Immunogenomic and behavioral consequences of diet and social stress in nonhuman primates (*Macaca fascicularis*)

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Abstract

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Aspects of the modern human environment, such as diet and the social environment, are highly variable and are associated with increased morbidity and mortality. One common dietary pattern, the Western diet, is associated with increased markers of inflammation, while the Mediterranean diet, another common human dietary pattern, is linked to anti-inflammatory markers. Similarly, two key aspects of the social environment, social status and social integration, have immunomodulary consequences across taxa. Proinflammatory polarization in the peripheral immune system, therefore, presents a mechanism that may link environmental stress of both sources to suboptimal health. To test this hypothesis, we conducted the first whole diet manipulation to compare Western vs Mediterranean diet patterns in a randomized preclinical trial design. This dissertation describes molecular and behavioral consequences of this dietary

manipulation and the social environment, as well as the interaction of the two sources of environmental stress. The first study (Chapter 2) utilizes RNA sequencing data to characterize gene expression in circulating monocytes and establishes genome-wide effects of diet that support the monocyte polarization hypothesis. Furthermore, we found evidence of multiple putative gene regulatory mechanisms, including a strong behavioral response to diet. The second study detailed here (Chapter 3) is an in-depth exploration of the behavioral response to diet. We showed an immediate and persistent behavioral phenotype in which Mediterranean diet-fed monkeys showed reduced isolation and anxiety in a social status-dependent manner. Chapter 4 relates an epigenetic analysis exploring DNA methylation in circulating monocytes. While limited in scope, these data suggested both dietary and social impacts on DNA methylation. When taken together, these studies support the protective role of the Mediterranean diet in immune and behavioral health, a finding with potentially important translational consequences.

Chapters 2 and 4 both contained large data files as result tables, which are contained in a data table submitted as supplementary material to this dissertation. Each sheet of the Microsoft Excel document corresponds to a different supplementary table referenced in this dissertation.

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I owe a debt of gratitude to many who have helped me on this journey. First, to my mentor Noah Snyder-Mackler, thank you for your insight, wisdom, compassion, and patience in training me to be a better thinker and scientist. I appreciate the risk you took on choosing me to be your first pancake. To the other members of the Snyder-Mackler lab, who have been both teachers and companions on my journey. To the other mentors that have guided me on my journey- Tom Register and Carol Shively who have walked this whole path with me; Joe Sisneros and Karin Bornfeldt who were local mentors to me; and the numerous others who have lent their time and wisdom to help me grow. To my parents, who have guided me from the start. Finally, to Tilly, Helen, and Anna – tá mé i mo chónaí nuair a bheidh mé libh.

PREFACE

The research conducted for each empirical chapter of this dissertation has been previously published, as in the case of Chapters 2 and 3 (Johnson et al., 2021, 2022), or will be submitted for publication (chapter 4). As such, each has been prepared in publication format and only modified here for format.

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Please excuse any redundancies in the text resulting from the publication-ready format of these empirical chapters.

DEDICATION

For Helen Margaret

Thug mo shúil aire dhuit,

Thug mo chroí taitneamh duit.

(Eibhlín Dubh Ní Chonaill, *Caoineadh Airt Uí Laoghaire*)

Chapter 1. Introduction

1.1 HUMAN DIETARY PATTERNS AND HEALTH

Modern human living conditions are widely variable across culture, geography, and socioeconomic strata. This heterogeneity contributes to disparities in health and survival, particularly when aspects of our modern environment do not match the environment in which we evolved, known as evolutionary mismatch. What we choose to eat, for example, is patterned by culture, geography, and socioeconomic status, and is associated with a vast array of comorbidities. The Western diet, prevalent in high income countries, has been long associated with adverse effects on health, particularly in relation to chronic diseases of aging (Cordain et al., 2005; Drake et al., 2018; Jacka et al., 2010; Manzel et al., 2014; Pontzer et al., 2018; Smil, 1989; Smyth & Heron, 2006). Western diets are high in simple sugars and saturated and omega-6 fatty acids, which increase sympathetic nervous activity, oxidative stress, and levels of inflammatory markers (Drescher et al., 2019; Giugliano et al., 2006; Holt et al., 2009; Lopez-Garcia et al., 2004; Nanri et al., 2007; Nettleton et al., 2006). Consequently, Western diets are associated with increased risk for metabolic syndrome (Drake et al., 2018), type II diabetes (Smyth & Heron, 2006), cardiovascular disease (Drake et al., 2018; Smil, 1989), nonalcoholic hepatosteatosis (Jump et al., 2015), autoimmune disorders (Manzel et al., 2014), depression (Jacka et al., 2010), and premature death (Cordain et al., 2005). Negative consequences associated with Western diets are hypothesized to be driven by an evolutionary mismatch between a human physiology – which evolved to subsist on a plant-based diet supplemented with fish and meat but no refined products – and the radically different nutritional environment of many human populations today (Eaton et al., 1988; Lieberman, 2014; Stearns & Koella, 2008).

The Mediterranean diet, in contrast, more closely resembles that of modern hunter-gatherer populations and presumed ancestral human populations in macronutrient composition and key dietary components (Mackenbach, 2007; Pontzer et al., 2018). The Mediterranean diet is also associated with numerous health benefits, such as an anti-inflammatory phenotype (O'Keefe et al., 2008), reduced risk of chronic disease, and reduced mortality (Farchi et al., 1994; Osler & Schroll, 1997; Romagnolo & Selmin, 2017; Trichopoulou et al., 1995). These two dietary patterns differ mainly in their nutritional composition, and not in the balance of macronutrients. For example, the overall proportion of calories derived from fat is remarkably similar between the two diets, as people consuming Mediterranean and Western diets obtain 32% and 33% of calories from fat, respectively (Bédard et al., 2012; USDA, 2016). The type of fat ingested is critical, however, as omega-6 fatty acids common in Western diets have been implicated in inflammation (Lopez-Garcia et al., 2004; O'Keefe et al., 2008), while omega-3 fatty acids characteristic of Mediterranean diets have demonstrated antioxidant and anti-inflammatory properties (Giugliano et al., 2006). Other components of Western diets (e.g. sugars, animal proteins) are also associated with inflammation (Freeman et al., 2018) and anti-inflammatory diets rely on plant-based nutrients (F. B. Hu, 2002), suggesting that comparing whole diets rather than individual nutrients is critical to understanding the connections between diet, inflammation, and health.

1.2 SOCIAL ADVERSITY AND HEALTH

Another aspect of the environment that can be characterized as an evolutionary mismatch is social adversity. In social species, interactions with conspecifics can be stressful. In contrast to other sources of stress, such as an attempted predation event, social relationships are persistent, and the acute stress of social interaction can become chronic. When a stressor persists beyond the duration

of the homeostatic mechanisms that evolved to deal with acute stress, it leads to negative fitness consequences. Persistent adverse social experiences in social animals overload the hypothalamic-pituitary-adrenal (HPA) axis that evolved to respond to acute stress, resulting in chronic psychosocial stress and its downstream effects of immune dysregulation (Gray et al., 2017; Kohn et al., 2016; McEwen, 2017; Snyder-Mackler & Lea, 2018). With dysregulation of the immune system in chronic stress conditions, the glucocorticoids that are produced in acute stress shift from anti- to pro-inflammatory. Two components of the social environment in particular—social isolation and low social status—are linked to mortality and morbidity across taxa (Cacioppo et al., 2015; Chetty et al., 2016; Cole et al., 2015; Eisenberger et al., 2017; Hawkley & Cacioppo, 2010; Nguyen et al., 2020; Snyder-Mackler et al., 2020; Taylor et al., 2018). In humans, social adversity is associated with increased mortality and chronic disease incidence (Hallman et al., 2001; Rosengren et al., 2004; Steptoe & Kivimäki, 2012, 2013; Stuller et al., 2012), and both human and mammalian studies have implicated markers of inflammation in the relationship between social adversity and health (Abbott et al., 2003; Sapolsky, 2005; Snyder-Mackler et al., 2020; Takahashi et al., 2018). Direct experimental manipulation of primate social status induced an inflammatory response (Snyder-Mackler et al., 2016), reinforcing the role of inflammation in the increased morbidity observed in situations of chronic stress. Inflammatory gene expression, therefore, represents a molecular mechanism that may be responsible for the health consequences of social adversity and stress.

1.3 MONOCYTE POLARIZATION IN ENVIRONMENTAL STRESS

Diet and social adversity are both drivers of inflammation in humans and animal models of health. One putative mechanism of the environmentally driven inflammatory response is polarization of circulating immune cells called monocytes. Monocytes and monocyte-derived macrophages are

part of the innate immune system that play a key role in responding to the molecular environment. In the case of bacterial infections, monocytes can alter gene expression to take on a proinflammatory (M1-like) phenotype. This results in production of inflammatory messenger proteins called cytokines, such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ, that induce classically-activated M1 monocytes to become macrophages (Mosser & Edwards, 2008). Conversely, following clearance of invading pathogens, monocytes can shift to be more regulatory/reparative (M2-like). M2 activated monocytes mobilize the tissue repair processes and release anti-inflammatory cytokines in response to IL-4, IL-13, and transforming growth factor (TGF)-β (Mosser & Edwards, 2008). An appropriate balance of these monocyte phenotypes is considered essential for a healthy immune system. Thus, dietary constituents or patterns may influence pathologic processes by altering the balance between these proinflammatory and anti-inflammatory monocyte subsets – a hypothesis that has yet to be tested (Devêvre et al., 2015).

Psychosocial stress resulting from social adversity is also known to impact immune phenotypes. Both low social status and poor social integration increase the expression of inflammatory genes in primary white blood cells in humans and other animals (Cole, 2013; Cole et al., 2015; Cole, 2019; Snyder-Mackler et al., 2016; Snyder-Mackler & Lea, 2018; Tung & Gilad, 2013). In addition, some foods can themselves drive behavioral changes, including in key social behaviors, suggesting the possibility of interaction between diet and the social environment in the regulation of immune cells (Hollis et al., 2018; J. R. Kaplan et al., 1991; Kasprowska-Liśkiewicz et al., 2017; Kougias et al., 2018; Warden & Fisler, 2008). This remains unresolved, as no study has yet directly compared the effects of whole diet patterns and social status in monocytes.

1.4 MACAQUES AS A MODEL FOR HEALTH

To date, long term effects of dietary patterns in human remain limited. This is due, in part, to the

difficulty of whole diet manipulations in humans, which are expensive and rely on strict voluntary adherence to a diet. What remains are either manipulations of single nutrients (F. B. Hu, 2002; Kimmig & Karalis, 2013; Ohlow et al., 2017; Steinhubl, 2008; Whelton et al., 1992), which cannot address the potentially important synergistic effects of the multiple nutrients that make up human diet patterns, or correlative studies that rely on self-reporting, which are unreliable (T. M. Miller et al., 2008; Suchanek et al., 2011). Diet effects may also be confounded by self-selective cohorts or consequences to health that may arise from dietary patterns, such as reduced physical activity or increased caloric intake associated with Western diets (Kraft et al., 2018; Lagranja et al., 2015; Snodgrass, 2013). Furthermore, measures of social adversity in human often rely on self-reported measures, during which people may conflate perceived and actual events or use differing definitions of key variables (Alcaraz et al., 2019; Evans et al., 2019; Friedler et al., 2015). The use of an appropriate model organism can overcome some of these obstacles.

Cynomolgus macaques (*Macaca fascicularis*) share physiological homologies and have an accelerated natural history (~ 3-4x that of humans), which situates them as good models for human health. Indeed, there is a long history of their use in understanding human health and disease (Adams et al., 1997; Clarkson, Anthony, et al., 2004; Clarkson, Appt, et al., 2004; Clarkson et al., 2013; Cline et al., 2001; Cline & Wood, 2006; Haberthur et al., 2010; Lees et al., 1998; Mikkola et al., 2004; Mikkola & Clarkson, 2006; Naftolin et al., 2004; Nagpal, Shively, et al., 2018; Nagpal, Wang, et al., 2018; Register et al., 2003; Register, 2009; Shively & Clarkson, 2009; Shively & Day, 2015; Sophonsritsuk et al., 2013; Walker et al., 2008; Willard & Shively, 2012; Wood et al., 2007). Their evolutionary proximity to humans confers a high degree of genetic identity, which allows for comparison to human patterns of gene regulation. They also develop chronic disease phenotypes, making them a useful model for understanding environmental impact on health

(Jarczok et al., 2018; Kromrey et al., 2016; Shively, 1998; Shively & Day, 2015; Willard & Shively, 2012). Female macaques naturally develop stable matrilineal hierarchies that persist, relatively unchanged, throughout their lifespan. Within their social groups, they have rich social interactions with conspecifics, leading to similarities with humans in key aspects of the social environment, such as social status and isolation (Abbott et al., 2003; Brent et al., 2017b; Sapolsky, 2005). These attributes make cynomolgus macaques an ideal model system for testing the immunogenomic and behavioral effects of environmental stress.

1.5 EXPERIMENTAL DESIGN

To better understand the mechanisms driving the health impacts of chronic stress from diet and the social environment, my collaborators at Wake Forest University designed the first long-term whole-diet manipulation in primates to directly compare Western versus Mediterranean diets. In this preclinical study design, female macaques were placed into social groups of four monkeys each while on a standard monkey chow diet during a baseline phase. The baseline phase provided an opportunity for behavioral and biological sample collection while the monkeys developed social hierarchies in their groups. At the end of the baseline phase, key physiological measurements such as body weight and serum triglyceride concentration were used to balance groups assigned to each of two experimental diets.

Whole groups were then transitioned to the experimental Western- or Mediterranean-like diets, which were formulated to match human dietary consumption patterns (see **Table 2.1** for comparison of experimental diets and human dietary patterns and **Table 3.1** for experimental diet formulations). Monkeys consumed the experimental diets for a total duration of 30 months, roughly equivalent to 7-8 years of a human lifespan. During this phase of the experiment, additional biological and behavioral samples were collected at various time points. In Chapter 2

of this dissertation, I describe efforts to characterize gene expression as a function of diet and social status from RNA collected at the midpoint of the experimental diet phase. In the same chapter, I detail behavioral changes associated with diet up to that point. Following the findings from Chapter 2, I conducted a more thorough behavioral analysis using behavioral data from the entire experiment, which is detailed in Chapter 3. Finally, to understand the regulatory mechanisms underlying the observed behavioral and gene expression changes, I conducted an epigenetic analysis of DNA collected at the same midpoint of the dietary intervention in Chapter 4. Two of these chapters have already been published (Johnson et al., 2021, 2022), and all three add to a growing body of literature coming from my collaborators' analyses of data derived from this experimental manipulation (Amick et al., 2021; Frye et al., 2021; Gonzalez-Armenta et al., 2019; Johnson et al., 2021, 2022; Nagpal et al., 2019; Nagpal, Shively, et al., 2018; Newman et al., 2021; Shively et al., 2018, 2019).

Chapter 2. Contrasting Effects of Western vs. Mediterranean Diets on Monocyte Inflammatory Gene Expression and Social Behavior in a Primate Model

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Abstract:

Dietary changes associated with industrialization substantially increase the prevalence of chronic diseases, such as obesity, type II diabetes, and cardiovascular disease, major contributors to the public health burden. The high prevalence of these chronic diseases is often attributed to an "evolutionary mismatch" between human physiology and modern nutritional environments. Western diets enriched with foods that were scarce throughout human evolutionary history (e.g., simple sugars and saturated fats) promote inflammation and disease relative to diets more akin to ancestral human hunter-gatherer diets, such as a Mediterranean diet. Peripheral blood monocytes, precursors to macrophages and important mediators of innate immunity and inflammation, are sensitive to the environment and may represent a critical intermediate in the pathway linking diet to disease. We evaluated the effects of 15 months of whole diet manipulations mimicking human Western or Mediterranean diet patterns on monocyte polarization using a well-established model of human health, the cynomolgus macaque (*Macaca fascicularis*). Monocyte transcriptional profiles differed markedly between the two diets, with 40% of transcripts showing differential expression (FDR < 0.05). Monocytes from Western diet consumers were polarized toward a more

proinflammatory phenotype. Compared to the Mediterranean diet, the Western diet shifted the co-expression of 445 gene pairs, including small RNAs and transcription factors associated with metabolism and adiposity in humans, and dramatically altered behavior. For example, Western-fed individuals were more anxious and less socially integrated compared to the Mediterranean-fed subjects. These behavioral changes were also associated with some of the effects of diet on gene expression, suggesting an interaction between diet, central nervous system activity, and monocyte gene expression. The results of this study provide new insights into evolutionary mismatch at the molecular level and uncover new pathways through which Western diets alter monocyte polarization toward a proinflammatory phenotype.

2.1 INTRODUCTION

Modern human diets vary across geography, cultures, and socioeconomic strata and have profound impacts on health, survival, and reproduction. The Western diet, prevalent in high income countries, has been long associated with adverse effects on health, particularly in relation to chronic diseases of aging (Cordain et al., 2005; Drake et al., 2018; Jacka et al., 2010; Manzel et al., 2014; Pontzer et al., 2018; Smil, 1989; Smyth & Heron, 2006). Western diets are high in simple sugars and saturated and omega-6 fatty acids, which increase sympathetic nervous activity, oxidative stress, and levels of inflammatory markers (Drescher et al., 2019; Giugliano et al., 2006; Holt et al., 2009; Lopez-Garcia et al., 2004; Nanri et al., 2007; Nettleton et al., 2006). Consequently, Western diets are associated with increased risk for metabolic syndrome (Drake et al., 2018), type II diabetes (Smyth & Heron, 2006), cardiovascular disease (Drake et al., 2018; Smil, 1989), nonalcoholic hepatosteatosis (Jump et al., 2015), autoimmune disorders (Manzel et al., 2014), depression (Jacka et al., 2010), and premature death (Cordain et al., 2005). From an evolutionary perspective, the negative health effects of Western diets are hypothesized to be driven

by a “mismatch” between human physiology – which evolved to subsist on a plant-based diet supplemented with fish and meat but no refined products – and the radically different nutritional environment of many human populations today (Eaton et al., 1988; Lieberman, 2014; Stearns & Koella, 2008).

In contrast to the Western diet, the Mediterranean diet derives most protein and fat from vegetable sources, which are enriched with antioxidants, monounsaturated and omega-3 fatty acids. This diet more closely resembles that of modern hunter-gatherer populations and presumed ancestral human populations in macronutrient composition and key dietary components (Mackenbach, 2007; Pontzer et al., 2018). Interestingly, the Mediterranean diet is also associated with an anti-inflammatory phenotype (O’Keefe et al., 2008), reduced incidence of chronic disease, and increased longevity, relative to a Western diet (Farchi et al., 1994; Osler & Schroll, 1997; Romagnolo & Selmin, 2017; Trichopoulou et al., 1995). At face value, the detrimental health effects associated with Western relative to Mediterranean diets are consistent with evolutionary mismatch. However, the mechanisms through which this mismatch may negatively and causally affect health, and conversely how the Mediterranean diet positively impacts health remains poorly understood. Disentangling these mechanisms is especially difficult in humans, as population shifts toward Western diets may be accompanied by other challenges to health such as reduced physical activity or increased total caloric intake (Kraft et al., 2018; Lagranja et al., 2015; Snodgrass, 2013).

One potential mechanism for dietary impacts on health is through changes to our immune system. Previous attempts to understand how Western versus Mediterranean diets impact the immune system have relied on correlational analyses of self-reported diet or short-term dietary interventions in humans, which are limited in their ability to address causality (Stice & Durant, 2014; Suchanek et al., 2011). Many experimental manipulations have focused on single nutrients

in animal models (F. B. Hu, 2002; Kimmig & Karalis, 2013; Ohlow et al., 2017; Steinhubl, 2008; Whelton et al., 1992), which cannot address the potentially important synergistic effects of the multiple nutrients that make up human diet patterns. Our study design employs whole diet manipulations in a randomized preclinical trial framework (Western versus Mediterranean) to address the role that monocytes play in sensing and responding to dietary inputs (Devêvre et al., 2015; Drescher et al., 2019; Holt et al., 2009; Nanri et al., 2007; Nettleton et al., 2006). Monocytes and monocyte-derived macrophages are innate immune cells that vary phenotypically along a spectrum, which ranges broadly from proinflammatory (M1-like) to regulatory/reparative (M2-like). An appropriate balance of these monocyte phenotypes is essential for a healthy immune system. Classically-activated M1 monocytes respond to proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ by becoming macrophages, which propagate the inflammatory response towards infection (Mosser & Edwards, 2008). In contrast, M2 activated monocytes mobilize the tissue repair processes and release anti-inflammatory cytokines in response to IL-4, IL-13, and transforming growth factor (TGF)-β (Mosser & Edwards, 2008). Thus, dietary constituents or patterns may influence pathologic processes by altering the balance between these proinflammatory and anti-inflammatory monocyte subsets – a hypothesis that has yet to be tested (Devêvre et al., 2015).

In addition to diet, psychosocial stress is also known to impact immune phenotypes. In particular, multiple sources of social adversity, such as low social status and poor social integration, have been shown to increase the expression of inflammatory genes in primary white blood cells in humans and other animals (Cole, 2013; Cole et al., 2015; Cole, 2019; Snyder-Mackler et al., 2016; Snyder-Mackler & Lea, 2018; Tung & Gilad, 2013). Given that some food constituents can directly alter social behaviors themselves (Hollis et al., 2018; J. R. Kaplan et al.,

1991; Kasprowska-Liśkiewicz et al., 2017; Kougias et al., 2018; Warden & Fisler, 2008), it is therefore possible that diet effects on immune cell regulation may, to some degree, be mediated through changes in these behaviors. It is also possible that diet-induced alterations in systemic inflammation may alter behavior. However, because no detailed studies of diet, social behavior, and immune cell phenotypes have been conducted, it remains unclear how these factors are linked and how, together, they impact health.

To overcome the limitations of human studies, we designed a randomized preclinical trail in cynomolgus macaques (*Macaca fascicularis*), a well-established model of dietary and behavioral influences on health in which we can carefully control diet and the environment. Macaques are excellent models for human health and disease as they share many core genetic, physiological, and behavioral phenotypes with humans (Jarczok et al., 2018; Kromrey et al., 2016; Shively, 1998; Shively & Day, 2015; Willard & Shively, 2012). In this study, we conducted a whole-diet manipulation to directly and simultaneously compare the behavioral and physiological effects of Mediterranean and Western diets, formulated to mimic human diet patterns. The randomized trial design allowed us to identify causal effects of realistic, complex diet patterns on one possible mechanism linking diet to chronic disease risk – polarization of immune cell populations toward a proinflammatory phenotype. Previous reports from this preclinical trial demonstrate that relative to the Mediterranean diet, the Western diet increased body weight, body fat, insulin resistance, and hepatosteatosis (Shively et al., 2019); exacerbated autonomic and hypothalamic-pituitary-adrenal responses to psychosocial stress (Shively et al., 2020); and altered brain neuroanatomy (Frye et al., 2020). Here, we report the effects of the Mediterranean and Western diet patterns on behavior and monocyte gene expression.

2.2 RESULTS

2.2.1 *Diet intervention*

Adult female cynomolgus macaques were fed either a Western-like (hereafter, "Western", *n* = 20) or a Mediterranean-like (hereafter, "Mediterranean", *n* = 15) diet for 15 months (approximately equivalent to 4 human years). The experimental diets were formulated to model human diet patterns and have been previously described (Shively et al., 2019). Briefly, the Western diet was designed to mimic the diet typically consumed by middle-aged Americans (USDA, 2016), whereas the Mediterranean diet reflected key aspects of the human Mediterranean diet (Kafatos et al., 2000). The experimental diets were matched on macronutrients and cholesterol content but differed in fatty acids. Fats and proteins were mostly plant based in the Mediterranean diet (Kafatos et al., 2000), and from animal sources in the Western diet. This resulted in high levels of monounsaturated fats in the Mediterranean diet, and saturated fats in the Western diet (Cordain et al., 2005; Kafatos et al., 2000). The Mediterranean diet was higher in complex carbohydrates and fiber, and had a lower omega-6:omega-3 fatty acid ratio (similar to a modern-day, traditional hunter-gatherer type diet (Cordain et al., 2005)), and lower sodium and refined sugars than the Western diet. Key Mediterranean ingredients included English walnut powder and extra-virgin olive oil which were the primary components provided to participants in the PREDIMED trial (Estruch et al., 2018). Macronutrient composition of experimental diets compared to monkey chow and human diet patterns can be found in **Table 2.1**, Methods.

2.2.2 *Diet induced major shifts in monocyte gene expression*

RNA sequencing was employed to measure genome-wide gene expression of purified CD14+ monocytes after 15 months on the experimental diets. Diet had a strong effect on monocyte gene

expression: the first principal component of the correlation matrix of normalized residual gene expression (see Methods), which explained 59% of the overall variance, was significantly associated with diet ($t_{(25.1)}$ = 4.4, p = 1.7 x 10^{-4}; **Figure 2.1A**). PC1 score was correlated with expression of known proinflammatory genes such as interleukin-6 (*IL6* Pearson's r = 0.77, p = 5.4 x 10^{-8}), interleukin-1α (*IL1A* Pearson's r = 0.69, p = 4.3 x 10^{-6}), and two subunits of the NF-κB protein (*NFKB1* Pearson's r = 0.61, p = 1.2 x 10^{-4}; *NFKB2* Pearson's r = 0.72, p = 1.3 x 10^{-6}). Approximately 40% of the 12,240 tested genes were significantly differentially expressed genes (DEGs) between the two diets (n = 4,900 genes, FDR < 0.05; for all detected genes and the effect size of diet, see **Table S2.2A**; for DEGs sorted by the effect size of diet, see **Table S2.2B**). The number of diet-responsive genes was roughly balanced between those that were more highly expressed in monkeys fed the Mediterranean diet (n = 2,664; hereafter "Mediterranean genes") and those that were more highly expressed in monkeys fed the Western diet (n = 2,236; hereafter "Western genes"). While balanced in direction, the effect sizes of diet in Western genes were, on average, 1.6-fold larger than in Mediterranean genes (Mann-Whitney U = 4.1 x 10^{6}, p = 6.1 x 10^{-117}; **Figure 2.1B**). Thus, the strongest effects were observed in genes that were either activated by a Western diet or suppressed by a Mediterranean diet.

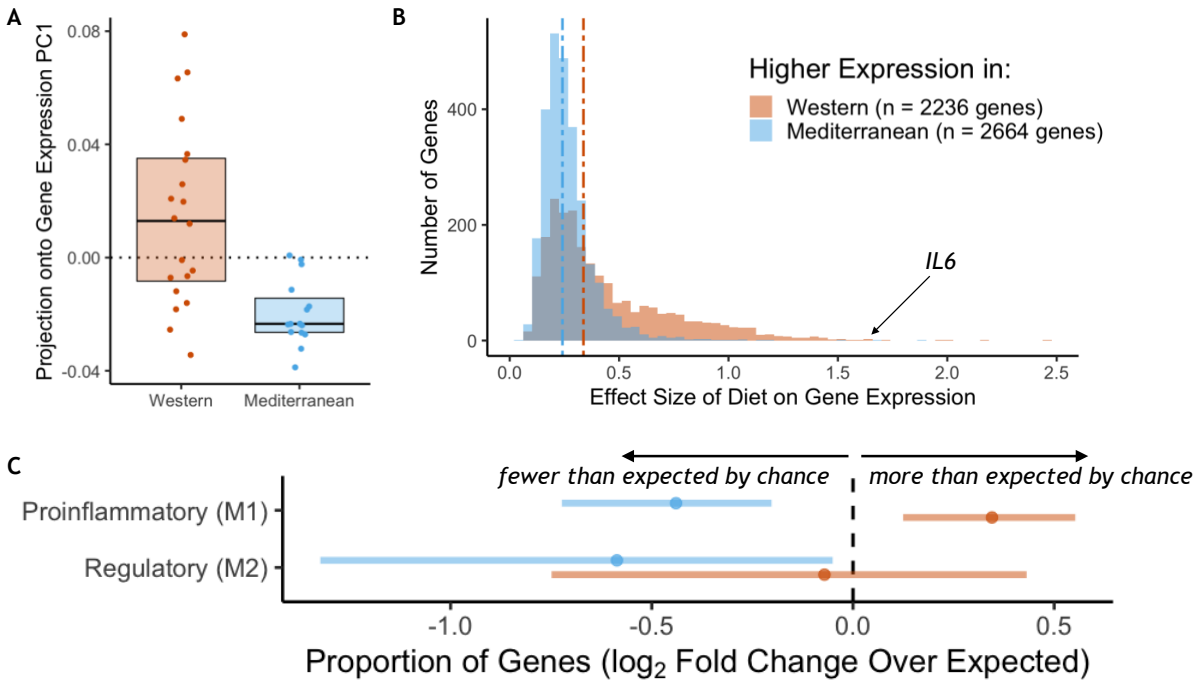

Figure 2.1 Diet effects on monocyte gene expression. A) Diet was significantly associated with the first principal component of gene expression (59% variance explained, $t_{(25.1)} = 4.4$, $p = 1.72 \times 10^{-4}$). **B)** The average effect size of diet on Western genes was 60% stronger than the effect size of diet on Mediterranean genes (Mann-Whitney $U = 4.1 \times 10^{6}$, $p = 6.1 \times 10^{-117}$). **C)** Western genes (orange) contained more M1 genes than expected by chance, indicating that the Western diet induced a shift towards a proinflammatory monocyte phenotype. Western genes were enriched for proinflammatory (M1-like) genes (fold-enrichment = 1.27, *95% CI* = 1.09, 1.46), while Mediterranean genes (blue) were depleted of these same M1-like genes (fold-enrichment = 0.74, *95% CI* = 0.61, 0.88). Regulatory (M2-like) genes were also under-represented in Mediterranean genes (fold-enrichment = 0.67, *95% CI* = 0.40, 0.97), but not in Western genes (fold-enrichment = 0.95, *95% CI* = 0.60, 1.35).

2.2.3 *Functional characterization of differentially expressed genes*

Monocytes from animals fed the Western diet had higher expression of a number of well-known inflammatory-related genes, including *IL6* (β_{diet} = 1.66, FDR = 8.9x10^{-3}; **Figure 2.1B**), *IL1A* (β_{diet} = 1.22, FDR = 0.033), and two subunits of the NF-κB protein (*NFKB1* β_{diet} = 0.30, FDR = 0.017; *NFKB2* β_{diet} = 0.42, FDR = 0.012). Western genes were more likely to be involved in replication and metabolic cellular processes, including response to growth factor (GO:0070848, weighted Fisher's Exact Test (FET) p = 4.6 x 10^{-3}) and response to insulin (GO:0032868, weighted FET p = 4.0 x 10^{-4}), suggesting that the Western diet also reprogrammed oxidative metabolic aspects of monocyte gene regulation. Conversely, Mediterranean diet monocyte expression patterns were involved in enhanced oxidation-reduction processes (GO:0055114, weighted FET p = 6.0 x 10^{-3}), a critical function in keeping proinflammatory monocytes in check (for all GO terms enriched in Western and Mediterranean genes, see **Table S2.3A-B**. When compared to genes causally implicated at the expression level in 103 complex human diseases and traits (Y. Zhang et al., 2020), we found that Western genes were enriched for genes involved in multiple human diet-associated diseases and traits (celiac disease: fold enrichment = 1.80, p = 0.016; body fat: fold enrichment = 0.26, p = 2.9 x 10^{-3}; and body mass index: fold enrichment = 0.20, p = 0.016; **Figure 2.2**), as well as genes associated with levels of important metabolites such as HDL cholesterol (fold enrichment = 0.61, p = 6.8 x 10^{-3}), LDL cholesterol (fold enrichment = 0.63, p = 0.012), and adiponectin (fold enrichment = 1.32, p = 7.7 x 10^{-3}). In contrast, Mediterranean genes were not enriched for any of the 103 complex traits tested (all FDR > 0.2).

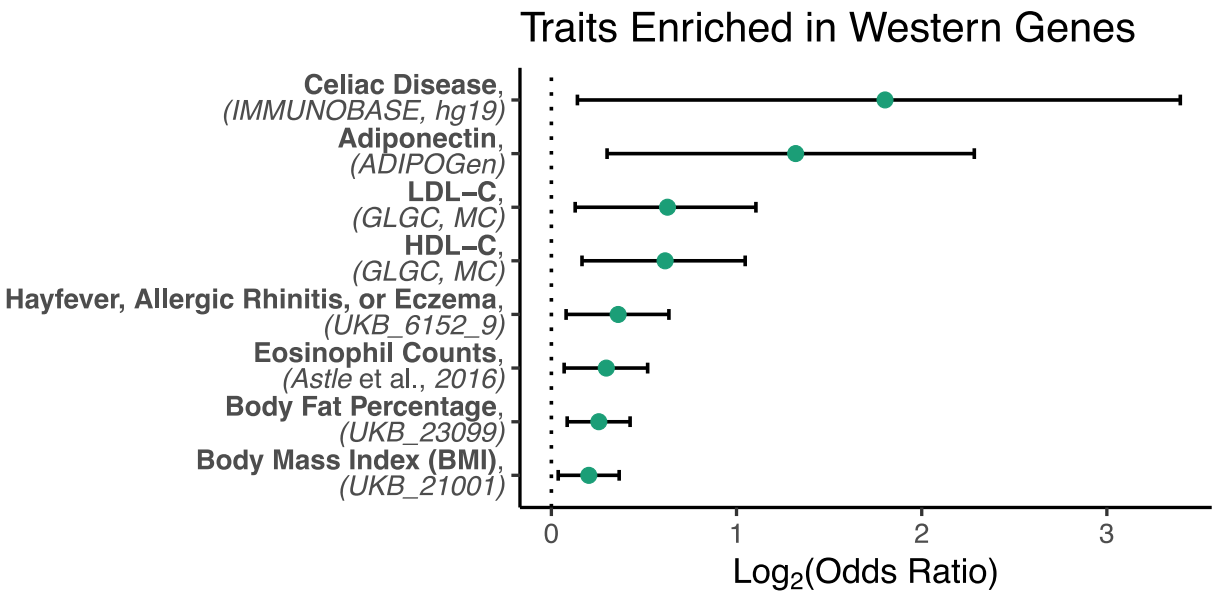

Figure 2.2 Enrichment of genes associated with human health. The sets of Western and Mediterranean genes (genes with significantly higher expression in monkeys fed the Mediterranean diet) were compared to genes implicated in 103 complex human diseases and traits (Y. Zhang et al., 2020). Fisher's Exact Tests were used to calculate the enrichment of trait-associated genes in Western genes that are depicted here (FDR < 0.02, 95% CI) and no traits were enriched in Mediterranean genes.

We next conducted a more targeted analysis of monocyte polarization by focusing on genes previously shown to be differentially expressed between induced proinflammatory (M1) and regulatory (M2) monocytes (Schmidl et al., 2014) (see **Table S2.2A** for polarization categories). Western genes contained more M1-associated genes than expected by chance (*n* = 162 genes, fold-enrichment = 1.27, *95% CI* = 1.09, 1.46; **Figure 2.1C**), but not M2-associated genes (*n* = 24 genes, fold-enrichment = 0.95, *95% CI* = 0.60, 1.35). Conversely, both M1-associated genes (*n* = 112 genes, fold-enrichment = 0.74, *95% CI* = 0.61, 0.88) and M2-associated genes (*n* = 20 genes, fold-enrichment = 0.67, *95% CI* = 0.40, 0.97) were underrepresented among Mediterranean genes.

2.2.4 *Association of transcription factors with differentially expressed genes*

To identify putative upstream gene regulatory mechanisms, we examined whether DEGs were associated with predicted *cis*-regulatory transcription factor binding sites. We identified 34 distinct transcription factor-binding motifs enriched within 2 kilobases of the transcription start sites of Mediterranean genes and one that was enriched near the transcription start sites of Western genes (FDR < 0.05; **Figure 2.3**, for all transcription factor binding motifs enriched in the regulatory regions of either set of diet genes, see **Table S2.4**). Diet altered expression of the genes encoding for seven of these 35 transcription factors, including *IRF3, IRF8, MEF2C*, and *SP1*, which drive monocyte fate and polarization in response to extracellular signals (Chistiakov et al., 2018; Günthner & Anders, 2013; Schuler et al., 2008; Scott et al., 1994; D. E. Zhang et al., 1994) (Chistiakov et al., 2018; Günthner & Anders, 2013; Schuler et al., 2008; Scott et al., 1994; D. E. Zhang et al., 1994). Thus, some of the diet-associated changes in monocyte transcriptional profiles may be mediated by changes in the expression and *cis*-regulatory binding of these key transcription factors.

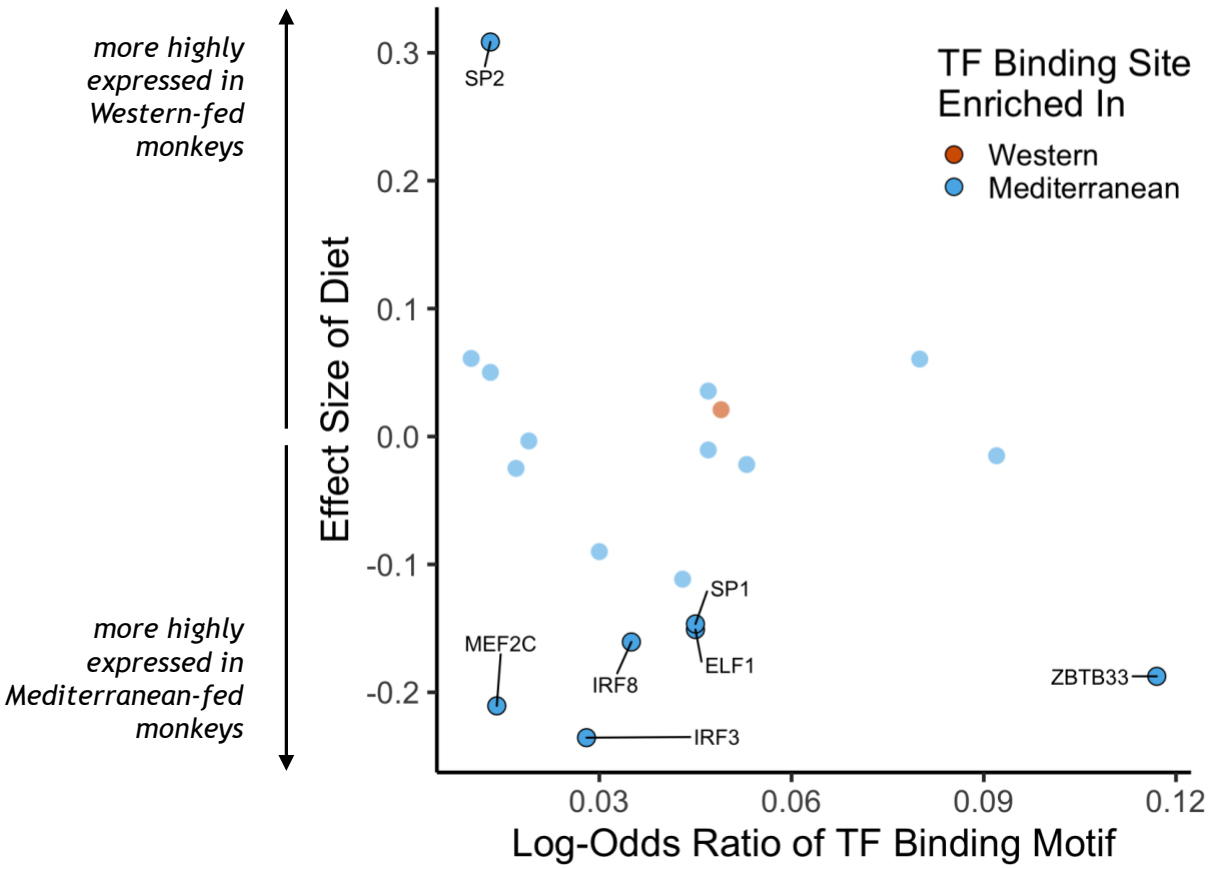

Figure 2.3 Transcription factor (TF) binding motifs correlated with diet effects on gene expression. The log-odds ratio of TF binding motif enrichment in Western genes (orange) or Mediterranean genes (blue) are depicted on the x-axis. The y-axis shows the effect size of diet on the expression of the gene that encodes for the TF. Only TFs with binding motifs significantly enriched in either gene set and that were detectably expressed in our samples are shown, with those significantly affected by diet (FDR < 0.05) outlined and labeled.

2.2.5 *Gene co-expression modules recapitulate functional role of diet-induced changes*

We employed a commonly-used bioinformatic approach, weighted gene co-expression network analysis (WGCNA) (Langfelder & Horvath, 2008) to group genes by pattern of transcription into co-expression modules. Overall, we identified 15 modules of co-expressed genes. Module 5 was more highly expressed in Mediterranean-fed animals (Welch-Satterthwaite $t_{(28.3)}$ = -3.9, Holm-Bonferroni-adjusted p (p_{HB}) = 8.1 x 10^{-3}; see **Table S2.5A** for all co-expression modules), and was depleted for M1 genes (n = 383 genes, log odds ratio = -0.6, *95% CI* = -0.8, -0.3, p_{HB} = 5.2 x 10^{-5}). Similar to the set of Mediterranean genes, module 5 was enriched with genes involved in the oxidation-reduction process (GO:0055114, weighted FET p = 3.1 x 10^{-7}; for all GO terms passing an adjusted p-value threshold of 0.05, see **Table S2.5B**). While only one module was associated with higher expression in the Mediterranean diet, we found two modules, modules 8 and 10, that were more highly expressed in Western-fed animals (module 8: $t_{(32.3)}$ = 3.5, p_{HB} = 0.020; module 10: $t_{(33.0)}$= 3.1, p_{HB} = 0.048). These two modules exhibited similar gene regulatory signatures, as both modules were enriched for regulation of transcription by RNA polymerase II (module 8: GO:0006357 (overall regulation), weighted FET p = 1.3 x 10^{-5}; module 10: GO:0045944 (positive regulation), weighted FET p = 3.1 x 10^{-7}). Module 10 also included more M1 genes than expected (n = 186, log odds ratio = 0.8, *95% CI* = 0.5, 1.0, p_{HB} = 1.9 x 10^{-7}). A third module that trended toward higher expression in the Western diet, module 9 ($t_{(32.2)}$ = 2.5, raw p = 0.019, p_{HB} = 0.19), was enriched for genes involved in the inflammatory response (GO:0006954, weighted FET p = 2.4 x 10^{-6}). Together, these results reinforce our findings that the Western diet contributes to proinflammatory polarization in a multi-faceted manner, while the Mediterranean diet can contribute to the reduction of oxidative stress. Interestingly, two modules, modules 4 and 12, were depleted for diet-associated genes (module 4: FET p = 2.1 x 10^{-17}, module 12: FET p = 3.9 x 10^{-}

^{19}) and were enriched for genes involved in the defense response to virus (module 4: GO:0051607, weighted FET p = 1.1 x 10^{-18}) and the adaptive immune response (module 12: GO:0002250, weighted FET p = 8.1 x 10^{-9}). This suggests that viral responses and adaptive immunity may be less affected by the diet.

2.2.6 *Diet differentially influenced gene co-expression patterns*

The effects of diet on the magnitude and direction of pairwise gene expression correlations were assessed for the most strongly diet-affected genes, as such effects could reveal key gene regulatory networks that are altered by diet, that may themselves be regulated by key upstream targets (de la Fuente, 2010; Gaiteri et al., 2014). To reduce the number of tests, we limited our analyses to the pairwise combinations of the top 140 DEGs (n = 9730 combinations). Of these gene pairs, many were significantly associated with each other in both diets, both positively (n = 714) and negatively (n = 332, $p < 0.05$; for all gene pairs tested and their correlations, see **Table S2.6A**), suggesting that while diet altered expression of these genes, it did not change their co-expression relationships. Drawing on a newly developed approach, "correlation by individual level product" (CILP) (A. Lea et al., 2019), we identified 445 other gene pairs that exhibited significant differences (FDR < 0.2) in their correlation between the Mediterranean- and Western-fed monkeys (**Table S2.6A**; **Figure 2.4A**), suggesting that one of the experimental diets altered the coherence between the genes (**Figure 2.4A**).

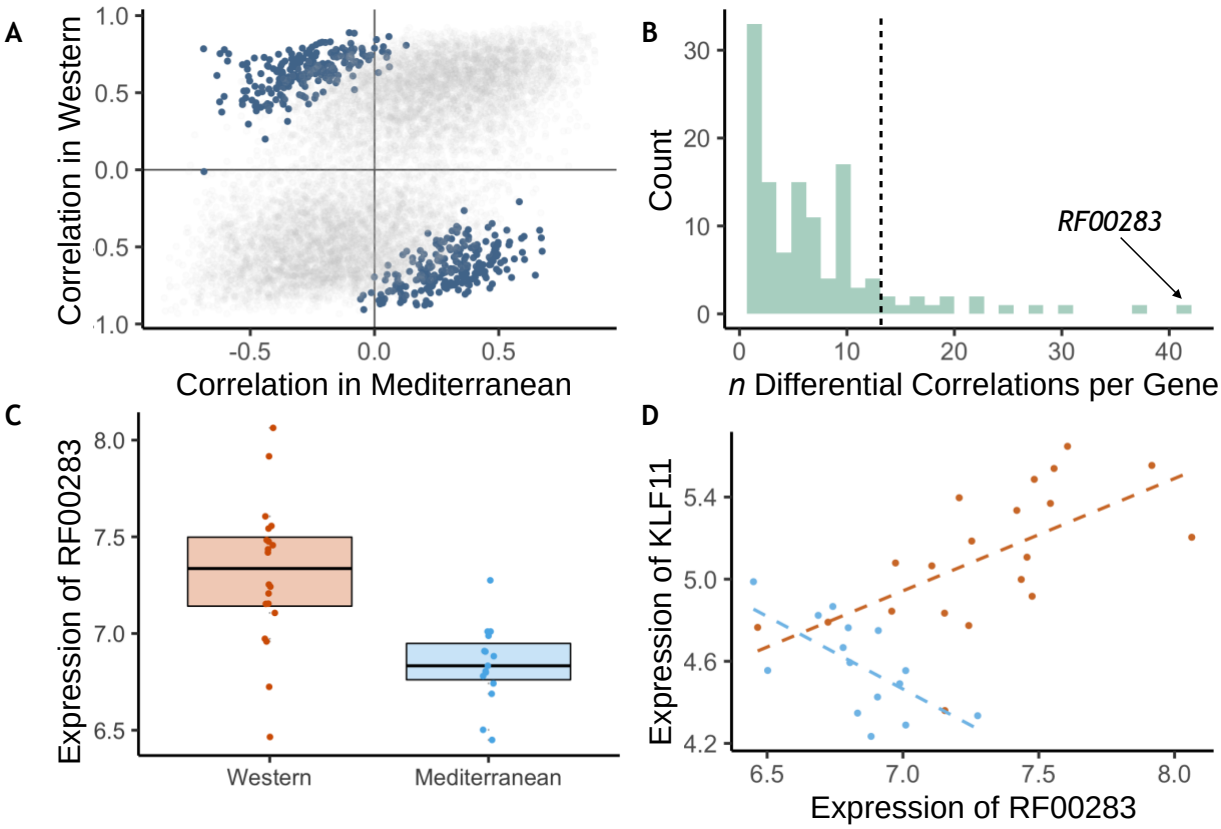

Figure 2.4 Diet altered monocyte gene co-expression. A) The Pearson correlation between each pair of genes within each of the experimental diets. Gene pairs that were significantly differently correlated between diets are highlighted in blue (n = 445 significant pairs, FDR < 0.2). **B)** Of the genes involved in significant pairs, some were paired with more genes than expected by chance, called "hub" genes (n = 16 hub genes; dotted black line is the maximum number of significant pairs expected by chance). The strongest hub gene was the non-coding RNA *RF00283*. **C)** Residual normalized expression of *RF00283* is significantly greater in Western- than Mediterranean-fed monkeys (β_{diet} = 0.51, FDR = 2.3 x 10^{-6}). **D)** Example of a differential correlation involving *RF00283*. Residual normalized expression of *RF00283* is plotted against expression of *KLF11*, a differentially-expressed transcription factor that regulates insulin and has been associated with type II diabetes in humans (Neve et al., 2005). The two genes were more highly expressed in Western monocytes, were positively correlated with one another in Western-fed monkeys (Pearson's r = 0.61, p = 4.2 x 10^{-3}), were negatively correlated in Mediterranean-fed monkeys (Pearson's r = -0.63, p = 0.011), and were differentially correlated between the two diets (p = 4.1 x 10^{-5}, FDR = 0.11).

We also identified 16 “hub” genes that exhibited differential correlations with more partner genes than expected by chance (**Figure 2.4B**, for all genes included in one or more differentially correlated gene pairs, see **Table S2.6B**). These hub genes were enriched for genes encoding transcription factors (OR = 7.40, FET $p = 7.0 \times 10^{-3}$), including *SOX4* (essential for normal insulin secretion and glucose tolerance) and *NR4A2* (involved in lipid, carbohydrate, and energy metabolism (Goldsworthy et al., 2008; Pearen & Muscat, 2010)), providing further support for immunological and metabolic reprogramming induced by our diet manipulation. Interestingly, the hub gene involved in the greatest number of differentially-correlated gene pairs was *RF00283, aka SCARNA18,* a non-coding RNA that has been associated with BMI, HDL cholesterol, and aging in human genome-wide association studies (J. P. Davis et al., 2017; Dluzen et al., 2018; Kanai et al., 2018; Tachmazidou et al., 2017) (**Figure 2.4B-D**). This small nucleolar RNA is thus a key transcriptional regulator that is altered by diet and has a cascading effect on other genes and pathways.

2.2.7 *Diet altered social and affective behavior*

In order to understand how diet may impact behavior and how both may interact to impact health, behavioral data were collected weekly during two 10-minute focal observations. These data were collected during both the baseline (2 hours/monkey total) and experimental phases (mean = 17.6 hours/monkey total) of the study. There were no significant differences in behavior between assigned diet groups during the baseline phase while consuming chow (**Figure 2.5A-B**). However, after 15 months on experimental diets, the two diet groups differed significantly in behavior. The Mediterranean group spent more time in body contact (Mann-Whitney $U = 284$, Holm-Bonferroni-adjusted p (p_{HB}) = 1.1×10^{-5}) and resting ($U = 269$, $p_{HB} = 1.6 \times 10^{-3}$), while those fed the Western diet spent more time alone ($U = 255$, $p_{HB} = 4.9 \times 10^{-3}$ **Figure 2.6A**; see **Figure 2.5C-D** for

behaviors during experimental diet consumption).

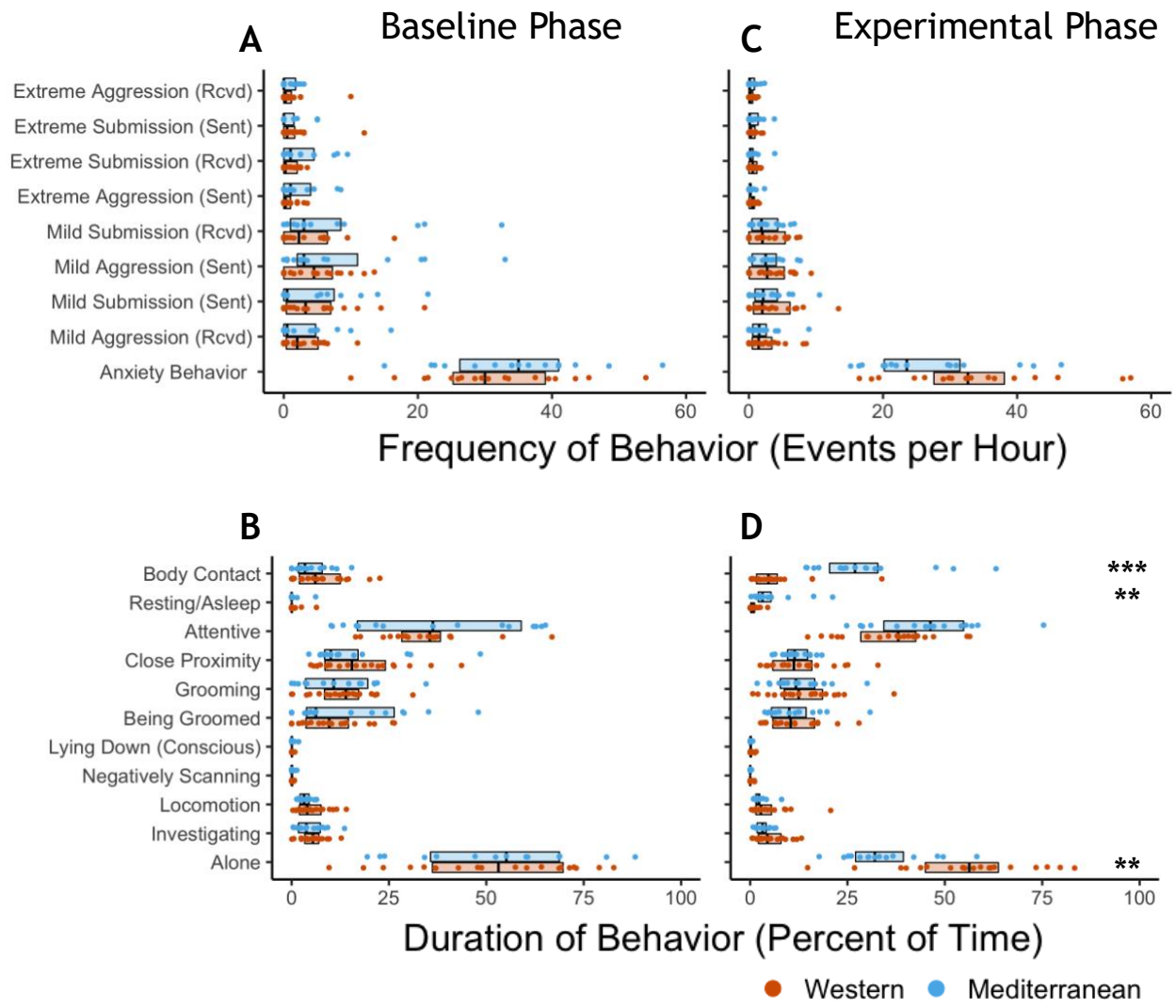

Figure 2.5 Behavior observed while monkeys consumed experimental diets. There were no differences between the Western- and Mediterranean-fed groups in the rates **(A)** or duration **(B)** of behaviors during the baseline phase, prior to diet manipulation. The boxplots depict the per-group medians and interquartile ranges for each behavior. Animals fed the Western diet are colored orange, and those fed the Mediterranean diet colored blue. Significant differences between the diet groups in the rates **(C)** or duration **(D)** of behaviors during the experimental phase are indicated (Mann-Whitney U test, Holm-Bonferroni adjusted $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***).

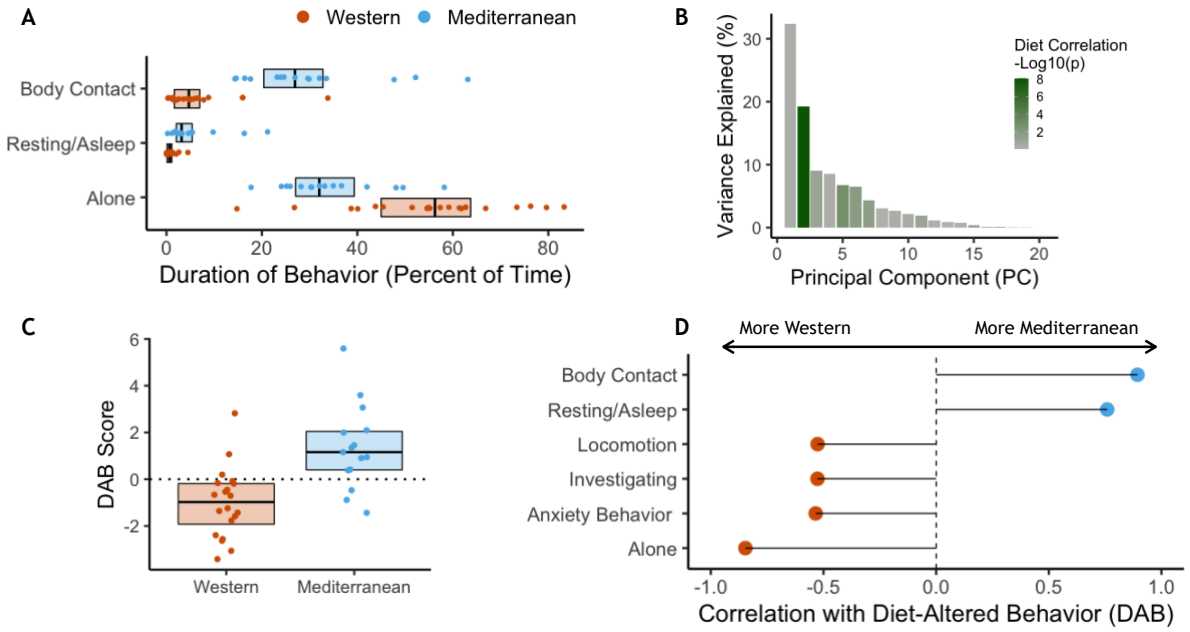

Figure 2.6 Diet alters behavioral phenotype. A) Three behaviors were significantly different between the two diet groups. Monkeys fed the Mediterranean diet spent more time in body contact (Holm-Bonferroni adjusted p (p_{HB}) = 1.1 x 10^{-5}) and resting (p_{HB} = 1.6 x 10^{-3}) than Western-fed monkeys. Monkeys eating the Western diet spent more time alone than Mediterranean-fed monkeys (p_{HB} = 4.9 x 10^{-3}). **B)** Principal component 2 (PC2) explained 19% of the variance in behavior and was the only PC significantly correlated with diet. **C)** PC2 represents a composite measure of diet-altered behavior, as individual loadings onto PC2 ("DAB scores"; 19% of all variance in behavior) were significantly higher in Mediterranean diet compared to Western diet animals ($t_{(26.8)}$ = 4.13, p = 3.2 x 10^{-4}). **D)** Six of the 20 behaviors observed are significantly correlated with DAB score (p_{HB} < 0.05). Here, significant correlations with DAB score in which behaviors are more frequent in Mediterranean diet or Western diet monkeys are indicated with blue or orange points, respectively.

Principal component analysis was conducted to identify key behaviors associated with one another (Benito et al., 2018; Seltmann et al., 2018). Behaviors associated with dominance interactions—including aggression, submission, and being groomed—all loaded heavily onto the first principal component, which explained 32% of the overall variance in behavior and did not differ between diets (Welch-Satterthwaite $t_{(30.4)}$= -0.3, p = 0.70; for relationship between dominance rank and PC1, see **Figure 2.7**; for further discussion of social status in these animals, see **Appendix B**).

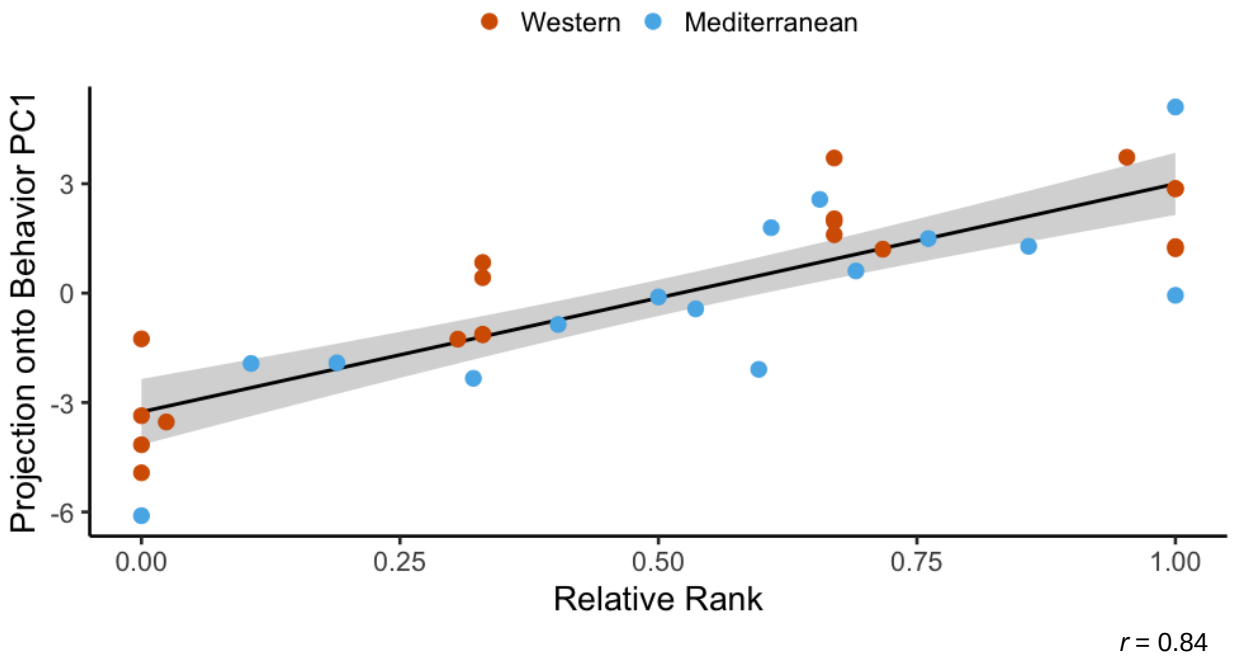

Figure 2.7 Correlation between behavior and social status. The first axis of variance in behavior—which explained 31% of the overall variance—was significantly positively correlated with dominance rank across diets (Pearson's $r = 0.84$, $p = 3.9 \times 10^{-10}$). All monkeys are assigned a rank between 0 and 1 based on the outcomes of dyadic interactions, where a higher rank indicates more dominant social status.

The second principal component explained 19% of the variance in behavior (**Figure 2.6B**) and differed significantly between the two diets ($t_{(26.8)}$ = 4.1, p = 3.2 x 10^{-4}; **Figure 2.6C**). No other principal component of behavioral phenotypes was significantly correlated with diet (**Figure 2.6B**). PC2 captured socially relevant behaviors that also differed between the diets and thus represents a composite of diet-altered behaviors (hereafter DAB). Specifically, DAB score (i.e., an individual's PC2 projection) was positively correlated with percent of time spent in body contact, indicative of social integration (Pearson's r = 0.89, p_{HB} = 1.0 x 10^{-11}; **Figure 2.6D**), and higher in Mediterranean-fed animals. Conversely, percent of time spent alone was associated with lower DAB scores (Pearson's r = -0.85, p_{HB} = 3.0 x 10^{-9}), and was higher in animals fed the Western diet. Previous work has validated a behavioral index of anxiety in nonhuman primates (rate of self-grooming and scratching) (Coleman et al., 2011; Maestripieri et al., 1992; Schino et al., 1996; Shively et al., 2015; Troisi, 2002; Troisi et al., 2000), which loaded heavily onto PC2 and is significantly negatively correlated with DAB score (Pearson's r = -0.53, p_{HB} = 0.019). Thus, PC2 (DAB) captured a measure of social integration associated with consuming a Mediterranean-like diet, and social isolation and anxiety associated with consuming a Western-like diet.

2.2.8 *Diet-altered behaviors and monocyte gene expression as mediators*

Given the effects of diet on both behavior and gene expression, we used mediation analyses to explore the potential influences of one on the other. Of the 4,900 DEGs, 29% were also significantly associated with the DAB score in a univariate model (n = 1,414, FDR < 0.05). Of these, DAB score significantly mediated the effect of diet on the expression of 1199 genes (24% of all DEGs, p < 0.05; **Figure 2.8A**). Among these DAB-mediated genes, DAB score mediation accounted for significantly more of the total effect of diet in Western genes (mean = 52.6%, s.d. = 12.6%), than Mediterranean genes (mean = 45.3%, s.d. = 10.1%; Mann-Whitney U = 1.1 x 10^5, p

= 6.4 x 10^{-25}; **Figure 2.8B**). These DAB-mediated genes were also significantly more likely to be Western genes than Mediterranean genes (*n* = 712 Western genes, 59%, two-sided binomial test *p* = 1.5 x 10^{-21}), and were enriched in regulation of inflammatory response (GO:0050727, weighted FET *p* = 2.9 x 10^{-3}; for all GO terms significantly enriched in DAB-mediated genes, see **Tables S2.7A-C**). Together, these observations suggest that the effect of diet on monocyte gene regulation may partially be due to diet-induced changes in key social behaviors.

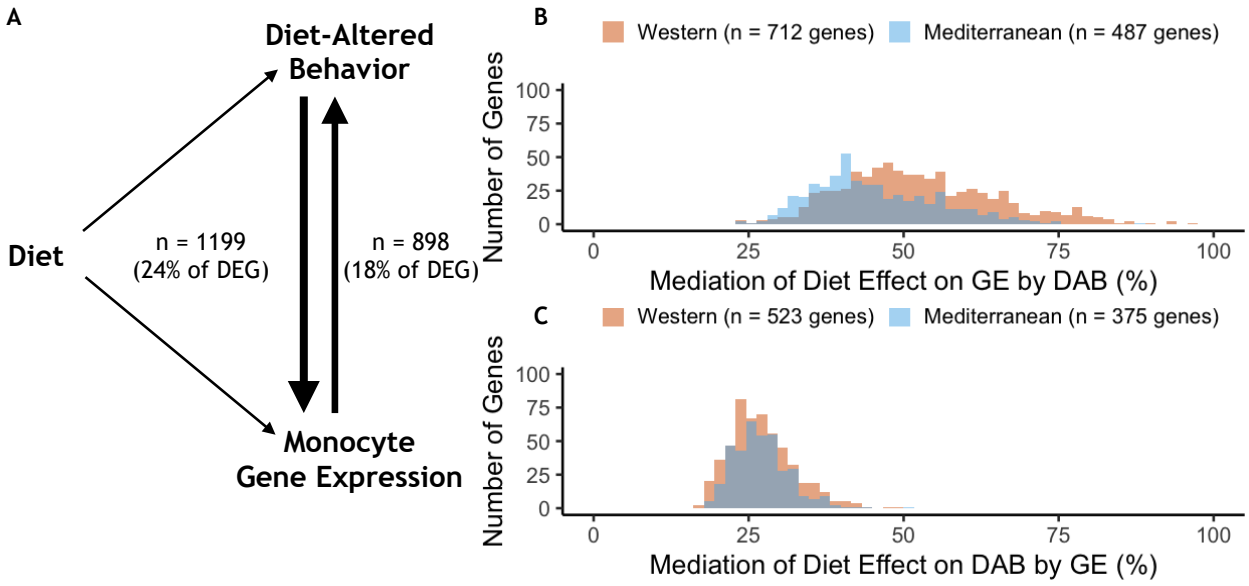

Figure 2.8 Behavior partially mediates the effect of diet on gene expression for 24% of diet-associated genes. A) Diet-altered behavior (DAB) mediated the effect of diet on gene expression for 24% (n = 1199) of genes for which diet had an effect (differentially expressed genes or DEGs). For 18% of DEGs, gene expression mediated the effect of diet on DAB score. **B)** DAB score mediated 23-97% of the total effect of diet on gene expression in 1199 genes (n = 712 Western genes, orange; n = 487 Mediterranean genes, blue). DAB score mediated a greater number of Western genes than Mediterranean genes ($p = 1.5 \times 10^{-21}$) and accounted for a greater portion of the effect size of diet ($p = 6.4 \times 10^{-25}$) in Western genes. **C)** In gene-by-gene models of DAB score as a function of diet + gene expression, gene expression mediated 17-51% of the total effect of diet on DAB in 898 genes (n = 523 Western genes; n = 375 Mediterranean genes). Gene expression mediated a greater number of Western genes than Mediterranean genes ($p = 4.6 \times 10^{-14}$), although expression of these genes did not account for more of the effect of diet on DAB score than Mediterranean genes (Mann-Whitney $U = 1.0 \times 10^{5}$, $p = 0.55$).

We also tested the hypothesis that peripheral immune cell gene expression mediated the effects of diet on behavior in the 27% of DEGs for which monocyte gene expression significantly predicted DAB in a univariate model (n = 1,324, FDR < 0.05). Gene expression mediated the effect of diet on DAB score in 898 genes (18% of all DEGs, $p < 0.05$; **Figure 2.8A**). Almost all of these genes (99%; 889/898) were in the set of genes for which behavioral changes mediated changes in gene expression. The genes that mediated the effect of diet on DAB score were more likely to be Western genes (n = 523 Western genes, 58%, two-sided binomial test $p = 4.6 \times 10^{-14}$), however the portion of the total effect of diet that was accounted for by gene expression did not vary between Western (mean = 27.1%, s.d. = 5.2%) and Mediterranean genes (mean = 27.1%, s.d. = 4.5%; Mann-Whitney $U = 1.0 \times 10^5$, $p = 0.55$; **Figure 2.8C**).

2.2.9 *Diet differentially induced expression of the conserved transcriptional response to adversity (CTRA) genes*

Additional analyses focused on expression of a well-studied set of social adversity-responsive genes known as the "conserved transcriptional response to adversity" (CTRA) (Cole et al., 2015) in the Western- and Mediterranean-fed animals in our study. Animals fed a Western diet exhibited significantly higher expression of pro-inflammatory genes included in the CTRA (Mann-Whitney $U = 222$, $p = 0.016$) and lower expression of antiviral- and antibody-related CTRA genes (Mann-Whitney $U = 82$, $p = 0.023$; **Figure 2.9**; for categorization of CTRA genes, see **Table S2.2A**).

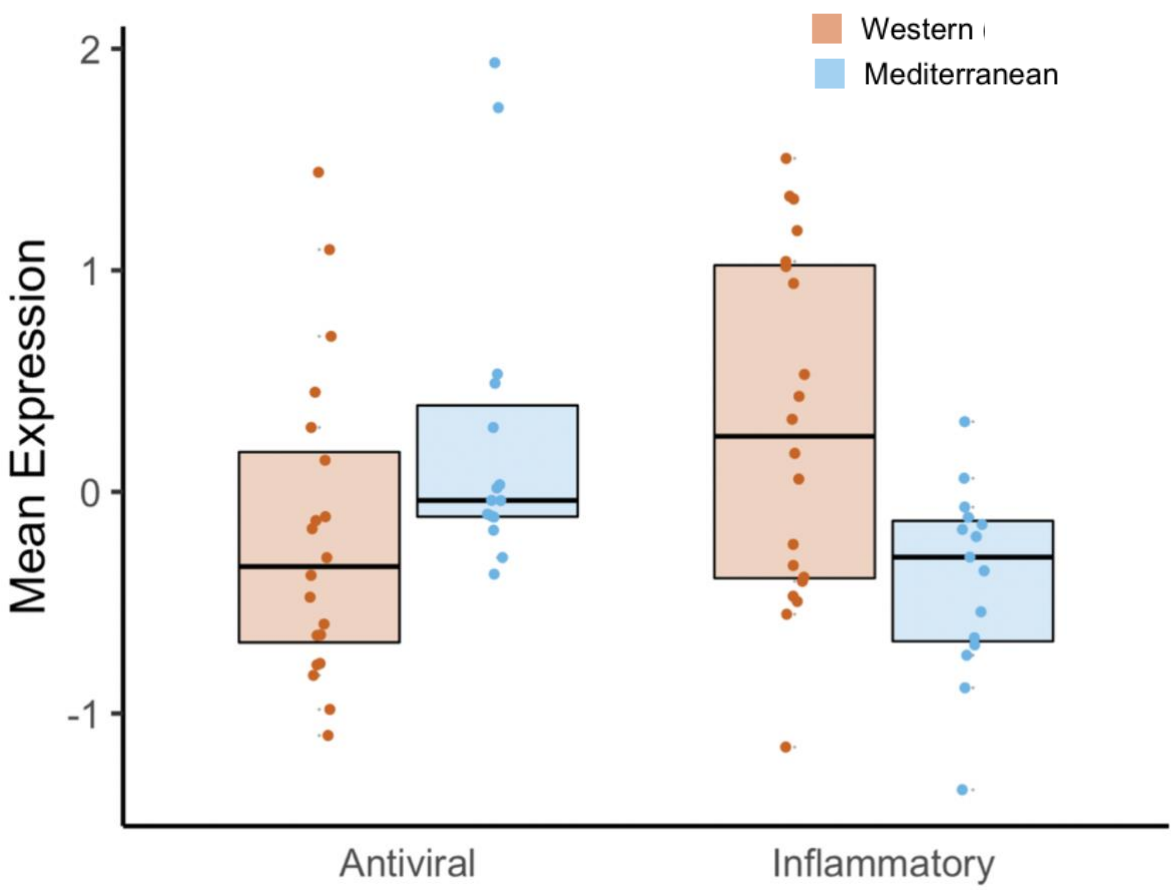

Figure 2.9 Expression of genes in the Conserved Transcriptional Response to Adversity (CTRA). Western-diet fed animals exhibited significantly higher expression of pro-inflammatory genes involved in the conserved transcriptional response to adversity (CTRA (Cole et al., 2015); Mann-Whitney $U = 222$, $p = 0.016$), and lower expression of antiviral- and antibody-related CTRA genes (Mann-Whitney $U = 82$, $p = 0.023$). See **Table S2.2A, B** for CTRA categories.

2.2.10 *Western diet induced a mosaic response*

Western diet induced substantial variation in multiple phenotypes, including body weight, gene expression, and behavior; consistent with previous studies demonstrating that some individuals may be more resistant (or susceptible) to the effects of a Western diet (Shively et al., 2009), presumably due to genetic variation or past environmental exposures. However, we were unable to identify consistencies in individual responsiveness across the phenotypes (**Figure 2.10**). For instance, monkeys that exhibited a strong gene regulatory response to the Western diet did not necessarily exhibit a large increase in body weight or a strong negative DAB score (all $p > 0.2$). Furthermore, change in body weight did not significantly predict gene expression in monocytes (all FDR > 0.2). Western diet fed individuals thus exhibited a mosaic response to diet across multiple phenotypes, presumably involving interactions between diet, stress, behavior, environment, microbiome, and genome/epigenome.

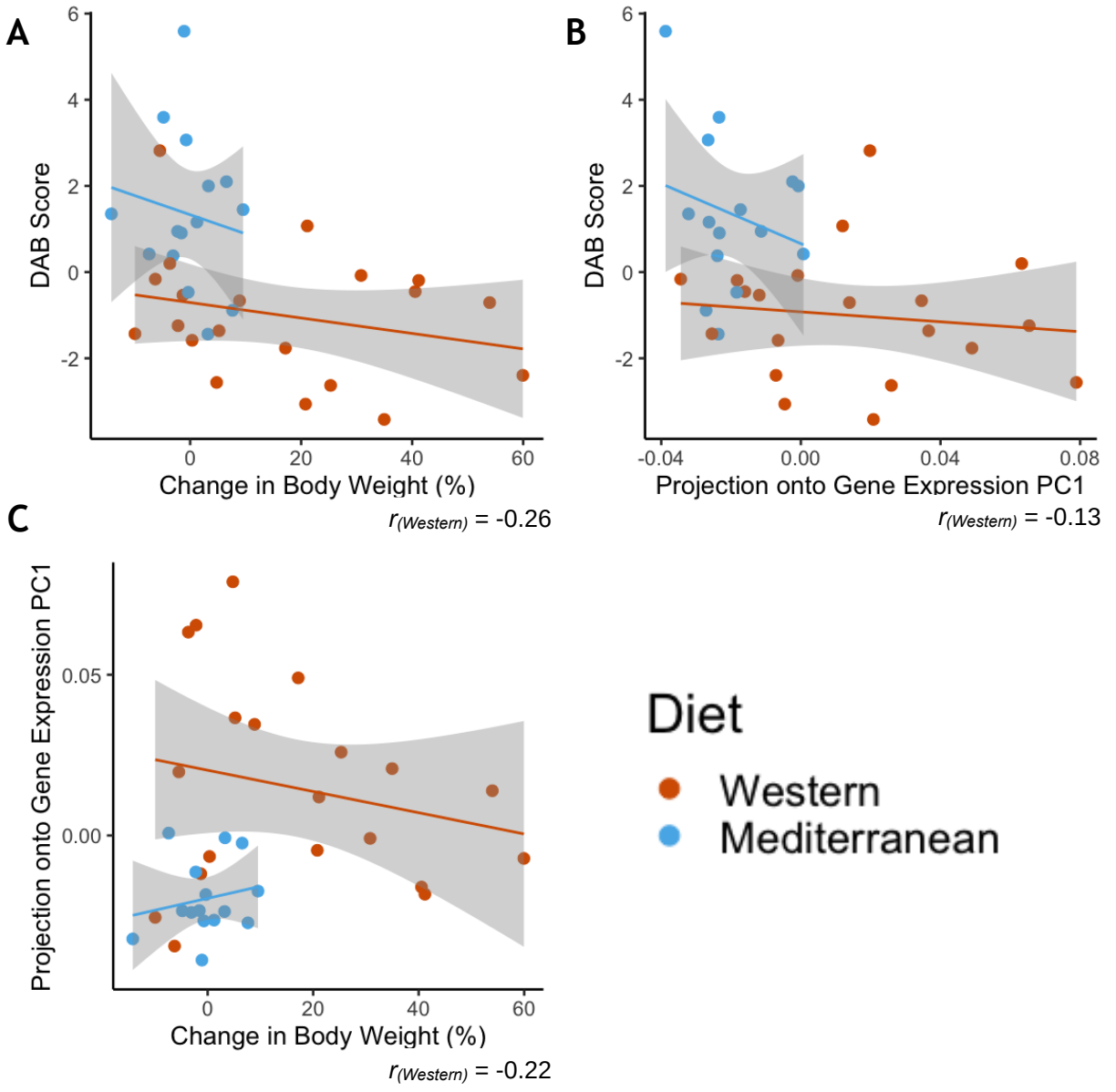

Figure 2.10 Greater phenotypic variability in Western diet fed monkeys does not show consistency in individual responsiveness across phenotypes. A) Monkeys fed the Western diet showed more variability than monkeys fed the Mediterranean diet in both diet-altered behavior (DAB) and change in body weight. However, the two phenotypes were not correlated within monkeys fed the Western diet (Pearson's $r_{Western}$ = -0.26, p = 0.28). **B)** Western fed monkeys also showed more variability in the first principal component (PC1) of gene expression than Mediterranean fed monkeys. DAB and PC1 of gene expression were not significantly correlated in Western fed monkeys (Pearson's $r_{Western}$ = -0.13, p = 0.60). **C)** PC1 of gene expression and

change in body weight were not significantly correlated in Western fed monkeys (Pearson's $r_{Western}$ = -0.22, p = 0.36).

2.3 DISCUSSION

This study shows, for the first time, that a whole-diet manipulation exerted profound effects on monocyte polarization and social behavior in primates. Forty percent of monocyte-expressed genes were differentially expressed between monkeys fed Western or Mediterranean diets, indicating that diet dramatically altered monocyte programming. Relative to monocytes from Mediterranean-fed subjects, monocytes from Western diet consumers exhibited increased expression of proinflammatory and monocyte polarization regulatory genes. Our findings extend previous studies, such as a randomized human cross-over trial that demonstrated changes in monocyte proinflammatory genes, including *IL6*, other interleukins, and NF-κB components, in elderly individuals consuming a Mediterranean like diet enriched in olive oil versus a diet more enriched in saturated fat (Camargo et al., 2012).

We identified a putative molecular mechanism, altered monocyte polarization, that may contribute to the established links between changes in human diets associated with industrialization and increases in chronic disease (Cordain et al., 2005; Drake et al., 2018; Jacka et al., 2010; A. J. Lea et al., 2020; Manzel et al., 2014; Pontzer et al., 2018; Smil, 1989; Smyth & Heron, 2006). Comparative studies of human health across different modern populations – namely those consuming traditional hunter-gatherer, forager-horticulturalist, or pastoralist diets versus modern, Western-like diets – lend support for the evolutionary mismatch hypothesis (Eaton et al., 1988; H. Kaplan et al., 2017; A. J. Lea et al., 2020; Pontzer et al., 2018). In particular, this work has found that traditional populations have much lower rates of non-communicable diseases, especially cardiometabolic diseases, relative to Western societies; however, because so many lifestyle factors differ between traditional societies and those in Western, high income countries, it has been difficult to understand the role of diet specifically in driving health variation or to

address causality (Kraft et al., 2018; Lagranja et al., 2015; Snodgrass, 2013). Additionally, it is difficult to collect samples appropriate for genomic analyses from subsistence-level groups, and consequently the molecular correlates of industrial transitions and evolutionary mismatch remain largely unexplored. Our preclinical randomized study design allows us to draw causal inferences about the role of Western diets in the development of chronic diseases of aging, and provides important data about cellular and molecular mechanisms that may contribute to evolutionary mismatch. These data set the stage for future studies that could compare the transcriptional response to diet in our preclinical study with gene regulatory variation observed between traditional and more market-integrated or Western-like human groups.

Beyond changes in gene expression, we also identified differences in gene co-expression and enrichment of transcription factor binding motifs, suggesting that diet exerts differential effects on gene regulatory networks. Many transcription factors appear to be involved in diet-regulated gene expression. Members of the E26 transformation-specific (ETS), specificity protein (Sp)/Krüppel-like family (KLF), myocyte-specific enhancer factor (MEF), and interferon-regulatory factor (IRF) families of transcription factors, which have all been linked to myeloid differentiation (Chistiakov et al., 2018; Schuler et al., 2008; Scott et al., 1994; D. E. Zhang et al., 1994), were overrepresented in regulatory regions of genes with higher expression in the Mediterranean diet group ("Mediterranean genes"). Three IRF family transcription factors had binding motifs enriched in Mediterranean genes: IRF-1 and IRF-8 are both linked to M1 monocyte polarization, while IRF-3 is associated with M2 polarization. The sole transcription factor with binding sites enriched in Western diet-associated genes, ATF2, is a key mediator of inflammatory pathways and diseases, including response to bacterial endotoxin, atherosclerosis, and obesity (Fledderus et al., 2007; Miyata et al., 2012; Reimold et al., 2001). Western genes were enriched for activation of the

MAPKK pathway, which lies upstream of ATF2 (Herlaar & Brown, 1999), supporting a role in monocyte polarization. Transcription factors were also overrepresented in the pairs of differentially co-expressed genes, further indicating that diet alters regulatory networks and monocyte differentiation and polarization.

It is also worth pointing out that changes in gene co-expression and network connectivity have been previously proposed as a response to novel or challenging environmental conditions, including Western diets. In particular, work on decanalization has hypothesized that gene regulatory networks evolve over many generations of stabilizing selection, and that novel environmental challenges (such as Western diets and lifestyles) may disrupt these fine-tuned connections leading to dysregulation, a breakdown in co-expression, and ultimately disease (Careau et al., 2014; Gibson, 2009a, 2009b; J. X. Hu et al., 2016; A. Lea et al., 2019). In support of this idea, we found diet-induced changes in the co-expression of transcription factors involved in insulin secretion and glucose tolerance (SOX4), lipid, carbohydrate, and energy metabolism (NR4A2), and BMI, HDL, and aging (*RF00283*) (J. P. Davis et al., 2017; Dluzen et al., 2018; Goldsworthy et al., 2008; Kanai et al., 2018; Pearen & Muscat, 2010). We also observed that the transcription factor *MEF2D*, which has previously been implicated in the transcriptomic response to insulin signaling (Samson & Wong, 2002; Solomon et al., 2008), is a hub gene identified in 22 differentially-correlated gene pairs. Hub genes like *MEF2D* may pinpoint optimized systems that break down as a result of mismatch and are thus intriguing targets for future analyses.

It is worth noting that the dichotomous M1/M2 paradigm of monocyte polarization is an oversimplification of the more complex heterogeneity of monocytes (Martinez & Gordon, 2014; Nahrendorf & Swirski, 2016). For example, there are at least three classes of monocytes in the circulation–classical, intermediate, and non-classical. We did not assess the relative abundance of

these subsets, thus the observed gene expression patterns could reflect either changes in the relative proportions of these subsets and/or shifts in monocyte polarization within subsets (Michalson et al., 2019; Wolf et al., 2017).

The diets also altered key behaviors. Monkeys consuming the Western diet exhibited more behaviors related to anxiety and social isolation, a phenotype remarkably similar to that observed in juvenile Japanese macaques born to mothers consuming a high-fat Western diet (Thompson et al., 2018). In that study, offspring behavior was associated with maternal levels of macrophage-derived chemokine (MDC), which showed higher expression in Western-diet fed animals in our study (β_{diet} = 0.243, FDR = 0.059). Our findings suggest that a Western diet may also exert similar behavioral effects when consumed during adulthood.

There are myriad pathways through which diet may affect behavior. Diet may induce changes in the central nervous system (CNS) by altering gut microbiota which alters vagal input to the brain (Bonaz et al., 2018). Previous results from our study demonstrated a strong diet effect on the gut microbiome (Nagpal, Shively, et al., 2018), and lower parasympathetic (vagal) activity in the Western diet group at the time the monocyte transcriptome was assessed (Shively et al., 2020). Taken together these observations suggest that diet-induced changes in vagal tone in the gut-brain axis may be one pathway through which diet impacted brain function, potentially affecting behavior.

Diet-altered behaviors were linked to changes in monocyte gene expression. For a subset (24%) of genes, the DAB score mediated the effect of diet on monocyte gene expression. Monocytes have been shown to be responsive to social isolation (Cole, 2019) and anxiety (Cole et al., 2015). Social isolation and anxiety, produced by Western diet consumption, may be accompanied by increased sympathetic outflow and increased hypothalamic-pituitary adrenal

production of cortisol, both of which modulate monocyte intracellular processes governing inflammatory molecule production (Cacioppo et al., 2015; Holwerda et al., 2018; Juruena et al., 2020). Supporting the involvement of these systems, we previously reported that the Western diet group had increased sympathetic activity, and increased cortisol concentrations (Shively et al., 2020). Western diet may contribute to inflammation by producing a more socially isolated or anxious animal with increased sympathetic and hypothalamic pituitary adrenal activity, which in turn alters monocyte function. Higher expression of genes in the conserved transcriptional response to adversity support this pathway ((Cole, 2019; Cole et al., 2015); **Figure 2.9**). Behavior is a functional assay for the CNS. Thus, this observation suggests that diet may alter CNS function, which may in turn alter circulating monocyte gene expression.

In a somewhat smaller and overlapping subset of genes (18%), diet-induced differences in monocyte gene expression significantly mediated the effect of diet on behavior (DAB). This observation suggests that diet alters monocyte gene expression, which in turn may affect CNS function. There are a variety of mechanisms through which diets differentially influence the nervous system. Western diet may disrupt the blood-brain barrier, increasing infiltration of Western-diet induced cytokines, chemokines, and myeloid cells from the periphery (Raison et al., 2006; Yang et al., 2019). Once in the brain these molecules can alter BDNF production, neurotransmitter systems, and hypothalamic-pituitary-adrenal function (Raison et al., 2006). Western diet induced inflammatory molecules also may affect the brain through direct effects on the afferent vagus nerve (Maier & Watkins, 1998), activation of glial cells (Graham et al., 2016), or alteration of neuronal membrane lipid composition affecting neurotransmission (Du et al., 2016), whereas a Mediterranean diet may have direct anti-inflammatory actions by increasing omega-3 fatty acids in the brain (Layé et al., 2018).

Together, these results support both mediation pathways, suggesting that multiple mechanistic pathways may contribute to these observations; however, we are unable to conclusively state that one mediation pathway is supported over the other or delineate the exact role of the CNS. As each gene is modeled independently in the mediation analyses, it is possible that the expression of a subset of genes in monocytes alters CNS function and induces behavioral change, while expression of another subset of genes is responsive to behavioral phenotypes and/or CNS function. These potential pathways present intriguing possibilities for future experiments.

Monkeys fed the Western diet displayed a heterogeneous response to the diet manipulation across physiological (e.g., body weight), gene regulatory, and behavioral measures. Rather than a single pattern of response to diet where the physiological changes are predictive of behavioral or gene regulatory changes in response to diet, there was no correlation between these measures in monkeys fed the Western diet. This suggests that physiological changes such as weight gain may not be the primary link between diet, poor immune function, and negative health consequences. Understanding both behavioral and gene regulatory responses to environmental mismatch, such as those introduced by dietary patterns, will help to understand the subsequent impact on health.

It is important to note the strengths and limitations of the current study. Macaques continue to be a critical model for understanding human health and disease, including on the influence of diet on numerous phenotypes including atherosclerosis and cardiovascular disease, bone metabolism, breast and uterine biology, and other physiological and pathological phenotypes (Adams et al., 1997; Clarkson, Anthony, et al., 2004; Clarkson, Appt, et al., 2004; Clarkson et al., 2013; Cline et al., 2001; Cline & Wood, 2006; Haberthur et al., 2010; Lees et al., 1998; Mikkola et al., 2004; Mikkola & Clarkson, 2006; Naftolin et al., 2004; Nagpal, Shively, et al., 2018; Nagpal, Wang, et al., 2018; Register, 2009; Register et al., 2003; Shively & Clarkson, 2009; Sophonsritsuk et al.,

2013; Walker et al., 2008; Wood et al., 2007). In a publication based on the same study animals, dietary manipulation produced changes in the gut microbiome similar to that seen in humans consuming Western and Mediterranean diets (Nagpal, Wang, et al., 2018), which further supports the translational relevance of our findings with diet-induced changes in gene expression in genes involved in human health and disease. Nevertheless, extrapolation of the current findings to human health should be done with caution, as the last common ancestor of humans and macaques lived 25 million years ago and evolution has shaped the physiology and natural diet of each species in distinct ways (Luca et al., 2010). A related complication is the difficulty in defining a "control" diet for both human and nonhuman primates. Macaques in the wild are omnivorous, and standard monkey chow derives most of its protein content from soy, which is rich in isoflavones such as genistein and daidzein known to have biological activity (Zaheer & Humayoun Akhtar, 2017). Thus, in the context of the evolutionary mismatch hypothesis, standard monkey chow does not recapitulate a natural macaque diet that could serve as a control for the current diet manipulation, leaving the interpretation of the current results ambiguous as to which diet is driving the changes observed in one diet group relative to the other, which could be addressed in future studies.

In summary, we found that diet significantly alters monocyte polarization and gene expression, and to a lesser extent behavior. The Western diet promoted a proinflammatory monocyte phenotype relative to a Mediterranean diet, which supports the role of monocyte polarization in diet-associated chronic inflammatory diseases. Thus, altered monocyte programming could represent one mechanism underlying an evolutionary mismatch between our past and current diets. The majority of the effects of diet are presumably mediated through direct or combined actions of saturated/polyunsaturated fats, omega-6:omega-3 ratios, pro- and anti-antioxidant characteristics, and other features of the Western diet inconsistent with the nutritional environment in which

humans and nonhuman primates evolved. Ongoing and future work will address interactions between social behavior (e.g., social status) and diet to further understand how environmental stressors may impact inflammation in the periphery and in the central nervous system.

2.4 MATERIALS AND METHODS

2.4.1 *Subjects*

Forty-three adult (age: mean = 9.0, range = 8.2-10.4 years, estimated by dentition), female cynomolgus macaques (*Macaca fascicularis*), were obtained (Shin Nippon Biomedical Laboratories, USA SRC, Alice, TX) and housed at the Wake Forest School of Medicine Primate Center (Winston-Salem, NC) (Shively et al., 2019). Briefly, the monkeys were socially housed in groups of 3-4 and consumed standard monkey chow (**Table 2.1**) during an eight-month baseline phase, after which pens were assigned to receive either the Western (5 groups, n = 21) or Mediterranean (6 groups, n = 22) diet, balanced on pretreatment characteristics that reflected overall health, including body weight, body mass index, circulating basal cortisol, total plasma concentrations, and plasma triglyceride concentrations (Shively et al., 2019). Two monkeys did not tolerate the experimental diet, and were switched to standard monkey chow, three animals died during the course of the study (discussed in (Frye et al., 2020)), and three samples were removed for insufficient CD14 purification (see "Removal of Batch Effects" below), resulting in a final sample size of 35 animals (Western n = 20, Mediterranean n = 15). All animal manipulations were performed according to the guidelines of state and federal laws, the US Department of Health and Human Services, and the Animal Care and Use Committee of Wake Forest School of Medicine.

2.4.2 *Experimental diets*

Experimental diets (**Table 2.1**) were formulated to be isocaloric with respect to protein, fat,

and carbohydrates, and identical in cholesterol content (~ 320mg / 2000 kilocalories (Cals)/day) as previously described (Shively et al., 2019).

Table 2.1 Comparison of Nutritional Contents of Diet Patterns in Human with Nonhuman Primate Diets Used in the Current Study

Diet Composition	Human		Nonhuman Primate		
	Western	**Mediterranean**	**Western[&]**	**Mediterranean[&]**	**Chow[#]**
% of Calories					
Protein	15[a]	17[b]	16[a]	16[b]	18
Carbohydrate[†]	51[a]	51[b]	54[a]	54[b]	69
Fat	33[a]	32[b]	31[a]	31[b]	13
% of Total Fats					
Saturated	33[a]	21[b]	36[a]	21[b]	26
Monounsaturated	36[a]	56[b]	36[a]	57[b]	28
Polyunsaturated	24[a]	15[b]	26[a]	20[b]	32
Other Nutrients					
ω6:ω3 Fatty Acids	15:1[c]	2.1-3:1[d]	14.8:1[c]	2.9:1[d]	12:01
Cholesterol mg/Cal	0.13[a]	0.16[b]	0.16[a]	0.15[b]	trace
Fiber g/Cal	0.01[a]	0.03[e]	0.02[a]	0.04[e]	0.01
Sodium mg/Cal	1.7[a,f]	1.3[b,e]	1.7[a,f]	1.0[b,e]	0.25

[&] Developed and prepared at Wake Forest School of Medicine

[#] LabDiet Chemical Composition Diet 5037/8. Type of fat known in 86% of total fat. Omega-6 from corn and pork fat.

[†] Human carbohydrate calories include alcohol.

[a] (USDA, 2016)

[b] (Bédard et al., 2012)

[c] (Simopoulos, 2006)

[d] (Cordain et al., 2005)

[e] (Kafatos et al., 2000)

[f] (Powles et al., 2013)

reprinted from Obesity *with permission* (Shively et al., 2019)

2.4.3 *Behavioral characterization*

Behavioral data were collected weekly during two 10-minute focal observations, balanced for time of day, for 6 weeks during the baseline phase (2 hours/monkey total) and for 14 months during the experimental phase (mean = 17.6 hours/monkey total). Behaviors recorded included the frequency of aggressive and submissive behaviors, time spent in positive social interactions such as sitting in body contact and grooming or alone, and anxious behavior defined as self-directed behaviors including self-grooming and scratching (Maestripieri et al., 1992; Schino et al., 1996; Shively et al., 2015; Troisi, 2002; Troisi et al., 2000). Behaviors were collected as previously described (Shively, 1998), and combined into summary behaviors (e.g., "aggression" was a combination of all total, noncontact, contact aggressive events). No significant differences in behavior were observed between the diet groups while consuming standard monkey chow diet during the baseline period (**Figure 2.5A, B**). In order to quantify the overall impact of diet on behavior, we conducted a principal component analysis using the R package *FactoMineR* (Lê et al., 2008). We corrected for multiple hypothesis tests using the Holm-Bonferroni adjusted p-values.

2.4.4 *Blood sample collection*

The monkeys were trained to run out of their social groups on voice command. Blood was drawn via venipuncture within 9 minutes of entering the building. Blood was collected into EDTA-containing tubes, mixed with an equal amount of PBS without calcium or magnesium, and overlaid on a 90% Ficoll-Paque Plus/10% PBS solution in LeucoSep tubes followed by centrifugation at 800 x g for 20 min. Isolated PBMCs were then immediately used for the collection of CD14+ monocytes by positive selection using a Miltenyi bead-based protocol following manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). After assessing cell viability and numbers, CD14+ monocytes were stored in 85% FBS, 15% DMSO sterile freezing media at -80°C

and transferred to liquid nitrogen for storage until RNA extraction. Blood samples were collected from all subjects in a given social group on the same day and collection order was alternated between diets and randomized by group.

2.4.5 *RNA extraction and sequencing*

RNA was extracted from monocytes using the AllPrep DNA/RNA Mini Kit (Qiagen, Inc., Hilden, Germany), and quantified using a NanoDrop spectrophotometer and Agilent 2100 Bioanalyzer with RNA 6000 Nano chips (Agilent Technology, Inc., Santa Clara, CA). RNA libraries were prepared for sequencing by the Cancer Genomics Shared Resource (Wake Forest School of Medicine, Winston-Salem, NC) using the TruSeq-stranded total RNA kit (Illumina), which includes a ribosomal depletion step. The RNA-seq libraries were then sequenced using single-end 76-bp reads on an Illumina NextSeq 500 to an average read depth of 34.5 million reads per sample (range 25.9 – 41.6 million reads). Reads were mapped to the *Macaca fascicularis* reference genome (Macaca_fascicularis_5.0, v 93, Ensembl) (Kersey et al., 2018; Kinsella et al., 2011) using HiSat2 (Kim et al., 2015) and then converted to a sample-by-gene read count matrix using featureCounts (Liao et al., 2014) (median = 38.0%; range 24.5 - 50.4% of reads mapped to exons). Sample processing order was randomized and where possible all samples were manipulated simultaneously so as to avoid introducing batch effects.

2.4.6 *Read count normalization and removal of batch effects*

First, we removed genes with low expression (median reads per kilobase per million reads mapped < 1), which resulted in 12,240 genes for downstream analyses. We normalized read counts using the *voom* function of the R package *limma* (Ritchie et al., 2015). While investigating monocyte purity, three samples differed in CD3 gene expression from the rest by several orders of

magnitude. We concluded that these samples were contaminated with CD3+ cells (i.e., inefficient CD14 purification, **Figure 2.11**) and excluded them from all analyses, leaving a final sample size of 35 monkeys (n = 20 fed the Western diet, n = 15 Mediterranean diet). To control for batch effects related to RNA quality and monocyte purity, we calculated the residual gene expression from a model of normalized gene expression as a function of CD14 expression, CD3 expression, RNA integrity, and RNA concentration. These residual gene expression values were used for all subsequent analyses.

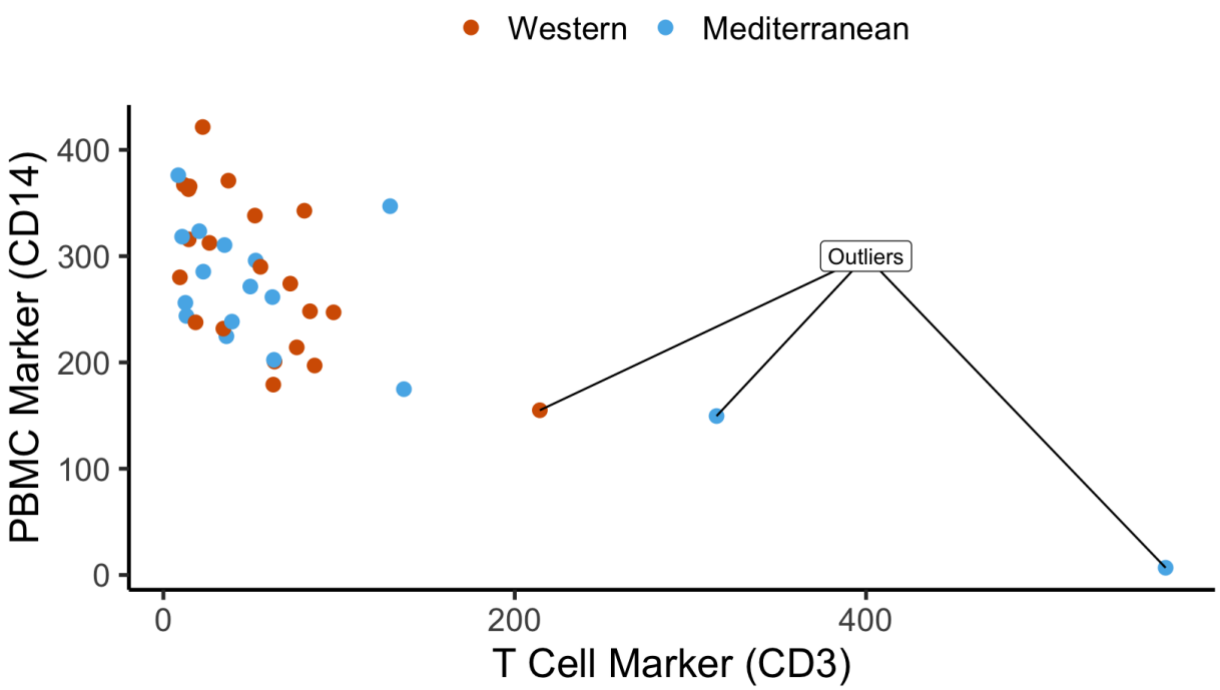

Figure 2.11 Quality control of cell purity by CD14 and CD3 expression levels: three samples were excluded due to lower CD14 and high CD3 – possible T cell contamination. Normalized expression (reads per kilobase million) of CD14 and CD3 are plotted as markers of monocytes and T cells, respectively. Three samples were excluded as outliers due to possible T cell contamination.

2.4.7 *Modeling effect of diet on gene expression*

In order to determine which genes were significantly affected by diet, we modeled the residual expression of each gene as a function of diet using a linear mixed effects model controlling for relatedness among monkeys using the R package *EMMREML* (Akdemir & Godfrey, 2015). Relatedness was estimated using the ngsRelate program (Hanghøj et al., 2019) with SNP genotypes inferred from the RNA-seq reads using bcftools mpileup (Li et al., 2009). We calculated an empirical false discovery rate (FDR) for each gene using a permutation-based approach (Snyder-Mackler et al., 2016), and report genes that passed at FDR < 0.05. To examine global patterns of variation in gene expression, we conducted principal component analysis on the correlation matrix of normalized residual gene expression using the *prcomp* function in R.

2.4.8 *Enrichment analyses*

Gene ontology (GO) enrichment analyses were conducted using Fisher's Exact Tests and the *weight01* algorithm to test for enrichment implemented in the R package *topGO* (Alexa & Rahnenfuhrer, 2019). For a more targeted analysis of M1 and M2 specific genes, we identified a set of DEGs in our data set that were previously found to be involved in monocyte polarization (Schmidl et al., 2014) (638 proinflammatory and 138 regulatory), which we used to explore monocyte polarization in the current study. We calculated the proportion of genes more highly expressed in the Mediterranean- and Western-fed animals in each polarization category and tested for significance using a permutation test (n = 100,000 permutations). To compare the DEGs identified to genes implicated in human health, we utilized gene sets associated with 103 complex human traits and diseases identified by a prior study (Y. Zhang et al., 2020). Fisher's Exact Tests were used to test for enrichment of these gene sets in our Western or Mediterranean DEGs.

We tested for enrichment of transcription factor binding motifs within 2 kb (upstream or downstream) of the transcription start sites of differentially expressed “Western genes” or “Mediterranean genes” (FDR < 0.05) using the program HOMER (Heinz et al., 2010) and equivalent regions around the transcription start sites of all genes expressed in these data as the background set for enrichment testing. We searched for known vertebrate transcription factor binding motifs and report the TF motifs passing a threshold of FDR < 0.05.

2.4.9 *Gene-gene co-expression analysis*

In addition to testing whether diet led to mean differences in gene expression between Western and Mediterranean animals, we also tested whether diet impacted the correlation structure among expressed genes (i.e., gene co-expression). Specifically, we employed ‘correlation by individual level product’ (CILP) (A. Lea et al., 2019) analyses to test whether diet affected the magnitude or direction of pairwise gene expression correlations among the top 140 DEGs (n = 9730 gene-gene pairs tested). To test whether a given pair of genes was differentially co-expressed as a function of diet, we first obtained a vector of products for each gene pair by multiplying the normalized gene expression values for two genes together. Normalization was performed by scaling expression values to mean 0 and unit variance within Mediterranean and Western subsets of the data respectively, to ensure that distributional differences between sample groups did not bias our results, following the CILP authors’ recommendations (A. Lea et al., 2019). Each of these vectors of products were used as the outcome variable in a linear mixed effects model implemented in the R package *EMMREML* (Akdemir & Godfrey, 2015), which included a fixed effect of diet and a random effect to control for genetic relatedness. To assess significance, we extracted the p-value associated with the diet effect for all 9730 gene pairs. We then repeated each linear mixed effects model 100 times after permuting diet, extracted the p-value associated with the diet effect, and

used these values to calculate an empirical FDR distribution (Snyder-Mackler et al., 2016).

Using this approach, we identified 445 gene pairs that were significantly differentially co-expressed as a function of diet at a 20% empirical FDR. Next, we performed two follow up analyses to understand their biological import. First, we tested for the existence of 'hub genes', defined as genes that displayed differential co-expression to their tested partner genes more so than expected by chance. To define the null distribution for identifying hub genes, we randomly sampled 445 gene pairs from the set of all 9730 tested gene pairs 1000 times and calculated the number of partners a focal gene had in each sample; we considered a gene to be a significant 'hub gene' if it fell outside the 95th percentile of this distribution, which was equivalent to a focal gene that displayed significant differential co-expression with 13 or more of its tested partner genes. Second, we asked whether the set of 'hub genes' we identified were enriched for transcription factors, relative to the background set of all 140 genes tested for differential co-expression. We performed this analysis because many of the proposed mechanisms to generate large scale changes in gene co-expression patterns involve changes in transcription factor function or activity (de la Fuente, 2010; Gaiteri et al., 2014). To implement the enrichment analysis, we used the TRRUST database of known mammalian transcription factors for annotation (Han et al., 2018) paired with hypergeometric tests.

2.4.10 *Weighted gene co-expression network analysis*

We employed the commonly used approach of weighted gene co-expression network analysis (WGCNA) to identify and characterize modules of co-expressed genes. We used the *WGCNA* R package (Langfelder & Horvath, 2008) with a minimum module size of 30 genes and minimum module dissimilarity threshold of 0.25 to identify co-expression modules, which were then used for downstream analyses.

2.4.11 *Mediation analysis*

To explore relationships between DAB score and differential gene expression, we conducted mediation analyses using a bootstrapping approach involving 10,000 bootstrap iterations of two models: (Model 1) the expression of each gene as a function of diet, and (Model 2) the expression of each gene as a function of diet and DAB score (Preacher & Hayes, 2004). For each bootstrap iteration, we then calculated the mediation effect (i.e., the indirect effect) of DAB score as the difference between the effect size of diet in Model 1 (β_{diet}) and Model 2 (β'_{diet}). We considered there to be a mediation effect when the 90% confidence interval for the indirect effect (β_{diet}-β'_{diet}) did not include zero.

A similar method was used to calculate the mediation of gene expression on DAB, testing the difference between the effect size of diet in two models: (Model 3) DAB as a function of diet, and (Model 4) DAB as a function of diet and the expression of each gene.

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Data Availability

All data and code used to complete these analyses can be found at https://github.com/cscjohns/diet_behavior_immunity. The raw data can be accessed from the gene expression omnibus repository from accession # GSE144314.

Chapter 3. Mediterranean Diet Reduces Social Isolation and Anxiety in Adult Female Nonhuman Primates

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Abstract:

Dietary composition is associated with differential prevalence of psychiatric disorders; the Western diet conferring increased risk, while the Mediterranean diet appears to reduce risk. In nonhuman primates, anxiety-like behaviors and social isolation have been linked to both Western diet consumption and increased inflammatory disease risk, and recent evidence suggests that diet composition may affect immune system function in part through its effects on behavior. This is particularly important in the context of the global COVID-19 pandemic in which social isolation has been associated with disease. Here, we examined the effects of Western- and Mediterranean-like diets on social behavior in a randomized, 34-month preclinical trial in middle-aged female

cynomolgus macaques (*Macaca fascicularis*). Diet induced rapid and persistent changes in a suite of behaviors. After just three months of experimental diet consumption, a composite measure of diet-altered behavior (DAB), significantly differed between the two diets ($p = 0.014$), and remained different throughout the 24-month experimental observation period ($p = 2.2 \times 10^{-8}$). Monkeys fed the Western diet spent more time alone (FDR = 4.4×10^{-5}) and displayed more anxiety behavior (FDR = 0.048), whereas monkeys fed the Mediterranean diet spent more time resting (FDR = 0.0013), attentive (FDR = 0.017), and in body contact with groupmates (FDR = 4.1×10^{-8}). These differences were largely due to changes in behavior of animals fed the Mediterranean diet, while Western-diet-fed-animals exhibited similar behaviors compared to the 8-month baseline period, during which all monkeys consumed a common laboratory diet. These observations provide experimental support in a nonhuman primate model demonstrating a potential therapeutic benefit of the Mediterranean diet consumption to reduce social isolation and anxiety, and thus mitigate social isolation associated disorders that often accompany illness and disability.

3.1 INTRODUCTION

Long standing evidence supports an association between diet composition and incidence of psychiatric disorders. Observational studies have demonstrated increased prevalence of anxiety in people self-reporting consumption of a Western diet, rich in simple sugars, and animal sources of saturated fats and proteins, and decreased prevalence in those self-reporting adherence to a Mediterranean diet, rich in plant sources of mono/polyunsaturated fats, proteins, and antioxidants (Bakhtiyari et al., 2013; Fond et al., 2020; Gibson-Smith et al., 2020; Jacka et al., 2010, 2017; Ventriglio et al., 2020). While psychiatric disorders have an independent and profound impact on human health, they are often comorbid with cardiovascular (Cohen et al., 2015; Goodwin et al.,

2009; Shively et al., 1997, 2009; Vogelzangs et al., 2010) and inflammatory diseases (Marrie et al., 2015, 2017), suggesting a shared underlying pathology. In support of this, a large body of evidence across taxa has supported a physiological response to social adversity (e.g. adverse events early in life, low social status, and social isolation), that is linked to reduced health span and lifespan (Snyder-Mackler et al., 2020). For instance, in both human and nonhuman primates (NHPs), individuals that are more socially integrated have longer, healthier lives than their more socially isolated counterparts (Berkman et al., 2000; Berkman & Syme, 1979; Campos et al., 2020; Hawkley & Cacioppo, 2010; Holt-Lunstad et al., 2010, 2015; House et al., 1988; Shively et al., 1989). Thus, understanding the effects of common human dietary patterns on socioemotional behavior, in particular social isolation and loneliness, may elucidate neurobiological pathways through which diet impacts human health.

Long term effects of diet composition on human behavior are difficult to ascertain as lengthy feeding trials are expensive, and self-reported food intake data are unreliable (T. M. Miller et al., 2008; Suchanek et al., 2011). Similarly, long term assessments of sociobehavioral variables in humans are most often based on self-report (Alcaraz et al., 2019; Friedler et al., 2015), confounding perceived with actual events. In addition, definitions of social isolation may vary across studies (Evans et al., 2019), and social isolation (objective physical separation from social contacts) and loneliness (distress from perceived social isolation) are often conflated in the literature (Holt-Lunstad et al., 2015). Macaques have long been used as models for human health and disease as they share many core genetic, physiological, and behavioral phenotypes, including omnivory, with humans (Jarczok et al., 2018; Kromrey et al., 2016; Shively, 1998; Shively & Clarkson, 2009; Shively & Day, 2015; Willard & Shively, 2012). Like humans, individual NHPs may vary in their degree of social integration and isolation, and multiple factors may influence sociality, including

age, sex, social status, kin networks, and familiarity with social partners (Brent et al., 2017a). These similarities suggest that NHPs may share mechanisms linking social isolation and health with humans, and direct observation of social isolation avoids the confounds inherent with human studies, thus supporting their use as a model system. Currently, little is known about how diet impacts social isolation and whether other measures of the social environment, like social status, can affect those relationships.

We drew on data from our randomized preclinical trial in socially housed cynomolgus macaques (*Macaca fascicularis*) to address these questions. The monkeys first consumed the lab-standard monkey chow during an 8-month baseline phase prior to assignment to groups that were fed either a Mediterranean or a Western diet, formulated to mimic human diet patterns, for 26 months (Shively et al., 2019). Our previous findings from this preclinical trial demonstrated that relative to the Mediterranean diet, the Western diet increased body weight, body fat, insulin resistance, and hepatosteatosis (Shively et al., 2019); altered gut microbiome composition (Nagpal, Shively, et al., 2018); exacerbated autonomic and hypothalamic-pituitary-adrenal responses to psychosocial stress (Shively et al., 2020); altered brain neuroanatomy (Frye et al., 2020); and drove inflammatory polarization of circulating immune cells (Johnson et al., 2021). In the latter article, we reported that the second axis of variance in a principal component analysis of behavior during the first 14 months of the study was strongly associated with diet (Johnson et al., 2021). High scores on this "diet-altered behavior" principal component were positively associated with Mediterranean diet consumption, time spent in body contact, and time resting. Low scores were associated with Western diet consumption, time spent alone, anxiety behaviors, and proinflammatory immune cell gene expression. This behavioral phenotype was remarkably similar to that seen in the juvenile offspring of Japanese macaques that consumed a Western diet in the

perinatal period (Thompson et al., 2018), and is concordant with a human study in which Western diet was a risk factor for loneliness (Kobayashi & Steptoe, 2018).

Here we present an in-depth analysis of long-term changes in socioemotional behavior that differed between those that consumed a Western versus a Mediterranean diet pattern. We demonstrate the relatively rapid appearance of diet-altered behavior after just three months of experimental diet consumption, and the persistence of diet-distinct patterns of socioemotional behavior throughout the entire 24 months observation. We show that diet induces changes in affiliation, activity, and anxiety, and that these behavioral differences between the two diet groups were driven largely by changes in behavior from the baseline phase in the Mediterranean-fed monkeys.

3.2 METHODS

3.2.1 *Subjects*

Adult (age: mean = 9.0, range = 8.2-10.4 years, estimated by dentition), female cynomolgus macaques (*Macaca fascicularis*), were socially housed in groups of 3-4 in 3 m × 3 m × 3 m enclosures, with daylight exposure, on a 12/12 light/dark cycle with monkey chow (**Table 2.1**) and water available ad libitum during an 8-month baseline phase. Social groups were then randomized to receive either the Western-like (hereafter "Western;" n = 5 groups, n = 21 monkeys) or Mediterranean-like (hereafter "Mediterranean;" n = 6 groups, n = 17 monkeys) diet. The two diet groups were balanced on markers of overall health measured during the baseline phase, including body weight, body mass index, circulating basal cortisol, total plasma cholesterol, and plasma triglyceride concentrations (Shively et al., 2019). All animal manipulations were performed according to state and federal laws, and guidelines from the US Department of Health and Human Services and the Animal Care and Use Committee of Wake Forest School of Medicine.

3.2.2 *Experimental diets*

Following the 8-month baseline period, macaques consumed one of two experimental diets formulated to match human Western and Mediterranean dietary patterns for 26 months. The experimental diets were isocaloric with respect to macronutrients and identical in cholesterol content (~320 mg/2000 kilocalories per day), but differed in composition. The Western diet was designed to mimic the diet typically consumed by middle-aged American women (USDA, 2016), resulting in a diet rich in saturated fats, sodium, and refined sugars with fats and proteins mostly from animal sources (Cordain et al., 2005). In contrast, the Mediterranean diet was formulated to reflect the human Mediterranean diet in high levels of monounsaturated fats and a lower omega-6:omega-3 fatty acid ratio than the Western diet, with protein and fat derived mostly from plant sources (Kafatos et al., 2000). Key Mediterranean ingredients included English walnut powder and extra-virgin olive oil, which were provided to participants in the Prevención con Dieta Mediterránea (PREDIMED) primary prevention trial (Estruch et al., 2018). Macronutrient composition of experimental diets compared to monkey chow and human diet patterns is shown in **Table 2.1**. The composition of experimental diets has previously been published (Shively et al., 2019), and is described in **Table 3.1**. Diet preparation is described in **Appendix C.** Monkeys were provided 120 kcal diet per kg of bodyweight per day (120 kcal/kg/day), which was enough so that 10% of the diet was left at the end of the day, ensuring all group members had adequate access.

Table 3.1 Experimental Diet Composition.

Western-like Diet		Mediterranean-like Diet	
Ingredient	**g/ kg**	**Ingredient**	**g/kg**
Casein, USP	85.0	Casein, USP	17.4
Whey Protein - 895 895	85.0	Whey protein - 895	17.4
		Dried Egg white	26.1
		Fishmeal (Menhaden)	26.1
		Walnuts	8.7
		Black Bean flour	43.5
		Garbanzo Bean flour	17.4
		Wheat Flour (all purpose)	243.5
Dextrin	260.0	Dextrin	96.6
Sucrose	180.0	Sucrose	34.8
High Fructose Corn Syrup 55	70.0	Banana	1304
		Applesauce	38.2
		Tomato paste	17.4
Cellulose (Alphacel)[1]	79.4	Cellulose (Alphacel) [1]	94.8
Lard	41.5	Olive Oil (Filippo Berio Extra Virgin)	61.7
Beef Tallow HHR	40.0	Menhaden Oil (Omegapure)	8.7
Butter, lightly salted	12.5	Butter, lightly salted	8.7
Corn Oil	35.0	Corn Oil	10.4
Flaxseed oil	3.0	Flaxseed oil	1.7
Dried Egg Yolk	6.0	Dried Egg Yolk	14.8
Crystalline Cholesterol	0.4		
Complete Vitamin Mix (Teklad)	25.0	Complete Vitamin Mix (Teklad)	21.7
Mineral Mix w/o Ca, P, NaCl	50.0	Mineral Mix w/o Ca, P, NaCl	43.5
Calcium Carbonate	4.3	Calcium Carbonate	3.7
Calcium Phosphate, Monobasic	7.5	Calcium Phosphate, Monobasic	6.5
NaCl (Table Salt)	16.0	NaCl (Table Salt)	6.3
TOTAL	1000	TOTAL	1000

[1] Total Fiber (% of diet): WEST: 7.94; MED: 12.7

3.2.3 *Behavioral characterization*

Behavioral data were collected weekly during two 10-minute focal observations (Altmann, 1974), balanced for time of day, for 6 consecutive weeks during months 3-4 of the baseline phase (2 hours/monkey total), and for 24 consecutive months beginning the third month of the treatment phase (approximately 200 behavior samples/monkey, mean = 31.0 hours/monkey, and 1178 observation hours total). Inter-rater reliability was maintained at ≥ 93% and no other research activities were ongoing during behavior observations. Behavioral data were not collected if the animal was ill or temporarily removed from the social group for evaluation. Thus, the length of observation varied from 0-80 minutes/monkey/month (median = 80 minutes/monkey/month). Data were collected on 38 animals, 21 in the Western group and 17 in the Mediterranean group (2 Mediterranean animals had zero observations for one month). All recorded behaviors are operationally defined in **Table 3.2**. Briefly, these behaviors included the frequency of aggressive and submissive behaviors differentiated by severity and directionality of behaviors; time spent in close proximity, alone, or in body contact to group mates; time spent giving or receiving grooming; time spent attending to the environment (attentive, investigating, or fearfully scanning); activity (time spent lying down with eyes open, resting with eyes closed, or in locomotion); and the frequency of anxious behavior defined as self-grooming and scratching (Maestripieri et al., 1992; Schino et al., 1996; Shively et al., 2015; Troisi, 2002; Troisi et al., 2000). Behaviors were combined into summary behaviors (e.g., “mild aggression”, defined as the sum of noncontact aggression events including display, displace, and threat), as previously described (Shively, 1998). The outcomes of agonistic interactions were also tabulated monthly to determine social status. Based on these win-loss interactions, females were assigned a relative dominance rank, which represented the proportion of females in their group that were submissive to them. Thus, the

highest-ranking female was given a relative rank value of 1, and the lowest-ranking female a relative rank value of 0. Relative ranks were stable over the experiment and significantly correlated between the baseline and treatment phases (Spearman's rho = 0.90, $p < 0.0001$) (Shively et al., 2020). Thus, mean relative ranks were calculated for the duration of the experiment, and animals classified as dominant if they had a mean relative rank > 0.5, subordinate if their mean relative rank was ≤ 0.5.

Table 3.2 Dependent Variables Measured or Calculated in Behavioral Characterization of Diet Trial.

Dependent Variable	Operational Definition
Affiliation Behaviors Observed	
Percent of Time Spent Alone	Percent of time spent out of monkey's arm's reach of conspecifics
Percent of Time Spent in Body Contact	Percent of time spent in contact with conspecific(s)
Percent of Time Spent in Close Proximity	Percent of time spent within monkey's arm's reach of conspecific(s)
Activity Behaviors Observed	
Percent of Time Spent Attentive	Percent of time monkey alert and attending to an object or event
Percent of Time Spent Fearfully Scanning	Percent of time visual scanning of the environment
Percent of Time Spent in Locomotion	Percent of time walking or running along the ground or over suspended surfaces (> 1 m/min)
Percent of Time Spent Investigating	Percent of time exploratory behavior directed at environment
Percent of Time Spent Lying Down	Percent of time lying down with eyes open
Percent of Time Spent Resting	Percent of time resting with eyes closed or asleep
Anxiety Behavior Observed	
Rate of Anxiety Behavior	Frequency of anxiety-related behaviors, including scratching, itching, and grooming self
Social Behaviors Observed	
Percent of Time Spent Being Groomed	Percent of time being groomed by conspecific(s)
Percent of Time Spent Grooming	Percent of time grooming conspecific(s)
Rate of Extreme Aggression	Frequency of extreme aggressive behaviors including bite, slap, grab, chase, charge, and lunge
Rate of Extreme Aggression Received	Frequency of receiving extreme aggressive behaviors including bite, slap, grab, chase, charge, and lunge
Rate of Extreme Submission	Frequency of extreme submissive behaviors including scream, squeal, scream threat, crouch, and flee
Rate of Extreme Submission Received	Frequency of receiving extreme submissive behaviors including scream, squeal, scream threat, crouch, and flee
Rate of Mild Aggression	Frequency of mild aggressive behaviors including display, displace, and threat (open mouth, stare, or yawn)
Rate of Mild Aggression Received	Frequency of receiving mild aggressive behaviors including displace and threat (open mouth, stare, or yawn)
Rate of Mild Submission	Frequency of mild submissive behaviors including lip smack, grimace, submissive present, crouch and move away
Rate of Mild Submission Received	Frequency of receiving mild submissive behaviors including lip smack, grimace, submissive present, crouch and move away
Composite Behavior Calculated	
Diet-Altered Behavior (DAB) Score	Linear combination of all 20 observed behaviors, scaled and multiplied by the loading of each variable onto PC2 (from Johnson et al., 2021) and weighted by its eigenvalue

3.2.4 *Statistical analyses*

The baseline and monthly treatment phase behavioral data were scaled and principal component analysis was conducted using the *PCA* function of the R package *FactoMineR* (Johnson et al., 2021; Lê et al., 2008). Similar to our previous report, the first principal component of behavior accounted for 59% of the variance and was related to relative rank but not diet. The second principal component was significantly associated with diet and accounted for 19% of the variance, and was used to calculate Diet-Altered Behavior (DAB) Scores (Johnson et al., 2021). Individual DAB scores were then calculated for each monkey for each month as a linear combination of the observed incidence of each behavior scaled and multiplied by the loading of each variable onto the second principal component (PC2, diet-altered behavior or DAB) weighted by the eigenvalue of PC2. This approach incorporated all 20 behaviors for which we collected data, but weighted behaviors by their contribution to the DAB score axis, such that time in body contact and resting had strong positive loading scores, while time alone and rate of anxiety behaviors had strong negative loading scores, and time in close proximity and grooming contributed little to an individual's DAB score.

We tested baseline differences between the two groups in each behavior or DAB scores using Welch-Satterthwaite t-tests. Analyses of variance or covariance were used to test the relationship between diet and behavior, where the behavior at baseline was included as a covariate in analysis of covariance (ANCOVA) if it was a significant predictor of the treatment phase behavior ($p < 0.05$); otherwise, ANOVA was used. ANCOVA was used to test the *a priori* hypothesis that DAB score changed in the first month of behavioral observations (third month of the treatment phase). A repeated measures ANCOVA was used to test for the stability of DAB score over time. Following this, ANCOVA was used as a *post-hoc* test to determine in which months the DAB

scores of the two diet groups were significantly different. P-values for the main effects of diet of each month were corrected for multiple hypothesis testing using the qvalue false discovery rate function from the *qvalue* R package (Storey & Tibshirani, 2003) and reported as the false discovery rate (FDR). Analysis of variance was used in the same manner to test for differences in each of the 20 behaviors collected.

3.3 RESULTS

We previously reported a significant difference between diet-altered behavior (DAB) scores of the two diet groups at 14 months experimental diet consumption and examined the association between DAB score and monocyte transcripts of immune function (Johnson et al., 2021). Here, we evaluated how quickly DAB score diverged between the diet groups and how long the effect persisted, and we examined the underlying differences in individual behavior.

3.3.1 *Diet groups did not significantly differ in any behaviors during the baseline phase*

There was no significant difference in DAB score between the two treatment groups during the baseline phase during which the monkeys consumed a standard "monkey chow" diet ($t_{[36.0]} = -0.3$, $p = 0.73$; **Figure 3.1**, **Table 3.3**). There were no significant differences between diet groups during the baseline phase in any of the twenty individual behaviors measured (all $p > 0.05$; see **Table 3.2** for a list of behaviors measured and their operational definitions).

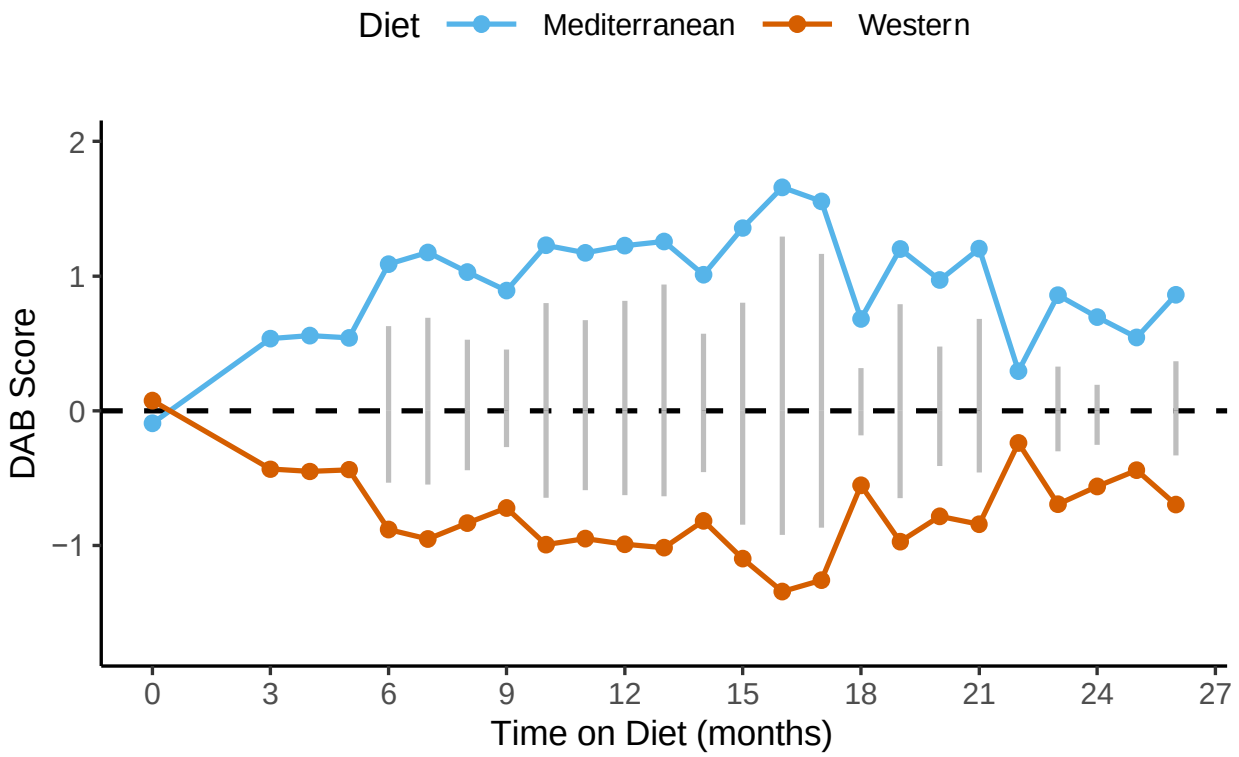

Figure 3.1 Diet induced rapid changes in DAB score that persisted throughout the experiment. Monkeys consuming the Mediterranean diets (blue points [mean] and ribbons [SEM]) exhibited higher DAB scores in the first month of behavioral observation (3 months on experimental diets; $F_{[1,35]} = 6.7$, $p = 0.014$), as well as consistently higher DAB scores during the treatment phase than did monkeys consuming the Western diets (orange points [mean] and ribbons [SEM]; $F_{[1,33]} = 53.3$, $p = 2.2 \times 10^{-8}$). There was a significant interaction between month and diet ($F_{[10.3,\ 341.2]} = 2.2$, $p = 0.019$). Holm-Bonferroni-adjusted *post-hoc* analyses demonstrated significant differences in 19/24 months at $p < 0.05$, as indicated by vertical lines between points for each month, and an additional 4/24 months at $p < 0.10$.

Table 3.3 Mediterranean vs. Western Group DAB Scores by Month.

Month of Observation (months on diet)	Mediterranean Group *Mean (S.D.)*	Western Group *Mean (S.D.)*	Statistical Analysis	Test Statistic	*p-value*	*Holm-Bonferroni adjusted p-value*
Baseline	-0.09 (1.4)	0.07 (1.6)	t-test (df = 36.0)	-0.3	0.73	-
1 (3)	0.54 (1.9)	-0.43 (1.5)	ANCOVA (df = [1, 35])	27.2	**0.014***	0.070
2 (4)	0.56 (2.0)	-0.45 (1.7)	ANCOVA (df = [1, 35])	29.1	0.016	0.070
3 (5)	0.54 (1.9)	-0.44 (1.6)	ANCOVA (df = [1, 35])	27.8	0.015	0.070
4 (6)	1.09 (1.7)	-0.88 (1.4)	ANCOVA (df = [1, 35])	15.9	1.8×10^{-5}	**3.0×10^{-4}**
5 (7)	1.18 (1.8)	-0.95 (1.6)	ANCOVA (df = [1, 35])	17.4	2.4×10^{-5}	**3.6×10^{-4}**
6 (8)	1.03 (1.9)	-0.83 (1.6)	ANCOVA (df = [1, 35])	14.7	2.0×10^{-4}	**0.0024**
7 (9)	0.89 (1.6)	-0.72 (1.8)	ANCOVA (df = [1, 35])	60.7	1.3×10^{-5}	**2.3×10^{-4}**
8 (10)	1.23 (1.6)	-1.00 (1.4)	ANCOVA (df = [1, 35])	10.6	4.3×10^{-6}	**8.6×10^{-5}**
9 (11)	1.17 (1.9)	-0.95 (1.4)	ANCOVA (df = [1, 35])	12.6	2.9×10^{-5}	**4.0×10^{-4}**
10 (12)	1.22 (1.5)	-0.99 (1.4)	ANCOVA (df = [1, 35])	4.4	1.8×10^{-5}	**3.0×10^{-4}**
11 (13)	1.26 (1.1)	-1.02 (1.5)	ANOVA (df = [1, 36])	26.4	9.9×10^{-6}	**1.9×10^{-4}**
12 (14)	1.01 (1.6)	-0.82 (1.4)	ANCOVA (df = [1, 35])	4.9	2.9×10^{-4}	**0.0032**
13 (15)	1.36 (2.1)	-1.10 (0.9)	ANCOVA (df = [1, 35])	10.0	2.6×10^{-6}	**5.4×10^{-5}**
14 (16)	1.66 (1.3)	-1.34 (1.7)	ANCOVA (df = [1, 35])	19.4	6.2×10^{-9}	**1.5×10^{-7}**
15 (17)	1.55 (1.4)	-1.26 (1.6)	ANCOVA (df = [1, 35])	7.0	2.4×10^{-7}	**5.4×10^{-6}**
16 (18)	0.68 (1.3)	-0.55 (1.5)	ANCOVA (df = [1, 35])	11.8	0.0020	**0.016**
17 (19)	1.20 (1.5)	-0.97 (1.3)	ANCOVA (df = [1, 35])	19.4	3.6×10^{-7}	**8.0×10^{-6}**
18 (20)	0.97 (1.8)	-0.78 (1.5)	ANCOVA (df = [1, 35])	8.9	6.1×10^{-4}	**0.0061**
19 (21)	1.20 (1.8)	-0.84 (1.5)	ANCOVA (df = [1, 35])	14.6	7.8×10^{-5}	**0.0010**
20 (22)	0.30 (2.1)	-0.24 (1.4)	ANCOVA (df = [1, 35])	10.0	0.23	0.23
21 (23)	0.86 (2.0)	-0.70 (1.6)	ANCOVA (df = [1, 35])	12.7	0.0020	**0.016**
22 (24)	0.70 (1.9)	-0.56 (1.2)	ANCOVA (df = [1, 35])	15.4	0.0030	**0.018**
23 (25)	0.54 (1.9)	-0.44 (1.3)	ANCOVA (df = [1, 35])	7.6	0.032	0.070
24 (26)	0.86 (1.8)	-0.70 (1.5)	ANCOVA (df = [1, 35])	15.9	7.4×10^{-4}	**0.0066**

**A priori* hypothesis, thus reported p-value was not corrected for multiple hypothesis testing.

3.3.2 *Diet induced changes in activity, affiliation, and anxiety*

In contrast with the baseline phase of the experiment, we observed differences in DAB scores between the two diet groups in the experimental phase. Over the 24 months in which monkeys were fed experimental diets, the Mediterranean group had significantly higher DAB scores than the Western group ($\bar{x}_{Mediterranean}$ = 0.98, $\bar{x}_{Western}$ = -0.79; ANCOVA $F_{[1, 33]}$ = 53.3, p = 2.2 x 10^{-8}; **Figure 3.1**).

We next determined which behaviors responded to diet. Of twenty behaviors measured, five (resting, body contact, attentive, anxiety, alone) were significantly different between diet groups during the treatment phase (**Figure 3.2A-C**). The Mediterranean group spent significantly more time resting ($\bar{x}_{Mediterranean}$ = 5.7%, $\bar{x}_{Western}$ = 1.2%; ANOVA $F_{[1,36]}$ = 15.6, FDR = 0.0013; **Figure 3.2A**), and were more attentive than the Western group ($\bar{x}_{Mediterranean}$ = 41.4% of time spent attentive, $\bar{x}_{Western}$ = 33.0%; ANCOVA $F_{[1,35]}$ = 8.6, FDR = 0.017; **Figure 3.2A**).

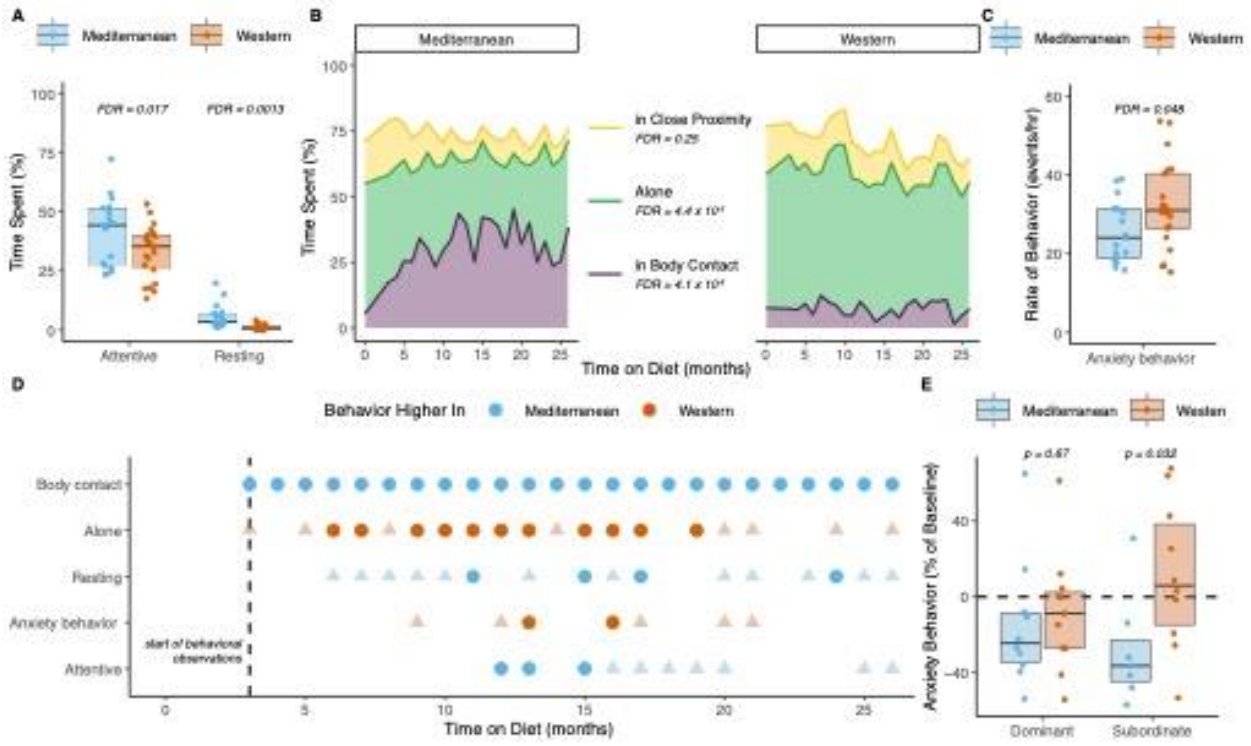

Figure 3.2 Changes in behavior following experimental diet treatments. A) Monkeys in the Mediterranean group (blue boxes and points) spent more time attentive (ANCOVA $F_{[1,35]}$ = 8.6, FDR = 0.017) and resting (AVOVA $F_{[1,36]}$ = 15.6, FDR = 0.0013) than those in the Western group (orange boxes and points). **B)** The left plot shows how monkeys in the Mediterranean group spent time in proximity to one another, while the right plot shows the same for monkeys in the Western group. Monkeys in the Mediterranean group spent more time in body contact (ANCOVA $F_{[1,35]}$ = 60.8, FDR = 4.1 x 10^{-8}; purple) and less time alone (ANCOVA $F_{[1,35]}$ = 27.4, FDR = 4.4 x 10^{-5}; green) than the Western group, while there was no significant difference in time spent in close proximity (ANCOVA $F_{[1,35]}$ = 0.8, FDR = 0.25; yellow). **C)** The rate of anxiety behaviors observed in the Western group was significantly higher than that of the Mediterranean group during the treatment phase (ANCOVA $F_{[1,35]}$ = 5.9, FDR = 0.048). **D)** Points indicate months in which there was a significant difference between diet groups for a particular behavior. Circular points are significant after correcting for multiple hypothesis test, whereas triangular points are those that do not pass the heightened threshold. Blue (Mediterranean) or orange (Western) points indicate which group displayed more of the behavior for that month. **E)** Dominant monkeys in both diet groups decreased anxiety from baseline (left), although there was no significant difference in change in anxiety between diet groups ($t_{(18.3)}$ = 0.67). Only subordinates fed the Mediterranean diet decreased

from baseline (right). Within subordinate animals, anxiety decreased significantly more in the Mediterranean group than in the Western group ($t_{(14.9)} = 2.37$, $p = 0.032$).

Time spent in body contact and proximity to groupmates (i.e., within arm's reach) in contrast to alone, are measures of social integration or affiliation. The Mediterranean group spent significantly more time in body contact than the Western group ($\bar{x}_{Mediterranean}$ = 31.6%, $\bar{x}_{Western}$ = 7.3%; ANCOVA $F_{[1,35]} = 60.8$, FDR = 4.1×10^{-8}; **Figure 3.2B**), whereas, the Western group spent more time alone (out of arm's reach of others) than the Mediterranean group ($\bar{x}_{Mediterranean}$ = 32.4%, $\bar{x}_{Western}$ = 51.4%; ANCOVA $F_{[1,35]} = 27.4$, FDR = 4.4×10^{-5}; **Figure 3.2B**). There was no significant difference in time spent in close proximity (within arm's reach) between the two diet groups ($\bar{x}_{Mediterranean}$ = 9.6%, $\bar{x}_{Western}$ = 11.6%; ANCOVA $F_{[1,35]} = 0.8$, FDR = 0.25; **Figure 3.2B**), suggesting that monkeys in the Mediterranean group were trading time alone for time in body contact, and shifting towards a more affiliative phenotype.

The Western group displayed more anxiety behavior than the Mediterranean group ($\bar{x}_{Mediterranean}$ = 25.4 events/hr, $\bar{x}_{Western}$ = 32.2 events/hr; ANCOVA $F_{[1,35]} = 5.9$, FDR = 0.048; **Figure 3.2C**). When taken together, these changes indicate that diet composition altered the physical activity, affiliation, and anxiety of monkeys.

3.3.3 *Diet-induced changes in DAB and affiliation were rapid*

To understand how quickly behavior changed in response to experimental diets, we compared DAB scores and the five significantly altered behaviors between diet groups for the first month of behavioral data collection. After 1 month of behavior observation (3 months of experimental diet consumption), the DAB scores of Mediterranean-fed monkeys were significantly higher than those of Western-fed monkeys ($\bar{x}_{Mediterranean}$ = 0.54 $\bar{x}_{Western}$ = -0.43; ANCOVA $F_{[1,35]} = 6.7$, p = 0.014; **Figure 3.1**), controlling for baseline DAB scores.

Of the behaviors that were significantly different throughout the experimental phase, only the changes in affiliation showed rapid changes. The Mediterranean group spent significantly less time

alone ($\bar{x}_{Mediterranean}$ = 41.0%, $\bar{x}_{Western}$ = 58.7%; ANCOVA $F_{[1,35]}$ = 8.14, Holm-Bonferroni adjusted p (p_{HB}) = 0.035; **Figure 3.2D**) and more time in body contact, on average ($\bar{x}_{Mediterranean}$ = 17.3%, $\bar{x}_{Western}$ = 7.3%; ANCOVA $F_{[1,35]}$ = 5.72, p_{HB} = 0.055), than the Western group.

3.3.4 *Diet-induced behavioral changes were persistent*

There was a significant interaction between diet and month of the treatment phase ($F_{[10.3, 341.2]}$ = 2.2, p = 0.019). To further understand this interaction, we compared DAB scores and the five significantly altered behaviors in each month. The behavioral changes that we observed persisted throughout the course of the experimental diet manipulation. Holm-Bonferroni-adjusted post-hoc corrections of monthly DAB scores revealed significant diet differences in 19 out of the 24 months of behavior observation at $p < 0.05$, and an additional 4/24 months at $p < 0.10$ (**Figure 3.1** and **Table 3.3** for monthly pairwise comparisons). These results indicate that the effect of diet on behavior occurred early and persisted over the two-year study.

Continuing with Holm-Bonferroni-adjusted post-hoc corrections at $p < 0.05$, the difference between diet groups in time that monkeys spent in body contact was significant at all 24 monthly timepoints of the observation period (**Figure 3.2D**). The two diet groups differed significantly in time spent alone starting in month 4 of observation (after 6 months of experimental diet consumption) and remained significantly different for 11 months of the treatment phase (**Figure 3.2D**). In contrast, differences in the other behaviors emerged later and were more variable over time. Time spent resting was significantly different between the Mediterranean- and Western-fed monkeys at months 9, 13, 15, and 22. The Western diet group showed significantly more anxiety behaviors at months 11 and 14. Time spent attentive was significantly different between diet groups in months 10, 11, and 13 (**Figure 3.2D**). At a nominal threshold of uncorrected $p < 0.05$, additional months showed differences between the two diet groups in each of the behaviors

described. While these months were not significant after correcting for multiple hypothesis testing, they nonetheless contribute to our understanding of the behavioral changes driven by diet (uncorrected $p < 0.05$; n = 24 months for time in body contact, n = 19 months for time alone, n = 16 months for time resting, n = 7 months for anxiety behavior, and n = 10 months for time attentive; indicated by the faded triangles in **Figure 3.2D**).

3.3.5 *Social status was associated with differences in fearful scanning, aggression, and submission*

We tested the relationship between social status and behavior during the baseline phase of the experiment, during which the monkeys consumed a standard "monkey chow" diet. Of the twenty individual behaviors measured, seven were significantly different between the dominant and subordinate monkeys. Dominant monkeys displayed higher rates of mild ($\overline{x}_{dominant}$ = 9.5 events/hr, $\overline{x}_{subordinate}$ = 2.5 events/hr; $t_{(25.8)}$ = 3.24, FDR = 0.012) and extreme ($\overline{x}_{dominant}$ = 2.3 events/hr, $\overline{x}_{subordinate}$ = 0.4 events/hr; $t_{(28.7)}$ = 3.11, FDR = 0.012) aggression than subordinates. Dominant monkeys also received more mild ($\overline{x}_{dominant}$ = 9.4 events/hr, $\overline{x}_{subordinate}$ = 1.3 events/hr; $t_{(21.8)}$ = 4.16, FDR = 0.003) and extreme submissions ($\overline{x}_{dominant}$ = 2.9 events/hr, $\overline{x}_{subordinate}$ = 0.5 events/hr; $t_{(26.4)}$ = 3.46, FDR = 0.009). Subordinate monkeys spent more time fearfully scanning their environment ($\overline{x}_{dominant}$ = 0.028% of time, $\overline{x}_{subordinate}$ = 0.34% of time; $t_{(17.0)}$ = -3.38, FDR = 0.012), showed more mild submission ($\overline{x}_{dominant}$ = 1.3 events/hr, $\overline{x}_{subordinate}$ = 8.7 events/hr; $t_{(21.7)}$ = -4.70, FDR = 0.002), and received more mild aggression ($\overline{x}_{dominant}$ = 0.9 events/hr, $\overline{x}_{subordinate}$ = 5.5 events/hr; $t_{(22.2)}$ = -4.56, FDR = 0.002) than dominant monkeys. These relationships between behavior and status observed in the baseline continued through the treatment phase, as previously reported (Shively et al., 2020).

3.3.6 *Social status altered the effect of diet on anxiety behavior*

We next examined if social status altered the effects of diet on socioemotional behaviors. During the baseline phase of the experiment, subordinate monkeys had higher levels of anxiety than dominants, but this was not a significant difference ($\bar{x}_{dominant}$ = 32.8 events/hr, $\bar{x}_{subordinate}$ = 37.6 events/hr; $t_{(21.4)}$ = 1.0, p = 0.33). On average, anxiety declined between the baseline and experimental phases, likely reflecting habituation to a novel environment ($\bar{x}_{baseline}$ = 34.8 events/hr, $\bar{x}_{experimental}$ = 29.0 events/hr; $t_{(68.9)}$ = 2.17, p = 0.039). However, decline between baseline and experimental phases was greatest in subordinates in the Mediterranean group, such that anxiety in Mediterranean group subordinates was reduced to the level of their dominant counterparts in the experimental phase ($\bar{x}_{dominant}$ = 25.7 events/hr, $\bar{x}_{subordinate}$ = 25.0 events/hr). Within subordinate animals, the Mediterranean group decreased anxiety from baseline to treatment phase significantly more than the Western group, which actually increased anxiety over the course of the experiment ($\bar{x}_{Mediterranean}$ = 28% reduction from baseline, $\bar{x}_{Western}$ = 11% increase over baseline; $t_{(14.9)}$ = 2.37, p = 0.032; **Figure 3.2E**). These results suggest that effects of diet on anxiety may be dependent on social status.

3.3.7 *Mediterranean diet drove most behavioral differences between diets*

To understand which experimental diet may be driving changes in behavior, we examined more closely the five behaviors that showed a significant difference between diet groups. Within each diet group, we conducted one-way repeated measures ANOVA between baseline and treatment phases of the study. There was no significant difference between baseline and treatment phase rates of behavior in the Western group in any of the five behaviors (all Holm-Bonferroni-adjusted p > 0.05). In contrast, there were significant differences between baseline and treatment phases in the Mediterranean group in time spent in body contact ($F_{[1,16]}$ = 60.4, Holm-Bonferroni-adjusted p

(p_{HB}) = 8.1 x 10^{-6}), time spent alone ($F_{[1,16]}$ = 18.2, p_{HB} = 0.0053), time spent resting ($F_{[1,16]}$ = 15.3, p_{HB} = 0.0080), and rate of anxiety behavior ($F_{[1,16]}$ = 13.4, p_{HB} = 0.014), but not time spent attentive ($F_{[1,16]}$ = 4.7, p_{HB} = 0.23). These observations suggest that most of the observed differences between diet groups in the treatment phase were due to changes in behavior of those in the Mediterranean group (**Figure 3.3**).

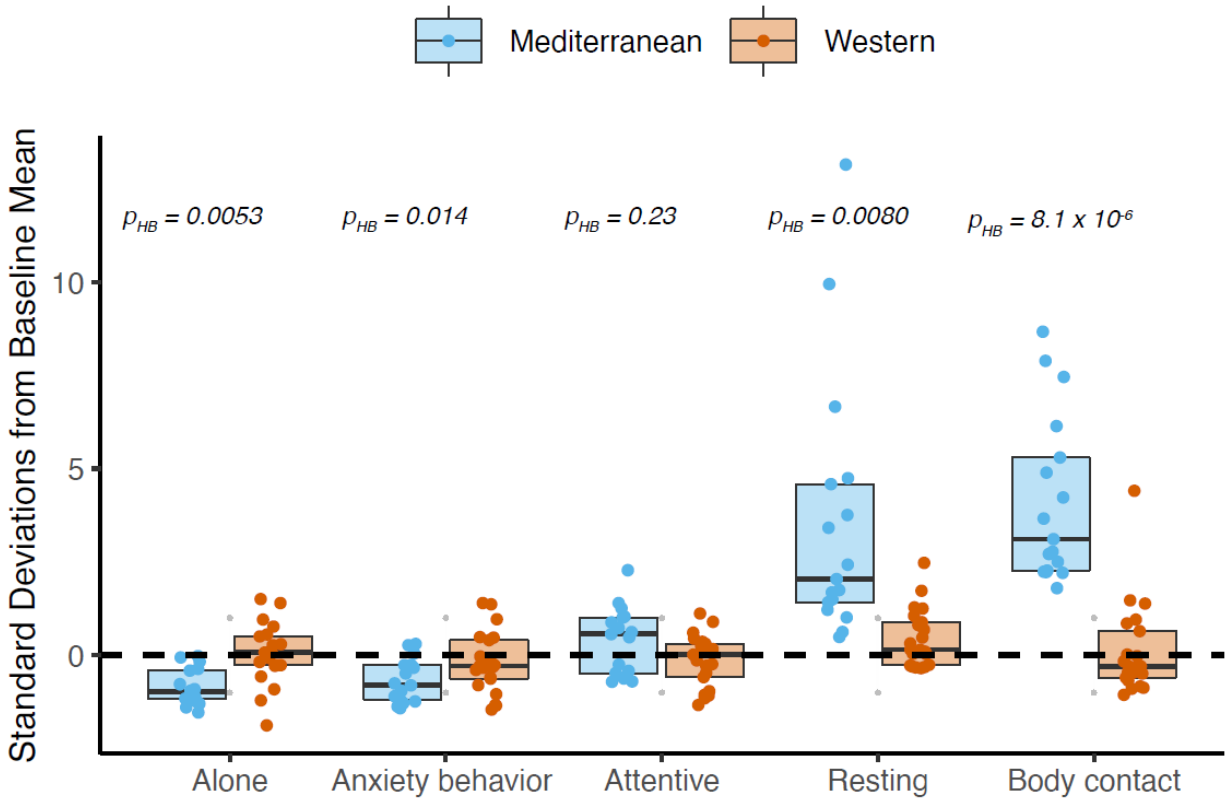

Figure 3.3 Diet driven changes in behavior are due to changes in Mediterranean-fed animals from baseline. Western-fed animals do not show a significant difference between baseline (time 0) and treatment phases (average of months 1-24) in any off the five behaviors that are affected by diet (all Holm-Bonferroni-adjusted $p > 0.05$). Mediterranean-fed animals show significant differences between baseline and treatment phase in all diet-altered behaviors except percent of time spent attentive. Reported Holm-Bonferroni-adjusted p (p_{HB}) are for ANOVA of Mediterranean group animals compared to their own baseline measurements. Blue (Mediterranean) and orange (Western) points indicate treatment phase values standardized against the across-group baseline mean (when all animals consumed a chow diet).

3.4 DISCUSSION

In this randomized preclinical trial in nonhuman primates, we found that two common human diet patterns had profoundly different effects on physiological and behavioral outcomes (Frye et al., 2020; Johnson et al., 2021; Nagpal, Shively, et al., 2018; Shively et al., 2019, 2020). This study expands on findings from a prior report, which showed that diet drove inflammatory polarization of circulating immune cells and altered behavior in ways that had both social (Mediterranean diet-fed animals were more socially integrated) and psychological (Mediterranean diet-fed animals exhibited fewer anxiety behaviors) consequences, and that behavioral changes were associated with some of the diet effects on gene expression (Johnson et al., 2021). Here, we delved more deeply into the character of the diet-induced behavioral changes, and found that the experimental diets rapidly and persistently shift affiliation, activity, and anxiety in cynomolgus macaques, and differently impact socially dominant and subordinate individuals.

The Mediterranean diet increased the time spent in body contact and decreased the time spent alone, indicating more time spent in affiliation. The diet-driven changes in affiliation were observed in the first month of behavior observations (the third month on the experimental diets) and persisted throughout the course of the study. Affiliation reflects a monkey's social integration or isolation, a key component of the social environment impacting health outcomes. In humans, social isolation has been associated with inflammation (Cacioppo et al., 2015; Cole et al., 2015; Eisenberger et al., 2017) and is a risk factor for psychiatric disorders (Hawkley & Cacioppo, 2010; Nguyen et al., 2020; Taylor et al., 2018). Thus, given the physiological and behavioral similarities shared between humans and NHPs (Shively & Clarkson, 2009), the higher isolation in Western diet-fed monkeys relative to Mediterranean diet-fed monkeys supports the link between components of the Western diet and psychiatric disorders. Reduced social interaction has been

observed in rodents fed a Western style diet (Peris-Sampedro et al., 2019, p.; Veniaminova et al., 2017), and in the offspring of rodents (Kang et al., 2014; Teixeira et al., 2020; Tsan et al., 2021) and female Japanese macaques (Thompson et al., 2018) that consumed a Western style diet during gestation. In human observational studies of self-reported diet and social characteristics, social isolation is associated with Western-diet consumption (Delerue Matos et al., 2021; Kobayashi & Steptoe, 2018), whereas adherence to a Mediterranean diet is correlated with decreased isolation (Ferrer-Cascales et al., 2018). The findings reported here are novel in the observation of the rapidity with which behavior changed in response to dietary changes, the longitudinal persistence of behavior change over two years, a timespan roughly equivalent to 7 human years, the observation that the Mediterranean diet drove most of the differences in behavior, and the randomized trial design which allows for causal inferences. These findings suggest that diet intervention may have a clinical application in the long-term treatment of adverse psychosocial effects of social isolation.

Another psychologically relevant behavioral change is that the Mediterranean diet decreased anxiety-like behaviors, resulting in lower levels of anxiety than Western diet-fed animals. This main effect of diet was largely due to an elevated rate of anxiety behaviors in subordinate animals, which was ameliorated by the dietary intervention in Mediterranean group subordinates. All diet-by-status groups except the socially subordinate Western animals exhibited average decreases in anxiety behaviors over time. In fact, Mediterranean group subordinates reduced anxiety behaviors to the level of their dominant counterparts ($\overline{x}_{dominant}$ = 25.7 events/hr, $\overline{x}_{subordinate}$ = 25.0 events/hr). This observation suggests that the Mediterranean diet could be an effective intervention on anxiety. Likewise, it may be that anxiolytics would be more efficacious on a Mediterranean than Western diet background, a hypothesis in need of testing.

Most of the diet-induced behavioral changes were driven by changes in the behavior of Mediterranean group monkeys. Behaviors of Western group monkeys did not change significantly from baseline. This implies that the Western diet resembles standard lab chow, at least with respect to effects on behavior and, by extension, central nervous system function (see **Table 2.1**). From this perspective, it is notable that the NHP Western diet and chow are similar in omega-6:omega-3 fatty acid ratios. Indeed, circulating omega-6:omega-3 fatty acid ratios are positively associated with anxiety and depression in humans (Berger et al., 2017). The data reported here suggest that those associations may be causal in nature. Likewise, changes in behavior in the Mediterranean group may be due to characteristics unique to that diet. In support of this, data from both humans and model organisms indicate a preventative role for omega-3 and polyunsaturated fatty acids in anxiety disorders (Su et al., 2015; Vaz et al., 2013).

The observed changes in behavior are suggestive of underlying physiological changes that may have important health consequences. There are multiple pathways through which diet may alter behavior—mediated or initiated through the CNS—some of which have already been supported by previous findings from this study. We previously demonstrated that, relative to the Mediterranean group, the Western group had reduced gut microbiota diversity (Nagpal, Shively, et al., 2018), which is a well-established modulator of CNS activity through the gut-brain axis (Cryan et al., 2019). The Western group exhibited increased caloric intake, body fat, insulin resistance, and sympathetic nervous system and hypothalamic-pituitary-adrenal activity relative to the Mediterranean group (Shively et al., 2019, 2020), all of which have been associated with either social isolation or anxiety or both (Baker et al., 2017; Foster & McVey Neufeld, 2013; Hoehn-Saric & McLeod, 1988; Juruena et al., 2020; Narita et al., 2008; Rivenes et al., 2009). We also demonstrated that the Western group had a pro-inflammatory monocyte transcriptome (Johnson et

al., 2021), consistent with numerous findings supporting links between Western diet and inflammation (Giugliano et al., 2006; Holt et al., 2009; Lopez-Garcia et al., 2004; Nanri et al., 2007; Nettleton et al., 2006), versus reduced inflammation with the Mediterranean diet and its components (Camargo et al., 2012; Layé et al., 2018; O'Keefe et al., 2008). The peripheral inflammatory milieu can alter CNS activation, either through modulation of vagal activity or direct infiltration by cytokines passing the blood-brain barrier (Fung et al., 2017; Prinz & Priller, 2017; Quan & Banks, 2007). Likewise, inflammatory cytokine production is sensitive to social isolation (Dunphy-Doherty et al., 2018; Hawkley & Cacioppo, 2010; Raison et al., 2006). Consistent with these plausible mechanisms, we previously reported that diet-altered behavior may mediate, or be mediated by, some pro-inflammatory shifts in immune cell gene expression (Johnson et al., 2021).

In addition to lasting negative psychosocial effects, social isolation and inflammation are both associated with worse outcomes from infections. In the ongoing COVID-19 global pandemic, patterns of differential mortality by country and region are associated with variation in dietary patterns (Bossak & Turk, 2021; Rajkumar, 2021; The ARIA group et al., 2020). Furthermore, lower mortality rates from COVID-19 have been observed in countries/regions that consume higher levels of antioxidants and seafood, and lower levels of sugar and animal products (Kamyari et al., 2021; Rajkumar, 2021), and omega-3 fatty acid supplementation was associated with reduced mortality and improved respiratory and renal function in a randomized clinical trial (Doaei et al., 2021). Thus, Mediterranean diet consumption may ameliorate COVID-19 infection severity via reduced levels of inflammation.

Taken together, diet composition may impact disease progression in the COVID-19 pandemic—exacerbation by Western diet or amelioration by Mediterranean diet—through both behavioral and inflammatory pathways, as our data suggest. Intriguingly, diet composition may

also be manipulated to mitigate the deleterious health effects of social isolation during the COVID-19 pandemic (Kumar & Salinas, 2021; Schou et al., 2021). Mediterranean diet consumption may also synergize with other COVID-19 therapeutic interventions, increasing favorable outcomes.

In summary, the data presented here and in our previous report from the same NHP preclinical trial strongly support the hypothesis that a Mediterranean-like diet beneficially influences health through behavioral as well as physiological pathways, and may be a critical component of a healthy lifestyle that increases positive social interactions while reducing anxiety and the risk of multiple chronic diseases of aging. Future studies should assess whether Mediterranean diet consumption in conjunction with other therapeutic interventions is more efficacious than standard therapies alone for treatment of anxiety, and inflammatory diseases such as COVID-19.

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Chapter 4. Epigenetic Responses to Diet, Social Status, and Behavior in Monocytes from Western vs. Mediterranean Diet-fed Adult Female Nonhuman Primates

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Abstract:

Environmental stressors can induce molecular changes that alter physiology and ultimately health and survival. Many common diseases are caused, in part, by inflammatory activation of the immune system by environmental stressors. Dietary composition is one such environmental stressor, with consumption of Western diets associated with more inflammation and Mediterranean-like diet linked to anti-inflammatory responses. Similarly, the chronic stress of low social status drives inflammation, resulting in peripheral inflammatory immune response. An

individual's environment can be biologically embedded through epigenetic changes, such as DNA methylation (DNAm), which can be responsible for transcriptomic changes through a *cis*-regulatory effect. DNAm in circulating monocytes—a key immune cell that can develop a pro- or anti-inflammatory phenotype—presents a potential avenue for understanding how these two environmental stressors may impact the immune system. To test this, we fed cynomolgus macaques (*Macaca fascicularis*) experimental diets mimicking the Western or Mediterranean diet of humans for 15 months, at which point we isolated monocytes from blood samples. Social status was associated with genome-wide patterns of methylation and differential methylation at 419 CpG sites (FDR < 0.2). Diet showed neither genome-wide nor CpG-specific methylation patterns, but instead stimulated differential methylation in the promoter region of 7 genes. 292 CpG sites showed a significant interaction between diet and social status, largely due to significant status effects in Mediterranean animals. We did not find evidence of DNAm regulating the expression of genes that were differentially expressed between the two diets, suggesting that another pre- or post-transcriptional mechanism may be driving the observed differential gene expression in response to diet. We did, however, find that some behaviors that changed with diet (anxiety, time attentive, time resting, time alone, and time in body contact) were also associated with methylation changes. These findings add to the growing body of literature demonstrating environmental effects on DNAm but suggest that environmental regulation of transcription in monocytes may be accomplished through varied mechanisms.

4.1 INTRODUCTION

Human diets vary across geography, cultures, and socioeconomic strata, and profoundly impact human health and survival. Consumption of Western diets (rich in animal food sources, starches, sugars, and saturated and omega-6 fatty acids) is associated with increased risk for cardiovascular

disease, metabolic syndrome, type 2 diabetes, autoimmune disorders, depression, and mortality (Cordain et al., 2005; Drake et al., 2018; Smil, 1989; Smyth & Heron, 2006). In contrast, Mediterranean diet consumption (characterized by raw fruits and vegetables, lean proteins, and monounsaturated and omega-3 fatty acids) is associated with lower risk of chronic disease and increased longevity (Farchi et al., 1994; Osler & Schroll, 1997; Romagnolo & Selmin, 2017; Trichopoulou et al., 1995). Studies in both humans (Holt et al., 2009; Nanri et al., 2007; Nettleton et al., 2006) and mammalian models (Drescher et al., 2019) have established that diets similar in composition to Western diets increase levels of circulating inflammatory cytokines—proteins that are both a product and driver of inflammation. The type of fat ingested is critical to the immune consequences of diet, as omega-6 fatty acids common in Western diets have been implicated in inflammation (Lopez-Garcia et al., 2004; O'Keefe et al., 2008), while omega-3 fatty acids characteristic of Mediterranean diets have demonstrated antioxidant and anti-inflammatory properties (Giugliano et al., 2006). Other components of Western diets (e.g. sugars, animal proteins) are also associated with inflammation (Freeman et al., 2018) and anti-inflammatory diets rely on plant-based nutrients (F. B. Hu, 2002), suggesting that comparing whole diets rather than individual nutrients is critical to understanding the connections between diet, inflammation, and health.

The social environment is another potent regulator of the immune system. The social environment can act to buffer stress when an individual has a high degree of support, or conversely social adversity can be a source of chronic stress. Two key sources of adversity in the social environment are an individual's status and their degree of affiliation or isolation. Chronic social adversity is known to drive activation of the hypothalamic-pituitary-adrenal axis and its downstream effects of immune dysregulation (Gray et al., 2017; Kohn et al., 2016; McEwen, 2017;

Snyder-Mackler & Lea, 2018). In humans, social adversity is associated with increased mortality and chronic disease incidence (Hallman et al., 2001; Rosengren et al., 2004; Steptoe & Kivimäki, 2012, 2013; Stuller et al., 2012), and both human and mammalian studies have implicated markers of inflammation in the relationship between social adversity and health (Abbott et al., 2003; Sapolsky, 2005; Snyder-Mackler et al., 2020; Takahashi et al., 2018). Indeed, direct experimental manipulation of primate social status induced an inflammatory response (Snyder-Mackler et al., 2016). Both of these social adversities are linked to mortality and morbidity across taxa (Cacioppo et al., 2015; Chetty et al., 2016; Cole et al., 2015; Eisenberger et al., 2017; Hawkley & Cacioppo, 2010; Nguyen et al., 2020; Snyder-Mackler et al., 2020; Taylor et al., 2018).

The effects of diet and social adversity are not mutually exclusive, as we demonstrated diet effects on social behavior. Primates fed a Mediterranean diet exhibited reduced social isolation compared to Western-fed counterparts (Johnson et al., 2022), which is consistent with findings in humans (Delerue Matos et al., 2021; Ferrer-Cascales et al., 2018; Kobayashi & Steptoe, 2018) and nonhuman models of human dietary patterns (Kang et al., 2014; Peris-Sampedro et al., 2019; Teixeira et al., 2020; Tsan et al., 2021; Veniaminova et al., 2017). Importantly, we showed that the diet effects on social isolation are status dependent (Johnson et al., 2022), suggesting that diet, social status, and social adversity may interact to impact organismal health.

It is clear that environmental stressors, such as diet and social adversity, can alter the biology of circulating immune cells. The peripheral immune system responds to environmental stimuli in part through the signaling of monocytes—immune cells that exhibit high phenotypic plasticity and can polarize their immune response toward either pro- or anti-inflammatory phenotypes (Mosser & Edwards, 2008). Monocyte polarization is achieved through epigenetic and transcriptomic changes (F. M. Davis & Gallagher, 2019; Li et al., 2018; Sica & Mantovani, 2012), and a proper

balance of monocyte phenotypes is thought to be important to maintaining health. We demonstrated differential polarization of monocytes in response to experimental Western vs. Mediterranean diets (Johnson et al., 2021). Inflammatory genes associated with social adversity, known as the conserved transcriptional response to adversity, overlap with those responsible for pro-inflammatory polarization of monocytes, meaning that diet and social adversity may both contribute to monocyte polarization (Cole, 2013, 2019; Cole et al., 2015). How exactly environmental stressors contribute to monocyte plasticity remains a mystery. Gene expression can be modified through multiple routes, and methylation of cytosines at CpG sites—known as DNA methylation (DNAm)—is a particularly well-understood epigenetic modification that is responsive to environmental conditions (Beetch et al., 2020; Bird, 2002; Cedar & Bergman, 2009; Dor & Cedar, 2018) (although the relative contributions of environmental and cellular conditions—as well as tissue type—to DNAm remain to be teased apart (Luo et al., 2018)), including diet (Corella et al., 2018; Howard et al., 2011; Maegawa et al., 2017; F. F. Zhang et al., 2011) and social adversity (Cunliffe, 2016; Tung & Gilad, 2013). Recent studies have identified differential patterns of DNAm across the genome in several chronic diseases (Beetch et al., 2020; Greenberg & Bourc'his, 2019; Jaenisch & Bird, 2003). Thus, DNAm is one environmentally responsive epigenetic change that may play a role in gene regulation, driving inflammation and health decline. Identification of methylation patterns associated with stress and diet will provide mechanistic insight into the observed differences in monocyte polarization and gene expression.

Long term effects of diet composition on human health are limited, as whole diet manipulations are expensive and self-reported food intake data are unreliable (T. M. Miller et al., 2008; Suchanek et al., 2011). Likewise, our understanding of social adversity in humans is often based on self-reported measures, which can conflate perceived and actual events and suffer from different

definitions of key variables (Alcaraz et al., 2019; Evans et al., 2019; Friedler et al., 2015). These confounds can be overcome with the use of appropriate model organisms. With a close phylogenetic relationship to humans and an accelerated life history (~3-4x faster than humans), cynomolgus macaques (*Macaca fascicularis*) can provide a useful model of human health and disease (Shively & Clarkson, 2009; Shively & Day, 2015; Willard & Shively, 2012). Their evolutionary proximity to humans confers a high degree of genetic identity, which allows for comparison to human patterns of gene regulation. Cynomolgus monkeys exhibit chronic disease phenotypes also observed in humans (Jarczok et al., 2018; Kromrey et al., 2016; Shively, 1998; Shively & Day, 2015; Willard & Shively, 2012). Further, macaques have complex social interactions leading to varying degrees of social isolation and they establish stable hierarchies, which can lead to chronic stress of low social status for some animals, similar to that seen in humans (Brent et al., 2017b; Sapolsky, 2005). These attributes make cynomolgus macaques a good model system for testing the effects of long-term dietary intervention and social adversity on DNAm within circulating immune cells.

The present study was designed to compare the effects of Western and Mediterranean whole diet manipulations in primates to examine the role of diet and the social environment on monocyte polarization and health. In this report, we measured DNAm in conjunction with previously reported transcriptomic data collected after 15 months on experimental diets. We tested the overall hypotheses that diet and social status alter patterns of DNA methylation in a non-mutually exclusive manner by modeling global, regional, and CpG site-specific patterns of methylation versus these two environmental stressors. We also measured correlations between methylation of CpG sites and the expression of nearby genes to assess the extent to which transcriptomic changes previously reported were driven by changes in DNAm. We modeled DNAm as a function of key

social behaviors to test the hypothesis that DNAm is correlated with previously reported behavioral changes. We found evidence for an interaction between diet and social status on DNAm, but only limited evidence for an epigenetic response to social status.

4.2 METHODS

4.2.1 *Experimental design*

We fed adult female cynomolgus macaques one of two experimental diets modeled after common human dietary patterns – the Mediterranean and Western diets – for 15 months (approximately equivalent to 4 years in humans), as previously described (Johnson et al., 2021; Shively et al., 2019). Following the experimental diet phase of the experiment, we extracted DNA from CD14+ monocytes and analyzed DNA methylation (DNAm). We used bioinformatic approaches to analyze these DNAm data to test the hypothesis that epigenetic mechanisms are driving the widespread effects of the dietary intervention that we previously reported (Frye et al., 2020; Johnson et al., 2021, 2022; Nagpal, Shively, et al., 2018; Shively et al., 2019, 2020), as well as additional hypotheses regarding the biological embedding of the social and nutritional environment in immune cells (detailed below).

4.2.2 *Study subjects*

Forty-three adult (age: mean = 9.0, range = 8.2-10.4 years, estimated by dentition) female cynomolgus macaques (*Macaca fascicularis*) were obtained (Shin Nippon Biomedical Laboratories, USA SRC, Alice, TX) and housed at the Wake Forest School of Medicine Primate Center (Winston-Salem, NC) under controlled conditions (Johnson et al., 2022; Shively et al., 2019). We placed monkeys into social groups of 3-4 in the baseline group-establishment phase, during which they consumed standard monkey chow (**Table 2.1**). After eight months, we

transitioned groups to either the Mediterranean-like (hereafter "Mediterranean," 6 groups, $n = 22$) or Western-like (hereafter "Western," 5 groups, $n = 21$) experimental diet for the duration of the experiment. Groups were balanced on key markers of health measured during the baseline phase, including body weight, body mass index, plasma triglyceride concentrations, total plasma cholesterol, and circulating basal cortisol (Shively et al., 2019). Due to some animals not adapting well to the experimental diets and issues in sample collection, the final sample size was 35 animals (Western $n = 20$, Mediterranean $n = 15$) (Frye et al., 2020; Johnson et al., 2021). All animal manipulations were performed according to the guidelines of state and federal laws, the US Department of Health and Human Services, and the Animal Care and Use Committee of Wake Forest School of Medicine.

4.2.3 *Experimental diets*

Following the eight-month baseline phase, we fed monkeys one of two experimental diets formulated to match the nutritional content of common human dietary patterns, extensively described elsewhere (Frye et al., 2020; Johnson et al., 2021, 2022; Nagpal, Shively, et al., 2018; Shively et al., 2019, 2020). Briefly, the experimental diets were isocaloric with respect to macronutrients and identical in cholesterol content (~320 mg/2000 kilocalories (Cals)/day), but different in their fatty acid composition (**Table 2.1**) and the ingredients from which they were formulated. The Mediterranean diet was rich in complex carbohydrates and fiber, contained high levels of monounsaturated fats, and had a omega-6:omega-3 fatty acid ratio similar to modern-day traditional hunter-gatherer diets (Cordain et al., 2005), all key characteristics of human Mediterranean diets (Bédard et al., 2012; Kafatos et al., 2000). In contrast, the Western diet was based on the dietary pattern observed in adult women in the United States of America (USDA, 2016), which is characterized by sugars, starches, and animal sources of proteins. Monkeys ate

these diets for the duration of the 15-month experimental diet phase of the study.

4.2.4 *Quantification of social status*

Animals developed stable social hierarchies during the baseline group establishment phase of the experiment and most animals (92.5%) did not change rank throughout the entire course of the study (Frye et al., 2020; Shively & Kaplan, 1991). Status was unknown prior to establishment of social groups but was determined during twice-weekly behavioral data collections for 6 weeks during the baseline phase (2 hours/monkey total) and for 14 months during the experimental phase (mean = 17.6 hours/monkey total). We determined rank through the outcomes of dyadic agonistic interactions. We operationalized social status as relative rank, which we defined as the ordinal position of each monkey within their social group, scaled to a maximum rank of 1 (e.g., a group with four monkeys would have ranks of 0, 0.33, 0.67, and 1).

4.2.5 *DNA extraction and methylation assay*

We trained monkeys to exit their social housing on command for blood sample collection at multiple stages of our study, including after 15 months on experimental diets for the current study. A technician drew blood via venipuncture within 9 minutes of entering the building. We randomized the order of blood sample collection by sample group, taking care to alternate between experimental diets and ensuring that all samples of a given group were collected on the same day. Blood was collected in EDTA-containing tubes and then mixed with an equal volume PBS without calcium or magnesium. The blood solution was then overlaid on a 90% Ficoll-Paque Plus/10% PBS solution in LeucoSep tubes and PBMCs were isolated through density centrifugation at 800 x g for 20 min. We then purified CD14+ monocytes from the isolated PBMCs by positive selection using a Miltenyi bead-based protocol following manufacturer's instructions (Miltenyi Biotec,

Bergisch Gladbach, Germany), which we then stored in 85% FBS, 15% DMSO sterile freezing media at -80°C and transferred to liquid nitrogen for storage until DNA extraction.

4.2.6 *Methylation probe selection and quality control*

We extracted DNA from CD14+ monocytes using the AllPrep DNA/RNA Mini Kit (Qiagen, Inc., Hilden, Germany). DNA methylation (DNAm) was measured at 866,091 CpG sites using the Illumina MethylationEPIC microarray (Illumina, San Diego, CA, United States). We performed DNA extraction and DNAm microarray analysis according to manufacturer's instructions. The Illumina MetthylationEPIC microarray was designed based on human DNAm sites. To minimize probe misalignment or multiple alignment in the *Macaca fascicularis* genome, we filtered probes to the 183,509 that singly aligned to the *M. fascicularis* genome (21% of original probes), following Nakachi and colleagues (Nakachi et al., 2020). We performed quality control on the remaining probes, removing an additional 22,425 with a detection *p*-value > 0.1 calculated with the *detectionP.minfi* function from the *ewastools* package in R (Heiss & Just, 2019). Using the *beadcounts* function from the *wateRmelon* R package (Pidsley et al., 2013), we removed 861 additional probes with a bead count < 3 in 5% or more of samples, resulting in DNAm data for 160,223 CpG sites. All samples passed QC thresholds for bisulfite conversion (>80%) and average detection *p*-value (< 0.05) and were retained. See **Appendix D** for additional filtration approaches considered.

4.2.7 *Methylation probe normalization and removal of batch effects*

Following quality control, we quantile normalized the DNAm measures using the *preprocessNoob* and *preprocessQuantile* functions from the *minfi* R package (Aryee et al., 2014), which is recommended for data sets of samples from the same species and tissue (Touleimat & Tost, 2012).

We calculated DNAm M-values (log2 ratio of the intensities of methylated probes versus unmethylated probes) using the standard analysis pipeline from the *minfi* R package (Aryee et al., 2014), which we used as input to PCA to determine the main axes of variance within the DNAm data and test for influence of covariates on variance in DNAm between samples. In addition to technical covariates, we included CD3, CD4, CD8, and CD14 expression (measured through bulk RNA sequencing) as a measure of CD14+ isolation purity. Cell type estimation is not recommended for the nearly homogenous populations expected from magnetic bead isolation, but this approach accounts for impurities in CD14+ isolation. The microarray slide batch and CD3 expression both loaded heavily in the first few principal components and we modeled M-values as a function of these two covariates in a linear model using the *limma* R package (Ritchie et al., 2015) to regress out their effect on DNAm. We used the residuals of this model in downstream analyses either as is (adjusted M-values) or converted back to β values where noted, which we calculated with the *getBeta* function of the *minfi* package in R (Aryee et al., 2014).

4.2.8 *Quantifying diet and status effects on DNA methylation*

We hypothesized that diet and chronic stress from the social environment alter DNAm, both individually and in an interactive manner, which we tested at numerous levels. Here, we describe the analysis plan, followed by detailed descriptions of each analysis. We first asked if the diet intervention or social environment were associated with differences in overall DNA methylation, using principal component analysis to analyze global patterns in DNAm. Next, we tested if diet and the social environment alter DNAm at individual CpG sites using linear mixed effects models, which we constructed for all CpG sites measured (regardless of location in genome) but then constricted to those in the regions of interest (promoters and first introns) of differentially expressed genes from this study. We tested for enrichment in three additional locations to test

specific gene regulation hypotheses that were generated from our prior work. We showed differential expression between diet groups of genes known to be associated with monocyte polarization (Schmidl et al., 2014), suggesting that diet may act on CpG sites near these genes to moderate monocyte polarization. Additionally, we identified "hub genes," which are genes that responded to diet by showing decanalized co-expression patterns, suggesting a disruption by diet of typical networks of gene expression. Finally, we found enrichment of putative transcription factor (TF) binding sites upstream of differentially expressed genes between diet groups, pointing to the role of TFs in regulating gene expression. We tested for enrichment in polarization-associated genes, "hub genes," and regions of likely TF binding to test these hypotheses. After testing for diet and status effects in individual CpG sites, we averaged methylation across the promoter regions of genes to test for differentially methylated regions in response to diet or social status. We repeated this test with the average methylation across the first intron for each gene.

We first conducted principal component analysis (PCA) using the *prcomp* function from the R *stats* package (*R: A Language and Environment for Statistical Computing.*, 2020) to reduce the dimensionality in two separate analyses: PCA on the raw M-values of DNAm to identify technical covariates through correlation tests (Pearson *r*) and PCA on the adjusted M-values of DNAm to test for global effects of diet and social status, through a linear model of PC loading ~ diet or status. In both instances, singular value decomposition was conducted on the translated (to form a 35 x 160,233 matrix of samples x CpG sites), centered (but unscaled) data within the *prcomp* function.

We fit linear mixed effects models of adjusted M-values of CpG site methylation as a function of diet and social status, controlling for relatedness among monkeys, using the R package *EMMREML* (Akdemir & Godfrey, 2015). Relatedness was estimated using the *ngsRelate* program (Hanghøj et al., 2019) with SNP genotypes inferred from RNA-seq reads using *bcftools mpileup*

(Li et al., 2009). For CpG sites that showed an interaction between diet and social status, we used the *lm* function from the R *stats* package (*R: A Language and Environment for Statistical Computing.*, 2020) to fit a model of DNAm (adjusted M-values) ~ social status within each diet group. The *EMMREML* package was also used to test the relationship between CpG site adjusted M-values and several behavioral measures—diet-altered behavior (DAB) scores, anxiety behavior, time attentive, time alone, time in body contact, and time resting—for each site. We conducted functional genomic analyses of CpG sites using the *gometh* function from the *missMethyl* R package (Phipson et al., 2016). We used each set of differentially methylated CpG sites as a focal set against all other CpG sites as a background set. We then tested sets of CpG sites directionally, where the differentially methylated sites in one direction were tested against all other sites.

As DNAm in the promoter region is known to be particularly relevant to gene expression, we tested for enrichment of diet effects in promoter regions of 1) all genes compared to non-promoter regions, 2) differentially expressed genes (DEGs) compared to all other genes. The proportion of CpG sites passing a nominal *p*-value threshold of 0.05 was compared to the proportion of CpG sites passing the threshold in the background set to which the set of CpG sites is being compared. After calculating the background proportion, a confidence interval was calculated around this proportion using 1000 iterations of bootstrap resampling from the background set of CpG sites, each time calculating the proportion of sites passing the nominal threshold. An enrichment *p*-value was calculated from comparing the observed proportion to this null distribution. We repeated this enrichment test for DNAm in the first intron of genes, as DNAm in this region has been demonstrated to be inversely correlated with gene expression across tissues and species (Anastasiadi et al., 2018). We used the same approach to tested for enrichment of diet effects in

"hub genes," and polarization genes, and likely TF binding sites, using predicted sites from the FANTOM5 database (Noguchi et al., 2017).

DNAm is canonically thought to interrupt or enable transcription factor binding in the promoter regions of genes. Methylation at multiple CpG sites within a stretch of DNA may act in concert to regulate gene expression, so we next tested for differentially methylated regions (DMRs) as a function of diet and social status. We first employed the *dmrCate* R package (Peters et al., 2015) to define and test *de novo* regions of DNA for univariate effects of diet or social status. Within the *dmrCate* package, parameters were loosened to include CpG sites that were not themselves differentially methylated. Notably, this increased the likelihood of Type I error. With this approach, we identified 19 and 110 putative *de novo* regions of differential methylation for diet and social status, respectively (ranging from 2-22 CpG sites per region). However, none of these regions were differentially methylated (all FDR > 0.7) so we abandoned this approach since it was both unreliable (high risk of Type I error) and produced no meaningful insights. Instead, we tested for DMR in regions of interest in the vicinity of genes: promoter regions (defined as the DNA 2 kb upstream of transcription start sites) or the first intron of genes. Of the 1,266 genes with at least one CpG site in the promoter region, 513 had 2 or more sites and we restricted our analyses to these genes, averaging DNAm (β values) across a promoter region for use in linear mixed effects models, as described above. 1,254 genes contained at least one CpG site in the first intron, 617 of which had 2 or more sites and were used for analysis. We repeated DMR analyses the region of DNA 5 or 10 kb upstream of genes, but results were not meaningfully different (see **Appendix D**).

4.2.9 cis-*regulatory changes*

To establish a regulatory link between DNAm of CpG sites and the gene expression, we tested if DNAm in the promoter region of genes predicted gene expression. For these analyses, we defined

the promoter regions of genes as the DNA 2 kilobases upstream of transcription start sites of genes in the *Macaca fascicularis* genome (see **Appendix D** for discussion of alternative approaches), which were gathered using tools from the *BEDtools* package (Quinlan & Hall, 2010). Of the 160,223 CpG sites in this data set, 2,509 fell in the promoter regions of 1,266 genes (out of the 12,240 genes with measurable detection in our prior work (Johnson et al., 2021)). Each of these genes had 1-18 CpG sites measured in their promoter region. We also tested for *cis*-regulatory effects of DNAm in the first intron of genes. 3,505 of the measured CpG sites were in the first intron of 1,254 genes (1-32 CpG sites per gene). For each of these CpG sites, we tested the correlation between DNAm at the site and expression of the associated gene as well as between methylation across a promoter region and gene expression.

4.2.10 *Statistical environment and false discovery rate correction*

We conducted all analyses in the R statistical computing environment (version 4.0.2) (*R: A Language and Environment for Statistical Computing.*, 2020), mostly on the Agave high performance computing cluster at Arizona State University. Where multiple hypotheses were tested, as in all models of DNAm at individual CpG sites, we calculated the false discovery rate (FDR) from *p*-values using the approach of Storey and Tibshirani with the *qvalue* function in the package of the same name (Storey & Tibshirani, 2003). In some cases where < 1000 hypothesis tests were conducted simultaneously and the data did not meet the assumptions necessary for the *qvalue* function, we calculated FDR using the *p.adjust* function in the R *stats* package (*R: A Language and Environment for Statistical Computing.*, 2020) to implement the Benjamini-Hochberg procedure of calculating FDR (Benjamini & Hochberg, 1995).

4.3 RESULTS

4.3.1 *Diet alters DNA methylation in promoter regions of a limited number of genes*

We found little evidence of global effects of diet on DNA methylation (DNAm). Principal component analysis revealed that the first principal component (PC) correlated with diet was PC12, which accounted for 3.5% of overall variance in DNAm across samples ($t_{(22.3)}$ = -3.14, Holm-Bonferroni adjusted p = 0.025). We then modeled DNAm as a function of diet and social status, and no CpG sites showed a significant association with diet (FDR < 0.05). At a loosened threshold, 38 CpG sites were associated with diet (FDR < 0.2; n = 4 CpG sites FDR < 0.1; **Table S4.1A**), which were balanced between those with higher methylation in Western- and Mediterranean-fed monkeys (n = 19 each). We conducted functional enrichment analyses (gene ontology) of these CpG sites, which yielded no significantly enriched biological processes.

As it is well known that CpG site methylation can have cis regulatory effects of nearby genes, we hypothesized that the effects of diet on DNAm may be more pronounced in CpG sites near genes, specifically those in the promoter regions of differentially expressed genes (DEGs) in which we previously identified an effect of diet (Johnson et al., 2021). To test this, we looked for enrichment of CpG sites with a nominal effect of diet (uncorrected p-value < 0.05) in the subset of CpG sites found in the promoter region of 1) all genes and 2) DEGs. Of the 2,509 CpG sites found in promoter regions of genes, 152 (6.1%) had a p-value < 0.05 for the main effect of diet, which is slightly higher than the set of all sites, but not significantly so (5.7% of all CpG sites had a diet effect p < 0.05; enrichment p = 0.45 calculated through bootstrapping). The promoter regions of DEGs (n = 517 genes, 1-10 CpG sites per gene) showed no enrichment of differential methylation by diet (n = 55 probes p < 0.05 out of 1,057 found in promoter regions of DEGs; 5.2%;

enrichment $p = 0.50$). These data suggest that there is not an increased likelihood of diet effects for CpG sites in promoter regions of genes or DEGs.

We repeated this analysis to test for enrichment of CpG sites in the first intron of genes, which has been shown to regulate gene expression. There was not enrichment of nominally significant diet effects at CpG sites in the introns of all genes (195 CpG sites p-value < 0.05 out of 3505; 5.6% compared to the background of 5.7% of all CpG sites; enrichment $p = 0.69$), nor was there enrichment of diet effects in the introns of DEGs (71 of 1405 CpG sites p-value < 0.05; 5.1%; enrichment $p = 0.30$).

Next, we tested if diet was associated with DNAm averaged across CpG sites within promoters (n = 513 genes with two or more CpG sites in their promoter regions). Promoter DNAm was significantly associated with diet for 7 genes at a relaxed significance threshold (FDR < 0.2; n = 1 FDR < 0.1, n = 0 FDR < 0.05). The size of this effect was small, ranging from a 0.8-4.8% difference in average methylation between diet groups and was split between those with higher methylation in the Mediterranean diet group (n = 3 genes) and those with higher methylation in the Western diet group (n = 4 genes), although all 7 genes had higher expression in the Mediterranean diet group (Johnson et al., 2021). Repeating this analysis for the first intron of 617 genes, diet was associated with DNAm in the first intron of two genes (FDR < 0.2, n = 1 FDR < 0.1, n = 0 FDR < 0.05). Thus, while diet does not have global effects or particularly strong effects at any given CpG site, there is a small group of genes for which diet is linked to changes in promoter DNAm.

4.3.2 *No evidence of epigenetic drivers of diet-driven monocyte polarization*

We previously found that diet induced transcriptomic changes reflective of monocyte polarization toward proinflammatory (M1) or regulatory (M2) states in these monkeys. We examined the CpG sites in the promoter regions of genes associated with monocyte polarization (Schmidl et al., 2014)

to find evidence of epigenetic changes driving polarization. There were 159 CpG sites in the promoter regions of 76 proinflammatory genes. There were no significant relationships between diet and DNAm at these sites (all FDR > 0.2), nor were there significant correlations between DNAm and gene expression (all FDR > 0.5). However, when we relaxed the multiple hypothesis test correction and examined the relationships that passed a nominal *p*-value threshold ($p < 0.05$), an interesting connection appeared. Eight CpG site-gene pairs had a suggestive relationship between DNAm and gene expression, four of which included the gene *RBM5*. The *RBM5* gene encodes the RNA-binding motif 5 protein, which is involved with differential RNA splicing as part of a spliceosome complex, and has been identified as a tumor suppressor gene for its role in cell death (Jackson & Kochanek, 2020). One of these CpG sites (probe cg05657651) had a significant main effect of both diet ($p = 0.043$) and social status ($p = 0.021$), such that there was higher methylation in Mediterranean-fed and low-status individuals. The expression of this gene was significantly higher in Western-fed animals, and there was an inverse relationship between DNAm and gene expression, matching the canonical relationship between DNAm and expression of a nearby gene. This suggests *RBM5* and the CpG site measured by the cg05657651 probe would be candidates for further analyses.

There were 45 CpG sites in the promoter regions of 16 genes known to drive regulatory monocyte polarization (M2 polarization). There was no enrichment of CpG sites in these genes and all relationships tested were not significant (all FDR > 0.4).

4.3.3 *No evidence of other regulatory mechanisms in monocyte response to diet*

Based on our prior findings, we tested two additional mechanisms of gene regulation. In our prior work, we identified a set of "hub genes" that showed dysregulation with other genes between the two diet conditions, which may be mediated by DNAm alterations. Of the 124 hub genes identified,

17 had CpG sites in their promoter regions and none of these 17 CpG sites had a significant diet effect (all raw $p > 0.05$). We previously showed an enrichment of transcription factor binding sites in the promoter regions of genes whose expression is altered by diet. This is an additional way in which DNAm may be modulating gene regulation, so we looked for the presence of significant diet effects in CpG sites found in transcription factor (TF) binding motifs. We extracted CAGE peaks that indicate likely TF binding from the FANTOM5 database (Noguchi et al., 2017), but found only 51 CpG sites in the likely TF binding sites. Of those 51 CpG sites, none showed a significant effect of diet on DNAm (all raw $p > 0.2$). These data provide no evidence in support of these two proposed mechanisms of gene regulation.

4.3.4 *Social status is associated with monocyte DNA methylation*

Social status was significantly associated with the first principal component (Pearson $r = -0.365$, $p = 0.031$; **Figure 4.1A**), which accounted for 8.2% of the overall variation. At the level of individual CpG sites, however, only 6 CpG sites (0.07%) were significantly associated with status (FDR < 0.05; $n = 68$ CpG sites FDR < 0.1; $n = 419$ CpG sites FDR < 0.2; **Table S4.1B**). In these 418 CpG sites, roughly half showed higher methylation in high-status individuals ($n = 197$ CpG sites; $n = 222$ sites with higher methylation in low status individuals). Functional enrichment analysis of these CpG sites showed no enrichment of gene ontology terms.

Next, we tested the promoter regions of 513 genes for differential methylation and found that none showed a main effect of social status (all FDR > 0.8). Of 617 genes with two or more CpG sites in the first intron, DNAm of one intronic region was significantly associated with social status (FDR < 0.05, $n = 3$ FDR < 0.1, $n = 3$ FDR < 0.2). Thus, social status affected methylation of a subset of individual CpG sites and provided some structure to the global pattern of methylation, but it did not alter regional DNAm, except in a few introns.

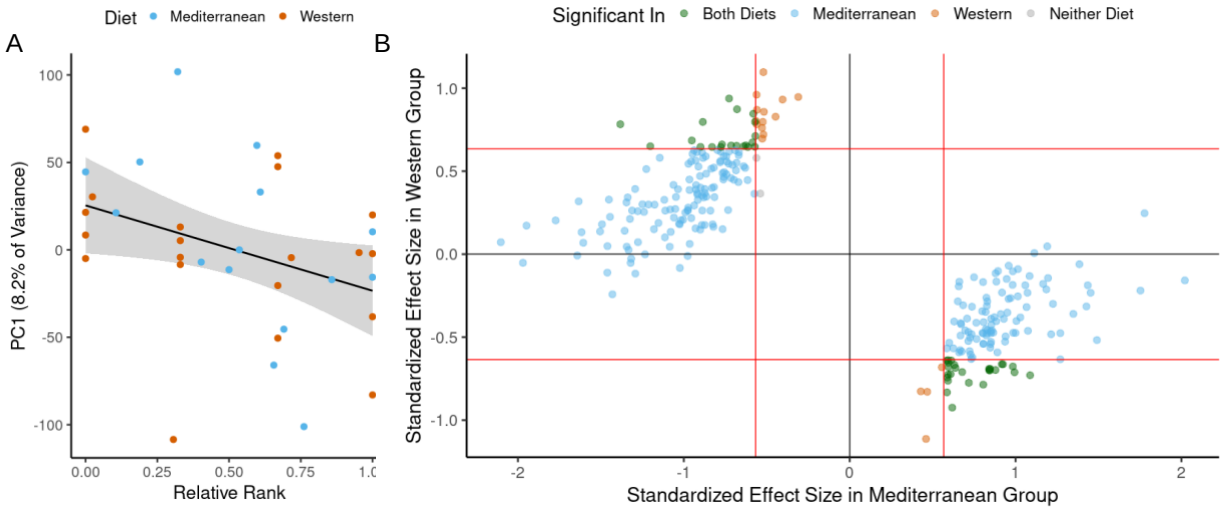

Figure 4.1 DNA methylation (DNAm) shows global and interactive effects of status (with diet). A) Points indicate individual score on the first axis of variance (PC1) in DNAm versus social status (relative rank in social group) for Mediterranean-fed (blue) and Western-fed (orange) monkeys. Social status is significantly correlated with the PC1 in DNAm (Pearson r = -0.365, p = 0.031). **B)** Standardized effect sizes of social status on DNAm within each diet group are plotted against each other for all CpG sites with a significant interaction effect between diet and status (n = 292 CpG sites). Red lines indicate the thresholds for significance (FDR < 0.05), and points are colored by which diet shows a significant effect of social status: both (green; n = 46 CpG sites), Mediterranean (blue; n = 227 CpG sites), Western (orange; n = 17 CpG sites), neither (gray; n = 2 CpG sites).

4.3.5 *Interaction of diet and social status is mostly driven by Mediterranean-fed animals*

In the model of CpG site DNAm by diet and status, a larger number of CpG sites (n = 31, 0.019%) showed an interaction between diet and status (FDR < 0.05; *n* = 93 CpG sites FDR < 0.1; *n* = 292 CpG sites FDR < 0.2; **Table S4.1C**) than did either main effect. For the set of CpG sites that passed a looser significance threshold (FDR < 0.2), we modeled DNAm (β values) as a function of status in each diet group separately and most had a significant effect of status in Mediterranean-fed animals and no significant effect in Western-fed animals (n = 128 higher methylation in low-status animals, n = 99 higher methylation in high-status animals, FDR < 0.05 in univariate models of DNAm ~ status nested within diet). DNAm at fewer CpG sites was associated with status in Western-, but not Mediterranean-fed animals (n = 13 higher methylation in high-status animals, n = 4 higher methylation in low-status animals). Only two CpG sites had a significant interaction without an effect of status in either diet subgroup and the remaining 46 CpG sites had a strong enough interaction that the effects of status were reversed in diet groups (n = 26 higher methylation in high-status Mediterranean-fed monkeys and low-status Western-fed monkeys; n = 20 higher methylation in low-status Mediterranean-fed monkeys and high-status Western-fed monkeys; see **Figure 4.1B** and **Table S4.1C** for the outputs of these models). There was no evidence of an interaction effect between diet and status on differential methylation of promoter regions (all FDR > 0.8), although two intronic regions were differentially methylated at a relaxed significance threshold (FDR < 0.1, *n* = 2 FDR < 0.2).

4.3.6 *No evidence of DNA methylation regulating gene expression*

We hypothesized that DNAm would mediate environmental effects on gene expression. To test mediation in a set of CpG sites, we would need to establish an effect of DNAm at that site on gene expression of a particular gene. We first tested this by measuring the correlation between DNAm

(β value) of a CpG site and expression of that gene for the 1,266 genes with at least one CpG site found in the promoter region. No sites meeting these criteria were found (highest correlation Pearson r = -0.532, all FDR > 0.4). Interestingly, when we examined all CpG site-gene pairs, we found 2 CpG site-gene pairs that were significantly correlated (FDR < 0.05, n = 4 FDR < 0.1; n = 12 FDR < 0.2). Both pairs were separated by more than 20,000 base pairs but had high inverse correlation (Pearson r < -0.7). These data show no evidence of cis regulation by DNAm, but limited evidence of transcription modulation from further upstream than the promoter region of a gene. One final test for DNAm mediating diet effects on gene expression is to test for similar effects of diet on both DNAm and gene expression. This allows us to examine effects without using arbitrary thresholds. We measured correlation between the effect sizes of diet on DNAm and gene expression, both in individual CpG site-gene pairs (n = 2,509 pairs) and promoter region-gene pairs (n = 513). DNAm at neither individual CpG sites (Spearman ρ = -0.009, p = 0.67), nor promoter regions (Spearman ρ = 0.025, p = 0.57) were correlated with gene expression. We were thus unable to find evidence that methylation in a region of DNA was driven by diet or status, nor were we able to link regional DNAm to gene expression.

4.3.7 *Behavior associated with DNA methylation at numerous CpG sites*

We previously reported a diet-associated behavioral phenotype that was partially responsible for diet-induced changes in gene expression. Specifically, monkeys fed the Mediterranean diet demonstrated increased social affiliation and reduced anxiety related to social status compared to the Western diet group of monkeys (Johnson et al., 2021, 2022). We asked if these behavioral changes that corresponded with changes in gene expression may be driven by—or be driving—changes in DNA methylation. We modeled DNAm across all CpG sites as a function of diet-altered behavior (DAB) scores and found no significant relationships (all FDR > 0.2). DAB was a

composite measure of all behaviors and may function in gene regulation differently than unique behaviors. Thus, we tested DNAm as a function of the five behaviors that were significantly different between diet groups in this study. Each CpG site was fit to a model of DNAm as a function of each behavior (one CpG site and one behavior per model). DNAm was significantly associated with anxiety behavior for 11 CpG sites, time attentive for 3 CpG sites, time resting for 78 CpG sites, time alone for 2 CpG sites, and time in body contact for 0 CpG sites (FDR < 0.05). These sites were all unique and none were significantly associated with diet or social status. There was no enrichment of biological processes in these sets of behavior associated CpG sites.

4.4 DISCUSSION

This study sought to measure the extent to which crucial aspects of the environment were biologically embedded through DNA methylation (DNAm) in circulating monocytes. We hypothesized that diet would have large global effects on DNA methylation (DNAm), given its sizeable effects on transcription that we previously described. In these same monocytes, diet was significantly correlated with PC1 (59% of variance in gene expression) and 40% of genes measured were differentially expressed between the two diet groups (Johnson et al., 2021). In addition to the transcriptional response to diet, we have shown diet to have widespread effects on microbiome composition (Nagpal, Shively, et al., 2018; Shively et al., 2018), brain morphology (Frye et al., 2020), stress physiology (Shively et al., 2020), and cellular and whole organism metabolic outcomes (Gonzalez-Armenta et al., 2019; Shively et al., 2019). In this report, we found evidence that diet altered methylation in a small number of gene promoter and intronic regions. This finding was surprising in its limited scope, given the previous results. However, the presence of strong status effects within diets suggests that diet plays an important, albeit confusing role in epigenomic regulation mediated in regulatory regions of genes.

We did, however, find the signature of social stress in both broad patterns of methylation and at individual CpG sites. We were unable to test for social status effects on gene expression at this same time point due to technical issues with RNA samples (see **Appendix B**), so we were not able to compare DNAm to gene expression as we were able to do with diet effects. We have demonstrated that status is associated with physiological effects in these monkeys, such as differential brain volumes (Frye et al., 2020), and thus the global DNAm response to social stress was not surprising. We were perplexed by the limited evidence of status effects at individual CpG sites, which was unexpected given how strongly social status loaded onto the principal axis of variance. However, the fact that PC1 accounted for so little of the overall variance (8.2%) suggests numerous sources of noise—either biological or technical—rather than a concerted response to experimental variables. The fact that DNAm at some CpG sites was significantly associated with behaviors other than those used to determine social status suggests that other aspects of the macaque social environment may be affecting the epigenome.

We sought to examine gene regulatory mechanisms through testing the relationship between DNAm and gene expression. To do this, we tested a few specific predictions. First, we expected there to be an enrichment of significant effects of diet and social status on DNAm in CpG sites found in the promoter region of differentially expressed genes, which we did not find. Second, we expected there to be a correlation between methylation of CpG sites in the promoter regions of genes and the expression of those genes. We found no CpG site-gene pairs that supported this prediction, nor were there relationships between methylation across promoter regions and the expression of those genes. Finally, we used these data to test hypotheses about epigenetic drivers of patterns that we observed. We previously reported that diet was associated with monocyte polarization (Johnson et al., 2021), and we predicted that DNAm would be related to diet near the

polarization genes. We found no enrichment of diet or status effects in the promoter regions of these genes. In the same study, we identified a set of "hub genes" that showed changes in co-expression patterns with more genes than expected, suggesting a role in gene regulation. We predicted that these genes may be impacted by DNA methylation, leading to their decanalization, but also found no evidence in support of this hypothesis. We also tested for—but found no evidence of—diet effects within purported transcription factor binding sites.

Given the size and scale of environmental impacts we have previously seen in these animals, these results are somewhat surprising. The small number of CpG sites and genes that show a significant effect of environmental stress may be due to technical issues stemming from mismatches in genomic sequences of *Homo sapiens*, for which the Illumina MethylationEPIC microarray was designed, and *Macaca fascicularis.* However, our group and others have previously had success measuring DNA methylation in nonhuman primate species on this microarray platform (Howard et al., 2011; Nakachi et al., 2020; Pichon et al., 2021). Indeed the approach we used to filter probes on sequence homology was based on a study that used this same platform in the same species (Nakachi et al., 2020). Our probe filtration, which increased our confidence in the results, likely contributed to the limited scale of the results, as 79% of probes were removed from the start of analysis. Future analyses could employ bisulfite sequencing, a technique that would circumvent the issues around microarray probe homology and avoid having to disregard nearly 80% of the data.

It is also possible that the limited scope of these epigenetic effects is biological in nature. There are additional sources of epigenetic variation, such as histone modification and differences in chromatin availability, all of which could be responsive to the environmental variables that we studied. Future work could make use of other sequencing methodologies, such as assaying open

chromatin through ATACseq to understand the various epigenetic responses to the environment. One could also examine the decay of messenger RNA or translational efficiency as a means of evaluating possible post-transcriptional mechanisms that could be responsible for the observed difference in gene expression as a result of experimental diet manipulation. Furthermore, it is worth considering the vast lifestyle changes that model organisms undergo during an experiment, including this study. These monkeys moved across states and experienced the subsequent changes to their climate. Following this move, the monkeys changed their daily routine, their environmental enrichment, and their social groups – all of which may be driving epigenomic changes that could mask the response to diet and social status. In support of this possibility, we found a comparatively large number of CpG sites were responsive to behaviors that were responsive to diet. Future work in this experimental paradigm and others will help to contextualize the findings of this study.

We found varied, albeit limited evidence suggesting epigenetic response within monocytes to both diet and social status. These data add to the growing understanding of how environmental stressors contribute to immune health and mortality. Two key aspects of this study expand upon the already substantial effects of environmental stressors observed in these animals. First, the strong correlation of social status with the primary axis of variance establishes a molecular effect of status in these monocytes that we were unable to address in transcriptomic data. Second, the strong effects of social status within Mediterranean-fed animals are reminiscent of the changes in anxiety behavior within subordinate Mediterranean-fed monkeys in this study. Both findings suggest that subordinate animals fed the Mediterranean diet face fewer consequences of social adversity. If true, this would have important translational ramifications on the ability of dietary modifications to ameliorate environmental stressors that are more intractable. The limited scope

of these findings prevents overarching conclusions, but still allows for hypothesis generation that will drive further research in this study system and others.

Chapter 5. Conclusion

In this dissertation, I sought to characterize the immunogenomic and behavioral consequences of two environmental stressors—diet and social adversity—in nonhuman primates. I hypothesized that both chronic social stress from low social status and nutritional stress from Western diet consumption would result in a pro-inflammatory polarization of circulating monocytes. I further hypothesized that the two environmental variables would act on overlapping molecular mechanisms, resulting in an interactive effect in key molecular phenotypes. I was able to leverage data from a preclinical trial of whole diet manipulations in female cynomolgus macaques (*Macaca fascicularis*) to address these hypotheses. The experimental paradigm is described in detail in Chapter 1 and in each of the empirical chapters of this dissertation. Briefly, monkeys were fed experimental diets matching Western or Mediterranean patterns of nutrient consumption in humans. Experimental diets were consumed for 30 months, during which time behavioral and biological data were collected. I characterized effects of environmental stressors on gene expression (Chapter 2) and DNA methylation (Chapter 4) in monocytes collected at the midway point of the dietary intervention to elucidate molecular response to diet and social adversity. Using behavioral data collected in a baseline phase and throughout the experimental diet phase of the experiment, I characterized behavioral patterns in these monkeys in Chapters 2 and 3. This chapter summarizes these findings.

5.1 DIET EFFECTS

5.1.1 *Monocyte transcriptional response to diet*

In Chapter 2 I demonstrated a genome-wide response in circulating monocytes to the dietary manipulations we carried out. Of the 12,240 genes measured, 40% were differentially expressed

between the two diet groups (n = 4,900 differentially expression genes (DEGs)). These included proinflammatory genes, such as the gene encoding interleukin-6 (*IL6*) and components of the transcription factor NF-κB, which were concordant with diet manipulations in humans that used components of the Mediterranean diet (Camargo et al., 2012). I showed differential expression of genes that were shown to be involved in human monocyte polarization *in vitro* (Schmidl et al., 2014), as well as higher expression of these genes in Western diet-fed monkeys. These data support our hypothesis about diet effects in monocytes.

I used a few approaches to functionally characterize the diet associated DEGs, starting with gene ontology. I showed that DEGs expressed higher in the Western group (referred to as "Western genes" in Chapter 2) were enriched for oxidative metabolic aspects of gene regulation, such as response to growth factor and response to insulin. Mediterranean genes (the DEGs with higher expression in the Mediterranean group) were more likely to be involved in oxidation-reduction processes than expected. Applying enrichment analyses to sets of genes previously associated with human diet-associated diseases and traits (Y. Zhang et al., 2020), I found that Western genes were enriched with genes linked to celiac disease, body fat, body mass index, HDL-cholesterol, LDL-cholesterol, and adiponectin.

5.1.2 *Gene regulatory response to diet*

I analyzed both transcriptomic (Chapter 2) and DNA methylation (Chapter 3) data to try to understand the regulatory mechanisms that might underpin the observed changes in gene expression. I did not observe large-scale effects of diet on DNA methylation (DNAm) as found in gene expression. At a relaxed significance threshold diet was significantly associated with DNAm in a small number of CpG sites (n = 38) and the promoter regions of seven genes were differentially methylated.

Returning to gene expression, I found that diet altered a few large co-expression networks of genes. Furthermore, numerous pairs of genes had significantly different co-expression patterns between the two diets, suggesting that diet may change the regulation of gene co-expression. Within the pairs of genes showing disrupted co-expression, there were some genes that appeared more frequently than expected by chance and these "hub genes" were enriched for transcription factors. Adding to this finding, I found enrichment of transcription factor (TF) binding motifs in the promoter regions of DEGs. Specifically, members of the E26 transformation-specific (ETS), specificity protein (Sp)/Krüppel-like family (KLF), myocyte-specific enhancer factor (MEF), and interferon-regulatory factor (IRF) families of TFs, which have all been linked to myeloid differentiation (Chistiakov et al., 2018; Schuler et al., 2008; Scott et al., 1994; D. E. Zhang et al., 1994), were overrepresented in regulatory regions of genes with higher expression in the Mediterranean diet group ("Mediterranean genes"). Additionally, the group of TFs included those involved in inflammatory pathways, metabolic disease, and monocyte polarization (Fledderus et al., 2007; Miyata et al., 2012; Reimold et al., 2001). Based on these results, I looked for enrichment of diet effects on DNAm in likely TF binding sites or in the regulatory regions of hub genes, neither of which were found. These findings suggest regulatory mechanisms that may be driving monocyte polarization in response to diet. However, the lack of substantiation in the DNAm data imply either different epigenetic mechanisms driving transcriptional changes, or technical issues reducing my ability to detect differential methylation in these samples.

5.1.3 *Behavioral response to diet*

I found that experimental diets altered key social behaviors in female monkeys. In Chapter 2, I analyzed behavior in the baseline phase and first half of the treatment phase of the experiment. Compared to the Mediterranean group, the Western group had more anxiety behavior and spent

more time in isolation, a phenotype previously observed in Japanese macaques born to mothers consuming a high-fat Western diet (Thompson et al., 2018). This diet-altered behavior (DAB) phenotype is relevant to organismal health, as social isolation has been associated with inflammation (Cacioppo et al., 2015; Cole et al., 2015; Eisenberger et al., 2017) and is a risk factor for psychiatric disorders (Hawkley & Cacioppo, 2010; Nguyen et al., 2020; Taylor et al., 2018). In Chapter 3 I extended the behavioral analysis to the whole experiment and sought to better understand how diet was shaping behavior.

Understanding the temporal dynamics of behavioral changes could help determine the role that they play in diet-associated health outcomes. DAB scores of Western and Mediterranean animals diverged immediately after introduction of experimental diets (the first behavioral data were collected 3 months after diet transitions) and stayed significantly different for most months of the treatment phase. At the level of specific behaviors, affiliative behavior changed immediately, where Mediterranean-fed monkeys traded time alone for time in body contact and monkeys in the Western group did not change patterns of affiliation. This was a consistent pattern across the five behaviors that were significantly different between diet groups: the behavior of Western-fed monkeys remained stable, but that of the Mediterranean-fed monkeys responded to diet manipulations.

I sought to examine molecular changes associated with behavioral changes. In Chapter 2, I conducted a mediation analysis where I demonstrated that DAB score mediated the effect of diet on gene expression for 24% of DEGs and conversely that differential gene expression mediated the effect of diet on behavior (DAB score) for 18% of DEGs. Interestingly, I found that the behaviors that showed diet-associated differences were predictive of DNAm at numerous CpG sites. These data suggest that diet-induced molecular changes in monocytes may 1) impact

behavioral phenotypes in nonhuman primates (perhaps through a central nervous system pathway), 2) be a product of behavioral changes associated with diet, or 3) share a common mechanism leading to each phenotype. We lack the controls to mechanistically test this statistical association, but literature supports either route of mediation (see section 2.2 for a thorough discussion of this).

5.2 SOCIAL ADVERSITY

I hypothesized that social adversity would lead to molecular changes in monocytes. When I tried to test this hypothesis in Chapter 2, I found that social status was significantly associated with RNA quality, which is a technical covariate that is commonly controlled for. This meant that I removed effects of social adversity on gene expression when I controlled for RNA quality, preventing their discovery in this experiment (see **Appendix B** for further discussion). Nonetheless, I was able to test for molecular and behavioral effects of social adversity in Chapter 4 and Chapter 3, respectively.

When I examined global effects of social status on DNAm, I found that it was highly correlated with the first axis of variance, suggesting a large effect. At the level of individual CpG sites, however, only a small fraction was significantly associated with social status at a relaxed significance threshold (n = 419 CpG sites FDR < 0.2, ~ 0.3% of all tested). There was a significant interaction between diet and social status in an additional 292 CpG sites. Interestingly, most of these sites showed a significant effect of status on DNAm in Mediterranean-fed monkeys, but not in Western-fed monkeys. This is striking considering the behavioral data from Chapter 3, in which the Mediterranean—but not Western—group changed behavior after changing diet. One possible explanation for this would be the potential similarity between standard chow monkey food (fed to monkeys during the baseline phase) and the Western diet (see **Table 2.1** for a comparison of nutritional contents).

As social groups are known to be quite stable in female cynomolgus macaques (Sapolsky, 2005; Shively & Kaplan, 1991), the effects of social status on behavior were not as temporally dynamic as those of diet. Behaviors typically associated with social status and maintenance of hierarchy were predicted by social status in both the baseline and treatment phases of the experiment. One effect of status on behavior stands out, which is the interaction between status and diet in anxiety behavior. I showed that the subordinate monkeys consuming a Mediterranean diet reduced anxiety behavior to levels commensurate with their dominant counterparts. Once again, this finding supports the conclusion that interactive effects between diet and social status appear in the Mediterranean group. This presents intriguing translational applications, as the Mediterranean diet may be able to alleviate the behavioral and molecular stress associated with low social status.

5.3 LIMITATIONS

This study, while robust in its design, does have some limitations. One critique from reviewers was the lack of a control diet during the treatment phase to compare experimental diets against. The extensive costs associated with maintaining a captive nonhuman primate population necessitated efficient use of resources and thus we were unable to include a third group consuming a control diet. This limitation was partially overcome by the preclinical design of the experiment with thorough randomization and controls, which allowed for causal inference to be made about differences between the diet groups. However, we are still limited in our ability to determine precisely which diet is causing physiological changes in instances where the two groups are directly compared. Those using standard chow diets in animal models of disease that may have an inflammatory component could consider direct comparison between chow diets and human dietary patterns.

It is worth noting that the dichotomous M1/M2 paradigm of monocyte polarization is an oversimplification of the more complex heterogeneity of monocytes (Martinez & Gordon, 2014; Nahrendorf & Swirski, 2016). There are at least three classes of monocytes in the circulation, and we limited our analyses to the dichotomous binary of M1/M2. We did not assess the relative abundance of these subsets; thus, the observed gene expression patterns could reflect either changes in the relative proportions of these subsets and/or shifts in monocyte polarization within subsets (Michalson et al., 2019; Wolf et al., 2017).

Relatedly, these studies were conducted in cells purified through CD14+ isolation and they ignore potential inflammatory changes in the rest of the immune compartment. While this allowed for direct evaluation of the M1/M2 polarization hypothesis, we may be missing important consequences of diet or social adversity in other cell populations. For example, experimental manipulation of social status in macaques led to differential gene expression across most immune cell subtypes but interestingly not monocytes (Snyder-Mackler et al., 2016). Furthermore, results presented here may be influenced by the purity of CD14+ isolation. As described in Chapter 2, samples had variable purity as a result of magnetic bead separation (see **Figure 2.11**). We controlled for this in our models of gene expression and DNA methylation, but isolating cell populations by cell surface markers and then controlling for differential separation success may mask important biological differences in cell populations. Future analyses within this experimental paradigm and studies that wish to further explore diet and social status immune consequences may wish to measure gene expression in multiple cell populations and/or account for variability in immune compartment proportions.

These studies employed a captive population of female macaques, which are an effective model of the human social environment due to their stable hierarchical social structures (Sapolsky,

2005; Shively & Day, 2015; Shively & Kaplan, 1991; Snyder-Mackler et al., 2020). The use of female macaques also served the aims of the larger project, which sought to understand drivers of cardiovascular health in women. However, there is ample reason to think that the findings here would not be replicated in male cynomolgus macaques. First, their social structure is more despotic and less stable than the females of their species. With this social organization chronic stress is not linearly related to social status as it is in females, nor is status stable across a lifetime, as measured by glucocorticoid levels in circulation across primate species (Abbott et al., 2003; Sapolsky, 2005; Shively & Day, 2015; Snyder-Mackler et al., 2016). Thus, we would expect effects of social status to more closely resemble that of the status effects on glucocorticoids in male macaque species. As social status both drives behavioral phenotypes and is itself mediated through conspecific interactions, we would also expect to see sex differences in behavior if this study were replicated in males. Furthermore, men and women show differential response to lipid metabolism and inflammation, due in part to the action of sex hormones (Palmisano et al., 2018; Varghese et al., 2017). Thus, we could predict that at least some of the differences between diet groups would be sex specific. Future analyses could address the role of diet and social status in males, although considerations would have to be made to account for the ethological differences between male and female nonhuman primates.

5.4 FUTURE DIRECTIONS

The results described in this dissertation are part of a larger experiment. There have already been numerous publications detailing results from this experimental manipulation (Amick et al., 2021; Frye et al., 2021; Gonzalez-Armenta et al., 2019; Johnson et al., 2021, 2022; Nagpal et al., 2019; Nagpal, Shively, et al., 2018; Newman et al., 2021; Shively et al., 2018, 2019) and analyses are ongoing.

A few projects are of particular interest to me that will extend the findings reported in this dissertation. First, we have collected RNA and DNA from the end of the baseline phase of the experiment before transitioning to experimental diets and at the end of the diet manipulations. Understanding how both gene expression and DNA methylation change over time will help to tease apart the effects of various environmental stressors, including diet and social adversity. The ability to compare an individual's molecular phenotypes to its own baseline data will provide insight as to which diets may be driving the observed changes. It will also help overcome technical limitations that prevented particular analyses. At the start and end of the treatment phase, we also conducted brief social stress tests to examine physiological and molecular response to acute stress. As chronic stress is thought to disrupt glucocorticoid signaling, we would expect to see altered response to acute stress after the diet manipulation and 38 months in social housing. My collaborators have already demonstrated altered stress physiology (Shively et al., 2020) and I hope to help understand the dynamics of gene expression responses to acute stress.

As the breadth of the publication history from this experiment would suggest, there have been biological samples collected across numerous tissue types in these animals. Collaborators are currently working to understand the varied response to environmental stress. Already, we have shown that a strong response to diet in one phenotype does not necessarily predict a strong response in a different diet-altered phenotype (see Chapter 2). This variable response to diet is an area of ongoing study. As more phenotypes are examined, the resulting picture becomes more complex. Future work will focus on identifying phenotypes that respond in kind, providing insight into health and disease mechanisms.

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APPENDIX A

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APPENDIX B

Because of the well-established effects of social status on atherosclerosis (Addo et al., 2012; Hallman et al., 2001; Rosengren et al., 2004; Shively et al., 1990; Steptoe & Kivimäki, 2012, 2013; Stuller et al., 2012; Yusuf et al., 2004), inflammation (Kiecolt-Glaser, 2010; Maes et al., 1998; Steptoe et al., 2007), and immune cell gene regulation (Brydon et al., 2005; Chen et al., 2008, 2011; Cole, 2013; G. E. Miller et al., 2008; Snyder-Mackler et al., 2016; Tung et al., 2012; Tung & Gilad, 2013), one of the goals of this study was to examine of social status interacted with diet to alter monocyte gene regulation. Specifically, we hypothesized that the promotion of regulatory polarization from the Mediterranean diet would attenuate the deleterious effects of social subordination. As with many RNA-sequencing data, the RNA integrity (RIN) in our data set was correlated with the first axis of variance in a PCA analysis of gene expression (Pearson's $r = 0.41$, $p = 0.020$; **Figure B.2A**) and thus needed to be controlled for. Unfortunately, in this dataset, RIN was significantly correlated with dominance (Pearson's $r = 0.49$, $p = 5.1 \times 10^{-3}$; **Figure B.2B**). Thus, when we controlled for RIN prior to downstream analysis, we also removed our ability to detect the effect of dominance rank on gene expression. Our subsequent analyses, and sampling at later timepoints in the study, led us to conclude this correlation was purely due to random and unidentified technical reasons (and that dominance rank does not influence RNA integrity). Samples collected at other timepoints in this study will allow us to address the potential interactions between diet and social status.

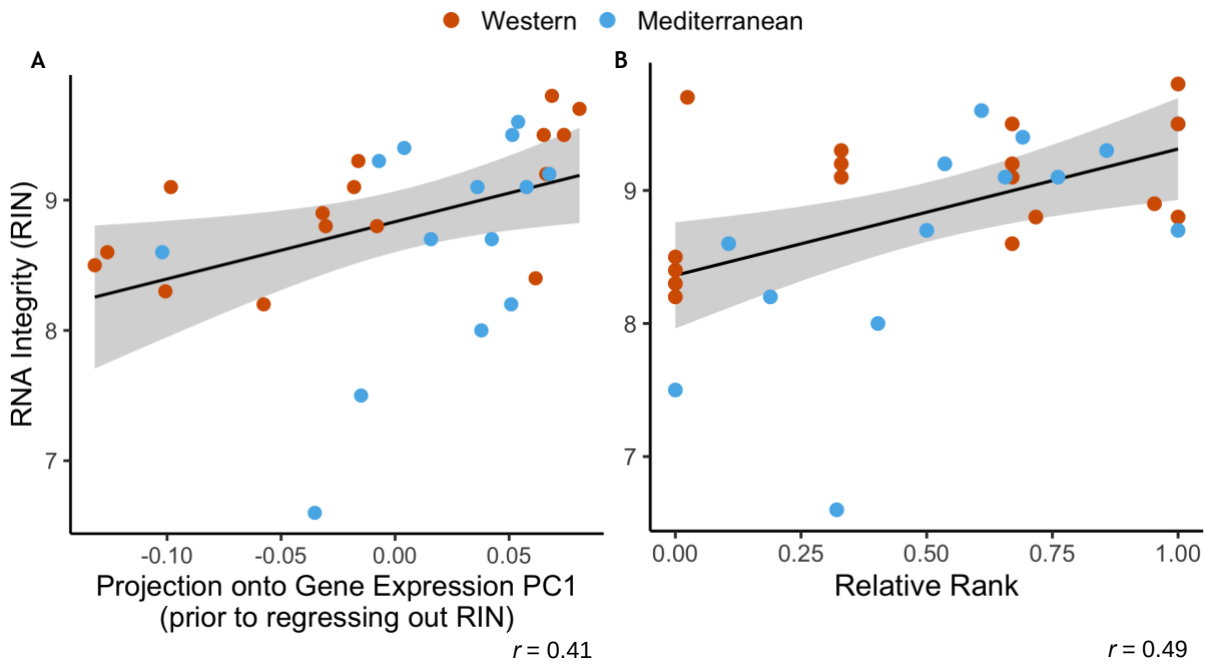

Figure B.2 RNA Integrity was correlated with both uncorrected gene expression and relative rank. A) RNA integrity (RIN) was correlated with PC1 of gene expression (62% of overall variance in gene expression) prior to correction for batch effects (Pearson's $r = 0.41$, $p = 0.020$). Because of this, RIN was included as a batch effect prior to downstream analysis. **B)** RIN was also correlated with relative dominance rank (Pearson's $r = 0.49$, $p = 5.1 \times 10^{-3}$). Points are colored to indicate Western (orange) or Mediterranean (blue) diet to show that diet did not have an interactive effect in either case.

APPENDIX C

Ten-kilogram batches of the experimental diets were prepared at a time. Batches were stored frozen at -20°C until thawing. Daily portions were thawed in the refrigerator prior to feeding. To prevent oxidation of lipids and polyphenols, extra-virgin olive oil was stored under argon. To ensure stability of each of the experimental diets, samples from three batches – one early, one midway, and one near study's end - were assayed for macronutrient and fatty acid content.

APPENDIX D

Some DNAm analyses reduce the search space by removing the probes that bind to constituently hypo- or hypomethylated CpG sites (either all β or median β between outside of the range of [0.1-0.9]). We considered using this approach, which would have resulted in either 75,834 or 103,003 probes, depending on if thresholding on all β values or the median β, but this did not meaningfully increase the statistical power to detect effects on individual CpG sites and reduced the data that would contribute to analysis of differentially methylated regions.

After conducting analyses on the 2kb of DNA upstream of transcription start sites, we extended our analyses to 5kb and 10kb upstream of genes. These analyses did not alter the overall findings of the study, so they were not detailed in the body of the manuscript. **Table D.2** shows a comparison of CpG sites in regulatory regions of all genes and differentially expressed genes in the three definitions of promoter region.

Table D.2 Comparison of CpG Sites in Regulatory Regions of Genes.

Measure	Upstream Region			1st Intron
	2 kb	5 kb	10 kb	
All Genes				
n genes	1266	2314	3604	1254
n CpG sites	2509	5744	11231	3505
n CpG sites/gene	1-18	1-23	1-61	1-32
n genes > 1 CpG site	513	1153	2044	617
n CpG sites diet p-val < 0.05	152	326	645	195
% CpG sites diet p-val < 0.05	6.1	5.7	5.7	5.6
enrichment p-val	0.44	0.87	0.88	0.69
Differentially Expressed Genes (DEGs)				
n genes	526	946	1456	477
n CpG sites	1057	2418	4671	1405
n CpG sites /gene	1-10	1-20	1-31	1-24
n genes > 1 CpG site	215	489	852	237
n CpG sites diet p-val < 0.05	55	132	246	71
% CpG sites diet p-val < 0.05	5.2	5.5	5.3	5.1
enrichment p-val	0.50	0.83	0.47	0.30

VITA

Corbin Scott Clarke Johnson was born Missoula, Montana and currently lives in Seattle, Washington. He earned his Bachelor of Science in Biology with a Minor in Irish Language and Literature from the University of Notre Dame in Notre Dame, Indiana in 2011. He completed his Master of the Arts in Teaching in 2013 from the University of Portland in Portland, Oregon. In 2022, he earned a Doctor of Philosophy in Psychology with an emphasis in Animal Behavior from the University of Washington in Seattle, Washington.