Dietary and metabolic influences on inflammatory markers of health

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Thesis Abstract

Recent availability and specificity of biological assessments coupled with a fast development in computational power and statistical methods have allowed for the efficient assessment of population health data. Aided by these advancements, perspectives are shifting from hypotheses based on gross physiology to those that are based on biochemistry and molecular biology. Accordingly, our understanding of adipose tissue is shifting from that of an inert organ for fat storage to that of a highly dynamic and interactive organ responsive to and promotor of many alterations in hormonal, metabolic, immune and neurological function. Further, we are just beginning to understand the role of diet in stimulating adipose accumulation and consequent disruption in inflammatory balance. Old notions about fat-induced arterial clogs are being replaced by the molecular understanding of complex biochemical events involving glycation products, oxidative stress, insulin dysfunction, *de novo* lipogenesis, adipose remodeling and adipokine release. However, many current studies linking nutrient metabolism, obesity and inflammation continue to perpetuate old hypotheses based on associations that did not account for confounding effects and do not match the evidence emerging from carefully designed experiments in biochemistry and molecular biology. Resolving complex relationships among the large number of variables that have been made available to epidemiology research requires the implementation of modern statistical methods that account for simultaneous, multidirectional, interactive and mediatory effects. We applied modern statistical methods to the analyses of epidemiological data from the Midlife in the United States (MIDUS) and National Health and Nutrition Examination Survey (NHANES – which year?) in order to assess the impact of body adiposity on two common markers of chronic inflammation, Interleukin-6 (IL-6) and C-Reactive Protein (CRP), and the extent to which adiposity mediates the effect of carbohydrates and lipids on inflammation at the dietary and metabolic levels. Our analyses indicate that body adiposity is a major contributor to baseline inflammation. BMI and specific measures of body composition explained a large portion of the variance in both IL-6 and CRP. Visceral fat also influenced IL-6 to a greater extent than non-visceral fat. While the opposite was true for CRP, the difference in effect was smaller. Visceral fat was the main mediator of the effect of BMI on IL-6 while non-visceral fat was the main mediator of the effect of BMI on CRP. BMI also influenced the heritability of both IL-6 and CRP. The additive genetic effects acting on IL-6 were all attributable to the heritable influences of BMI. The additive genetic effect acting on CRP was split between the influences of BMI and other factors. While IL-6 mediated some of the effects of BMI on CRP, IL-6 did not influenced the heritability of CRP. BMI also exerted greater effect on inflammation than other markers of metabolic function, including total cholesterol. It also mediated a large proportion of the effect of diet on inflammation, specifically, the effect of dietary carbohydrates. Accordingly, dietary carbohydrates, especially sugar and fiber, exerted the largest dietary effects on both body adiposity and CRP. Dietary lipids made small contributions to either BMI or CRP. Specifically, saturated fat did not influence BMI or CRP; dietary cholesterol also did not influence BMI, and only influenced CRP before accounting for metabolic factors. Similarly, carbohydrate, not lipid, metabolism was found at the origin of the metabolic models linking nutrient metabolism to body adiposity and inflammation. The strongest effects linked glucoregulation to body adiposity to inflammation. These effects are not

usually observed in epidemiological studies, especially those that have used simplified statistical methods and which did not account for the full data variance, confounding factors and simultaneous effects. Unlike similar studies, the results from our population analyses support or are consistent with the conclusions obtained in controlled experimental studies that carefully examined the biochemistry and molecular biology linking diet, nutrient metabolism, adipose accumulation and inflammatory physiology.

Thesis Introduction

While the role of adipose tissues in inflammatory physiology is becoming better appreciated, the extent of its influence is still under investigation. Concurrently, there still is much debate over the effects of diet and metabolism on obesity and inflammatory health. Many controversies exist over the effect of excess caloric intake, dietary carbohydrate and lipid balance, as well as the significance of blood cholesterol and fat accumulation in mediateing the effects of diet on health. In particular, there is ample disagreement on the order of effects that link diet, glucodysregulation, dyslipidemia, obesity and inflammation. Further, there are major discrepancies between the conclusions derived from human correlational studies and those derived from biochemistry and molecular biology. We applied modern statistical methods, including general linear modeling, structural equation modeling, and bootstrapping mediation methods, to analyse epidemiological data and assess the directionality of effects according to the physiological premises that have been set by controlled experimental studies. The studies comprising this thesis sought to narrow the gap that separates epidemiological and experimental biology. Specificlaly, we sought to assess the extent to which body adiposity affects the physiological levels of common markers of inflammation and their heritabilities, and the extent to which body adiposity mediates the effect of carbohydrates versus lipids on such markers, both at the dietary and metabolic levels.

This project is comprised of four studies, presented in two parts. In the first part of this project, I sought to assess the impact of body adiposity on inflammation. To this end, I investigated how various measures of body adiposity predict two biomarkers of systemic

inflammation, Interleukin-6 (IL-6) and C-Reactive protein (CRP), and the extent to which the heritabilities of these biomarkers are influenced by Body Mass Index (BMI). The first two studies of this dissertation address the first goal. In the first study, I applied general linear modeling and bootstrapped mediation methods to determine how various measures of body adiposity predict inflammatory status using a subsample of MIDUS participants from whom Dual-Energy X-Ray Absorptiometry (DXA) data was obtained. I examined the extent to which BMI reflects on total fat mass, visceral and non-visceral fat. I also examined how specific measures of body composition differentially contribute to systemic levels of IL-6 and CRP. Finally, I examined how the effects of BMI on systemic IL-6 and CRP are differentially mediated by those fat depots. In a second study, I used a special application of structural equation modeling for the estimation of the inter-trait heritability in order to determine the extent to which the heritabilities of these inflammatory markers were influenced by BMI. For these analyses, I used a co-twin subsample of MIDUS participants, that included monozygotic and dizygotic twins. Specifically, I estimated the shared heritability between BMI and baseline levels of IL-6 and CRP, as well as the co-heritability between IL-6 and CRP. In addition, I tested whether the intra-class correlation in IL-6 between unrelated controls matched on BMI, age and gender is similar to that of monozygotic co-twins.

In the second part of the project, I sought to assess the mediative role of body adiposity on inflammation with a focus on carbohydrate and lipid metabolism. To this end, I investigated how lipids and carbohydrates affect BMI and inflammatory status, at the dietary and nutrient metabolism level. The following two studies of this dissertation address this goal. In the third study, I applied general linear modeling and bootstrapping

mediation methods to determine the effect of dietary lipids and carbohydrates on body adiposity and inflammation, and the extent to which body adiposity mediates the effect of diet on inflammation, using dietary survey and biomarker data from combined NHANES cohorts. I examined how dietary carbohydrates and lipids influence BMI, including macronutrient interactions, non-linear relationships and confounding factors. Likewise, I examined the extent to which dietary lipids and carbohydrates influence systemic CRP, and whether such effects are mediated by BMI and other markers of metabolic status. I also used this model to compare the effect of BMI on CRP to that of other markers of metabolic status. In addition, I specifically estimated the proportion of the effect of carbohydrates and lipids on CRP that are mediated through BMI. In the fourth and last study, I applied structural equation modeling to assess the effect pathways underlying the complex relationships between carbohydrate and lipid metabolism, adiposity and inflammation, using the MIDUS participant data. This was accomplished by designing, testing and revising multiple mathematical models and using Bayesian statistics to select the models that best fit the data. This method allowed for testing of the order and directionality of effects. This model allowed me to examine the pathways linking nutrient metabolism to various markers of inflammation, thereby determining the metabolic pathways mediated by body adiposity. Finally, we tested the veracity and clinical relevance of this model in two metabolically distinct populations: diabetics and users of statin medications.

Inflammation

Immune function involves the defense of the body against disease, which typically entails guarding against infection. However, the immune system is also involved in the interface with commensal microorganism (Goto & Kiyono, 2012), in anti-oncogenic processes (De Visser, Eichten, & Coussens, 2006), wound healing (Park & Barbul, 2004) and adipose remodeling (Suganami & Ogawa, 2010). It also interacts with brain and metabolic systems to orchestrate complex physiological shifts, affecting feeding behavior (Dantzer, 2001) and energy balance (Demas, 2004). Hence, proper physiological function and health rely on the meticulous control of pro- and antiinflammatory balance. When high proinflammatory activity is chronically activated or sustained, damage and disease often ensue (Pawelec, Goldeck, & Derhovanessian, 2014). While chronic inflammation and related diseases are often attributed to genetics, gene association studies have failed to predict baseline levels of proinflammatory agents (Balistreri, Colonna-Romano, Lio, Candore, & Caruso, 2009; de Maat et al., 2004; Grimaldi et al., 2007; Lee, Bae, Choi, Ji, & Song, 2012). Reports often convey relative effects and omit or de-emphasize the small absolute effects. On the other hand, the effect of anthropomorphic and life-style variables, such as obesity, diet, physical activity and stress, are well documented and explain a significant portion of the individual difference in inflammatory physiology (Debnath, Agrawal, Agrawal, & Dubey, 2016; Fleshner, 2013; Huang, Zourdos, Jo, & Ormsbee, 2013). Chronic inflammation is associated with almost all diseases of the Western civilization, and plays significant role in the progression of

neurological and cardiovascular disease (Libby, 2006; Perry, 2004). Inflammation can be measured by many different biomarkers of immune function or activation.

Two markers are commonly used to describe individual differences in inflammatory status in the population: Interleukin-6 (IL-6) and C-Reactive Protein (CRP). The pleiotropic cytokine, IL-6, is produced by many cells and tissues, including adipocytes, and plays a major role in normal physiology and in orchestrating inflammatory responses (Castell et al., 1989). It has been widely employed in research on population health and aging because circulating levels of IL-6 tend to rise in old age, with obesity, and following stressful life events (Fried, Bunkin, & Greenberg, 1998; Friedman et al., 2005; Kiecolt-Glaser et al., 2003). C-Reactive Protein is an acute phase reactant produced by hepatocytes, endothelial cells and adipocytes (Anty et al., 2006; Calabro, Chang, Willerson, & Yeh, 2005). High levels of CRP have been associated with many inflammatory conditions, including infection, smoking and cardiovascular disease (Gupta et al., 2012; Saijo et al., 2004). Hence, its use has been adopted in research and in clinical practice as an independent marker of liver and vascular disease. CRP production in the liver and adipose tissue is also readily stimulated by IL-6 (Anty et al., 2006; Calabro et al., 2005; Heinrich, Castell, & Andus, 1990; Volanakis, 2001). Therefore, this project focused on these two biomarkers of inflammation as a measure of inflammatory status.

Metabolic syndrome and obesity

According to American Medical Association, the average middle-aged American adult is overweight and shows signs of metabolic syndrome (Ford, Giles, & Dietz, 2014). In addition, the incidences of obesity and metabolic syndrome are increasing at alarming

rates (Flegal et al., 2009; Mokdad et al., 2003). Metabolic syndrome describes a set of symptoms that characterize system-wide dysfunction in the regulation of nutrient metabolism, energy balance and adipose accumulation. These conditions include hyperglycemia, hypertriglyceridemia, hypercholesterolemia, hypertension and obesity. The main complications of metabolic syndrome are type 2 diabetes mellitus and cardiovascular disease. Worse yet, once considered a disease of aging, type 2 diabetes and metabolic syndrome now affect all age groups, including young adolescents (Krolewski et al., 1987; Liese et al., 2006; Pinhas-Hamiel & Zeitler, 2005; Sardinha et al., 2016). A number of biomarkers have been used to assess metabolic status and to diagnose metabolic syndrome. They include fasting glucose and insulin, Hemoglobin A1c (HA1c), triglycerides, blood cholesterol measures, blood pressure and obesity. Fasting glucose is a typical measure of glycemic control, which involves balance between two pancreatic hormones: glucagon and insulin. Glucagon stimulates gluconeogenesis, a process by which glucose is produced by the liver. Insulin stimulates lipogenesis and the uptake of circulating glucose by various tissues, including fat stores. Together, they keep blood glucose within a narrow physiological range. However, with frequent glucose consumption, as is typical in the American diet, and subsequent chronic insulin release, many tissues develop insulin resistance, giving rise to conditions such as diabetes mellitus. Hence, hyperglycemia is a common symptom of diabetes. High glucose in circulation has also been shown to induce glycation products, including glycated hemoglobin (Makita et al., 1992). If not cleared, these damaged products may become further glycated by further glucose exposure, giving rise to Advanced Glycation End-Products (AGEs), which are highly proinflammatory and add to the inflammatory burden

associated with metabolic syndrome and cardiovascular disease (Fu et al., 1994; Turk, Mesić, & Benko, 1998; Yan, Ramasamy, Naka, & Schmidt, 2003). Because glycated hemoglobin (HA1c) levels, i.e., the percentage of hemoglobin molecules that are glycosylated, reflects long-term prevailing blood glucose concentration during the life of red blood cells, it serves as a biomarker for glucose regulation and diabetes in research and medical practice. Fasting glucose, insulin and HA1c are often used together in medical practice to assess glucoregulatory status.

Triglycerides are produced by the liver and adipose tissue in the process of *de novo* lipogenesis as a function of high carbohydrate intake (Hofmann & Tschöp, 2009). Because liver and muscles have limited glycogen stores, excess glucose is converted to triglycerides, then oxidized or stored as fat. Because the body cannot store fructose, it is immediately converted to triglycerides for storage or oxidation. Hypertriglyceridemia reflects sustained high blood triglycerides and is often associated with diabetes.

Cholesterol is synthesized in the liver or obtained from diet. There is evidence that, like *de novo* lipogenesis, cholesterol synthesis is stimulated by high glycemic carbohydrate intake (Hite, Berkowitz, & Berkowitz, 2011). Cholesterol is a substrate for cellular components and hormones, and is carried by lipoproteins. Low- and high-density lipoprotein cholesterol (LDL and HDL or LDL-C and HDL-C) reflect the amount of cholesterol carried by LDL and HDL particles. Total and HDL cholesterol are easily measured, and LDL typically calculated. These three cholesterol measures are often used as clinical and research markers of lipid metabolism. Hypercholesterolemia reflects high total blood cholesterol or high LDL cholesterol.

While the human body readily converts excess carbohydrates to lipids, the capacity to convert lipids to carbohydrates does not exist in human physiology. In the absence of dietary carbohydrates, amino acids serve as the substrate for gluconeogenesis, thereby satisfying physiological needs.

Hypertension is reflective of arterial stiffening, which is directly associated to the atherogenic physiology. Blood pressure also fluctuates to meet nutrient and oxygen demand and is readily influences by physical activity and psychological stress. Adipose accumulation presents increases in proinflammatory processes, exarcebating cardiosvascular inflammation and disrupting hormones and energy metabolism. Accordingly, chronic inflammatory conditions are often comorbid with obesity and metabolic dysfunction (Raman et al., 2013; Shrivastava, Singh, Raizada, & Singh, 2015; Willerson & Ridker, 2004). Likewise, the above metabolic factors are all involved in inflammatory processes in some way. Glucose-induced glycation and oxidative damage may trigger proinflammatory processes (Yan et al., 2003). Insulin has been reported to exert acute anti-inflammatory effects. Triglycerides-rich lipoproteins have been shown to trigger proinflammatory pathways and to contribute to directly to atherosclerosis (Rosenson, Davidson, Hirsh, Kathiresan, & Gaudet, 2014; Welty, 2013). While blood cholesterol receives most of the attention in the literature on cardiovascular inflammation and atherosclerogenic progression, controversy persists regarding the validity or basis for this relationship (Ordovas, 2005; Weinberg, 2004). High blood pressure may contribute to arterial lesions, though stiffened arteries are likely a side effect of atherosclerosis. Here, there is discussion on which factor, or factors, are the major contributors to inflammation, especially in the context of metabolic syndrome.

Obesity and body adiposity is commonly measured as Body Mass Index (BMI; body weight in kilograms divided by height in meters square) in research and institutional reports because of the general availability of height and weight information and ease of calculation. It is used as a proxy for body composition and even fat distribution. Because visceral adiposity closely associated with metabolic syndrome, cardiovascular disease and other chronic inflammatory conditions, much of which is thought to be mediated by adipokine secretion (Fontana, Eagon, Trujillo, Scherer, & Klein, 2007; Saijo et al., 2004), we included a comparison of BMI to specific assessments of visceral and non-visceral fat masses. Nonetheless, BMI is accepted as a direct reflection of fat weight gains in adulthood and is consistently associated with many diseases and disorders, especially those of inflammatory nature (Faith, Matz, & Jorge, 2002; Kredel et al., 2013; Lau, Dhillon, Yan, Szmitko, & Verma, 2005; Vazquez, Duval, Jacobs, & Silventoinen, 2007).

Adipose tissue is a major site of proinflammatory activity, and a significant source of proinflammatory signals (Calabro, Chang, Willerson, & Yeh, 2005; Coppack, 2001; Frühbeck, Gómez-Ambrosi, Muruzábal, & Burrell, 2001; Goode & Watson, 2012; Lau et al., 2005; Mohamed-Ali et al., 1997; Peyrin-Biroulet et al., 2012; Trayhurn & Beattie, 2001). Adipose tissues participate in acute phase response to infections and in immune regulation (Calabro et al., 2005). Conversely, adipose tissue is dependent on macrophages for adipose remodeling, the process of adipose expansion involving high rates progenitor cell proliferation and adipocyte death (Cinti et al., 2005; Strissel et al., 2007). Systemic IL-6 is largely influenced by body adiposity (Crichton et al., 1996; Mehra et al., 2006; Mohamed-Ali et al., 1999; Pini et al., 2012). Adipocytes, as well as the macrophages embedded in fat tissue, are known to be the major source, especially in the

non-stimulated state (Simon W. Coppack, 2007; Fried et al., 1998; Suganami & Ogawa, 2010; Weisberg et al., 2003). The adipokine leptin also stimulates the release of IL-6 from leukocytes and macrophages (Agrawal, Gollapudi, Su, & Gupta, 2011; Behrendt et al., 2010; Kredel et al., 2013). Although these biological pathways often interact in a bidirectional and reciprocal manner, there is considerable evidence to suggest that adiposity exerts a far greater influence on inflammatory physiology (Miller, 2003; Welsh et al., 2010). Recent research has also shown that adipocytes produce large quantities of CRP and adipose tissue is a major source of systemic CRP levels (Anty et al., 2006; Calabro et al., 2005; Forouhi et al., 2001). Moreover, high body adiposity can also increase production of CRP by the liver and other sites (Anty et al., 2006; Calabro, Chang, Willerson, & Yeh, 2005). Accordingly, obesity may exacerbate proinflammatory processes and fuel inflammatory diseases across physiological systems. At the same time, which factors exert the greatest influence on inflammatory biology is still a topic of much debate. This project took advantage of these biomarkers to assess metabolic status and to examine the mechanisms driving obesity and inflammation, as well as to test the influence of BMI as a mediator of inflammatory status.

Diet, metabolism, inflammation and disease

The metabolic deterioration in the American health was concomitant with a major shift in dietary patterns since the 1950s, based on governmental guidelines and incentives favoring a carbohydrate based diet over the consumption of dietary cholesterol and saturated fats (Aranceta & Pérez-Rodrigo, 2012; Flegal, Carroll, Kuczmarski, & Johnson, 1998; Gifford, 2002; Layman, 2009; Teicholz, 2015). The over-simplistic gross-anatomic

notion was that high dietary cholesterol and saturated fats intake would lead to high blood cholesterol and high blood lipids, which in turn would passively clog arteries (Jensen et al., 2014). This hypothesis is known as the “Diet-Heart Hypothesis”. However, we now appreciate the complexities of atherosclerogenesis and atherosclerotic plaque formation, which involve specific molecular events: LDL-modification (Ahmed, 2005; Angelica & Fong, 2008; Itabe et al., 2011; Yoshida & Kisugi, 2010), vascular infiltration of LDLs (Nordestgaard & Tybjaerg-Hansen, 1992), reactive oxygen species (ROS) generation by mitochondria (Bonnefont-Rousselot, 2002; Figueroa-Romero, Sadidi & Feldman, 2008; Schleicher & Friess, 2007; Yu, & Bennett, 2014), the formation of foam cells (Huh, Pearce, Yesner, Schindler, & Silverstein, 1996), chronic inflammatory processes taking place at the vascular (Corti, Hutter, Badimon, & Fuster, 2004; Libby, 2006) and systemic levels (Shrivastava *et al.*, 2015), as well as arterial calcification (Kalampogias et al., 2016; Zieman, Melenovsky, & Kass, 2005). Yet, outdated hypotheses based on cholesterol-clogged arteries persist (see Figure 1, Jensen et al., 2014).

With the advancement and greater availability of biomolecular methods of greater specificity, our modern understanding of the diet-obesity-inflammation relationships has shifted focus to excess sugar consumption. The traditional “Diet-Heart Hypothesis” was first popularized by Ancel Keys’ Seven Country Study, presumably showing that nations with higher percent fat-calorie intake predicted greater number of deaths (Blackburn & Labarthe, 2012; Keys, Anderson, & Grande, 1965; Kritchevsky, 1998). However, it was later shown that Keys picked six out of the original 22 nations in order to prove his hypothesis, as the inclusion of all 22 nations voided his conclusions (Yerushalmy & Hilleboe, 1957). The six countries included were (in order of increasing death dates and

percent fat calories): Japan, Italy, England/Wales, Australia, Canada, and the USA. The countries excluded from the analyses were Austria, Ceylon, Chile, Denmark, Finland, France, German, Ireland, Israel, Mexico, Netherlands, New Zealand, Norway, Portugal, Sweden and Switzerland. Keys' research eventually led to the funding of The Framingham Study, which is a large longitudinal study that has produced many major publications further supporting the Diet-Heart Hypothesis. However, an unofficial release of internal research documentations by a Framingham Study statistician exposed early data which did not support the Diet-Heart Hypothesis and therefore was omitted from publication (Kannel & Gordon, 1970). The studies showed that, in fact, caloric, fat or cholesterol intake did not predict higher blood cholesterol or disease.

Additional studies offered further support to the Diet-Heart Hypothesis, often based on bivariate associations and outdated statistical methods that simplified the data to allow for manual calculations (e.g., dichotomization of continuous variables into categorical factors). Only very recently, statistical methods were developed that are able to parse the variance of effects to account for simultaneous, multidirectional, interactive and mediatory effects. These methods require computer-intensive processing and are still in the process of adoption. Accordingly, many studies linking diet, metabolism, obesity, inflammation and disease continue to rely on data simplification. As a consequence, many of these analyses have led to concluions that do not match the evidence emerging from carefully designed experiments in biochemistry and molecular biology.

Results from various studies that previously supported the Diet-Heart Hypothesis have been brought to question and a few have been re-evaluated. Recent re-analysis of the data from the Minnesota Coronary Experiment (a double blind randomized trial to test

the substitution of saturated fat by polyunsaturated fat) and meta-analyses showed no reductions in cardiovascular outcomes or death (Frantz et al., 1989; Ramsden et al., 2016; Veerman, 2016). Another re-evaluation was carried out for the Sydney Diet Heart Study based on omitted data, which when included, showed opposite effects in the substitution of saturated by polyunsaturated fats (Ramsden et al., 2013).

When high blood cholesterol is observed in cardiovascular disease, it may be product of glucodysregulation and hypertriglyceridemia, rather than the actual cause of disease. Dietary cholesterol only modestly increases blood cholesterol (Ginsberg et al., 1995; Griffin & Lichtenstein, 2013; Hopkins, 1992), cholesterol is dynamically controlled at various levels that may exhibit dysfunction (Dietschy et al., 1993; Lecerf & de Lorgeril, 2011; Ono, 2012). Cholesterol is either absorbed from the diet or produced in the liver by sugar ingestion (Jameel et al., 2014; Feingold & Moser, 1985; Schaefer et al., 2009). Dietary cholesterol is not only selectively absorbed, but also excreted by erythrocytes in the intestinal lumen (Lammert & Wang, 2005). Excess systemic cholesterol is also reconverted to bile for excression, much of which is also lost in feces (Lewis & Rader, 2005). Hence, higher blood cholesterol may be reflective of abnormal cholesterol synthesis or clearance. More likely, it is induced by carbohydrate-induced synthesis and hypertriglyceridemia induced impairment of hepatic reabsorption of lipoproteins, generating an accumulation of cholesterol-containing remnant particles (Skeggs & Morton, 2002). Altogether, the current body of evidence seems to suggest that triglycerides and blood cholesterol are not the cause of atherosclerosis, but instead another by-product of carbohydrate metabolism. Accordingly, recent studies point to carbohydrate metabolism as the root problem driving lipid metabolism dysfunction,

obesity and inflammation, contributing to the rapid increase in development of diabetes, metabolic syndrome, chronic diseases (Ballard et al., 2013; Boden, 2009; Bueno et al., 2013; Douris et al., 2015; Hussain et al., 2012; Kirk et al., 2009; Volek et al., 2009). Notwithstanding, there is still much debate over whether excess carbohydrates or lipids drive metabolic syndrome, obesity and inflammation, at the dietary or nutrient metabolism level. Hence, this a secondary goal of this project was to assess the contribution of carbohydrate and lipids to adipose accumulation and inflammation.

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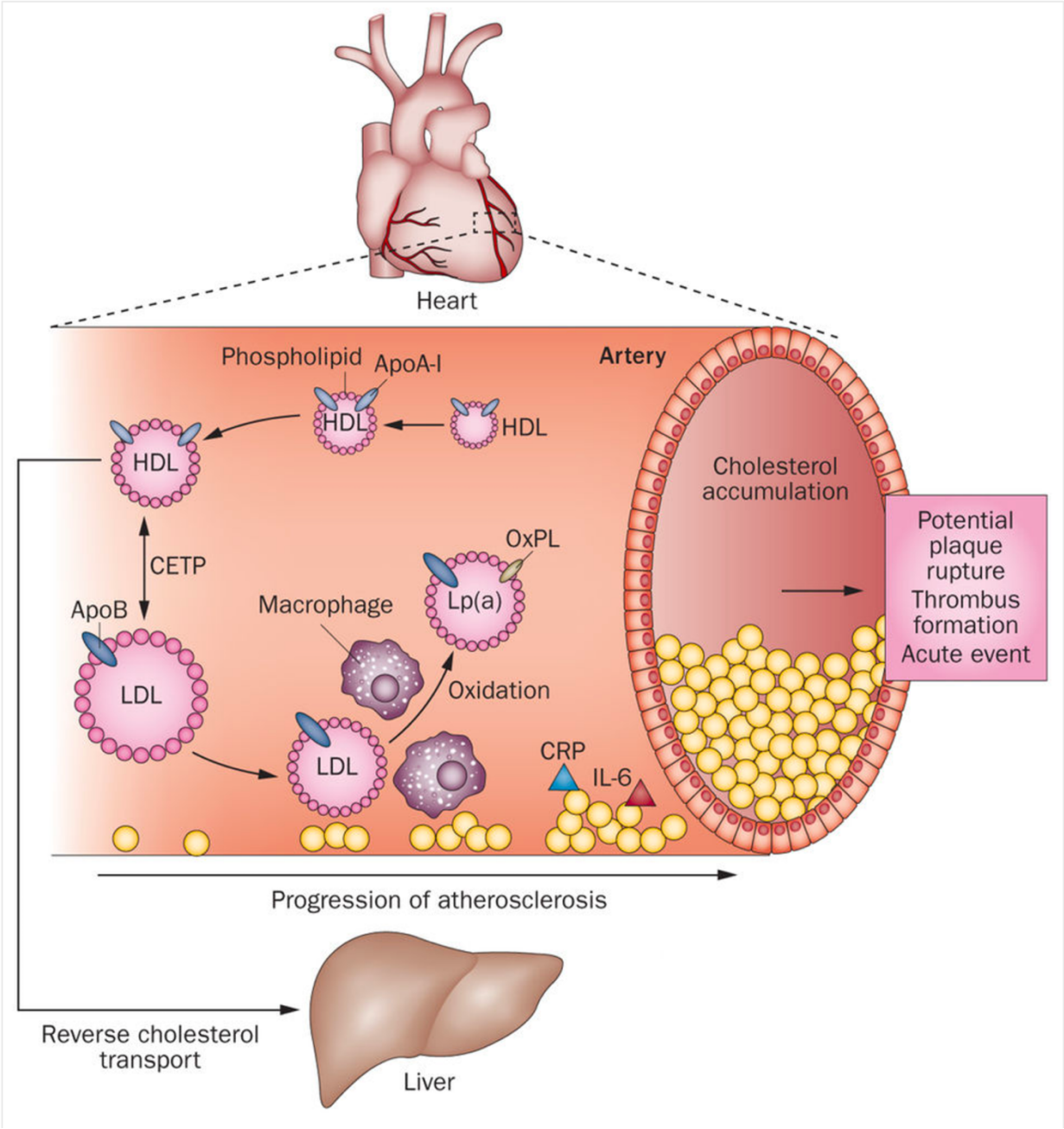

Figure 1. 2014 Nature Reviews Endocrinology article illustrating atherosclerosis as arteries clogged by cholesterol molecules.

Project hypotheses and significance

While the effect of body adiposity on inflammatory processes has been well characterized experimentally, there is question to the significance of its effect, especially in comparison to blood cholesterol. There is also significant debate over the order of effects linking glucoregulation, dyslipidemia, obesity and inflammation. Broadly, I hypothesized that body adiposity would exert major influence over both IL-6 and CRP, above and beyond diet and other metabolic factors, mediating a large proportion of the genetic, dietary and metabolic effects on CRP. Secondarily, I hypothesized that carbohydrates, at the dietary and metabolic levels, have greater influence on adiposity and CRP than lipids. In support of this thesis, more specific hypotheses follow for each study:

Study 1.1. There is still debate over the use of BMI as a measure of body adiposity, and especially whether it reflects specific fat depots. And while it has been proposed that visceral fat exerts greater impact on health, the mechanisms by which visceral fat differs from non-visceral fat are not well characterized, especially in the obese. I hypothesized that, in the context of the Western civilization, BMI would be a good proxy for total body fat, and an acceptable predictor of visceral and non-visceral fat depots, especially in the context of its inflammatory effects. I further hypothesized that visceral body fat would exhibit the greatest influence over both IL-6 and CRP. Similarly, I expected that visceral fat would best reflect the effect of BMI on both IL-6 and CRP.

Study 1.2. The extent of the impact of BMI on IL-6 and CRP is still ill-defined in the literature. In addition, many studies attribute the heritability of IL-6 to its own genetic influences. The extent of the heritable influence of BMI on both IL-6 and CRP is still ill-

defined. Based on the small genetic effects detected in the literature, I hypothesized, contrary to similar studies, that the additive genetic effect unique to IL-6 is small or insignificant. Rather, that the additive genetic effect observed in the heritability of IL-6 is inflated by the influence of BMI. According to the hypothesis that BMI exerts defining influence over baseline levels of IL-6, I expected matched controls to exhibit an intra-class correlation that is similar in magnitude to that of monozygotic co-twins. I also hypothesized that both IL-6 and BMI would exert influence over the heritability of CRP. However, I expect the additive genetic effect on CRP to be influenced to a lesser extent by BMI than IL-6.

Study 2.1. There is ample debate over the effect of diet on BMI, whether it is simply a matter of energy imbalance (excess caloric intake and/or low expenditure), or whether relative macronutrient intake (carbohydrates vs. lipids) drives adipose accumulation. The majority consensus is still that dietary lipids matter most, especially dietary cholesterol and saturated fats. The relative effect of the various markers of metabolic status on CRP (e.g., BMI vs blood cholesterol) is still ill-characterized. Here too the consensus points to blood cholesterol as the main target of treatment in metabolic syndrome and inflammatory disease. There is also discussion on whether dietary macronutrients exhibit direct or indirect effect on inflammation. I hypothesized that diet would explain a significant proportion of the variance in BMI, and that dietary carbohydrates would exert greater effect on adipose accumulation than lipids. Similarly, I hypothesized greater direct and indirect effect of carbohydrates on CRP, as well as a greater proportion of the effect of carbohydrates than lipids to be mediated through BMI. I also hypothesized that BMI would exert a greater effect on CRP than other markers of metabolic status.

Study 2.2. There is much debate on whether carbohydrate or lipid metabolism sits at the origin of metabolic dysfunction, and considerable disagreement on the order of effects. Path analyses of a structural equation model linking carbohydrate and lipid metabolism, adiposity and inflammatory markers can potentially disambiguate the order and directionality of effects. Further, such model may be used to analyze system-wide shift in metabolic-adipose-inflammatory interactions across conditions. Such model may help assess the alterations in physiological effects as a function of a condition or pharmaceutical manipulation. Accordingly, there currently are many controversies regarding the use of statin medication to lower blood cholesterol. It has been proposed that the beneficial effect of statins is due to its antiinflammatory effects rather than to its cholesterol lowering effects. There is also suggestion that statin medications may cause susceptibility to diabetes. Both hypotheses can be tested in our model, such that we may assess not only the effect of statins on the biomarker levels but also assess how the medication alter the relationship among those biomarkers. I hypothesized a model where carbohydrate metabolism fed onto lipid metabolism and body adiposity and where the three systems would feed onto inflammatory factors. I further hypothesized that this model would better fit the data than one that places lipid metabolism at the origin of the system. I also hypothesized that the magnitude of effects in the model would demonstrate that the flux of effects would point to BMI as a major mediator of the effects of carbohydrate metabolism on the inflammatory biomarkers. In addition, I expected that when diabetics and non-diabetics are compared in this model that we would observe alterations in path effects that are consistent with insulin resistance and diabetes. I also expected similar path effect alterations when users and non-users of statin medications

are compared in the model, and that these analyses would reflect a weakening of the inflammatory pathways.

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Part 1. The impact of body adiposity on two common population markers of chronic inflammation

Section 1.1.

Body Fat Distribution Differentially Predicts Interleukin-6 and C-Reactive Protein Levels

Abstract

Body Mass Index (BMI) is a surrogate measure of body adiposity, a marker of metabolic dysfunction and a strong predictor of inflammation. Cumulative evidence shows that visceral fat exerts greater inflammatory potential than other fat depots. However, BMI does not specifically measure body composition or distribution. The extent to which the effects of BMI on inflammatory markers reflect the proinflammatory capacity of specific body fat depots has not been thoroughly characterized. In our study, we sought to assess the impact of body adiposity on two common makers of chronic inflammatory physiology, Interleukin-6 (IL-6) and C-Reactive Protein (CRP). We determined the extent to which BMI reflects total body fat, visceral and non-visceral fat depots. We evaluated the value of specific DXA measurements of body composition in predicting IL-6 and CRP compared to BMI, and the extent to which specific fat depots mediate the effect of BMI on these proinflammatory biomarkers. Because IL-6 also stimulate acute phase reactants, we also assessed the extent to which IL-6 mediates the effect of BMI on CRP. We found that BMI was highly reflective of total body fat and non-visceral fat depots. While DXA measure of total body fat was no better than BMI in predicting IL-6 and CRP, parsing body fat by visceral and non-visceral fat depots better predicted IL-6 and CRP than BMI. Further, visceral fat was a better predictor of IL-6 than non-visceral fat, and the opposite was true for CRP. IL-6 was also a significant mediator of the effect of fat depots on CRP. Our analyses of mediation suggest that the use of BMI in predicting IL-6 and CRP reflect distinct anatomical effects. Notwithstanding, our analyses showed that BMI is an efficient

alternative to the DXA measure of total body fat, especially in the prediction of IL-6 and CRP.

Highlights:

- BMI was highly correlated to total body fat and non-visceral body fat.
- Total body fat was not better than BMI in estimating IL-6 and CRP.
- Visceral fat exerted greater influence on IL-6 than non-visceral fat
- Non-visceral fat exerted greater influence on CRP.
- The effect of BMI on IL-6 was mediated by visceral fat.
- The effect of BMI on CRP was mediated by non-visceral fat.
- The effect of visceral and non-visceral fat on CRP were partially mediated by IL-6.

INTRODUCTION

Body Mass Index as a measure of body adiposity

Body Mass Index (BMI) is a common surrogate measure of body adiposity in most research studies and institutional reports on diet, obesity, metabolic syndrome and disease. BMI is a simple function calculated from an individual's height and weight, thus it imprecisely represents body composition, and includes non-adipose tissues (e.g., skeletal muscles, organ tissues, bones) as well as a different types of fat tissue. However, it is widely accepted that increased BMI is a directly reflective of fat gains, especially in western civilizations that are marked by obesity and metabolic syndrome. Notwithstanding, discussions persist over the best measure of adiposity as well as the validity, utility and clinical applicability of BMI, especially within specific populations (Geliebter, Atalayer, Flancbaum, & Gibson, 2013; Lam, Koh, Chen, Wong, & Fallows, 2015; Okorodudu et al., 2010; Pietrobelli et al., 1998; Sardinha et al., 2016; Shah & Braverman, 2012; Stolic et al., 2002). Even within a typical western population (e.g., the United states), the validity and utility of BMI may depend on the goals of the study, as well as the biological relationships under investigation. This may be true even when BMI is a good representative measure of body adiposity. The physiological and pathological roles of body fat tissue may vary by histology and by body compartment (Cannon & Nedergaard, 2004; Fontana, Eagon, Trujillo, Scherer, & Klein, 2007; Nicholls & Locke, 1984; Vazquez et al., 2007). Body adiposity can be measured or estimated by various means, Including: BMI, waist-to-hip ratio, skin caliper, bioelectrical impedance, hydrostatic weighing, air-displacement plethysmography, computer tomography, or dual-

energy x-ray absorptiometry (DXA, or DXA). The latter two can estimate fat mass and lean mass by body location, which is useful in accessing the physiological roles of specific adipose depots.

Cross-system interactions

Contrary to conventional wisdom, body adiposity is not physiologically inert and does not serve solely as energy storage. Fat tissues produce various endocrine factors, affect brain function, normal metabolism and immunity, and is involved in complex cross-talk within each of those systems (Bartness, Kay Song, Shi, Bowers, & Foster, 2005; Galic, Oakhill, & Steinberg, 2010; Pénicaud, Cousin, Leloup, Lorsignol, & Casteilla, 2000; Romijn & Fliers, 2005; Vázquez-Vela, Torres, & Tovar, 2008). For example, adipose tissue receives sympathetic and parasympathetic innervation, responds to neuroendocrine factors, and participants in the conversion of cortisone to cortisol (Lee et al., 2008; Stimson et al., 2009). Adipose tissue is also regulated by multiple systems, and may become deregulated and dysfunctional in the context of obesity and metabolic syndrome (Dorresteijn, Visseren, & Spiering, 2012; Monteiro & Azevedo, 2010; Stolic et al., 2002; Strissel et al., 2007).

Adipose tissue, immunity and inflammation

Fat tissues have the ability to exhibit active immune functions such as participating in acute phase responses to infections and in immune regulation (Calabro et al., 2005). Adipose tissue is also dependent on macrophages for adipose remodeling, the process of adipose expansion involving high rates progenitor cell proliferation as well as adipocyte

death (Cinti et al., 2005; Strissel et al., 2007). Additionally, adipose tissue may exhibit two histological profiles: brown fat and white fat, the former which is involved in thermoregulatory functions (Cannon & Nedergaard, 2004; Nicholls & Locke, 1984) and the latter which presents greater cross-system complexity and which is more often associated with inflammation and disease (Trayhurn & Beattie, 2001b; Vázquez-Vela et al., 2008).

Recent evidence also points to differing disease impact based on adipose tissue distribution (Forouhi, Sattar, & McKeigue, 2001; Kang et al., 2011; Wajchenburg, 2014). Visceral adiposity is most commonly associated with metabolic syndrome, cardiovascular disease and other chronic inflammatory conditions, much of which is thought to be mediated by adipokine secretion (Fontana et al., 2007; Saijo et al., 2004). Interleukin-6 (IL-6) is a known mediator of many inflammatory processes, including T- and B-cell growth and differentiation, as well as a potent stimulant of acute phase protein production (Dienz & Rincon, 2009; Shrivastava et al., 2015). IL-6 is produced by various cell and tissue types, including various immune cells, non-immune hepatocytes, synoviocytes in synovial fluid, intestinal enterocytes, neurons and astrocytes in the brain, skeletal myocytes, and adipocytes (Fried et al., 1998; Gadient & Otten, 1994; Gagari, Tsai, Lantz, Fox, & Galli, 1997; Guerne, Zuraw, Vaughan, Carson, & Lotz, 1989; Hope et al., 1995; Jones et al., 1993; Pedersen & Febbraio, 2008; Tiggelman et al., 1995; Van Wagoner, Oh, Repovic, & Benveniste, 1999).

Adipose tissue is a major source of systemic IL-6 secretion, most notably within the obese population. In addition, studies have shown greater activity or release of IL-6 by visceral than subcutaneous adipose tissue in culture (Fried et al., 1998). Studies have

reported stronger associations for metabolic and cardiovascular complications to visceral adiposity compared to non-visceral (Fontana et al., 2007; Item & Konrad, 2012; Kuk et al., 2006; Matsuzawa et al., 1995). C-Reactive Protein (CRP) is an acute phase reactant that has been thought to be produced in large by hepatocytes, and in minor proportion by endothelial cells (Castell et al., 1989; Devaraj, Torok, Dasu, Samols, & Jialal, 2008). However, we now know that adipocytes produce large quantities of CRP and that adipose tissue is a major source of systemic CRP levels (Anty et al., 2006; Calabro et al., 2005; Forouhi et al., 2001). Although CRP production and release has not been experimentally shown to vary by adipose tissue location, systemic levels of CRP are sometimes, but not always, associated with visceral fat in cross-sectional studies (Carroll et al., 2009; Forouhi et al., 2001; Tsuriya et al., 2011). In one study, systemic CRP correlated with IL-6 values measured from the portal vein, which drains the viscera, indicating a possible mediated effect of adiposity by IL-6 (Fontana et al., 2007). Nonetheless, IL-6 and CRP are considered major mediators of chronic inflammation, largely produced by fat tissue. Accordingly, these measures are consistently associated with various measures of obesity, metabolic syndrome and cardiovascular disease (Malavazos et al., 2007; Wisse, 2004).

Goals

Because many studies utilize BMI as a proxy for body adiposity, we compared BMI to a DXA body composition assessment for a subsample of middle age adults in the Midlife in the United States (MIDUS) study. We sought to examine the extent to which BMI predicts total fat mass, visceral and non-visceral tissue composition. The relevance

of utilizing detailed body composition measures over BMI likely depends on the context of the study and adipose function under investigation. Therefore, we specifically examined the value of utilizing DXA body composition scans over BMI in predicting systemic IL-6 and CRP levels. Because, IL-6 and CRP are produced and released from adipose tissues, muscle and organs, we sought to determine the effect exerted by specific measures of body composition. In addition, we estimated the proportion of the effect of BMI on those proinflammatory markers that can be attributed to specific fat depots. Given that CRP production and secretion may be stimulated by IL-6, we also estimated the proportion of the observed effects of IL-6 on CRP that is mediated by each DXA measure, and tested the interaction between IL-6 and each DXA component in predicting CRP levels.

METHODS

Participants and clinical information

DXA scan, biomarker, anthropomorphic and demographic data were retrieved for 215 participants from the Biomarker Project in 2004-2009, from the original MacArthur Foundation Survey of Midlife Development in the United States (MIDUS II). Our analyses included 204 individuals who participated in the full DXA body composition assessment. Clinical and biological measures for these were assessed for participants who consented to the overnight hospital stay in Madison, WI. Participants arrived on Day 1 and were admitted and escorted to their rooms. That same evening, the participants completed their prior medical history and a physical exam with the help of staff, as well as a self-

administered questionnaire. Participants who participated in the biological assessment were demographically similar to the larger survey with respect to age, gender, marital status, and were slightly more educated (Love et al., 2010). All sample collections and analyses were approved by the Health Sciences Institutional Review Board (IRB) at the University of Wisconsin-Madison, as well as by the IRBs at UCLA and Georgetown University. All participants provided informed consent.

Dual-Energy X-ray Absorptiometry

Scan data were obtained on GE Healthcare Lunar (Madison, WI) using an iDXA densitometer. The entire body of all volunteers was contained within the scan field and positioning was per standard recommendations. All scans were acquired and data generated with the GE enCORE software. Total fat percentage was computed as follows: (total fat mass / (fat mass + lean soft mass + bone mineral content)) × 100. For measuring android fat, a region was defined between the top of the iliac crest and 20% of the distance from the top of the iliac crest to the base of the skull. Android fat percentage was calculated as follows: (android fat mass / (android fat mass + android lean mass + android bone mineral content)) × 100. Visceral fat was estimated by a proprietary algorithm, which is incorporated into the GE CoreScan software (Ergun, et al., 2013). The width of the subcutaneous fat layer along the lateral extent of the abdomen was calculated, and the anteroposterior abdominal thickness of the abdomen was derived from basis set transformation. The subcutaneous fat width and anteroposterior abdominal thickness were used to calculate subcutaneous fat in the

android region. Visceral fat was calculated by subtracting subcutaneous fat from the total fat mass in the android region.

Specimen Collection

Fasted blood samples were obtained between 05:00 and 07:00 AM on Day 2. Whole blood was used to determine HA1c; aliquots of serum and plasma were frozen for later analysis. All sample collections and analyses were approved by the Health Sciences Institutional Review Board at the University of Wisconsin-Madison, as well as by the IRBs at UCLA and Georgetown University. All participants provided informed consent.

Biological assessments

Glucose: Blood glucose was measure by the standard clinical methods performed at Meriter Labs (GML) in Madison, WI. Insulin: This assay is performed on a Siemens Advia Centaur analyzer. The assay performed at GML in Madison, WI. Glycosylated Hemoglobin (%) (HA1c): Hemoglobin A1c assay performed at GML in Madison, WI. Triglycerides (TG), total cholesterol (TC), and High-Density Lipoprotein-Cholesterol (HDL-C): A standard lipid panel was performed at GML in Madison, WI. The HDL-C assay was re-standardized by Roche Diagnostics on August 6, 2007. The results of assays done after that date were adjusted to bring the new values in line with the existing data: Adjusted value = 1.1423(new value) - 0.9028. Low Density Lipoprotein-Cholesterol (LDL-C): An estimation of LDL-C was calculated using the Fridewald formula from direct measurements of TC, TG, and HDL-C. When TG values were above 400 mg/dl, the Biocore used 400 mg/dl as the upper limit for calculating LDL-C. Interleukin-6 (IL-6): IL-

6 was measured using the Quantikine® High-sensitivity ELISA kit #HS600B (R & D Systems, Minneapolis, MN) at the MIDUS Biocore Laboratory at the University of Wisconsin, Madison, WI. C-reactive protein (CRP): CRP was measured using the BNII nephelometer from Dade Behring utilizing a particle enhanced immunonepholometric assay. The CRP assay was performed at the Laboratory for Clinical Biochemistry Research at the University of Vermont, Burlington, VT. Body Mass Index: BMI was computed by dividing weight (in kilograms) by height squared (in meters. Height measure (in centimeters) was multiplied by 100 to get the height in meters. The measures were obtained by the clinical staff according to a standardized protocol. Medication: Respondents were instructed to bring all their home medications, in the original bottles, to the GCRC (General Clinical Research Center) when they come for their visit. We ask them to do this to ensure that we are able to record medication names and dosages accurately. Completed forms were sent to the University of Wisconsin-Madison (central coordinating site) for review prior to data entry. The review included specification of medication codes based on the medication name, as well as information about route, or reason for taking the medication, as appropriate. The medication codes were selected from our master list of medication codes derived from the UW Hospital Formulary which utilizes the American Hospital Formulary System (AHFS) Pharmacologic-Therapeutic classification system. For our analyses, the only relevant medication information was whether the participant took medications for blood pressure (i.e., beta-blockers) or corticosteroid medications.

Statistics

Correlation was run in R (R Project for Statistical Computing) to inspect the associations among BMI and the various DXA measures. A simple regression model regressed IL-6 or CRP on all independent continuous variables of interest in order to check model assumptions and regression outliers. All analyses were carried out in R. Regressions were analyzed with the lmSupport package, standardized coefficients were computed with the ml.beta package, and the causal mediation models were analyzed with the "mediation" package, by a nonparametric bootstrapped confidence interval method running 1000 simulations. Variance Inflation Factors (VIFs) were checked before assessing model results in order to detect multicollinearity issues that could lead to the misestimations of regression coefficients. Regression coefficients were expressed as per-unit-change or per-standard-deviation-change, as indicated in the results. When regression dependent variables were transformed to normalize the regression residual distributions, natural log transformations were chosen for ease of interpretability. In those regressions, regression coefficients are predictive of the geometric and not arithmetic mean, and therefore may be interpreted as relative changes from the mean (x100 = percent change from the mean of regressed variable). ETA-R-squared were computed to estimate the unique proportion of the variance explained by each predictor in regression models. Linear regression models were used to estimate total effect of DXA components on IL-6 and CRP. Mediation analysis models adjusted for concurrent effect of other DXA components.

RESULTS

This MIDUS participant subsample was comprised of male and female participants of Caucasian and African-American descent, averaging 50 years or age, and metabolically representative of the larger MIDUS cohort. They were overweight and almost obese (BMI ≥25), pre-diabetic (fasting blood sugar levels from 100-125 mg/dL, and HA1c between 5.7-6.5), and exhibited low grade inflammation (CRP and IL-6 ≥ 3.0 mg/L) (Table 1).

Model assumptions and outlier analyses

IL-6, CRP and BMI were natural logged transformed when used as dependent variables in regressions to achieve normal distributions of regression residuals. No regression outliers were detected.

BMI and DXA body composition

BMI, Total body fat and non-visceral fat were highly correlated, while BMI was only moderately correlated with visceral fat or lean mass (Table 2). Unadjusted regression analyses showed that BMI predicted 76.4% of the variance in total body fat such that every one unit increase in BMI predicted a 195.1 g increase in total body fat ($B=195.1$, $F(1, 202)=25.55$, $p<0.001$). BMI predicted 34.1% of the variance in visceral fat mass, such that a one unit increase in BMI predicted a 89.7g increase in visceral fat ($B=89.7$, $F(1, 202)=10.21$, $p<0.001$). BMI predicted 74.1% of the variance in non-visceral fat, such that a one unit increase in BMI predicted a 186.1g increase in non-visceral fat ($B=186.1$, $F(1,202)=24.05$, $p<0.001$). Finally, BMI predicted 9.59% of the variance in lean body

mass, such that a one unit increase in BMI predicted a 70.1g increase in lean body weight (B=70.1, F(1,202)=4.63, p<0.001). One-standard deviation increase in BMI predicted a 58.3, 0.86 and 0.31 standard deviation increase in visceral fat, non-visceral fat and lean body mass, respectively. Hence, higher in BMI values predicted greater gains in non-visceral fat mass.

Effects of BMI or DXA body composition in predicting IL-6

BMI predicted 13.5% of the variance in IL-6 values (F(1, 202)=32.67, p<0.001), while parsing body composition into total fat and lean masses predicted 14.48% of the variance in IL-6 concentrations (F(1, 201)=17.02, p<0.001). Regression differences using ANOVA showed that the two models were not significantly different from one another (F(1,2)=0.53, p=0.25). Parsing body composition by visceral and non-visceral fat and lean body mass predicted 18.8% of the variance in IL-6 (F(3,200)=15.46, p<0.001). Using the DXA scan data over BMI explained an extra 4.9% of the variance in IL-6 values (F((1,2)=6.04, p=0.003). Visceral fat explained 5.18% of the variance in IL-6 (β=0.3061, F(1, 200)=12.76, p<0.001), while non-visceral fat explained 3.86% of the variance (β=0.2300, F(1,200)=9.52, p=0.002) and lean body mass did not predict IL-6 (β=-0.1054, F(1,200)=1.85, p=0.17). Accounting for other effects, a standard deviation increase in visceral fat predicted a 30.61% increase from average IL-6 concentration, while a standard deviation increase in non-visceral fat predicted a 23.00% increase (Figure 1). Our analyses showed that visceral and non-visceral fat depots contributed unequally to systemic IL-6 and that visceral fat exhibited the greater effect. Further, mediation analyses indicate that 32.0% (95% C.I. 11.4-65.9%, p<0.001) of the effect of BMI on IL-6

is mediated through visceral fat, while non-significant proportions were mediated through non-visceral fat (p=0.21) or lean body mass (p=0.27) (Figure 2). We concluded that when measuring the effect of BMI on IL-6, most of that effect is mediated by visceral fat, even though both fat depots contribute to overall increasing systemic IL-6 levels. BMI is a good, albeit incomplete, proxy for visceral fat when predicting IL-6. Our IL-6 analyses support previous findings that the differential effects of adipose tissue on systemic IL-6 depend on depot location.

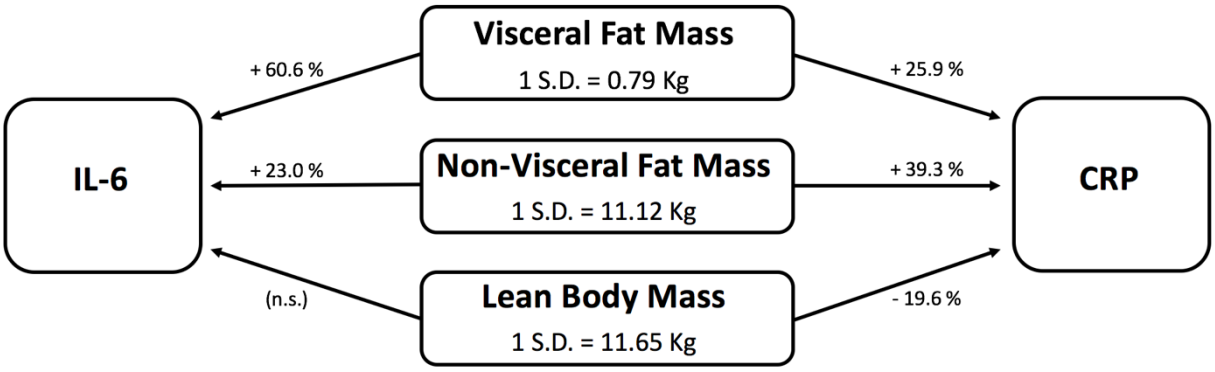

Figure 1. Body composition estimates unequally contribute to systemic IL-6 and CRP levels. Effects indicate the differences in circulating IL-6 or CRP levels per standard deviation change in specific body composition measure.

Effects of BMI or DXA body composition in predicting CRP

BMI predicted 25.31% of the variance in CRP (β=0.2531, $F(1, 202)$=68.46, $p<0.001$), while parsing body composition into total fat and lean masses predicted 27.59% of the variance in CRP (β=0.2759, $F(1, 201)$=38.29, $p<0.001$). Regression differences using ANOVA showed that the two models were significantly different from one another

($F(1,2)=6.31$, $p=0.01$); where the total body fat DXA measurement predicted an extra 2.27% of the variance in CRP. Analyzing body composition by visceral and non-visceral fat and lean body mass predicted 30.35% of the variance in CRP ($\beta=0.3035$, $F(3,200)=29.05$, $p<0.001$). Using DXA scan data over BMI explained an extra 5.04% of the variance in CRP ($F((1,2)=7.23$, $p<0.001$). Using said model, visceral fat explained 3.71% of the variance in CRP ($\beta=0.2589$, $F(1, 200)=10.64$, $p=0.001$), while non-visceral fat explained 11.29% ($\beta=0.3931$, $F(1,200)=32.42$, $p<0.001$) and lean body mass explained 2.59% of the variance ($\beta=-0.1956$, $F(1,200)=7.439$, $p=0.006$). Accounting for other effects, one standard deviation increase in visceral or non-visceral fat predicted 25.89% and 39.31% increases in CRP over the average, respectively (Figure 1). On the other hand, one standard deviation increase in lean body mass predicted 19.56% lower CRP. Contrary to IL-6, a greater effect on CRP was observed for non-visceral fat than by visceral fat, while greater lean body mass exhibited potentially protective effects. Further, the effect of BMI was mostly mediated by non-visceral fat. Non-visceral adiposity was estimated to mediate 52.34% (95%C.I. 16.6-100%) of the effect of BMI on CRP (Figure 2). Visceral fat did not appear to mediate a significant effect of BMI on CRP ($p=0.37$).

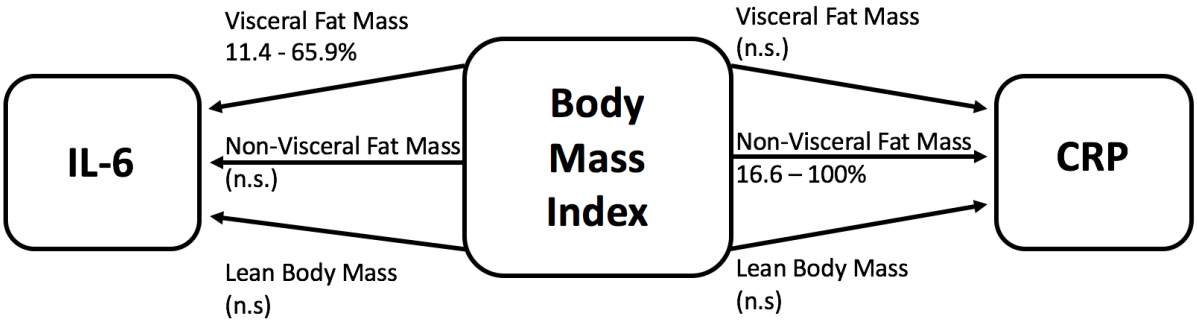

Figure 2. The proportion of the effect of BMI on IL-6 or CRP that is mediated through specific body composition measure.

Direct and indirect effects of body adiposity on CRP: mediation by IL-6

When IL-6 was added to the CRP regression, it explained an extra 10.75% of the variance ($\beta=0.1075$, $F(1,2)=36.32$, $p<0.001$), with only a small loss in significance from the effects of the three subcategories. Hence, some of the effects of adiposity on CRP might be mediated by IL-6. Mediation analyses estimated that 24.1% (95% C.I. 7.9-56.1%) of the effects of visceral fat on BMI was mediated by IL-6. Whereas 17.7% (95% C.I. 6.2-33.2%) of the effects of non-visceral fat were shown to mediated by IL-6. Hence, one might infer that IL-6 increases the effect of adiposity on CRP disproportionately based on location of body adiposity and where greater effects were mediated through visceral fat. These results were in accordance with the previous analyses which showed that higher IL-6 levels were more associated with visceral than non-visceral fat. Multiple regression analyses were used to test the interaction between IL-6 and each DXA component in the prediction of CRP; no interactions were detected.

DISCUSSION

Comparison of BMI and DXA measures in the prediction of IL-6 and CRP

Our participant sample exhibited multiple signs of metabolic syndrome and were, on average, over-weight. Hence, these results must be interpreted in the context of such population. Previous studies have suggested that the representation of body composition and distribution by BMI depends on age, gender, and metabolic status (Daniels, Khoury, & Morrison, 1997; Goh, Tain, Tong, Mok, & Wong, 2004; Goulding et al., 1996; Lambert et al., 2012; Rothman, 2008). We showed that, in this middle-age population, BMI is on average near-equivalent to DXA measures of total body fat. BMI moderately corresponded differences in non-visceral fat, while visceral adiposity and lean mass were both underrepresented. Consistent with the current literature, BMI was a significant predictor of both IL-6 and CRP (Aleksandrova, Mozaffarian, & Pischon, 2017; Debnath, Agrawal, Agrawal, & Dubey, 2016), but the latter to a greater extent. In our sample, nothing was gained by utilizing the DXA measure of total body fat over BMI in the prediction of IL-6 or CRP. However, by specifically estimating visceral and non-visceral fat and lean mass, we gained approximately 5% explanatory power in the prediction of these inflammatory markers.

Effect of fat depots on IL-6 and CRP

Our results also showed that visceral fat predicted higher circulating levels of IL-6 and explained a greater proportion of the variance than non-visceral fat. These finding are in keeping with the literature that predict greater IL-6 with increases with abdominal

fat accumulation (Fried et al., 1998; Pou et al., 2007; Wajchenburg, 2014). The opposite was true for CRP, which was better predicted by non-visceral fat. Previous studies have also shown that while visceral fat is consistently associated with IL-6, mixed results are observed for CRP (Forouhi et al., 2001; Pou et al., 2007; Saijo et al., 2004). Similarly, we found that that the effect of BMI on IL-6 largely represents the effect of visceral fat, while the effect of BMI on CRP represents the effect of non-visceral fat. Together, these results suggest that the effect of BMI on IL-6 and CRP are differentially mediated by distinct anatomical and physiological pathways. Although we did not detect interactions between IL-6 and DXA components in predicting CRP, we showed that the effects of BMI on CRP are partially mediated through IL-6. While CRP can be produced independently of IL-6 stimulation, its production may be enhanced by IL-6 (Anty et al., 2006). Greater mediative effects of IL-6 on CRP were observed through visceral rather than non-visceral fat. Accordingly, a study by Anty *et al*. (2006) showed that visceral fat exhibited association to systemic CRP indirectly through greater IL-6 released at the portal vein. Further, while lean mass did not predict IL-6, it predicted lower circulating levels of CRP, perhaps reflecting a buffering effect. The same buffering effect was not observed for IL-6, likely because skeletal muscle itself is a major source of IL-6 (Hiscock, Chan, Bisucci, Darby, & Febbraio, 2004; Pedersen & Febbraio, 2008).

Significance

Given the high cost of DXA examination, its use may only be warranted when discrimination between specific body fat depots are necessary. Until now, the relative value of utilizing DXA data over BMI in predicting IL-6 and CRP was as of yet ill-defined.

Our analyses allowed us to determine the value of utilizing DXA body composition measurements over BMI in assessing these proinflammatory markers in a small population sample. Our analyses indicate that DXA total body fat estimates were highly correlated with BMI and that non-visceral fat also closely represented by BMI, to a further extent than visceral fat or lean mass. Further, we showed that specific fat depots exerted distinct effects on IL-6 and CRP. The effect of visceral fat on IL-6 was greater than the effect on non-visceral fat. The opposite was true for CRP, although the difference by compartment were smaller for CRP than for IL-6. These findings are in keeping with studies which have shown that visceral fat may exert greater inflammatory influence. We also assessed the extent to which specific body fat depots mediated the effect of BMI on IL-6 and CRP. According to the previous analyses, the effect of BMI on IL-6 was largely mediated by visceral fat, while the effects on CRP were mediated by non-visceral fat. These finding suggest that the use of BMI in predicting inflammatory markers may represent distinct anatomical and physiological pathways. Our analyses also showed that part of the effect of visceral and non-visceral fat depots on CRP were also mediated by IL-6, suggesting that fat depots affect systemic levels of CRP both directly and, by a lesser extent, indirectly through IL-6 stimulation. While the use of specific measures of body adiposity may be favored in the prediction of specific inflammatory biomarkers, the use of BMI is well warranted in assessing the general inflammatory effect of obesity. To our knowledge, our study was the first to assess the utility of DXA over BMI in predicting inflammatory biomarkers and to determine the extent to which specific fat depots mediate the effect of BMI on inflammation.

TABLES

Table 1. Descriptive Statistics

	Mean or Frequency	Std. Dev.
Demographics		
Age	50.0	11.3
Gender		
Males	79	
Females	125	
Race		
Caucasian	104	
African American	100	
Anthropomorphic Measures		
Body Mass Index (kg/m^2)	29.5	5.2
Total lean mass (g)	49987.8	11653.3
Total adipose tissue mass (g)	31165.6	11499.9
Visceral adipose tissue mass (g)	1147.5	792.0
Non-visceral adipose tissue mass (g)	30018.1	11137.0
Pro-Inflammatory Biomarkers		
Serum IL6 (pg/mL)	3.3	3.0
Blood C-Reactive Protein (ug/mL)	3.2	4.1
Neuroendocrine measures		
Urine Cortisol adj for Urine Creat (ug/g)	14.5	11.5
Urine Epinephrine adjusted for Urine Creat(ug/g)	1.7	1.2
Metabolic Biomarkers		
Blood Hemoglobin A1c %	6.3	1.7
Blood Total Cholesterol (mg/dL)	183.0	40.2
Blood Triglycerides (mg/dL)	123.0	81.7
Blood HDL Cholesterol (mg/dL)	57.8	19.0
Blood Fasting Glucose levels mg/dL	108.1	42.1
Blood Fasting Insulin levels uIU/mL	13.9	13.8

Table 2. Correlations between BMI and body composition components

	BMI	Total Body Fat	Visceral Fat	Non-Visceral Fat
BMI				
Total Body Fat	0.87			
Visceral Fat	0.58	0.49		
Non-Visceral Fat	0.86	1.00	0.43	
Lean Body Mass	0.31	0.00	0.49	-0.04

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Section 1.2.

Shared Heritability Between Adiposity and Inflammation

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Abstract

The previous study showed that while the effect of BMI on inflammatory physiology was reflective of distinct anatomical effects, that BMI was also an appropriate surrogate of total body adiposity and a comparable predictor of Interleukin-6 (IL-6) and C-Reactive Protein (CRP) in population data. To further assess the impact of body adiposity on inflammatory physiology, we determined the extent to which IL-6 and CRP share heritable influences with BMI. Previous research has claimed large heritable effects on baseline levels of IL-6 and CRP. However, gene association studies do not efficiently predict individual differences in unstimulated proinflammatory levels. On the other hand, the heritability of body adiposity and obesity in the context of a fixed diet is well established. Given the strong relationship between adipose tissue and inflammatory physiology, it is likely that previously reported estimates of IL-6 and CRP have been inflated by the heritable influence of BMI. In our study, we used twin heritability models to estimate the proportional effect of heritable and non-heritable influences on IL-6 and CRP and their covariance with BMI. Like the previous study, BMI predicted distinct effects on IL-6 and CRP. Our models indicated a greater genetic effect on IL-6 than on CRP. However, the additive genetic effect of IL-6 was dominated by the heritable influence of BMI. On the other hand, the additive genetic effect on CRP was split between influences by BMI and influences independent of BMI. The genetic covariance between BMI and IL-6 was 83% while between BMI and CRP was 54.5%. On the other hand, IL-6 and CRP were not co-inherited to any degree. Rather, they shared common environmental effects.

Nonetheless, BMI exhibited powerful influences on unstimulated blood levels of both IL-6 and CRP.

Highlights:

- The additive genetic effects of BMI influenced the heritability of IL-6 and CRP.
- The heritability of IL-6 was completely shared with BMI.
- The heritability of CRP was split between the influence of BMI and genetic influences independent of BMI.
- The BMI x IL-6 correlation between matched controls was similar to that of monozygotic twins.
- The Heritability of CRP was not influenced by IL-6, but they shared common environmental influences.

INTRODUCTION

The genetics of Interleukin-6

The pleiotropic cytokine, interleukin-6 (IL-6), is produced by many cells and tissues, and plays a major role in normal physiology and inflammatory responses. It has been widely employed in research on population health and aging because circulating levels of IL-6 tend to rise in old age, with obesity, and following stressful life events (Fried, Bunkin, & Greenberg, 1998; Friedman et al., 2005; Kiecolt-Glaser et al., 2003). It has also been associated with chronic stress and vulnerability to depression (Bob et al., 2010; Lutgendorf et al., 1999; G. E. Miller, Stetler, Carney, Freedland, & Banks, 2002). It is known that the magnitude of the IL-6 response in inflammatory conditions can be affected by different single nucleotide polymorphisms (SNPs) associated with the IL-6 gene (Bruunsgaard et al., 2004; Sen, Paine, & Chowdhury, 2011; Walston et al., 2007). Accordingly, an examination of IL-6 responses to a strong inflammatory stimulus yielded high heritability estimates (de Craen et al., 2005). However, this general conclusion is often, and inappropriately, overgeneralized to all aspects of IL-6 synthesis and release. In the absence of inflammatory stimuli, SNPs have a weak or no association with baseline levels of IL-6 in the blood stream (Bagli et al., 2003; Bennermo et al., 2004; Brull et al., 2001; Burzotta et al., 2001; Herbert, Liu, Karamohamed, & Liu, 2006; Lieb et al., 2004; Markus Nauck et al., 2002; Sen et al., 2011; T. Shah et al., 2013; van Oijen et al., 2006; Wernstedt et al., 2004).

IL-6 and body adiposity

Serum IL-6 is largely influenced by body adiposity (Crichton et al., 1996; Mehra et al., 2006; Vidya Mohamed-Ali et al., 1999; Pini et al., 2012). Adipocytes, as well as the macrophages embedded in fat tissue, are known to be a major source of the IL-6 found in blood, especially in the non-inflammatory, healthy state (Simon W. Coppack, 2007; Fried et al., 1998; Khaodhiar, Ling, Blackburn, & Bistrian, 2004; Suganami & Ogawa, 2010; Weisberg et al., 2003; Wisse, 2004). The adipokine leptin also stimulates the release of IL-6 from leukocytes and macrophages (Agrawal, Gollapudi, Su, & Gupta, 2011; Behrendt et al., 2010; Kredel et al., 2013). Although these biological pathways often interact in a bidirectional and reciprocal manner, there is considerable evidence to suggest that adiposity exerts a far greater influence on inflammatory physiology (G. Miller, 2003; Welsh et al., 2010). Given the strong association between obesity and IL-6, it is likely that heritable factors influencing weight gain would also have a parallel effect on IL-6, a hypothesized linkage specifically tested in our analyses.

C-Reactive Protein

Likewise, the acute phase reactant, C-reactive protein (CRP) has been consistently associated with adiposity and inflammatory conditions, including cardiovascular disease (Carroll et al., 2009; N. K. Gupta et al., 2012; Saijo et al., 2004). Obesity can increase production of CRP by the liver as well as in adipose tissue (Anty et al., 2006; Calabro, Chang, Willerson, & Yeh, 2005b). Although previous genetic and heritability studies have demonstrated direct and independent effects on CRP levels (de Maat et al., 2004; Dehghan et al., 2011; Pankow et al., 2001; Wörns, Victor, Galle, & Höhler, 2006), there is evidence suggesting a causal relationship between the genetics

of obesity and circulating CRP levels. SNPs associated with body mass index (BMI) can influence blood levels of CRP, while the converse has not been demonstrated (Holmes et al., 2014; Welsh et al., 2010). Thus, to further probe the unique nature and strength of the association between adiposity and IL-6, we also considered the relationship between adiposity and CRP. It is known that both CRP and IL-6 are involved in inflammatory response pathways, but CRP production appears to be more readily stimulated by IL-6, even within adipose tissue (Anty et al., 2006; Calabro et al., 2005b; Heinrich, Castell, & Andus, 1990; Volanakis, 2001). In addition, circulating levels of CRP and IL-6 are regulated independently by different genes (T. Shah et al., 2013). By analyzing these associations in identical and fraternal twins, we were able to directly compare and contrast the relative influence of adiposity on both CRP and IL-6, and to consider the reciprocal relationship between CRP and IL-6.

Possibility of co-heritability

Previous twin studies have determined that adiposity, measured as BMI, is highly heritable (Hjelmborg et al., 2008; Schousboe et al., 2003; Segal, Feng, & McGuire, 2008). Associations between a genetic score, consisting of 14 SNPs related to BMI, and multiple cardiovascular and inflammatory traits including both IL-6 and CRP, were recently assessed (Holmes et al., 2014). However, it is still not known whether the heritable influence of obesity on IL-6 and CRP are coordinated. We probed these relationships by comparing IL-6 and CRP levels in identical and fraternal adult twins, who also varied in adiposity and anthropometric concordance.

Inter-trait heritability models

Twin studies take advantage of the different degree of genetic relatedness between monozygotic (MZ) and dizygotic (DZ) twins to estimate the relative contribution of genetic and environmental effects contributing to the phenotypic variance of a trait, as well as to the covariance between traits. Typical twin studies rely on the assumption that MZ twins share 100% and DZ twins share 50% of their genes, while both types of twins, regardless of zygosity, share a common rearing environment (Hall, 2003). In addition to the shared environment, it is also possible to discern effects of the non-shared environment, reflecting individual experiences not shared in common. Greater phenotypic similarity for MZ twins than found in DZ twins would be indicative of higher heritable contributions. On the other hand, when MZ and DZ twins present a similar phenotype, more variance attributable to life style and common environmental processes is assumed. Likewise, when monozygotic twins are discordant, it is usually attributed to unshared environmental influences (Boomsma, Busjahn, & Peltonen, 2002; Christensen, Støvring, & McGue, 2001; Duffy, Mitchell, & Martin, 1998; Hjelmborg et al., 2008).

Hypotheses

We utilized bivariate ACE heritability models (**A**, additive genetics; **C**,common environment; and **E**, unique environment) based on structural equation modeling (SEM) to estimate the proportion of IL-6 and CRP variation accounted for by genetic, shared and unshared environmental factors, as well as the heritable influences shared with adiposity. Our *a priori* hypothesis was that obesity would have a large effect on IL-6. Secondarily, the *a posteriori* hypothesis was tested: obesity would exert a heritable influence on CRP, but one that was only moderately associated with the genetics of IL-6. We also compared IL-6 intra-class correlations (ICCs) between co-twins in order to confirm the greater similarity between MZ co-twins than between DZ co-twins, indicative of heritable influences. To further test this hypothesis, we compared the ICC for MZ co-twins with that of genetically unrelated control participants matched to each MZ twin case. Assuming that environmental and lifestyle factors, BMI in particular, play a

greater role in accounting for IL-6, we predicted that ICCs between the actual MZ co-twins would be no higher than for control adults who were closely matched on age, gender, BMI and a socio-economic index (SEI). By matching control subjects to the twin cases on these attributes, we simulated 4 of the major environmental and host factors known to influence IL-6 (Ershler & Keller, 2000; Friedman et al., 2005; Hjelmborg et al., 2008; Johnson & Krueger, 2005; O'Connor, Motivala, Valladares, Olmstead, & Irwin, 2007; Wisse, 2004).

Study population

These analyses were possible because the recruitment strategy of a large survey of health and aging in the United States, Midlife Development in the United States (MIDUS), which included an over-sampling of twin siblings. It provided the unique opportunity to determine the heritability of circulating IL-6 and CRP, as well as the heritable contribution of obesity.

METHODS

Participants

Participants were drawn from the MIDUS II Biomarker project, 2004-2009, a continuation of an earlier MIDUS 1 survey supported by the MacArthur Foundation in 1995-96. In addition to a representative probability sample, MIDUS 1 recruited a national sample of twin pairs, from which the current cohort was selected. Between 2003-2005, blood specimens were obtained, enabling the determination of cytokines and other biomarkers for each twin pair (Love et al., 2010). The twin sample was comprised of 73 monozygotic and 32 dizygotic same-sex twin pairs, as well as 37 matched controls. In addition, we took advantage of BMI and IL-6/sIL-6r data on 830 unrelated participants.

The same dataset was utilized for the CRP analyses, with information available on 72 monozygotic twins, 31 dizygotic twins, and 826 unrelated participants.

Twin Recruitment

Twin pairs were recruited by asking randomly selected correspondents from about 50,000 households across 48 states whether there were twin pairs in the immediate family. With their permission, twin pairs were referred to MIDUS II recruiters. The recruited twin pairs were related to original correspondents, reared together but living apart as adults, ranging from 25 to 74 years in age, residing in the continental U.S., English speakers, possessing a residential telephone number, and were mentally and physically capable of participating in interviews and questionnaires. They had to be healthy enough to travel to one of three Institutes for Clinical and Translational Research (ICTR) for the Biomarker project, where they spent the night before sample collection on the following morning.

Socio-Demographics, Zygosity, Clinical and Biological Measures

Zygosity was determined by self-report in MIDUS 1. Similarity of eye and hair colors, as well as the degree to which their identity was confused by others during childhood, were among the criteria for twin determination. This approach is more than 95% accurate when compared to blood tests (Nichols & Bilbro, 1966). Each sibling's Socioeconomic Index (SEI) was derived using income, educational attainment and occupation categories from the 1990 Census classification (Hauser & Warren, 1997) and incorporated into MIDUS II, 2004-2006.

Clinical and biological measures were assessed for a total of 1255 participants in the Biomarker project who consented to the overnight stay, either in Madison, WI, Los Angeles, CA, or Washington DC. Participants arrived on Day 1 at one of the three sites where they were admitted to the hospital research unit. They completed a medical history and physical exam, as well as a self-administered questionnaire. Smoking can affect IL-6 and could interact with genetic factors that influence transcription and release of IL-6 (Bruunsgaard et al., 2004; Semlali, Witoled, Alanazi, & Rouabhia, 2012; Zhou, An, & Chen, 2014). Therefore, we also examined the concordance of the smoking history between MZ and DZ co-twins.

Smoking history was assessed on the clinical questionnaire by asking: “Have you ever smoked cigarettes regularly?“. Fasted blood samples were obtained between 0500 and 0700, and sera frozen until analyzed. All sample collections and analyses were approved by the Health Sciences Institutional Review Board at the University of Wisconsin-Madison, as well as by the IRBs at UCLA and Georgetown University. All participants provided informed consent. Nursing staff followed standardized procedures detailed in a general “Manual of Procedures”, as well as specific “Guidelines for Collecting and Processing Biomarkers” in order to maintain consistency.

Cytokine Assessment

Serum IL-6 levels were determined for all 1255 biomarker project participants by high-sensitivity enzyme-linked immunosorbent assay (ELISA) (Quantikine, R&D Systems, Minneapolis, MN), with a lower sensitivity of detection at 0.16 pg/mL. All values were quantified in duplicate; any value over 10 pg/mL was re-run with sera diluted to fall

on the standard curve. The laboratory intra-assay coefficient of variance (CV) was 4.1% and the inter-assay CV was 12.9% (generated by inclusion of a low and high IL-6 serum pool in each assay). Sandwich ELISA kits were also employed to quantify sIL-6r levels (Quantikine, R&D Systems). Sera were diluted 1:100 so values would fall on the standard reference curve from 7 to 2000 pg/mL. Thus, the effective assay range for sIL-6r was 0.7–200 ng/mL. The intra-assay and inter-assay CVs was 2.0%. Serum CRP levels were determined for all subjects via particle-enhanced immunonephelometric assay, and high values used as exclusion criteria.

Statistical Analysis

Nine twin pairs were excluded when a sibling had CRP levels indicative of sickness (above 10 mg/L), or they were discrepant on smoking status or chronic illness. In addition, because there were insufficient numbers of African-Americans (only 2 twin pairs), and there are known race differences in cytokine and CRP levels (Carroll et al., 2009; Coe et al., 2011; Crawford et al., 2006), both pairs were excluded. Opposite-sex, dizygotic twin pairs were also excluded from the analysis because there were too few pairs to include in the model. In order to achieve normal distributions, IL-6, CRP and BMI were natural log-transformed before statistical calculations.

Linear regressions were employed to examine the effect of BMI on IL-6 and CRP for singleton birth participants in the main Biomarker sample, after excluding the twin participants. Including the twins in these regression analyses would have violated assumption of independent observations. Similarly, these regression analyses were run

on only the Caucasian participants of European descent, because of the known differences in African-Americans (Coe et al., 2011).

In order to estimate the additive genetic and environmental effects contributing to the IL-6 and CRP variance, including the covariance with factors affecting BMI, we fitted bivariate ACE models to these data by using Cholesky's decomposition approaches in SEM (Karmakar, Malkin, & Kobyliansky, 2012). SEM allows for testing whether covariance matrices of hypothetical models fit the covariance matrix of the actual data. They further allow for distinguishing the submodels that fit the data most parsimoniously. Classical ACE models decompose the phenotypic variance into three categories, A (Additive Genetic Effects), C (Common Environmental Effects) and E (Unshared Environmental Effects plus residual/error variance). Bivariate ACE models estimate A, C and E effects for two phenotypic traits and, in addition, estimate the covariance between each effect across phenotypes. We defined a saturated ACE covariance model as well as submodels for AE covariance (negligible sharing of common environmental effects) and CE covariance (negligible sharing of additive genetic effects) which were tested against the data covariance matrices, providing the bases upon which one may identify the models that best fits the data. The Cholesky's decomposition of phenotypic effects allowed us to parse out the additive genetic effects unique to IL-6 or CRP from those shared with BMI by comparing the various covariance submodels, while allowing univariate variance components to vary freely. This same approach was used in order to test the genetic covariance shared between CRP and IL-6.

These biometric models were run in MX using a script adapted from file rawVC4a.mx, provided with the MX software (Neale, Boker, Xie, & Maes, 2003; Posthuma

& Boomsma, 2005). Because MZ and DZ twins share 100% and 50% of their independently segregating genes, respectively, SEM covariance coefficients for additive genetic effects were set to 1 for MZ twin pairs and to ½ for DZ pairs. Covariance coefficients for common environment effects were set to 1 and those attributed to unshared environment effects were set to 0. Age and Gender were included in Cholesky's decomposition models as covariates in order to prevent indirect inflation of ICCs that could potentially confound heritability estimates (McGue & Bouchard, 1984). Saturated and nested covariance submodels (ACE, AE and CE) were fit to the data and submodels were tested against the saturated ACE models. Non-significant differences in Chi-square probabilities allowed us to discern nested submodels that provided fit of the data. The models that best described our data were identified on the basis of maximum likelihood estimates, -2LL, (-2*log-likelihood = $-2*\log(C - \chi^2/2)$), and relative fit indices, Akaike's Information Criterion, AIC, (AIC = $\chi^2 - 2*d.f.$), and Bayesian Information Criterion, BIC (BIC= $\chi^2 - d.f. * \ln(n)$). Lower AIC and BIC values indicate models that fit the observed data most parsimoniously.

Intra-class correlations for IL-6 and smoking history were calculated for the MZ and DZ co-twins (Hawkins, 1989; Hotelling, 1953; Sedgwick, 2013). One-tailed Fisher's r-to-z tests assessed whether IL-6 ICCs for MZ twins were significantly greater than for DZ twins. To further evaluate the influence of obesity on IL-6, one-tailed Fisher's r-to-z tests were used to compare the ICCs between MZ co-twins to the ICCs between 37 MZ twins and unrelated controls matched for age, gender, BMI and SEI. Smoking history was assessed using a two-tailed Fisher's r-to-z test to examine the presence of possibly

confounding differences between MZ and DZ ICCs. Except for the biometrical models, analyses were determined with SPSS 19.

RESULTS

Twin Sample

The MIDUS Biomarker project has been shown previously to be comparable to the larger MIDUS participants for most socio-demographic, health status, and health behavior indicators, but not race (Love et al., 2010). The latter was not an issue for the current analyses because the 2 African-American twin pairs were excluded. Table 1 presents the socio-demographic and clinical information, and mean cytokine values for MZ and DZ twins and the unrelated controls who were matched with the cases, as well as for the remaining Biomarker participants from which the controls were selected.

Effect of BMI on IL-6 and CRP

After adjusting the regression models for age and gender by including them as covariates, BMI accounted for 7.8% of the variance in IL-6 levels ($\beta = 0.28$, $F[1,829] = 42.4$, $p < 0.001$) and 18.0% of the variance in CRP levels ($\beta = 0.43$, $F[1,826] = 70.0$, $p < 0.001$). In keeping with our predictions, BMI exerted a stronger effect on IL-6 than on the soluble IL-6 receptor among unrelated MIDUS participants, as well as had a substantial effect on CRP.

Heritability Estimates

Non-significant chi-square difference tests indicated that CE and AE covariance submodels for BMI X IL-6, BMI X CRP and CRP X IL-6 presented an equivalent fit for the data as did the saturated ACE covariance models. In addition, lower relative fit indices (AIC and BIC) identified the AE submodels as most parsimoniously fitting all three sets of BMI covariance data, and the CE submodel as a better fit for the CRP X IL-6 data (Table 3). In the three bivariate BMI models, additive genetic effects and unshared environmental effects comprised almost all of the influences acting independently on BMI, thus providing two available pathways for covariance with IL-6 and CRP (Table 4). Covariance was notably high in accounting for the heritable associations between BMI and IL-6 (83.0%). The proportions presented in Table 4 were calculated based on the SEM path coefficient estimates.

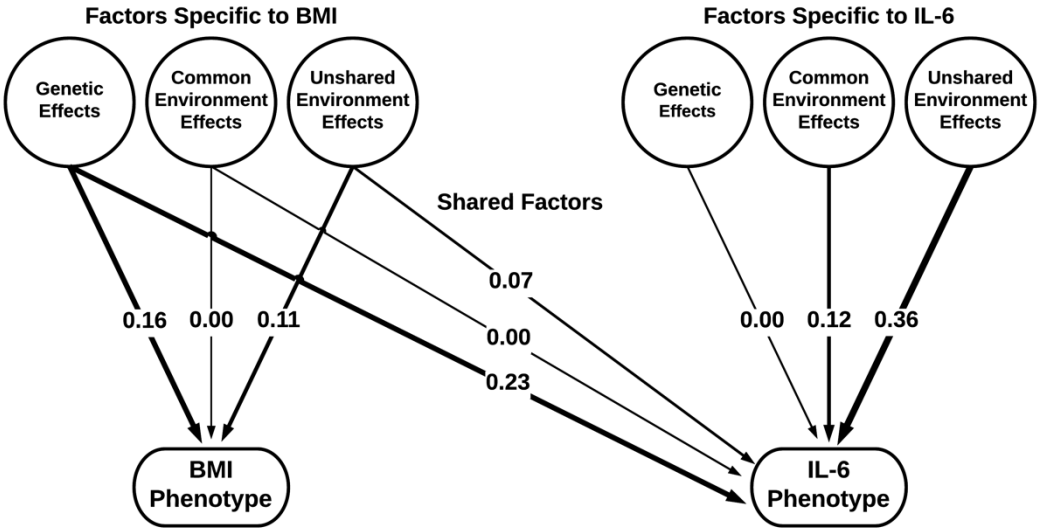

Fig. 1 Path coefficient estimates for bivariate BMI X IL-6 model, including the corresponding percent contributions of additive genetic effects, and common and unshared environmental effects affecting IL-6. The model also parses effects specific to IL-6 from those shared with BMI. The most parsimonious model indicated that BMI and IL-6 share both genetic and unshared environmental effects. However, model estimates further indicated that the additive genetic effect acting on IL-6 is mostly shared with BMI.

These effects are also portrayed in Figure 1 for IL-6, which show the discrete effects parsed by specific sources of variation. These estimations indicated further that the suggestion of a genetic constraint on IL-6 levels was not attributable specifically to the genetics of IL-6, but rather was driven more indirectly by factors shared with the predisposition for BMI (Figure 1). Even when inflated by considering the high covariance with BMI (Table 4), the total genetic effect estimated for IL-6 was still small (averaging 26.2%). In contrast, the heritable influences on the soluble receptor for IL-6 were significantly stronger (averaging 42.2%). CRP also exhibited a high genetic covariance with BMI (54.5%, Table 4); however, not to the same extent as the IL-6 covariance (83.0%). Further, the additive genetic effects on CRP were almost evenly divided between those shared with BMI and factors unique to CRP (Figure 3). Moreover, our models did not reveal shared genetic effects between CRP and IL-6. The covariance between CRP and IL-6 phenotype was split between shared and unshared environmental effects (Table 4).

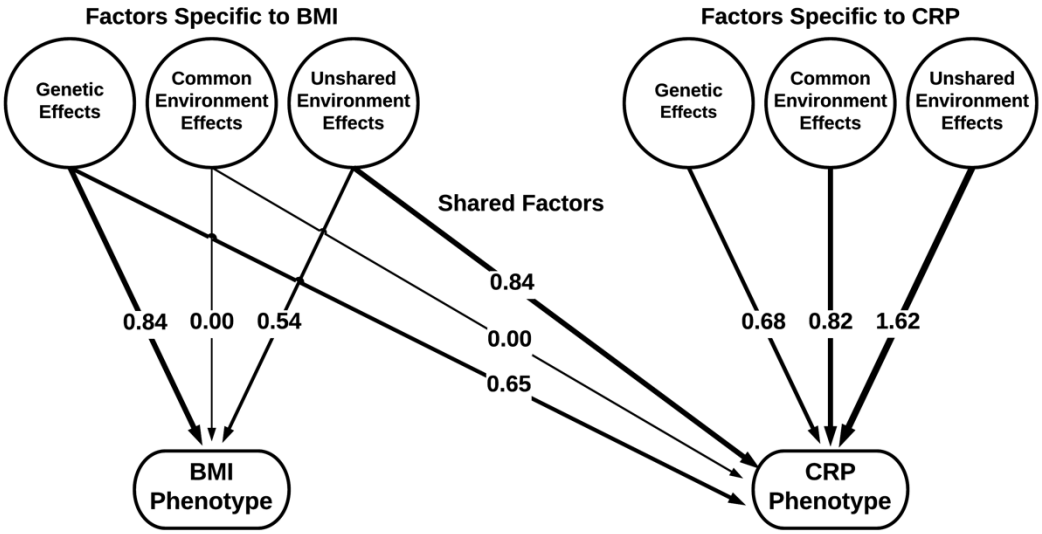

Fig. 3 The most parsimonious path coefficient estimates for the bivariate BMI X CRP model also indicated shared covariance between genetic and unshared environmental effects. Unlike the bivariate model for IL-6, however, the phenotypic variance attributed to the additive genetic effects was split between those shared with BMI and some independent of BMI.

Intra-Class Correlations

This case/control analysis confirmed the importance of heritable pathways related to obesity, and indicated that the 4 matching criteria resulted in IL-6 values similar to the twins, whereas there was no evidence that these 4 attributes increased the likelihood of a correlation in sIL-6r between cases and controls. In addition, as shown in Table 2, the ICC for IL-6 between the MZ twins was not greater than for the DZ twins, demonstrating that the degree of relatedness did not influence IL-6 more than the concordance for obesity. When a MZ participant was matched to an unrelated control by age, gender, BMI and SEI, the resulting ICC for IL-6 was nearly the same as for the MZ co-twin (Figures 5a & 5b). On the other hand, the sIL-6r of a twin was not significantly correlated with the value seen in the unrelated, matched control (Figures 5c & 5d). MZ and DZ twins were highly concordant for smoking history ($r = 0.57$, $p < 0.001$ and $r = 0.41$, $p = 0.02$, respectively). However, a history of smoking or abstinence did not significantly affect IL-6.

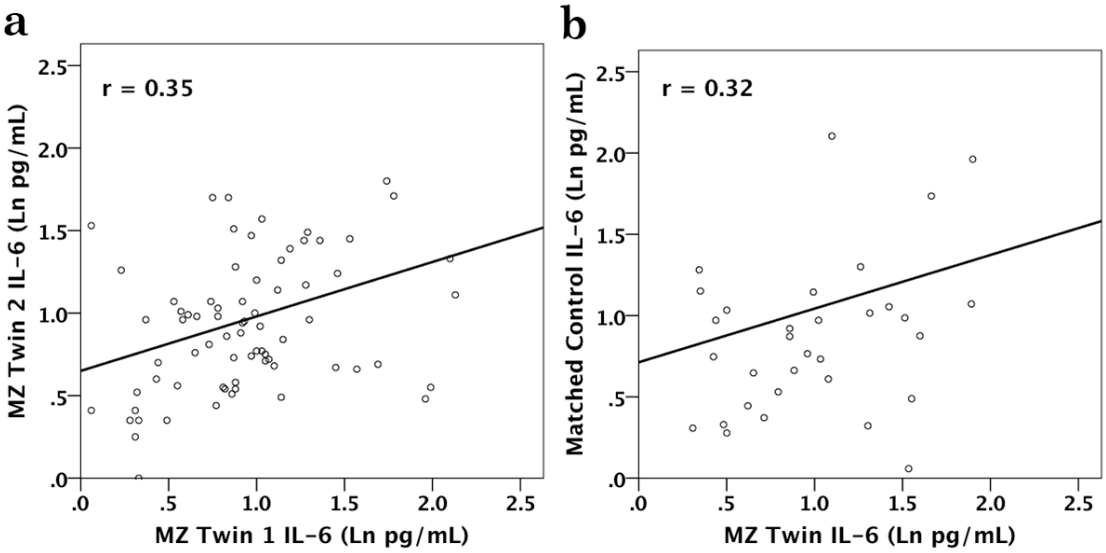

Fig. 5 Intra-class correlations are shown for IL-6 between MZ co-twins and between MZ twins and unrelated, matched controls, as well as for the sIL-6r associations. The correlation for IL-6 for MZ co-twins (A) was nearly identical to the correlation between MZ twins and unrelated controls matched on age, gender, BMI and SEI (B).

DISCUSSION

Co-heritability between BMI, IL-6 and CRP

By fitting bivariate ACE models, we calculated variance and covariance components, parsing out genetic and environmental effects specific to IL-6 and CRP from those linked to the heritability of BMI. The approach more clearly revealed the degree of covariance in the additive genetic effects shared by IL-6 and BMI. Optimized path coefficients did not expose unique effects specific to the genetic control of IL-6, but rather an overlap of the heritable processes influencing both IL-6 and BMI in adults. These analyses also extend our understanding of the differential influence exerted by BMI on CRP. While the regression analyses did show that BMI affected serum CRP levels to a greater extent than IL-6, the heritability models indicated that the effects of BMI on IL-6 were largely due to shared genetics. In contrast, the effect of BMI on CRP was split between shared genetics and environmental influences. The genetic effects acting on CRP were evenly distributed between those shared with BMI and those unique to CRP genetics, whereas the additive genetic effects influencing IL-6 phenotype appeared to be more exclusively tied to BMI. In spite of the close relationship between CRP and IL-6, our heritability estimates point to environmental factors as the main source of covariance. Hence, in the unstimulated state without infection or chronic disease, the genetics underlying body adiposity appears to influence IL-6 and CRP levels in the blood through independent pathways.

Matched Controls

In order to further validate our conclusions on the differential constraints regulating IL-6, we conducted a case/control analysis, with each twin matched to an unrelated individual on the basis of gender, age, BMI, and education. Given the strong influence these 4 variables have on IL-6, the ICCs attained for the matched controls were of the same magnitude as for the actual co-twin siblings.

Genetics of IL-6

Our heritability estimates for IL-6 in blood do contrast with some of the commonly held assumptions derived from other approaches. For example, it has been reported previously that both CRP and IL-6 levels are similar in twins, a finding that will emerge when the influence of adiposity is not taken into account, nor statistically considered as a contributing factor (Rooks, Veledar, Goldberg, Bremner, & Vaccarino, 2012; Wörns et al., 2006). In addition, there is a substantial literature reporting that allele polymorphisms affect IL-6 release, but that effect is most apparent in the context of inflammatory disorders, or when cells are activated *in vitro* by a proinflammatory stimulant (Bennermo et al., 2004; Brull et al., 2001; Burzotta et al., 2001; T. Shah et al., 2013). IL-6 gene-related polymorphisms include the SNPs rs1800795 and rs1800796 (Chatzikyriakidou, Voulgari, Lambropoulos, & Drosos, 2013; S. Chen, Chen, Lai, & Chen, 2012; Vaughn, Ochs-Balcom, & Nie, 2013). Although these polymorphisms do affect inflammatory responses in patients (Bruunsgaard et al., 2004; Sen et al., 2011; Walston et al., 2007), they do not appear to have a strong influence on basal IL-6 in the blood of a healthy individual. In addition, these SNPs do not have a strong effect on IL-6 transcription at baseline or even a large influence when the cells are activated by proinflammatory

stimulants (A. Smith, Zheng, Palmen, & Pang, 2012). That may help to explain why studies of the association between the -176C/G SNP and cardiovascular disease have been inconsistent (Brull et al., 2001; Burzotta et al., 2001; Lieb et al., 2004; Markus Nauck et al., 2002). Similar concerns have also been raised about the predictive power of IL-6 related SNPs in meta-analyses of the literature on inflammatory disease (Dai, Liu, Guo, Wang, & Bai, 2012; Di Bona et al., 2009; Y. H. Lee et al., 2012; Nikolopoulos, Dimou, Hamodrakas, & Bagos, 2008; Y. Yang et al., 2012).

IL-6 and adiposity

IL-6 is secreted by many cells, including fibroblasts, hepatocytes, endothelial cells, leukocytes, and in large systemic quantities by adipocytes (Hamzic et al., 2013; Lepiller, Abbas, Kumar, Tripathy, & Herbein, 2013; Saiki et al., 2013; Salman, Vahabi, Movaghar, & Mahjour, 2013). The diversity of these tissue sources is key to understanding the heritability of IL-6, especially given the strong genetic and familial influences on adiposity (Schousboe et al., 2003; Segal et al., 2008). Although some previous twin studies considered the influence of adiposity on IL-6 heritability by adjusting IL-6 values by BMI or waist-to-hip ratios, they did not account for the shared genetic covariance (de Maat et al., 2004; Sas et al., 2012; Su, Snieder, Miller, & Ritchie, 2008). Therefore, those genetic estimates were likely inflated by the shared influence with BMI.

Limitations

Twin analyses have a number of assumptions, including that there is a sharing of 100% of the genetic load by MZ twins versus 50% for DZ twins. It is also assumed that

MZ and DZ siblings both share a similar influence of the common environments, and gene-environment interactions are not modeled in the standard twin model (e.g., ACE estimates are estimated as constant for the sample because potential moderator variables are not modeled). Some investigations have questioned the latter assumption based on the view that epigenetic modifications and individual perceptions may lead to a differential experience of even minor environmental events (Stenberg, 2012). Assuming a perfectly homogeneous and common environmental influence is probably not reasonable, so it will be important to verify our conclusions with a larger-scale gene-association study (Purcell, 2002; Tan, Ohm Kyvik, Kruse, & Christensen, 2010). In addition, a substantial portion of the variance in IL-6 and CRP still remains to be accounted. Some variance may be attributed to reliability issues when relying on a single blood sample (Navarro et al., 2012). Determining the stability of IL-6 levels over time through multiple samples, would be of value for replicating and extending our heritability estimates. It should also be acknowledged that our participants were entirely American adults of European descent. IL-6 tends to be higher in certain races, including in those of African backgrounds, whereas it tends to be lower in some Asian populations (e.g., Japanese) (Coe et al., 2011). That was one reason why we opted not to include the small number of African-American twins in the current analyses. Similarly, body adiposity, IL-6 and CRP, as well as the relationships between adiposity and these inflammatory markers, vary not only by race, but also by gender (Carroll et al., 2009; Clifton, 2003; Coe et al., 2011). Hence, it is likely that estimates of shared genetic effects will vary by race and gender. Further validation for the driving effects of obesity on IL-6 and CRP would be

achieved by controlled interventional studies targeting improvements in diet, exercise and weight reduction.

Significance

Variation in IL-6 and CRP levels has been associated with metabolic dysfunction, obesity and various inflammatory processes relevant to cardiovascular disease and cross-systems interactions. However, the extent to which baseline levels of IL-6 and CRP are determined by body adiposity remains unknown. Previous twin studies have shown that systemic levels of IL-6 and CRP are to some extent heritable. However, that data contradicts genetic association studies which have shown only a minor influence by genetic polymorphisms. In the context of the Western civilization, the body mass has been consistently shown to exhibit high heritability. Hence, it is likely that the heritability of BMI inflates the estimated heritability for both IL-6 and CRP. In this study, we sought to determine the extent to which the additive genetic effect on IL-6 and CRP were influenced by BMI. We found high shared genetic covariance between BMI and both IL-6 and CRP. In support of these finding, matched control analyses showed that unrelated individuals show similar baseline levels of IL-6 when simple matched for age, sex, race and BMI. While the heritable influence of BMI on IL-6 was greater than for CRP, the heritability of CRP was also influenced by IL-6. This is in keeping with molecular biology studies, which have shown that IL-6 is a potent stimulation of acute phase reactants. Our results were in keeping with assessments of adipose tissue which have shown that adiposity is a major determinant factor of unstimulated IL-6 and CRP, but contradicts studies gene association studies that clam independent genetic effect on systemic levels

of these proinflammatory markers. While genetic polymorphisms are likely important in defining proinflammatory response to stimuli, baseline levels are likely under the influence of metabolic factors, especially body adiposity in an overweight population. While other studies have shown high heritability for BMI, IL-6 and CRP, our study was the first to show the extent to which the additive genetic effect of BMI affects the heritability of both IL-6 and CRP.

Tables

Table 1. Sample descriptives for primary variables used in the twin analyses

	MZ		DZ		Control		Larger Sample	
Age	52.8	(11.2)	50.2	(9.1)	52.0	(8.5)	56.0	(11.9)
BMI (Kg/m²)	27.6	(5.4)	29.6	(5.7)	25.6	(3.4)	29.4	(6.1)
IL-6 (pg/mL)	2.1	(1.7)	2.7	(2.1)	2.5	(1.7)	2.9	(3.0)
sIL-6r (pg/mL)	37401	(10582)	33611	(7826)	34600	(9407)	36412	(10351)
CRP	2.10	(2.22)	2.54	(2.26)	2.12	(1.87)	3.02	(4.78)
SEI	44.6	(13.9)	44.8	(15.8)	44.2	(10.7)	43.0	(14.1)
Ever Smoked	36.0%		39.0%		43.3%		46.5%	
Males	62		16		20		394	
Females	84		48		17		436	
Total Participants	146		64		37		830	

Mean values and standard deviations, or counts for numbers of participants.

MZ, Monozygotic Twins; DZ, Dizygotic twins; the Larger Sample was comprised of all other subjects in Biomarker project, after excluding the twins and African-American participants.

Table 2

Intra-class correlations and statistical difference between coefficients demonstrating MZ and DZ twins had similar values for IL-6, but differed for sIL-6r

	Intra-Class Correlation Coefficients and 95% C.I.						Fisher's r-to-z, z-values	
	MZ		DZ		MC		MZ vs. DZ	MZ vs. MC
IL-6	0.35 (.17 - .50)	P<0.001	0.32 (-.02 - .60)	P=0.03	0.36 (.02 - .63)	P=0.02	0.15 n.s.	0.05 n.s.

MZ, Monozygotic Twins; DZ, Dizygotic Twins; MC, Matched Controls

Table 3
Comparison of nested model statistics

Covariance Models		-2LL	D.F.	AIC	BIC	Difference χ^2	p-value
BMI X IL-6	ACE	39.22	405	-770.77	-922.81		
	CE	41.52	406	-770.48	-924.00	2.29	0.13
	AE	**40.69**	**406**	**-771.31**	**-924.41**	**1.46**	**0.23**
BMI X CRP	ACE	1396.80	401	594.80	-232.80		
	CE	1398.10	402	594.10	-234.47	1.31	0.25
	AE	**1396.81**	**402**	**592.81**	**-235.12**	**0.01**	**0.91**
CRP X IL-6	ACE	1415.15	401	613.15	-223.63		
	CE	**1415.46**	**402**	**611.46**	**-225.79**	**0.31**	**0.57**
	AE	1415.48	402	611.47	-225.78	0.33	0.57

-2LL (-2 * Log-Likelihood), maximum likelihood estimate; AIC, Akaike's Information Criterion; BIC, Bayesian Information Criterion; Lower -2LL, AIC and BIC indicate models that more parsimoniously fit the data; Non-significance in chi-square (χ^2) values between saturated and nested models indicate equivalent fit of data; Bold highlighted font indicates the model that most parsimoniously fit the data, based on AIC and BIC.

Table 4
Proportion of genetic and environmental effects for best-fit models

Variance / Covariance Components	Estimated Effects (95% C.I.)		
	Additive genetic (A)	Common Environment (C)	Unshared Environment (E)
BMI	68.8% (41.4-78.5)	0.0%	31.2% (21.5-44.8)
IL-6	26.1% (12.5-51.8)	7.6% (0-7.6)	66.3% (48.2-84.3)
Covariance	83.0%		17.0%
BMI	70.6% (27.2-79.8)	0.0%	29.4% (20.2-42.7)
CRP	18.2% (1.2-50.0)	13.7% (0.0-36.6)	68.1% (50.0-88.2)
Covariance	54.5%		45.5%
CRP	15.2% (0.0-31.7)	14.7% (2.8-44.2)	70.1% (52.8-90.0)
IL-6	0.0% (0.0-35.0)	37.8% (6.4-53.0)	62.2% (45.4-79.9)
Covariance		51.3%	48.7%

Best-fit models were chosen on the basis of the AIC and BIC.

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Part 2.

The mediatory role of body adiposity on population markers of chronic inflammation with a focus on lipid and carbohydrate metabolism

Section 2.1.

The Effect of Dietary Carbohydrates and Lipids on Adiposity and Inflammation in the Context of the American diet

Abstract

Results from epidemiological nutrition studies vary greatly. Further, clinical and dietary guidelines have been recently criticized for the lack of methodological vigor, misinterpretation of data, or exclusion of important evidence that would challenge decades of dietary dogma. These dogmas include: the “Diet-Heart Hypothesis”, which holds that foods high cholesterol and saturated fats cause obesity and heart disease; the “Calories-in, Calories-out Hypothesis”, which holds that obesity and heart disease are caused by excess caloric consumption and/or low caloric expenditure; and the “Lipid Hypothesis”, which holds that dietary lipid-induced high blood cholesterol clogs arteries thereby causing cardiovascular disease. Studies supporting these hypotheses often are poorly controlled and often do not account for simultaneous confounding effects and interactions, often utilize antiquated statistical methods aimed at simplifying calculations and almost always test the effect of dietary lipids in the context of high carbohydrate diets. These studies often do not match the effects that are expected from the biochemistry, molecular biology and system biology approaches. Consequently, few dietary factors have produced consistent effects on weight gain, inflammation and heart disease (e.g., dietary fiber). Hence, we sought to apply modern statistical methods (relying on computationally-intensive estimations, such as generalized linear regressions and bootstrapping mediation analyses) to analyze the effect of dietary intake on Body adiposity (measured as Body Mass Index, BMI) and on a proinflammatory biomarker closely associated with metabolic syndrome and cardiovascular disease, C-Reactive Protein (CRP). We analyzed data from the National Health and Nutrition Examination

Survey (NHANES 2003-2004, 2005-2006, 2007-2008 and 2009-2010) by utilizing modern statistical methods that account the full variance of the data and for simultaneous and interactive effects. Specifically, we examined the extent to which dietary carbohydrates and lipids influenced BMI. We compared the effect of BMI on CRP to the effect of other stable markers of metabolic status (HA1c, total cholesterol, HDL and blood pressure), and assessed the proportion of the effect of dietary carbohydrates and lipids that are mediated through BMI. We also assessed the direct and indirect effect of dietary carbohydrates and lipids on CRP. Our analyses indicated that macronutrients accounted for almost 4% more variance in BMI than caloric intake. This effect was dominated by carbohydrates, especially sugar and fiber. Monounsaturated fat was a significant predictor but multicollinearity indicated that its estimated was inaccurate. On the other hand, other saturated and polyunsaturated fats were not predictive of BMI. Macronutrients accounted for 1.17% more variance in CRP than caloric intake. The effect on CRP was also dominated by carbohydrates. While starch was a significant predictor, its effect could not be accurately estimated. Further, dietary cholesterol did not predict CRP after accounting for metabolic status; saturated fat was not a significant predictor, before or after. Mediation analyses also showed that BMI mediated the effects of dietary carbohydrates but not lipids on CRP. In addition, BMI exerted greater effect on CRP than all other markers of metabolic status. Our results challenge the notion that dietary lipids, especially cholesterol and saturated fat, drive obesity and inflammation, and show that body adiposity is the main driver of inflammation, not blood cholesterol. Our results support that dietary carbohydrates, not lipids, drive adipose accumulation and systemic

inflammation, both directly and indirectly. Our results also closely conform to those obtained by biochemistry and molecular biology approaches.

Highlights:

- Macronutrients accounted for more variance in BMI and CRP than caloric intake.
- The effects of diet on BMI and CRP were dominated by carbohydrates.
- Saturated and polyunsaturated fats were not predictive of BMI.
- Dietary cholesterol did not predict CRP after accounting for metabolic status.
- Saturated fat was not a significant predictor, before or after accounting for metabolic status.
- BMI mediated the effects of dietary carbohydrates but not lipids on CRP.
- BMI exerted greater effect on CRP than all other markers of metabolic status.

INTRODUCTION

Metabolic and inflammatory comorbidities

Comorbidity is often observed between diseases that are traditionally categorized as metabolic or inflammatory (Chen, Chen, Wang, & Liang, 2015; Giugliano, Ceriello, & Esposito, 2006; Monteiro & Azevedo, 2010; Straub, 2014; Yu & Bennett, 2014). Unsurprisingly, most chronic diseases exhibit characteristics of both metabolic and inflammatory dysfunctions with ample evidence for bidirectional influence (Carrera-Bastos et al., 2011; Franceschi & Campisi, 2014; Pawelec, Goldeck, & Derhovanessian, 2014). Although diet has been implicated in the both, there is debate over which specific dietary factors affect the progression of metabolic syndrome and the inflammatory disorders that drive many chronic diseases including cardiovascular, neurological and oncological ailments (Carrera-Bastos et al., 2011; Hou, Abraham, & El-Serag, 2011; La Vecchia et al., 1990; Myles, 2014; Yerushalmy & Hilleboe, 1957). C-reactive protein (CRP) is an acute phase reactant and proinflammatory agent that has been consistently associated with metabolic syndrome and inflammatory conditions, and is an independent predictor of cardiovascular disease (Carroll et al., 2009; Gupta et al., 2012; Saijo et al., 2004). It is also known that obesity can increase the production of CRP by the liver as well as in adipose tissue (Anty et al., 2006; Calabro, Chang, Willerson, & Yeh, 2005). Hence, CRP has become a common marker of inflammation and an independent risk factor for casrdiovascular disease in population studies and in the clinical setting.

The American diet

The American diet is often described in scientific outlets as a “high fat diet” (Buettner, Schölmerich, & Bollheimer, 2007), which is misleading and may drive inaccurate conclusions and inferences about the development and management of metabolic diseases. The American diet is more appropriately defined as a high-fat/high-carbohydrate diet, in distinction to high-fat/low-carbohydrate diets, typically consisting of 5-10% of calories by glycemic carbohydrates and near 70% of calories by fats (Westman et al., 2007). The former is implicated with the initiation and progression of diabetes, obesity and other chronic diseases, whereas the latter refers to carbohydrate-restricted dietary practices associated with strong anti-obesogenic and antiinflammatory effects as well as the amelioration of metabolic markers of disease (Dupuis, Curatolo, Benoist, & Auvin, 2015; Gasior, Rogawski, & Hartman, 2006; Ruskin, Kawamura, & Masino, 2009; Yang & Cheng, 2010). This distinction is necessary for the interpretation of dietary data that is characterized by high carbohydrate consumption. Controlled studies, in both animals and humans, have characterized mechanisms by which high blood glucose affects metabolism, inflammation and the storage or oxidation of lipids (Altannavch, Roubalová, Kučera, & Anděl, 2004; Ceriello et al., 2008; Fu et al., 1994; Greene, Stevens, Obrosova, & Feldman, 1999; Russell et al., 2002; Schaefer, Gleason, & Dansinger, 2009; Sidossis, Stuart, Shulman, Lopaschuk, & Wolfe, 1996). Therefore, our findings should be read in the specific context of a High-Fat High-Carbohydrate diet, as well as in the context of specific foods that make up this diet (highly processed). While nutritional guidelines have recently improved by de-emphasizing the constraints on dietary fats and

cholesterol in favor greater restriction of sugars and alcohol, such warnings persist and continue to advise towards carbohydrate-based diets (U.S. Department of Health and Human Services and U.S. Department of Agriculture & Agriculture, 2015). Accordingly, many researchers criticize the current guidelines for deviating from the scientific evidence by omitting important studies that contradict decades of dietary advice and by making recommendations based on inaccurate or incomplete interpretation of the literature (Aracenta and Pérez-Rodrigo, 2012; Marshall, 2011; Teicholz, 2015). As such, many questions and controversies remain about the health impact of carbohydrates and lipids on adipose accumulation, inflammation and disease in the context of the current American diet. Therefore, we assessed the direct and indirect impact of dietary carbohydrates and lipids on C-reactive Protein (CRP), controlling for simultaneous effects and interactions. CRP is a potent proinflammatory mediator, which can be produced in large quantities by hepatic, endothelial and adipose tissues (Anty et al., 2006; Calabro et al., 2005; Castell et al., 1989; Devaraj, Torok, Dasu, Samols, & Jialal, 2008; Forouhi et al., 2001). Hence, we also assessed the extent to which body mass mediated the effects of dietary carbohydrates and lipids on CRP.

The effect of dietary carbohydrates and lipids on obesity

Experimental comparisons in humans have shown that high fat diets, in the context of low-carbohydrates produce greater weight loss than isocaloric low-fat high-carbohydrate diets, both short- and long-term. (Gardner et al., 2007; Volek et al., 2004; Volek, Quann, & Forsythe, 2010). In addition, high-fat low-carbohydrate diets also produce favorable markers of metabolic health than low-fat high carbohydrate diets,

including lower blood levels of triglycerides, saturated fat, glycated hemoglobin, fasting glucose and insulin, as well as improved LDL particle size and greater HDL cholesterol (Buyken et al., 2010; Forsythe et al., 2010; Gardner et al., 2007; Khatana, Taveira, Dooley, & Wu, 2010; Phinney, Bistrian, Wolfe, & Blackburn, 1983; Pradhan, 2001; Reaven, 1997; Volek et al., 2009; Volek, Sharman, & Forsythe, 2005). On the other hand, obesogenic and metabolically detrimental effects of dietary fats are observed in the context of high carbohydrate diets (Hopper et al., 2012). Similarly, animal models have shown that higher proportions of dietary fat cause weight gain and metabolic dysfunction in the presence of high carbohydrates (where the caloric intake from fat usually ranges from only 10 to 35% of the caloric intake), but where weight gain and effects on metabolic function vary greatly across species when fat constituted most of the caloric content of the diets (65-75% of the calories) (West and York, 1998). Because the American diet is typically a high-carbohydrate high-fat diet, epidemiological studies are likely to find pro-obesogenic effects of fats, especially when not accounting for the simultaneous effect of carbohydrates. In our study, we assessed the effect of dietary carbohydrates and lipids while accounting for simultaneous effect in order to assess the extent to which each uniquely explained body mass in the population.

Common issues with studies on diet and nutrition

Due to the high costs associated with controlled human diet studies as well as the difficulties associated with dietary compliance, much of the diet research has relied on small controlled experimental designs in humans or nonhuman primates, rodent models, or large epidemiological studies. While it is essential to address specific hypotheses,

experimental human diet studies are typically small, exhibiting low statistical power, and often limited to short-term effects. Although rodent models are essential for assessing biological mechanisms, translation to human health must be carefully interpreted given the adaptive differences in digestive and metabolic systems, reflecting varying thresholds of carbohydrate and lipid tolerance (Buettner et al., 2007; Dietschy, Turley, & Spady, 1993). Nonhuman primate models are closet to the human condition, but studies are scarce. While most have focused largely on caloric intake and diabetes, some have demonstrated the impact of fructose intake on metabolic and inflammatory markers of disease (Bremer et al., 2011; Mattison et al., 2014). One has examined the effect of low-carbohydrate high fat-diet on glucoregulation (20% carbohydrate, 65% fat), showing no disturbance of glucose metabolism (Fabbrini et al., 2013). In turn, epidemiological diet and nutrition studies often present major methodological issues. In probing the effects of specific macronutrients, many population studies have omitted important confounding macronutrients and interactions as model covariates. Variable selection has also been a common issue that may take various forms. For instance, LDL cholesterol is often used as a measure of cardiovascular disease risk when many studies have shown that high levels of circulating LDL cholesterol is not necessarily atherosclerogenic (Fernandez, 2012; Hite et al., 2011). In addition, many population analyses have resorted to the simplification of the data by dichotomization of continuous variables when the categorization is theoretically unwarranted (Dawson & Weiss, 2012; Naggara et al., 2011; Preacher, Rucker, MacCallum, & Nicewander, 2005). Statisticians have often criticized dichotomization practices for the amplification of biases, often yielding in deceptive results (Altman & Royston, 2006; MacCallum, Zhang, Preacher, & Rucker, 2002; Shentu & Xie,

2010). An associated conundrum is imposed when studies unnecessarily utilize odd-ratios of dichotomized outcome variables to emphasize relative probability while downplaying or omitting absolute effect sizes (Ravnskov et al., 2015). A recent examination of the relationship between diet, CRP and lipid responses presented different findings depending on whether CRP was treated as a continuous variable or dichotomized (St-Onge, Zhang, Darnell, & Allison, 2009). Similar effects have also been observed in the dichotomization of BMI in the NHANES (Janssen, Katzmarzyk, & Ross, 2004). Another possible source of bias in nutrition research is the source of funding and conflicts of interest (Chen, Gluud, & Kjaergard, 2017; Moher, Tetzlaff, Tricco, Sampson, & Altman, 2007; Shah et al., 2005). Many nutrition studies are funded by private industries or by institutions organized to defend and advance their interests, including some that used the NHANES data (O'Neil et al., 2011; Sun, Anderson, Flickinger, Williamson-Hughes, & Empie, 2011). Further, many nutrition epidemiology studies may contain confirmatory biases driven by perpetuated dogmas on what has historically been considered a healthy or unhealthy diet, often ignoring the underlying biochemistry or molecular biology of nutrient metabolism, adipose accumulation and inflammatory diseases. Thus, epidemiological diet and nutrition studies have produced overwhelmingly inconsistent results in predicting weight loss, biomarkers of health and disease outcomes. Our hypotheses were based on findings from controlled studies carried out in either humans or animal models, emphasized the biological mechanisms linking diet, nutrient metabolism, adiposity and inflammation, and our analyzes were designed to minimize known sources of methodological bias. While a few studies have already examined the relationship between dietary nutrients and CRP in various NHANES cohorts, all of which

dichotomized key variables (Ford, Mokdad, & Liu, 2005; King, Egan, & Geesey, 2003; Mazidi, Gao, Vatanparast, & Kengne, 2017; Mazidi, Kengne, P. Mikhailidis, F. Cicero, & Banach, 2017; Qureshi, Singer, & Moore, 2009; Stewart, Mainous III, & Gilbert, 2002). In our study, we treated all variables in their original continuous form and applied simultaneous regressions and mediation models in order to parse out independent effects and the extent to which body mass mediates them, thereby minimizing statistical sources of bias.

Goals and Hypotheses

The primary goals of our study were to assess the effects of dietary carbohydrates and lipids on body adiposity and CRP in the context of the American diet, and to assess the extent to which those effects are mediated by body adiposity. Our study also allowed us to compare the effect of body adiposity to that of other markers of metabolic.

Based on controlled experimental studies, especially those that assessed biochemical and biomolecular pathways, we expected to confirm the more established relationship between dietary fiber and inflammation, especially given that some research has brought to question whether the benefits of fiber are due to the fiber itself or to the absence of high-glycemic carbohydrates (Alessa et al., 2016; Jenkins et al., 2000). We also sought to examine the effect of carbohydrate and lipid macronutrients for which recent guideline recommendations are shifting (fats, cholesterol, sugars and alcohol) (Grundy et al., 2016), and to assess the effect of starch, a broad carbohydrate category for which mixed results exist due to their heterogeneous composition or food source (Akande, Doma, Agu & Adamu, 2010; Pasiakos, Agarwal, Lieberman & Fulgoni, 2015;

Rehman & Shah, 2005; Pereira, Buzati & Leonel, 2014; Tovar & Melito, 1996). We expected to find an antiinflammatory effect of fiber and polyunsaturated fats, a neutral effect of saturated and monounsaturated fats, and a proinflammatory effect of sugar, alcohol and starch, all of which may be partly mediated by body adiposity. Further, we tested for non-linear relationships and specific macronutrient interactions that may affect adipose accumulation and inflammatory physiology.

Current experimental research suggests that higher dietary fiber may attenuate the glycemic impact of sugars (Alessa et al., 2016; Bednar et al., 2001; Jenkins et al., 1987; Neuhouser et al., 2012; Slavin, 2013). Studies on the gut microbiome further suggest that the balance of digestible and non-digestible carbohydrates may alter the energetic harvesting potential of the gut microflora influencing metabolic health (Bednar et al., 2001; Payne, Chassard, & Lacroix, 2012). Similarly, dietary carbohydrate composition may alter the absorption or production of meat-derived metabolites that influence inflammation either directly by dilution in fiber or by alterations in gut microbiota composition (Humphreys et al., 2014; Le Leu et al., 2015; Winter et al., 2011). Various studies have suggested interactions between fats and carbohydrates (Gulliford, Bicknell, & Scarpello, 1989; Owen & Wolever, 2003). While dietary fats appear to slow the absorption of glycemic carbohydrates, these carbohydrates affect fat oxidation and deposition (Coyle, Jeukendrup, Wagenmakers, & Saris, 1997; Gulliford et al., 1989; Sidossis et al., 1996). Hence, we tested the interactive effects between fiber and sugar, starch and fiber, starch and protein, starch and fats, and sugar and fats. We also explored bivariate correlations and confounding multicollinearity issues that may explain some of the discordant results in the nutrition epidemiology literature.

METHODS

Participants

Our study made used of publicly data available on 14,997 adult participants with ages 20 to 85 years old from multiple cohorts of the National Health and Nutrition Examination Survey for which all variables of interest were available, 2003-2010 period ("Centers for Disease Control and Prevention (CDC). National Center for Health Statistics (NCHS). National Health and Nutrition Examination Survey Laboratory Protocol," 2003-2004, 2005-2006, 2007-2008 and 2009-2010, "Centers for Disease Control and Prevention (CDC). National Center for Health Statistics (NCHS). National Health and Nutrition Examination Survey Questionnaire," 2003-2004, 2005-2006, 2007-2008 and 2009-2010).

Demographics

Demographic information was collected at home prior to in-person examinations by computer assisted personal interviewing methodology, and answered directly or aided by a proxy if the participant was unable to answer questions themselves. After data collection, interview records were reviewed by staff for accuracy and completeness. Gender was coded -0.5 for males and +0.5 for females, so that results can be interpreted as the difference in each measure when moving from males to females. Race/ethnicity was derived from race and Hispanic origin questions and categorized as Mexican American, Other Hispanic, Non-Hispanic White, Non-Hispanic Black and Other races (including multi-racial). The variable was dummy coded to reflect variable differences in

reference to Non-Hispanic White which comprised the numeric majority of this study population. Income and Education information was collected as stratified categories and used as such by dummy coding in reference to the lowest level available. Education and Income were only included in the regression models as control covariates, since socioeconomic status reportedly affects measures of systemic inflammation (Koster et al., 2006; Steptoe, Owen, Kunz-Ebrecht, & Mohamed-Ali, 2002).

Diet

Detailed dietary intake information were collected from NHANES participants by the What We Eat in America (WWEIA) dietary interview, conducted in partnership with the U.S. Department of Agriculture (USDA) and the U.S. Department of Health and Human Services (DHHS). The National Center for Health Statistics of the DHHS was responsible for the design and data collection methods of the WWEIA interview, as well as the databases used to code, process, generate nutrient data and quality-review the dietary data utilized in our study. The interview was conducted in-person in the NHANES Mobile Examination Center (MEC), consisting of private rooms, and by phone interview based on 24-hour recalls. The phone interview was conducted 3 to 10 days after the in-person interview. The nutrient data utilized in our analyses were calculated by the average of the first day and second day from the total nutrients data file where both days were available. Otherwise, the measures for the one day were available and used (1502 cases). Macronutrient and micronutrient components used in our study were available in NHANES databases, except for calculated starch. NHANES databases parsed carbohydrate intake into total carbohydrates, total sugars (which included both naturally

occurring and added sugars) and dietary fiber (including soluble and insoluble). Total carbohydrates were comprised of sugars and complex carbohydrates. This was calculated by the difference between the total weight of the food and the weights of the protein, fat, ash, and water. We calculated starch values for the diet by subtracting total sugars and dietary fiber from total carbohydrates. In the current data, starch was comprised complex carbohydrates, which may include resistant and non-resistant starches as well as processed and unprocessed starches. These vary greatly in absorption rate and glycemic index. Hence, results from this carbohydrate category must be interpreted cautiously.

Alcohol content of the diet was also available for the two-day interview. However, because reporting of licit or illicit drugs are likely to be under-consumed, underestimated or omitted during a health diary survey, we utilized NHANES interview variables that were specific to alcohol use. We calculated the average daily alcohol consumption, measured as drinks per day (based on frequency of drinking and number of drinks per day when drank), reported for the preceding month. Although the number of drinks consumed reflect not only alcohol but also sugar composition, the regression estimates reflect the effects of drinking while adjusting for the effect of total sugar consumption.

Variables for dietary fat content were available at two classification levels, as specific fatty acids (categorized by carbon chain length and saturation), and by the 3 saturation-based categories: Saturated Fatty Acid (SFA), Monounsaturated Fatty Acid (MUFA) and Polyunsaturated Fatty Acid (PUFA). The latter was utilized in the main analyses. Analyses of the formaer were included in the Appendix to this study. The dietary trans-

fats composition was not available for the selected cohorts, therefore not included in this study.

Metabolic Status

Metabolic syndrome refers to a set of risk factors including excess over-weight, high blood triglycerides, low levels of high-density lipoproteins (HDL), high blood pressure and high fasting blood sugars. These risk factors are related to most chronic diseases associated with post-agricultural civilizations. In the current analyses, we accounted for the meditative and moderate effects of metabolic status by including stable physiological measures available in the NHANES that best reflected metabolic syndrome: Body Mass Index (BMI), total cholesterol, HDL, systolic and diastolic blood pressures (SBP and DBP), and glycated hemoglobin (HA1c). BMI was used as a measure of body adiposity, calculated from weight and height and reviewed by NHANES staff for accuracy and plausibility according to other anthropometric measures. Lipid panels provided total cholesterol (TC), triglycerides, high-density Lipoprotein cholesterol (HDL) and estimated-low-density lipoprotein cholesterol (LDL) measures. We only considered stable metabolic markers, TC and HDL, as metabolic status covariates in our analysis.

While NHANES blood collections were expected in the morning and in the fasting state, the fasting length varied greatly or were not assessed in many individuals. Previous studies have shown evidence of instability for glucose, triglycerides and LDL measures as a function of feeding and short fasting times, whereas TC and HDL have been shown to provide more stable measures of systemic lipid metabolism (Davis, 2013). TC and HDL were quantified in serum by standard enzymatic and absorptiometry methods. Due

to changes in equipment and observed measurement biases between major cohorts, HDL values were corrected for compatibility by NHANES staff according to control samples.

Further details about the specific procedures and adjustments are available on the NHANES web site (Centers for Disease Control and Prevention (CDC). National Center for Health Statistics (NCHS). National Health and Nutrition Examination Survey Laboratory Protocol, 2003-2004, 2005-2006, 2007-2008 and 2009-2010). Systolic and diastolic blood pressure measures were averaged from 3 to 4 determinations by certified examiners after a 5-minute rest in the seated position. HA1c was measured by standard boronate affinity high performance liquid chromatography, and the data was presented as percent glycohemoglobin.

CRP

Blood was obtained by standardized methods and serum specimens were frozen to -20°C until thawed for analyses, and high sensitivity C-reactive protein was measured by latex-enhanced nephelometry as part of the NHANES laboratory examinations. Further details about the specific method used in collection and laboratory procedures are available on the NHANES web site (Centers for Disease Control and Prevention (CDC). National Center for Health Statistics (NCHS). National Health and Nutrition Examination Survey Laboratory Protocol, 2003-2004, 2005-2006, 2007-2008 and 2009-2010). There were no changes in equipment, lab method or analysis site for all cohorts included in our analyses.

Statistics

Base regression models (which included all variables, but no interaction or nonlinear effects) were built in order to test the assumptions that model parameters were linear, residuals are normally distributed and have constant variance. Case analysis tests were conducted to identify and remove regression outliers of high influence; that is, cases that exhibit large residuals and high leverage. Dietary variables were fit to regression models predicting BMI, and in models with and without variables of metabolic status in order to access the direct and indirect effects of diet on CRP. All model models adjusted for common covariates associated with metabolic and inflammatory health including total caloric intake, salts (sodium and magnesium), demographics (age, gender and race) and socioeconomic status (income and education, categorically stratified as available in NHANES database), nicotine use status (current smoker versus non-smoker), as well as logistic variables of possible influence (time session of the blood collection and the year of cohort sampling). All regression models were analyzed in the lmSupport package in R.

Further, the Variable Inflation Factor (VIF) was computed using the “car” package in R and presented for each regression factor in order to determine the extent to which the variance associated with each model prediction was overestimated by multicollinearity effects. Variables with high inflation indices (VIF>5) exhibit high multicollinearity and variance, and therefore, may render unreliable effect estimates. VIF values between 2.5 and 5 are acceptable, while values below 2.5 may be considered reliable, with 1 being perfectly reliable (no multicollinearity).

Hypothetical interactions were tested and retained in the final models if statistically significant in either model, including or excluding metabolic factors. The final models also

included explorative quadratic relationships and interactions if statistically significant. Exploratory interactions included those among nutrients, demographic factors, as well as cohort year, in order to access changes in macronutrient effect across years which may suggest changes in nutrient quality over time. All model interactions were interpreted according to the directionality of effects that was expected from the physiological literature.

Three conditions must be met in order to conclude mediation of effects: (1) a predictor or set of predictors must explain variation in the mediating factor, (2) the mediating factor must explain variation in the outcome variable, and (3) the effect of a set of predictors is reduced when accounting for the mediating factors. If diet explains significant variance in BMI and other metabolic factors, if those explain significant variance in CRP, and if the dietary factors exhibit at least partial reduction in variance and non-variance based effect sizes when metabolic factors are included in the model, partial mediation of the effects of diet on CRP by those factors may be concluded. When these conditions were met, a non-parametric bootstrapping method was applied to calculate the proportion of the effect of the macronutrient on CRP that was mediated by BMI. Mediation models were carried out by the Yamamoto method (Tingley *et al*., 2014) in the “mediate” package in R in order to calculate the proportion of the effect variance mediated by BMI.

Models also tested total caloric intake as the only dietary predictor of BMI and CRP in order to compute the amount of variance explained by nutrient intake over and above total caloric intake. Analysis of difference in model delta R-squared was computed by the lmSupport package in R.

Because the consumption of dietary nutrients varies greatly by order of magnitude, standardized regression coefficients were produced in order to compare across the estimated effects.

RESULTS

Descriptives

On average, NHANES participants consumed approximately 2110 kcal a day, which was comprised of 82 grams of protein, 257 grams of carbohydrates and 79 grams of fat (Table 1). Table 2 and Figure 1 describe the caloric intake parsed by the macronutrient source. We found that carbohydrates comprised about half of the caloric content of the diet in these population samples, whereas sugars comprised almost half of the carbohydrate calories. Fats made up little over 30% of the caloric intake. Hence, it must be emphasized that the results that follow must be interpreted in the context of a high-carbohydrate/high-fat diet, and marked by high sugar intake (Figure 1). The two-day dietary diary also indicated a daily intake of 10 grams of alcohol, or per interview data, an average of a half of an “alcoholic drink a day” (Table 1). According to the National Institute on Alcohol Abuse and Alcoholism, one standard drink consists of 12 fl oz of beer, 5 fl oz of wine or 1.5 fl oz of distilled spirit. Alcohol intake also made significant contribution to the caloric make-up of this diet, averaging 71 kcal/day, or 3% of the caloric intake. In addition, participants were, on average, both overweight and pre-diabetic (Table 1).

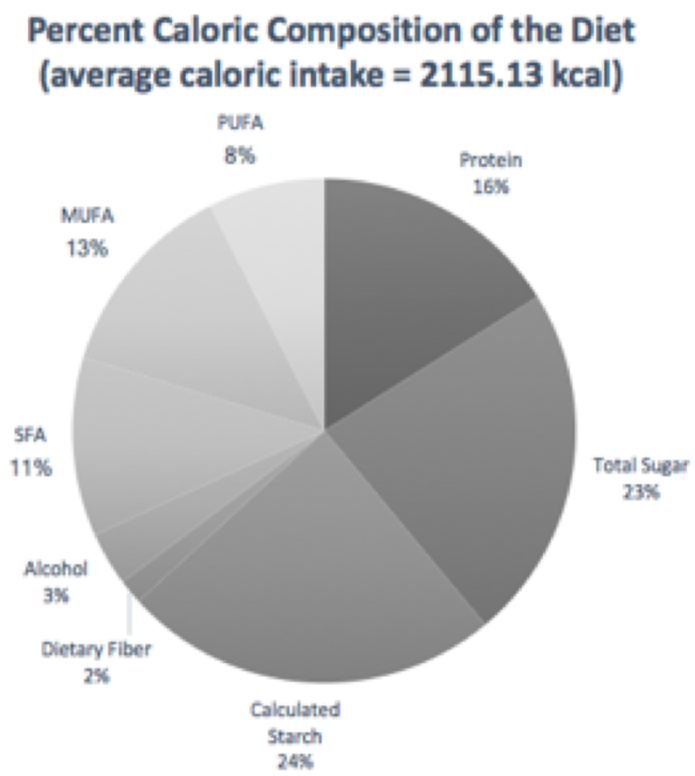

Figure 1. Average dietary intake by NHANES participants across all cohorts.

Bivariate Correlations

Bivariate correlations showed that all macronutrients, as well as total calories, were highly and positively correlated with one another (Table 3) suggesting that the average diet contains high content of all macronutrients, rather than being comprised of one macronutrient group over another; excluding alcohol. As the intake of one macronutrient group increased, the intake of other macronutrients also increased. Interestingly, CRP and almost all metabolic factors are negatively correlated to all dietary variables when not adjusted by simultaneous regressions. The number of drinks per day and the estimated alcohol content of the diet exhibited the same correlation pattern across other variables (Table 3) and were moderately correlated with each other ($r=0.56$, $p<0.001$).

Analytical model, assumptions, case analyses and coding

BMI and CRP (mg/dL) were transformed to meet the General Linear Model assumptions when used as outcome variables. Specifically, they were natural log transformed in order to also facilitate the conversion of the regression estimates to percent change from the sample mean (geometric mean estimates). Next, case analyses were conducted and two high leverage outliers were removed from the data. Leaving 14,995 total participants who remained for all analyses. Categorical variables were centered when made binary and dummy coded, thus, comprising multiple categories. Gender was coded to reflect changes from male to female, race coded for White Caucasian as reference, and other categorical variables were coded for the lowest level as a reference. The predicted effect of macronutrients described below were derived from two regression models: one model that did not include metabolic factors in order to access the full effect of nutrients on CRP (including the portion of the effect that may have been mediated by metabolic factors); and a second model that included BMI and the metabolic factors to access the direct effects of macronutrients on CRP above and beyond those mediated by BMI and other markers of metabolic status. All model estimates described account for the simultaneous effects of all other model variables. Covariate and interaction effects that were included in the models for the purpose of control were not reported unless they were relevant to the conclusions of this manuscript.

The overall effect of diet on BMI

10.04% of the variance in BMI (model adjusted-R^2) was accounted by diet, demographics, smoking status and interactions. Model comparison analyses showed that diet alone explained 2.64% of the variance in BMI $F(28,14923)=21.931$, $p<0.001$, $\Delta R^2=2.64\%$). Further, nutrient intake explained 3.69% more variance in BMI than a similar model where caloric intake was the only dietary predictor ($F(25,14923)=17.606$, $p<0.001$, $\Delta R^2=3.69\%$), showing that the influence of diet on adipose accumulation is not a simple function of excessive caloric intake.

The overall effect of diet and metabolic status on CRP

Our full model explained 27.62% of the variance in CRP, accounting for all dietary, metabolic, demographic and logistic factors, including non-linear relationships and interactions (model adjusted-R^2). Model comparison analyses showed that nutrient intake explained 1.20% of the CRP variance above and beyond metabolic status and other covariates ($F(30,14892)=8.234$, $p<0.001$, $\Delta R^2=1.20\%$), while metabolic status accounted for 18.72% of the variance above all other factors ($F(24,14892)=161.23$, $p<0.001$, $\Delta R^2=18.72\%$). Further, nutrient intake explained 1.17% more variance in CRP than a similar model where caloric intake was the only dietary predictor ($F(29,14892)=8.26$, $p<0.001$, $\Delta R^2=1.17\%$), showing that the influence of diet on inflammatory biology is also not a simple function of excessive caloric intake.

The effect of BMI and other metabolic markers on CRP

Adjusted for all covariates, including dietary factors, increases in all markers of metabolic status predicted greater CRP. BMI explained for the most variance in CRP,

accounting for 5.05% of the variance. HDL, total cholesterol, HA1c and systolic blood pressure explained 0.83%, 0.69%, 0.16% and 0.06%, respectively. As expected, CRP was largely predicted by BMI. One standard deviation increase in BMI (SD=6.39 Kg/m²) predicted a 47.57% increase in CRP levels (β=47.57, $F(1,14888)$=1044.53, $p<0.001$, ΔR^2=5.05%, VIF=2.78). However, the effect of BMI became weaker as BMI increased (β=-4.01, $F(1,14888)$=61.28, $p<0.001$, ΔR^2=0.30%). Accordingly, greater HDL levels predicted lower CRP, such that one standard deviation increase in HDL (SD=16.51 mg/dL) estimated a 16.45% decrease in CRP (β=-16.45, $F(1,14888)$=171.79, $p<0.001$, ΔR^2=0.83%, VIF=2.02). On the other hand, one standard deviation increase in total cholesterol in blood (SD=42.58 mg/dL) predicted a 12.33% increase in CRP (β=12.33, $F(1,14888)$=143.18, $p<0.001$, ΔR^2=0.69%, VIF=1.36), with a weakening of effect as total blood cholesterol increased (β=-1.28, $F(1,14888)$=8.47, $p=0.004$, ΔR^2=0.04%). One standard deviation increase in HA1c (SD=0.99%) was predictive of 9.88% higher CRP (β=9.88, $F(1,14888)$=32.23, $p<0.001$, ΔR^2=0.16%, VIF=3.886), also with a small decrease in effect as HA1c levels increased (β=-0.84, $F(1,14888)$=6.642, $p=0.010$, ΔR^2=0.03%). Finally, higher systolic, but not diastolic, blood pressure predicted 5.40% higher CRP (β=5.40, $F(1,14888)$=13.17, $p<0.001$, ΔR^2=0.06%, VIF=2.72 and β=-2.51, $F(1,14888)$=3.19, $p=0.074$, ΔR^2=0.02%, VIF=1.42 respectively) per standard deviation increase (SD=18.75 mmHg).

The effects of carbohydrates on BMI

At average sugar intake, sugar did not predict greater BMI (β=-0.42, $F(1,14923)$=1.099, $p=0.295$), but with every standard deviation increase in sugar

(SD=69.7 gram), its effect on BMI increased by 0.08% (β=0.08, F(1,14923)=26.008, p<0.001, ΔR^2=0.16%, VIF=2.485). Increased dietary fiber predicted lower BMI. For every standard deviation increase in dietary fiber consumption (SD=8.67 grams), BMI decreased by 1.28% (β=1.28, F(1,14923)=22.832, p<0.001, ΔR^2=0.14%, VIF=2.714). Starch did not predict differences in BMI (β=0.18, F(1,14923)=0.156, p=0.692). Alcohol intake, measured as average drinks per day, predicted lower BMI (β=2.26, F(1,14923)=56.381, p<0.001, ΔR^2=0.34%, VIF=3.402). At average intake, one drink per day predicted 2.26% lower BMI. However, this negative relationship between BMI and alcohol was predicted to decreased by 0.17% with every additional drink per day (β=0.17, F(1,14923)=30.500, p<0.001, ΔR^2=0.18%, VIF=2.430).

The effects of lipids on BMI

At average intake, saturated fat did not predict differences in BMI (β=0.48, F(1,14923)=1.095, p=0.295). However, with every standard deviation increase in saturated fat intake, its effect on BMI was estimated to become more negative by 0.19% (β=0.19, F(1,14923)=6.697, p=0.010, ΔR^2=0.04%, VIF=2.789). Monounsaturated fat predicted greater BMI, however multicollinearity analyses indicated that the regression coefficient could not be reliably estimated (β=1.69, F(1,14923)=10.694, p=0.001, ΔR^2=0.06%, VIF=10.099). Polyunsaturated fat did not predict differences in BMI (β=-0.09, F(1,14923)=0.089, p=0.765). Dietary cholesterol also did not predict differences in BMI (β=0.20, F(1,14923)=0.667, p=0.414). Compared to carbohydrates, dietary lipids were very poor predictors of BMI (ΔR^2=0.04% and 0.06%, compared to 0.01%, 0.16%, 0.14%, 0.34% and 0.18%).

The effect of macronutrient interactions on BMI

We detected significant macronutrient interactions among dietary carbohydrates but not lipids, accounting for another 0.15% of the variance in CRP over lipids. We found an interaction between protein and starch (β=0.68, $F(1,14923)$=16.251, $p<0.001$, ΔR^2=0.10%) and between sugar and magnesium (β=-0.51, $F(1,14923)$=7.842, $p=0.005$, ΔR^2=0.05%). For every standard deviation increase in starch intake, the effect of protein on BMI increases by 0.68%, and for every standard deviation increase in magnesium intake, the effect of sugar on BMI decreases by 0.51%.

The effects of diet on each marker of metabolic status

The dietary factors and interactions that significantly predicted CRP in the main full CRP model were used to estimate the total variance explained by diet for the other markers of metabolic status. Diet, as a whole, explained 5.30% of the variance in HDL, 3.26% of the variance in HA1c, 2.64% of the variance in BMI, 1.66% of the variance in systolic blood pressure, 0.71% of the variance in total blood cholesterol and 0.62% of the variance in diastolic pressure (adjusted model R^2).

The effects of carbohydrates on CRP and mediation by BMI

According to out regression model, for every standard deviation increase in sugar intake (SD=69.7 grams), CRP was expected to increase by 8.35% (β=8.35, $F(1,14918)$=19.18, $p<0.001$, ΔR^2=0.12%). After accounting for metabolic status, the effect of sugar remained significant but was reduced (β=4.75, $F(1,14888)$=7.65, $p=0.006$,

ΔR^2=0.04%), suggestive that its effects on CRP were partially mediated by metabolic factors. Sugar predicted greater BMI and CRP, hence we bootstrapped the proportion of the effect of sugar mediated by BMI. Our mediation analyses estimated that 42.39% (95% C.I.=27.93-61.03, p<0.01) of the effect of sugar was mediated by BMI. Conversely, dietary fiber intake predicted significantly lower CRP. For every standard deviation increase in fiber intake (SD=8.67 grams), CRP was predicted to decrease by 11.93% (β=-11.93, F(1,14918)=53.56, p<0.001, ΔR^2=0.32%). After accounting for metabolic factors, fiber intake was still predictive of an 8.71% decrease in CRP levels (β=-8.71, F(1,14888)=35.05, p<0.001, ΔR^2=0.17%). Dietary fiber predicted lower BMI and CRP, which is also indicative of a partial mediation by metabolic factors. Specifically, our mediation analyses showed that 17.84% (95% C.I.=13.58-22.22, p<0.01) of the effect of fiber is mediated through its effect on BMI. Calculated starch intake also predicted lower CRP before and after accounting for the metabolic factors (β=-2575.00, F(1,14918)=8.13, p=0.004, ΔR^2=0.05% and β=-2220.00, F(1,14888)=7.56, p0.006, ΔR^2=0.04%, respectively). Notably, our models calculated discrepantly high coefficient for the effect of starch. These results were corroborated by multicollinearity analyses: while the effect estimates for both sugar and fiber intake were statistically reliable (VIP=3.79 and 2.78, respectively), the effect of starch on CRP exhibited unacceptable reliability (VIP=8.37x10^5), and therefore should not be interpreted. Because starch did not predict BMI, we also cannot conclude mediation of its effects by BMI. Average alcohol intake (measured in drinks per day) predicted higher CRP after accounting for metabolic factors with very high reliability; For every one daily drink of alcoholic beverage ingested, CRP is estimated to increase by 3.68% (β=3.68, F(1,14888)=12.34, p<0.001, ΔR^2=0.06%,

VIF=1.41). While the number of drinks per day predicted greater CRP, it non-linearly predicted lower BMI. Our mediation analyses indicated that 42.68% (95% C.I.=34.07-54.08, $p<0.01$) of the effect of alcohol was mediated through BMI.

The effects of lipids on CRP and mediation by BMI

Saturated fat did not significantly predict CRP before or after accounting for metabolic status ($\beta=2.17$, $F(1,14924)=0.71$, $p=0.401$, and $\beta=1.40$, $F(1,14894)=0.368$, $p=0.544$, respectively). Monounsaturated fat predicted higher CRP before accounting for metabolic status but not after ($\beta=9.08$, $F(1,14924)=8.38$, $p=0.004$, $\Delta R^2=0.05\%$, VIF=10.10 and $\beta=2.20$, $F(1,14894)=0.62$, $p=0.432$, respectively), indicative of mediation. However, monounsaturated fat exhibited high multicollinearity, indicating that its coefficient could not be reliably estimated. On the other hand, higher intake of polyunsaturated fat significantly and reliably estimated lower CRP with little difference in effect before and after accounting for metabolic status ($\beta=-4.29$, $F(1,14924)=5.08$, $p=0.024$, $\Delta R^2=0.03\%$ and $\beta=-3.83$, $F(1,14894)=5.075$, $p=0.024$, $\Delta R^2=0.02\%$, VIF=3.71, respectively), such that for every standard deviation increase in polyunsaturated fat intake (SD=17.0 grams), CRP is predicted to decrease by 4.29% before and 3.83% after accounting for metabolic status.

Dietary cholesterol predicted greater CRP. For every one standard deviation increase in cholesterol intake (SD=199.43 milligrams), CRP is predicted to increase by 3.25% before accounting for metabolic status ($\beta=3.25$, $F(1,14918)=4.91$, $p=0.027$, $\Delta R^2=0.03\%$). Dietary cholesterol did not predict CRP levels after accounting for metabolic status, suggesting that metabolic factors may have mediated its effects on CRP ($\beta=1.83$,

F(1,14918)=1.95, p=0.162). VIF for dietary cholesterol indicates that it is within acceptable reliability (VIF=2.20).

BMI did not mediate the effects of any dietary lipids. Polyunsaturated fat and dietary cholesterol did not predict BMI; therefore, their effects could not be mediated by BMI. Mediation analyses indicated that BMI did not significantly mediate the effect of saturated or monounsaturated fat (p=0.91 and p=0.26, respectively).

Carbohydrate and lipid interactions

We tested the following hypothetical interactions between nutrients in the BMI and both CRP regression models: fiber and sugar, starch and fiber, and starch and protein, starch and fats, and sugar and fats. The interaction of protein and starch only was predictive of BMI. For every standard deviation increase in starch intake, the effect of protein on BMI increased by 0.68% (β=0.68, F(1,14918)=16.251, p<0.001, ΔR^2=0.10%). All other macronutrient interactions were non-significant (statistics not shown). Before accounting for metabolic status, we found significant interactions between fiber and sugar, starch and fiber, and starch and protein. An increase in dietary fiber intake was associated with an attenuated effect of sugar on CRP (β=-2.50, F(1,14918)=7.13, p=0.008, ΔR^2=0.04%). On the other hand, increasing starch consumption predicted a decrease in the beneficial effect of dietary fiber on CRP (β=3.77, F(1,14918)=16.55, p<0.001, ΔR^2=0.10%). Greater starch consumption was also suggestive of an increased proinflammatory effect of protein (β=2.79, F(1,14918)=6.16, p=0.013, ΔR^2=0.04%). After accounting for metabolic status, only the interaction between fiber and starch remained significant (β=3.19, F(1,14888)=14.82, p<0.001, ΔR^2=0.07%), suggesting that the

interactive effects of fiber and sugar and starch and protein on CRP may have been mediated by metabolic status (β=-1.09, $F(1,14888)=1.71$, $p=0.192$, $\Delta R^2=0.01\%$ and $\beta=1.40$, $F(1,14888)=1.95$, $p=0.163$, $\Delta R^2=0.01\%$, respectively). SFA, MUFA and PUFA did not exhibit interaction with fiber, starch or sugar (statistics not shown).

DISCUSSION

NHANES has collected data on the U.S. Population for decades, making nationally representative data available on diet, eating behaviors, demographics and health. By utilizing available data on nearly fifteen thousand adults, we examined the macronutrient composition of the American diet, analyzed the effects of dietary carbohydrates and lipids on BMI and CRP, tested the mediation by BMI as well as specific carbohydrate and lipid interactions. Because of the uniformity in macronutrient intake ratios and high multicolinearity we were not able to reliably assess the effect of few dietary variables. By inspecting simple bivariate correlations, it was evident that their simple associations were spurious and illogical – all macronutrients were negatively correlated with CRP. An overall positive association amongst almost all macronutrients was also a strong indication that Americans eat more or less of every macronutrient without significant differences in macronutrient ratios, which may explain inconsistent health predictions that have been found by many epidemiological studies on diet that have simplified the data and failed to account for simultaneous effects.

The effect of dietary carbohydrates and lipids on BMI

Our analyses showed a nonlinear relationship between sugar and BMI where the higher sugar consumption the greater the effect of sugar on BMI such that sugar intake increases BMI for individuals who eat above average sugar. While sugars may include many subtypes, most of which have been not well characterized, the majority of sugars consumed in the Western Diet is comprised of fructose and sucrose. Fructose and sucrose exert important effect on energy metabolism and have been shown to differentially affect metabolic processes (Jameel et al., 2014; Schaefer et al., 2009). Fructose has been shown to drive triglyceride production in the liver (Kazumi et al., 1986), cause mitochondria dysfunction and cell damage in the liver and the brain systems that regulate peripheral energy metabolism, glucoregulation, and eating behaviors (Cigliano et al., 2017; Lopes et al., 2014; Lustig, 2013; Rayssiguier, Gueux, Nowacki, Rock, & Mazur, 2006), all of which may contribute to adipose accumulation. On the other hand, dietary fiber predicted lower BMI. Various types of dietary fiber have been shown to exert beneficial effects on gut microbiome and metabolite generation (Bednar et al., 2001; Lattimer & Haub, 2010). Fiber may exert anti-obesogenic effects through short-chain fatty acid generation (Chakraborti, 2015; Lu et al., 2016) or by the modulation of the gut microbiome: A number of studies have shown that microbiota composition affects metabolism and adipose accumulation independent of macronutrient composition of the diet (Rosenbaum, Knight, & Leibel, 2016; Tilg & Kaser, 2011). Additionally, starch did not predict changes in BMI, which may be due to the heterogeneity of the starch macronutrient group, which includes resistant and non-resistant starches. Resistant and non-resistant starch may play distinctive roles and metabolic effects (Lockyer & Nugent, 2017; Lyte et al., 2016). These are perhaps the most difficult to measure, since food

preparation has significant effect on starch structure and absorption. Resistant starches may exert effects similar to that of dietary fiber, however, cooking complex carbohydrates increase their digestibility and absorption, hence, greater glycemic load more closely resembling simple carbohydrates (Atkinson, Foster-Powell, & Brand-Miller, 2008; El-hady & Habiba, 2003; Rehman & Shah, 2005). The specific composition of starches (amylose content), cooking methods and cooling (retrogradation), may also modify the digestibility properties of resistant starches (El-hady & Habiba, 2003; Tovar & Melito, 1996). These modifiable properties are difficult to account for in a dietary survey and may partially explain the mixed claims regarding this macronutrient. Ultimately, the balance between digestible and non-digestible carbohydrates may influence an individual's metabolism directly and indirectly. For example, by providing specific metabolism modulating phytonutrients, by providing raw materials for carbohydrate-derived short chain fatty acid (butyric, propionic and acetic acids) production by the gut flora, by influencing bacteria-derived metabolites that affect hormones, or by dilution effect on other dietary factors. Conversely, sugars, non-resistant starches, cooked and processed starches, may inflict high glycemic impact and lead to dysfunctional metabolism. Most of the carbohydrates consumed in the American diet are highly glycemic carbohydrates, as evident by the top dietary sources of non-fiber carbohydrates (sugars and starches) and the top sources of dietary fiber, according from a 2003-2006 NHANES report (O'Neil, Keast, Fulgoni, & Nicklas, 2012; O'Neil et al., 2012). These reports also showed that the top-ranking sources of sugar in the American diet were soft drinks and soda, while the top-ranking source of fiber was yeast bread and rolls. While the effect of ethanol on hepatic adipogenesis is well documented (Nagy, Ding, Cresci, Saikia, & Shah, 2016), lesser is

known about whole body adipose accumulation as a function of alcohol consumption. Our analyses showed that, at average consumption, alcoholic drinks were associated with lower BMI, but also that this effect decreased with increased intake. Alcohol may induce lipolysis and adipose tissue shrinkage, which results in fatty acid mobilization and liver fat accumulation, thereby aggravating fatty liver disease (Zhao *et al.*, 2015). Our analyses also showed that, at average intake, saturated fats did not affect BMI. However, a nonlinear relationship estimated a decrease in BMI with greater than average intake of saturated fat. Polyunsaturated fat and dietary alcohol also did not predict BMI. While Monounsaturated fat seemed to predict higher BMI, analyses of multicollinearity indicated that the effect its effect could not be accurately estimated because of high overlap with other variables. Because our models adjusted for the simultaneous effects of carbohydrates and other nutrients, including their interaction, it is possible that we were able to isolate the effect of dietary lipids above and beyond the effects of carbohydrates. As such, these results were in agreement with human studies showing that dietary lipids do not themselves drive adipose accumulation (Volek et al., 2004; Volek, Quann, & Forsythe, 2010; West & York, 1998). As a whole, the dietary effects on BMI were governed by carbohydrates, not lipids, where dietary carbohydrates accounted for more cumulative variance in BMI than lipids, including interactions (0.97% versus 0.10%).

In predicting BMI, we found interactions between magnesium and sugar and between starch and protein. We found that magnesium intake attenuates the effect of sugar on adipose accumulation. Magnesium is essential for many physiological processes and enzymatic reactions, many of which are essential to glucoregulation and immune function (Nielsen, 2010; Weglicki & Phillips, 1992; Weglicki, Phillips, Freedman,

Cassidy, & Dickens, 1992), and may thereby influence adipose accumulation. We also found an interaction between protein and starch. While we do not understand how such interaction may directly affect adiposity, it is known that starches alter the processing of animal derived proteins and may affect microbiome-gut interaction and metabolite generation (Bingham, 1997; Le Leu et al., 2015; Paturi et al., 2012; Toden, Bird, Topping, & Conlon, 2006).

Effects and Mediation by BMI and other markers of metabolic status

Our analyses showed that diet affected all markers of metabolic status to some extent. BMI, HDL and HA1c were most affected. As a whole, diet predicted 2.64%, 5.30% and 3.26% of the variance in BMI, HDL and HA1c, respectively, but less than one percent of the variance in total cholesterol and blood pressure. In turn, BMI explained 5.35% of the variance in CRP, while HDL, HA1c and total cholesterol explained less than one percent each, and blood pressure explained a negligible amount. While we may also conclude partial mediation by the other markers of metabolic status, their effects were very small compared to that BMI. Adipose tissue may exhibit local CRP production and may further stimulate the hepatic production of CRP through adipokines, and hence is a major driver of proinflammatory processes (Anty et al., 2006; Calabro et al., 2005; Goode & Watson, 2012; Lau et al., 2005; Peyrin-Biroulet et al., 2012). In our analyses, carbohydrates explained most of the dietmetabolic factors. Our mediation analyses also showed that large proportions of the effect of the carbohydrates, sugar, fiber and alcohol, were mediated by BMI. At the same time, the effect of lipids on BMI was dismal. Accordingly, BMI did not mediate the effect of any dietary lipids on CRP.

The effect of caloric Intake on CRP

As a whole, diet explained a unique 1.20% of the variance in CRP above and beyond the effects of metabolic status, while metabolic status explained 18.72%. The difference in variance based effect sizes makes sense given that metabolic status is a more proximal effector on inflammatory physiology while diet likely represents a mode distal influence. The nutrient intake model explained significantly more variance in CRP than a calories-only model, which is in keeping with the up-to-date notion that the effect of diet on health is more complex than expected from hypotheses that obesity and disease are a function of excess calories (Hite et al., 2010). Further, caloric intake was not a reliable predictor of CRP due to evident multicollinearity, and also not a significant predictor after adjusting for metabolic status. These results were expected since caloric intake is a non-physiological measure of macronutrient consumption and does not distinguish among the various pathways of nutrient metabolism. According to our analyses, the population consumes more or less of everything, maintaining similar a High-Fat High-Carbohydrate macronutrient dietary profile, which may be the reason studies often detect high caloric intake as a key factor in health and disease.

The effect of dietary carbohydrates on CRP

The dietary fiber content of foods affects their glycemic impact but also exerts direct antiinflammatory effects through gut bacteria products (Andoh, Bamba, & Sasaki, 1999). The short chain fatty acid, butyrate, is a major fiber-derived metabolite that has been shown to exert potent antiinflammatory effects (Andoh et al., 1999). In our model, dietary

fiber intake was not only predictive of lower CRP but it also exhibited the largest variance based effect size among the dietary factors, before or after adjusting for the metabolic factors.

On the other hand, excessive sugars intake may exert proinflammatory effects (Jameel, Phang, Wood, & Garg, 2014; Jena et al., 2014). High sugar consumption is also associated with glucose dysregulation, dyslipidemia and diabetes (Giuglino et al., 2006; Hofmann & Tschöp, 2009; Yan, Ramasamy, Naka, & Schmidt, 2003). At a cellular level, it also exerts its effects leading to dysfunctional mitochondria and oxidative stress, which are major drivers of inflammation (Fu et al., 1994; Jameel et al., 2014; Robertson, 2004). The Western Diet is also typically high in fructose. Fructose has been shown to have worse effects than sucrose alone, since it is not easily utilized in beta-oxidative processes and therefore directly converted to triglycerides for storage (Jameel et al., 2014; Kazumi, Vranic, & Steiner, 1986; Schaefer et al., 2009; Sun et al., 2011). In our models, sugar predicted higher CRP and exhibited the second largest effect, and especially before adjusting for metabolic factors.

Alcohol consumption has also been shown to cause liver and brain tissue damage, affecting both metabolic and inflammatory systems by processes similar to that of fructose (Lieber, 2004; Mandrekar & Szabo, 2009; Topiwala et al., 2017; Wang, Zakhari, & Jung, 2010). In the past, many epidemiological studies have suggested non-linear effects for alcohol on cardiovascular health, albeit with speculations but not proof of mechanisms by which small amounts of alcohol intake could exert health benefits (Averina, Nilssen, Arkhipovsky, Kalinin, & Brox, 2006; Fekjær, 2013; Stockwell et al., 2016). Contrary to popular belief, there is little consensus among governmental agencies on how much

alcohol is considered to be “healthy drinking.” Governmental guidelines on alcohol consumption vary greatly across different countries (Dufour, 1999; Furtwængler & De Visser, 2013). It is also noteworthy that most studies have dichotomized alcoholic drinks intake by “non-drinkers,” “moderate” and “high” consumers, rather than conserving the variable in its original continuous format. The use of dichotomous variables has allowed many studies on alcohol to avoid reporting absolute risk estimates in favor of relative risk (Jackson & Beaglehole, 1995). Many studies also did not include the obvious confounding variable, socioeconomic status, as a covariate, which has been shown to alter conclusions regarding moderate alcohol consumption (Averina et al., 2006; Towers, Philipp, Dulin, & Allen, 2016). Further, as it has been more recently disclosed that studies that claim health benefits of moderate alcohol consumption have been systematically confounded by the inclusion of non-drinkers who do not consume alcohol because of sickness or poor health conditions, thereby shifting the effect on most measures of metabolic, inflammatory and cardiovascular function between non-drinker and moderate drinker categories (Holahan et al., 2010). Such errors of great social impact could have been avoided by not-dichotomizing and by accounting for all the relevant covariates. The selection of alcohol consumption variables and their ability to estimate alcohol intake may also affect results – estimating the number of alcoholic drinks consumed may be more accurate in a survey study than the estimation of alcohol content (Chick & Kemppainen, 2007; Rehm et al., 2003). Hence, we opted for using the number of alcoholic drinks per day over the estimated volume of alcohol in the diet. In these analyses, the average number of alcoholic drink consumed per day predicted higher CRP, above and beyond the effects of sugar, other macronutrients, metabolic status and demographics, including

socioeconomic status. Our analyses detected few non-linear regression effects, however the effect of alcohol on CRP was linear, as expected from previous studies that carefully addressed the physiological effects of alcohol.

The effect of dietary lipids on CRP

While many studies have shown direct potent antiinflammatory effects of polyunsaturated fats, especially of DHA and EPA (Oh et al., 2010; Serhan et al., 2000; Singer et al., 2008) and consistently harmful effects of trans-fatty acids (Kris-Etherton, 2010; Mozaffarian, Aro & Willett, 2009), the effects of other fatty acids in the context of high carbohydrate diets are still under examination and heavy debate (Nettleton, Koletzko, & Hornstra, 2011). Polyunsaturated fats exhibit their effects though antioxidant and antiinflammatory actions, by contributing to cell membrane fluidity and architecture (Abbott et al, 2012; Richard et al., 2008; Shaikh and Edidin, 2006; Shaikh and Edidin, 2008). In our data, the polyunsaturated fat predicted lower CRP before and after accounting for metabolic status, though it only accounted for a small fraction of the variance in CRP, compared to other macronutrients.

Controlling for all other dietary effects, we did not find an effect of saturated fats on CRP before or after accounting for metabolic status. Controlling for all dietary effects, we only found an effect of monounsaturated fats before accounting for metabolic status. While it may have been mediated by metabolic status, our mediation analyses showed that its effects were not mediated by BMI. These results are in agreement with recent changes in dietary guidelines that de-emphasize harmful effects by dietary fats. It is likely that the detrimental effects of fats on inflammation and cardiovascular health may have

been previously overstated in the literature. Many clinical and population studies measure the impact of dietary lipids on “cardiovascular disease risk” (e.g., elevation in LDL) rather than on actual disease physiology. Studies in molecular biology have shown that LDL is only atherosclerogenic in dysfunctional conditions, for example: in the form of remnant very-low-density lipoproteins (vLDL), which is associated with hypertriglycemia (Lewis & Steiner, 1996; Lewis, Uffelman, Szeto, & Steiner, 1993; Parks, Krauss, Christiansen, Neese, & Hellerstein, 1999); when carrying oxidized lipids or exposed reactive oxygen species (Chen, Azhar, Abbasi, Carantoni, & Reaven, 2000; Itabe, Obama, & Kato, 2011; Lercker & Rodriguez-Estrada, 2000; Perrin-Cocon et al., 2001; Sobal, Menzel, & Sinzinger, 2000); and when glycated by high glucose exposure (Angelica & Fong, 2008; Lyons, 1992; Sobal et al., 2000; Tero & Errera, 2002). Fats have also been shown to exert potent antiinflammatory effects, but usually in the context of carbohydrate-restricted diets (Dupuis et al., 2015; Gasior et al., 2006; Ruskin et al., 2009; Yang & Cheng, 2010). Many of the antiinflammatory effect of fats appear to be mediated by a fat-derived liver metabolite, beta-hydroxybutyrate, which is structurally similar to the fiber-derived bacterial metabolite butyrate. The liver produces beta-hydroxybutyrate from fats after the exhaustion of circulating glucose and liver glycogen during periods of carbohydrate restriction, as imposed by ketogenic diets, intermittent fasting protocols and very low caloric diets (Ballard et al., 2013; Hussain et al., 2012; Pan, Rothman, Behar, Stein, & Hetherington, 2000; Westman et al., 2007).

Likewise, dietary cholesterol exhibited only a very weak proinflammatory effect and only before accounting for metabolic status, accounting for a relatively negligible proportion of the variance in CRP, compared to other dietary factors. This finding was

also in accord with recent de-emphasis of dietary cholesterol restriction in the most recent dietary guidelines. The experimental evidence that dietary cholesterol induces atherosclerosis was modeled in rabbits, which are obligate herbivores, and therefore do not naturally consume animal-derived sterols and exhibit distinct cholesterol metabolism from humans. Mammals vary in their need for dietary cholesterol. Accordingly, liver cholesterol synthesis is regulated and differ greatly by species (Dietschy et al., 1993). Rabbits, rodents and humans do not share similar diets and hence, do not present the same mechanical, digestive or metabolic adaptations to diets. Rabbits are often used to model atherosclerosis because it can be easily and quickly induced, however it is not an acceptable model for the etiology of atherosclerosis and inflammation, the early mechanisms of atherosclerogenic development, or even for interventions that depend on metabolic mechanisms that differ across species. Likewise, rodent models of atherosclerosis have important limitations. Transgenic mice deficient for the LDL receptor have elevated LDL-cholesterol, but do not form lesions, and in models where lesions form, these present characteristics that differ from the human pathology (Buettner et al., 2007; Jawień, Nastałek, & Korbut, 2004; Joven et al., 2007). Hence, differences in metabolism need to be considered very carefully while translating animal model findings in nutrition to human health. The effect of dietary cholesterol in nonhuman primate studies have indicated accelerated atherosclerosis as well as hypercholesteremia, but all in the context of high carbohydrate or high sugar diets (Cox, et al., 1958; Srinivasan, et al., 1979; Vesselinovitch, et al., 1974). Further, controlled studies in humans show that dietary cholesterol intake itself does not raise blood cholesterol levels in most individual, but rather in a subgroup of the population only (Fernandez, 2012; Ginsberg et al., 1995;

Griffin & Lichtenstein, 2013). Conversely, hepatic cholesterol synthesis has been shown to be dependent on the intake of fructose and other sugars (Jameel et al., 2014; Moser, 1985; Schaefer et al., 2009; Silbernagel et al., 2012). Cholesterol is essential in the maintenance of cellular structure as well as in the biosynthesis of steroid hormones, vitamin D and bile acids, and may also serve as an anti-oxidant (Lecerf & de Lorgeril, 2011; Smith, 1991). In addition, inflammation and phase reactants and alter cholesterol metabolism (Gierens et al., 2000; Heldenberg et al., 1980; Lindhorst, Young, Bagshaw, Hyland, & Kisilevsky, 1997; Pfohl, Schreiber, Liebich, Häring, & Hoffmeister, 1999). Studies have shown the crystallized cholesterol can be formed by the inflammatory process in atherosclerosis, requiring macrophage recruitment to remove the cholesterol crystals from underneath the vascular endothelium (Duewell et al., 2010; Rajaṁaki et al., 2010). While there are pathways by which cholesterol may elicit proinflammatory responses, we do not yet know how, or if, those processes contribute to chronic inflammation. Dietary carbohydrates also accounted for more cumulative variance in CRP than lipids before accounting for metabolic status (0.75% versus 0.11%). After accounting for metabolic status, carbohydrates still accounted for greater cumulative variance in CRP than did lipids (0.32% versus 0.02%). These results support recent experimental evidence in humans that dietary carbohydrates exhibit greater effect on proinflammatory physiology than do fats and cholesterol, both through and independently of body adiposity (Aljada et al., 2006; Buyken et al., 2010; Dhindsa et al., 2004; Forsythe et al., 2008, 2010; Khatana, Taveira, Dooley, & Wu, 2010; Pradhan, 2001; Seshadri et al., 2004).

Carbohydrate and lipid interactions predicting CRP

We detected significant interactions only among dietary carbohydrates, and none among lipids. These interactions accounted for another cumulative 0.18% of the variance explained in CRP over lipids. We detected a significant interaction between protein and starch, such that the higher the starch content o the diet, the higher the inflammatory potential of protein. This effect may reflect varying effects for meat in the context of different carbohydrates or a variation in types of starchy vegetables and their protein content/composition. In terms of foods, these possibilities may reflect meat-starch combinations or the presence of inflammatory proteins in legumes (e.g. wheat and similar proteins) (Brouns, Van Buul, Shewry, Buul, & Shewry, 2013; Le Leu et al., 2015; Winter et al., 2011). Further, interaction between meats and resistant or non-resistant starches may affect gut bacteria composition affecting metabolic and inflammatory processes (Bingham, 1997; Le Leu et al., 2015; Paturi et al., 2012; Toden, Bird, Topping, & Conlon, 2006). In addition, we also found interactions between starch and fiber, as well as between fiber and sugar. The fiber-starch interaction remained significantly predictive of CRP after adjusting for metabolic status. Given the direction of the effects, we might interpret that higher starch content undermines the antiinflammatory effect of fiber, and that fiber attenuates the effect of sugar. These effects may be a reflection of the effect of resistant starches and fibers verus non-resistant starches and sugars as they affect gut bacteria products, glycemic and insulinemic loads (Bednar et al., 2001; Behall & Howe, 1995; Jenkins et al., 2000; Lattimer & Haub, 2010; Raben et al., 1994; Topping & Clifton, 2001). On the other hand, we did not detect interactions between fats and carbohydrates in predicting CRP.

Multicollinearity issues

Analyses of multicollinearity indicated that, albeit significant, the effect of starch, protein and monounsaturated fat overlapped with other variables to the extent that their unique effects could not be calculated. Their regression estimates likely depict simultaneous effects of various nutrients. These results were not surprising given that starch (legumes and grains), protein (meats, legumes and grains) and monounsaturated fats (processed vegetable oils) are staples of the American diet. And unlike sugar or fiber, which showed large distinct effects on CRP, starches are also a heterogeneous macronutrient group and likely exerts mixed effects depending on their solubility and digestibility. Our models indicated that the only dietary factors that were reliable and significantly predictive of CRP were sugar, fiber, consumption of alcoholic drinks, polyunsaturated fat and dietary cholesterol.

Similar studies of the NHANES data

Similar studies that examined the effect of the American diet on CRP in the NHANES data either using the same dataset, a subgroup of the same dataset, or the data from a younger cohort, arrived at some similar results and conclusions but also at important differences. All these studies dichotomized the outcome variable (CRP) and/or the independent variables and used ANOVA, ANCOVA and Chi-Square tests, logistic regressions, or other methods that were referenced to outside sources but not explained in the methods. None of them presented the rationale for their method choices. In the

studies, the authors dichotomized CRP, its predictors, or both. Their cutoffs for CRP stratification also varied greatly where authors categorized it by quarters, two cutoffs at 1.0 and 3.0 mg/L, one cutoff at 3.0mg/L or a percentile cutoff at 85% of sex-specific distribution (4.4mg/L for males and 7.0mg/L for females). The dietary data was also organized by various dissimilar methods: (1) summarizing the dietary data into a discretionary "Healthy Eating Index" that was computed from scoring the dietary composition based on the number of servings of specific foods in each food group (Ford et al., 2005); (2) combining dietary data into five "food group" categories (dairy, grains, fruits, vegetables and meat/other proteins) in servings per day, isolating specific foods (Qureshi et al., 2009); (3) and by utilizing micro- and macronutrient nutrient composition, but varying by which nutrients were included or excluded in the analyses and as covariates (Mazidi, Gao, et al., 2017; Mazidi, Kengne, et al., 2017).

One recent examination of this NHANES data was published in two manuscripts (Mazidi, Gao, et al., 2017; Mazidi, Kengne, et al., 2017). This study closely resembled ours, except for various stratifications of the data, statistical approach and the exclusion various important covariates. Mazidi and co-authors focused the first manuscript on all micro- and macronutrients and a second manuscript on the effects of specific dietary fatty acids. In the first article, while adjusting by age, sex, race, BMI and total caloric intake, the authors found that sugar increased while dietary fiber and PUFA decreased across CRP quarters. Their results were in agreement with only the more robust effects detected in our analyses (sugar, fiber and PUFA). They also omitted important nutrients from their study, starch, alcohol (or alcoholic drinks) and cholesterol. Adjusted for age, sex, race, BMI and total caloric intake, they found that total PUFA, PUFA 18:2 and PUFA 18:3

decreased and total SFA, SFA 4:0, SFA 6:0, SFA 8:0, SFA 10:0, SFA 14:0 and SFA 18:0 increased across CRP quarters. We did not find associations between PUFA 18:2 and PUFA 18:3, nor for SFA 4:0 and SFA 14:0 after accounting for all confounding factors. While we found similar associations between the SFAs 6:0, 8:0, 10:0 and 18:0, we also showed that these nutrients were not reliable predictors because of high multicollinearity in the data (Appendix 1.3).

A second study dichotomized CRP as well as the dietary factors into quartiles (dichotomized dependent and independent variables) and included age, race, gender, body mass index, smoking status, alcohol consumption, exercise, medications, and total caloric intake as covariates (King et al., 2003). This analytical approach maximizes the chances of finding differences "somewhere and anywhere" among any of the macronutrient quartiles that can associate to any of the CRP quartiles. Here, the authors concluded that total energetic intake, protein, carbohydrate, fish and cholesterol were not associated with CRP. Fiber was associated with lower CRP and saturated fat was associated with elevated CRP, unless adjusted for fiber intake. With adjustments, only the effect of dietary fiber was detected in their study.

A third study examined diet in five "food groups" and also percent caloric weight for the three major macronutrient types adjusting the data for stratification and clustering (Qureshi et al., 2009). The authors found a significant association of CRP to grains and vegetables, as well as a very weaker association to dairy, but not to fruits or meats and other proteins. Whether or not statistically significant, all their dietary variables were associated with lower CRP, similar to the bivariate correlations in our study. The percent caloric weight for carbohydrates, fats, protein and dietary fiber (adjusted for caloric intake)

did not predict CRP in their study. The association of CRP to grains and vegetables paralleled the direction of effects usually found for dietary fiber in other studies, although dietary fiber itself was not significantly associated with a decrease in CRP.

A fourth study focused on “Healthy Eating Index” but also included analyses for specific food groups and macronutrients, adjusted for age, sex, race, smoking, education, BMI, diabetes, caloric intake and others (Ford et al., 2005). Total cholesterol was associated with higher CRP, while alcoholic drinks, energetic intake, grains and vegetables were associated with lower CRP, and no association to dairy, meats, or legumes were found. These results differed from ours where we found no effect of cholesterol after accounting for metabolic status, the opposite effect for alcoholic drinks (unlike our bivariate correlations), and statistical evidence for redundancy in negative correlation for caloric intake.

Three other studies using overlapping NHANES datasets are notable for their highly controversial conclusions by using similarly biased statistical methods. One study examined the association of various markers of metabolic syndrome, including CRP to candy consumption (O’Neil et al., 2011). They found that CRP and other markers of metabolic syndrome were significantly reduced in consumers of candy and chocolate candies. Contrary to many studies, including ours that show robust proinflammatory effects of sugar on metabolic factors and CRP, the authors of this manuscript claimed that the “current level of candy consumption was not associated with adverse health effects.” A similar study also reported that the estimated fructose and non-fructose sugar contents of foods did not affect various markers of metabolic syndrome (CRP was not included in their analyses) (Sun et al., 2011). This study dichotomized fructose as well as non-

fructose sugar intake into 12 categories and did not account for confounding variables. It should also be noted that O'Neil *et al.*'s and Sun *et al.*'s studies, reported funding from the National Confectioners Association and Archer Daniels Midland Company, respectively. Their study results were far from the experimental evidence and more in keeping with the commercial interests of their research sponsors. Another study using the NHANES data showed that nondrinkers had elevated CRP (31%) more often than moderate drinkers (21%) or high frequency drinkers (18%) (Stewart et al., 2002). This study also only adjusted for age, sex, race, BMI, smoking status, two autoimmune disease diagnoses (but no other diagnoses) and antiinflammatory use. This was a typical study on alcohol consumption that does not account for confounding factors that were available in the dataset such as diet, other metabolic factors, socioeconomic status or diagnoses that may have kept participants from drinking. In our study, bivariate correlations showed that alcohol intake (g), as well as number of daily alcoholic drinks, were positively correlated with all other nutrients (except sugar), and negatively correlated with CRP (seemingly antiinflammatory when unadjusted for confounding factors). However, when all appropriate variables are accounted for, our regression analyses showed a linear proinflammatory effect of alcoholic drinks on CRP. This instance exemplifies how all relevant covariates need to be accounted. If a study focuses specifically on dichotomized alcohol consumption, and it must also account for the reasons why non-drinkers did not drink – which they did not do. Studies such as this have been under the spotlight for systematic errors, specifically highlighting that most studies on alcohol did not account for individuals who did not drink because of health conditions (Averina, et al., 2006; Fekjaer, 2013; Fillmore et al., 2007; Stockwell et al., 2016).

Significance

Similar epidemiological and human studies on diet and health, including previous analyses of the NHANES diet data, have produced results that diverge from those produced by molecular biology and biochemistry research. By applying simultaneous regression models, avoiding variable dichotomization, and by including in our models all relevant confounding factors that were available in the dataset, we estimated the effects of dietary carbohydrate and lipids on BMI and CRP. We tested interactions and non-linear relationships, and identified which predictors reliably estimated those outcome variables. In addition, we compared BMI to other metabolic factors and tested for mediation effects linking diet, adiposity and inflammation. Unlike similar epidemiological nutrition studies, we were able to detect effects that mirror what is expected from molecular biology, biochemistry and carefully controlled human and animal studies. Specifically, we showed that macronutrients predicted BMI and CRP better than caloric intake, and that total caloric intake became insignificant and unreliable when accounting for macronutrient composition of the diet, thereby defeating common notions that obesity and inflammatory disease is a simple function of excess caloric intake. We showed that dietary carbohydrates influence BMI and CRP to a much greater extent than dietary lipids, defeating the popular belief that dietary fats and cholesterol are drivers of obesity and inflammation. Dietary carbohydrates exhibited the greatest influence on both BMI and CRP, mostly though the detrimental effect of sugar and the beneficial effect of dietary fiber. Dietary lipids exhibited relatively little effect on either BMI or CRP. Importantly, we detected macronutrients that exhibited significant effects but which did not yield reliable

estimates due to the overlap of their effects with other components of the diet, among them: monounsaturated fats in predicting BMI; and starch, monounsaturated fat and protein in predicting CRP. Among the metabolic factors, BMI exerted the greatest direct effect on CRP and was the main mediator of the effect of the macronutrients. A large proportion of the effects of carbohydrates and none of the effects of lipids were mediated through BMI. Additionally, all macronutrient interactions pertained to relationships with carbohydrates, but none with lipids. We detected interactions between sugar and magnesium, and starch and proteins in predicting BMI, as well as between starch and protein, sugar and fiber, and sugar and starch in predicting CRP, all in the expected directions. The American diet, as it reflected on the NHANES data, did not vary greatly in the proportion of macronutrients consumed. The study population ate more or less of everything, as evidenced by high bivariate correlations among the macronutrient types. The high correlation among macronutrients and the similar correlation between macronutrients and BMI or CRP may also explain why epidemiological studies of the American diet may be so easily confounded, and why simultaneous effects, interactions of continuous variables and the inclusion of relevant confounding factors are necessary for conserving data variance and parsing of specific effects. Our study adds significantly to the epidemiological nutrition literature by replicating effects that are usually found only in small controlled studies, thereby minimizing the discrepancies often found between those approaches. By applying modern statistical methods in our study, we contributed by challenging decades of dietary dogma in favor of the recent evidence indicating that carbohydrates are the major determinants of obesity and inflammation and by showing

that body adiposity, not blood cholesterol, is the main mediator of the inflammatory physiology.

TABLES

Table 1. Descriptive Statistics

	Frequency	Percent
Total Participants	14997	
NHANES Collection Periods		
2003-2004	3265	32.7%
2005-2006	3396	34.0%
2007-2008	4037	40.4%
2009-2010	4299	43.0%
Availability of Food Diary Data		
Both Days	13495	90.0%
One Day Only	1502	10.0%
Gender		
Male	7978	53.2%
Female	7019	46.8%
Race/Ethnicity		
Mexican American	2765	18.4%
Other Hispanic	1041	6.9%
Non-Hispanic White	7915	52.8%
Non-Hispanic Black	2730	18.2%
Other, incl.multiracial	546	3.6%
Annual Household Income		
$0-4999	582	3.9%
$5000-14999	1070	7.1%
$15000-19999	1029	6.9%
$20000-24999	1190	7.9%
$25000-34999	1854	12.4%
$35000-44999	1448	9.7%
$45000-54999	1310	8.7%
$55000-64999	933	6.2%
$65000-74999	778	5.2%
$75000-99999	815	5.4%
> $100000	1198	8.0%
Refused, don't know, missing	2549	17.0%
Education Level		
< 9th Grade	1649	11.0%
9th-11th Grade	2336	15.6%
HS or GED	3618	24.1%
Some College or AA	4293	28.6%
College Graduate or Above	3088	20.6%
Total	14984	99.9%
Missing	13	0.1%
Snoker Status		
Non-smokers	11354	75.70%
Smokers	3643	24.30%
Cigarrettes Smoked per Day (mean and S.D.)		
Non-smokers	0	(0.00)
Smokers	13.27	(11.71)

	Mean	Std. Dev.
Number of Alcoholic Drinks per Day	0.53	1.21
Age at Screening	49.23	17.93
C-reactive protein(mg/dL)	4.34	8.40
Metablic Factors		
Weight (kg)	81.98	20.28
Body Mass Index (kg/m2)**	28.81	6.39
Glycohemoglobin (%)	5.65	0.99
Total Cholesterol(mg/dL)	198.72	42.58
Direct HDL-Cholesterol (mg/dL)	53.37	16.51
Systolic	123.67	18.75
Diastolic	69.75	13.11
Total Energetic Intake (kcal)	2115.13	878.43
Protein (gm)	82.28	36.52
Carbohydrates		
Total Carbohydrates (gm)	257.31	112.48
Total sugars (gm)	117.05	69.68
Calculated Starch (g)	123.97	57.80
Dietary Fiber (g)	16.29	8.67
Alcohol (g)	10.14	24.26
Dietary Cholesterol (mg)	295.54	199.42
Fat Categories		
Total fat (gm)	79.15	40.01
Total saturated fatty acids (gm)	26.01	14.44
Total monounsaturated fatty acids (gm)	29.19	15.53
Total polyunsaturated fatty acids (gm)	16.99	9.59
Fatty Acids		
SFA 4:0 (Butanoic) (gm)	0.54	0.48
SFA 6:0 (Hexanoic) (gm)	0.29	0.26
SFA 8:0 (Octanoic) (gm)	0.24	0.22
SFA 10:0 (Decanoic) (gm)	0.43	0.36
SFA 12:0 (Dodecanoic) (gm)	0.72	0.93
SFA 14:0 (Tetradecanoic) (gm)	2.15	1.59
SFA 16:0 (Hexadecanoic) (gm)	14.13	7.54
SFA 18:0 (Octadecanoic) (gm)	6.70	3.74
MFA 16:1 (Hexadecenoic) (gm)	1.26	0.80
MFA 18:1 (Octadecenoic) (gm)	27.24	14.56
MFA 20:1 (Eicosenoic) (gm)	0.25	0.19
MFA 22:1 (Docosenoic) (gm)	0.03	0.11
PFA 18:2 (Octadecadienoic) (gm)	14.96	8.63
PFA 18:3 (Octadecatrienoic) (gm)	1.49	0.92
PFA 18:4 (Octadecatetraenoic) (gm)	0.01	0.03
PFA 20:4 (Eicosatetraenoic, EPA) (gm)	0.15	0.11
PFA 20:5 (Eicosapentaenoic) (gm)	0.04	0.11
PFA 22:5 (Docosapentaenoic) (gm)	0.02	0.04
PFA 22:6 (Docosahexaenoic, DHA) (gm)	0.08	0.17
Salts		
Magnesium (mg)	293.84	146.60
Sodium (mg)	3475.52	1845.53
Potassium (mg)		

Table 2. Caloric Intake by Macronutrient (kcal)

	Mean (g)	Std. Dev.
Protein	329.13	146.06
Total Sugar	468.20	278.70
Calculated Starch	495.88	231.20
Dietary Fiber	32.58	17.34
Alcohol	71.00	169.83
SFA	234.13	129.93
MUFA	262.70	139.77
PUFA	152.87	86.29
Total Energy	2115.13	878.43

able 3. Bivariate Correlations Among Macronutrients, Total Calories, Metabolic Factors and C-Reactive Protein

	Protein (gm)	Total sugars (gm)	Calculated Starch	Dietary fiber (gm)	Alcohol (gm)	Number of Alcoholic Drinks per Day	Total saturated fatty acids (gm)	Total monounsaturated fatty acids (gm)	Total polyunsaturated fatty acids (gm)	Cholesterol (mg)
rotein (g)										
otal sugars (g)	.386**									
alculated Starch (g)	.631**	.426**								
ietary fiber (g)	.478**	.293**	.592**							
lcohol (g)	.164**	-.035**	.212**	-.020*						
lumber of Alcoholic Drinks per Day	.128**	-.024**	.155**	-.007	.559**					
otal saturated fatty acids (g)	.727**	.472**	.569**	.317**	.106**	.088**				
otal monounsaturated fatty acids (g)	.742**	.430**	.617**	.399**	.127**	.093**	.882**			
otal polyunsaturated fatty acids (g)	.603**	.357**	.571**	.438**	.091**	.055**	.641**	.804**		
holesterol (mg)	.656**	.238**	.326**	.161**	.116**	.082**	.599**	.594**	.449**	
nergy (kcal)	.816**	.675**	.821**	.514**	.314**	.200**	.831**	.856**	.737**	.551**
ody Mass Index (kg/m**2)	-.003	-.046**	-.049**	-.058**	-.077**	-.080**	-.001	.010	.004	.037**
ilycohemoglobin (%)	-.052**	-.115**	-.075**	-.022**	-.071**	-.056**	-.068**	-.051**	-.047**	.010
otal Cholesterol(mg/dL)	-.017*	-.032**	-.039**	-.012	.045**	.042**	-.015	-.023**	-.037**	.003
irect HDL-Cholesterol (mg/dL)	-.084**	-.119**	-.094**	-.018*	.115**	.103**	-.064**	-.067**	-.028**	-.067**
ystolic	-.072**	-.094**	-.072**	-.034**	.034**	.039**	-.074**	-.065**	-.064**	-.020*
iastolic	.059**	.032**	.044**	.001	.084**	.067**	.058**	.072**	.057**	.049**
-reactive protein(mg/L)	-.075**	-.023**	-.082**	-.086**	-.051**	-.033**	-.047**	-.052**	-.059**	-.026**

* Significant at the 0.01; * Significant at the 0.05 level.

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Section 2.2.

Pathways Linking Carbohydrate and Lipid Metabolism, Adiposity and Inflammation

Abstract

Assessing causality in human disease is a major challenge, especially in conditions characterized by multiple dysfunctional systems like metabolic syndrome. While many clinical association studies point to dysfunctional lipid metabolism as the main target of treatment, recent evidence has challenged that notion. For example, lipid targeted therapies have not reversed the development of metabolic syndrome, nor reduced mortality. Similarly, dietary guidelines to reduce fat and cholesterol intake have not reduced obesity or the development of cardiovascular disease in the population. With the advancement of modern biological techniques and greater computational power, recent research in biochemistry and molecular biology has elucidated pathways by which excess glycemic carbohydrate intake disrupts glucodysregulation, causes dyslipidemia, weight gain and inflammation. Our previous study showed that dietary carbohydrates, rather than lipids, exerted the greater effect on obesity and inflammation and that body adiposity is the main mediator between diet and inflammation. In further support of those analyses, we applied structural equation models to test and revise hypothetical models fit to the biomarker data available from the Midlife in the United States (MIDUS) to determine the path and direction of effects liking carbohydrate and lipid metabolism to body adiposity and inflammation. In keeping with the effects expected from the biochemistry and molecular biology literature, and in contrast to many clinical and epidemiological studies, our analyses place glucodysregulation at the origin of the system, driving dyslipidemia and weight gain, and affecting inflammation indirectly through lipid metabolism and adiposity, where body adiposity serves as the major path mediating the effect of

carbohydrate and lipid metabolism on systemic inflammation. The resulting model was further tested across levels of diabetic status. The alterations observed in path effects were consistent with metabolic shift expected during diabetes. Further, we tested this model across users and non-users of statin medication, and found evidence that the beneficial effects of statins may be due to a weakening of proinflammatory pathways rather than by necessarily lowering blood cholesterol. In addition, this analysis suggest that statin medication may cause systemic changes that resemble the alterations previously observed across diabetic status. These findings support recent evidence that statins exert antiinflammatory action but may also exhibit pro-diabetic side-effects. These systems-level analyses effectively placed carbohydrate metabolism at the genesis of metabolic dysfunction and obesity as the main path to inflammation.

Highlights:

- Carbohydrate metabolism was at the origin of the system, affecting lipid metabolism and body adiposity, which in turn affected the systemic and vascular inflammation
- The main path of effects linked carbohydrate metabolism to inflammation through BMI.
- Comparisons between non-diabetics and diabetics indicated alterations in path effects that are consistent with insulin resistance and type-2 diabetes
- Pre-diabetics show alterations that are already indicative of diabetic physiology.
- Comparisons between users and non-users of statin medications indicate similarities to the diabetic physiology.

- Comparisons between users and non-users of statin medications indicated a weakening of the inflammatory pathways

INTRODUCTION

Shifting away from traditional dogmas

The scientific literature is slowly moving away from blaming cardiovascular disease on dietary fats and cholesterol and abandoning longstanding, over-simplified views on blood lipids. As more careful observations and specific assessments accrue, the spotlight is turning to the excessive consumption of highly processed foods and highly glycemic carbohydrates that are common staple in the traditional American diet (Baker & Friel, 2014; Hofmann & Tschöp, 2009; Lustig, 2013; Myles, 2014). Our understanding of the "Diet-Heart Hypothesis" is being reshaped by biological assessments of greater specificity as well as the integrations among the various fields of science (Hite et al., 2011; Hu, 2010; Kiecolt-Glaser, 2010; Malhotra, Redberg, & Meier, 2017). Molecular approaches and improvements in statistical modeling have allowed for the elucidation of biological networks regulating energy balance and inflammatory processes and for more comprehensive analyses of epidemiological data. Nevertheless, headlines still reference fat-clogged arteries, even though lipids have never been shown to clog blood vessels. In clinical settings, a formula-based estimation of LDL cholesterol is still used as the main basis of dyslipidemia diagnosis and main criterium in the prescription of statin treatment. In research, LDL cholesterol estimation also serves as the main outcome measure of cardiovascular risk. The artificial lowering of LDL cholesterol has only been shown to prevent cardiovascular events in a small subgroup of the population who already have inflammatory disease, while naturally low levels are often associated with higher, not lower, disease and mortality (Bae et al., 2012; Berger, Raman, Vishwanathan, Jacques,

& Johnson, 2015; Eichholzer et al., 2000; Nago et al., 2011; Ravnskov et al., 2015). In addition, over half of the hospital admission for cardiovascular cases do not present elevated LDL cholesterol levels (Horwich, Hernandez, Dai, Yancy, & Fonarow, 2008; Sachdeva et al., 2009). In epidemiological research, causal relationships are often assumed based on prior associations without careful examination of directionality, mediation and biological mechanisms. We carried out a thorough review of the literature linking nutrient metabolism, glucoregulation, lipoprotein turnover, adipose accumulation and the proinflammatory processes involved in the atherosclerogenic process. The key aspects of this physiology are described below and were the bases used to build and test structural equation models that delineate the sequence of events linking nutrient metabolism, obesity and inflammation.

The physiology of cardiovascular disease

Metabolic syndrome is closely tied to lifestyle factors such as diet, physical activity and stress, and is the main driver of cardiovascular disease in the Western World. Although cardiovascular disease encompasses multiple illnesses, many are closely related to the atherosclerotic process. Until recently, atherosclerosis was considered a result of passive fat and cholesterol accumulation in the vascular wall (Jensen et al., 2014). However, we now appreciate the complexity of this process, which requires specific molecular events: LDL-modification (Ahmed, 2005; Angelica & Fong, 2008; Itabe et al., 2011; Yoshida & Kisugi, 2010), vascular infiltration of LDLs (Nordestgaard & Tybjaerg-Hansen, 1992), reactive oxygen species (ROS) generation by mitochondria (Bonnefont-Rousselot, 2002; Figueroa-Romero, Sadidi, & Feldman, 2008; Schleicher &

Friess, 2007), the formation of foam cells (Huh, Pearce, Yesner, Schindler, & Silverstein, 1996), chronic inflammatory processes taking place at the vascular (Corti, Hutter, Badimon, & Fuster, 2004; Libby, 2006) and systemic levels (Shrivastava et al., 2015), as well as arterial calcification (Kalampogias et al., 2016; Zieman, Melenovsky, & Kass, 2005). Understanding this process also sheds light on the potential effect of diet on metabolic dysregulation, chronic inflammation and disease.

Blood cholesterol, triglycerides and the role of lipoproteins

The pathophysiology of cardiovascular inflammation also depends on briefly disambiguating the identity and the assumptions involving the measurement most commonly associated with cardiovascular health: the Low-Density Lipoprotein Cholesterol (LDL, or LDL-C). The simple lipid panel commonly ordered for both clinical or research use includes concentrations of Total Cholesterol (TC), High Density Lipoprotein Cholesterol (HDL-C, or HDL, by common usage), Low Density Lipoprotein Cholesterol (LDL-C, or just LDL, by common usage) and Triglycerides (or technically correct, triacylglycerides). HDL-C and LDL-C refer to the cholesterol content within those lipoproteins, which is measured by the common panel. This is to differentiate the assessment from that of the HDL-Particle (HDL-P) and LDL-Particle (LDL-P), referring to the lipoproteins' particle counts, which are important for the discussion. Further, LDL-C is rarely assessed. Rather, it is estimated by the Friedewald Formula; said formula was a product of an important advance in lipoprotein measurements — the ability to measure cholesterol-triglyceride ratios within lipoproteins. Cholesterol molecules, triglycerides and other lipids are hydrophobic; hence they are mainly carried by lipoproteins in circulation.

Each lipoprotein has a characteristic ratio of cholesterol and triglycerides. Hence, LDL-C is estimated by the subtraction of HDL-C and 1/5 of the triglyceride concentration (vLDL particles typically present a cholesterol-to-triglyceride ratio of 1:5) from the TC concentration. HDL-C and TC are easy to assay, which is not the case for LDL-C and other cholesterol subfractions. The formula disregards other lipoproteins, which present cholesterol in negligible quantities during the fasted state in non-insulin resistant individuals, and therefore will misestimate LDL as triglycerides increase (Cordova, Schneider, & Juttel, 2004; Wagner et al., 2000, Whitting, Shephard, & Tallis, 1997). The expected cholesterol-to-triglyceride ratio of lipoproteins do not hold for insulin resistant individuals. The lesser cholesterol and greater triglyceride content, the smaller and denser the lipoproteins particles are (Goldberg, Eckel, & McPherson, 2011; Verges, 2015). They are, in increasing order of density and decreasing particle size: chylomicrons, vLDL (very Low-Density Lipoprotein), IDL (Intermediate Density Lipoprotein), LDL and HDL. Chylomicrons particles carry triglycerides, cholesterol and lipids from the small intestines into circulation and to tissues for utilization. While vLDL and LDL particles carry both triglycerides and cholesterols from the liver into circulation and to tissues, vLDL particles carry mostly triglycerides and LDL particles carry mostly cholesterol. IDL particles are intermediaries between vLDL and LDL. As triglycerides in vLDL are transferred to tissue or other lipoproteins, it becomes smaller and denser (Koi, Ohnishi, & Yokoyamaff, 1994; Skeggs & Morton, 2002). With the transfer, the vLDL particle becomes IDL, which may lose further triglycerides and proteins to become an LDL particle (Biggerstaff & Wooten, 2004). In such state, this lipoprotein is referred to as small dense LDLs, or sdLDL particles. Other lipoprotein remnants (depleted of their lipid

core) are also small and dense. High blood triglycerides interfere with the hepatic reabsorption of lipoproteins and thereby may increase circulating levels or remnants (Kissebah, Alfarsi, & Adams, 1981; Peterson, Olivecrona, & Bengtsson-Olivecrona, 1985). Hypertriglyceridemia also interferes with the lipoprotein structure, thereby affecting normal cholesterol transfer, susceptibility to oxidation, as well as the feedback regulation of cholesterol synthesis (Callow, Samra, & Frayn, 1998; Deckelbaum, Granot, Oschry, Rose, & Eisenberg, 1984; Skeggs & Morton, 2002). When lipoproteins are small enough may they infiltrate the vascular endothelia. Hence, cholesterol-rich (large and fluffy) LDL particles do not infiltrate behind endothelial walls in large quantities. Accordingly, various studies have shown evidence that LDL-P count, vLDL or sdLDL are better predictors of atherosclerosis than LDL-C (Austin, 2000; Aviram, Lund-Katz, Phillips, & Chait, 1988; Campos et al., 1992; Martin et al., 2009; Prenner et al., 2014; Rajman, Eacho, Chowienczyk, & Ritter, 1999; Vakkilainen et al., 2000; VanderLaan, Reardon, Thisted, & Getz, 2009). Hence, two individuals may present the same total LDL-C concentration, while discordant in LDL-P. One individual may present fewer larger cholesterol-rich LDL particles (a healthy profile) while another individual may present greater numbers of small dense cholesterol-poor triglyceride-rich LDL particles carrying the same total concentration of LDL-C. Notwithstanding, the commonly calculated LDL still shows a positive association with atherosclerosis in the American population. The high LDL-C observed in a proportion of the cardiovascular disease cases may be product of dysregulated glucose metabolism and hypertriglyceridemia, but not the actual cause of disease. Dietary cholesterol only modestly increases blood cholesterol (Ginsberg et al., 1995; Griffin & Lichtenstein, 2013; Hopkins, 1992), cholesterol is dynamically

controlled at various levels that may exhibit dysfunction (Dietschy et al., 1993; Lecerf & de Lorgeril, 2011; Ono, 2012). Cholesterol is either absorbed from the diet or produced in the liver by sugar ingestion (Jameel et al., 2014; Moser, 1985; Schaefer et al., 2009). Excess cholesterol is readily excreted by erythrocytes in the intestines or converted to bile, much of which is lost in feces (Lammert & Wang, 2005). Hence, higher LDL-C may be reflective of an abnormal cholesterol absorption, synthesis or clearance. More likely, it is induced by carbohydrate-induced synthesis and hypertriglyceridemia induced impairment of hepatic reabsorption of lipoproteins, generating an accumulation of cholesterol-containing remnant particles (Skeggs & Morton, 2002). Altogether, the current body of evidence seems to suggest that LDL-C is not the cause of atherosclerosis, but instead a by-product of carbohydrate metabolism. Based on this literature, our hypothetical model tested the influence of glucoregulatory pathwyas and triglycerides on LDL. In addition, we expected weak effects of LDL on inflammation.

High blood glucose, Reactive Oxygen Species and glycosylation

Glucose-induced Reactive Oxygen Species (ROS) is generated by mitochondria as well as through Protein Kinase C (PKC) pathways (Figueroa-Romero et al., 2008; Lee, Yu, Song, & Ha, 2004; Rolo & Palmeira, 2006). Lipoproteins may become glycated, the damaging non-enzymatic glycosylation by high glucose exposure. High glucose in circulation has also been shown to induce glycation products, such as the glycated hemoglobin (HA1c), commonly used as a biomarker of glucoregulation and diabetes (Makita et al., 1992). If not cleared, these damaged products may become further glycated by further glucose exposure, giving rise to Advanced Glycation End-Products

(AGEs), which are highly proinflammatory and add to the inflammatory burden associated with metabolic syndrome and cardiovascular disease (Fu et al., 1994; Turk et al., 1998; Yan et al., 2003). LDLs may also become oxidized by glycation or exposure to ROS (Giugliano, Ceriello, & Paolisso, 1996). Based on this literature, we expected glucoregulatory pathways to also affect inflammatory biomarkers directly.

Lipoprotein oxidation and clearance

When lipoproteins are oxidized or glycated behind the vascular endothelium, they elicit potent proinflammatory responses (Yan et al., 2003). Soluble proinflammatory products such as the Intercellular Adhesion Molecule 1 (ICAM-1) are produced in atherosclerotic inflammation and facilitate the recruitment and infiltration of macrophages (Davies et al., 1993). The vascular inflammatory process, including the ICAM-1 induction, may be further amplified by the presence of high circulating levels of C-Reactive protein (CRP), a proinflammatory acute phase reactant (Devaraj, Kumaresan, & Jialal, 2004; Montecucco et al., 2008; Pasceri, Cheng, Willerson, & Yeh, 2001; Woollard, Phillips, & Griffiths, 2002). Through phagocytosis, macrophages absorb these damaged lipoproteins, including their lipid content. In abnormal conditions when endothelial accumulation of lipoproteins is high, overwhelmed macrophages form foam cells. Their accumulation marks the initiation of atherosclerosis and elicits further inflammatory processes and cellular dysfunctions. High glucose adds to the atherosclerogenic process by driving the calcification of vascular tissue by calcium accumulation and chondrocyte induction, thereby making the vasculature stiff and brittle (Yoshida & Kisugi, 2010).

HDL particles are known as good or protective lipoproteins because they may accept triglycerides from vLDLs, retrieve lipids from the endothelial vasculature, exert anti-oxidant and antiinflammatory properties, and shuttle cholesterol cargo back to the liver for reabsorption and recycling (Barter et al., 2004; De Nardo et al., 2014; Drew, Rye, Duffy, Barter, & Kingwell, 2012; Gomaraschi et al., 2005; Mertens & Holvoet, 2001; Nofer et al., 2002). Though, HDL can also become dysfunctional and atherosclerotic (Bindu G, Rao, & Kakkar, 2011; Navab et al., 2006). And while the “reverse cholesterol transport” [to the liver] is seen as characteristic of HDL particles, LDL particles not only play this same role, but actually accounts for about 90% of the reverse cholesterol transport (Spady, 1992). Based on this literature, we expected HDL cholesterol to reflect beneficial effects on inflammation and the possibility that LDL cholesterol may not always exert detrimental effects.

Glucose, *de novo* lipogenesis and fat accumulation

Hepatic triglycerides and cholesterol are produced in small part by lipoproteins that are retrieved from blood circulation and lymphatic tissue, which absorbs dietary lipids, while in individuals feeding on high carbohydrate diets, these lipids are formed by a process known as *de novo* lipogenesis (Ginsberg, Zhang, & Hernandez-Ono, 2005; Parks et al., 1999). Hepatic cholesterol and triglycerides syntheses are stimulated by insulin and produced from sugars, especially fructose (Jameel et al., 2014; Moser, 1985; Schaefer et al., 2009). Fructose and glucose are often absorbed together as a sucrose disaccharide. Almost all fructose in the body is obtained from dietary consumption. Glucose on the other hand, may be released from the liver where it is stored as glycogen,

or produced from amino acids in the liver. Because a high concentration of glucose is toxic, it is readily stored as glycogen in the liver and muscles. In the absence of dietary glucose and exhaustion of glycogen, the body's glucose needs are met by hepatic gluconeogenesis. When the system is overwhelmed, glucose is also converted to and stored as triglycerides in adipose tissue, thereby adding to body adiposity. Under normal physiological conditions, this glucoregulatory process is meticulously controlled by hormones insulin and glucagon. Glucagon raises blood glucose and fatty acids, is regulated by insulin, and stimulated by gluconeogenic amino acids. Insulin is a major orchestrator of energy balance and its release is readily stimulated by glucose and a few amino acids. Insulin regulates glucose absorption by tissues, the conversion of glucose to triglycerides, and lipolysis. High insulin signals the deposition of triglycerides in adipose tissue and lipogenesis, while low insulin signals lipolysis and lipid beta-oxidation. Hence, insulin is a major force regulating the accumulation and expenditure of adipose fat storage. Based on this literature, we expected glucose to drive triglycerides.

Adipose tissue and inflammation

Body fat accumulation requires a process known as adipose remodeling. Adipose remodeling involves the proliferation of new adipocytes as well as cell death (Strissel et al., 2007; Suganami & Ogawa, 2010). With repetitive or sustained insulin stimulus, precursor stem cells proliferate at high rates. Insulin stimulates the uptake of glucose, the deposition of lipids by LDL-P, and the inhibition of fatty acid release in the adipose tissue. Adipocytes grow in number and size, altering the adipose tissue profile from the metabolically healthy brown fat to the proinflammatory white fat. Stem cell survival and

maturation to adipocytes are incomplete. The associated cell death stimulates cytokine and adipokine release and the recruitment of macrophages. Adipocytes and the resident macrophages are major producers of proinflammatory signaling molecules, including Interleukin-6 (IL-6) and CRP (Anty et al., 2006; Calabro et al., 2005; Mazurek et al., 2003). Studies also show that IL-6 induces CRP production in the liver, adding to systemic inflammatory load (Castell et al., 1989). In turn, systemic inflammation may also affect systemic oxidative stress, glucoregulation and lipoprotein metabolism, further developing the metabolic syndrome complex and aggravating cardiovascular disease (Dandona, Aljada, & Bandyopadhyay, 2004; Donath & Shoelson, 2011; Shoelson et al., 2006; Yan et al., 2003). Based on this literature, we expected adiposity to mediate the effects of glucoregulatory pathways on inflammatory pathways.

Carbohydrate metabolism at the origin of metabolic dysregulation

Recent experimental data convincingly points to carbohydrate metabolism as the root problem driving lipid metabolism dysfunction and inflammatory processes, contributing to the rapid increase in development of diabetes, metabolic syndrome, chronic inflammation and cardiovascular disease (Ballard et al., 2013; Boden, 2009; Bueno et al., 2013; Douris et al., 2015; Hussain et al., 2012; Kirk et al., 2009; Volek et al., 2009). Our culture has moved from a much more varied diet to guidelines that advise the substitution of saturated fats by carbohydrates. These recent changes in dietary guidelines in the U.S. coincide with the increase in incidence of obesity and metabolic and inflammatory diseases. While common genetic differences do not determine disease, they may represent different susceptibilities to the current diet, characterized by

excessive amounts of highly processed and highly glycemic foods. Still, many epidemiological studies on diet and metabolism, as well as governmental guidelines, continue to rely on correlations and imply causations based on notions conceived from antiquated methods and correlational studies (Binnie et al., 2014; Fernandez, 2012; Gifford, 2002; Hite et al., 2010; Teicholz, 2015). While earlier studies lacked technological means, we now enjoy a surplus of data produced by highly specific assessments. While many of these assessments are too expensive for large population studies, we can analyze the clinical biomarkers that we do have available in the context of biomolecular pathways. Our study was designed to test whether the metabolic and inflammatory data available in the Midlife in the United States (MIDUS) project fits a mathematical model that was based on the current understanding of human molecular biology and nutrient metabolism.

Goals and hypotheses

We built a hypothetical structural equation model based on the accruing literature in the molecular biology and biochemical sciences, linking glucoregulation to the lipid metabolism and inflammatory processes that are relevant to the progression of cardiovascular disease, as above described. We tested how well this model fit the data of a subsample of non-diabetic, White Caucasian Americans, who were not taking insulin or cholesterol targeted medications. This original model was then revised by removing non-significant relationships. Given that most biological systems present regulatory feedback loops, the relationship between two biomarkers may be dominated by one direction over another. For instance, changes in blood glucose elicit insulin responses

while the variation in blood insulin levels also regulates glucose production, release and absorption. Model variations were created in order to test the dominating directionality of effects and to compare with other directional possibilities. Thus, structural equation models can also help support hypotheses about the directionality of effects. Because age may influence the efficiency of synthesis or clearance of metabolic and inflammatory components, thereby affecting the sensitivity of a system, the effect of age on each biomarker was tested and retained when significant. We hypothesized that our model would detect the effect of carbohydrate metabolism at the origin of the system, feeding on lipid metabolism and body adiposity (BMI), directly and indirectly affecting the biomarkers of systemic and vascular inflammation. Our model was then used to predict how these biological systems were affected by specific conditions of metabolic interest. This was accomplished by fitting this model to the data of participant subsamples that varied by diabetic status (diabetes mellitus) and statin usage. Because experimental studies point to glucodysregulation as a principal cause of dyslipidemia, inflammation and cardiovascular disease, we compared the model effects across diabetic status. In order to further validate such model, the differences in model effects by diabetic status should be reflective of the physiological alterations that are expected in type-II diabetes. Recent evidence also suggests that the pharmacological family of statin medications benefit individuals who already present cardiovascular disease but does little to prevent such disease (Hayward, Hofer, & Vijan, 2006). Recent studies also suggest that the benefits of statins are due to their antiinflammatory effects rather than to their cholesterol lowering effect (Blake & Ridker, 2000; Montecucco et al., 2009; Sukhova, Williams, & Libby, 2002). Further, the benefits of lowering blood cholesterol in preventing cardiovascular disease

has also been brought to question, especially as concerns accrue about the side-effects of statins, which may include insulin resistance, mitochondrial and cognitive dysfunction (Deichmann, Lavie, & Andrews, 2010; Ganda, 2016; Golomb & Evans, 2008; Riordan, 2012; Seneff, 2009). By comparing statin users to non-users, we provided insights to the effects of statins on the relationships among the various biomarkers of metabolism and inflammation. Further, by comparing the results from the statins model to the diabetic model, we may infer whether our data and models support the suggestion that stating use may drive metabolic alterations that resemble the diabetic status. Through group-mean analyses, we also assessed the general effects of those metabolic conditions on the biomarker levels, thereby confirming the effects of our path analysis models.

METHODS

Participants and Clinical Information

Participants were drawn from the Biomarker Project (2004-2009) stemming from the original MacArthur Foundation Survey of Midlife Development in the United States (MIDUS II). Clinical and biological measures were assessed for 1,255 participants in the Biomarker Project who consented to the overnight hospital stay, either in Madison, WI, Los Angeles, CA, or Washington DC. Participants arrived on Day 1 at one of the three sites where they were admitted and escorted to their rooms. That same evening, the participants completed their medical history and a physical exam with the help of staff, as well as a self-administered questionnaire. The subject participating in the biological assessment were demographically like the larger survey with respect to age, gender,

marital status, and generally had a higher level of education (Love, Seeman, Weinstein, & Ryff, 2010). In order to increase the representation of African–American participants during this second assessment, a sample was added from Milwaukee, WI. All sample collections and analyses were approved by the Health Sciences Institutional Review Board (IRB) at the University of Wisconsin-Madison, as well as by the IRBs at UCLA and Georgetown University. All participants provided informed consent.

Specimen Collection

Fasted blood samples were obtained between 05:00 and 07:00 AM on Day 2. Specimens were frozen until analyzed. All sample collections and analyses were approved by the Health Sciences IRB at the University of Wisconsin-Madison, as well as by the IRBs at UCLA and Georgetown University. All participants provided written informed consent.

Assessments

Glucose: Blood glucose was measured by the standard clinical methods performed at Meriter Labs (GML) (Madison, WI). Insulin: This assay was performed on a Siemens Advia Centaur analyzer. The assay was performed at Meriter Labs (GML) in Madison, WI. Glycosylated Hemoglobin (%) (HA1c): Hemoglobin A1c was measured using monoclonal antibodies, binding the ß-N-terminal fragments of HbA1c. The quantity of bound glycopeptides was measured turbidometrically at 552 nm. Percent HA1c is then determined by dividing the concentration of HA1c by the total hemoglobin. The assay was performed at GML in Madison, WI. Triglycerides, total cholesterol, and HDL-C: A standard

blood lipid panel was performed at GML in Madison, WI. The HDL-C assay was re-standardized by Roche Diagnostics on August 6, 2007. The results of assays done after that date were adjusted to bring the new values in line within the existing data: Adjusted value = 1.1423(new value) - 0.9028. LDL-C: An estimation of LDL-C was calculated by using the Fridewald formula from direct measurements of total cholesterol (TC), triglycerides (TG), and HDL-C. When triglyceride values were above 400 mg/dl, the MIDUS Biocore used 400 mg/dl as the upper limit for calculating LDL-cholesterol. Interleukin-6 (IL-6): IL-6 was measured using the Quantikine® high-sensitivity ELISA kit #HS600B (R & D Systems, Minneapolis, MN) at the MIDUS Biocore Laboratory at the University of Wisconsin, Madison, WI. C-reactive protein (CRP): CRP was measured using the BNII nephelometer from Dade Behring utilizing a particle enhanced immunonepholometric assay. The CRP assay was performed at the Laboratory for Clinical Biochemistry Research at the University of Vermont, in Burlington, VT. Human Soluble Intercellular Adhesion Molecule-1 (ICAM-1): ICAM-1 was measured by an ELISA assay (Parameter Human sICAM-1 Immunoassay; R&D Systems, Minneapolis, MN) and performed at the Laboratory for Clinical Biochemistry Research at the University of Vermont, Burlington, VT. Body Mass Index: BMI was computed by dividing weight (in kilograms) by height squared (in meters. Height measure (in centimeters) was multiplied by 100 to get the height in meters. The measures were obtained by the clinical staff per standardized protocol. Medication: Respondents were instructed to bring all their medications in the original bottles to the General Clinical Research Center (GCRC) during clinic visits. We asked them to do this to ensure that medication names and dosages were recorded accurately. Completed forms were sent to the University of Madison (central

coordinating site) for review prior to data entry. Said review included specification of medication codes based on the medication name, as well as information about route, or reason for taking the medication, as appropriate. The medication codes were selected from our master list of medication codes derived from the UW Hospital Formulary, which utilizes the American Hospital Formulary System (AHFS) Pharmacologic-Therapeutic classification system. Diabetic Status: HA1c was dichotomized according to clinical cutoffs in order to compare the model effects across three diabetic statuses: non-diabetics (HA1c < 5.7%), pre-diabetics (5.7% ≤ HA1c < 6.5%), and diabetics (HA1c ≥ 6.5%). Statin Use: For comparing model effects across users and non-users of statin medications, participants were categorized based on whether they took HMG-CoA reductase inhibitor at any dosage and frequency for at least the past three weeks.

SEM Data Preparation

Participants with incomplete data were not considered in the analyses and were removed from the dataset. Due to lack of participants from other racial ethnicities, only Caucasian and African American participants were retained in the dataset. The categorical variables: sex, race, diabetic status and statin use were dummy coded. Ill-scaled variances were checked and HA1c, IL-6 and ICAM-1 were re-scaled for analyses, yet, model estimates were converted back to their original units. Our hypothetical model was tested and revised based on data that excluded extreme cases. Five percent of the most extreme multivariate covariances were removed in order to conform the data to a sample distribution of Mahalanobis Distance (MD) scores that did not deviate significantly from the chi-square distribution. MD scores were calculated using the R-Project software,

and the data distribution was checked by plotting MD scores against quantiles of the chi-squared distribution using the chisq.plot package in R. The data was further checked for positive definite variance-covariance matrix (i.e., acceptable eigenvalues greater than 0).

Structural Equation Modeling

The R package lavaan was used to fit SEM models to the data. SEM assumptions were modified by selecting the Wishart likelihood function, requiring the covariance matrix of the data follow a central Wishart distribution rather than full multivariate normality. Robust Maximum Likelihood estimator (MLR) for non-normality was also selected to adjust chi-square and standard error estimates. Huber White “sandwich” estimator was used as the default standard errors adjustment for MLR.

The original model tested the following regressions: HA1c was regressed on glucose; glucose was regressed on insulin; insulin was regressed IL-6, CRP and glucose; triglyceride values were regressed on glucose and insulin; HDL was regressed on IL-6, CRP and ICAM-1; LDL was regressed on triglycerides, IL-6, CRP and ICAM-1; BMI was regressed on glucose, insulin, triglycerides, LDL and HDL; IL-6 was regressed on BMI, glucose, triglycerides, HDL and LDL; CRP was regressed on IL-6, BMI, glucose, triglycerides, HDL and LDL; ICAM-1 was regressed on IL-6, CRP, glucose, triglycerides, HDL and LDL. Model regressions were revised by stepwise removal and by adding residual correlations as non-causal model covariances when, and only when, compatible with observed physiology. Individual regression predictors in the SEM were retained based on their p-values and the regression estimates were presented in raw units (allowing for the comparison of regression coefficients across subsamples) and in

standardized units (allowing for comparisons between regression coefficients within the model). Model variations were also tested by inverting the directionality or route of specific regression effects, when in conformity with physiological alternatives. Various indices of absolute fit were used to test whether our revised hypothetical model fit the data, including Chi-square, Root Mean Square Error of Approximation (RMSEA), Standardized Root Mean Square Residual (SRMR), [Robust] Tucker-Lewis Index (TLI) and [Robust] Comparative Fit Index (CFI). The model Chi-Square analysis tests whether there is a statistically significant difference between model and data covariance matrices, hence, non-significant p-values indicate that the model fits the data. As a result of chi-square tests tending to produce statistically significant p-values for data containing many cases (>400), other fitness indices were also examined and considered. The RMSEA test requires large degrees of freedom; and the null hypothesis is that the hypothetical model fits no worse than the close-fitting model (which is an RMSEA = 0.05). A value of RMSEA > 0.05 is indicative of a good global fit of the model. When RMSEA values are lesser than 0.05, 95% confidence intervals overlap with zero, and the RMSEA p-value is greater than 0.05, it is concluded that the model fits the data better than the null model. The RMSEA penalty for complexity is the Chi-Square to DF ratio. Like RMSEA, SRMR is an absolute measure of fit, where SRMR = 0 indicates the perfect fit. However, unlike RMSEA, SRMR has no penalty for model complexity. RSMR < 0.08 is generally considered a good fit. The TLI indicates good fitting models at low Chi-Square to degrees of freedom ratios. CFI is like, and highly correlated to TLI, but imposes a penalty for every parameter estimated. Good fitting models should exhibit TLI and CFI > 0.95. Power

analyses consisted of computing power for RMSEA. The analysis was set to alpha=0.05, null hypothesis RMSEA=0, and the alternative hypothesis at RMSEA = 0.01.

Alternative model variations were tested by placing the dyslipidemia at the origin of the system, and feeding forward onto inflammatory and glucoregulatory biomarkers. Various permutations of effects were tested, keeping lipid biomarkers at the origin of the system according to perpetuating claims that dyslipidemia cause obesity and inflammation, which, in turn can cause diabetes. A final model was optimized for best fit based on mathematical associations and the order of effects without necessarily complying with known physiological mechanisms. This final model was tested for the absolute fit to the data, then compared to our hypothetical model, which places glucoregulatory biomarkers at the origin of the system. Those models were compared based on relative fit indices, such as negative-Loglikelihood, Akaike Information Criterium (AIC) and Bayesian Information Criterium (BIC) and Sample-Adjusted Bayesian Information Criterium (SABIC). AIC and SABIC may be less biased for data with large number of cases, while BIC may be more appropriate for data with lesser cases, hence greater weight should be place on the former two. Lower values indicate a better fit, therefore, the model with lowest LL, AIC, BIC or SABIC values is the best fitting model.

Chi-Square difference tests were used to decide whether subsamples that reflect conditions of metabolic interest (diabetic status, statin use, race and sex) should be modeled together or separate. If the chi-square difference test is nonsignificant, modeling the subsamples separately is not any worse, statistically speaking, than modeling the subsamples together as a single sample. In cases of nonsignificant differences, the most parsimonious model should be chosen — the model that treats all participants as a single

sample. Conversely, when the Chi-Square difference test is significant, the model should treat the subsamples separately (e.g., the specified model does not equally fit all subgroups). SEM differences by diabetic status was carried out on participants who did not use insulin targeted medications or statins. Differences by statins usage were tested in participants who were not diabetic. To test the effect of race and sex, insulin targeted and statin medication users as well as diabetics were excluded from analysis.

Group-Mean Analyses

Group-mean analyses of the biomarkers of glucoregulation, lipid balance and inflammation were also carried out. Each biomarker was tested by t-test or ANOVA, without adjustments, across the metabolic conditions of interest (diabetic status and statin use) to aid in the interpretation of their effects on the SEM relationships and the system as a whole.

RESULTS

Descriptive statistics

A total of 1161 Caucasian and African-American participants had complete data, including 57.2% females and averaging 54.8 years of age (Table 1). On average, these participants were also pre-diabetic (fasting blood sugar levels from 100-125 mg/dL, and HA1c between 5.7-6.5). Sixteen percent of them had HA1c levels indicative of type 2 diabetes. The majority were also overweight and almost obese (BMI> 30). By clinical standards, their CRP were indicative of high risk for cardiovascular disease (CRP> 3.0

mg/L). Yet, per standard clinical guidelines, most their blood lipids were within optimum or near optimum ranges: triglycerides (<150 mg/dL), total cholesterol (<200 mg/dL) and estimated LDL-C (<100 mg/dL); and HDL (40-59 mg/dL) were considered within acceptable range. However, these averages included participants taking statins, which amount to approximately 24.0% of participants. Although most participants in this study presented blood lipid biomarkers within controlled ranges, they still presented with a high inflammatory profile.

SEM path analyses

In order to test and revise the hypothetical model, a more homogeneous subsample was generated. Five percent of the most extreme cases were removed based on MD scores and chi-square distributions; 1103 remaining cases were filtered for Caucasian participants who were not users of insulin or cholesterol targeted medications and who did not present HA1c levels indicative of diabetes (greater than 6.7%), yielding a total of 626 cases to be considered for analysis. The covariance matrix and eigenvalues for the full complete dataset are presented in Table 2. All Eigenvalues were acceptable (>0). The original model was fit to the data and revised by path analyses as described (see example in Table 3). The final model was estimated for 626 cases at 27 degrees of freedom, producing a nonsignificant chi-square, indicating that the model fit the data (Table 4). Chi-square analyses of SEMs tend to produce statistically significant values for data containing over 350 cases. The fact that our hypothetical model produced a nonsignificant chi-square value despite a large number of cases indicates a strong fit for the data. Other indices of absolute model fit, RMSEA, SRMR, CFI and TLI, all support

this conclusion (Table 4). Although, there were residual correlations not accounted by the model (Figure 1), those associations did not produce significant covariances (regression predictors or non-causal covariances), nor rendered the model unfit of the data.

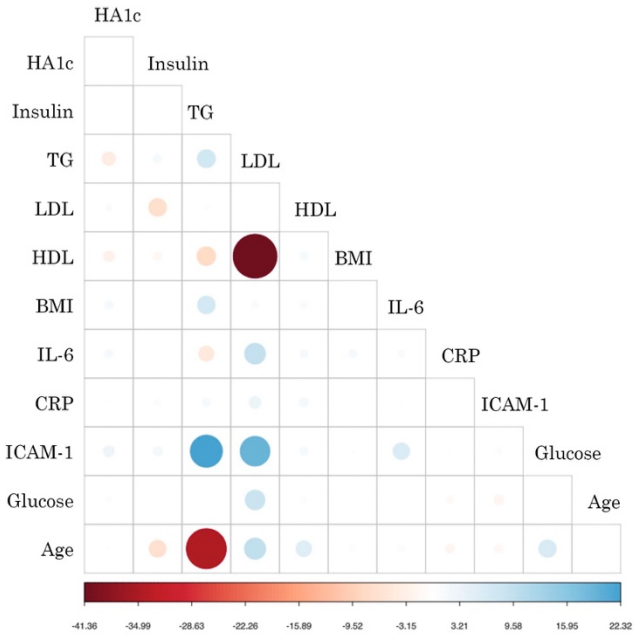


Figure 1. Model Residual Correlation. Faint colors indicate that little covariance was not accounted for given two factors in this model. Conversely, darker colors/larger circles indicate significant residual covariances that were not accounted for in the model.

The final model also provided many significant regression estimates that were in keeping with our hypotheses, pointing to glucodysregulation at the main physiological driver affecting lipid balance and inflammation (Table 5). Specifically, we observed large effects of glucose on HA1c and insulin; insulin on triglycerides; triglycerides on LDL, insulin and HDL on BMI; LDL, HDL and BMI on IL-6; BMI and IL-6 on CRP; and moderate effects of CRP and HDL on ICAM-1; lastly, IL-6 on insulin (Table 5). In all cases, HDL exhibited protective effects. While LDL added to BMI, it also contributed to lower IL-6. The largest biomarker effects in each regression were: glucose on insulin; insulin on triglycerides; insulin on BMI; BMI on IL-6; IL-6 on CRP; and similar effects for CRP and HDL on ICAM-1. Age exerted a positive effect on HA1c (expected with slower hemoglobin turn over in aging). Age predicted higher HDL, IL-6 and ICAM-1 (Table 5). Our model did not detect the effect of age on glucose, insulin, LDL or CRP, as expected. Direct effects of glucose on inflammation were not detected as expected. Through inspection of the relative effect sizes in the model (indicated by standardized coefficient estimates), we concluded that the major route of effect followed from glucose to insulin to BMI to both IL-6 and CRP, from IL-6 to CRP, and CRP to ICAM-1 (Figure 2). This effect pathway reflects a proinflammatory drive. A secondary, but weaker, proinflammatory route was exhibited from triglycerides to LDL to BMI (Figure 2). HDL exhibited lowering effects on BMI, IL-6 and ICAM-1. All routes support our hypothesis that glucodysregulation is the root of dyslipidemic metabolism and body adiposity, ultimately affecting systemic inflammation and vascular inflammation. However, we did not detect direct deleterious effects of glucose metabolism, triglycerides or LDL on systemic or vascular inflammation markers.

Our SEM explained approximately 16% of the variance in HA1c, 18% of the variance in insulin, 21% of the variance in triglycerides, 6.1% of the variance in LDL, less than 1% of the variance in HDL (by age), 35% of the variance in BMI, 12% of the variance in IL-6, 29% of the variance in CRP and 8% of the variance in ICAM-1. Based on 626 cases, 27 degrees of freedom, as well as the specifications described in Methods, RMSEA power analyses indicated that we had a 97.30% probability of rejecting a false model.

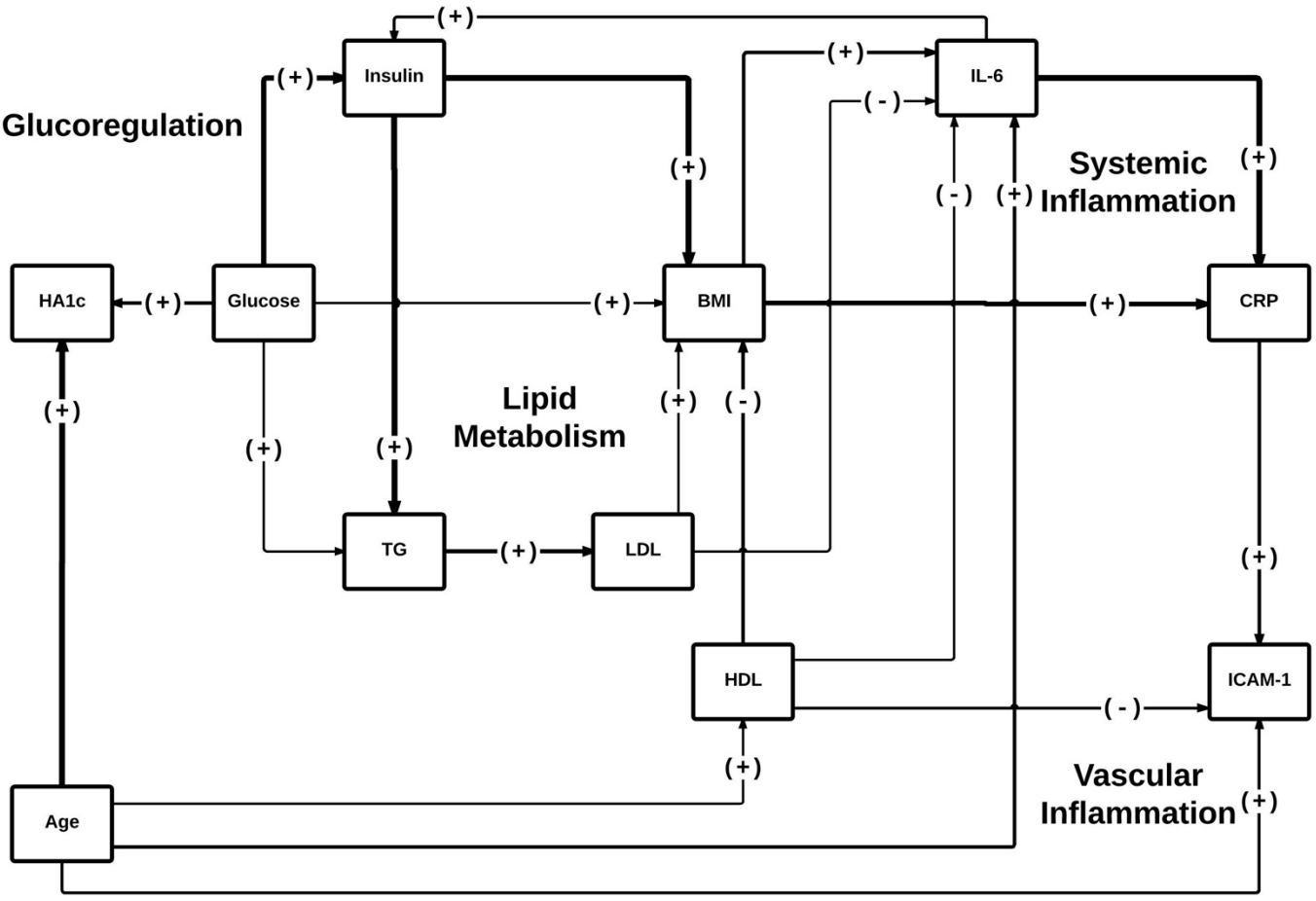


Figure 2. Causal modeling of metabolic-inflammatory relationships. Significant

parameter estimates in final model are shown. The directionality of effects is indicated by the arrow head. Sign in parenthesis indicate positive and negative effects. The relative magnitude of effects is indicated by the weight of the path line. Greater weight indicates greater relative effect.

An alternative model, placing dyslipidemia at the origin of the system, was also tested and revised to produce the best feeling model. Model revisions discarded nonsignificant regressions and added residual correlations as non-casual relationships, while preserving the general directionality of effects from lipid to inflammatory to glucoregulatory biomarkers as per a well-known hypothesis that is still claimed by many epidemiological studies on cardiovascular disease risk. The resulting model contained the following significant regression estimates: HDL was predicted by age; BMI was predicted by triglycerides and HDL; IL-6 was predicted by age, BMI, HDL, and LDL; CRP was predicted by BMI and IL-6; ICAM-1 was predicted by age, CRP and HDL; HA1c was predicted by age, glucose, IL-6 and BMI; and finally, insulin was predicted by glucose, IL-6, BMI, triglycerides and HDL. Other causal covariances were not significant. Many components of this model that remained significant overlapped with our hypothetical model. Other causal effects, while mathematically associated, do not have physiological precedents. The resulting model also did not pass the Chi-Square test, and produced a statistically significant p-value ($\chi^2(28)=42.409$, p=0.040), indicating that the model covariance matrix is significantly different from the data covariance matrix. However, the significant Chi-Square value could be a product of the large number of cases. While the RMSEA analysis (RMSEA=0.30, p=0.987, 95% C.I. 0.007 - 0.047) was non-significant, the 95% confidence interval indicated a near close-fitting model (null model). On the contrary, other indices of absolute fit were within acceptable values (SRMR=0.026, CFI=0.988 and TLI=0.976). Overall, the alternative model fits the data well enough to proceed to model comparisons. The relative fit indices were produced and compared between the hypothetical (see Table 4) and alternative model (-LL=25915.152,

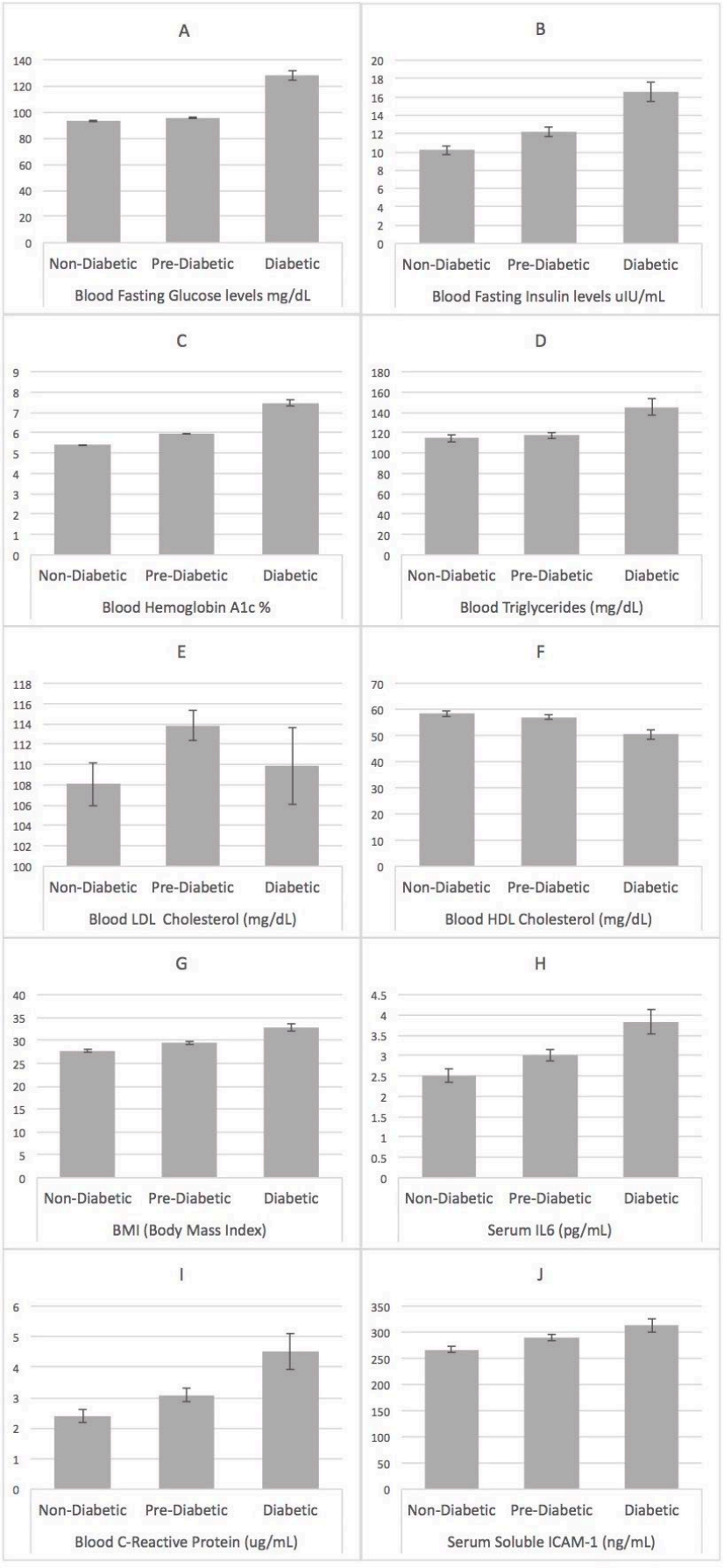
AIC=51928.305, BIC=52145.755 and SABIC=51990.186). The alternative model produced larger indices of relative fit, indicating that it does not fit the data as well as our hypothetical model.

Moderation by diabetic status

Data from participants who were not being treated with insulin targeted or statin medications were compared across categories of diabetes status, based on their HA1c. Because the hypothetical model was revised using data from non-diabetic and pre-diabetic participants, it would be less likely to find differences in the model fit between those two categories (the number of non-diabetic participants was too low to use them alone as originally intended). Nevertheless, we expected to observe differences in regression effects between the two categories. Yet, differences between non-diabetics and pre-diabetics were large enough to produce a statistically significant difference in the model fit. We found distinctions in model fit across all three categories (Table 6). Further, the differences in regression effects produced were in keeping with physiological changes expected in diabetes (Table 7), as further validating the use of our model. Specifically, we observed an increased effect of glucose on HA1c and triglycerides, while insulin was less influenced by glucose and exerted a diminished effect on triglycerides and BMI. Accordingly, we observed increases in mean glucose, insulin, HA1c and triglycerides (Table 8, and Figure 2A-D). All causal relationships to and from LDL were decreased among diabetics (Table 7). There were no significant differences in LDL levels across diabetic statuses (Table 8), but there was an apparent increase in variance (Figure 2E). These findings may be a product of a wrong estimation of LDL-C by Friedewald's equation

for individuals with diabetes or dyslipidemia. We also observed weakened effects (both positive and negative) on HDL, BMI and IL6 in diabetics (Table 7), also with worsened mean concentration levels (Table 8 and Figure 2F, G, H), which may be indicative of lipid and inflammatory deregulation. On the other hand, diabetics presented higher mean levels on CRP with intensified effects (Table 7 and Figure 2I). While the effect of CRP on ICAM-1 was diminished among diabetics (Table 7), ICAM-1 levels increased across diabetic statuses (Figure 2J) again indicating possible deregulation of the system or the effect of other physiological component not assessed in our study. In keeping with previous studies demonstrating that HDL may exert antiinflammatory effects on the vasculature, we observed a largely increased effect of HDL on ICAM-1 levels (Table 7), perhaps indicative of an antiinflammatory response.

Figure 3. Mean differences by diabetic status on the biomarkers of metabolism and inflammation

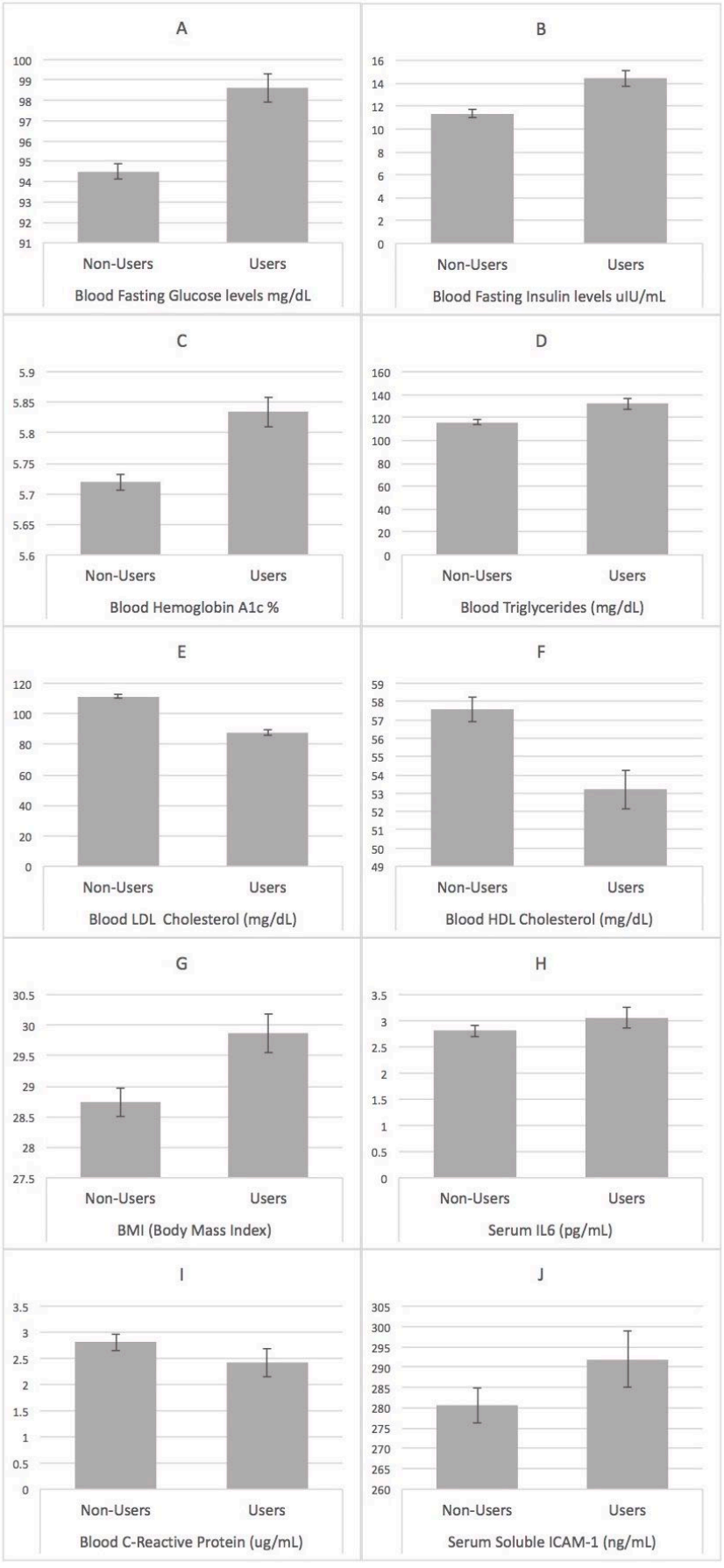


Moderation by statins

Data from non-diabetic participants were compared between non-users and users of statins. The Chi-Square difference test indicated a statistically significant difference between modeling non-users and users of statins separately, rather than modeling them without distinction (Table 9). The effects acting on HA1c, insulin, LDL and BMI among statin users resembled those found among diabetic participants, except for a greater effect of glucose on BMI (Table 10). Accordingly, statin users also presented with higher levels of glucose, insulin, HA1c, triglycerides, as well as greater BMI (Table 11 and Figure 3A-D). Therefore, these results indicate a higher risk of diabetes among statin users. While statin users may have presented pre-existing, pro-diabetic profiles, we attempted to remove such confounds by excluded participants who exhibited a diabetic HA1c value from these analyses. Further, there is growing evidence that statin medication may induce diabetes. Moreover, glucose also exerted a weakened effect on triglycerides, while insulin exerted a stronger effect. It is important to note that the effect of insulin on triglycerides were of much higher magnitude than the effects of glucose (Table 5). Despite higher triglyceride levels, statin users exhibited lower LDL (Figure D and E), accompanied by a weakening of the triglyceride-LDL relationship. These effects are expected because of the use of statins, which specifically target cholesterol. However, amongst statin users, we also observed a sizable reduction in HDL levels (Figure 3F). Statin users presented with 22.35% lower LDL and 7.64% lower HDL when compared to non-statin users. Additionally, we observed that except for the increased effect of HDL on IL-6, all effects acting on the proinflammatory biomarkers were largely decreased (often to nonsignificance) among statin users in relation to non-users (Table 10). This finding may

suggest that statins potentially attenuate the links connecting dysregulated metabolism to inflammation, as well as the pathways connecting the inflammatory biomarkers (Table 10). Accordingly, statin users did not show statistically higher or lower levels of IL-6, CRP or ICAM-1 (Table 11). Our finding is in keeping with previous studies that suggest that statins may benefit individuals presenting metabolic syndrome through antiinflammatory effects. Appropriately, statin users also exhibited lower effect of IL-6 on insulin (Table 10).

Figure 4. Mean differences by statin medications use on the biomarkers of metabolism and inflammation



DISCUSSION

Much of the population sample in the MIDUS Biomarker project was on the verge of obesity. According to HA1c and fasting glucose values, the majority was, at least, pre-diabetic. Although, their lipid panels were almost optimal by most measures, they present CRP levels that placed them in a clinical category of “moderate risk.” Further, nearly a quarter of the sample was on statin drugs. While cholesterol levels for both users and non-users of statins were well controlled, their glucoregulatory and inflammatory systems were not. Hence, our models, their effects and the difference in their effects across subgroups should be interpreted in context of a population that show signs of metabolic syndrome.

Path and directionality of effects

We used structural equation modeling to infer causal relationships among various biomarkers of health based on known metabolic and inflammatory physiology. We assessed the fit and effects of a hypothetical model that placed glucoregulatory biomarkers at the causal root of a system, affecting markers of markers of lipid metabolism, systemic and vascular inflammation. Through analyses of the mediation and directionality of effects, we optimized the model to a near homogeneous participant subset, confirming our primary hypothesis that such a model better fits the data. These analyses also indicate that the main route of deleterious effects linking glucoregulation to vascular inflammation follows primarily through the effect of glucose and insulin on BMI, BMI on IL-6 and CRP, IL-6 on CRP, and CRP on ICAM-1. While we detected the effect

of triglycerides on LDL, the effects of LDL were either weak (on BMI) or antiinflammatory [on IL-6]. The weak effects of LDL were indicative of a few possibilities: (1) LDL cholesterol is not a main driver of inflammation as once thought, in keeping with the more recent literature; (2) That most of the LDL is not proinflammatory, and rather a subtraction that is oxidized or glycated; (3) or that the estimated LDL cholesterol is not a useful measure of lipid metabolism for a population that shows strong signs of metabolic syndrome, as suggested by previous studies. On the other hand, HDL exhibited beneficial relationships to BMI, IL-6 and ICAM-1, acting as an antiinflammatory factor towards the markers of inflammation. While we found a positive effect of glucose and insulin on triglycerides, we did not find any direct inflammatory effects of glucose on inflammatory markers, as we expected from multiple studies (Kim, Kim, & Kim, 2011; Rizzo, Barbieri, Marfella, & Paolisso, 2012; Yan et al., 2003).

Carbohydrate metabolism at the genesis of obesity and inflammation

Our application of Structural Equation Modeling to this data allowed us to assess the directionality and mediation of effects and to compare competing models in order to better understand how the relationships within a biological system change across different participant samples. This strategy generated a system-wide view of the biomarker physiology, provided insight into the systems-level effect of type 2 diabetes and statins treatment. We compared the fit of competing mathematical models on population data, selecting the model that best represents the parsing of variances found within that population sample, thereby testing the path and directionality of effects through mathematical means. Our hypothetical model indicates that glucoregulation and

adiposity may be the two major components driving systemic and vascular inflammation. To further support our hypotheses, we compared the relative fit of our model to a mathematically optimized model placing dyslipidemia at the causal root of the model. We found that our hypothetical model better fits this data than this alternative model, confirming that a causal effect of glucodysregulation better explains the metabolic-inflammatory profiles than lipid dysregulation. Our model also points to body adiposity as one of the main mediator of the inflammatory effects. Although BMI is an imperfect measure of body adiposity, it is an appropriate marker in a population characterized by overweight, pre-obese profiles. Additionally, our model indicates that blood cholesterol pathways are secondary to other effect routes that exert stronger inflammatory pathways: glucose, insulin and triglycerides. Accordingly, recent studies point to triglycerides as an important predictors of Insulin resistance, LDL particle size and cardiovascular disease (Boizel et al., 2000; da Luz, Favarato, Faria-Neto, Lemos, & Chagas, 2008; Frohlich & Dobiášová, 2003; Gonzalez-Chavez, Simental-Mendia, & Elizondo-Argueta, 2011; Maruyama, Imamura, & Teramoto, 2003)

Diabetic status

We compared the model effects across three levels of diabetic status according to clinical HA1c cutoffs and found differences in the relationships among the various biomarkers between non-diabetics and diabetics, and also between non-diabetics and pre-diabetics and pre-diabetics and diabetics. The differences in effects across diabetic statuses were consistent with the biological alterations as expected in the pathophysiology of Diabetes Mellitus. For instance, all relationships to and from insulin

are weaker among diabetics then non-diabetics, reflective of reduced insulin sensitivity and greater insulin resistance. These effects suggest that metabolic dysfunction takes place far earlier than clinical diagnosis, as pre-diabetics already exhibited pro-diabetic profiles. In addition, specific and system-wide differences in lipid and inflammatory effects were evident from one level of diabetic status to the next. These differences in model effects were also accompanied by increasing mean levels of inflammatory biomarkers and BMI. These results are in keeping with the recent research that favors low glycemic diets and addressing insulin sensitivity over low fat diets and cholesterol target treatments (Boden, 2009; Hite et al., 2011; Volek et al., 2009; Westman et al., 2007).

Statin Treatment

In assessing the differences in effects between non-users and users of statin medications, we found that glucoregulatory alterations were similar to those found in the assessment of diabetes. These differences may suggest a greater risk of diabetes among statin users, especially considering that they also present higher mean levels of the glucoregulatory biomarker than non-users. This difference reflects recent evidence that statin medications may drive diabetic processes (Ganda, 2016; Riordan, 2012). In addition, we found weaker relationships amongst the biomarkers of inflammation within those statin users. This finding would support recent evidence that suggests the benefit of statins may come from antiinflammatory effects (Blake & Ridker, 2000; Li et al., 2010; Liao & Laufs, 2009; Sukhova et al., 2002). However, statin users did not exhibit lower mean levels of inflammatory biomarkers than non-users. That could be an indication that

statins weaken some of the proinflammatory drivers in the system but may not necessarily exert antiinflammatory effects that would reduce inflammation.

Significance

There is still controversy in the scientific literature and governmental guidelines on the dietary and metabolic causes of obesity and inflammatory disease. In opposition to biochemistry and molecular biology based studies, many population studies still support the notion that dietary lipids are the main drivers of obesity, metabolic and inflammatory disease. Many population and clinical studies also point to total cholesterol or LDL bound cholesterol as the main markers of inflammatory and cardiovascular disease. Structural equation modeling allowed us to build systems-level pathway models connecting biomarkers of glucose and lipid metabolism, body adiposity, systemic and vascular inflammation, to test and compare different hypothetical models, and to statistically decide which best fit the data. We sought to test whether carbohydrate or lipid metabolism drive glucodysregulation, dyslipidemia, obesity and inflammation, and the pathway of effects connecting those physiological systems. Our analyses showed that carbohydrate and not lipid metabolism or inflammation sit at the genesis of metabolic and inflammatory dysfunction. The best fitting model indicated that glucose and insulin directly feed onto triglycerides and BMI, that triglycerides feed on LDL then BMI, both LDL and HDL exerted antiinflammatory effect on IL-6, while BMI exerted proinflammatory effect, HDL also exerted antiinflammatory effect on ICAM-1, CRP was driven by both BMI and IL-6, and ICAM-1 was driven by CRP. The strongest pathways effects followed from glucoregulation to BMI to IL-6 and CRP. According to our predictions, BMI was the main

gateway linking macronutrient metabolism and inflammation. By testing across diabetic status, we detected changes in biomarkers and pathways that were consistent with insulin resistance and diabetes, which serve as further support for our theoretical model. Biomarker and pathways were also affected by statin medications use, and showed a number of similarities to the alterations observed across diabetic status. Testing by statin use also indicated that the beneficial effects of statins may not be due to its cholesterol lowering effect, but rather by weakening proinflammatory pathways. Both findings regarding statins are in keeping with the recent examinations on the specific effects of statins. The results from these analyses corroborate with those of previous studies included in this thesis. It provides support that carbohydrate and not lipid metabolism is the main driver of obesity and inflammation and that body adiposity exerts important mediative effect and directly affecting inflammatory status. While these pathways have been individually characterized in small experimental models, our study was the first to examine them using a systems level perspective with epidemiological data.

TABLES

Table 1. Descriptive Statistics

	Mean	Std. Dev.
Age	54.76	11.79
Markes of glucoregulation		
Glucose (mg/dL)	101.61	27.22
Insulin (uIU/mL)	13.34	13.18
Glycated Hemoglobin (HA1c, %)	6.08	1.12
Markers of lipid metabolism		
Total Cholesterol (mg/dL)	185.39	38.67
HDL Cholesterol (mg/dL)	55.77	34.79
Triglycerides (mg/dL)	123.52	66.53
Calculated LDL Cholesterol (mg/dL)	104.91	18.03
BMI (kg/m²)	29.62	6.57
Markers of systemic inflammation		
Serum IL6 (pg/mL)	3.04	3.06
Blood C-Reactive Protein (mg/L)	3.04	4.86
Marker of vascular inflammation		
Serum Soluble ICAM-1 (ng/mL)	287.47	116.74

Total Participants with complete data	1161
Gender	
Females	664
Males	497
Race	
Caucasian	945
African-Descendent	216
Diabetic Status	
Non-Diabetic (HA1c < 5.7%)	373
Pre-Diabetic (5.7% ≤ HA1c < 6.5%)	599
Diabetic (HA1c ≥ 6.5%)	189
Medication	
Taking insulin targeted medications	34
Taking cholesterol absorption inhibitors	17
Taking statins (HMG-CoA reductase inhibitors)	279

Table 2. Data Covariance Matrix and eigen values

	Insulin	HA1c	BMI	Triglyceride	LDL	HDL	IL-6	CRP	ICAM-1	Glucose	Age
Insulin	104.149656										
HA1c	16.182745	52.219821									
BMI	30.466825	10.762017	37.694016								
Triglyceride	281.096277	69.303009	113.394859	4331.68797							
LDL	0.719136	-14.287648	13.627523	357.155137	1165.01441						
HDL	-61.222996	-11.456359	-33.001651	-557.3563	-77.277505	320.786208					
IL-6	45.009208	33.872335	36.936232	161.273317	-48.22518	-61.892308	538.26327				
CRP	7.624076	3.868063	7.950574	20.083907	7.20291	-5.823218	30.82105	10.058721			
ICAM-1	12.971023	8.876279	5.448138	107.531428	18.732993	-29.608438	29.7502	4.523658	110.013561		
Glucose	55.384904	79.602934	29.08475	313.467097	-19.129446	-49.562313	42.11489	5.966753	11.883751	286.274853	
Age	-7.12037	20.142007	-2.648277	-2.919548	-36.096637	9.140927	38.44178	-1.156954	18.469901	19.92459	137.617433
Eigen values (acceptable > 0)	4505.44055	1139.95076	547.853459	285.754142	242.618214	141.321215	98.218447	75.313942	26.911369	23.570085	6.827746

Table 3. Example of model selection

Model specification differences	Coefficient Estimate	S.E.	Z-value	P-value	Standardized Estimate
Final Model (Prefered)					
Insulin ~ Glucose	0.303	0.047	6.384	p<0.001	$0.350
Model Variation (Alternative)					
Glucose ~ Insulin	0.411	0.057	7.216	p<0.001	$0.355

SEM Statistics	Prefered	Alternative
Chi-Square	34.418	36.295
Degrees of freedom	27	27
P-value (Chi-square)	0.154	0.109
-Loglikelihood (H0)	25911.195	25912.268
Akaike (AIC)	51922.39	51924.535
Bayesian (BIC)	52144.277	52146.423

Physiologically, both effects are possible. Glucose elicits insulin responses which feedback by stimulating liver, muscle and adipose tissue to absorb glucose from the blood stream, thereby decreasing blood glucose. In comparing these two models, the directionality of the glucose-insulin relationship only makes physiological sense by considering the effet of glucose on insulin (prefered model). Futher, relative indices of model fit indicate better fit (smaller LL, AIC, BIC values) of the model which specified an effect of glucose on insulin (prefered model).

Table 4. Model fit statistics

Chi-Square	
Number of observations	626
Estimator: MLR, Robust	
Minimum Function Test Statistic	34.418
Degrees of Freedom	27
P-value	0.154
Scaling correction factor for the Yuan-Bentler correction	1.075
Root Mean Square Error of Approximation:	
RMSEA (90% C.I.)	0.021 (0.000-0.039)
P-value	0.998
Robust RMSEA	0.022
Standardized Root Mean Square Residual:	
SRMR	0.024
User model versus baseline model:	
Robust Comparative Fit Index (CFI)	0.993
Robust Tucker-Lewis Index (TLI)	0.987
Relative fit indices	
-Loglikelihood user model (H0)	25911.195
-Loglikelihood unrestricted model (H1)	25892.667
Akaike (AIC)	51922.390
Bayesian (BIC)	52144.277
Sample-size adjusted Bayesian (SABIC)	51985.534

Table 5. Model Parameter Estimates (robust Huber White)

Model regressions and R-square	Coefficient Estimate	S.E.	Z-value	P-value	Standardized Estimate
HA1c (15.7%)					
Glucose	0.007	0.001	5.161	<0.001	0.204
Age	0.010	0.001	9.810	<0.001	0.341
Insulin (17.5%)					
Glucose	0.303	0.047	6.384	<0.001	0.350
IL-6	0.590	0.190	3.181	0.001	0.136
Triglycerides (21.2%)					
Glucose	0.445	0.227	1.957	0.050	0.074
Insulin	2.962	0.302	9.795	<0.001	0.425
LDL (6.1%)					
Triglycerides	0.137	0.023	5.890	<0.001	0.252
HDL (0.06%)					
Age	0.119	0.055	2.144	0.032	0.076
BMI (35.0%)					
Insulin	0.265	0.038	6.896	<0.001	0.424
Glucose	0.053	0.019	2.773	0.006	0.098
HDL	-0.056	0.010	-5.550	<0.001	-0.182
LDL	0.013	0.006	2.239	0.025	0.076
IL-6 (11.7%)					
BMI	0.066	0.017	3.982	<0.001	0.179
Age	0.036	0.007	5.034	<0.001	0.205
HDL	-0.013	0.005	-2.869	0.004	-0.113
LDL	-0.006	0.003	-2.404	0.016	-0.102
CRP (29.1%)					
BMI	0.144	0.022	6.540	<0.001	0.286
IL-6	0.530	0.090	5.837	<0.001	0.389
ICAM-1 (7.9%)					
Age	1.500	0.350	4.286	<0.001	0.175
CRP	4.960	1.610	3.078	0.002	0.140
HDL	-0.820	0.230	-3.621	<0.001	-0.149

Non-causal covariances	Coefficient Estimate	Std.Err	Z-value	P-value	Standardized Estimate
Triglyceride					
HDL	-349.492	35.287	-9.904	<0.001	-0.351
Insulin					
HDL	-46.151	6.595	-6.998	<0.001	-0.315
HDL					
Glucose	-41.846	9.569	-4.373	<0.001	-0.225
HA1c					
Insulin	0.193	0.101	1.905	0.057	0.079
IL-6	7.143	2.667	2.678	0.007	0.123
LDL	0.751	0.409	1.835	0.066	0.076
ICAM-1	0.215	0.115	1.874	0.061	0.074

This model was build based on physiological relationships and revised based on the data for caucasian americans who were not diabetic (but including pre-diabetics), not being treated with insulin or cholesterol medications, and after 5% of cases exhibiting the most extreme Mahalanobis Distances were excluded.

Table 6. Relative fit statistics for differences by diabetic status

	Non- vs Pre-Diabetic		Pre- vs Diabetic		Non- vs Diabetic	
	Independent Parameter Estimates	Equal Parameter Estimates	Independent Parameter Estimates	Equal Parameter Estimates	Independent Parameter Estimates	Equal Parameter Estimates
Number of observations						
Non-Diabetic		304				304
Pre-Diabetic		436		436		
Diabetic				93		93
Chi-Square						
Minimum Function Test Statistic	82.091	114.932	72.318	140.103	94.395	161.649
Degrees of Freedom	54	75	54	75	54	75
P-value	0.008	0.002	0.049	<0.001	0.001	<0.001
Scaling correction factor for the Yuan-Bentler correction	0.992	1.081	0.932	1.013	0.921	1.000
Chi-Square for each group						
Non-Diabetic	50.902	70.945			54.833	60.137
Pre-Diabetic	31.189	43.988	33.212	38.297		
Diabetic			39.106	101.806	39.561	101.512
ANOVA for model fit						
Chi-Square Difference		32.682		60.983		62.078
Chi-Square DF Difference		21		21		21
P-value		0.050		<0.001		<0.001

Table 7. Parameter estimates by diabetic status (robust Huber White)

	Non-Diabetic			Pre-Diabetic			Diabetic			
Model regressions	Coefficient Estimate	S.E.	P-value	Coefficient Estimate	S.E.	P-value	Coefficient Estimate	S.E.	P-value	Difference in effect
HA1c										
Glucose	-0.001	0.001	0.570	0.004	0.001	<0.001	0.027	0.005	<0.001	Greater
Age	0.001	0.001	0.329	0.003	0.001	0.001	-0.003	0.009	0.698	Lesser
Insulin										
Glucose	0.267	0.058	<0.001	0.350	0.061	<0.001	0.100	0.043	0.020	Lesser
IL-6	0.800	0.360	0.027	0.180	0.180	0.311	0.030	0.320	0.915	Lesser
Triglycerides										
Glucose	0.404	0.389	0.298	0.746	0.253	0.003	1.019	0.384	0.008	Greater
Insulin	3.214	0.529	<0.001	2.298	0.402	<0.001	2.267	0.832	0.006	Lesser
LDL										
Triglycerides	0.154	0.039	<0.001	0.101	0.023	<0.001	0.099	0.042	0.019	Lesser
HDL										
Age	0.074	0.097	0.450	0.153	0.067	0.022	-0.065	0.134	0.628	Lesser
BMI										
Insulin	0.241	0.066	<0.001	0.279	0.034	<0.001	0.162	0.104	0.121	Lesser
Glucose	-0.006	0.028	0.824	0.058	0.023	0.011	0.031	0.027	0.242	Lesser
HDL	-0.044	0.015	0.004	-0.064	0.013	<0.001	-0.011	0.038	0.764	Lesser
LDL	0.017	0.008	0.024	0.010	0.008	0.210	0.014	0.017	0.412	Lesser
IL-6										
BMI	0.077	0.023	0.001	0.096	0.017	<0.001	0.063	0.031	0.040	Lesser
Age	0.014	0.008	0.105	0.034	0.009	<0.001	0.015	0.020	0.444	Lesser
HDL	-0.003	0.006	0.658	-0.009	0.006	0.124	-0.022	0.013	0.088	
LDL	0.000	0.003	0.976	-0.011	0.003	0.001	0.004	0.008	0.669	Lesser
CRP										
BMI	0.192	0.045	<0.001	0.149	0.027	<0.001	0.231	0.085	0.006	Greater
IL-6	0.440	0.140	0.001	0.510	0.090	<0.001	0.450	0.220	0.040	Greater
ICAM-1										
Age	1.690	0.540	0.002	1.540	0.480	0.001	-0.820	1.170	0.484	Lesser
CRP	2.200	1.470	0.136	6.350	2.060	0.002	1.780	1.670	0.286	Lesser
HDL	-0.670	0.310	0.031	-0.950	0.300	0.001	-1.760	0.690	0.011	Greater

Table 8. Differences in biomarkers by diabetes status

	One-Way Anova			Bonferroni Post-hocs (P-Values)		
	F-statistic	DFs	P-value	Non- vs Diabetic	Non- vs Pre-Diabetic	Pre- vs Diabetic
Glucose (mg/dL)	205.983	(2,863)	p<0.001	p<0.001	0.098	p<0.001
Insulin (uIU/mL)	16.076	(2,863)	p<0.001	p<0.001	0.017	p<0.001
Glycated Hemoglobin (HA1c, %)	518.885	(2,863)	p<0.001	p<0.001	p<0.001	p<0.001
Blood Triglycerides (mg/dL)	9.452	(2,863)	p<0.001	p<0.001	1.000	p<0.001
Blood LDL Cholesterol (mg/dL)	2.711	(2,863)	0.067	1.000	0.680	0.873
Blood HDL Cholesterol (mg/dL)	7.347	(2,863)	0.001	p<0.001	1.000	0.003
BMI (kg/m^2)	23.479	(2,863)	p<0.001	p<0.001	0.001	p<0.001
Serum IL6 (pg/mL)	8.076	(2,863)	p<0.001	p<0.001	0.066	0.033
Blood C-Reactive Protein (ug/mL)	8.499	(2,863)	p<0.001	p<0.001	0.116	0.013
Serum Soluble ICAM-1 (ng/mL)	6.608	(2,863)	0.001	0.003	0.030	0.238

Diabetic status was categorized by non-diabetics, pre-diabetics and diabetics by traditional clinical HA1c cutoffs.

Table 9. Relative fit statistics for differences by statin usage

	Independent Parameter Estimates	Equal Parameter Estimates
Number of observations		
Non-Users		740
Statin Users		205
Chi-Square		
Minimum Function Test Statistic	92.417	130.440
Degrees of Freedom	54	75
P-value	0.001	0.000
Scaling correction factor for the Yuan-Bentler correction	1.011	1.088
Chi-Square for each group		
Non-Users	48.369	54.282
Statin Users	44.048	76.158
ANOVA for model fit		
Chi-Square Difference		37.702
Chi-Square DF Difference		21
P-value		0.014

Table 10. Parameter estimates by statin usage (robust Huber White)

	Non-Users			Statin Users				
	Coefficient			Coefficient			Difference	
Model regressions	Estimate	S.E.	P-value	Estimate	S.E.	P-value	in effect	
HA1c								
Glucose	0.006	0.001	<0.001	0.007	0.002	0.001	Greater	*
Age	0.008	0.001	<0.001	0.003	0.002	0.224	Lesser	*
Insulin								
Glucose	0.327	0.045	<0.001	0.298	0.074	<0.001	Lesser	*
IL-6	0.410	0.160	0.011	0.010	0.290	0.972	Lesser	*
Triglycerides								
Glucose	0.603	0.213	0.005	0.413	0.435	0.343	Lesser	
Insulin	2.573	0.327	<0.001	2.634	0.576	<0.001	Greater	
LDL								
Triglycerides	0.126	0.021	<0.001	0.035	0.027	0.191	Lesser	*
HDL								
Age	0.108	0.052	0.039	0.124	0.095	0.191	Lesser	
BMI								
Insulin	0.271	0.032	<0.001	0.157	0.033	<0.001	Lesser	*
Glucose	0.042	0.019	0.024	0.071	0.029	0.012	Greater	
HDL	-0.054	0.010	<0.001	-0.035	0.021	0.090	Lesser	*
LDL	0.014	0.006	0.012	0.006	0.012	0.636	Lesser	*
IL-6								
BMI	0.089	0.014	<0.001	-0.022	0.047	0.638	Lesser	*
Age	0.031	0.007	<0.001	0.007	0.018	0.676	Lesser	
HDL	-0.007	0.004	0.097	-0.033	0.012	0.007	Greater	
LDL	-0.006	0.002	0.021	-0.007	0.006	0.262	Lesser	*
CRP								
BMI	0.164	0.024	<0.001	0.066	0.036	0.067	Lesser	
IL-6	0.490	0.080	<0.001	0.420	0.140	0.002	Lesser	
ICAM-1								
Age	1.670	0.340	<0.001	1.400	0.470	0.003	Lesser	*
CRP	4.950	1.450	0.001	4.360	3.160	0.168	Lesser	*
HDL	-0.830	0.210	<0.001	-0.570	0.380	0.138	Lesser	

*** Statin medication usage mimiced many key differences detected in diabetes**

Table 11. Differences in biomarkers by statin medication use

	T-statistic	DF	P-value
Glucose (mg/dL)	5.084	970	p<0.001
Insulin (uIU/mL)	3.957	970	p<0.001
Glycated Hemoglobin (HA1c, %)	4.140	970	p<0.001
Blood Triglycerides (mg/dL)	3.198	970	0.001
Blood LDL Cholesterol (mg/dL)	-9.364	970	p<0.001
Blood HDL Cholesterol (mg/dL)	-3.143	970	0.002
BMI (kg/m^2)	2.381	970	0.017
Serum IL6 (pg/mL)	1.115	970	0.265
Blood C-Reactive Protein (ug/mL)	-1.172	970	0.241
Serum Soluble ICAM-1 (ng/mL)	1.271	970	0.204

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Thesis Conclusion

Hypotheses and conclusions

The overarching goals of this dissertation were to assess the impact of body adiposity on two common population markers of chronic inflammation and to assess the mediating role of body adiposity on those markers of inflammation with focus on lipid and carbohydrate metabolism. I hypothesized that body adiposity would exert a major influence on both IL-6 and CRP, above and beyond diet and other metabolic factors, mediating a large proportion of the genetic, dietary and metabolic effects on CRP. Secondarily, I hypothesized that carbohydrates, at the dietary and metabolic levels, would have greater influence of adiposity and CRP than lipids. These questions were addressed through four studies, and determined:

1.1 <u>How various measures of body adiposity predict inflammatory status:</u> I hypothesized that BMI would be a good proxy for total body fat, and an acceptable predictor of visceral and non-visceral fat depots, especially in the context of its inflammatory effects. I further hypothesized that visceral body fat would exhibit the greatest influence over both IL-6 and CRP. Similarly, I expected that visceral fat would best reflect the effect of BMI on both IL-6 and CRP. Our results indicated that BMI is indeed a good proxy of total body fat in the study population. However, body fat non-uniformly reflected body fat depots; it better represented non-viscera than visceral stores.

Further, visceral and non-visceral fat depots differed in their effect on IL-6 and CRP. While visceral fat exerted a greater effect on IL-6 as hypothesized, non-visceral fat exhibited a greater influence on CRP than did visceral fat. That said, the differences in the contribution of visceral and non-visceral compartments was smaller for CRP than for IL-6. Further, visceral fat mediated the effect of BMI on IL-6 while non-visceral fat mediated the effect of BMI on CRP, meaning that the effect observed for BMI on IL-6 and CRP may reflect different anatomical and physiological pathways. In addition, we detected the effect of visceral and non-visceral fat on CRP was partially mediated by IL-6, suggesting the fat tissues influence CRP directly and indirectly through IL-6. These analyses support the role of body adiposity as a major predictor of baseline inflammation, and elucidate the proportional contribution of visceral and non-visceral fat depots. They also support the use of BMI as a measure of body adiposity, with the caveat that BMI reflects distinct fat depots in the prediction of IL-6 and CRP.

1.2. <u>The extent to which the heritability of inflammation is associated with body adiposity:</u> I hypothesized that the additive genetic effect unique to IL-6 is small or insignificant. Rather, that the additive genetic effect observed in the heritability of IL-6 is inflated by the influence of BMI. According to the hypothesis that BMI exerts defining influence over baseline levels of IL-6, I expected matched controls to exhibit an intra-class correlation that is similar in magnitude to that of monozygotic co-twins. I also hypothesized that both IL-6 and BMI would exert influence over the heritability of CRP. However, I expected the additive genetic effect on CRP to be influenced to a lesser extent by BMI than IL-6. We found no additive genetic effect acting uniquely on IL-6. Rather, the heritability of IL-6 was explained by its co-inheritance with BMI. BMI also influenced

the heritability of CRP. However, the additive genetic effects acting on CRP were only partially influenced by BMI. The heritability of CRP was not influenced by IL-6. Rather, they shared common environmental influences. While this may seem surprising given that BMI exerts strong heritable influence over both of them, according to the previous study, the effect of BMI on IL-6 and CRP were reflective of different anatomical pathways (visceral vs non-visceral), which may exhibit differential heritable effects. These analyses study build on the first study by elucidating the shared heritability between BMI, IL-6 and CRP, thereby demonstrating the extent to which body adiposity affects inflammatory biology.

2.1. The effect of dietary lipids and carbohydrates on body adiposity and inflammation, as well as the extent to which body adiposity mediates the effect of diet on inflammation: I hypothesized that diet would explain a significant proportion of the variance in BMI, and that dietary carbohydrates would exert a greater effect on adipose accumulation than lipids. Similarly, I hypothesized greater direct and indirect effect of carbohydrates on CRP, as well as a greater proportion of the effect of carbohydrates than lipids to be mediated through BMI. I also hypothesized that BMI would exert a greater effect on CRP than other markers of metabolic status. We found that carbohydrates accounted for the largest proportion of the effect of diet on BMI and CRP, while lipids accounted for very little. Specifically, dietary fiber and sugar exerted greatest effects among the macronutrients, while the effect of dietary cholesterol and saturated fat were relatively minor. We also found that diet exerted a direct as well as indirect effect on CRP, and that BMI mediated the effects of dietary carbohydrates but not lipids. Further, we showed that BMI is a potent predictor of CRP, far more than the other markers of

metabolic status, including blood cholesterol. Our analyses also identified dietary factors that were high in multicollinearity in the prediction of CRP – starch, monounsaturated fat and protein — while significant, their effects could not be reliably estimated. These analyses corroborate on the past two studies by further supporting the role of body adiposity as the major metabolic influence on inflammation and the major mediator of the effects of diet. Further, these analyses support that carbohydrates, not lipids, are major contributors to obesity and inflammation.

2.2. The pathways underlying the relationship between lipid and carbohydrate nutrient metabolism, adiposity and inflammation: I hypothesized a model in which carbohydrate metabolism would drive lipid metabolism and body adiposity and where the three systems would directly affect the measures of inflammation. I further hypothesized that this model would better fit the data than one that places lipid metabolism at the origin of the system. I also hypothesized that the magnitude of effects in the model would demonstrate that the flux of effects would point to BMI as a major mediator of the effects of carbohydrate metabolism on the inflammatory biomarkers. In addition, I expected that when diabetics and non-diabetics are compared in this model that we would observe alterations in path effects that are consistent with insulin resistance and diabetes. I also expected similar path effect alterations when users and non-users of statin medications are compared in the model, and that such model would reflect a weakening of the inflammatory pathways.

Our model showed that carbohydrates do indeed sit at the origin of the system, directly influencing lipid metabolism and adiposity, but only indirectly affecting inflammatory status. Path analyses showed that the indirect effect of carbohydrate

metabolism on inflammation was largely mediated by body adiposity. Our model better fitted the data than a model which was optimized with lipid metabolism at the origin of the system. Comparing across diabetic status also showed path effect alterations that were consistent with insulin resistance. In addition, our model indicated a weakening of the proinflammatory pathways as well as key resemblances to the diabetic profile. In further support to the thesis, and in accord to our previous studies, these analyses showed that carbohydrate metabolism sits at the origin of lipid dysfunction, obesity and inflammation and places body adiposity as the main mediator of those effects.

In conclusion, this thesis emphasizes the role of body adiposity as a proximal and defining influence on inflammatory status, and carbohydrates as the main driver behind metabolic dysregulation, adipose accumulation, and, ultimately, inflammation.

APPENDICES 1.

ANALYSES NOT INCLUDED IN THE DISSERTATION

Appendix 1.1.

Body Fat Distribution Differentially Predicts Interleukin-6 and C-Reactive Protein

Neuroendocrine interactions

Neuroendocrine factors exert metabolic and inflammatory effects at the systems level, orchestrating responses to stress, injury, infection, as well as energetic shifts (Straub, 2014). Cortisol and epinephrine exhibit varied regulatory effects on adipose tissue, including lipolysis, glucose uptake, cellular proliferation, differentiation and cell death (Hauner, Schmid, & Pfeiffer, 1987; Nomellini, Gomez, Gamelli, & Kovacs, 2009; Peckett, Wright, & Riddell, 2011; Romijn & Fliers, 2005; Straub, 2014). Systemically, the evidence suggests that cortisol and epinephrine can exert pro- or antiinflammatory effects depending on the magnitude and duration of the stimulus (Engdahl, Opperman, Yerrum, Monroy, & Daly, 2007; Yeager, Pioli, & Guyre, 2011), but their effects could also vary by tissue. Epinephrine has been shown to increase IL-6 production and release in fat (Goossens et al., 2008; Keller, Keller, Robinson, & Pedersen, 2004; V Mohamed-Ali et al., 2000; Vidya Mohamed-Ali et al., 2011) and muscle tissues during exercise (Frost, Nystrom, & Lang, 2004; Goossens et al., 2008; Steensberg, Toft, Schjerling, Halkjaer-Kristensen, & Pedersen, 2001), contributing significantly to systemic levels. However, this is not observed in peripheral blood cells (V Mohamed-Ali et al., 2000). Conversely, cortisol has been shown to suppress stimulated IL-6 in both muscle and fat tissues (Fried et al., 1998). To our knowledge, acute changes in either epinephrine or cortisol have not been shown to up- or down-regulate CRP in fat or muscle tissue. Additionally, it is not

known whether individual differences in cortisol and epinephrine at baseline levels might influence the regulation of IL-6 or CRP in those tissues.

Because catecholamines and glucocorticoids are known to influence IL-6 release as well as tissue function, we determined whether measures of systemic epinephrine and cortisol moderate the effect of body composition on the circulating levels of IL-6. To our knowledge, the effect of catecholamines and glucocorticoids on CRP production across body composition measures has not been reported. However, because neuroendocrine factors exert powerful effects over various tissues, we explored the possible interactions between each body composition measure and epinephrine or cortisol in predicting on CRP.

Methods

Urine Cortisol: Urine was collected overnight during the hospital stay. Urine specimens were aliquoted and frozen until analyses in the -60° to -80° freezer. High-Pressure Liquid Chromatography (HPLC) coupled with electrochemical detection was used for quantification. The assay was performed at Mayo Medical Laboratories (MN). Urinary cortisol was adjusted for creatinine concentration to control for blood filtration rate, thus estimating the concentration in the blood. Epinephrine: Urine was collected overnight during the hospital stay. Urine species were stabilized to ph ≤ 5, then frozen until analyses in the -60° to -80° freezer. HPLC mass spectrometry was used for quantification. The assay was performed at MN. Urinary epinephrine was adjusted for creatinine excretion to control for blood filtration rate and therefore estimate the concentration in the blood.

To test the interactive effects of neuroendocrine factors and DXA body composition components, the regression models were adjusted for potentially confounding covariates (i.e., age, gender, race, HDL-cholesterol, non-HDL-C, and the use of blood pressure or corticosteroid medications).

Results

Simultaneous multiple regression models were designed where the interaction of systemic measures of cortisol and epinephrine with each of the three DXA components were tested in predicting IL-6 and CRP. These models also included covariates for model adjustments. VIFs were checked in each regression model for all independent variables. All variables exhibited low multicollinearity, indicating acceptable accuracy in coefficient estimations. In the regression of IL-6 in the adjusted model, we found two significant interactions. These were observed between cortisol and lean body mass (β=-7.816x10^-7, $F(1,190)=8.34$, $p=0.004$), as well as non-visceral fat and epinephrine (β=-4.281x10^-6, $F(1,190)=5.83$, $p=0.017$). While these interactions predicted small changes in tissue effect on IL-6, they explained 3.07% and 2.15% of the variance, respectively. The main effect of cortisol was shown to be non-significant, therefore considered negligible ($F(1,190)=1.568$, $p=0.212$). As baseline cortisol levels increased in the population sample, the effect of lean body mass on IL-6 became increasingly negative. However, these analyses indicate that the effect of increased cortisol may work, in part, through lean tissue to lower IL-6. As with cortisol, the main effect of epinephrine in predicting IL-6 was negligible ($F(1,190)=0.129$, $p=0.720$). However, as baseline epinephrine levels increased in the sample, the effect of non-visceral fat on IL-6 was attenuated. This may

reflect antiinflammatory actions of epinephrine on non-visceral adipose tissue. Notwithstanding, the non-variance based effects sizes for both interactions were relatively small, which may be expected at non-stimulated systemic levels. No interactions were found to act on visceral fat's effect on IL-6. In the regression of CRP, adjusted for the aforementioned covariates, we did not detect any neuroendocrine interactions in regard to visceral fat, non-visceral fat or lean tissue.

Discussion

Our test for neuroendocrine interactions showed that higher levels of baseline cortisol predicted a greater antiinflammatory effect of lean body mass on IL-6. An interaction effect was not detected between cortisol and adipose tissues. This may be reflective of higher cortisol sensitivity by muscle in the production or release of IL-6. One study has suggested that muscle expression of glucocorticoid receptors is elevated in metabolic syndrome, which may help explain our findings (Whorwood, Donovan, Flanagan, Phillips, & Byrne, 2002). In turn, higher levels of epinephrine were associated with an attenuation of the proinflammatory effect of non-visceral fat on IL-6. This finding is in keeping with another study that showed beta-adrenergic receptor sensitivity in subcutaneous adipose tissue, was greater in individuals with high visceral adiposity (Wajchenburg, 2014). Other interactions of cortisol and epinephrine with DXA body composition measures did not predict differences in IL-6 levels. In addition, no interaction between these neuroendocrine biomarkers and DXA measures predicted differences in systemic CRP. Small or absent effects for both cortisol and epinephrine are similar to results from other studies showing likewise neutral effects of neuroendocrine factors at

baseline. Cortisol and epinephrine are powerful stimulants and orchestrators of energy metabolism, immunologic and neurological alteration. While acute changes may bring about easily detectable physiological alterations, baseline levels are likely to contribute to fine adjustments to homeostatic balance. In this study, we did not find other interaction effects due to a relatively small number of participants, given measures (neuroendocrine) that usually show a high individual variability. Our measures of cortisol and epinephrine were also distal, urine samples that were adjusted for creatinine. Direct blood neuroendocrine analyses would likely yield more reliable measures that may help disclose other interactive effects with fat or lean tissues.

Appendix 1.2.

Shared Heritability Between Adiposity and Inflammation

The Soluble Interleukin-6 Receptor (sIL-6r)

Although blood levels of IL-6 are often quantified in isolation, its biological actions are determined by two distinct membrane bound glycoproteins expressed on the surface of target cells: 1) a classical transmembrane IL-6 receptor (mIL-6r), and 2) a signal-transducing non-ligand binding subunit, gp130, which is activated by a complex formed by IL-6 and the soluble form of the IL-6 receptor (sIL-6r) (Kallen, 2002; Peters, Müller, & Rose-John, 1998). Further, the sIL-6r has also been shown to moderate central actions of IL-6 within the brain (Schöbitz, Pezeshki, & Pohl, 1995). Therefore, to more completely understand variation in IL-6 synthesis and responses across individuals, it is important to also quantify the soluble receptor, which was done in the following study. IL-6 and sIL-6r are coded by different genes and controlled by distinct mechanisms of expression (Crichton, Nichols, Zhao, Bulun, & Simpson, 1996; S. A. Jones, Horiuchi, Topley, Yamamoto, & Fuller, 2001; Lust et al., 1992). In addition, sIL-6r may be produced either by alternative mRNA splicing or by proteolytic cleavage and shedding from the surface of cells (S. A. Jones et al., 2001; Müllberg et al., 1993). Thus, it is of significance to determine the extent and similarity of the genetic constraints on IL-6 and its soluble receptor. SNPs affecting sIL-6r production appear to be more consistently associated with its levels in systemic circulation (Galicia et al., 2004; Rafiq, Frayling, Murray, & Hurst, 2007; Sasayama et al., 2012; van Dongen et al., 2014). sIL-6r can serve as an independent biomarker for certain pathological conditions. It does not always react to the

same physiological stimuli as IL-6; it is not always responsive to changes in IL-6 levels, and it does not play a role in all IL-6 pathways (Hurst et al., 2001; Kallen, 2002; Lamas et al., 2013; Mehra et al., 2006; Montero-Julian, 2001; Peters et al., 1998). Hence, we could infer whether BMI and demographics (e.g., age, gender and SEI) accounted for the concordance between matched controls and co-twins. Given the likelihood of strong genetic constraints on sIL-6r, we anticipated small or negligible ICCs for sIL-6r with the unrelated, matched controls.

Methods

Sandwich ELISA kits were also employed to quantify sIL-6r levels (Quantikine, R&D Systems). Sera were diluted 1:100 so values would fall on the standard reference curve from 7 to 2000 pg/mL. Thus, the effective assay range for sIL-6r was 0.7–200 ng/mL. The intra-assay and inter-assay CVs was 6.9%. sIL-6r was natural log-transformed before statistical calculations. The same approach described for IL-6 and CRP was used in order to test the genetic covariance shared between BMI and sIL-6r.

Results

After adjusting the regression models for age and gender by including them as covariates, BMI accounted for only 0.3% of the variance in sIL-6r ($\beta = 0.07$, $F[1,829] = 3.2$, $p = 0.02$). Although sIL-6r also showed some covariance with genetic factors associated with BMI (33.7%, Table 4), path coefficients indicated the effects attributable to BMI were minimal (Figure 2). In addition, a much larger portion of the total phenotypic variance was unique to sIL-6r (Figure 2). In contrast to IL-6, the sIL-6r levels were more

similar for MZ twins, while DZ twins were divergent, supporting the greater heritability of sIL-6r. Stronger genetic constraints on sIL-6r were also clearly evident when comparing the trait ICCs between the twins and unrelated adult controls who had been matched on BMI, gender, age, and SEI (Table 2).

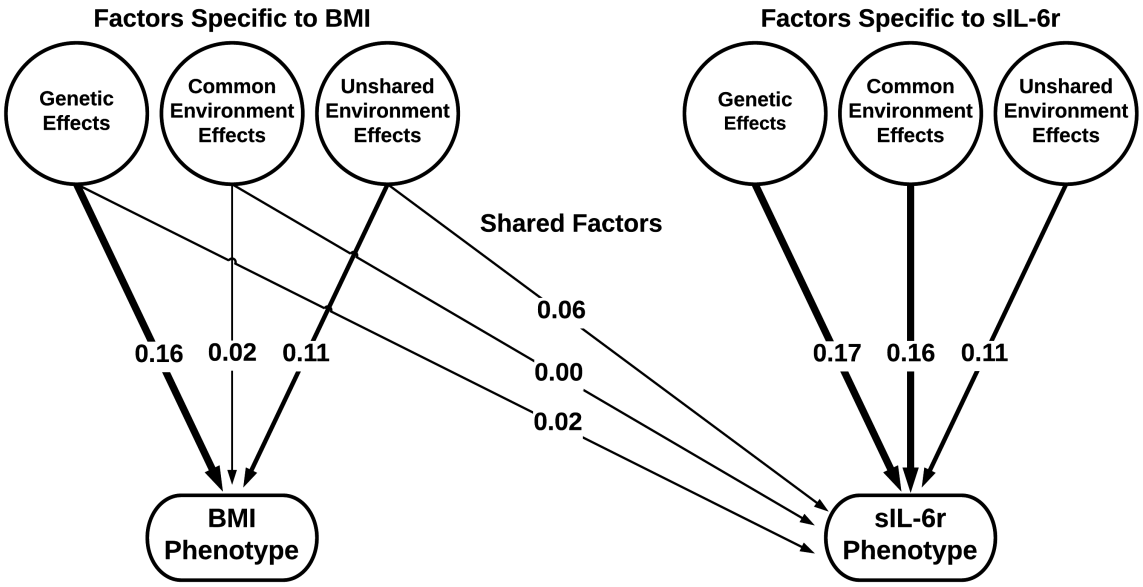

Fig. 2 The path coefficient estimates for the bivariate BMI X sIL-6r model also parsed the genetic effects specific to sIL-6r from those shared with BMI. This model similarly points to a shared covariance between genetic and unshared environmental effects. However, most of the additive genetic effects explaining the sIL-6r phenotype were independent of BMI, thus very distinct from the pattern of genetic constraints found for IL-6.

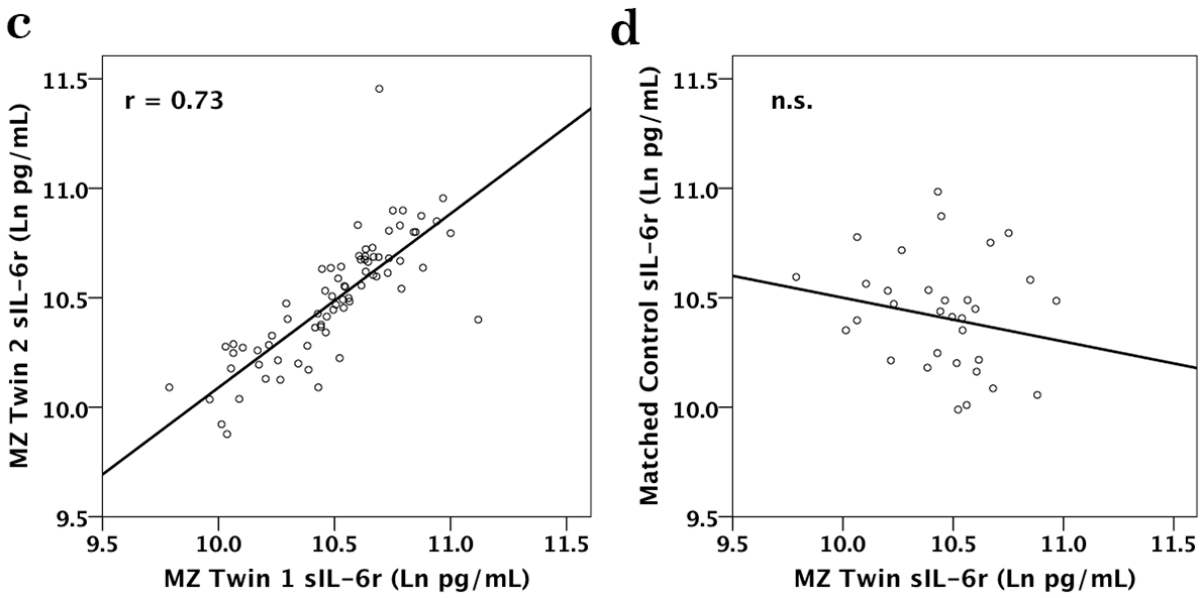

Fig. 5 Levels of sIL-6r in MZ co-twins were highly correlated (C), while sIL-6r values were not significantly correlated between MZ cases and their unrelated, matched controls (D).

Discussion

By taking advantage of the fact that monozygotic twins are genetically more similar than dizygotic twins, our analyses demonstrated that the genetic constraints on IL-6 and its soluble receptor are clearly distinct. In addition, the association between BMI and IL-6 was more evident than for sIL-6r. This differential relationship with adiposity affected the heritability estimates. In contrast to IL-6, the sIL-6r values were markedly discordant, confirming that the genetic constraint on the soluble receptor was less influenced by life style and other environmental factors.

Several polymorphisms that affect the production and shedding of the soluble IL-6 receptor, including rs2228145, rs2228146, rs2229238, rs4072391, rs4537545 and rs8192284, reliably account for variation in basal levels (Ferreira et al., 2013; Lamas et al., 2013; Marinou, Walters, Winfield, Bax, & Wilson, 2010; Rafiq et al., 2007; Reich et al.,

2007; Rodríguez-Rodríguez et al., 2011; Sasayama et al., 2012; H. Wang et al., 2005). These SNPs have already been linked to poor prognosis in clinical conditions, including chronic inflammation, metabolic and psychological disorders (Ferreira et al., 2013; Hamid et al., 2004; Huth et al., 2006; Lamas et al., 2013; Marinou et al., 2010; Sasayama et al., 2012; Stephens et al., 2012; Zhang, Feng, Wu, & Xie, 2013). Genetic admixture mapping also indicates robust correlations of the sIL-6r variation in blood to ancestry (Reich et al., 2007). Although both IL-6 and its soluble receptor play important roles in inflammatory processes, the production of sIL-6r seems to be more tightly regulated, while IL-6 is more sensitive to life style, diet and other factors associated with adiposity.

Table 2

Intra-class correlations and statistical difference between coefficients demonstrating MZ and DZ twins had similar values for IL-6, but differed for sIL-6r

	Intra-Class Correlation Coefficients and 95% C.I.						Fisher's r-to-z, z-values	
	MZ		DZ		MC		MZ vs. DZ	MZ vs. MC
sIL-6r	0.73 (.62 - .80)	p<0.001	0.48 (.17 - .70)	P=0.002	-0.20 (-.51 - .16)	n.s.	1.84 p=.03	5.41 p<0.001

MZ, Monozygotic Twins; DZ, Dizygotic Twins; MC, Matched Controls

Table 3
Comparison of nested model statistics

Covariance Models		-2LL	D.F.	AIC	BIC	Difference χ^2	p-value
BMI X sIL-6r	ACE	-216.23	405	-1026.23	-1050.54		
	CE	-215.63	406	-1027.63	-1052.57	0.60	0.44
	AE	**-215.99**	**406**	**-1027.99**	**-1052.75**	**0.25**	**0.62**

-2LL (-2 * Log-Likelihood), maximum likelihood estimate; AIC, Akaike's Information Criterion; BIC, Bayesian Information Criterion; Lower -2LL, AIC and BIC indicate models that more parsimoniously fit the data; Non-significance in chi-square (χ^2) values between saturated and nested models indicate equivalent fit of data; Bold highlighted font indicates the model that most parsimoniously fit the data, based on AIC and BIC.

Table 4
Proportion of genetic and environmental effects for best-fit models

Variance / Covariance Components	Estimated Effects (95% C.I.)		
	Additive genetic (A)	Common Environment (C)	Unshared Environment (E)
BMI	68.2% (21.1-78.9)	1.3% (1.1-44.7)	30.5% (21.1-44.0)
sIL-6r	42.2% (7.7-82.9)	35.6% (0-67.9)	22.2% (15.3-32.2)
Covariance	33.7%		66.3%

Best-fit models were chosen on the basis of the AIC and BIC.

Appendix 1.3.

The Effect of Dietary Carbohydrates and Lipids on Adiposity and Inflammation in the Context of the American Diet

The effect of Protein on BMI and CRP

At average intake, protein intake predicts greater BMI. However, that relationship becomes smaller with increasing protein intake (β=1.98, $F(1, 14923)$=21.973, $p<0.001$, ΔR^2= 0.13%, VIF=6.746). One standard deviation increase in protein intake (SD=36.52 grams) predicted 1.98% greater BMI, but for every standard deviation increase in protein intake, its effect dropped by 0.68% (β=0.68, $F(1, 14923)$=22.981, $p<0.001$, ΔR^2= 0.14%, VIF=4.321).

Protein also predicted higher CRP values before and after accounting for metabolic status (β=12.79, $F(1,14918)$=26.75, $p<0.001$, ΔR^2=0.16% and β=6.11, $F(1,14918)$=7.57, $p=0.006$, ΔR^2=0.04%, respectively). Although protein showed a quadratic relationship where its effect increased with higher protein intake, this relationship was negligibly small for both before and after accounting for metabolic status (β=0.00, $F(1,14918)$=16.34, $p<0.001$ and β=0.00, $F(1,14888)$=4.35, $p=0.04$, respectively). However, the estimated effects of protein intake on CRP showed low reliability due to multicollinearity (VIF=6.28), hence these estimates should be very cautiously considered. Mediation analyses showed that BMI did not mediate the effect of protein on CRP ($p=0.65$).

The effect of specific fatty acids on CRP

When specific fatty acids were tested by VIF-based stepwise backward elimination, only eicosapentaenoic acid (EPA) (PUFA 20:5) remained (VIF=1.102). One standard deviation increase in EPA (SD=0.11 grams) intake predicted 2.66% lower CRP (β=-2.66, F(1,14923)=6.65, p=0.010, ΔR^2=0.04%) before accounting for metabolic status, but not after (β=-1.36, F(1,14888)=2.21, p=0.138, ΔR^2=0.01%), indicating that most of its effect may have been mediated by metabolic factors. When specific fatty acids were tested by significance-based stepwise backward elimination, five fatty acids remained significant: docosahexaenoic acid (DHA) (PUFA 22:6), caproic acid (SFA 6:0), caprylic acid (SFA 8:0), capric acid (SFA 10:0) and stearic acid (SFA 18:0). One standard deviation increase in DHA (SD=0.17 grams) intake reliably predicted 2.15% lower CRP before but not after accounting for metabolic status (β=-2.15, F(1,14918)=3.91, p=0.048, ΔR^2=0.02% and β=-1.19, F(1,14888)=1.39, p=0.238, ΔR^2=0.01%, respectively, and VIF= 1.21), indicative of mediation by metabolic factors. While these effect estimates seem low, it is noteworthy that the average consumption of EPA and DHA in these population samples were very low (0.04 and 0.08 grams, respectively). Non-standardized effect sizes indicated that for every one gram increase in EPA and DHA intake, CRP is predicted to decrease by 23.8% and 12.7%, respectively. The medium chain fatty acids, caproic acid, caprylic acid and capric acid were significant before (β=8.89, F(1,14918)=7.66, p=0.006, ΔR^2=0.05%, β=6.56, F(1,14918)=5.62, p=0.018, ΔR^2=0.03% and β=-14.69, F(1,14918)=9.83, p=0.002, ΔR^2=0.06%, respectively), as well as after accounting for metabolic factors (β=13.50, F(1,14888)=22.16, p<0.001, ΔR^2=0.0.11%, β=2.47%, F(1,14888)=9.34, p=0.002, ΔR^2=0.05% and β=-17.68, F(1,14888)=17.88, p<0.001, ΔR^2=0.09%, respectively). However, none of them were reliable predictors based on their VIFs (10.56,

7.85 and 22.45, respectively). Stearic acid was significant before but not after accounting for metabolic status, though this fatty acid too exhibited low reliability (β=7.73, F(1,14918)=8.82, p=0.004, ΔR^2=0.05% and β=0.79, F(1,14888)=0.11, p=0.74, ΔR^2=0.00%, VIR=7.37, respectively). Overall, when saturated fatty acids appeared to significantly predict CRP, their VIFs exhibited high degrees of multicollinearity, indicating that their effect estimates were not sufficiently reliable for interpretations.

Exploratory effects and interactions predicting CRP

Adjusted for the intake of macronutrients, total caloric intake only exhibited a significant effect before, but not after, accounting for metabolic status (β=-18.79, F(1,14918)=12.94, p<0.001, ΔR^2=0.08% and β=-4.31, F(1,14888)=0.84, p=0.358, ΔR^2=0.00%, respectively). However, analyses of multicollinearity indicated unacceptable estimate reliability (VIF=28.22).

Sodium, potassium and magnesium have previously been associated to metabolic health and inflammatory physiology, hence these were also included in our models. Sodium predicted higher CRP, while magnesium predicted lower CRP before accounting for metabolic status (β=5.52, F(1,14918)=12.63, p<0.001, ΔR^2=0.08%, VIF=2.47, and β=-10.33, F(1,14918)=26.74, p<0.001, ΔR^2=0.16%, VIF=4.11, respectively). Magnesium, but not sodium, remained significant after accounting for metabolic status (β=-6.49, F(1,14888)=13.16, p,<0.001 ΔR^2=0.06% and β=2.25, F(1,14888)=2.64, p=0.104, ΔR^2=0.01%, respectively). The decrease in variance and non-variance based effect sizes for sodium intake were suggestive of mediation by metabolic status. Potassium exhibited no effect on CRP (statistics not shown).

The only non-linear macronutrient effect found was for protein intake after accounting for metabolic status, such that the effect of protein on CRP decreased with higher protein intake (β=-1.44, $F(1,14888)$=4.35, p=0.037, ΔR^2=0.02%).

Certain macronutrients also seemed to affect individuals differently by gender and race. The effect of protein and starch on CRP were more proinflammatory (or less antiinflammatory) for women than for men before accounting for metabolic status (β=7.90, $F(1,14918)$=6.87, p=0.009, ΔR^2=0.04% and β=7.50, $F(1,14918)$=6.29, p=0.012, ΔR^2=0.04%, respectively). Gender continued to predict a more proinflammatory effect of protein on CRP for females then for men after accounting for metabolic factors (β=8.92, $F(1,14888)$=9.83, p=0.002, ΔR^2=0.05%). That was not the case for the effect of starch which seemed to have been mediated by metabolic status (β=-1.07, $F(1,14888)$=0.60, p=0.438, ΔR^2=0.00%). The effect of starch on CRP was also less antiinflammatory for Caucasians then for Non-Hispanic blacks before and after accounting for metabolic status (β=-8.89, $F(1,14918)$=6.57, p=0.010, ΔR^2=0.09% and β=-7.28, $F(1,14888)$=5.48, p=0.019, ΔR^2=0.08%, respectively).

Our regression models also revealed an interaction between magnesium intake and one marker of metabolic health, as well as interactions between various metabolic factors. Increased magnesium intake was associated with the attenuation of HA1c's proinflammatory effect on CRP (β=2.61, $F(1,14888)$=10.54, p=0.001, ΔR^2=0.05%). Higher HDL was also predictive of a lessened effect of HA1c on CRP (β=-3.27, $F(1,14888)$=10.469, p=0.001, ΔR^2=0.05%). Conversely greater total blood cholesterol was associated with a diminished effect of HDL on CRP (β=3.61, $F(1,14888)$=15.288, $p<0.001$, ΔR^2=0.07%). Further, greater BMI predicted a smaller effect of systolic blood

pressure on CRP (β=-7.55, $F(1,14888)=59.07$, $p<0.001$, $\Delta R^2=0.29\%$), perhaps indicating that higher systolic blood pressure at higher BMI is at least partially due to the increased demand of a larger body mass, rather than a sole product of metabolic dysfunction. Greater BMI also predicted a lowering effect of diastolic blood pressure on CRP (β=4.09, $F(1,14888)=17.95$, $p<0.001$, $\Delta R^2=0.09\%$).

We also found a significant interaction between starch and the year of data collection both before and after adjusting for metabolic status (B=1.29, $F(1,14918)=8.137$, $p=0.004$, $\Delta R^2=0.05\%$ and β=1.11, $F(1,14918)=7.546$, $p=.006$, $\Delta R^2=0.4\%$, respectively), where the effect of starch on CRP is more proinflammatory across time than other macronutrients. NHANES reports that there were no changes in CRP assay technique, equipment or location, and no intra-assay differences, nor changes in dietary data survey strategies for the cohorts included in this study. Hence, our results raise the possibility of a change in the starch quality or composition (resistant versus non-resistant starch) over time in the population.

Appendix 1.4.

Pathways Linking Carbohydrate and Lipid Metabolism, Adiposity and Inflammation

Differences by race and sex

Model effects were also compared across sex and race. Prior investigations of this and other datasets have shown differences in adiposity and inflammation between Caucasian and African Americans, but the systems-levels assessment of the relationship between those biomarkers has not been examined. To test the effect of race and sex, insulin targeted and statin medication users as well as diabetics were excluded from analysis.

Results

A subsample of non-diabetic participants who were not being treated with insulin or statin medications were used to assess SEM differences by race and sex. Chi-Square difference tests show that fitting our hypothetical model to the data parsed by either race or sex was significantly different than fitting the model to all participants without distinction of race or sex (Tables 12 & 14). Thus, SEM regression estimates were computed and compared between Caucasian and African Americans, and between Males and Females.

Compared to Caucasians, African Americans exhibited a markedly greater effect of glucose on insulin and triglycerides, but a lower effect of insulin on triglycerides (Table 13). A greater effect of glucose on insulin may be indicative of greater insulin sensitivity. However, a greater effect of glucose on triglycerides may be indicative of consumption of

high glycemic loads. While most effects on BMI were weaker in African Americans than in Caucasians, they exhibited a slightly greater effect of insulin (Table 13). On the other hand, BMI exhibited much greater effect on IL-6 and CRP in African Americans, whereas other pathway effects feeding onto those IL-6 ad CRP were weaker (Table 13). ICAM-1 was also under greater influence of CRP and lesser influence of HDL among African-Americans. In general, HDL and LDL exerted weaker outward path effects amongst African Americans (Table 13). In this subsample, the major route of effects was mediated through insulin and BMI, reflecting effect routes of vulnerability. In addition, African Americans presented greater average levels of all metabolic and systemic inflammation biomarkers, except for having lower triglycerides and LDL (Table 16). Despite the unfavorable biomarker profile, there were no mean difference in ICAM-1, possibly indicating greater vascular tolerance to metabolic stress and systemic inflammation.

When looking at gender, females exhibited a higher effect of glucose on insulin, which may be indicative of greater insulin sensitivity (Table 15). In comparison to the effect of IL-6 on insulin, glucose and insulin on triglycerides, and biomarkers on ICAM-1 - females exhibited overall greater sensitivity throughout systems of metabolism and systemic inflammation (Table 15). While females presented with more favorable mean biomarker levels than males, they also presented greater indices of systemic inflammation (Table 17). According to our results, ICAM-1 appeared less sensitive in females, which could also reflect greater inflammatory tolerance at the vascular level.

Discussion

We also found differences across race as well as sex. Compared to Caucasian participants, African American participants presented with more pro-diabetic profiles, as well as greater adiposity and higher levels of systemic inflammation. However, they presented with better blood lipid profiles and no sign of vascular inflammation. Accordingly, our SEM path analyses indicated multiple differences in model effects that resembled diabetes and a greater effect of BMI on both IL-6 and CRP, in metabolic and inflammatory systems. While the effect of CRP on ICAM-1 were found to be higher compared to Caucasian participants, their ICAM-1 levels were no different. Altogether, this data indicates a greater risk of metabolic syndrome and greater proinflammatory potential of adiposity among African Americans, but with lesser vascular inflammatory response. While this finding could indicate greater vascular resilience, reports show a greater incidence of cardiovascular disease among African-Americans than among Caucasians (Dariush Mozaffarian et al., 2016). While African American showed higher mean HA1c levels, path analyses showed that the effect of glucose on HA1c were smaller than for Caucasians. This may be a reflection that HA1c assessment were biased by race because of differences in the molecular structure of hemoglobin by race, which interfere with accurate assay detectability of the glycated hemoglobin molecules (Herman & Cohen, 2012; Ziemer et al., 2010). Females exhibited greater overall sensitivities throughout whole systems, some of which may be beneficial (e.g., greater insulin sensitivity to glucose) and others that may pose higher risk (e.g., greater effects of age and BMI on downstream components, and greater general effects on BMI, IL-6 and CRP). Accordingly, females exhibited healthier metabolic profiles and worse inflammatory profiles. In spite of higher systemic inflammatory markers, females also exhibited smaller

effects on ICAM-1, while exhibiting similar concentrations, which may indicate greater tolerance to systemic inflammation. Females also showed greater HA1c while predicted to be under less of an impact of glucose when compared to males, which is in accordance with the sex differences in blood hemoglobin levels (J. C. Bae et al., 2014).

Table 12. Relative fit statistics for race differences

	Independent Parameter Estimates	Equal Parameter Estimates
Number of observations		
Caucasian		626
African-Descendent		114
Chi-Square		
Minimum Function Test Statistic	74.469	108.009
Degrees of Freedom	54	75
P-value	0.034	0.008
Scaling correction factor for the Yuan-Bentler correction	0.997	1.010
Chi-Square for each group		
Caucasian	37.126	41.605
African-Descendent	37.343	66.404
ANOVA for model fit		
Chi-Square Difference		33.388
Chi-Square DF Difference		21
P-value		0.042

Table 13. Relative fit statistics for sex differences

	Independent Parameter Estimates	Equal Parameter Estimates
Number of observations		
Male		303
Female		437
Chi-Square		
Minimum Function Test Statistic	100.801	135.576
Degrees of Freedom	54.000	75.000
P-value	0.000	0.000
Scaling correction factor for the Yuan-Bentler correction	1.041	1.117
Chi-Square for each group		
Male	42.984	64.945
Female	57.817	70.631
ANOVA for model fit		
Chi-Square Difference		35.437
Chi-Square DF Difference		21
P-value		0.025

Table 14. Parameter estimates by race (robust Huber White)

Model regressions and R-square	Caucasian Americans Coefficient Estimate	S.E.	P-value	African Americans Coefficient Estimate	S.E.	P-value	Difference in effect	
HA1c								
Glucose	0.007	0.001	<0.001	-0.001	0.004	0.899	Lesser	
Age	0.010	0.001	<0.001	0.002	0.004	0.584	Lesser	*
Insulin								
Glucose	0.303	0.047	<0.001	0.402	0.123	0.001	Greater	
IL-6	0.590	0.190	0.001	-0.300	0.350	0.400	Lesser	*
Triglycerides								
Glucose	0.445	0.227	0.050	1.505	0.479	0.002	Greater	*
Insulin	2.962	0.303	<0.001	1.629	0.688	0.018	Lesser	*
LDL								
Triglycerides	0.137	0.023	<0.001	0.027	0.039	0.497	Lesser	*
HDL								
Age	0.119	0.055	0.032	0.190	0.168	0.258	Lesser	*
BMI								
Insulin	0.265	0.038	<0.001	0.273	0.053	<0.001	Greater	
Glucose	0.053	0.019	0.006	-0.029	0.058	0.617	Lesser	*
HDL	-0.056	0.010	<0.001	-0.063	0.026	0.016	Greater	
LDL	0.013	0.006	0.025	0.035	0.018	0.058	Lesser	*
IL-6								
BMI	0.066	0.017	<0.001	0.113	0.025	<0.001	Greater	
Age	0.036	0.007	<0.001	0.029	0.021	0.154	Lesser	*
HDL	-0.013	0.005	0.004	0.005	0.011	0.655	Lesser	
LDL	-0.006	0.003	0.016	0.004	0.006	0.544	Lesser	*
CRP								
BMI	0.144	0.022	<0.001	0.233	0.062	<0.001	Greater	*
IL-6	0.530	0.090	<0.001	0.310	0.130	0.018	Lesser	
ICAM-1								
Age	1.500	0.350	<0.001	1.460	1.290	0.260	Lesser	*
CRP	4.960	1.610	0.002	6.660	3.470	0.055	Greater	
HDL	-0.820	0.230	<0.001	-0.430	0.590	0.474	Lesser	

*** Dissimilarities that mimic some of the key differences detected in diabetes**

Table 15. Parameter estimates by sex (robust Huber White)

Model regressions and R-square	Male Coefficient Estimate	S.E.	P-value	Female Coefficient Estimate	S.E.	P-value	Difference in effect	
HA1c								
Glucose	0.072	0.024	0.002	0.064	0.015	<0.001	Lesser	
Age	0.056	0.018	0.002	0.097	0.012	<0.001	Greater	
Insulin								
Glucose	0.243	0.061	<0.001	0.382	0.072	<0.001	Greater	
IL-6	0.057	0.022	0.008	0.030	0.022	0.172	Lesser	*
Triglycerides								
Glucose	0.644	0.298	0.031	0.344	0.287	0.230	Lesser	
Insulin	2.994	0.406	<0.001	2.189	0.468	<0.001	Lesser	*
LDL								
Triglycerides	0.115	0.030	<0.001	0.137	0.030	<0.001	Greater	
HDL								
Age	-0.029	0.072	0.686	0.173	0.066	0.009	Greater	
BMI								
Insulin	0.228	0.044	<0.001	0.303	0.048	<0.001	Greater	
Glucose	0.022	0.026	0.381	0.058	0.027	0.032	Greater	
HDL	-0.053	0.016	0.001	-0.069	0.015	<0.001	Greater	
LDL	0.011	0.007	0.111	0.015	0.008	0.066		
IL-6								
BMI	0.799	0.232	0.001	0.914	0.177	<0.001	Greater	
Age	0.226	0.103	0.028	0.375	0.092	<0.001	Greater	
HDL	-0.028	0.084	0.740	-0.157	0.061	0.010	Greater	
LDL	-0.043	0.030	0.147	-0.072	0.035	0.038	Greater	
CRP								
BMI	0.121	0.029	<0.001	0.188	0.032	<0.001	Greater	
IL-6	0.041	0.007	<0.001	0.051	0.011	<0.001	Greater	
ICAM-1								
Age	0.148	0.047	0.002	0.169	0.047	<0.001	Greater	
CRP	1.125	0.446	0.012	0.265	0.133	0.046	Lesser	*
HDL	-0.108	0.037	0.003	-0.089	0.029	0.002	Lesser	

Table 16. Differences in biomarkers by race

	T-statistic	DF	P-value
Glucose (mg/dL)	1.898	762	0.058
Insulin (uIU/mL)	3.178	762	0.002
Glycated Hemoglobin (HA1c, %)	2.760	762	0.006
Blood Triglycerides (mg/dL)	-3.034	762	0.002
Blood LDL Cholesterol (mg/dL)	-3.053	762	0.002
Blood HDL Cholesterol (mg/dL)	2.311	762	0.021
BMI (kg/m^2)	5.798	762	p<0.001
Serum IL6 (pg/mL)	5.542	762	p<0.001
Blood C-Reactive Protein (ug/mL)	3.286	762	0.001
Serum Soluble ICAM-1 (ng/mL)	-1.133	762	0.258

Adjusted by age and sex

Table 17. Differences in biomarkers by sex

	T-statistic	DF	P-value
Glucose (mg/dL)	-5.465	762	p<0.001
Insulin (uIU/mL)	-1.556	762	0.120
Glycated Hemoglobin (HA1c, %)	3.347	762	0.001
Blood Triglycerides (mg/dL)	-5.783	762	p<0.001
Blood LDL Cholesterol (mg/dL)	-0.774	762	0.439
Blood HDL Cholesterol (mg/dL)	11.103	762	p<0.001
BMI (kg/m^2)	-0.288	762	0.774
Serum IL6 (pg/mL)	2.736	762	0.006
Blood C-Reactive Protein (ug/mL)	3.795	762	p<0.001
Serum Soluble ICAM-1 (ng/mL)	1.354	762	0.176

Adjusted by age and race

APPENDIX 2. PhD Projects

Published Projects

Amaral, W.Z., Lubach G.R., Kapoor, A., Proctor, A., Phillips, G.J., Lyte, M., Coe, C.L. (2017). Low *Lactobacilli* abundance and polymicrobial diversity in the lower reproductive tract of female rhesus monkeys do not compromise their reproductive success. *American Journal of Primatology*, e22691.

Amaral, W.Z., Lubach, G.R., Proctor, A., Lyte, M., Phillips, G.J., & Coe, C.L. (2017). Social Influences on Prevotella and the Gut Microbiome of Young Monkeys. *American Psychosomatic Medicine.* 79(8):888-897.

Amaral, W. Z., Krueger, R. F., Ryff, C. D., & Coe, C. L. (2015). Genetic and environmental determinants of population variation in interleukin-6, its soluble receptor and C-reactive protein: Insights from identical and fraternal Caucasian twins. *Brain, Behavior, and Immunity*. 49:171-81. **

Amaral, W. Z., Lubach, G. R., Bennett, A. J., & Coe, C. L. (2013). Inflammatory vulnerability associated with the rh5-HTTLPR genotype in juvenile rhesus monkeys. *Genes, Brain, and Behavior*. 12(3): 353-60.

Unpublished Projects

Amaral, W.Z., Lubach, G.R., Proctor, A., Lyte, M., Phillips, G.J., & Coe, C.L. (In preparation). Profiling of the female rhesus monkey microflora indicates differences across reproductive phases in the cervicovaginal compartment but not in the distal rectum.

Amaral, W.Z. & Coe, C.L. (In preparation) Pathways Linking Carbohydrate and Lipid Metabolism, Adiposity and Inflammation. **

Amaral, W.Z., Galles, N.C. & Coe, C.L. (In preparation) The Effect of Dietary Carbohydrates and Lipids on Adiposity and Inflammation in the Context of the American diet. **

Amaral, W.Z. & Coe, C.L. (In preparation) Peripheral catecholamines interact with visceral fat and blood glucose to influence metabolic and inflammatory status in subsample of MIDUS adults. **

Amaral, W.Z., Davidson, R.J. & Coe, C.L. EEG brain asymmetry predicts systemic interleukin-6 in a MIDUS subsample. (revising analyses)

Amaral, W.Z. & Coe, C.L. Negative affectivity predicts daily rhythms and weekly patterns of saliva cortisol in the MIDUS population sample. (revising analyses)

Amaral, W.Z., Mayneris-Perxachs J., Swann, J.R., Lyte, M., Phillips, G.J., & Coe, C.L. Long-term influence of iron deficiency anemia on the gut microbiota and metabolome of developing rhesus monkeys. (Pending metabolomics from collaborators)

** Denote studies included in the dissertation project.