 (SRB-1).¹³⁴ These enzymes convert carotenoids into retinoic acid and binds it to retinoic acid receptors in nucleus of enterocytes, which helps to regulate the gene expression of carotenoid intake.¹³⁴ Compared to vitamin A, carotenoids have low toxicity caused by this negative feedback system in the small intestine where absorption and accumulation of β -carotene can be controlled as well as its metabolites.^{134,135} Carotenoids are further metabolized in the liver, either stored in hepatocytes or converted into retinol for biofunctionality.¹³⁶ The liver can pack carotenoids into very-low-density lipoproteins (VLDL) to distribute it to adipose tissue where CD36 transporter protein uptakes them into the peripheral tissue.^{137,138} Adipose serves as a long term reserve for non-provitamin A carotenoids like lutein and lycopene as they accumulate in lipid droplets and are gradually mobilized as needed.¹³⁸ Any unabsorbed carotenoids pass into the large intestine, where they may be metabolized by gut microbiota before eventual excretion in feces.¹³⁸

Plant derived foods that are red may have high levels of lycopene as its double conjugated bonds allows it to absorb blue light and exert the opposite color in the spectrum.¹³⁹ Orange plants such as carrots, pumpkin and sweet potatoes are rich sources of β -carotene.¹⁴⁰ Leafy greens like spinach and kale are rich in lutein and zeaxanthin that help to protect plants by dispersing excess light energy.¹⁴¹ As chlorophyll levels in leaves decline in autumn, the once hidden carotenoids such as lutein, zeaxanthin, and β -carotene emerge and give leaves their characteristic yellow and orange pigments.¹⁴² Stress, temperature, and exposure to sunlight all have a significant impact on the production of carotenoid pigments in plants.¹⁴³ More light tends to raise carotenoid quantities as a

protective measure against oxidative damage, therefore makes them essential for photosynthesis just like other phytochemicals.¹⁴³ These compounds are highly effective not only in plants but for humans as well, since they provide a lot of benefits in immunity, have anti-cancerogenic effects, and aid in disease prevention as antioxidant agents, particularly after their absorption in the small intestine and circulation in the body. A research published in the Journal of National Cancer Institute showed that carotenoids decrease the risk of tumor formation in cancer progression¹⁴⁴, which was previously suggested in Journal of Clinical Oncology as well that plasma total carotenoid quantities had direct positive effect in inhibiting reoccurrence of certain types of cancer.¹⁴⁵ This was also revealed by a case control study conducted in China where they found that higher consumption of lycopene, β -carotene, and β -cryptoxanthin was significantly linked to a reduced risk of breast cancer.¹⁴⁶ Other studies show that subjects who ate a diet rich in carotenes and xanthophylls had a considerably decreased incidence of lung cancer¹⁴⁷, ovarian cancer¹⁴⁸, prostate cancer¹⁴⁹, inflammation and oxidative stress¹⁵⁰, asthma¹⁵¹ and serum carotenoids levels were found to be inversely correlated with risk of death from all other causes.¹⁵² Additionally, studies have found that dietary carotenoids can enhance DNA repair and have protective properties due to their antioxidant functions.^{153–155} Higher carotenoid intake is also correlated with better cognitive functions in elderly compared to other antioxidants.¹⁵⁶ Insufficient level of carotenoids was also shown to lead to a 30-45% higher risk of cardiovascular diseases such as myocardial infarction.¹⁵⁷ Some other diseases such as osteoporosis were shown to occur less with higher carotenoid serum levels which was linked lower risk in bone loss.¹⁵⁸ Antioxidant properties of β -carotene and β -cryptoxanthin showed positive effects in improvements in bone mineral density in women.¹⁵⁸ Complementary to this data, a Harvard study also concluded that great total quantities of carotenoids help to improve sclerosis.¹⁵⁹ Studies were also done on the relationship with insulin

resistance and carotenoid intake. Research showed that higher serum β -carotene levels lowered blood glucose levels in men and women and improved insulin resistance and higher fruit and vegetable intake was correlated with higher plasma carotenoid levels that resulted in lower risk for risk for type 2 diabetes.^{160,161}

A 27 year study done in Sweden with subjects 50 years old or older revealed that the specific function that β -carotene contributed towards lowering levels of oxidative stress while improving insulin sensitivity were independent of other physiological and lifestyle variables.¹⁶² HOMA-IR and serum β -cryptoxanthin in men and lycopene and β -cryptoxanthin in women were shown to be inversely correlated.¹⁶³ Research on animals also demonstrated that β -carotene enhanced insulin sensitivity and decreased oxidative stress in rats with diabetes and had benefits in the processing of glucose.¹⁶⁴ These findings show that even with genetic predispositions, a diet high in carotenoids reduces the probability of health concerns determined by age and gender and delay the onset of neurological¹⁶⁵, cardiovascular¹⁶⁶, immune related¹⁶⁷, cellular¹⁶⁸ and skeletal diseases. Carotenoids also have a positive impact in the preventative measures for fatty liver diseases. Increased intake from diet and blood concentrations of α -carotene, β -carotene and lutein were substantially linked to decreased risks of non-alcoholic fatty liver disease, according to a broad observational study that examined a decade worth of public health data.¹⁶⁹ In a two week nutritional program, thirty-seven women raised their mean intake of vegetables and fruit to around 12 portions per day and their lipid peroxidation markers were assessed.¹⁷⁰ There was an inverse relationship between indicators of liver impairment and greater plasma levels of xanthophyll carotenoids. An investigation into the impact of lycopene intake on rats with NASH caused by a diet rich in fat, revealed that lycopene decreased steatosis and inflammation and lowered markers of fatty liver such as ALT, AST, and TNF- α as well as blood sugar levels.¹⁷¹ The beneficial effects of tomato

extract and lycopene on liver cancer were investigated in rat model research.¹⁷² The findings demonstrated that by lowering levels of inflammation and oxidative stress and regulating distinct biological processes, both supplements prevented liver carcinogenesis caused by a high-fat diet (HFD). In a comparable rat study, lycopene supplementation of ≤ 2 mg/kg body weight (BW) demonstrated reduced oxidative stress symptoms, ALT and triglycerides, liver inflammation and steatosis in comparison to the HFD group.¹⁷³ A study with guinea pigs that were divided into two groups where the control group was fed with a high cholesterol diet and the experimental group with the same diet was supplemented with lutein (0.1 g/100 g) for 3 months. The lutein supplemented group had lower levels of oxidative stress markers, decreased inflammation with 32% less TNF- α levels and significantly less nuclear factor kappa β (NF- κ B) DNA binding activity which is a key regulator of inflammation.¹⁷⁴ Most of the studies done with carotenoids use encapsulation and a supplementation method, using extracted and pure forms of carotenes and xanthophylls. When current research is analyzed, not many experiments are seen with a whole food intervention of carotenoids in relation to its impact on metabolic diseases. A study done on the intake of β -carotene on cancer incidence in males that was continued for eight years was done with a 20 mg supplement intervention.¹⁷⁵ The results were negative as mortality rates were 8% higher due to cancer and cardiological diseases in those who had β -carotene pills. A systematic review based on such trials showed that in six more similar trials with β -carotene pills that were used singly compared to placebo revealed more incidences of higher mortality in subjects.¹⁷⁶ The review also analyzed over 20 other studies where β -carotene was supplemented together with other antioxidants, and still significantly increased mortality rates compared to control placebo groups.¹⁷⁶ On the other hand, many studies found associations between low carotenoid levels and increased chances of developing chronic diseases when the intake of vegetables such as leafy

greens, carrot and pumpkin is low¹⁷⁷, or it can be linked that adults in the U.S. have low fruit and vegetable consumption and therefore have low phytonutrient blood concentrations, including β -carotene.¹⁷⁸ High carotenoid plasma concentration has been correlated with better health indexes across populations¹⁷⁹, however this should not conclude that carotenoids should be primarily marketed in supplementation form when a whole food approach may be more beneficial in improving its concentration in blood. Studies show that supplementation of β -carotene to prevent diseases may have been a misguided approach, as it could have been interventions with fruits and vegetables that were studied in randomized control trials to have convincing benefits.¹⁸⁰ Food synergy concept suggests that the idea of supplementation may be intriguing for higher bioavailability and bio-functionality of studied compounds and can be observed in certain effects in the subjects; however, in most research studies, an isolated nutrient should be considered as a drug where they are not studied or regulated as one.¹⁸¹ A whole food approach may be greater than just analyzing some of its components even though today scientific approach is more on “reductionist” strategies, either supplements or eliminating parts of food when testing.¹⁸¹ To get benefits from plant-based foods, it is important to incorporate multiple servings in the daily diet¹⁸² as fruits and vegetables have been shown to promote health and prevent many illnesses.¹⁸³ However, it is very challenging to persuade individuals to alter their diets, even in the face of widely distributed public education campaigns about healthy eating.¹⁸³ Reintroducing the phytonutrients that are absent from a lot of easy access meals is one suggested approach.¹⁸³ However it was observed that with this solution, food engineering and genetic modification of foods may come with side effects that can cause dietary problems and toxicity in certain populations.¹⁸⁴ Research shows that it is important to promote whole food approaches for phytonutrient intake. Numerous studies on carotenoids have predominantly investigated their

effects using supplementation by elevated fat percentages to induce notable diseases in control groups and aimed to discover substantial alterations in intervention groups. A research study including β -carotene supplementation (300 mg/kg BW) in male Wistar rats on a 60% high-fat diet indicated improvements in liver weight index, liver enzymes, and reduction in steatosis and inflammation.¹⁸⁵ Furthermore, mice fed a 60% fat diet and lutein supplementation (300 and 500 nM) demonstrated that lutein reduced obesity and visceral fat buildup.¹⁸⁶ Another research investigation experimented with mice that were fed a 45% fat diet at a lower β -carotene dosage (3 mg/kg/day) and saw notable reductions in body weight, visceral fat, and liver lipid levels.¹⁸⁷ A three month study done with rats that were fed a low fat diet, a high-fat diet (45% fat), or a high-fat diet supplemented with lycopene (25 or 50 mg/kg/day), showed positive results with lycopene supplementation as it reduced body weight, liver weight, abdominal fat, cholesterol, and liver damage in rats¹⁸⁸. An additional study using the same animal model using a high-fat diet with 32% fat, which is lower than the 45% fat commonly used in other studies, showed that lycopene supplementation still showed beneficial effects such as reduced abdominal fat, improved liver function, and lower inflammation markers.¹⁸⁹ The majority of this research concentrates on use of supplements which have higher bioavailability and bioaccessibility therefore can be effective with lower fat diets. A study that examined carotenoids from carrot pomace powder in high-fat diets, while using whole-food sources, yet still used fat levels of over 60%, which is typically seen in whole food carotenoid experiments and found that the intervention decreased cholesterol levels, body weight and markers of oxidative stress in rats with elevated cholesterol.¹⁹⁰ Supplementation based treatments can be used in research examining the effects of carotenoids even at lower dietary fat levels, whereas whole-food methods are more frequently studied in relation to higher-fat diets.

In this study, we aimed to provide data to test how different carotenoid types would affect NAFLD conditions in the liver. Experiments were designed to fill the gap regarding a whole food approach in the research of carotenoids by intervention with dietary intake of carrots with various colors while using mice models to compare the negative effects of a high fat diet to their health.

Abbreviations:

AASLD: American Association for the Study of Liver Diseases; **ACOX1**: Acyl-CoA Oxidase 1; **ACC**: Acetyl-CoA Carboxylase; **ADA**: American Diabetes Association; **ALT**: Alanine Aminotransferase; **AST**: Aspartate Aminotransferase; **BCO1**: β -carotene Dioxygenase 1; **BMI**: Body Mass Index; **BPD-DS**: Biliopancreatic Diversion with Duodenal Switch; **CD36**: Cluster of Differentiation 36; **CPT-2**: Carnitine Palmitoyltransferase 2; **DASH**: Dietary Approaches to Stop Hypertension; **DHA**: Docosahexaenoic Acid; **DGAT2**: Diacylglycerol O-Acyltransferase 2; **DNL**: De Novo Lipogenesis; **EASL**: European Association for the Study of the Liver; **ELF**: Enhanced Liver Fibrosis; **EPA**: Eicosapentaenoic Acid; **FAST**: FibroScan-AST Score; **FAS**: Fatty Acid Synthase; **FFAs**: Free Fatty Acids; **FIB-4**: Fibrosis-4; **FLI**: Fatty Liver Index; **GLP-1**: Glucagon-Like Peptide-1; **HOMA-IR**: Homeostasis Model Assessment of Insulin Resistance; **IGF**: Insulin-like Growth Factor; **IL-1 β** : Interleukin-1 Beta; **IL-6**: Interleukin-6; **IHTG**: Intrahepatic Triglyceride; **IU**: International Unit; **LAGB**: Laparoscopic Adjustable Gastric Band; **LPS**: Lipopolysaccharide; **LVSG**: Laparoscopic Vertical Sleeve Gastrectomy; **LXR α** : Liver X Receptor Alpha; **NAFLD**: Non-Alcoholic Fatty Liver Disease; **NASH**: Non-Alcoholic Steatohepatitis; **NFS**: NAFLD Fibrosis Score; **NF- κ B**: Nuclear Factor Kappa B; **NHANES**: National Health and Nutrition Examination Survey; **PCOS**: Polycystic Ovary Syndrome; **PGC1**: Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1; **PPAR- α** : Peroxisome Proliferator-Activated Receptor Alpha; **PPAR- γ** : Peroxisome Proliferator-Activated Receptor Gamma; **PUFAs**: Polyunsaturated Fatty Acids; **RYGB**: Roux-en-Y Gastric Bypass; **SCD1**: Stearoyl-Coenzyme A Desaturase 1; **SREBP-1c**: Sterol Regulatory Element-Binding Protein 1c; **SRB-1**: Scavenger Receptor Class B Type 1; **TLR4**: Toll-Like Receptor 4; **TNF- α** : Tumor Necrosis Factor Alpha; **TGF- β** : Transforming Growth Factor Beta; **Treg**: Regulatory T Cells; **VLDL**: Very-Low-Density Lipoprotein

CHAPTER 2

2. Materials and Methods

2.1. Preparation and Formulation of Diet Pellets

White (carotenoid-control), orange (β -carotene, α -carotene), red (lycopene) and yellow (lutein) carrots were grown by and bought from a local producer. Carrots were then lyophilized at the NC State Food Innovation Lab in Kannapolis, North Carolina. Diet pellets were made by Research Diets Inc. (New Brunswick, NJ, USA). Six diet pellet varieties were adjusted in terms of fat content where low-fat diet pellets (LFD, D12450J) were prepared so that 10% calories came from fat and high-fat diet pellets (HFD, D12492) had 45% calories from fat. Final carrot products were presented in HFD diet pellets only, with 20% weight/weight (w/w) in powder form. After formulation, all diets were irradiated (10–20 kGy) for sterility. Composition of diet pellets can be found in the Supplementary Table 3.8.2.

2.2. Animals

The Institutional Animal Care and Use Committee (IACUC) at North Carolina State University approved all animal protocols which previous Eroglu lab members conducted. A total of 120 male C57BL/6J mice were obtained from Jackson Laboratory located in Bar Harbor (ME, USA). The controlled temperature and humidity room contained two co-housed mice per cage with a standard chow diet in cages under a 12-hour light/dark cycle. At 6 weeks of age the mice received random dietary distribution into six treatment groups (each group included $n = 20$ animals): low-fat diet (LFD, D12450J, 10% calories from fat), high-fat diet (HFD, D12492, 45% calories from fat), HFD with 20% w/w white carrot (HFD+WC), HFD with 20% w/w orange carrot (HFD+OC), HFD with 20% w/w red carrot (HFD+RC), HFD with 20% w/w yellow carrot (HFD+YC). The use of 20% (w/w) carrots in this study was followed due to the minimal bioavailability of carotenoids.¹⁹² The

conversion of carotenoids into apocarotenoids through cleavage exhibits stronger efficiency within the mouse system above the human system.^{193,194} Since this study investigates carotenoids instead of their metabolites, the 20% carrot concentration remains suitable despite its possible supraphysiological (approx. equivalent to 2–2.5 dietary servings /day in humans) nature for human consumption. The study subjects received food and water without any restrictions. The entire dietary treatment period lasted twenty weeks. Research personnel documented both the mouse body weight, and the food consumption levels during each week. Researchers obtained blood through submandibular collection during the entire study period. The research concluded by performing an isoflurane anesthetization followed by cardiac puncture on mice. After blood collection in a capillary tube, the specimen remained on the room temperature surface for half an hour. The blood centrifugation process at 1,500 x g with 4 °C temperature control generated serum needed for collection. Saline was used to wash the liver specimens before measurement of their weight. Liver tissue pieces were fixed in 10% formalin obtained from Thermo Fisher Scientific (Waltham, MA, USA) for subsequent histopathological assessment. All the tissues were harvested and snap-frozen in liquid nitrogen, later to be stored in a –80 °C freezer. Body composition data collection was performed utilizing EchoMRI-100 (EchoMRI, Houston, TX, USA) at both study initiation and week 20. The procedure was done by putting mice inside restraining cylinders without anesthesia, while using Velcro straps to limit movement. The chamber unit of the EchoMRI-100 measured total body composition which included fat mass, lean mass, free water and total water mass using cylinders that contained the mice.

2.3. Biochemical Assays

2.3.1. Serum Lipopolysaccharide (LPS)

The endotoxin assessment in serum samples followed the protocol supplied with the PyroGene™ Recombinant Factor C Endotoxin Detection Assay from Lonza Bioscience (Walkersville, MD, USA, Cat# 50-658U). The LAL Reagent Water provided the 1:1,000 dilution solution to analyze serum samples through two successive dilutions (1:10 and then 1:100). The preparation of Dilution 1 included 2 µL of serum combined with 18 µL LAL water and Dilution 2 consisted of 2 µL of Dilution 1 mixed with 198 µL LAL water. A 96-well plate received 100 µL solutions of samples and standards that underwent 37 °C pre-incubation for ten minutes. The combination solution containing fluorogenic substrate and assay buffer and recombinant Factor C enzyme at a ratio of 5:4:1 (100 µL/well) made the working reagent. The separation between initial and one-hour readings of the fluorescence data occurred at 37 °C. Research analysts used known concentrations of E. coli O55:B5 endotoxin to develop a standard curve for endotoxin concentration calculations.

2.3.2. Serum Lipopolysaccharide Binding Protein (LBP)

The Mouse LBP ELISA kit was used to measure LBP concentrations according to its manufactured protocol (Lonza Bioscience, Walkersville, MD, USA). The serum dilution rate was set at 1:50 using the sample diluent. The plate contained 100 µL of diluted samples before the incubation started at 37 °C for 90 minutes. The addition of biotinylated anti-Mouse LBP antibody (100 µL) to each well proceeded for a second 60-minute incubation at 37 °C before wash procedures. The solution received another set of washes before the addition of 100 µL ABC working solution and a 30-minute incubation period. TMB substrate development was carried out in the dark using 90 µL solution for 25 minutes. The measurement of absorbance took place at 450 nm after the addition of 100 µL stop solution.

2.3.3. Serum C-Reactive Protein (CRP)

The measurement of CRP levels followed procedures outlined in the Mouse C-Reactive Protein/CRP Quantikine ELISA Kit (R&D Systems, Cat# MCRP00) according to manufacturer guidelines. Serum samples were diluted 1:2,000. The assay procedure included combining 50 μ L of diluted sample with 50 μ L of assay diluent RD1W inside each well while maintaining room temperature for 2 hours. The four washing phases preceded a two-hour solution incubation period using 100 μ L of Mouse CRP Conjugate. The 100 μ L Substrate Solution incubation step took 30 minutes at room temperature with light protection. The reaction was ended using 100 μ L Stop Solution before absorbance readings at 450 nm using 540 nm wavelength correction.

2.3.4 Fecal Calprotectin

The Mouse S100A8/S100A9 Heterodimer DuoSet ELISA Kit (R&D Systems, Cat# DY8596-05) was used to measure fecal calprotectin. The extraction buffer utilized 0.1M Tris along with 0.15M NaCl and 1.0M urea and 10 mM CaCl₂ and 0.1M citric acid monohydrate and 0.5 g/L BSA at pH 8.0 to homogenize 50 mg fresh feces placed in a 1.5 mL Eppendorf tube. The extraction buffer solution contained Tris buffer at 20 mL combined with NaCl at 6 mL along with urea at 12.012 g and CaCl₂ at 2 mL and citric acid monohydrate at 421.8 mg and BSA at 100 mg that was brought to a final volume of 20 mL. The mixture underwent homogenization until all visible particles disappeared before a 10-minute ride at 10,000 \times g and 4 °C temperature. The supernatants were obtained from this mixture and utilized for further analysis.

The ELISA plate preparation process started with a 100 μ L liquid addition of capture antibody solution into each well and involved overnight incubation at room temperature. Each well received a 30 μ L blocking buffer treatment lasting throughout one hour. The plate received 100 μ L of both sample solutions and standard solutions which were then maintained at room temperature while

incubating for 2 hours. The wells received 100 μL of detection antibody solution for a 2-hour incubation step. Next, each well received 100 μL of Streptavidin-HRP solution which was incubated for 20 minutes and then exposed to substrate solution for a period of 20 minutes. The reaction process ended using 50 μL stop solution before measuring absorbance at 450 nm but wavelength correction was used at 540 nm.

2.4. Analysis of Carotenoid Concentrations Using High Pressure Liquid Chromatography (HPLC)

The extraction process of carotenoids and vitamin A from the liver tissue was done with Ultimate 3000 HPLC (Thermo Fisher Scientific, USA). A Thermo Fisher Scientific Acclaim C30 $5\mu\text{m}$ 4.6 x 150 mm column was used to perform the carotenoid separation at 25 $^{\circ}\text{C}$ inside a TCC-3000 column oven (Thermo Fisher Scientific, USA). The instrument method used nine minutes of equilibration followed by sample injection using a mixture of acetonitrile: methanol (25:75 v/v ratio at -9 through -0.5 min at 1.5 mL/min, then 25:75 v/v ratio at -0.5 to 0 min at 1.0 mL/min flow rate). The mobile phase started with a linear gradient of methyl tert-butyl ether: acetonitrile: methanol (v/v/v: 0:25:75 to 50:15:35) from time zero to 20 minutes, then operated at 50:15:35 at time 20–25 minutes at 1.0 mL/min. The fluorometric channels for tracking carotenoids and retinoids used all-trans- β -apo-8'-carotenal at 464 nm, α -carotene at 444 nm, β -carotene at 450 nm and retinol at 325 nm and retinyl acetate at 325 nm. A dilution series of β -apo-8'-carotenal internal standard was prepared by dissolving its powder in HPLC-grade acetone. The Thermo Fisher Scientific USA spectrophotometer with UV-Vis functionality measured concentration levels using an absorbance peak of 458 nm within 0.1-1 range. Beer-Lambert Law ($A = \epsilon \cdot b \cdot c$) was used with $\epsilon = 109,800$ and $b = 1$ for calculations using absorbance readings and proper internal standard

concentration was determined for addition to homogenized samples. The same procedure was followed to prepare retinyl acetate as an internal standard for vitamin A extraction using HPLC grade ethanol as solvent ($\epsilon = 51,500$ and $b = 1$). The final concentrations of internal standards were calculated to be $3.0 \mu\text{M}$ β -apo-8'-carotenal in acetone, and $1.0 \mu\text{M}$ retinyl acetate in ethanol through dilution calculations corresponding to their reconstitution volume of sample extractions. Each liver sample was weighed around 60 mg and was mixed with 1 mL of HPLC-grade acetone solution containing proper amounts of internal standards. A rotor stator homogenizer (Fisher Scientific, USA) was operated for around one minute to mix the solution. After centrifugation at $16000 \times g$ for 10 minutes at 4°C , each sample's supernatant was transferred into a new sterile eppendorf tube. Samples were dried with nitrogen gas and later reconstituted with $200 \mu\text{L}$ acetone, then $100 \mu\text{L}$ of diluted solution was added to HPLC injection tubing with a set injection volume of $20 \mu\text{L}$.

For serum samples, $180 \mu\text{L}$ of HPLC-grade hexane/acetone/ethanol solution (50:25:25) was mixed with $65 \mu\text{L}$ of serum containing $1.0 \mu\text{M}$ β -apo-8'-carotenal and retinyl acetate internal standards. The centrifugation process was at 4°C with $4,000 \text{ rpm}$ for 1 minute duration. Supernatants from each sample were separately dried using nitrogen gas. Reconstituted samples using $65 \mu\text{L}$ acetone were then used for HPLC analysis. For fecal samples, 100 mg from each group was weighed and mixed with grade acetone along with β -apo-8'-carotenal to achieve $3.0 \mu\text{M}$ in the appropriate solvent volume. The mixture was homogenized using ZR bashing bead tubes (Zymo Research, USA) by vortexing and each sample was centrifuged at $16,000 \times g$ for 5 minutes at 4°C . Using nitrogen gas, supernatants from the samples were dried and reconstituted using $400 \mu\text{L}$ HPLC-grade acetone. For each group $100 \mu\text{L}$ aliquot of the reconstituted sample was transferred into the injection vial for HPLC analysis.

2.5. Protein Expression Analysis from Liver Tissues Using Western Blot

The Western Blot technique was used to detect differences in liver protein expression through analysis of liver tissues. Radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher, USA) was added to 100 mg of liver tissue for rotor stator homogenization. Samples underwent 10 minutes of centrifugation at 12,000 rpm at 4 °C. After supernatants were removed, liver samples were diluted 1:100, 1:10 and then again 1:10 if needed, respectively. For protein quantification, 80 µL of diluted samples were added to the bicinchoninic acid (BCA) mixture in tubes, vortexed and read at 480 nm wavelength using the prerecorded method with the standard curve. Each sample was standardized by adding bio-grade water. Novex by Life technology Bolt sample reducing agent and 10 µL lithium dodecyl sulfate (LDS) sample buffer were added to each standardized protein sample and the mixture was denatured at 70°C for 10 minutes. Electrophoresis was conducted using NuPAGE Bis-Tris premade gels, running with 1X MOPs buffer. The selected protein marker ranged from the tested protein weights. The electrophoresis ran for two hours at 110 Volts. Membrane protein transfer was done using iBlot 2 Transfer nitrocellulose membranes (Thermo Fisher Scientific, USA). The cutting process for membranes was done based on specific molecular weight requirements needed for protein measurements. Membranes underwent non-specific blocking for one hour using 5% bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (TBST) solution followed by three rounds of TBST washing (each 10 minutes). Primary antibodies were diluted, then poured on membranes, followed by overnight incubation on the shaker in a cold room (4°C). Membranes were then washed three times with TBST solution before a one-hour incubation with secondary antibodies. Membrane washing was followed by analysis using UVP ChemStudio from Thermo Fisher Scientific (USA) to visualize chemiluminescent signals from western blot analysis.

2.6. Gene Expression Analysis from Liver Tissues with Quantitative Polymerase Chain Reaction (qPCR)

PureLink RNA Kit (Thermo Fisher Scientific, Cat# 12183020) was used in order to extract total RNA from liver tissue. Liver samples were weighed around 70 mg and homogenized with Lysis Buffer and 1% 2-mercaptoethanol mixture. The mixture was centrifuged at 2,600 x g for 5 minutes and 70% EtOH was then added to the supernatant to get rid of precipitate. Samples were added to spin cartridges to bind RNA to membranes. Samples were then washed three times using wash buffers, centrifuging at 12,000 × g for 15 seconds each time. The RNA was eluted into recovery tubes by adding 30 µL of RNase-free water to the center, incubating at room temperature for 1 minute and centrifuged again for 2 minutes to elute all of the RNA. The RNA concentration analysis was done using QuickDrop Spectrometer (ThermoFisher Scientific, USA) to quantify nucleic acid concentration in the purified RNA. The RNA samples were then standardized using nuclease-free water and a total of 9 µL mixture was prepared from each sample. Applied Biosystems™ High-Capacity RNA-to-cDNA™ Kit (ThermoFisher Scientific, 4387406) was used to synthesize cDNA from RNA samples. 2X RT Buffer Mix (10 µL for each reaction) and 20X RT Enzyme Mix (1 µL for each reaction) were mixed separately with each RNA mixture. The samples were loaded into the Biometra Thermal Cycler Applied Biosystems™ Real-Time PCR system where they were incubated at 37 °C for 60 minutes, followed by 95 °C for 5 minutes, and then at 4 °C. The cDNAs were then diluted 1:10 with RNase free water depending on the targeted gene cDNA concentration. In each well of a 96-well plate, 10 µL SybrUP Green Master Mix, 1 µL forward primer, 1 µL reverse primer, 3 µL RNase-free water and 5 µL of the diluted samples were then mixed specific to the target gene and sample. The plate was sealed centrifuged at 1000 x g for 2 min at 4 °C. Plates were placed in qTOWER 3 Thermal Cycler (ThermoFisher

Scientific,USA) and cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 seconds, 60 °C for 1 minute; then 95 °C for 15 sec, 60 °C for 1 min and 95 °C for 15 seconds.

2.7. Quantitative proteomics

2.7.1. Sample preparation for quantitative bottom-up colon proteomics

Peptide samples were prepared for proteomic analysis with the EasyPep Mini MS Sample Prep kit (#A40006, ThermoFisher). In brief, the liver (approximately 50 mg; n = 4) samples of mice were harvested using the EasyPep lysis buffer containing 1% nuclease. The Rapid Gold BCA kit determined the protein concentration according to manufacturer instructions, and samples were standardized to 100 µg protein with the EasyPep lysis buffer. Reduction and alkylation buffers were added to the standardized protein and incubated at 95 °C on a heat block for ten minutes. Digestion into peptides was achieved by adding a trypsin/Lys-C protease mix and incubation with shaking for three hours at 37 °C. After that, a stop solution was added to terminate the digestion. Hydrophilic/hydrophobic contaminants (i.e., buffer salts, detergents, and other biomolecules) were removed from the peptides by the clean-up columns from the EasyPep kit according to the manufacturer's instructions. The resulting cleaned peptides were then dried by nitrogen gas.

2.7.2. Tandem Mass Tag pro (TMTpro) 16-plex Labeling

The dried peptides (100 µg) were reconstituted in 100 µL of 100 mM triethylamine bicarbonate (TEAB). TMTpro reagents (TMTpro 16-plex Label Reagent Set 0.5 mg #A44521, ThermoFisher) were dissolved in 20 µL of 100% LC/MS-grade anhydrous acetonitrile following equilibration to

room temperature and the entire volume was added to the peptide samples of their respective treatment groups as shown in Table 2.1.

Table 2.1. TMTpro 16-Plex Labeling Scheme for Peptide Samples by Treatment Group

SAMPLES	DESCRIPTION	TMTpro-16plex LABEL
1-3	Low fat diet (LFD)	TMTpro-126, TMTpro-127N, TMTpro-127C
4-6	High-fat diet (HFD)	TMTpro-128N, TMTpro-128C, TMTpro-129N
7-9	High-fat diet + white carrot (HFD+WC)	TMTpro-129C, TMTpro-130N, TMTpro-130C
10-12	High-fat diet + orange carrot (HFD+OC)	TMTpro-131N, TMTpro-131C, TMTpro-132N
13-15	High-fat diet + red carrot (HFD+RC)	TMTpro-132C, TMTpro-133N, TMTpro-133C
--	--	TMTpro-134N (not used)

2.7.3. Fractionation of TMT-labeled peptides

Proteolytic digests of proteins extracted from liver tissue samples were loaded onto an equilibrated, high-pH, reversed-phase fractionation spin column. Peptides were bound to the hydrophobic resin under aqueous conditions and desalted by washing the column with water by low-speed centrifugation. A step gradient (Table 2.2.) of increasing acetonitrile concentrations in a volatile high-pH elution solution was then applied to the columns to elute bound peptides into eight different fractions collected by centrifugation.

Table 2.2. Fractionation Gradient for Peptide Elution

Fraction #	ACN %	ACN (μL)	Triethylamine (0.1%) (μL)
Wash	5.0	50	950

1	10.0	100	900
2	12.5	125	875
3	15.0	150	850
4	17.5	175	825
5	20.0	200	800
6	22.5	225	775
7	25.0	250	750
8	50.0	500	500

Each of the 1-8 eluting fraction was then dried under nitrogen gas and stored until analysis by mass spectrometry. During LC-MS analysis, peptides in each high-pH fraction were further separated using a low-pH gradient, thus reducing the overall sample complexity and improving the ability to identify low-abundant peptides.

2.7.4. Nano-liquid chromatography-mass spectrometry (nanoLC-MS/MS)

The following LC-MS/MS experiment was conducted by the Molecular Education, Technology, and Research Innovation Center (METRIC) at North Carolina State University. Materials were purchased from Fisher Scientific (Wilmington, DE): LC/MS grade water, LC/MS grade acetonitrile, LC/MS grade formic acid, PepMap™ Neo trap cartridge (C18, 5 µm particle, 300 µm × 5 mm). An Aurora Frontier analytical column (1.7 µm particle, 75 µm × 60 cm) was purchased from Ionopticks (Fitzroy, Victoria, AU).

Fractionated, TMTpro-labeled peptides were reconstituted in 100 µL water containing 2% acetonitrile and 0.1% formic acid. A 10 µL injection was analyzed by reversed-phase nano-liquid chromatography-mass spectrometry (nanoLC-MS/MS) using a Vanquish™ Neo UHPLC system (Thermo Scientific™, San Jose, CA, USA) interfaced with an Orbitrap Eclipse™ Tribrid (Thermo

Scientific™) mass spectrometer. Peptides were concentrated, desalted, and separated using a trap and elute column configuration consisting of a PepMap Neo trap cartridge (Thermo Scientific™) in line with an Aurora Frontier analytical column held at 45 °C. Mobile Phase A (MPA) consisted of water containing 0.1% formic acid, and Mobile Phase B (MPB) consisted of acetonitrile containing 20% water and 0.1% formic acid. The trap cartridge loading program used combined pressure (800 bar maximum) and flow control (100 µL/min maximum) with the loading volume automatically determined. For the analytical gradient, MPB increased from 2% at 0 min to 4% at 1 min, increased to 22% at 51 min, increased to 32% at 72 min, increased to 44% at 95 min, and increased to 50% at 105 min. The cartridge and column were washed for 10 min at 95% MPB. Mass spectrometer parameters were optimized for the detection and fragmentation of TMT-labeled peptides in a data-dependent experiment. Mass spectrometer parameters were set as follows: 1.8 kV positive ion mode spray voltage, ion transfer tube temperature of 275 °C, master scan cycle time of 3 s, m/z scan range of 400 to 1,600 at 120 K resolution, standard AGC Target, automatic MS1 injection time, RF lens of 30%, intensity threshold for MS2 scan of 5.0e4, theoretical precursor fit threshold of 70% and m/z window of 0.7, dynamic exclusion for precursors applied for 90 s, mass resolving power of 50 K for data-dependent MS2 scans, m/z isolation window of 0.7, 38% normalized HCD collision energy, 200% normalized AGC Target, and 105 ms maximum injection time.

2.7.5. Mass spectrometry data analysis

Mass spectrometer raw data files were processed by Proteome Discoverer version 3.1 (PD3.1, Thermo Scientific™) using a non-nested study design and a TMTpro quantification method. Within Chemical Modifications of PD, the TMTpro delta mass was defined as 304.207146 with possible modification at the protein N-terminus, any N-terminus, and possible residue target K.

The TMTpro parameters were set according to specifications on the product data sheet for the TMTpro 16plex Isobaric Label Reagent Set, as shown in Supplementary Table 2.

Reporter ion masses and their isotope correction factors were recorded in the Quan Channels dialog box. Residue modification of K and N-terminal modifications were selected. Mouse samples were categorized into 5 exposure groups: a low-fat diet, high-fat diet, high-fat diet red carrots, high-fat diet white carrots, and high-fat diet orange carrots, and were associated with TMT quantification channels.

A *Mus musculus* protein database (92,954 sequences, taxonomy 10090) from Swiss-Prot and a custom home-built contaminants database containing human keratins and porcine trypsin were used as database. Processing and consensus workflows were optimized for searching and quantifying TMTpro-labeled peptides. The Spectrum Files RC node used the following settings: *Mus musculus* protein database; trypsin (full) enzyme; 10 ppm precursor and 0.6 Da fragment mass tolerances; dynamic modification of TMTpro on any N-terminus; static modifications of TMTpro on K and carbamidomethyl on C; and non-linear regression with coarse tuning. The Spectrum Selector node used the following settings: MS1 precursor selection; isotope pattern re-evaluation; mass range from 350 – 5000 Da; FTMS mass analyzer; MS order of MS2; HCD activation; and scan type of full. The Reporter Ions Quantifier node used a 20 ppm integration tolerance and the most confident centroid integration method. The SEQUEST HT database search node used the following settings: *Mus musculus* and contaminants protein databases; trypsin (full) enzyme; maximum of 2 missed cleavage sites; minimum peptide length of 6 amino acids; 10 ppm precursor mass tolerance; 0.6 Da fragment mass tolerance; maximum of 3 equal dynamic modifications, which were N-terminal addition of acetyl group on methionine, oxidation or N-terminal loss of methionine, or loss of acetylated methionine; static modifications of

carbamidomethyl on cysteine, TMTpro on any N-terminus, and TMTpro on lysine. Peptides were validated by the Percolator node with a q-value set to 0.05 and a strict false discovery rate (FDR) set to 0.01. The MSF Files node in the consensus step filtered peptide spectral matches with a maximum delta Cn of 0.05 and xCorr score of 1. The Reporter Ions Quantifier node used the following settings: use unique plus razor peptides; automatic reporter abundance, no quantitation value corrections; co-isolation threshold of 50; reporter signal-to-noise threshold of 10; SPS mass matches threshold of 65%; normalization using total peptide amount; scaling using “on controls average”; protein abundance-based ratio calculations; no imputation; and ANOVA hypothesis testing.

2.8. Immunohistochemistry

The Anatomical Pathology Histology Lab located in the North Carolina State University College of Veterinary Medicine handled liver specimen preparation for processing. The liver tissues were fixed with 10% formalin solution and proceeded with 70% ethanol washing. A tissue processor was then used to dehydrate and infiltrate the tissues with paraffin wax. The samples were then sectioned, fixed in paraffin, and ready for examination. Five-micrometers thick tissue sections were stained with hematoxylin and eosin (H&E). Analysis for each test group was done through ZEISS Axio Observer microscope (Carl Zeiss Microscopy, White Plains, NY, USA) that utilized AxioVision software.

The evaluation of liver steatosis required measuring macrovesicular and microvesicular fat vacuoles percentage through four different areas, using ImageJ software (NIH, Bethesda, MD, USA) under a 20X magnification according to a standardized method. The pictures were inverted to black and white after first being transformed into an 8-bit grayscale format. The processed photos were subjected to a particular upper threshold for grayscale intensity. The "Analyze

Particles" feature of ImageJ was used for particle analysis through setting a circularity range between 0.5–1.0 to eliminate background noise and non-lipid droplet artifacts.

2.9. Fiber Analysis

Diet pellets of carrot groups HFD+WC (white carrot), HFD+OC (orange carrot), HFD+RC (red carrot), HFD+YC (yellow carrot) were sent to Beaconpoint Labs (Kannapolis, NC, USA) to perform a total fiber analysis.

2.10. Statistical Analysis

The data distribution test was performed using the D'Agostino-Pearson omnibus normality test where p values greater than 0.05 indicated normal distribution. The equality of data variance was tested through an F test where a result with $p > 0.05$ indicated equal variances. One-way ANOVA with post-hoc Tukey HSD served to detect differences between multiple population groups. Kruskal-Wallis test and post-hoc Tukey HSD determined the differences of nonparametric parameters between multiple groups. Data analysis used Two-way mixed ANOVA with post-hoc Tukey HSD to show the differences across multiple groups while incorporating time as a factor. The statistical significance threshold was established at p values less than 0.05. Statistical analysis was performed through GraphPad Prism 9 (San Diego, CA, USA) for all work. The presented values consist of text and figure data shown as mean values \pm standard error of the mean (SEM).

Abbreviations:

HFD: High-Fat Diet; **LFD:** Low-Fat Diet; **WC:** White Carrot; **OC:** Orange Carrot; **RC:** Red Carrot; **YC:** Yellow Carrot; **w/w:** Weight/Weight; **IACUC:** Institutional Animal Care and Use Committee; **CRP:** C-Reactive Protein; **LPS:** Lipopolysaccharide; **LBP:** Lipopolysaccharide-Binding Protein; **ELISA:** Enzyme-Linked Immunosorbent Assay; **TMB:** 3,3',5,5'-Tetramethylbenzidine; **HRP:** Horseradish Peroxidase; **HPLC:** High Performance Liquid Chromatography; **UV-Vis:** Ultraviolet–Visible Spectroscopy; **BSA:** Bovine Serum Albumin; **DTT:** Dithiothreitol; **LDS:** Lithium Dodecyl Sulfate; **TBST:** Tris-Buffered Saline with Tween 20; **BCA:** Bicinchoninic Acid; **SDS-PAGE:** Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; **RNA:** Ribonucleic Acid; **cDNA:** Complementary DNA; **qPCR:** Quantitative Polymerase Chain Reaction; **EtOH:** Ethanol; **RT:** Reverse Transcription; **TEAB:** Triethylammonium Bicarbonate; **TMTpro:** Tandem Mass Tag Pro; **LC-MS/MS:** Liquid Chromatography–Tandem Mass Spectrometry; **MS:** Mass Spectrometry; **MS/MS:** Tandem Mass Spectrometry; **FTMS:** Fourier Transform Mass Spectrometry; **AGC:** Automatic Gain Control; **HCD:** Higher-energy C-trap Dissociation; **FDR:** False Discovery Rate; **xCorr:** Cross-Correlation Score; **LC:** Liquid Chromatography; **MSF:** Mass Spectrometry File; **METRIC:** Molecular Education, Technology, and Research Innovation Center; **SEM:** Standard Error of the Mean; **NIH:** National Institutes of Health; **ImageJ:** Image Processing and Analysis Software, NIH.

CHAPTER 3

3. Results

3.1. Carotenoid Concentration in Diet Pellets, Serum, Liver, Fecal and White Adipose Tissue

As mentioned in the methods section HPLC, analyses of different types of carotenoids (β -carotene, α -carotene, lycopene and lutein) and vitamin A concentrations were made in various sample types. Low-fat diet (LFD) and high-fat diet (HFD) pellets did not have any carotenoids as they were control groups for carrot/carotenoid interventions (Table 3.1A, Figure 3.8.1). Diet pellets for the high-fat diet with white carrot powder (HFD+WC) were made with the same composition as the other intervention groups and were added to act as a carotenoid-deficient food matrix control, as they had the properties of carrots without the presence of any carotenoids. As expected, the LFD, HFD and HFD+WC groups did not contain any detectable carotenoids present in diet pellets, serum samples, liver and adipose tissues or in feces of mice. Orange carrot powder added high fat diet pellets (HFD+OC) had 102.64 ± 1.3 nmol/g of β -carotene (0.276 mg/g in OC powder), 76.76 ± 0.3 nmol/g of α -carotene (0.206 mg/g in OC powder), 0 nmol/g of lycopene and 1.65 ± 0.05 nmol/g of lutein (0.005 mg/g in OC powder). Red carrot powder added high-fat diet pellets (HFD+RC) had a lower amount of β -carotene with 22.78 ± 1.7 nmol/g (0.061 mg/g in RC powder), and α -carotene present was found to be 0.96 ± 0.03 nmol/g (0.003 mg/g in RC powder); additionally, lycopene in this diet was 95.19 ± 14.05 nmol/g (0.255 mg/g in RC powder) and there was 0.96 ± 1.12 nmol/g lutein detected (0.003 mg/g in RC powder). Yellow carrot powder added high-fat diet pellets (HFD+YC) contained the lowest values in all carotenoid amounts as β -carotene concentration was found to be 1.16 ± 0.03 nmol/g (0.003 mg/g in YC powder), α -carotene was detected as 0 nmol/g; furthermore, there was 0 nmol/g lycopene present in the diet pellet and lutein concentration was analyzed as 2.06 ± 0.13 nmol/g (0.006 mg/g in YC powder).

Circulating carotenoids found in serum samples were only present in red carrot and orange carrot intervention groups (Table 3.1B, Figure 3.8.2). Serum samples for HFD+OC intervention group had a higher amount of α -carotene (32.09 ± 4.45 nM) compared to β -carotene (7.76 ± 0.01 nM). Lycopene and lutein were not present in serum samples of HFD+OC group. Red carrot added high-fat diet group only had lycopene present in the serum samples. Circulating β -carotene, α -carotene and lutein levels were too small to be detected in HFD+RC group, however lycopene was found to be present in the amount of 33.89 ± 5.80 nM. HFD+YC intervention group did not have any detectable carotenoids present in serum samples.

Liver samples were analyzed to detect any storage of the carotenoids that were found in the diet pellets (Table 3.1C, Figure 3.8.3). HFD+OC liver samples had 10.15 ± 2.50 nmol/g of β -carotene, and 42.03 ± 0.1 nmol/g of α -carotene. Vitamin A (retinol) was also detected as 51.47 ± 0.12 nmol/g in HFD+OC group liver samples; however, no lycopene or lutein was detected. HFD+RC group liver samples had an average of 2.55 ± 0.57 nmol/g of β -carotene and 7.30 ± 1.80 nmol/g of lycopene present and there was no α -carotene or lutein that were detected. Vitamin A in HFD+RC liver samples were found to be 33.32 ± 3.10 nmol/g which was a little lower than orange carrot intervention group. Liver samples of HFD+YC intervention did not have any detectable carotenoids present, including lutein. Vitamin A levels that were found in HFD+YC liver samples were 21.45 ± 1.35 nmol/g, which was the lowest in all interventions.

Fecal samples were also analyzed to see if any carotenoids were not absorbed properly by the specimen and were excreted after digestion and absorption (Table 3.1.D, Figure 3.8.4). HFD+OC group had 47.74 ± 2.90 nmol/g of α -carotene, 62.19 ± 4.46 nmol/g of β -carotene and 3.7 ± 0.8 nmol/g of lutein present in feces samples. HFD+RC group had 13.23 ± 1.10 of β -carotene, and a much lower amount of α -carotene (1.88 ± 0.25 nmol/g) present in fecal samples. There was 12.03

± 3.50 nmol/g of lycopene found in HFD+RC group fecal samples and a little amount of lutein was present (1.41 ± 0.25 nmol/g). HFD+YC groups fecal samples had similar amounts of different carotenoids with 2.03 ± 0.30 nmol/g of α -carotene, 2.15 ± 0.40 nmol/g of β -carotene, and no lycopene was found in fecal samples of this group; however, lutein was a lot higher compared to other carotenoids found in HFD+YC fecal samples and was assessed as 33.14 ± 4.60 nmol/g.

Adipose tissue was also analyzed as it is a potential reserve for carotenoids, and after running HPLC for the carotenoids found in adipose tissues, there weren't any detectable or quantifiable peaks for β -carotene, α -carotene, lycopene or lutein (Figure 3.8.5). Therefore, concentrations for carotenoids were either under the limit of detection or not found in white adipose tissues collected from each group of interventions.

Table 3.1. α -Carotene, β -carotene, lycopene and lutein concentrations in a) diet pellets (n=6), b) serum (LFD: n=20; HFD, HFD+WC, HFD+OC, HFD+RC, HFD+YC: n=20), c) liver and d) fecal (LFD: n=20; HFD, HFD+WC, HFD+OC, HFD+RC, HFD+YC: n=20). n.a.: not available. Values are means \pm SD. Diet pellets were administered ad libitum.

Treatment Groups	a) Diet pellets (nmol/g)				b) Serum (nM)			
	α -carotene	β -carotene	lycopene	lutein	α -carotene	β -carotene	lycopene	lutein
LFD	0	0	0	0	0	0	0	0
HFD	0	0	0	0	0	0	0	0
HFD+WC	0	0	0	0	0	0	0	0
HFD+OC	76.76 \pm 0.3	102.64 \pm 1.3	0	1.65 \pm 0.05	32.09 \pm 4.45	7.76 \pm 0.01	0	0
HFD+RC	0.96 \pm 0.03	22.78 \pm 1.7	95.19 \pm 14.05	0.96 \pm 1.12	0	0	33.89 \pm 5.8	0
HFD+YC	0	1.16 \pm 0.03	0	2.06 \pm 0.13	0	0	0	0
Treatment Groups	c) Liver (nmol/g)				d) Fecal (nmol/g)			
	α -carotene	β -carotene	lycopene	lutein	α -carotene	β -carotene	lycopene	lutein
LFD	0	0	0	0	0	0	0	0
HFD	0	0	0	0	0	0	0	0
HFD+WC	0	0	0	0	0	0	0	0
HFD+OC	42.03 \pm 0.1	10.15 \pm 2.5	0	0	47.74 \pm 2.9	62.19 \pm 4.46	0	3.7 \pm 0.8
HFD+RC	0	2.55 \pm 0.57	7.3 \pm 1.8	0	1.88 \pm 0.25	13.23 \pm 1.1	12.03 \pm 3.5	1.41 \pm 0.25
HFD+YC	0	0	0	0	0	2.15 \pm 0.4	0	33.14 \pm 4.6

3.2. Effect of Carotenoids on Body Weight Change

The study groups displayed no initial differences in their body weight measurements, along with fat mass and lean mass values (Figure 3.2 A, C). Body fat mass distribution and lean mass

measurements matched consistently between groups according to EchoMRI results initially (Figure 3.2 A, C).

3.2.1. Body Weight Change

During this initial stage the HFD-fed mice exhibited similar body weight patterns to one another. The rapid body weight increase for all high-fat diet groups exceeded low-fat diet levels during Week 2 ($p < 0.0001$). The carrot-supplemented groups demonstrated no distinctions compared to one another during this weight measurement period. The body weight differences between each of the HFD groups started to separate clearly during Week 10. The HFD+WC and HFD+OC groups displayed the greatest body weights, yet the HFD+RC and HFD+YC groups had slightly lower weights than other HFD-fed groups which all were significantly higher than the LFD group ($p < 0.0001$). HFD+WC along with HFD+OC demonstrated the maximum cumulative weight gain at Week 10 but HFD+RC displayed statistically significant lower weight gain compared to both HFD+WC and HFD+OC ($p < 0.05$). The Week 20 final body weight measurements provided distinct results which are displayed in Figure 3.1.B. The groups of HFD+WC and HFD+OC produced the most significant body weight increase whereas HFD+RC and HFD+YC consumption resulted in slightly lower body weights among HFD-fed subjects. Week 20 body weight analysis included HFD+WC as the highest weight gain group among the other experimental groups (Figure 3.1.F). The supplement of red carrots within HFD consumption reduced body weight gain to the lowest level among all HFD-fed groups. The body mass of all high-fat-diet-fed groups exceeded that of the low-fat-diet group ($p < 0.0001$). The HFD+RC group maintained lower weights as compared to HFD+WC according to multiple comparison results ($p < 0.05$).

3.2.2. Fat Mass and Fat Mass Percentage

At Week 2, HFD-fed mice already displayed substantial rises in fat mass compared to LFD ($p < 0.001$), but the carrot-supplemented groups showed no significant differences in their fat mass accumulation. The excessive fat mass accumulation at Week 20 reached its peak in HFD+WC and HFD+OC but HFD+RC demonstrated lower fat accumulations that were statistically different from HFD+WC and HFD+OC ($p < 0.05$). The results from Figure 3.2.D show that all HFD-fed groups exhibited a higher percentage of fat mass than LFD at Week 20 ($p < 0.0001$). HFD+RC demonstrated the minimum weight in fat among all HFD-fed groups, but HFD+WC held the greatest weight in fat. Findings from the statistical analysis showed HFD+RC produced lower fat mass compared to both HFD+WC and HFD+OC ($p < 0.05$).

3.2.3. Lean Mass and Lean Mass Change

At Week 20 the lean mass reached its highest value in HFD+OC and HFD+RC demonstrated slightly lower lean mass than both HFD+WC and HFD+OC (Figure 3.2. B, E) All groups consuming high fat diets and carrot powders maintained higher lean mass levels when compared to LFD while significant differences in lean mass existed between LFD and HFD ($p < 0.001$).

3.2.4. Food Consumption

The amount of food consumption remained constant among all groups starting from Week 6. Food intake during the first five weeks exceeded 60g/week in both the HFD+YC and HFD+WC groups above other experimental conditions. By the end of Week 16 every pair within a cage consumed 35–45g/week with no significant differences in food intake observed. Initial variations in diet consumption level equalized during this period.

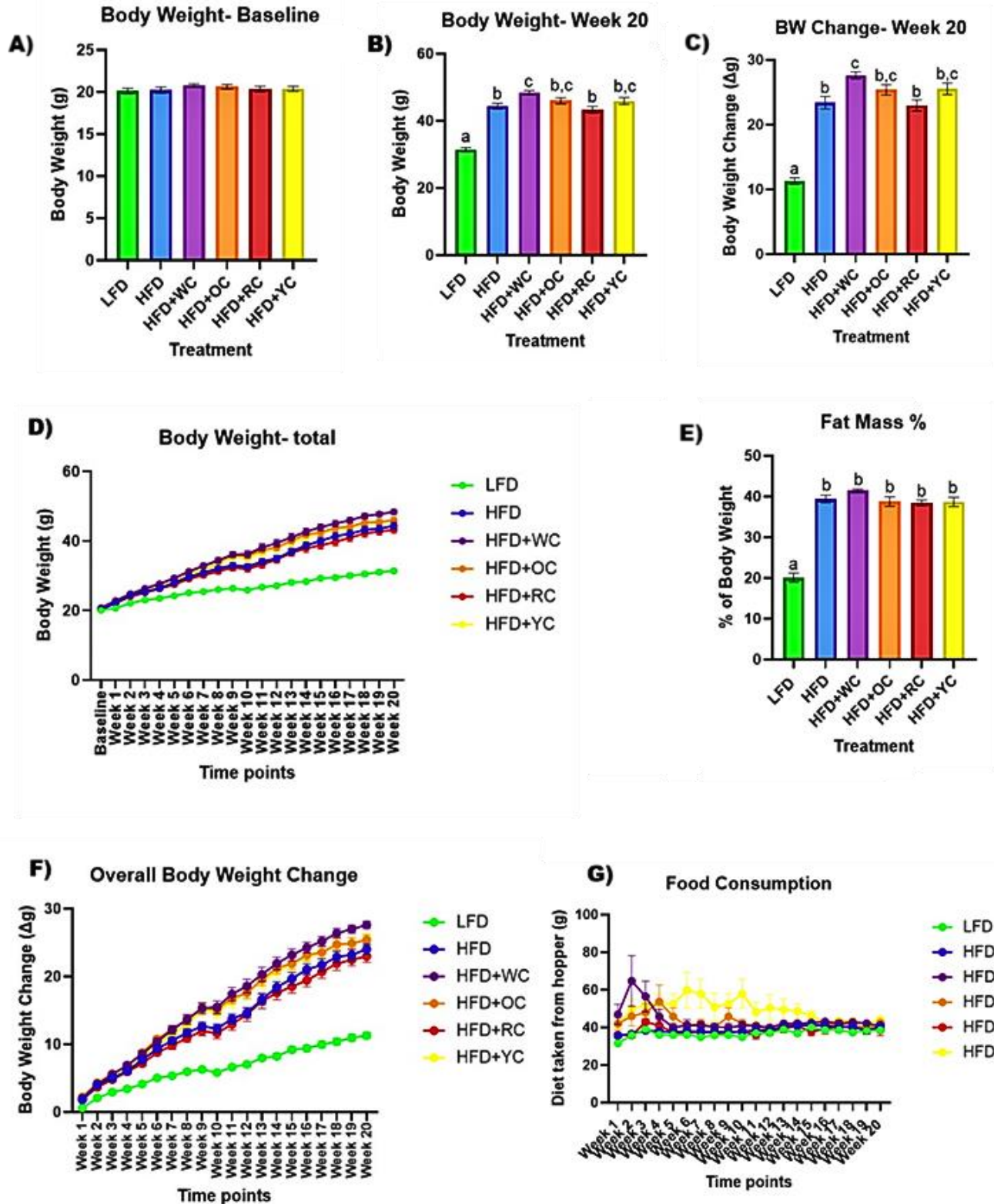


Figure 3.1: Effects of dietary treatments on body weight, fat mass, and food consumption over 20 weeks in mice. A) Baseline body weights. B) Body weights at week 20. C) Body weight change (Δg) from baseline to week 20. D) Body weight progression over time. E) Fat mass (% of body weight) at week 20. F) Overall body weight gain (Δg) over time. G) Weekly food consumption (g). Values are means \pm SEMs. Different letters above bars indicate significant differences between groups ($p < 0.05$). Abbreviations: LFD – Low-Fat Diet; HFD – High-Fat Diet; WC – White Carrot; OC – Orange Carrot; RC – Red Carrot; YC – Yellow Carrot.

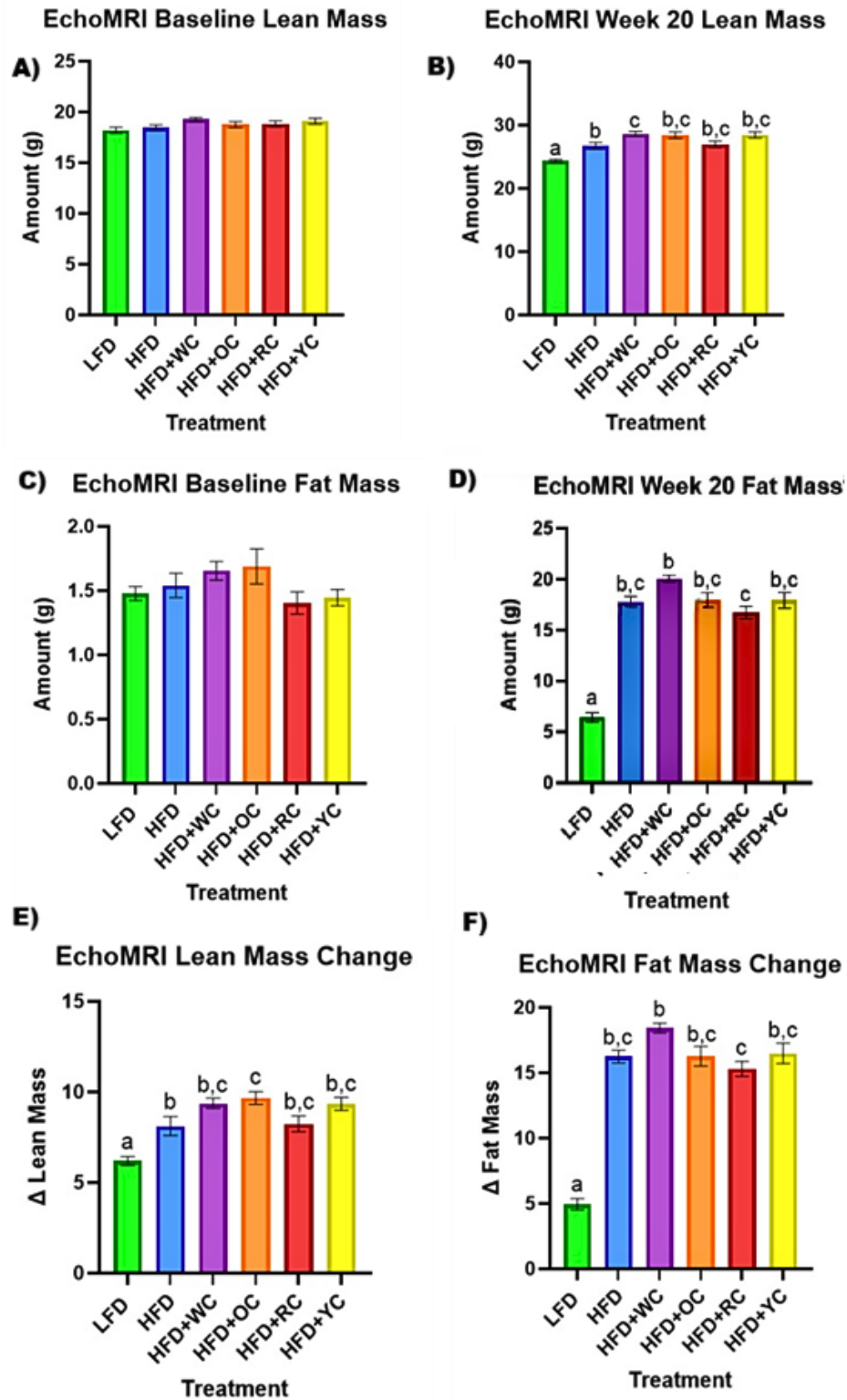


Figure 3.2: EchoMRI analysis of lean and fat mass at baseline and after 20 weeks of dietary treatment in mice. A) Baseline lean mass. B) Lean mass at week 20. C) Baseline fat mass. D) Fat mass at week 20. E) Change in lean mass (Δ lean mass). F) Change in fat mass (Δ fat mass). Values are means \pm SEMs. Different letters above bars indicate significant differences between groups ($p < 0.05$). Abbreviations: LFD – Low-Fat Diet; HFD – High-Fat Diet; WC – White Carrot; OC – Orange Carrot; RC – Red Carrot; YC – Yellow Carrot.

3.3. Effect of Carotenoids on Inflammatory Markers

Systemic and gut inflammation was evaluated through multiple analysis of the inflammatory markers calprotectin (feces), lipopolysaccharide-binding protein (LBP, serum), C-reactive protein (CRP, serum) and lipopolysaccharide (LPS, serum).

3.3.1. Calprotectin (Fecal)

Fecal calprotectin testing occurred four times to examine the extent of intestinal inflammation in the subjects (Figure 3.3A). Early diet induced inflammation was found to be reduced in carrot interventions according to Week 5 calprotectin measurements which showed statistically lower levels in HFD+WC ($p < 0.0001$), HFD+OC ($p < 0.0001$), and HFD+YC ($p = 0.0231$) compared to high fat diet group, except HFD+RC ($p = 0.2725$). HFD+OC mice exhibited lower calprotectin levels when compared to HFD mice at Week 10 and the results were only statistically significant for HFD+OC ($p = 0.0308$). There were no substantial differences found between any of the tested groups at Week 15. Only the HFD+OC, HFD+YC and HFD+RC groups showed more anti-inflammatory effects over the Weeks 20 period as demonstrated through reduced calprotectin levels when compared to HFD ($p = 0.0423$, $p = 0.047$ and $p = 0.0255$ respectively). Supplementing with orange or red carrots led to a reduction in HFD triggered intestinal inflammation and the most substantial benefits occurred during Weeks 5 and 20.

3.3.2. LBP (Serum)

LBP is a marker of metabolic endotoxemia. At Week 10, comparing with the HFD+YC group, LBP in the HFD group was significantly lower ($p = 0.0114$), while none of the other comparisons were significant (Figure 3.3B). From Week 20 on, all HFD groups, including those supplemented with carrots, had significantly higher circulating LBP levels compared to the LFD group ($p < 0.0001$ for all). However, no differences were observed between HFD groups and carotenoid

supplementation did not seem to influence the progression of diet induced endotoxemia according to this circulatory marker.

3.3.3. LPS (Serum)

During Week 10 the examined groups showed equal serum LPS levels and differences did not reach statistical significance (Figure 3.3C). This indicates that dietary changes had no impact on circulating endotoxin levels at this early time point. Week 20 outcomes revealed that the HFD+YC and HFD+RC diet groups demonstrated elevated LPS serum levels compared to the LFD group resulting in $p = 0.0041$ and $p = 0.0113$ significance respectively. LFD had no statistically different results with other HFD fed groups and different HFD groups remained non-significant to one another.

3.3.4. CRP (Serum)

In Figure 3.3D, HFD and HFD+WC diet groups during Week 10 displayed elevated CRP concentrations above the LFD group levels ($p = 0.0234$ and $p = 0.0134$). The results showed that carotenoids possibly reduced CRP elevation during HFD consumption, yet this suppression did not reach statistical significance compared to HFD+OC, HFD+RC and HFD+YC groups. CRP measures from all HFD fed subjects exceeded LFD levels ($p < 0.0001$ for all) throughout Week 20 and no essential variation existed among HFD-only and carrot supplemented groups. No significant reductions of CRP concentrations emerged at the later stages of Week 20, despite the introduction of carrot powder into the high-fat diet.

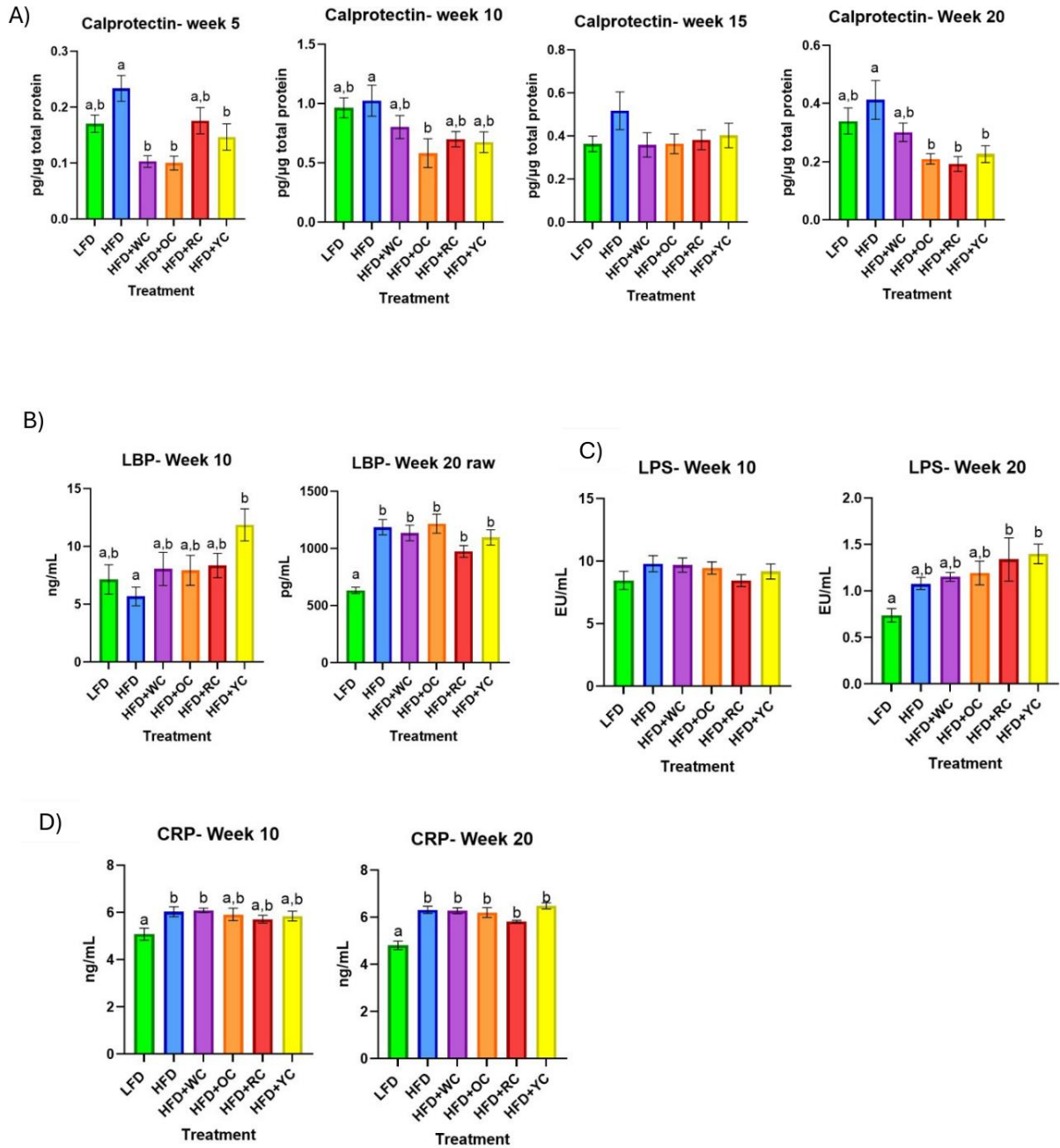


Figure 3.3: Effects of dietary treatments on gut and systemic inflammatory markers across 20 weeks. A) Fecal calprotectin levels at weeks 5, 10, 15, and 20 (pg/μg total protein). B) Serum LBP (lipopolysaccharide-binding protein) levels at weeks 10 and 20 (ng/mL and pg/mL, respectively). C) Serum LPS (lipopolysaccharide) levels at weeks 10 and 20 (EU/mL). D) Serum CRP (C-reactive protein) levels at weeks 10 and 20 (ng/mL). Values are means ± SEMs. Different letters above bars indicate significant differences between groups ($p < 0.05$). Abbreviations: LFD – Low-Fat Diet; HFD – High-Fat Diet; WC – White Carrot; OC – Orange Carrot; RC – Red Carrot; YC – Yellow Carrot

3.4. Hepatic Steatosis and Carotenoid Intervention

The amount of fat accumulation in the livers was shown by Hematoxylin and Eosin (H&E) staining (Figure 3.4.1). Histology of the HFD liver revealed a significant buildup of fat ($17.08 \pm 2.46\%$). LFD livers, on the other hand, showed much less fat accumulation ($5.10 \pm 0.80\%$, $p < 0.001$ vs. HFD). Fat accumulation was significantly lower in the HFD+WC ($6.49 \pm 1.20\%$) and HFD+OC ($6.90 \pm 1.50\%$) groups than in the HFD group ($p < 0.01$). Likewise, the fat content of HFD+RC ($6.20 \pm 2.80\%$) and HFD+YC ($5.98 \pm 2.46\%$) were substantially lower than that of HFD ($p < 0.05$). There were no overall apparent distinctions among the carotenoid treatment groups compared to HFD and all carrot groups were comparable to LFD liver histology results. According to these outcomes, no intervention was shown to be more effective than the others in reducing the formation of hepatic fat when compared to HFD, and all were effective in improving hepatic steatosis.

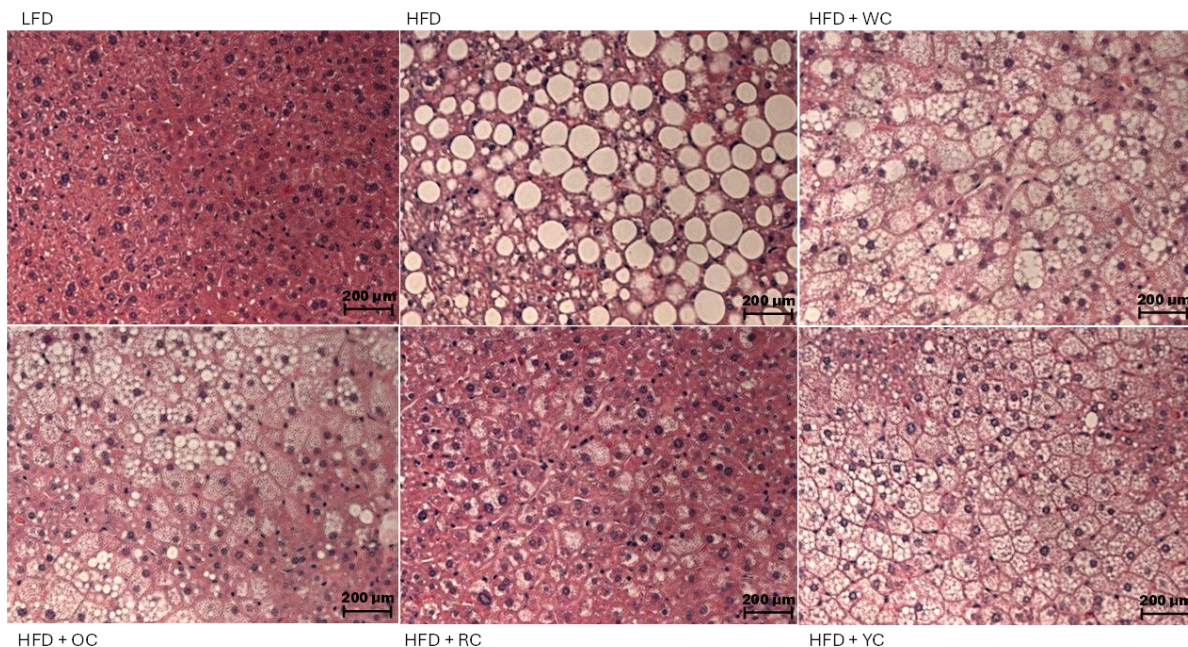


Figure 3.4.1: H&E-stained liver sections showing fat accumulation after 20 weeks of dietary treatment. Scale bar: 200 μ m. Abbreviations: LFD – Low-Fat Diet; HFD – High-Fat Diet; WC – White Carrot; OC – Orange Carrot; RC – Red Carrot; YC – Yellow Carrot.

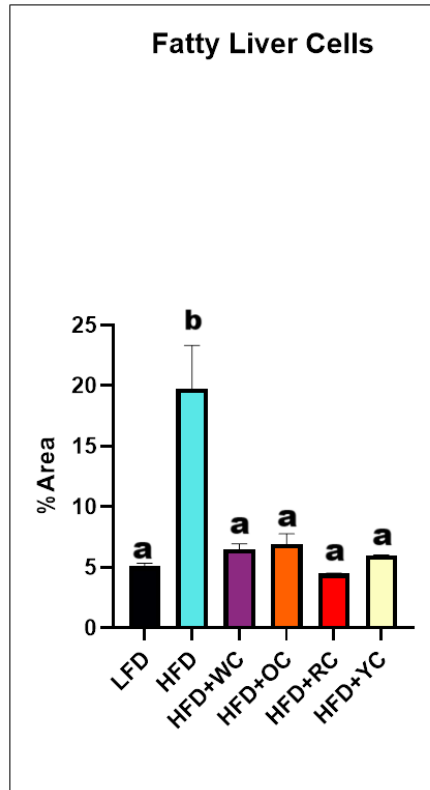


Figure 3.4.2: Percentage of fatty liver area measured from H&E-stained liver sections. HFD group showed significantly greater fat accumulation compared to all other groups ($p < 0.05$). LFD, WC, OC, RC, and YC groups showed similarly low levels of fat accumulation, with no significant differences among them. Values are means \pm SEMs. Different letters indicate statistically significant differences ($p < 0.05$). Abbreviations: LFD – Low-Fat Diet; HFD – High-Fat Diet; WC – White Carrot; OC – Orange Carrot; RC – Red Carrot; YC – Yellow Carrot.

3.5. Impact of Carotenoids on the Protein and Gene Expressions of Lipid Metabolism Regulators

Using Western Blot analysis, expressions of different proteins involved in lipid metabolism and transportation in the liver were analyzed for all six groups. Acyl-CoA oxidase 1 (ACOX-1) has peroxisomal fatty acid oxidation properties and breaks down fatty acids.³⁵ One-way ANOVA statistical analysis did not show significant differences among any of the intervention groups for protein expression (Figure 3.5.1A). Even though HFD group and HFD groups with carrot interventions showed higher qPCR data trends for *acox-1* gene expression (Figure 3.5.2A)

compared to LFD, it did not show any significant differences between treatment groups at mRNA level.

Peroxisome proliferator activated receptor alpha (PPAR α) is a major transcription factor which activates oxidation genes such as carnitine palmitoyltransferase 2 (CPT2), which is essential for transporting long-chain fatty acids into mitochondria for β -oxidation.²⁹ PPAR- α protein expression did not significantly change between intervention groups (Figure 3.5.1B). HFD+YC showed the highest mean protein expression and HFD+WC the lowest, though these differences were not statistically significant. There were no significant differences that were examined at the mRNA level for *ppara* in between groups (Figure 3.5.2B).

Fatty acid synthase (FAS) produces fatty acids that help maintain the balance of fat concentration.³² The Western Blot analysis of FAS expression of different dietary groups showed significant differences (Figure 3.5.1C); post-hoc Tukey's multiple comparisons test identified that the LFD group had significantly higher FAS expression compared to the HFD, HFD+ OC, and HFD+YC groups (p -values = 0.0065, 0.006 and 0.0123, respectively). Orange carrot, red carrot and white carrot interventions didn't reveal any significant differences compared to each other and between only high-fat diet fed group (all $p > 0.05$). Therefore, these diets do not have markedly distinct effects on FAS protein expression. Additionally, qPCR experiments did not result in significant changes in gene expression of *fas* in between treatment groups (Figure 3.5.2C).

PGC1- α (peroxisome proliferator-activated receptor gamma coactivator 1 alpha) is involved in the breakdown of fatty acids in the mitochondria of hepatocytes for energy use.^{30,31} The LFD group had significantly higher PGC1- α expression compared to the HFD+WC group ($p = 0.0094$), but no significant differences were observed between LFD and other treatment arms (all p -values > 0.05 , (Figure 3.5.1D). Similarly, comparisons between the other intervention arms (HFD vs. WC, OC

vs. RC, RC vs. YC.) did not reach any significance. LFD influenced PGC1- α expression but the effects among the other diet arms were less distinct. Gene expression of *pgc-1 α* was not different significantly between experimental groups (Figure 3.5.2D).

Another important transporter protein of fatty acids is cluster of differentiation 36 (CD36) and functions in moving them into the liver cells or adipose tissue and helps with signaling of cell metabolism pathways.^{33,34} In Figure 3.5.1E, the only significant difference of CD36 protein expression was found when comparing LFD and HFD+OC ($p = 0.011$). Other comparisons (HFD+WC, HFD+RC, and HFD+YC) did not yield significant differences ($p > 0.05$) and diets affected CD36 expression similarly when compared. Furthermore, qPCR analysis revealed significant differences in *cd36* gene expression between dietary groups and the significance was the highest between LFD vs. HFD+OC ($p < 0.0001$, Figure 3.5.2E). There was also an observed significant difference between LFD vs. HFD+WC ($p = 0.0039$). HFD+OC group had significant differences when compared to red carrot ($p = 0.018$) and yellow carrot treatments ($p = 0.003$). Other carotenoid treatments did not show significant differences compared to control groups (LFD vs. HFD+RC, $p = 0.0814$; LFD vs. HFD+YC, $p = 0.2668$).

CPT2 is essential for transporting long-chain fatty acids into mitochondria for β -oxidation.²⁹ LFD showed lower CPT2 expression compared to HFD ($p = 0.0165$) and HFD+WC ($p = 0.0144$) groups (Figure 3.5.1F). No significant differences were observed when LFD was compared to orange carrot ($p = 0.0857$), red carrot ($p = 0.0532$), or yellow carrot ($p = 0.2581$) interventions. Similarly, there were no significant differences between HFD and high fat diet with carrot added groups (HFD+WC, HFD+OC, HFD+RC, HFD+YC; all $p > 0.05$). These results show that LFD significantly reduced CPT2 expression compared to HFD and HFD+WC, but other treatments did

not show significant effects. There were no significant differences that were examined at the mRNA level for *cpt2* between groups (Figure 3.5.2F).

Thioredoxin (TRX) is a redox protein that plays a role in reducing oxidative stress and inflammation in the liver. The HFD+OC group showed significantly higher protein expression compared to HFD+RC ($p = 0.0491$), but other groups (LFD, HFD+WC, HFD, and HFD+YC) did not have significant differences ($p > 0.05$), meaning these diets do not strongly affect TRX expression compared to each other (Figure 3.5.1G).

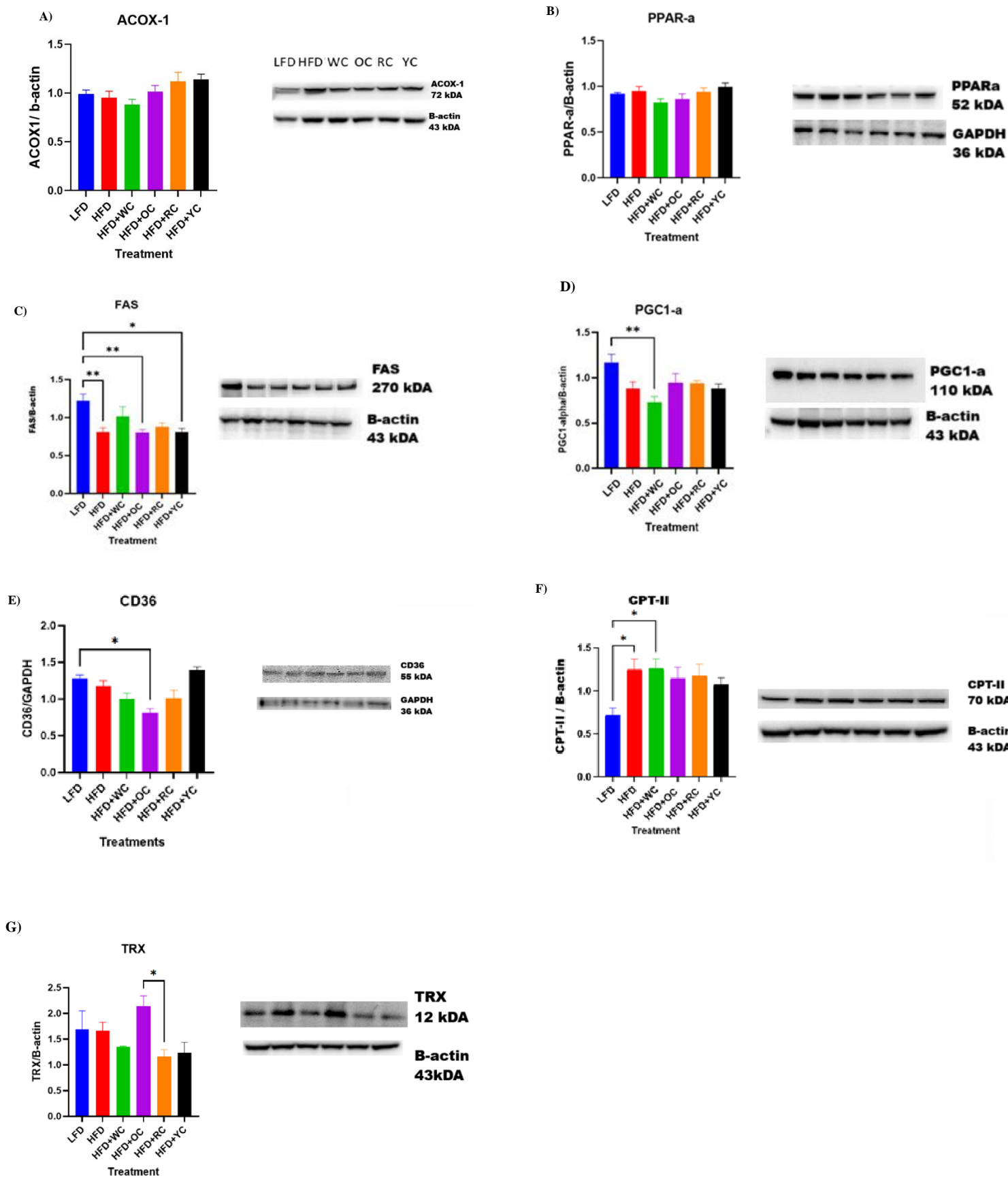


Figure 3.5.1: Hepatic expression of lipid metabolism and redox-related proteins assessed by Western blot analysis. A) ACOX-1, B) PPAR- α , C) FAS, D) PGC1- α , E) CD36, F) CPT-II, and G) TRX protein levels in liver tissues across treatment groups. Protein expression levels were normalized to β -actin or GAPDH and presented as relative fold change. Asterisks indicate statistically significant differences between groups (* $p < 0.05$, ** $p < 0.01$). Abbreviations: LFD – Low-Fat Diet; HFD – High-Fat Diet; WC – White Carrot; OC – Orange Carrot; RC – Red Carrot; YC – Yellow Carrot.

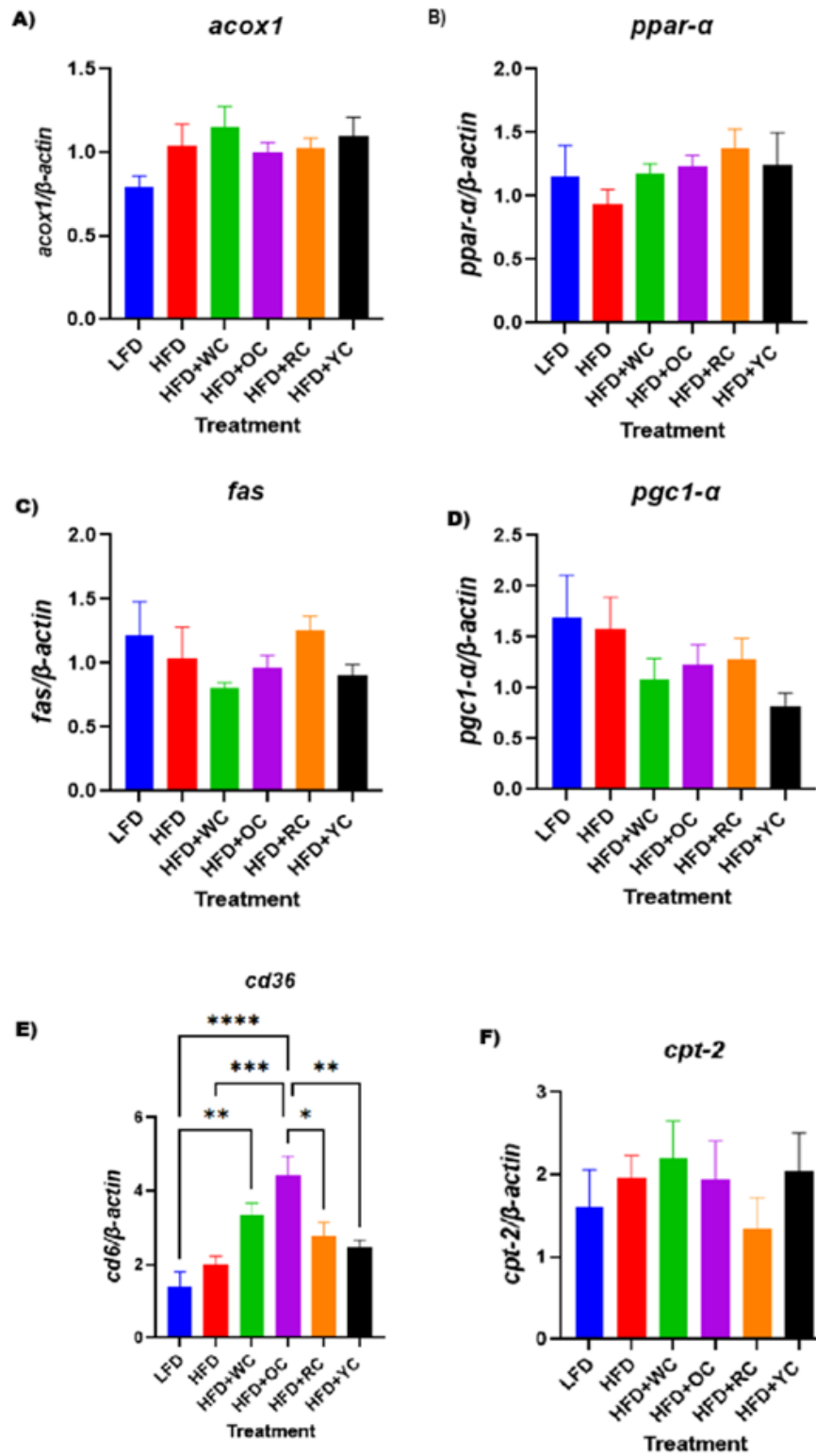


Figure 3.5.2: Hepatic gene expression of lipid metabolism regulators assessed by qPCR. A) *aco1*, B) *ppar-α*, C) *fas*, D) *pgc1-α*, E) *cd36*, and F) *cpt-2* mRNA levels in liver tissue normalized to β-actin. Significant increases in *cd36* expression were observed in HFD+WC and HFD+OC groups compared to LFD and other treatment groups. Asterisks indicate statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Abbreviations: LFD – Low-Fat Diet; HFD – High-Fat Diet; WC – White Carrot; OC – Orange Carrot; RC – Red Carrot; YC – Yellow Carrot.

3.6. Proteomics Analysis

3.6.1. HFD+RC vs HFD

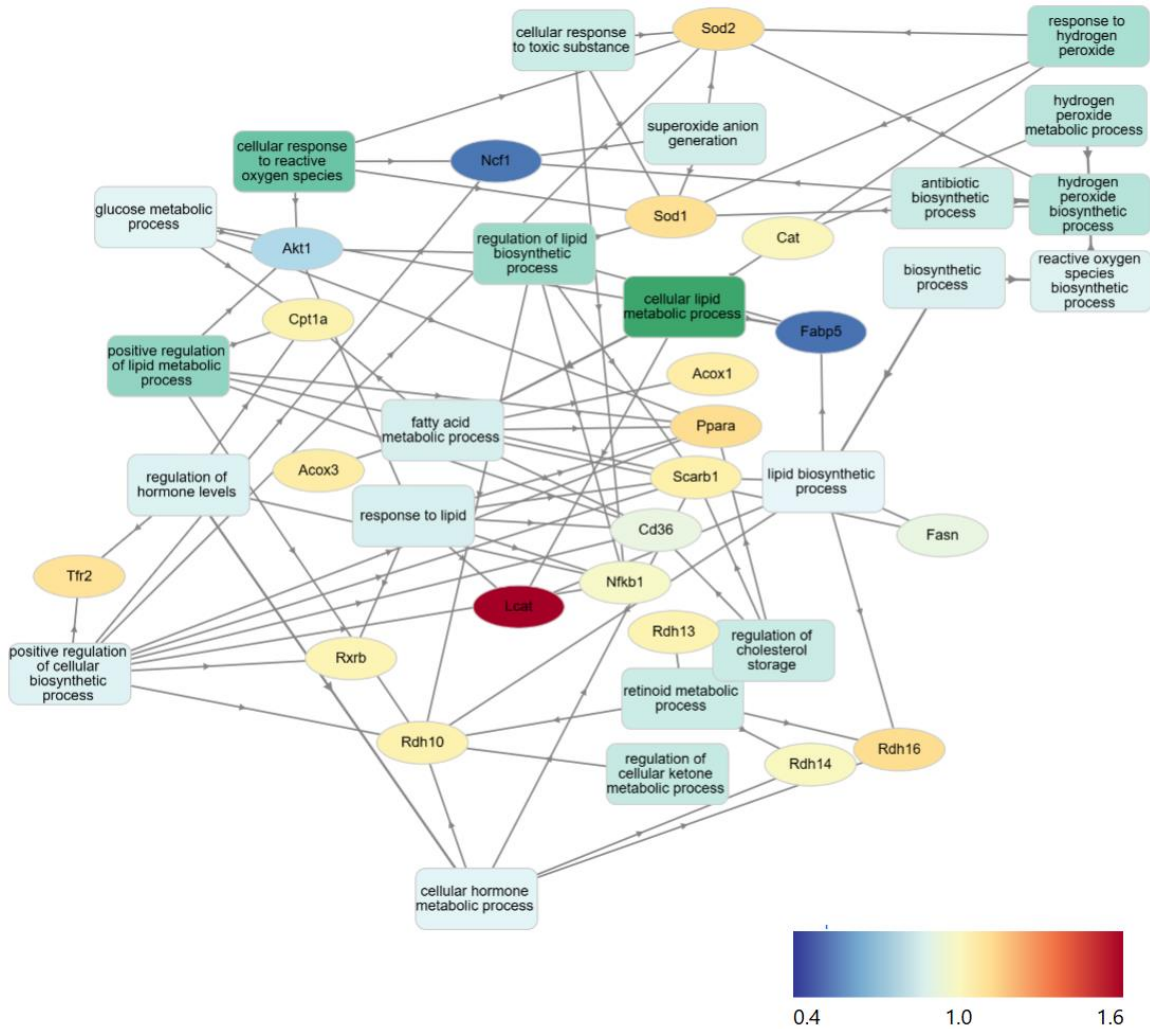


Figure 3.6.1: Gene ontology (GO) enrichment network of differentially expressed hepatic proteins in RC vs. HFD groups. Functional enrichment analysis was performed using GO terms related to metabolic and oxidative processes. Nodes represent GO biological processes (boxes) and associated genes (blue to red, based on fold change), with darker blue or red indicating greater downregulation or upregulation, respectively; in the RC group. Key enriched processes include lipid biosynthetic process, fatty acid metabolism, response to reactive oxygen species, and cholesterol storage regulation. Color scale represents normalized expression (abundance ratio fold change).

The gene ontology network (GOnet) analysis was used to elaborate on proteomics data, where it shows the networks of metabolic and regulatory pathways and several proteins that relate to them with significant differential expressions. Proteins can be seen in different colors based on their expression levels and relative fold change amongst this particular comparison (HFD+RC/HFD) where blue represents downregulation and red represents higher fold change as indicated by the heatmap scale. This network shows how red carrot intervention showed different protein expression compared to only high-fat diet (Figure 3.6.1). Lecithin-cholesterol acyltransferase (LCAT) had the highest upregulation (dark red) ($p = 0.027$). Downregulated proteins were identified as neutrophil cytosolic factor 1 (NCF1) and fatty acid-binding protein 5 (FABP5) ($p = 0.018$) which were connected to oxidative stress responses and lipid metabolic processes, however NCF1 was not significant. Several key lipid metabolism regulators were identified but were not significantly affected in terms of expression intensity were CD36, PPAR α , and SCARB1 which were shown to be involved in interactions with multiple biological processes in response to lipids and fatty acid metabolism. Oxidative stress-related enzymes such as superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), and catalase (CAT) were involved within pathways related to reactive oxygen species metabolism, although were not significantly expressed in a different manner. The detection of lipid biosynthesis and cholesterol storage is also seen with retinol dehydrogenase 13 (RDH13), retinol dehydrogenase 14 (RDH14), and retinol dehydrogenase 16 (RDH16) which are involved in retinoid metabolic processes.

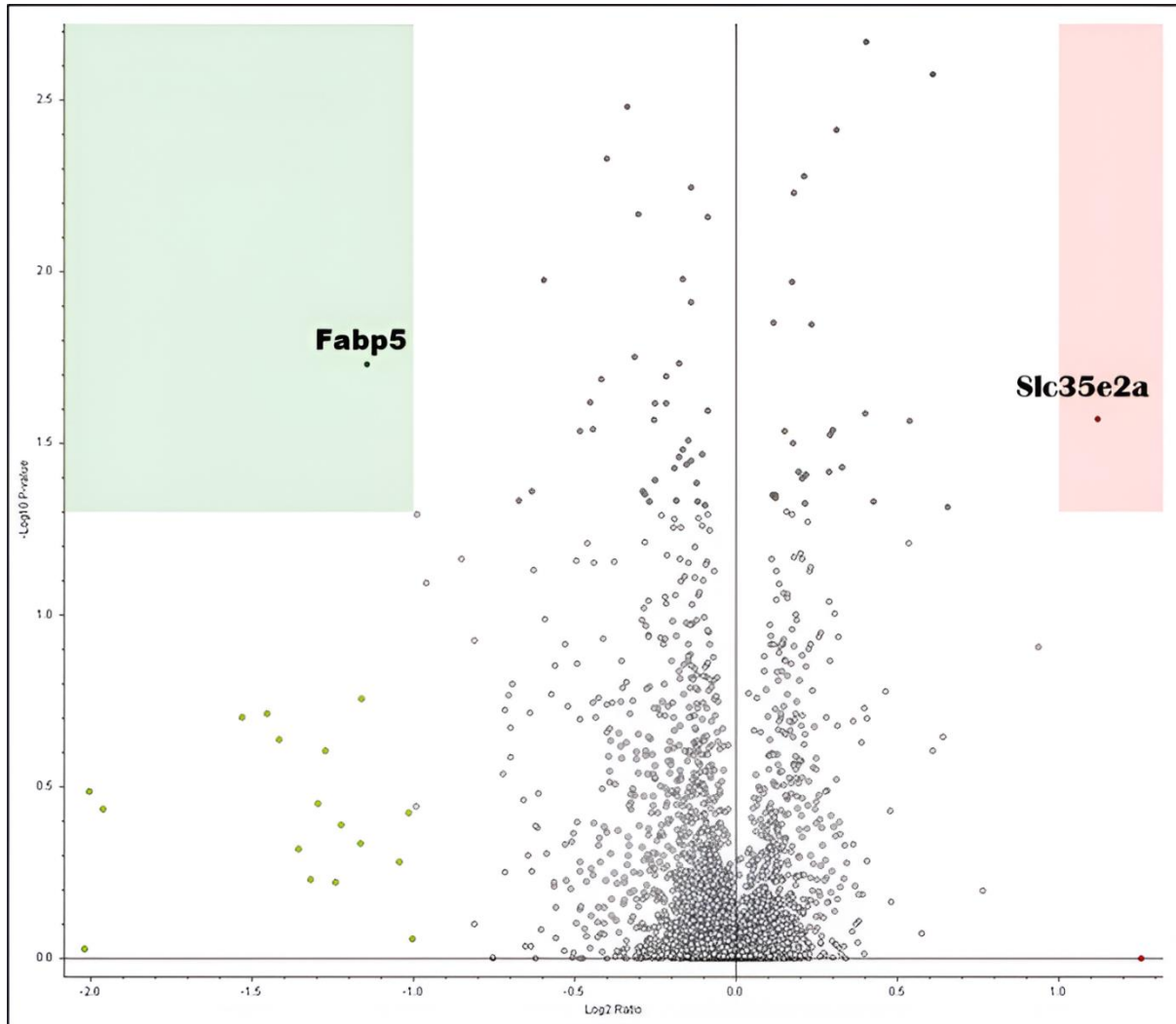


Figure 3.6.2: Volcano plot of differentially expressed hepatic proteins in HFD+RC vs. HFD groups. \log_2 fold change is plotted against $-\log_{10}$ p-value to visualize significance and magnitude of differential expression. Each point represents an individual protein; shaded regions indicate thresholds for significance and fold change.

The volcano plot further showed different protein expressions between the red carrot intervention and high-fat diet control groups (Figure 3.6.2). Here the x-axis represents the \log_2 fold change and the y-axis depicting statistical significance ($-\log_{10}$ p-value). Most proteins remained close to the center as they had little to no differential expression. FABP5 (fatty acid-binding protein 5) ($p=0.018$) was significantly downregulated in the RC group relative to HFD (green region, left side). This suggests the same pattern as GOnet diagram where it had reduced involvement in lipid transport under red carrot diet conditions. Solute carrier family 35 member E2A (SLC35E2A) is a

solute carrier protein that was upregulated in HFD+RC compared to HFD (red region, right side) ($p = 0.026$), which has a potential metabolic shift in glycosylation or related pathways

3.6.2. HFD+RC vs HFD+WC

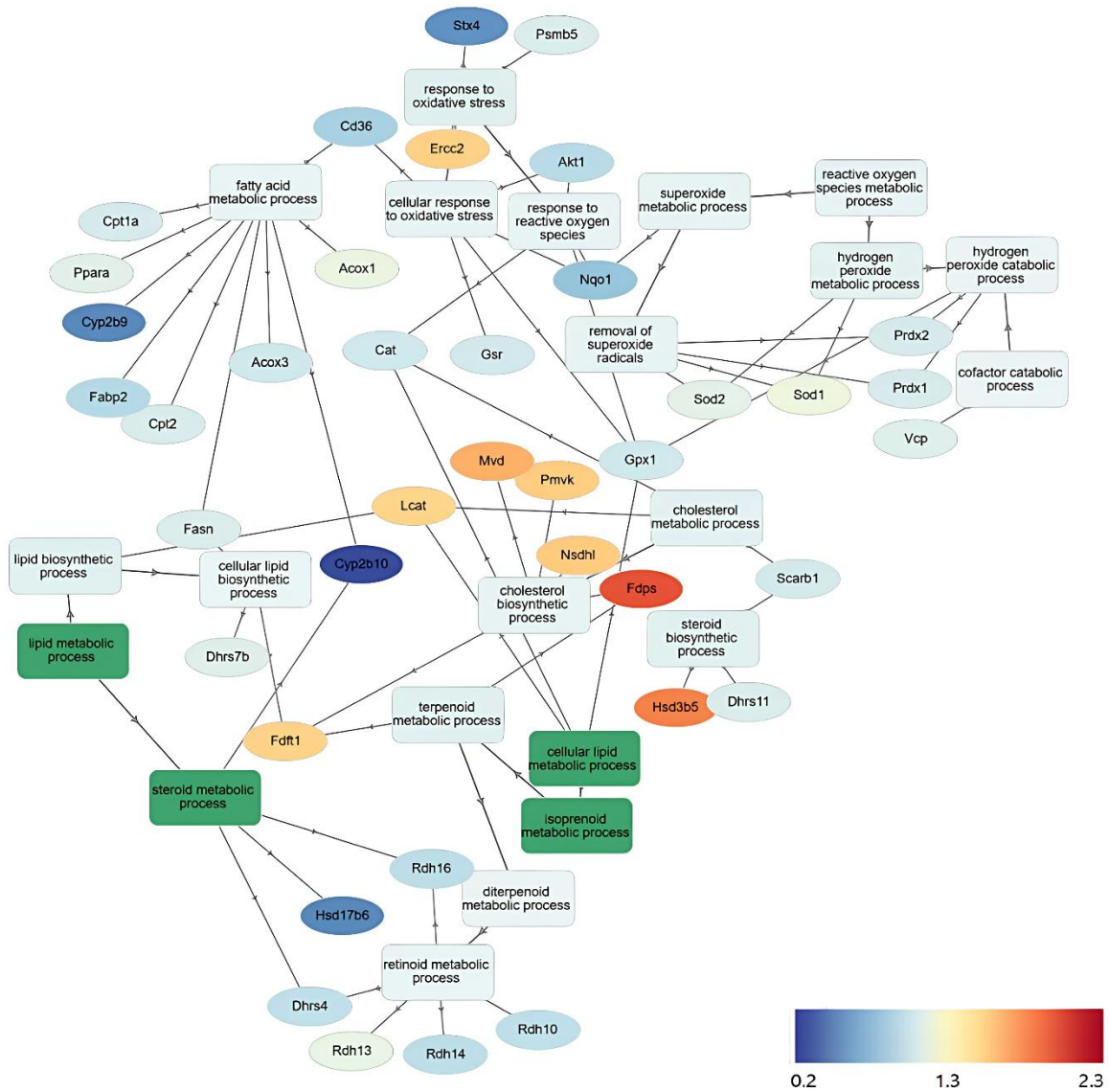


Figure 3.6.3: GO enrichment network of differentially expressed hepatic proteins in HFD+RC vs. HFD+WC groups. Network visualization shows Gene Ontology biological processes (boxes) and associated genes (blue to red nodes) altered by RC treatment compared to WC. Node color represents normalized expression (abundance ratio fold change).

When red carrot intervention was compared to white carrot intervention (Figure 3.6.3), farnesyl diphosphate synthase (FDPS) ($p = 0.02$) and 3β -hydroxysteroid dehydrogenase type 5 (HSD3B5) ($p = 0.03$) displayed the highest fold changes which showed significant upregulation. These proteins were centrally linked to steroid biosynthetic and isoprenoid metabolic processes which related to their functions in activation of cholesterol and hormone biosynthesis pathways. Other upregulated proteins were mevalonate diphosphate decarboxylase (MVD) ($p = 0.015$), phosphomevalonate kinase (PMVK) ($p = 0.015$), and LCAT ($p = 0.018$), and they were shown to be connected to the cholesterol biosynthetic and terpenoid metabolic processes and the upregulation of sterol metabolism. Cytochrome P450 2B10 (CYP2B10) ($p = 0.027$) and syntaxin 4 (STX4) ($p = 0.03$) were the most downregulated proteins which indicated potential suppression of xenobiotic metabolism and vesicle transport, respectively. Antioxidant response elements such as excision repair cross-complementation group 2 (ERCC2) ($p = 0.057$) were downregulated compared to the white carrot group, which was related to the cellular response to oxidative stress. Other antioxidant enzymes such as superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), glutathione-disulfide reductase (GSR), and NAD(P)H quinone dehydrogenase 1 (NQO1) showed slightly increased expression, though not significantly with the exception of SOD1 ($p = 0.027$), and were analyzed as clustering around reactive oxygen species detoxification pathways.

3.6.3. HFD+OC vs HFD

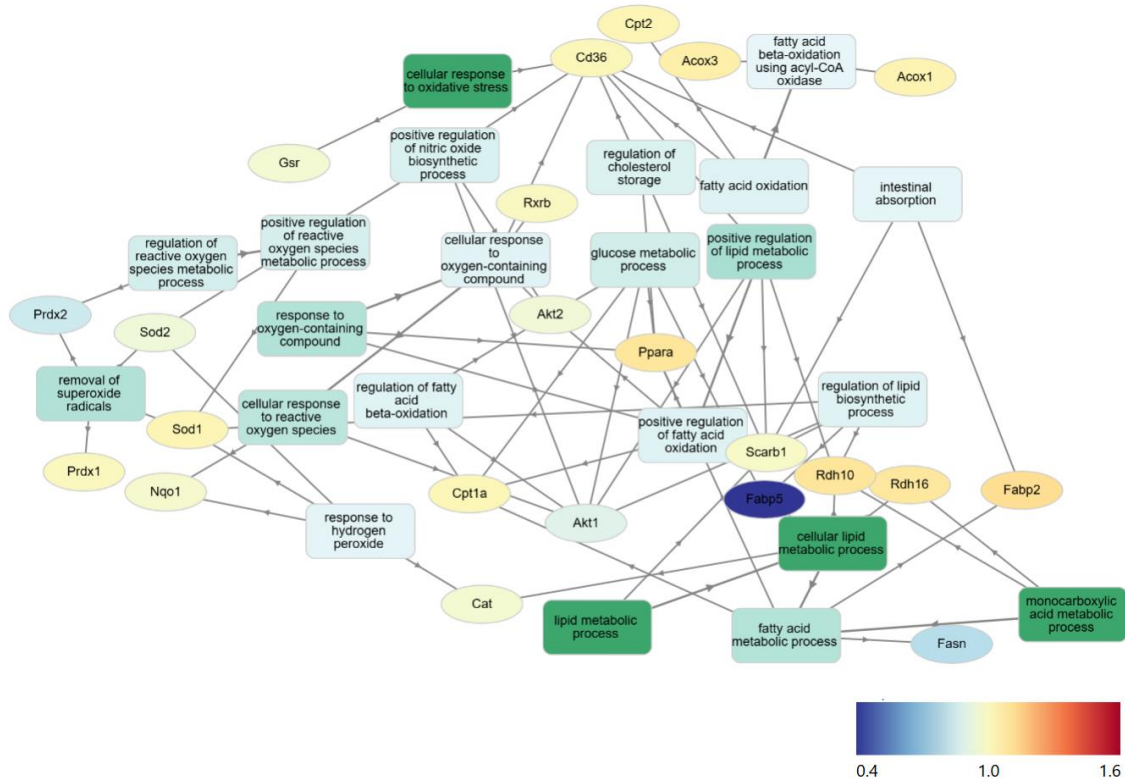


Figure 3.6.4: GO enrichment network of differentially expressed hepatic proteins in HFD+OC vs. HFD groups. Network shows enriched Gene Ontology (GO) biological processes (boxes) and related genes (blue to red nodes, colored by fold change). Color scale represents normalized expression (abundance ratio fold change).

Orange carrot intervention compared to a high fat diet showed identification of some regulators such as CD36, PPAR α , and SCARB1, as they are involved in lipid metabolism and are related to fatty acid oxidation, glucose metabolism, and cholesterol storage (Figure 3.6.4). However, even though there was upregulation in all, fold change of these proteins was not high and no significance was seen. FABP5 ($p = 0.01$) was significantly downregulated and was very evident in the network. CPT2 and ACOX3, which is acyl-CoA oxidase 3, were upregulated, but significant fold change was not achieved. Oxidative stress-related processes were also identified with SOD1, SOD2, NQO1, and peroxiredoxin 1 (PRDX1), peroxiredoxin 2 (PRDX2), but did not meet the threshold

of significance. AKT serine/threonine kinase 1(AKT1), also showed downregulation patterns and was shown to be related to lipid and glucose metabolic pathways but was not statistically significant.

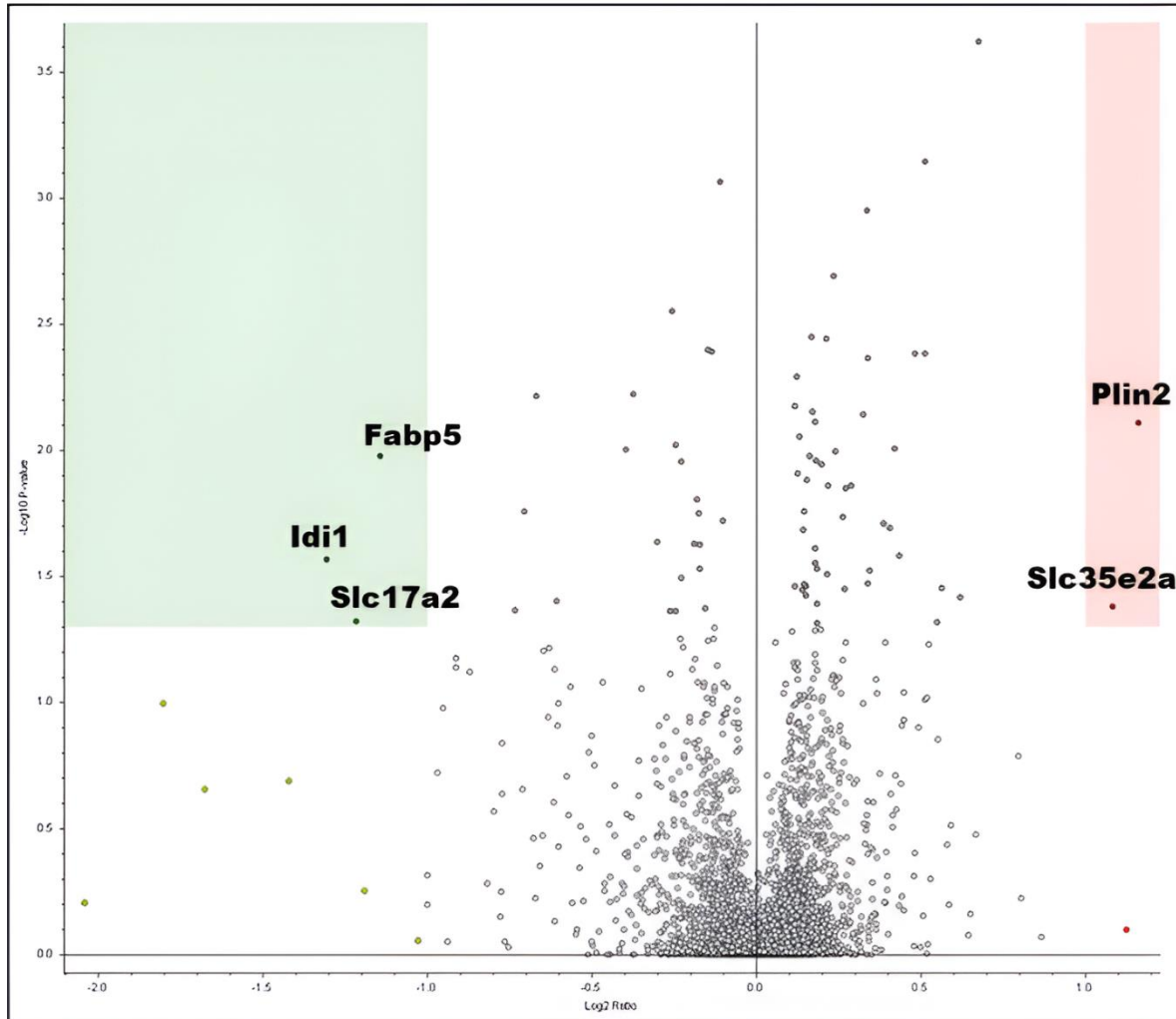


Figure 3.6.5: Volcano plot of differentially expressed hepatic proteins in HFD+OC vs. HFD groups. Log₂ fold change is plotted against $-\log_{10}$ p-value to identify significant protein expression differences. Fabp5, Idi1, and Slc17a2 were significantly downregulated in the OC group (green zone), while Plin2 and Slc35e2a were upregulated (red zone). Each point represents an individual protein; shaded regions indicate thresholds for significance and fold change.

According to the volcano plot, key proteins that were significantly downregulated were isopentenyl-diphosphate delta-isomerase 1 (IDI1) ($p = 0.02$), and solute carrier family 17 member 2 (SLC17A2) ($p = 0.047$), along with FABP5 ($p = 0.01$) in the HFD+OC group. Upregulated

proteins were perilipin 2 (PLIN2) ($p = 0.007$), which is a lipid droplet-associated protein, and solute carrier family 35 member E2A (SLC35E2A) ($p = 0.041$). These proteins can cause changes in lipid storage and glycosylation pathways.

3.6.4. HFD+OC vs HFD+WC

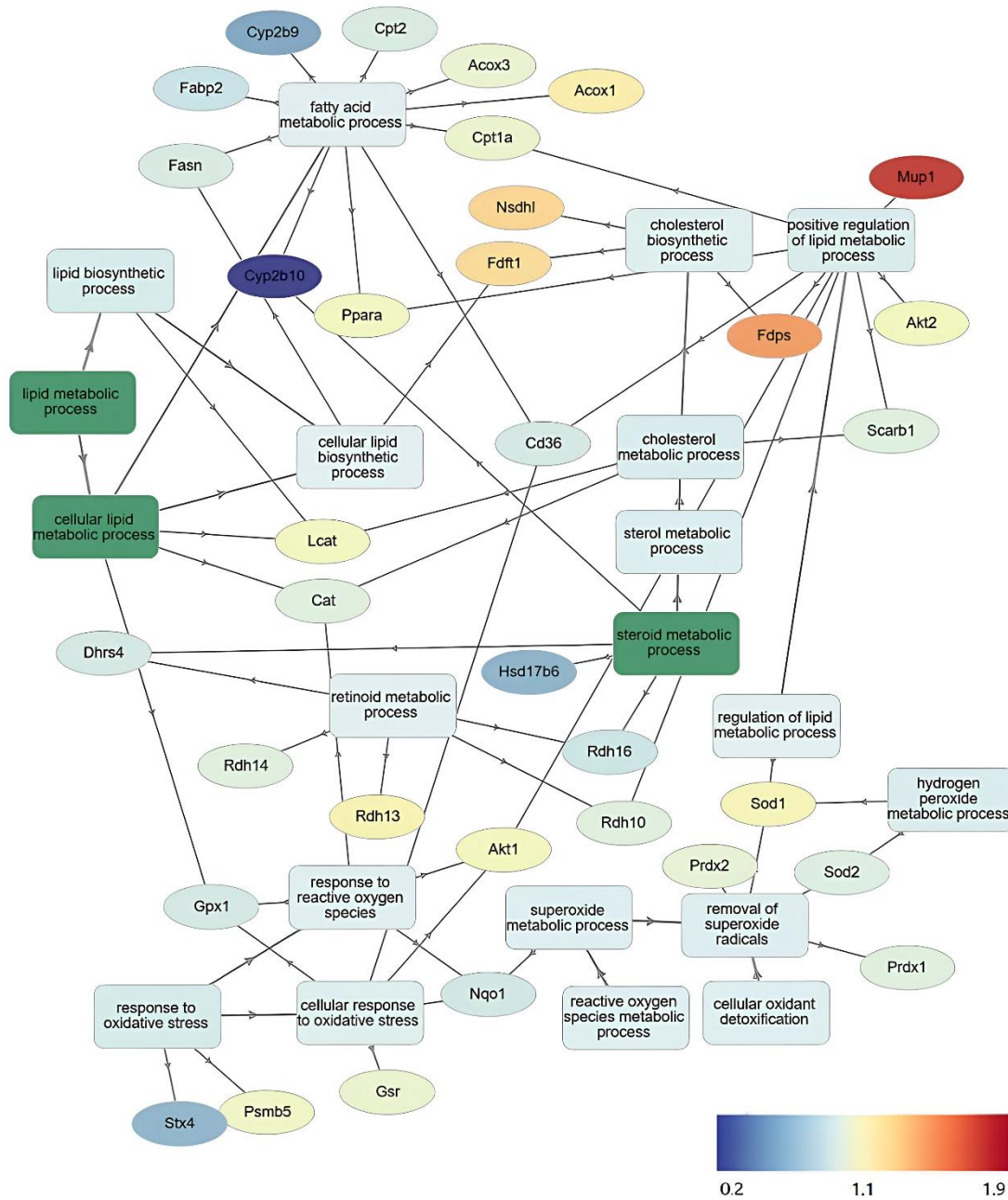


Figure 3.6.6: GO enrichment network of differentially expressed hepatic proteins in HFD+OC vs. HFD+ WC groups. Network visualization shows Gene Ontology biological processes (boxes) and associated genes (blue to red nodes) altered by OC treatment compared to WC. Node color represents normalized expression (abundance ratio fold change).

When orange carrot intervention was compared to the carotenoid-deficient white carrot control group, CD36, PPAR α , and SCARB1 had more downregulation patterns although it was moderate and not significant (Figure 3.6.6). The protein that was significantly downregulated was cytochrome P450 2B10 (CYP2B10) ($p = 0.000001$), which showed strong upregulation linked to lipid metabolic regulation, and in the same pathway, major urinary protein 1 (MUP1) had upregulation changes although significance was not observed. Upregulation patterns in steroid metabolism and cholesterol biosynthetic pathways were also seen with hydroxysteroid 17-beta dehydrogenase 6 (HSD17B6) and farnesyl diphosphate synthase (FDPS) protein expressions, while not statistically significant. Other proteins such as SOD1, PRDX1, PRDX2, STX4, and NQO1 were within pathways associated with reactive oxygen species detoxification and cellular oxidative responses but did not show significant fold changes.

3.6.5. HFD+WC vs HFD

HFD+WC compared to the only HFD group showed several protein expressions that were significantly different in the proteomics analysis (Figure 3.6.7). IDI1 ($p = 0.001$), FABP5 ($p = 0.0044$), selenium-binding protein 2 (SELENBP2) ($p = 0.01$), FDPS ($p = 0.002$), MVD ($p = 0.013$), and SWAP switching B-cell complex subunit 70 (SWAP70) ($p = 0.03$) were significantly downregulated with white carrot intervention. These proteins are commonly associated with lipid metabolism and oxidative processes. Upregulated proteins were CYP2B10 ($p < 0.0001$), PLIN2 ($p = 0.002$), SLC35E2A ($p = 0.007$), and HSD17B6 ($p = 0.018$). CYP2B10 showed the highest ratios of both significance ($p < 0.0001$) and upregulation fold change, compared to other proteins.

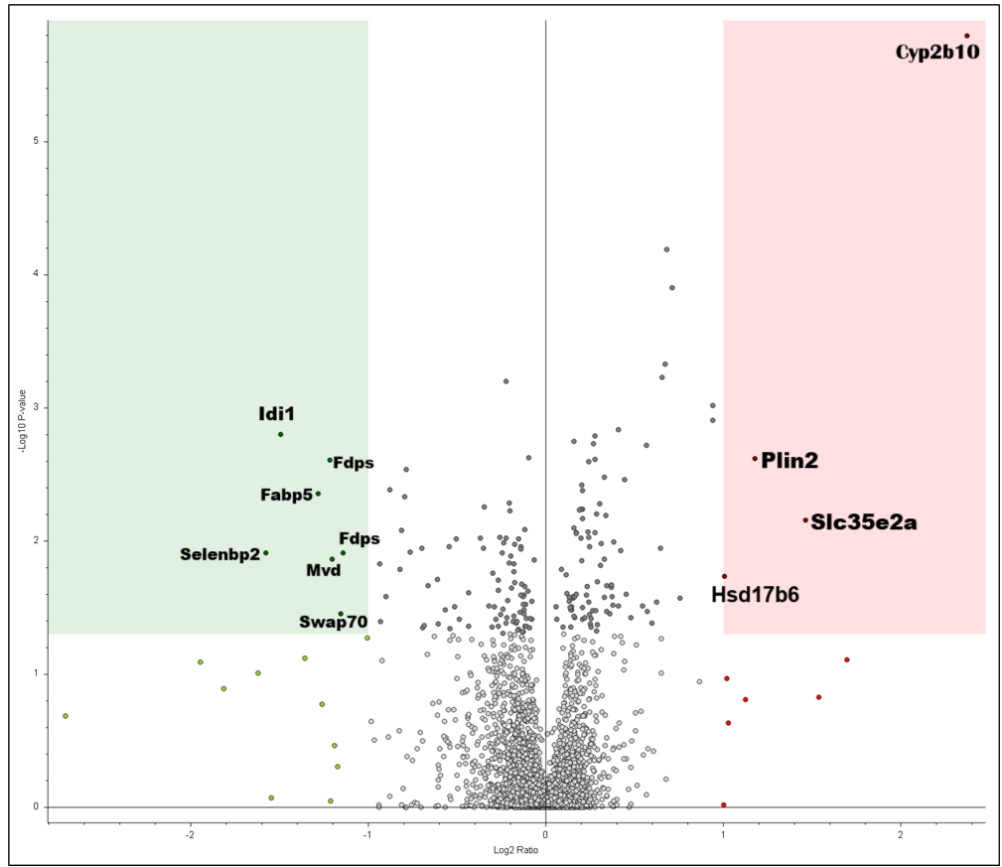


Figure 3.6.7: Volcano plot of differentially expressed hepatic proteins in HFD+WC vs. HFD groups. Log₂ fold change is plotted against -log₁₀ p-value to show protein expression differences. Proteins such as Idi1, Fdps, and Selenbp2 were significantly downregulated in WC (green zone), while Cyp2b10, Plin2, Slc35e2a, and Hsd17b6 were significantly upregulated (red zone). Each dot represents an individual protein; shaded areas highlight significant expression changes.

3.6.5. LFD vs HFD

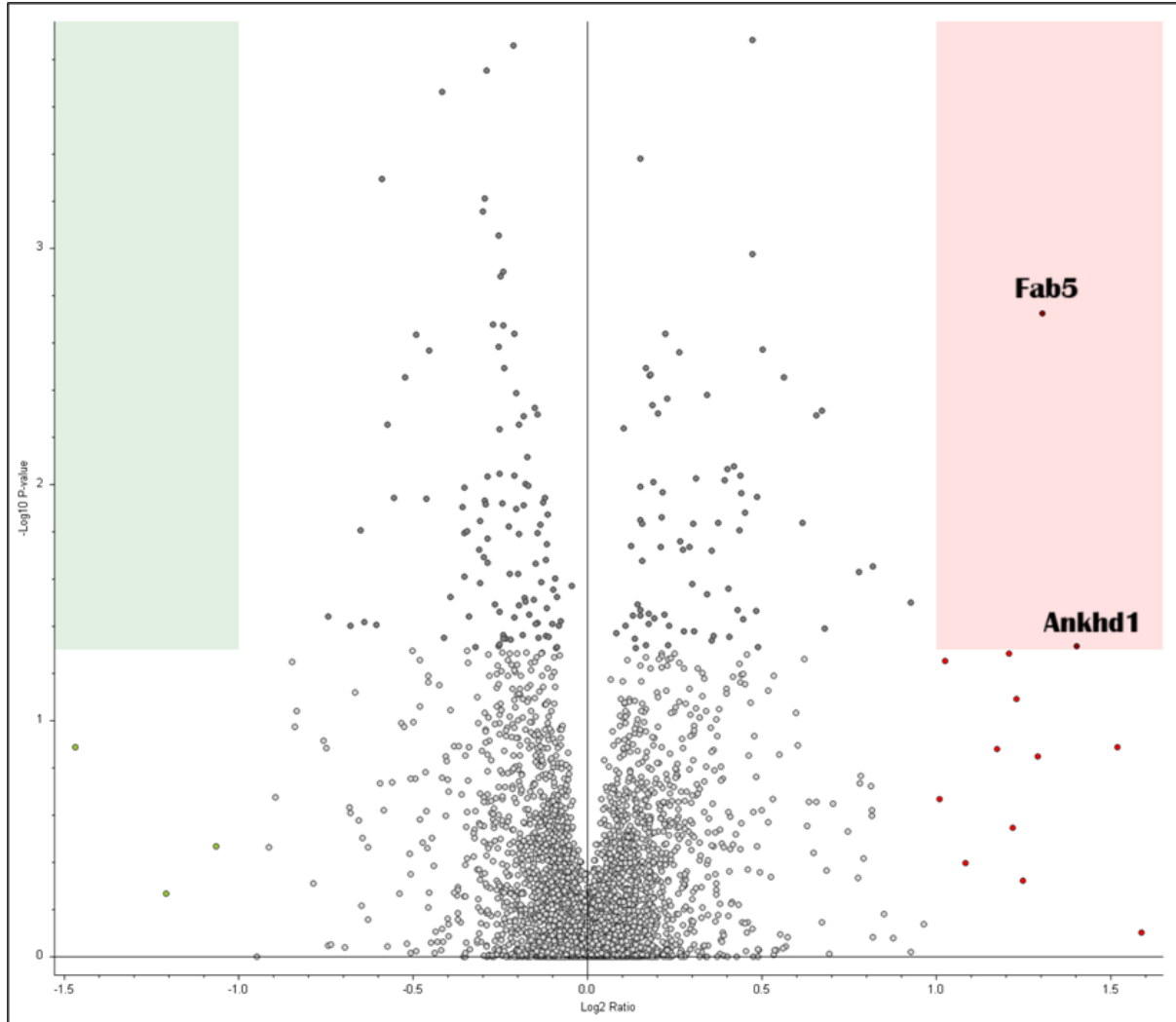


Figure 3.6.8: Volcano plot of differentially expressed hepatic proteins in LFD vs. HFD groups. Log₂ fold change is plotted against $-\log_{10}$ p-value to illustrate differences in protein expression. Fab5 and Ankhd1 were significantly upregulated in the HFD group (red zone). Each dot represents an individual protein; shaded areas indicate statistically significant expression changes.

The low-fat diet comparison to the high-fat diet (Figure 3.6.8) showed a significant upregulation of FABP5 ($p=0.001$) and ANKHD1 ($p=0.01$). FABP5 only showed upregulation with the low-fat diet group which might be caused by an adaptive response to lower dietary fat availability.

3.7. Fiber Analysis

Table 3.7. Total Dietary Fiber Content of Diet Pellets

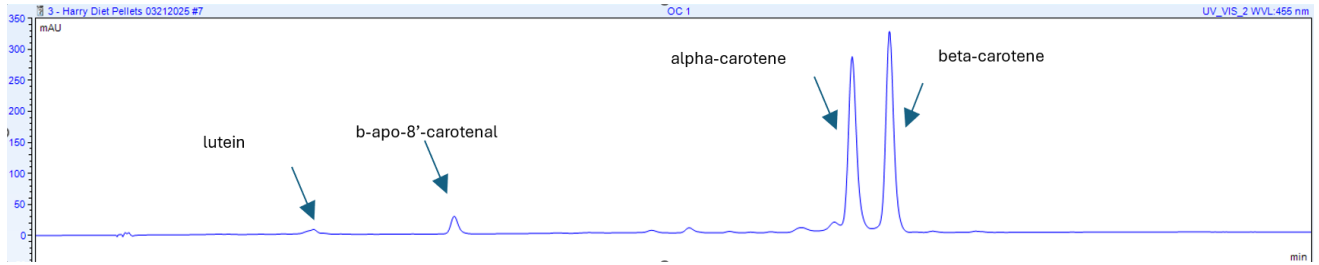
Sample Description	Analysis Method	Result (%)
HFD-WC Diet Pellet – White Carrot+ HFD	AOAC 991.43 (Total Fiber)	6.97
HFD-OC Diet Pellet – Orange Carrot+ HFD	AOAC 991.43 (Total Fiber)	6.88
HFD-RC Diet Pellet – Red Carrot+ HFD	AOAC 991.43 (Total Fiber)	6.27
HFD-YC Diet Pellet – Yellow Carrot+ HFD	AOAC 991.43 (Total Fiber)	6.31

Total dietary fiber (TDF) analysis of all diet pellets that had carrot powders was done using the AOAC 991.43 method by Beaconpoint Labs (Table 3.7), which is an enzymatic-gravimetric method that calculates TDF amounts in food that contain both soluble and insoluble fiber types. The aim was to see if the fiber content would differ between white, orange, red, and yellow carrot diet pellets. The white carrot-based diet pellet had the highest fiber content with 6.97% total fiber, the orange carrot-based pellet had 6.88% total fiber, while the red carrot diet pellet exhibited the lowest fiber content as 6.27% and the yellow carrot pellet contained 6.31% fiber. These differences were shown to be relatively small to cause a major difference in between interventions as the maximum variation was 0.7 percentage points in between groups.

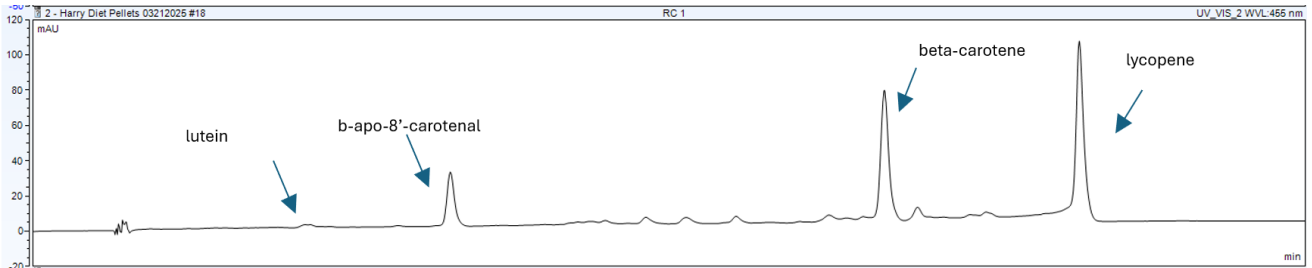
3.8. Supplementary Materials

3.8.1. Diet Pellets HPLC Chromatograms

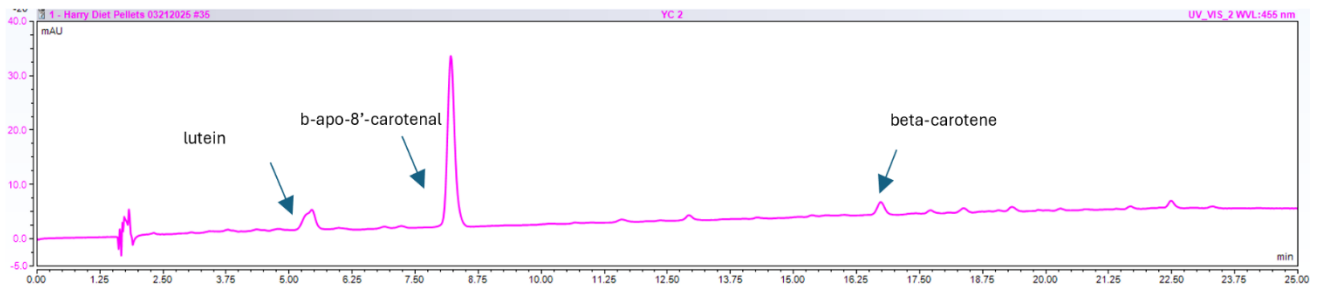
3.8.1.1 Orange Carrot Diet Pellets



3.8.1.2 Red Carrot Diet Pellets

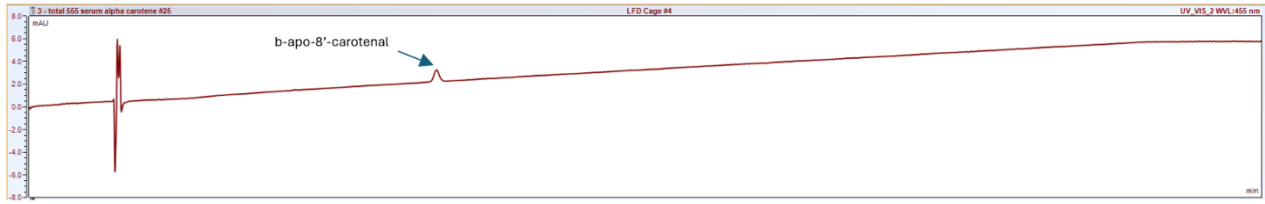


3.8.1.3 Yellow Carrot Diet Pellets



3.8.2. Serum HPLC Chromatograms

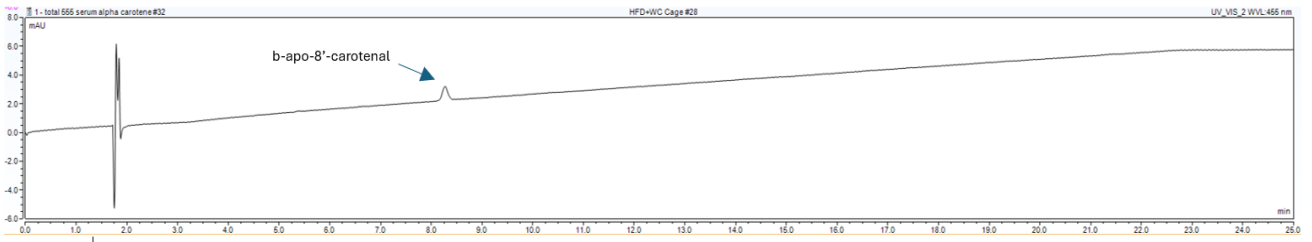
3.8.2.1. Low Fat Diet Serum



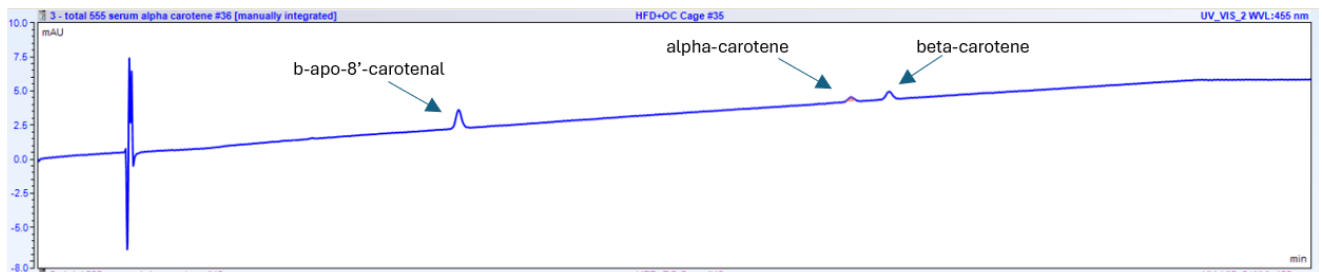
3.8.2.2. High Fat Diet Serum



3.8.2.3. White Carrot Serum



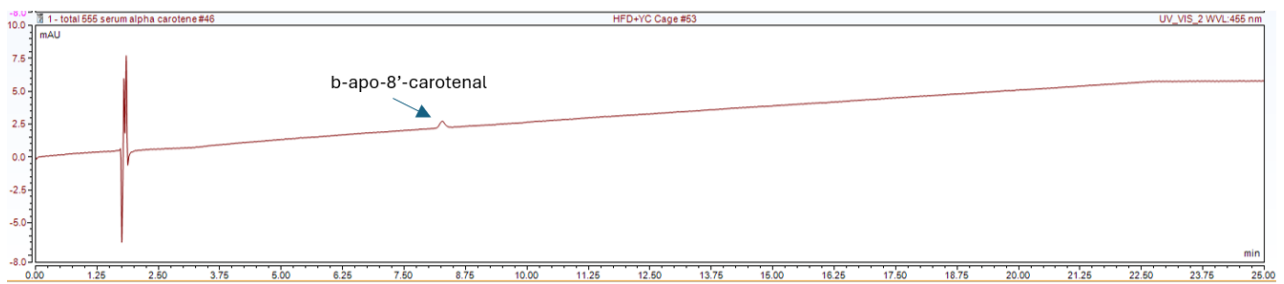
3.8.2.4. Orange Carrot Serum



3.8.2.5. Red Carrot Serum

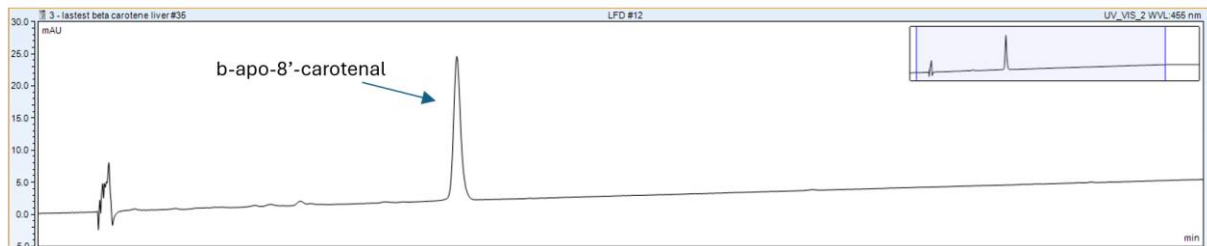


3.8.2.6. Yellow Carrot Serum

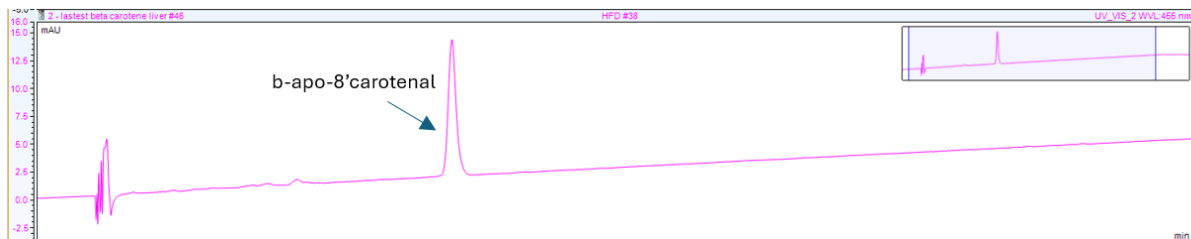


3.8.3. Liver HPLC Chromatograms

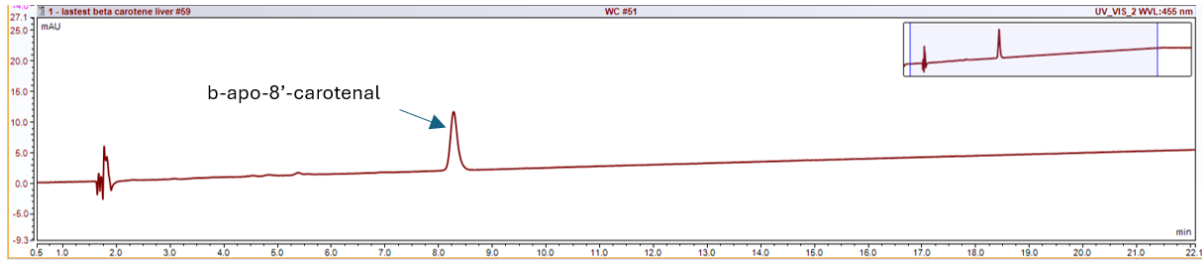
3.8.3.1. Low Fat Diet Liver



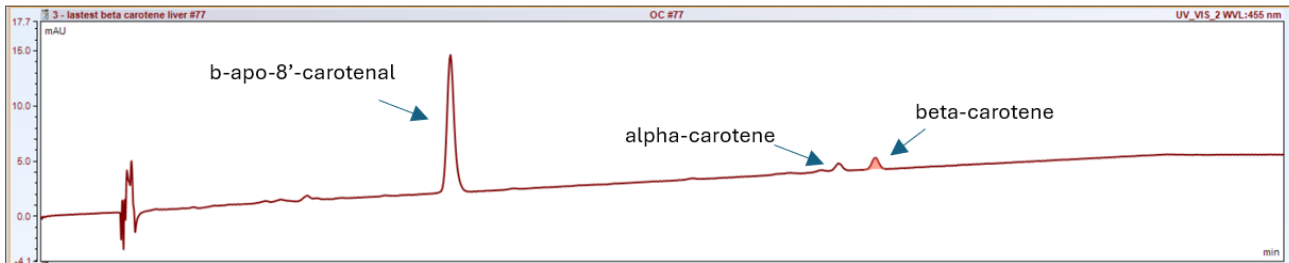
3.8.3.2. High Fat Diet Liver



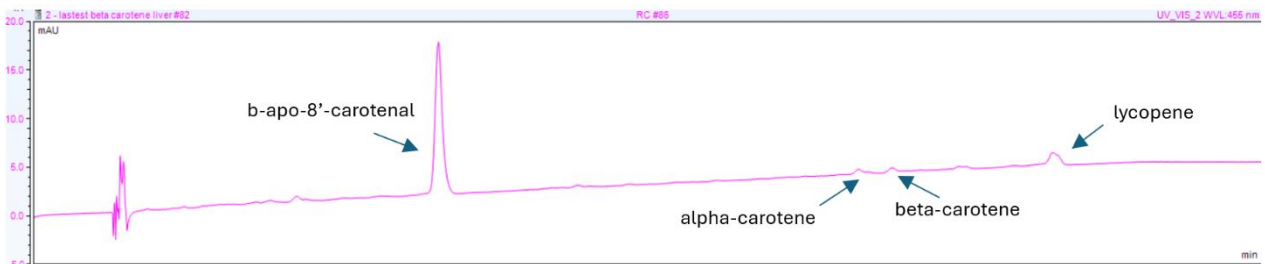
3.8.3.3. White Carrot Diet Liver



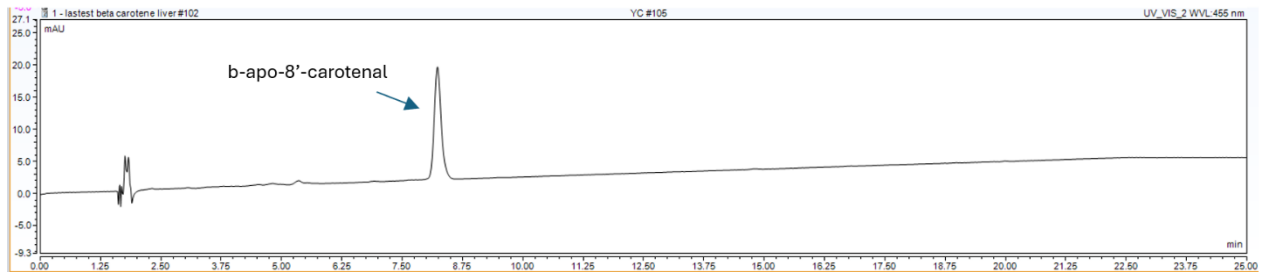
3.8.3.4. Orange Carrot Diet Liver



3.8.3.5. Red Carrot Diet Liver

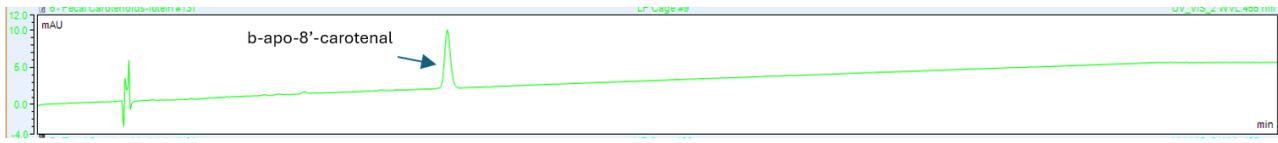


3.8.3.6. Yellow Carrot Diet Liver



3.8.4. Fecal HPLC Chromatograms

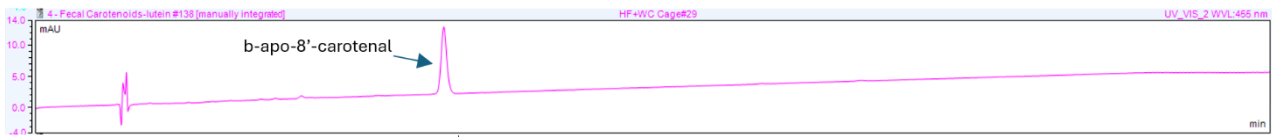
3.8.4.1. Low Fat Diet Fecal



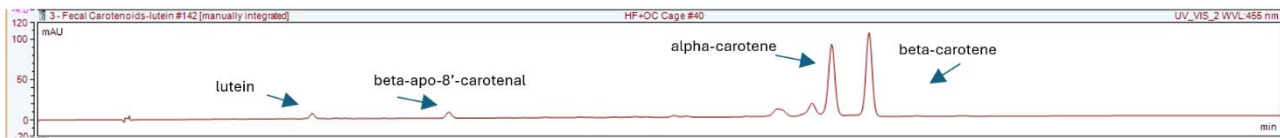
3.8.4.2. High Fat Diet Fecal



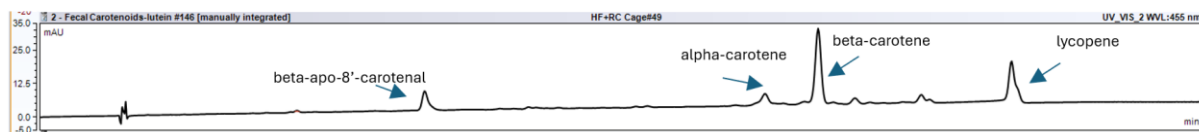
3.8.4.3. White Carrot Diet Fecal



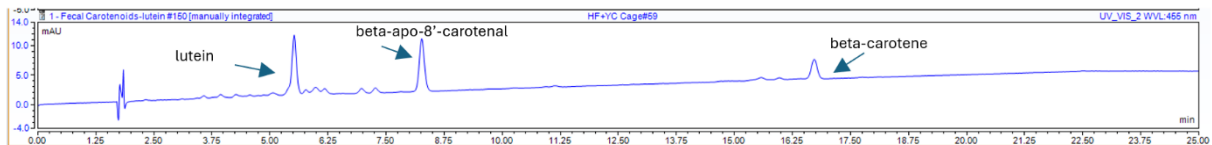
3.8.4.4. Orange Carrot Diet Fecal



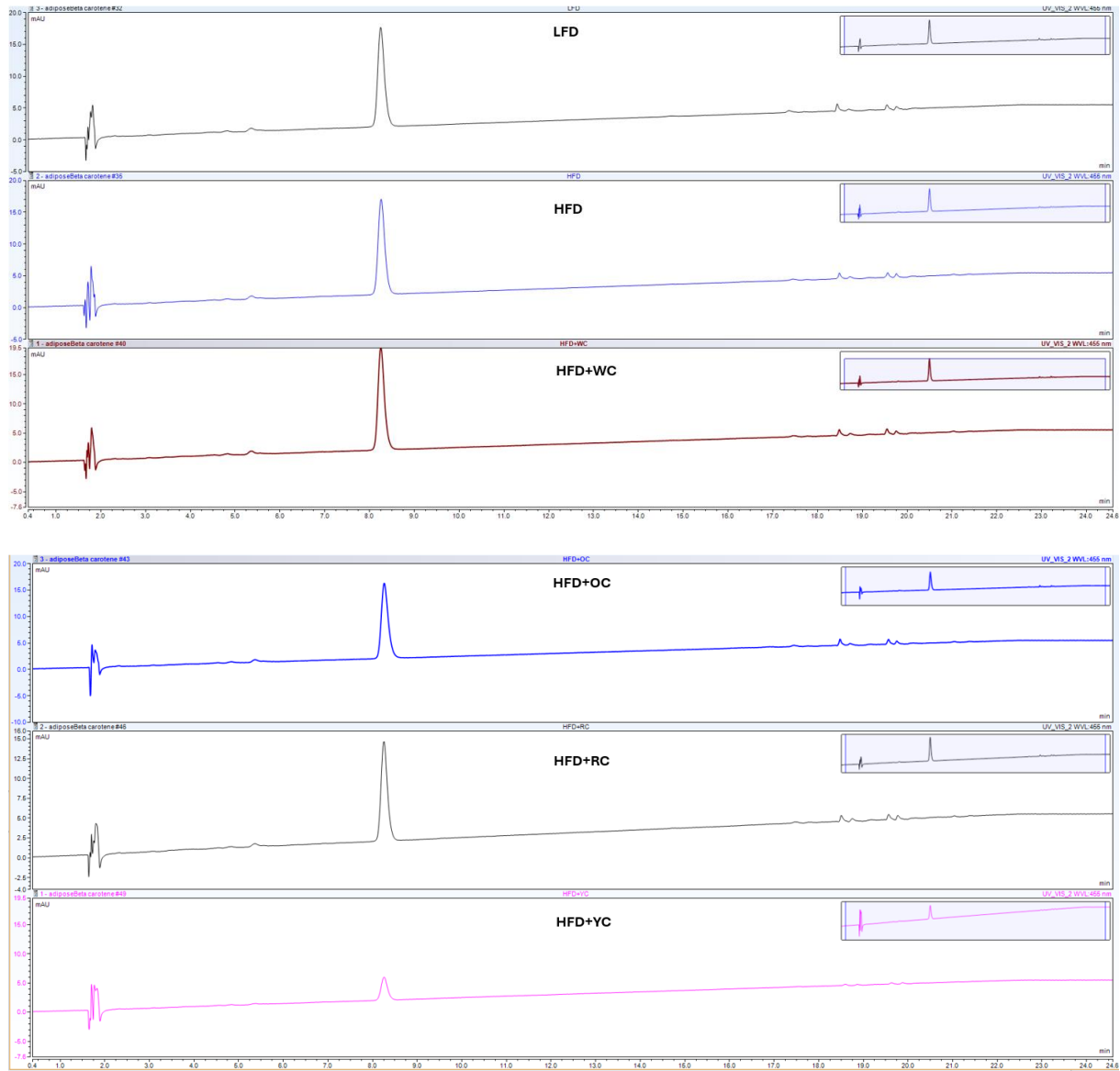
3.8.4.5. Red Carrot Diet Fecal



3.8.4.6. Yellow Carrot Diet Fecal



3.8.5. Adipose Tissue HPLC Chromatograms



Supplementary Table 3.8.1: Primer sequences used in qPCR experiments

Forward and reverse sequences generated using the NCBI BLAST tool.

Genes:	Forward:	Reverse:
<i>Acox1</i>	TAACTTCCTCACTCGAAGCCA	AGTTCATGACCCATCTCTGTC
<i>β-Actin</i>	CTTTTCCAGCCTTCCTTCTTGG	CAGCACTGTGTTGGCATAGAGG
<i>Cd36</i>	GCGACATGATTAATGGCACA	CCTGCAAATGTCAGAGGAAA
<i>Cpt</i>	GCACTGCAGCTCGCACATTACAA	CTCAGACAGTACCTCCTTCAGGAAA
<i>Fas</i>	GCTGCGGAAACTTCAGGAAAT	AGAGACGTGTCACTCCTGGACTT
<i>Ppara</i>	GTACCACTACGGAGTTCACGCA	CATTGTGTGACATCCCGACAG
<i>Pgc1α</i>	TATGGAGTGACATAGAGTGTGCT	CCACTTCAATCCACCCAGAAAG
<i>Scd1</i>	CCGGAGACCCCTTAGATCGA	TAGCCTGTAAAAGATTTCTGCAAACC
<i>Srebp1</i>	TAGAGCATATCCCCCAGGTG	GGTACGGGCCACAAGAAGTA

Supplementary Table 3.8.2: Composition of dietary pellets

	D12450HB	D124518	D23051601	D23051602	D23051603	D23051604
	LF Control	HF Control	White Carrot	Yellow Carrot	Orange Carrot	Red Carrot
<i>Ingredient</i>	gm	gm	gm	gm	gm	gm
Casein	200	200	186.7	186.7	186.7	186.7
L-Cystine	3	3	3	3	3	3
Corn Starch	452.2	72.8	0	0	0	0
Maltodextrin 10	75	100	72.7	72.7	72.7	72.7
Sucrose	172.8	172.8	172.8	172.8	172.8	172.8
White carrot powder	0	0	175.6	0	0	0
Yellow carrot powder	0	0	0	175.6	0	0
Orange carrot powder	0	0	0	0	175.6	0
Red carrot powder	0	0	0	0	0	175.6
Cellulose BW200	50	50	9.1	9.1	9.1	9.1
Soybean Oil	25	25	25	25	25	25
Lard	20	177.5	177.5	177.5	177.5	177.5
Mineral Mix. S10026	10	10	10	10	10	10
Dicalcium Phosphate	13	13	13	13	13	13
Calcium Carbonate	5.5	5.5	5.5	5.5	5.5	5.5
Potassium Citrate, 1 H2O	16.5	16.5	16.5	16.5	16.5	16.5
Vitamin Mix. V10001	10	10	10	10	10	10
Choline Bitartrate	2	2	2	2	2	2
FO&C Blue dye #1	0.05	0.05	0.05	0.05	0.05	0.05
<i>Total</i>	1055.05	858.15	879.45	879.45	879.45	879.45
<i>gm</i>						
Protein	177.0	177.0	177.0	177.0	177.0	177.0
Carbohydrate	710.0	355.0	355.0	355.0	355.0	355.0
Fat	45.0	202.5	202.5	202.5	202.5	202.5
Fiber	50.0	50.0	50.0	50.0	50.0	50.0
<i>gm%</i>						
Protein	16.8	20.6	20.1	20.1	20.1	20.1
Carbohydrate	67.3	41.4	40.4	40.4	40.4	40.4
Fat	4.3	23.6	23.0	23.0	23.0	23.0
Carrot Powder	0.0	0.0	20.0	20.0	20.0	20.0
<i>kcal</i>						
Protein	708	708	708	708	708	708
Carbohydrate	2840	1422.4	1422	1422	1422	1422
Fat	405	1822.5	1823	1823	1823	1823
<i>Total</i>	3953	3953	3953	3953	3953	3953
<i>kcal%</i>						
Protein	18	18	18	18	18	18
Carbohydrate	72	36	36	36	36	36
Fat	10	46	46	46	46	46
<i>kcal/gm</i>	3.75	4.61	4.49	4.49	4.49	4.49

Supplementary Table 3.8.3. Vitamin Mix V10001

TYPE	INGREDIENT	AMOUNT
CARBOHYDRATE	Sucrose, Fine Granulated	78.42 g
VITAMIN	Vitamin E Acetate, 50%	10.00 g
VITAMIN	Niacin (a.k.a. B3, Nicotinic Acid)	3.00 g
VITAMIN	Biotin, 1%	2.00 g
VITAMIN	Pantothenic Acid, d, Calcium (a.k.a. B5)	1.60 g
VITAMIN	Vitamin D3, 100,000 IU/gm	1.00 g
VITAMIN	Vitamin B12, Cyanocobalamin, 0.1%	1.00 g
VITAMIN	Vitamin A Acetate, 500,000 IU/gm	0.80 g
VITAMIN	Pyridoxine HCl (a.k.a. B6)	0.70 g
VITAMIN	Riboflavin (A.K.A. B2)	0.60 g
VITAMIN	Thiamine HCl (a.k.a. B1)	0.60 g
VITAMIN	Folic Acid	0.20 g
VITAMIN	Menadione Sodium Bisulfite	0.08 g
	Total:	100.00 g

Supplementary Table 3.8.4. TMTpro 16plex Isobaric Labeling Parameters

Mass Tag	Reporter Ion Mass	-2		-1		M+	+1		+2	
		-2x 13C	-13C -15N	-13C	-15N		-	+15N	+13C	+15N +13C
TMTpro-126	126.12772 6	N/A	N/A	N/A	N/A	100%	0.05 %	10.40 % (127C)	0.02 %	0.34 %
TMTpro-127N	127.12476 1	N/A	N/A	N/A	0.57% (126)	100%	N/A	9.79% (128N)	N/A	0.33 %
TMTpro-127C	127.13108 1	N/A	N/A	0.84% (126)	N/A	100%	0.23 %	8.40% (128C)	0.02 %	0.27 %
TMTpro-128N	128.12811 6	N/A	0.00%	0.68% (127N)	0.52%	100%	N/A	5.23% (129N)	N/A	0.20 %
TMTpro-128C	128.13443 6	0.00%	N/A	1.44% (127C)	N/A	100%	0.34 %	6.26% (129C)	0.00 %	0.17 %
TMTpro-129N	129.13147 1	0.00%	0.14%	1.30% (128N)	0.89%	100%	N/A	7.52% (130N)	N/A	0.12 %
TMTpro-129C	129.13779	0.13%	N/A	2.59% (128C)	N/A	100%	0.32 %	6.07% (130C)	0.01 %	0.09 %
TMTpro-130N	130.13482 5	0.13%	0.00%	2.41% (129N)	0.27%	100%	N/A	5.58% (131N)	N/A	0.10 %
TMTpro-130C	130.14114 5	0.25%	N/A	3.22% (129C)	N/A	100%	0.28 %	5.06% (131C)	0.00 %	0.06 %
TMTpro-131N	131.13818	0.04%	0.01%	2.73% (130N)	0.49%	100%	N/A	3.13% (132N)	N/A	0.06 %
TMTpro-131C	131.1445	0.09%	N/A	4.02% (130C)	N/A	100%	1.17 %	3.62% (132C)	0.02 %	0.03 %
TMTpro-132N	132.14153 5	0.07%	0.01%	3.14% (131N)	0.73%	100%	N/A	3.40% (133N)	N/A	0.03 %
TMTpro-132C	132.14785 5	0.13%	N/A	5.14% (131C)	N/A	100%	1.16 %	1.92% (133C)	0.00 %	0.00 %
TMTpro-133N	133.14489	0.15%	0.01%	3.58% (132N)	0.72%	100%	N/A	1.80% (134N)	N/A	0.00 %
TMTpro-133C	133.15121	0.18%	N/A	4.14% (132C)	N/A	100%	0.40 %	1.11% (134C)	0.00 %	N/A
TMTpro-134N	134.14824 5	0.28%	0.10%	5.52% (133N)	0.35%	100%	N/A	1.12% (135N)	N/A	N/A

Abbreviations:

HFD: High-Fat Diet; **LFD:** Low-Fat Diet; **WC:** White Carrot; **OC:** Orange Carrot; **RC:** Red Carrot; **YC:** Yellow Carrot; **H&E:** Hematoxylin and Eosin; **SEM:** Standard Error of the Mean; **qPCR:** Quantitative Polymerase Chain Reaction; **ACOX1:** Acyl-CoA Oxidase 1; **PPAR α :** Peroxisome Proliferator-Activated Receptor Alpha; **CPT2:** Carnitine Palmitoyltransferase 2; **FAS** or **FASN:** Fatty Acid Synthase; **PGC1 α :** Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha; **CD36:** Cluster of Differentiation 36; **TRX:** Thioredoxin; **CRP:** C-Reactive Protein; **LPS:** Lipopolysaccharide; **LBP:** Lipopolysaccharide-Binding Protein; **FABP5:** Fatty Acid Binding Protein 5; **LCAT:** Lecithin-Cholesterol Acyltransferase; **NCF1:** Neutrophil Cytosolic Factor 1; **SCARB1:** Scavenger Receptor Class B Type 1; **SOD1/2:** Superoxide Dismutase 1 and 2; **CAT:** Catalase; **RDH13/14/16:** Retinol Dehydrogenase 13, 14, 16; **SLC35E2A:** Solute Carrier Family 35 Member E2A; **IDI1:** Isopentenyl-Diphosphate Delta-Isomerase 1; **SLC17A2:** Solute Carrier Family 17 Member 2; **PLIN2:** Perilipin 2; **CYP2B10:** Cytochrome P450 2B10; **MUP1:** Major Urinary Protein 1; **HSD17B6:** Hydroxysteroid 17-Beta Dehydrogenase 6; **FDPS:** Farnesyl Diphosphate Synthase; **HSD3B5:** Hydroxy-Delta-5-Steroid Dehydrogenase 3 Beta- and Steroid Delta-Isomerase 5; **MVD:** Mevalonate Diphosphate Decarboxylase; **PMVK:** Phosphomevalonate Kinase; **STX4:** Syntaxin 4; **ERCC2:** Excision Repair Cross-Complementation Group 2; **GSR:** Glutathione Reductase; **NQO1:** NAD(P)H Quinone Dehydrogenase 1; **PRDX1/2:** Peroxiredoxin 1 and 2; **AKT1:** AKT Serine/Threonine Kinase 1; **ANKHD1:** Ankyrin Repeat and KH Domain Containing 1; **AOAC:** Association of Official Analytical Collaboration.

CHAPTER 4

4. Discussion

The aim of this study was to examine how major dietary carotenoids sourced from different colored carrots affected liver health, especially in the context of non-alcoholic fatty liver disease (NAFLD). To induce stress in mice and to initiate the progression of NAFLD, a high fat diet was introduced as 45% of composition of the diet stemmed from fat over the course of 20 weeks. This way obesity induced liver disease could occur, and the control group with a low-fat diet of 10% could be compared in terms of liver health and leanness of mice. The 45% fat diet was used in all carrot intervention arms and was compared to determine if carotenoids would decrease the negative effects associated with the high fat diet treatment. To ensure the effect of dietary carotenoids were analyzed, independent from the whole food benefits of carrots, the white carrot intervention was administered in order to have a carotenoid deficient food matrix control group. Therefore, the overall experimental design included a C57BL/6J animal model to test whether various colored carrot powders would be effective against deleterious effects of obesity-induced NAFLD facilitated by a high-fat diet, as compared to a positive control group fed a white carrot powder supplemented high-fat diet, and a negative control group of a low-fat diet. In order to test this hypothesis, concentrations of different carotenoids were examined via high-performance liquid chromatography (HPLC) in samples, starting from diet pellets and mice tissues including serum, liver, and adipose in addition to fecal samples. Serum carotenoid concentration was analyzed to observe if carotenoids were absorbed from the gut and to measure circulatory carotenoid levels. The liver is known to be the main site for lipid metabolism,¹³⁶ and as carotenoids are fat soluble compounds high concentrations of carotenoids in the tissue were expected. Adipose tissue is also another site for fat accumulation therefore it was tested to see if any carotenoid storage was

metabolized in adipocytes by looking at their concentrations.¹³⁸ Fecal samples were analyzed to observe if carotenoids were metabolized efficiently by liver and other organs, or if they were more likely to be excreted as to having low bioavailability and bioaccessibility especially when introduced in whole food form.¹⁸¹ Diet pellets made with orange carrot powder (HFD+OC) showed the highest amounts of β -carotene (102.64 ± 1.3 nmol/g) and α -carotene (76.76 ± 0.3 nmol/g) compared to red carrot and yellow carrot powders. Therefore, in liver samples α -carotene and β -carotene were expected to be higher in orange carrot intervention groups, which could be linked to any positive effects seen in that group specifically. Red carrot powder added high-fat diet pellets (HFD+RC) had the highest amount of lycopene (95.19 ± 14.05 nmol/g) and liver tissues from this groups could show specific outcomes from the effects of lycopene. Through our dietary analysis, we observed that the yellow carrot powder supplemented high-fat diet pellets (HFD+YC) contained the lowest total carotenoid levels, with the exception of lutein, which was the highest across all groups at 2.06 ± 0.13 nmol/g. This concentration appears low when compared to α -carotene, β -carotene, or lycopene levels that were found in diet pellets of other carrot groups. A mice study demonstrated that a 4.39 nmol/g dose of lutein (0.25 mg per 100 g of diet) was effective in raising plasma lutein concentrations to 43 nM, therefore bioavailability of lutein from whole food sources can be sufficient enough to be absorbed by the gut.¹⁹¹ However, in this current study, the concentration of lutein in HFD+YC diet pellets was less than a half of this value. Furthermore, serum and liver lutein levels were undetectable across all groups, which raises questions about either absorption, transportation, or possible interactions with the fat content of the diet. This discrepancy suggests that even though lutein is known to be effective at low doses, its bioavailability may still depend heavily on the dietary matrix, fat composition, or individual metabolic conditions. This concern is further supported by fecal analysis, which revealed that the

HFD+YC group excreted a significantly high amount of lutein (33.14 ± 4.6 nmol/g), which was higher than the levels of other carotenoids in the same group and indicated that a substantial portion of dietary lutein was not absorbed. In terms of β -carotene, α -carotene, and lycopene, distinct differences were observed among the carrot intervention groups that highlight both the carotenoid profiles of the specific carrot varieties and their varying degrees of absorption and tissue accumulation. The HFD+OC group had the highest dietary levels of both β -carotene and α -carotene, which translated into detectable concentrations in serum (7.76 ± 0.01 nM and 32.09 ± 4.45 nM, respectively) and substantial liver concentration (10.15 ± 2.5 nmol/g for β -carotene, 42.03 ± 0.1 nmol/g for α -carotene) which shows an efficient uptake and tissue storage. The HFD+RC group was lower in β -carotene (22.78 ± 1.7 nmol/g) and α -carotene (0.96 ± 0.03 nmol/g) compared to orange carrot diet pellets. Lycopene was highly concentrated in the red carrot diet pellet samples (95.19 ± 14.05 nmol/g) and was the only carotenoid detected in both serum (33.89 ± 5.8 nM) and liver (7.3 ± 1.8 nmol/g) of this group. This can be translated as lycopene having sufficient bioavailability from red carrots. In contrast, the HFD+YC group showed minimal levels of β -carotene (1.16 ± 0.03 nmol/g), undetectable α -carotene and lycopene, and no carotenoids found in serum or liver, suggesting that the total carotenoid content was too low to have measurable systemic effects, aside from the unusual excretion pattern of lutein. Fecal analysis revealed that HFD+OC showed the highest excretion of β -carotene and α -carotene (62.19 ± 4.46 and 47.74 ± 2.9 nmol/g, respectively) which indicated a good absorption with a portion remained unutilized. These results reveal the importance of both carotenoid concentration in the diet and the specific chemical nature of each carotenoid in determining absorption efficiency and tissue distribution. The study results also point to the potential of red carrot-derived lycopene as a bioavailable antioxidant, even when present alongside lower amounts of other carotenoids.

Body composition measurements showed that supplementation with carrots did not stop weight gain in any group, however HFD+RC and HFD+YC subjects experienced a tendency to decreased body mass and fat parameters as compared to HFD+OC and HFD+WC subjects. Among the carrot supplemented groups, the administration of HFD+RC showed the most success in lowering both total body weight and stored fat. The data implied that the lycopene concentrated carrot consumption affected both energy regulation and lipid metabolic processes and test subjects gained the least weight compared to other groups, as it was shown that body weight and fat gain measurements indicated no weight reduction or fat control ability within HFD+OC and HFD+WC groups.

A previous study that was conducted at Eroglu lab showed that intervention with orange carrots at 20% w/w in a HFD with 60% kcals from fat over 15 weeks established noteworthy inhibitory effects on body weight gain, when compared to the HFD diet alone.¹⁹⁵ That research study showed that white carrot intake also led to a decreased body weight gain compared to the HFD control group, but these results were not considered statistically significant. Diets containing 60% fat are included in metabolic research to cause obesity and metabolic dysfunction, since this fat level causes obesity-related metabolic changes and inflammatory responses in a shorter amount of time. Based on the intended level of metabolic disturbance and its progression, experiments may include diets with 45% or 60% fat.¹⁹⁶ Using diets with high-fat contents ($\geq 60\%$ kcals from fat) might decrease the relevance of physiological processes outcomes.¹⁹⁶

The selected 45% fat diet regimen produced measurements that differed from the original study. The higher dietary fat levels in HFD pellets likely enhanced how well the fat-soluble carotenoids were absorbed by the body through the use of 60% fat content. High lipid content in the 60% kcals from fat diet of the prior study could have boosted the uptake of fat-soluble carotenoids in carrot

arms and produced additional anti-obesity benefits. Such findings suggest that pathways linked to obesity-related inflammation in the liver did not develop in this model although other studies triggered liver dysfunctions by feeding mice 45% fat calories during the same timeframe.

Inflammatory measurements using fecal and serum samples did not show significant outcomes throughout the experiment. The blood protein levels of lipopolysaccharides (LPS) did not demonstrate any protective impact from carotenoid supplementation according to results since the week 20 lipopolysaccharide-binding protein (LBP) measurements from all HFD-fed groups including carrot-fed groups exceeded LFD levels but showed no differences between the HFD groups. Local suppression of intestinal inflammation by carotenoid interventions failed to bring sufficient changes to metabolic endotoxemia that developed because of the high-fat diet at the systemic level. An equal pattern emerged in the changes of C-reactive protein (CRP) values found in mice blood as the supplement of carrot powders showed limited effects that did not result in significant CRP reduction when compared to HFD at Week 10. All HFD-fed groups showed steady elevation of CRP levels from Week 20 irrespective of carrot supplementation indicating carotenoids did not largely modify systemic low-grade inflammation. The analysis of Week 20 serum LPS levels indicated elevated LPS concentrations only in the HFD+RC and HFD+YC groups as compared to LFD while no differences were detected in Week 10 LPS measurements. Endotoxemia results even worsened according to these research results during advanced stages when carotenoids are combined with a HFD.

Some carotenoids affected gut inflammation by reducing it at certain time points, but did not consistently reduce systemic inflammation caused by the high fat diet intake. This imbalance between gut and other tissue markers may be due to the limited effects of carotenoids under metabolic stress or how different tissues respond. Stronger effects might be seen if earlier

intervention is made with a higher carotenoid implementation, or strategies that directly target the gut barrier.

Histological analysis of liver samples showed significant differences between HFD group and other experimental arms. As expected, compared to the LFD group's fat accumulation in liver cells ($5.10 \pm 0.80\%$, $p < 0.001$), HFD treatment had pronounced hepatic steatosis, with a significantly higher percentage of fatty liver cells ($17.08 \pm 2.46\%$). The main finding was that all carrot intervention groups exhibited significantly reduced fat accumulation in the liver compared to the HFD only group (all $p < 0.05$). The percentage area of fatty liver cells in all intervention groups consisted of 6-7% and were statistically similar to the LFD group whereas none of the carrot groups differed significantly from each other. The liver histology images support these results as large fat buildup can be seen in the HFD group, while the carrot groups and LFD group had healthier tissue with less fat. Even though white carrot supplementation had no carotenoids detected, this intervention still helped to reduce liver fat possibly due to other plant compounds or fiber. All carrot types helped prevent fat buildup in the liver, therefore carrot intervention without a distinction of the carotenoid type aided to protect the liver compared to a high-fat diet.

Protein expression and gene expression data from Western Blot analysis and qPCR were used to identify lipid metabolism changes modulated by different carotenoid interventions. Changes in expressions of several targets were observed, though with limited significance. Acyl-CoA oxidase 1 (ACOX1), peroxisome proliferator-activated receptor alpha (PPAR α), and carnitine palmitoyltransferase 2 (CPT2) protein expressions were analyzed to reveal regulatory changes in peroxisomal and mitochondrial fatty acid oxidation; however, these targets remained largely unaltered across all groups at both the mRNA and protein levels. This indicates that main β -oxidation pathways were not significantly impacted by dietary carotenoids. On the other hand,

fatty acid synthase (FAS) protein expression was significantly reduced in all HFD groups compared to the LFD control and carotenoid sources did not change the significance of the results. Therefore, the suppression of hepatic lipogenesis was not further modified by carrot supplementation but varied due to the fat percentage of the diets. At the transcription level peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*pgc1-α*) expression was highest in the LFD group and was significantly downregulated in the HFD+WC group. This trend showed a decline in mitochondrial biogenesis in response to a high-fat diet but again there was no significant improvement observed with carotenoid interventions. The most notable molecular response was with cluster of differentiation 36 (CD36) with the intervention of HFD+OC group. β-carotene in orange carrots can be modified into retinol, retinal and retinoic acid in the gut.¹³³ Retinoic acid receptor RAR/RXR regulates CD36 transcription and may influence gene expression through nuclear signaling pathways and therefore is linked to β-carotene concentration.¹³⁴ On the other hand, CD36 also functions in facilitating fatty acid uptake into hepatocytes and its upregulation may suggest that orange carrot supplementation may have increased hepatic lipid transport and may have caused the upregulation of transportation of fatty acids into liver cells. In physical parameters such as fat change percentage orange carrot intervention was not effective enough to create positive outcomes, though the intervention induced molecular changes. Increased CD36 expression could favor lipid storage rather than oxidation or mobilization. As a result, any metabolic benefits from carotenoid intake may have been limited by the simultaneous increase in hepatic fat uptake which may have prevented reductions in overall adiposity. This shows that molecular activation of lipid transport and redox pathways did not translate into measurable systemic benefits in this group. Lycopene in red carrots showed different effects on CD36 compared to the HFD+OC group as indicated by significantly less expression, which may suggest

reduced hepatic fatty acid uptake that aligns with lower fat mass and hepatic steatosis observed in this group. Although no major differences were observed in main fatty acid oxidation markers such as ACOX1, PPAR α , or CPT2 at the protein level, the maintenance of lower CD36 levels may be related to the notion that lycopene may have attenuated lipid influx into hepatocytes rather than modulating oxidative metabolism directly. In qPCR results, *cd36* mRNA expression in HFD+RC was also significantly lower than in HFD+OC. Therefore, the regulatory effect was also expressed at the transcriptional level. This combination of reduced fatty acid transport and some gene expression preservation may explain why HFD+RC had the most consistent reductions in fat accumulation, inflammatory markers, and body weight gain among all HFD carrot intervention groups. HFD+YC intervention showed slightly lower body weight and fat mass outcomes compared to HFD+WC and HFD+OC groups, but the differences were not statistically significant. Lutein did not cause the expression of β -oxidation genes (*acox1*, *ppara*, *pgc-1 α*) at either the mRNA or protein level and *pgc-1 α* mRNA was lowest in HFD+YC which might reflect reduced mitochondrial biogenesis, but this was not confirmed at the protein level.

To assess changes in protein expression with a high-throughput analysis, discovery quantitative proteomics was utilized. Proteomics analysis showed further insights into several protein expressions. For HFD+RC group, GOnet network analysis showed that fatty acid-binding protein 5 (FABP5) was downregulated significantly, and neutrophil cytosolic factor 1 (NCF1) had a negative fold change pattern although it was not significant. These proteins are connected to reactive oxygen species (ROS) and macrophage being present in oxidative stress conditions.^{197,198}

There was a mild suppression of oxidative stress response elements such as excision repair cross-complementation group 2 (ERCC2) as well. These shifts are highly relevant to NAFLD progression as oxidative stress plays a key role in liver inflammation. While it did not meet

significance, slight upregulation of retinoid metabolizing enzymes retinol dehydrogenase 13 (RDH13), retinol dehydrogenase 14 (RDH14), and retinol dehydrogenase 16 (RDH16) were also observed. This pattern shows that liver might be interacting with vitamin A metabolism related pathways. ACOX1, CPT2 and CD36 proteins were slightly more expressed but the fold change was not significant except CD36, which indicated the same results as previous data from qPCR and Western Blots. Proteins involved in cholesterol biosynthesis and steroid regulation (hydroxysteroid 17-beta dehydrogenase 6 (HSD17B6), farnesyl diphosphate synthase (FDPS), phosphomevalonate kinase (PMVK), and mevalonate diphosphate decarboxylase (MVD) also had elevated expressions in HFD+RC compared to HFD+WC groups. Such data shows that lycopene may drive a coordinated remodeling of sterol and hormone metabolism under metabolic stress. This was followed by downregulation of proteins like cytochrome P450 2B10 (CYP2B10) and syntaxin 4 (STX4), linked to xenobiotic metabolism and vesicle transport, respectively, shown in GOnet diagrams. Activation of these proteins in mice has been shown to cause hepatic inflammation due to increased lipogenesis and increased uptake of fatty acids by CD36 pathways.¹⁹⁹ Red carrot intervention was effective in downregulating these proteins significantly, compared to the white carrot group which indicates the role of lycopene in improving NAFLD conditions. FABP5 was significantly downregulated in HFD+OC group according to proteomics data, despite upregulation patterns of CD36, which was significant in Western Blot analysis and may reflect a dysregulated feedback loop in lipid transport dynamics. Proteins involved in ROS regulation and oxidative stress response such as superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), NAD(P)H quinone dehydrogenase 1 (NQO1), and peroxiredoxin 1 and 2 (PRDX1/2) had moderate positive fold changes however it did not reach significance in HFD+OC when compared to HFD, which might show a small pattern towards antioxidant response to lipid accumulation. There was

an upregulation of mitochondrial β -oxidation proteins including CPT2 and ACOX3 though these were not expressed significantly with an high enough fold change to suggest enhanced fatty acid catabolism. Even so, these positive outcomes could be offset by the strong increases in perilipin 2 (PLIN2) expression as it is involved in fatty acid uptake which is associated with NAFLD progression. For orange carrot intervention GOnet analysis showed PPAR α and scavenger receptor class B type 1 (SCARB1) were present but were not significantly upregulated to be effective in lipid metabolism or cholesterol storage networks. β -Carotene and α -carotene concentration in orange carrot intervention could not inhibit the upregulation of solute carrier family 35 member E2A (SLC35E2A), which is a transporter protein potentially involved in glycosylation and endoplasmic reticulum (ER) stress that was caused by the high fat diet. The expression of isopentenyl-diphosphate delta-isomerase 1 (IDI1) and solute carrier family 17 member 2 (SLC17A2) were also significantly downregulated which indicates shifts in isoprenoid biosynthesis and nutrient transport. The upregulation of AKT serine/threonine kinase 1 and 2 (AKT1/2) was not sufficient to translate into reductions in fat accumulation or liver steatosis.

Overall, red carrot intervention resulted in reduced expression of FABP5, which is involved in hepatic fatty acid uptake and transport; the downregulation of this protein shows a potential decrease in lipid accumulation in the liver.²⁰⁰⁻²⁰² In parallel, there was a detection of antioxidant enzymes SOD1, SOD2, and catalase (CAT) expressions with a pattern towards upregulation, although not significant, which might have improved oxidative stress regulation under high-fat dietary conditions. Similar patterns were observed with sterol and retinoid metabolism proteins including HSD17B6, FDPS, RDH13, RDH14, and RDH16 which also had increased fold changes. Even though there was a positive pattern of hepatic lipid metabolism, this may not be accurate enough to reflect a broader metabolic effect relevant to red carrot supplementation, as these

changes did not meet significance threshold. In the orange carrot group, proteins related to fatty acid β -oxidation pathways such as ACOX3 and CPT2 were detected; however, this was not significant enough to make positive claims on improvement of fat breakdown in liver. These trends occurred alongside significantly increased levels of CD36 and PLIN2, which are proteins associated with fatty acid uptake, lipid storage, and cholesterol transport; this may suggest a continued drive toward hepatic lipid accumulation. Additionally, the transporter SLC35E2A was significantly upregulated, which may indicate altered glycosylation activity and a possible increase in ER stress.

These results show that red and orange carrot interventions influence hepatic protein expression in different ways. Red carrot, which is high in lycopene, showed consistent trends towards reduced lipid uptake, improved antioxidant defense, and modulation of retinoid and sterol metabolism. Orange carrot, which is rich in β -carotene, activated both lipid oxidation and storage related pathways, but may reduce its overall effectiveness in preventing hepatic lipid accumulation under high-fat conditions.

The main aim of this study was to provide a more realistic approach to a high-fat diet by utilizing 45% fat content as that is physiologically relevant, and incorporating a food synergy aspect to the experimental design could show the outcomes of the idea of carotenoids showing positive effects in the body when taken in the form of its original source. However, there were several limitations to this study that should be acknowledged.

First, most of the changes we observed in gene and protein expression were not statistically significant. Although patterns were observed that pointed in certain directions, the variability across individual animals and the modest effect sizes meant confirmation of those differences

could not be made with confidence. These inconsistencies made it harder to explain the physiological outcomes using molecular data.

A possible explanation lies in the diet composition. The high-fat diet used in this study contained 45% fat, which is widely accepted in metabolic research, but it may not have been high enough to drive more severe liver changes. In comparison, an earlier preliminary study done in the Eroglu lab used a 60% fat diet and observed significantly different effects between orange carrot diet arm and other groups.¹⁹⁵ The outcomes were present both at the tissue and gene/protein expression levels. In the case of the current study, the lower fat content here may have limited the degree of metabolic stress, which may have made it more difficult to detect intervention effects. The study lasted 20 weeks, which is a reasonable timeframe for NAFLD development, yet a longer intervention may have produced more advanced features like inflammation or fibrosis, especially when using reduced fat content of 45%. With the previous study, the 60% fat diet showed changes in body weight and fat accumulation in 15 weeks and physical changes were noticeable.

Another limitation was using whole carrots instead of isolated carotenoids, which of course was intentional as research was limited in terms of incorporating whole foods as carotenoid sources and a carotenoid deficient food matrix control, but this aspect introduced more complexity. Carrots contain many bioactive compounds beyond various carotenoids as this food source includes various types of fiber, which were not separately quantified. Although total fiber content was almost the same in intervention arms, soluble versus insoluble fiber content could have changed the effects of the whole food carrot addition to diet pellets. These dietary factors could have affected digestion, absorption, and gut liver interactions in ways that couldn't be pinpointed with experiments conducted in this project.

Another complexity is derived from using obesity as a stressor for liver disease as this disease state is driven by a complex relation of metabolic, inflammatory, hormonal, and environmental factors which can make it hard to attribute changes between groups to a single pathway or biomarker. As stated in the introduction section, the progression of simple liver inflammation into more chronic steatohepatitis and scarring involves lipid accumulation in liver cells as well as other contributors such as insulin resistance, immune signaling, chronic inflammation, oxidative stress, and gut disruption. In this study, mainly hepatic tissue was analyzed while systemic metabolic markers or other tissue responses were not assessed in detail. This current focus limits the ability to analyze the full picture of obesity related stress and its impact on liver health. Metabolomics and other gut studies could compliment these aspects of the complexity of NAFLD. As stated, the 45% high-fat diet may not have been sufficient to fully activate the closely related stress responses, therefore as a result, even if subtle changes occurred in upstream regulators or peripheral tissues, may not have been reflected at the liver level or through the specific molecular markers selected. The key limitation was that the disease model used may not have fully captured the physiological complexity of obesity-associated liver pathology which indicates the necessity to evaluate the full impact of dietary interventions on NAFLD progression.

Even though there are other studies that use 45% or less fat percentage to initiate the progression of liver disease, the intervention of carotenoids was done using supplementation with their pure forms. This approach makes carotenoids more bioaccessible and increases their bioavailability and also eliminates other effects that can root from whole food plant intervention. Therefore, a lower fat percentage used in diet pellets with the combination of whole food sources to obtain carotenoids may not have been sufficient to detect more subtle effects in liver metabolism. Carotenoid

transportation and absorption can be affected by the fat found in the diet, due to their hydrophobic nature and their transportation through fat vesicles.

Overall, the study used carrots instead of purified carotenoids resulting in improved dietary connection at the cost of added experimental considerations. The combination of phytochemicals and polyphenols along with fiber in carrots enables them to possibly modify lipid metabolic processes and inflammatory course. The diets maintained equal total fiber amounts, yet it was difficult to determine the soluble and insoluble fiber distribution. The multiple fiber types present in the carrots could affect gut function and metabolic responses, but these effects were not part of the study measurements. The evaluation of gene and protein expression was limited to liver tissue as the only analysis site. The actual development of NAFLD involves combined systematic processes between the intestine, and immune system responses. The ability to determine effects that extend outside the liver becomes limited when multiple tissues are not subjected to measurement. The widespread use of high-fat diets to create NAFLD in mice does not address all disease aspects so researchers utilize non-dietary experimental approaches which produce specific disease characteristics other than obesity development. The hepatic inflammation and fibrosis development in LDL receptor knockout ($Ldlr^{-/-}$) mice is induced through oxLDL administration because these mice exhibit enhanced oxLDL uptake properties. This research model enables researchers to study inflammatory aspects of NAFLD without obesity factors because it produces sustained hepatic inflammation and fibrosis.²⁰³ Targeting CD98 with siRNA-loaded nanoparticles causes liver damage and reduces lipid accumulation via inflammation and immune modulation functions as another model for NAFLD research when obesity plays no significant role.

For future research, a useful approach could be to use a more aggressive dietary model, extend the duration of the intervention, analyze fiber composition in more detail, and expand tissue collection

to include adipose tissue. Insulin sensitivity and glucose tolerance markers can be studied in depth to analyze diabetes progression, as well. These additions would provide a more complete view of how whole food sourced carotenoids influence NAFLD and related metabolic processes.

Abbreviations:

NAFLD: Non-Alcoholic Fatty Liver Disease; **HFD:** High-Fat Diet; **LFD:** Low-Fat Diet; **WC:** White Carrot; **OC:** Orange Carrot; **RC:** Red Carrot; **YC:** Yellow Carrot; **HPLC:** High-Performance Liquid Chromatography; **LBP:** Lipopolysaccharide-Binding Protein; **CRP:** C-Reactive Protein; **LPS:** Lipopolysaccharide; **ACOX1:** Acyl-CoA Oxidase 1; **PPAR α :** Peroxisome Proliferator-Activated Receptor Alpha; **CPT2:** Carnitine Palmitoyltransferase 2; **FAS** or **FASN:** Fatty Acid Synthase; **PGC1 α :** Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha; **CD36:** Cluster of Differentiation 36; **RAR:** Retinoic Acid Receptor; **RXR:** Retinoid X Receptor; **FABP5:** Fatty Acid Binding Protein 5; **SOD1/2:** Superoxide Dismutase 1 and 2; **CAT:** Catalase; **RDH13/14/16:** Retinol Dehydrogenase 13, 14, and 16; **ERCC2:** Excision Repair Cross-Complementation Group 2; **HSD17B6:** Hydroxysteroid 17-Beta Dehydrogenase 6; **FDPS:** Farnesyl Diphosphate Synthase; **PMVK:** Phosphomevalonate Kinase; **MVD:** Mevalonate Diphosphate Decarboxylase; **CYP2B10:** Cytochrome P450 2B10; **STX4:** Syntaxin 4; **NQO1:** NAD(P)H Quinone Dehydrogenase 1; **PRDX1/2:** Peroxiredoxin 1 and 2; **SLC35E2A:** Solute Carrier Family 35 Member E2A; **SLC17A2:** Solute Carrier Family 17 Member 2; **AKT1/2:** AKT Serine/Threonine Kinase 1 and 2; **PLIN2:** Perilipin 2; **qPCR:** Quantitative Polymerase Chain Reaction; **Ldlr $^{-/-}$:** LDL Receptor Knockout.

CHAPTER 5

5. Conclusion

In conclusion, this research analyzed how dietary carotenoids from various carrots affected NAFLD conditions induced by obesity utilizing mice as subjects. The results indicated that there were positive patterns related to the effects of dietary carotenoids on hepatic lipid accumulation and oxidative stress; however, none of the treatment arms showed significant outcomes to support the original hypothesis. High fat diet with orange carrot intervention (HFD+OC) was higher in α -carotene and β -carotene and had measurable hepatic carotenoid accumulation, yet significant improvements in metabolic markers or body composition were not observed. The intake of orange carrots elevated CD36 levels in the liver which may be related to fatty acid transport into the hepatocytes. Proteomics data showed moderate elevation patterns of the β -oxidation proteins such as CPT2 and ACOX3 which have a role in enhanced fatty acid catabolism, though the data was not significant. The significant upregulation of PLIN2 and SLC35E2A might indicate that in HFD+OC group, ER stress and hepatocyte lipid accumulation could be elevated due to high fat diet intake, and orange carrot intervention may not have been effective enough to reverse these adverse conditions. The proteins involved in the oxidative stress response which included SOD1, SOD2, NQO1 and PRDX1/2 exhibited minimal, non-significant upregulation trends in regulated expression.

The research showed that red carrot supplementation containing lycopene (HFD+RC) was effective in lowering body fat as well as positive indications towards reduction in hepatic lipid storage and decreased lipid uptake into the liver. Oxidative stress-related proteins NCF1 and ERCC2 showed modulated suppression by HFD+RC intervention; however, the results were not statistically significant as the proteomics data revealed. The proteins responsible for cholesterol

production and steroid metabolism showed moderate elevation by HFD+RC intervention relative to the HFD+WC group through the analysis of HSD17B6, FDPS, PMVK and MVD and these changes were statistically significant but their impact on cholesterol and steroid metabolism were not observed to be sufficient to conclude in positive claims. Finally, the HFD+RC group exhibited a decrease in hepatic inflammatory protein CYP2B10 along with STX4 compared to the HFD+WC group, though multiple protein measurements fell short of statistical significance to demonstrate definitive benefits.

The HFD+YC supplementation had low lutein content and demonstrated poor bioavailability since it resulted in high excretion levels in feces along with no detectable lutein levels in serum or liver tissues. Results from yellow carrot intervention showed weak effects on metabolic proteins and minimal gene expression along with other regulatory components involved in lipid metabolism in liver. The protein analysis data failed to validate positive results statistically.

White carrots (HFD+WC) demonstrated hepatic lipid lowering effects to a similar degree as other carrots with carotenoids. Genomic and metabolic analysis showed the HFD+WC and other carrot arms shared similar protein regulation of key metabolic proteins, inflammatory proteins and oxidative stress proteins. The similar effects between white and colored carrots favor the explanation that hepatic changes arise from compounds independent of carotenoids and could be related to the other compounds in carrots as a whole.

The use of a 45% high-fat diet was chosen over a more intense 60% high-fat diet, as 45% fat diet is more physiologically relevant to a modern diet and models of a Western-type diet; however, the use of 45% fat content could have restricted how severely NAFLD developed thus decreasing the ability to observe important intervention variations. The results of previous studies in the Eroglu Lab using a 60% fat diet provided clearer effects on carotenoid uptake and the physiological

responses following carotenoid consumption, as 60% fat is more reflective of a diet-induced obesity model.

The protein expression patterns identified in the hepatic effects of carotenoid interventions derived from whole carrots showed positive patterns, but their significance was not established due to insufficient data in order to link these effects exclusively to carotenoids. Therefore, the original hypothesis was rejected.

A research path for the future can include implementing a more severe dietary-induced obesity model (e.g. 60% fat) and performing detailed fiber compositions followed by collecting tissues from the adipose tissues. The expansion of animal models with additional modifications on stress inducers specifically targeting the liver could illustrate how whole food-sourced carotenoids impact NAFLD development in addition to related metabolic processes.

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