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Low-grade inflammation is a systemic response to infection and is implicated in a plethora of chronic conditions. This response is exacerbated due to excess production of reactive oxygen species and inflammatory cytokines, which can impair micronutrient status and lead to negative health outcomes. Antioxidant foods/food components including nuts and phytates are potential vehicles for mitigating inflammation and its adverse effects. Yet, data on their effects on health outcomes are scanty. The main aim of this project was to identify antioxidant foods/food components that can reduce inflammation and related health outcomes and improve micronutrient status. To achieve this, we investigated the association between phytate intake and cognition in adults ≥ 60 years using NHANES 2013-2014. Phytate intake was estimated using published data on phytate content of food groups. Regression analysis was used to determine the association between cognitive function scores and phytate intake. We also determined the association between COVID-19 severity and dietary intake among individuals diagnosed with COVID-19. As part of the Nutrition and COVID-19 in North Carolina (NC-NC) study, subjects completed online health and dietary assessment surveys. A COVID-19 severity index (CSI) was developed from reported symptoms. Regression analysis was used to investigate the relationship among COVID-19 severity index (CSI), nutrient intake, and dietary patterns developed using cluster analysis. Lastly, we studied the effect of almond intake on inflammation and iron status in a mouse model of aging. Hepcidin, IL-6, and iron status biomarkers were measured from plasma and tissue samples of aged C57BL/6 mice fed an almond diet (15% calories from almonds) for 13 weeks compared to control mice. Findings from these studies revealed that phytate was positively associated with cognition among adults ≥ 60 years while selenium (Se) intake was

inversely associated with CSI among individuals with Se and zinc (Zn) intake below the median. Additionally, among mice fed an almond-supplemented diet, iron status (higher hemoglobin, liver, and spleen iron stores) improved in mice fed an almond-supplemented diet compared to those fed a control diet without almonds. In conclusion, our study showed that antioxidant foods/food components may contribute to improvements in inflammation-related health outcomes.

THE ROLE OF DIETARY COMPONENTS IN MITIGATING INFLAMMATION AND RELATED HEALTH CONDITIONS

by

Doreen Y Larvie

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Committee Chair

DEDICATION

I dedicate this dissertation to my mom, Janet Davordzi, dad, Jasper Larvie and my brothers, Prince Larvie and Joy Larvie who continue to be a pillar of support and a model of greatness for me to follow. I would not be where I am without them. Thank you for your encouragement and teaching me the value of hard work.

APPROVAL PAGE

This dissertation written by Doreen Y Larvie has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair

Dr. Seth Armah

Committee Members

Dr. Jigna Dharod

Dr. Deborah Kipp

Dr. Keith Erikson

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Date of Final Oral Examination

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CHAPTER I: INTRODUCTION

Background

Inflammation has long been viewed as a necessary response to infection or injury. The innate and adaptive immune system are recruited to fight off offending pathogens and drive tissue regeneration. However, in cases where the pathogen is not eliminated, a chronic state of systemic damage ensues (Thurnham, 2015). Excess production of reactive oxygen species (ROS) by myeloid cells, such as macrophages exacerbates inflammation (El-Kenawi & Ruffell, 2017). This magnified and prolonged inflammatory response is composed of inflammatory cytokines, IL-6, TNF-alpha and IL-1 (Thurnham, 2015). The adverse health impacts of inflammation are evident in various health conditions such as poor cognition in aging, anemia of inflammation and viral infections such as the Coronavirus disease 19 (COVID-19).

Aging can initiate a persistent and systemic inflammatory response called inflammaging which is associated with increased levels of IL-6, TNF-alpha and NF-kB (McCranor et al., 2013; Sanada et al., 2018). IL-6 increases the progression from acute to chronic inflammation (Thurnham, 2015). Deterioration of the gut microbiota, cell debris accumulation, increasing number of senescent cells and dysregulation of the innate immune system have been attributed to inflammaging (Sanada et al., 2018). For instance, changes to innate immune system biomarkers are linked with neurodegeneration (Thurnham, 2015).

The older generation forms a large segment of the population globally and in the year 2015 in the US, 61.7 million people were 65 years or older. It is estimated that 12% (1 billion) of the world's population will be comprised of the elderly in 2030 with a projected increase of 5% by 2050 (Roberts et al., n.d.). In line with this increase is the associated burden of disease. In 2017, according to the Centers for Disease Control and Prevention, 22% of adults 65 years and

above lived with fair or poor health (*FastStats*, 2020). Aging and age-related disorders further accelerates brain neuronal dysfunction and loss resulting in decline in processing speed, attention and executive function (Cohen et al., 2016; Murman, 2015). Cognitive decline in aging is multifaceted (Murman, 2015) and is attributed to factors such as impaired calcium homeostasis, mitochondrial dysfunction, oxidative damage, inflammation and increased susceptibility to stress leading to epigenetic modifications that affect learning, memory and synaptic processes in the brain (Harman & Martín, 2020). Aging is also associated with anemia due to increased inflammation. This is because inflammation upregulates IL-6 and hepcidin leading to impaired erythropoiesis and consequently anemia of inflammation (AI). AI is the most common anemia in hospitalized patients and the second most frequent anemia worldwide (Kassebaum et al., 2014). Hepcidin is a peptide hormone that drives anemia in inflammatory states by inhibiting iron release from the liver and macrophages leading to hypoferremia (McCranor et al., 2013; Steinbicker & Muckenthaler, 2013a). In an aged mice model of anemia, iron sequestration was attenuated in hepcidin knock out mice (McCranor et al., 2013).

Beyond poor cognition and anemia, inflammation also plays a role in COVID-19 progression. COVID-19 became an epidemic in 2019 with over 95 million cases and 1 million deaths in the US as at September 2022 (CDC, 2020c). It is characterized by activation of the innate immune system, progressive inflammation, and a cytokine surge. The entry of SARS-CoV-2 virus into lung cells can initiate cellular oxidative stress because the virus uses the host cell machinery to hinder cellular processes. COVID-19 is thereby an imbalance in the cell redox state with excessive reactive oxygen species production and further activation of inflammatory signaling pathways (Alexander et al., 2020).

Current literature suggests that diet may play a role in minimizing inflammation and hence contribute to mitigating inflammation-related conditions. For example, inadequate intake of certain food groups including fruits, vegetables, cereals and grains, as well as nutrients such as zinc, selenium, copper, fiber and some vitamins have been linked with cognitive decline (Bruins et al., 2019; S. Li et al., 2019). In addition to nutrients and food groups, phytochemicals such as phytates and some polyphenols are potential agents for improving cognitive health in aging due to their antioxidant and anti-inflammatory properties (8). Trace element consideration are also needful (Retondario et al., 2021). Selenium (Se), for example, is a cofactor in cellular inflammatory pathways and its deficiency is linked with inflammation, oxidative stress, platelet aggregation, impaired immunity and viral infections (Bae & Kim, 2020; Hiffler & Rakotoambinina, 2020; Khatiwada & Subedi, 2021). Se regulates CD4+ and CD8+ T cells, natural killer cells as well as antibody production to effect antiviral action (Bae & Kim, 2020). Consequently, Se, an NF-kB inhibitor may reduce the oxidative stress and underlying low-grade inflammation seen with the progression of COVID-19 (Hiffler & Rakotoambinina, 2020). A deficiency of zinc is also associated with increased risk of viral infections (Jayawardena et al., 2020). There is evidence that adequate zinc (Zn) status in COVID-19 patients may be associated with a short duration of consistent diarrhea, and common colds (Alexander et al., 2020). These antioxidant nutrients can act through enzymes such as SOD (superoxide dismutase; copper and zinc isoforms), and GPX (glutathione peroxidase) (Kuhn et al., 2017) to repair and reduce the effects of oxidative stress in red blood cells (RBCs) and other tissues via reduction of myeloid-derived reactive oxygen species produced in inflammation-related conditions (El-Kenawi & Ruffell, 2017; Thurnham, 2015).

Despite the potential role of antioxidant foods/food components in inflammation-related conditions, data are still minimal in this area. For instance, although Alzheimer's and Parkinson's disease mice models as well as cell studies have shown an association between phytate and cognition, few studies have been done in humans due to the lack of individual phytate intake data. Also, while the impact of micronutrient status on COVID-19 outcomes have been determined, data on the role of dietary patterns in COVID-19 severity are scanty. Additionally, current treatment for AI have focused on pharmaceutical approaches but there is a dearth of data on the use of whole foods in mitigating the underlying inflammation in AI. Therefore, this proposed study aims to determine the relationship between antioxidant foods/food components such as nuts and phytate and these inflammation-related conditions.

Objectives

1. To investigate the association between daily phytate intake and cognition in older adults 60 years and above.
2. To investigate the association between COVID-19 severity and dietary intake among individuals diagnosed with COVID-19.
3. To determine the effect of almond meal on hepcidin and iron status in an aging mice model.

Dissertation Organization

This dissertation is organized into six chapters. It contains an introduction (Chapter I), literature review (Chapter 2) and three research manuscripts (Chapters 3, 4 and 5) with an epilogue (Chapter 6). The introduction provides a description of the significance of the topic and highlights connections with the research studies conducted. The literature review addresses the role of antioxidant foods/food components in three inflammation-related health conditions. One

of the manuscripts (Chapter 3) has been published and two are yet to be submitted. All references are cited according to the APA format.

CHAPTER II: LITERATURE REVIEW

Inflammation is a necessary response to biological, chemical, and physical stimuli (Germolec et al., 2018). It can be a double-edged sword when it is unable to mount a defense against microbial invasion or persistent and sustained resulting in a chronic state of inflammation and organ damage. Depending on the hormonal response a diet elicits, it can be pro-inflammatory or anti-inflammatory. This is because these hormonal responses and specific nutrients in the diet are linked with the innate immune system – where inflammatory processes originate (Sears, 2015). Poor nutrition thereby increases infectivity and impairs the immune response and western-style diets associated with calorically dense and processed foods have been linked with immune system activation and chronic inflammation (Christ et al., 2019; Morais et al., 2020; Thurnham, 2015).

An optimal nutritional status is fundamental to modulate inflammatory processes via the immune system (Gabriele & Pucci, 2017). Micronutrients such as Zn, Se, vitamin C, vitamin A and E present in anti-inflammatory foods may exert added benefits and modulate the formation of free radicals, and consequently, oxidative stress (Kuczmarski et al., 2013). Supplementation with Zn, vitamin C and vitamin D may also modulate the immune system and minimize susceptibility to infection (14). Other dietary and nutritional constituents known to exert anti-inflammatory and antioxidant properties include bioactive components such as quecertin, flavonoids, terpenes, and resveratrol, and polyunsaturated fatty acids such as omega-3 fatty acids. For instance, the fatty acid profile of the diet may alter the fatty acid structure of the cell membrane to upregulate anti-inflammatory resolvins (lipid protectants responsible for tissue regeneration) and eicosanoids to modulate B and T lymphocyte activity (Calder, 2008; Spite et al., 2014). Additionally, in a review, probiotics, omega-3 but not vitamin D and resveratrol

supplementation significantly reduced IL-6 and CRP among adults with chronic low-grade inflammation (Custodero et al., 2018). The synergistic effect of Vitamin C and quercetin may also modulate interleukins, upregulate interferons (IFN) and impair replication and enzyme activity (Colunga Biancatelli et al., 2020).

Flavonoids, such as resveratrol, quercetin, apigenin, and luteolin may have a therapeutic potential in the treatment of inflammation-related diseases, similar to anti-inflammatory cytokine modulators. Polyphenols and terpenes may reduce cyclooxygenase 1 and 2, reactive oxygen species and nitric oxide production in liposaccharide activated macrophages (Leyva-López et al., 2016). As such, these bioactive compounds act as anti-inflammatory compounds and could be important in inflammatory processes and oxidative stress. Polyphenols (flavonoids, hesperidin, quercetin), Vitamin D, antioxidant nutrients, polyunsaturated fatty acids may also improve chronic disease-related fatigue (Haß et al., 2019). It has been shown that high glycemic index diets may increase post-prandial inflammatory response and the implications of dietary fiber on the gut microbiota for improving CD8+ T cell function has been shown in animal models (Iddir et al., 2020). A review by Chin, 2016 summarized the evidence of the use of curcumin as an anti-inflammatory agent in osteoarthritis. There was an increase in chondrogenesis and decreased inflammatory and oxidative stress markers (Chin, 2016). Together, these anti-inflammatory active compounds and dietary components could be important in the overall homeostasis of inflammation and oxidative stress and could potentially be useful in mitigating inflammation-related diseases.

Although several foods and nutrients may contribute to the anti-inflammatory properties of the diet, this review will focus on selected dietary components, namely nuts (with an emphasis on almonds), phytate, Zn, and Se as specific anti-inflammatory dietary factors. It will also

address three selected conditions (AI, COVID-19, aging-related cognitive decline) that may illicit the release of inflammatory cytokines and/or that result from a pro-inflammatory state and how the anti-inflammatory foods/food components discussed may mitigate these inflammatory states.

Diet Quality and Health

Diet quality refers to the extent to which a diet minimizes the risk of non-communicable diseases (Asghari et al., 2017; Harrison et al., 2020; Waijers et al., 2007). A diet has a high diet quality if it contains beneficial foods such as vegetables, fruits, lean meat, whole grains and low dairy while a poorer quality diet is high in animal foods, refined grains and sweets (Waijers et al., 2007). Plant based foods may have a high diet quality due to the presence of vitamin A (Jahns et al., 2018; Rubin et al., 2017), vitamin C (Colunga Biancatelli et al., 2020), as well as a variety of phytochemicals, such as polyphenols (Haß et al., 2019) and phytate (Buades Fuster et al., 2017). Although plant-based diets may have a high diet quality, it must be noted that not all vegetarian diets may lead to improved health outcomes as while they exclude or limit animal sources, may not require the consumption of whole grains or restrict sugar or fat. On the other hand, plant-based diets high in whole grains, fruits, vegetables, seeds and nuts may reduce blood pressure, lower BMI and blood sugar levels (Tuso et al., 2013). Similarly, among individuals at risk of metabolic syndrome, a high whole grain diet (≥75g/day) compared to a refined grain diet (≤10g/day whole grains) resulted in lower inflammatory cytokines and body weight but not changes in the gut microbiome or blood glucose control (Roager et al., 2019).

Diet quality is linked with nutrient adequacy. Among Black adults 60 years and above in South Africa, low food and dietary diversity scores were associated with low mean adequacy ratio (MAR) (Wilna H. & Rozanne, 2008). MAR is an overall measure of nutrient adequacy of a

population and is based on the nutrient adequacy ratio (NAR, an individual's intake as a percentage of the recommended allowance for that nutrient). MAR is calculated from the sum of the NAR for each nutrient of interest divided by the number of nutrients expressed as a percentage (Madden & Yoder, 1972; M'Kaibi et al., 2015). In a lifestyle intervention study, mean adequacy ratio was positively associated with diet quality scores (Ojeda-Rodríguez et al., 2018) while in a low-income urban population, a 10 % increase in MAR from 15 nutrients including Zn, folate, niacin, Vitamin C and Vitamin A was associated with a 4 % decrease in CRP levels (Kuczmarski et al., 2013).

Diet quality can also be measured from dietary patterns and are linked to improved health outcomes. In the PREDIMED plus randomized control study among elderly Spanish individuals, adherence to the Mediterranean diet was associated with a high nutrient quality (Cano-Ibáñez et al., 2020). The Mediterranean diet composed mainly of whole grains, fruit and vegetables, milk products, legumes, polyphenol-rich vegetables, fish, nuts, olive oil, and lean meat has been suggested as an anti-inflammatory dietary pattern while there is some evidence, albeit inconclusive, that protein-rich foods, root plants – such as ginger and ginseng – and probiotics are some examples of anti-inflammatory foods (Cherian et al., 2019).

Studies show a positive association between diet quality, physical performance, and nutrient adequacy and an inverse relationship with inflammation. Conversely, in a 10-year prospective study among a Flemish population, although an unhealthy plant-based diet index was positively associated with total cholesterol, waist circumference and BMI, this association did not remain after adjustment for covariates (Waterplas et al., 2020). However, a positive association has been observed between diet quality and muscle mass and strength (Bloom et al., 2018). Ward et al. also showed that diet quality measured via indices, Dietary Approaches to

Stop Hypertension (DASH), Mediterranean (MED) diet score and Alternate Healthy Eating Index (aHEI) were associated with a lower odds of frailty (Ward et al., 2020). A review also showed an inverse association between healthy eating index (HEI) and obesity indicators such as waist circumference or BMI (Asghari et al., 2017). With the observed association between diet quality and health in a number of studies, there is a need to focus on specific foods such as nuts, which due to their nutritional composition may improve health outcomes.

Nuts, Diet Quality and Health Outcomes

Nuts account for approximately 2% of total energy intake in the US, higher than in Australia (1.6%) and Denmark (0.5%) (Auestad et al., 2015). The health promoting components of nuts are largely contributed by plant stanols, fiber, polyphenols, phytonutrients, and essential fatty acids (Waijers et al., 2007).

Nuts can increase energy intake, and portion sizes by displacement of other foods as it can contribute 50-120% of nutrient intake. Suggested ways to increase nut intake includes using them as nut butters and pure oils and timing its intake in between meals as opposed to with meals to reduce its effect on energy intake and meal displacement (Tan et al., 2018). In a study that determined total tree nut consumption, that is, including that from nut-containing foods such as breads, cereals, and muffins, tree nut consumers made up only 6% of the study population with a usual intake of nuts at 44 g/day. Nevertheless, tree nut consumers had higher diet quality (HEI-2005) and nutrient adequacy for select nutrients compared to non-tree nut consumers (O'Neil et al., 2015). In an elderly population consuming a Mediterranean diet, both supplementation with nuts (30g/day) or 4 tablespoonful olive oil/day was associated with a 30% lower risk of a major cardiovascular event. However, nut consumers compared to the group consuming olive oil had a higher nutrient and diet quality (Del Mar Bibiloni et al., 2013).

There is a potential benefit to the use of whole foods such as nuts as anti-inflammatory foods. Synergistic effects from polyphenols present in nuts such as lignans, proanthocyanidins, flavonoids, stilbenes and phytates as well as the non-phenolic components (oleic acid, linolenic acid, choline, niacin, Vitamin E) may promote axonal and dendritic growth, and neurotransmitter metabolism via oxidative stress pathways (Gorji et al., 2018). Other nutritional components such as polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA), phytosterols, antioxidants, protein, magnesium, potassium, zinc, and iron found in almonds, walnuts and pistachios have been linked with the growth of a healthy gut microbiota, improved cardiovascular health, lipid profile, insulin resistance and glycemic control (de Souza et al., 2019). Phytosterols found in tree nuts exhibit direct, indirect and co-antioxidant activity to modulate cellular cytoprotective signaling pathways, neutralize free radicals, block lipid peroxidation and regenerate essential vitamins (Rusu et al., 2019). In Male Wistar rats fed a 5% Brazil nut supplemental diet (containing 302µg Se/100g), there was a decrease in plasma lipid peroxidation and an increase in high density lipoprotein compared to the control group receiving a standard chow diet (da Silva Costa et al., 2022). Among women at cardiometabolic risk, an energy restricted diet containing brazil nuts and cashew nuts (15 g + 30g) resulted in a decrease in total fat and the soluble adhesion molecule, VCAM-1 compared with women receiving an energy restricted diet with no nuts (Caldas et al., 2022). Hazel nuts, almonds and walnuts have also been suggested to have brain-protective properties among individuals with Alzheimer's disease through oxidative stress and anti-inflammatory pathways (Gorji et al., 2018). Walnut polyphenols may also exert antioxidant activity and are implicated in a healthy gut microbiota and reducing the risk of cardiovascular disease (CVD), Type II DM, and cognitive decline (Pan et al., 2013).

A review by Rusu et al. (Rusu et al., 2018) highlights the association between tree nuts and the gut microbiota, cognition, glycemic control, cardiometabolic disorders, endothelial function and inflammation and cancer. With regards to the gut microbiota, a meta-analysis revealed that nuts may broadly confer beneficial effects on the genus but not at the level of the phyla or bacterial diversity (Creedon et al., 2020). However, Dhillon and colleagues showed that almond compared with crackers for 8 weeks resulted in increased diversity of the gut microbiome among College Freshmen (Dhillon et al., 2019). In a five-year prospective study, nut intake was associated with a 5% reduced risk of obesity and a 2.5% reduction in body weight compared to non-nut consumers after adjustment for smoking and physical activity (Freisling et al., 2018). Data from the Nurse's Health Study showed that among women, increased intake of all nuts for at least 3x/week compared to less than a month at midlife (30-55 years) was associated with a higher odds of healthy aging (measured by women who after 65 years had intact mental health, cognition, no physical disabilities and no history of chronic diseases) (Freitas et al., 2017).

While understanding the health benefits of nuts is crucial, it is imperative to understand the contribution of individual nuts such as almonds to health due to its increasing popularity in the general population (Barreca et al., 2020).

Contribution of Almonds to Health Indicators/Outcomes

Almonds (*Prunus dulcis*) remain one of the most cultivated nuts producing over 3 million tons worldwide (Roncero et al., 2020). They are a good source of polyphenols, vitamins (B, E), riboflavin, minerals (manganese, zinc, iron, copper, phosphorus), MUFA and dietary fiber (Barreca et al., 2020). Despite its compositional benefits and large-scale production, only about 2.93 grams of almond is consumed per day in the US. With the FDA recommendation of 42.5

g/day, 2.93 g meets only 7% of these recommendations (Eslampour, Moodi, et al., 2020; Lee-Bravatti et al., 2019).

Due to its fatty acid, vitamin E, mineral, fiber content, and other nutrient composition, epidemiological studies show that almonds may be protective against obesity, diabetes, and CVD via body weight, blood glucose, and lipidemic control respectively (Asbaghi et al., 2021; Barreca et al., 2020; Kalita et al., 2018). In addition, Eslampour and colleagues in meta-analyses showed that almond may reduce diastolic blood pressure, and fat mass but not systolic blood pressure and fat-free mass (Eslampour, Asbaghi, et al., 2020; Eslampour, Moodi, et al., 2020). In-vivo studies show that almond may improve learning and memory and in rats fed a 150-600 mg/kg almond meal for 2 weeks as part of an anti-dementia diet, there was lowered brain cholinesterase activity, cholesterol, and triglyceride concentration (Kulkarni et al., 2010). In Wistar rats fed an 8 % pistachio diet versus a 7.5 % mixed nut that includes almond, a lower concentration of CRP and improved lipid profile were observed in both nut groups compared to the control group while a higher catalase and superoxide dismutase (SOD) activity were observed in the mixed nut compared to the pistachio group (Hong et al., 2018). In Brazil, hemodialysis patients provided with 5 g baru almond oil versus placebo (mineral oil) had significantly lower hs-CRP concentrations after 12 weeks supplementation. However, no differences in body composition, anthropometry, other inflammation markers and lipid profile were observed (Schincaglia et al., 2020). In women with overweight or obesity, 20 g baru almond plus advice on following a normocaloric diet for 8 weeks significantly increased glutathione peroxidase (GPX) concentrations compared to those receiving advice only (de Souza et al., 2019). Among healthy 50-80 year old Australians with overweight/obesity, an almond enriched versus a nut-free diet

providing 15 % of Estimated Energy Requirement for 12 weeks improved systolic blood pressure, triglyceride but not composites of mood and cognition (Coates et al., 2020).

Anti-inflammatory/Antioxidant Food Components

There is growing evidence regarding the effect of food components and nutrients on the immune and antioxidant system and in reducing viral load (Alexander et al., 2020; Khatiwada & Subedi, 2021). In this section, the importance of phytate and antioxidant nutrients, Zn, and Se and the mechanisms involved in eliciting anti-inflammatory response will be discussed.

Phytate is one of the phytochemicals predominantly found in nuts, legumes, cereals and grains (Fulcher et al., 1981; O'Dell et al., 1972). It is a reservoir of phosphorus that enables the usage of its stored nutrients such as phosphorus for the germination of plants and seeds (Beal & Mehta, 1985; Raboy, 2007). Phytate has long been considered an antinutrient because humans lack the phytase enzyme required for endogenous breakdown. Research however shows that with a well-balanced diet, phytate has minimal impact on the absorption of trace minerals (Schlemmer et al., 2009). The absorption of non-heme iron is in part dependent on the individual's iron status and the presence of iron absorption enhancers in the diet (Lynch et al., 2018). Recent studies have shown the antioxidant role of phytate (60,61) via the reduction of the catalytic activity of major divalent metals through chelation (61). Phytic acid accelerates the oxidation of Fe^{2+} to Fe^{3+}, thus minimizing availability of Fe^{2+} for iron catalyzed hydroxyl radical formation via the Fenton reaction (Silva & Bracarense, 2016). This inhibits downstream lipid peroxidation and DNA damage (Shamsuddin et al., 1997). Phytate has also been found to be protective against inflammatory bowel disease, colonic cancer (Graf & Eaton, 1993), neurodegenerative disease (Anekonda et al., 2011b) and platelet aggregation (Vucenik & Shamsuddin, 2003). Phytate upon hydrolysis and dephosphorylation may illicit anti-inflammatory activity, inhibit tumor

proliferation, induce differentiation/enhance apoptosis in tumor cells, inhibit neovascularization/angiogenesis and prevent tumor metastasis (Schlemmer et al., 2009). In a Kenyan diet, phytic acid was found to have over 60 percent antioxidant activity and inhibited α-glucosidase activity downregulating the rise in post-meal blood glucose concentration seen in type II diabetes (Kunyanga et al., 2011).

The role of Zn in health was recognized in the 20th century and two billion people worldwide are estimated to be Zn deficient. Over 300 enzymes require zinc for activation and/or stability of structure. For example, Zn inhibits nicotinamide adenine dinucleotide phosphate (NADPH) oxidase – which drives superoxide production from oxygen. It is also a co-factor for SOD production hence catalyzes the formation of hydrogen peroxide from superoxide consequently minimizing the action and potency of reactive oxygen species (Prasad, 2014). Zinc deficiency is therefore associated with reduced non-specific immunity and decreased antibody production with supplementation associated with a decrease in TNF-alpha and markers of oxidative stress (Gammoh & Rink, 2017).

Selenium influences the inflammatory response by the down-regulation of pro-inflammatory cytokines (Y. Shen et al., 2015; Yu et al., 2015). When the incorporation of Se at the active sites of enzymes such as GPX is impaired due to Se deficiency, the inflammatory response in chronic conditions is exacerbated. Suboptimal selenium status is associated with an increase in IL-6 while Se supplementation may reduce cholesterol concentration among individuals with hypercholesterolemia (Duntas & Hubalewska-Dydejczyk, 2015; Zhao et al., 2021). Selenium deficiency in chronic inflammatory conditions also results in the decreased synthesis of selenoproteins and antioxidants such as glutathione (GSH), increasing the production of CRP and the inflammatory state (Asemi et al., 2015). Selenium supplementation

may thereby restore serum Se levels resulting in the down-regulation of CRP attenuating the inflammatory response (Ferrari et al., 2015; Mutakin et al., 2013; Tseng et al., 2013).

Selected Health Conditions

Persistent low-grade chronic inflammation is a contributory factor in a number of conditions and may lead to death, endothelial damage, and vascular calcification (Dai et al., 2017; Ferrucci & Fabbri, 2018). An important predictor of mortality, high CRP and IL-6 seen in low-grade inflammation, are key in the pathogenesis of CVD, diabetes, cancer, and age-related diseases. Behavioral pathways, oxidative stress, pro-inflammatory cytokines, and mitochondrial health are implicated in this systemic inflammation (Dai et al., 2017; Rohleder, 2019). Low-grade inflammation is different from a response to infection or injury as it is systemic and non-localized, of a lower magnitude but longer duration, with no specific stimuli compared to the inflammation seen in acute infections (Thurnham, 2015). Nevertheless, acute stress responses may predict long-term inflammatory pathways. In a study to determine the role of inflammation in the association between obesity and heart failure outcomes, obesity with high inflammation and no/low obesity with high inflammation were both associated with approximately two times the risk of all-cause hospitalization and death (Saleh et al., 2020).

While some conditions illicit a pro-inflammatory state, a high concentration of inflammatory cytokines may also cause disease states (Jiang et al., 2021). For example, COVID-19 has been characterized to illicit a cytokine storm and hyper-inflammatory state and in China, higher levels of inflammatory cytokines (IL-6, TNF-alpha) and lymphocyte subsets were observed in deceased patients with COVID-19 compared to those who survived from the condition (Jiang et al., 2021). Additionally, Leonard et al. (Leonard, 2018) surmised that chronic low-grade inflammation may trigger changes that play a role in patients with depression. In this

meta-analysis, one in six subjects with depression were estimated to have high CRP (>3 mg/L) levels – upon exclusion of subjects with suspected infections – and 46% higher odds than healthy non-depressed controls (Osimo et al., 2019).

Partly because the cause of chronic inflammation is multifactorial, there is minimal consensus on how to handle the underlying chronic inflammation that causes chronic conditions or conditions that illicit a pro-inflammatory state. Anti-inflammatory therapies have been evaluated in end stage renal disease (ESRD) for instance and include lifestyle modifications, immuno-nutrition with Vitamin E and Probiotics, and anti-cytokine therapy (Dai et al., 2017). Nevertheless, there is existing evidence to suggest that anti-inflammatory supplements, drugs and dietary patterns due to their antioxidant effects may prevent or modify the pro-inflammatory state (Ferrucci & Fabbri, 2018).

Anemia of Inflammation

Inflammation is a cause of anemia in hospitalized patients, individuals with critical illnesses and the elderly. It is commonly seen in diseases associated with persistent immune activation, autoimmune diseases, and cancer (Weiss et al., 2019). Anemia of chronic disease (ACD) or anemia of inflammation (AI) is a multifactorial malignancy from elevated concentrations of pro-inflammatory cytokines and hepcidin, iron retention and impaired erythropoiesis (Cheng et al., 2013; Weiss et al., 2019).

It is not fully known whether AI is solely a marker seen with the progression and increased severity of chronic diseases or a cause of disease outcomes (Weiss et al., 2019). This is because, red blood cells are susceptible to immune system damage accelerating its destruction which may trigger a pro-inflammatory response. The increased levels of TNF-alpha, IL-1B, IFN-gamma leads to erythrophagocytosis, and impaired erythroid progenitor regeneration. This

response may lead to AI and is characterized by a non-regenerative and mild to moderate decrease in the number of RBCs. The consequent reduction in iron limits oxidation and free radical formation, and bacterial growth (Germolec et al., 2018). Some evidence on the other hand suggests that AI-related symptoms are caused by underlying disease conditions (Weiss et al., 2019).

Inflammatory cytokines and oxidative stress are associated with AI (Lynch et al., 2018). During inflammation, IL-6 binds to glycoprotein l30 and the activation of this ligand increases the expression of hepcidin. Induced by the cytokines, IL-6 and IL-22, the HAMP gene (encodes for hepcidin) is upregulated via STAT-3 and may also feed into the BMP-6 pathway resulting in ferroportin endocytosis and decreased iron export (Camaschella, 2015; Nairz et al., 2016). This functional iron deficiency results in re-trafficking of iron from sites of use such as erythron and mucosal surfaces to storage sites such as the liver and macrophage (Nairz et al., 2016).

Hepcidin, a 25-peptide is produced primarily in the hepatocytes and regulated by erythropoietic needs, iron homeostasis, and inflammation (via BM6-SMAD and IL-6 stimulating the JAK/STAT pathway). Hepcidin may also be regulated by cellular proliferation, and growth factors (Nairz et al., 2016). Increased hepcidin production inhibits ferroportin-mediated release of iron from macrophages and intestinal absorption of iron (Germolec et al., 2018). As a result, there is a lowered proliferative capacity of erythroid progenitor cells due to decreased availability of iron for erythropoiesis from the increased iron sequestration. The anemia in AI is further exacerbated by increased erythrophagocytosis culminating in insufficient erythropoietin (EPO) response and resistance (Nairz et al., 2016). This may be evidenced by the elevated ferritin levels sometimes seen in AI. This increased cytokine release results in increased levels of hepcidin, decreased iron absorption and a consequent mild iron deficiency (Camaschella, 2015;

Wang et al., 2013). Consequently, a hepcidin knock-out mice treated with B abortus to induce AI showed improved erythropoiesis with high mean corpuscular volume, and hemoglobin and effective mobilization of iron from tissue stores with higher serum iron compared to hepcidin knock-out controls without AI (Kim et al., 2014).

Anemia of inflammation is thereby a condition of disordered iron homeostasis characterized by extremely low levels of erythropoietin, high serum ferritin (>12ng/mL), low transferrin, low transferrin saturation (<15%), low serum iron (<60µg/dL), elevated levels of inflammatory markers (IL-6, CRP) and high hepcidin concentration (Fraenkel, 2017). While current approaches to manage AI include, erythoropoietin treatment, IV iron therapy, and hepcidin antagonists, phytonutrients and antioxidant rich foods may also be helpful in slowing the onset of or mitigating AI (Macciò & Madeddu, 2012).

Natural products such as almonds may improve health outcomes via reducing inflammation, oxidative stress, and blood pressure, regulating gut microbiota and lipid profile concentration. With the observed association between inflammation, oxidative stress and AI, almonds laden with antioxidant compounds such as MUFA, minerals, phytochemicals and vitamin E may prevent or ameliorate AI via these mechanisms of action (Zhou et al., 2021). The reduction of oxidative stress may occur via the increase in antioxidant enzymes such as SOD, catalase (CAT), and GPX while decreasing lipid peroxidation via malondialdehyde (MDA). Through NF-kB, the levels of pro-inflammatory cytokines such as IL-6, and TNF-alpha may also be decreased (Zhou et al., 2021). Studies show that almonds, in amounts ranging from 30-170 g/day may decrease the levels of CRP, and MDA without increasing the levels of SOD and GPX (Barreca et al., 2020; Jia et al., 2006). Among male smokers however, vitamin E, SOD, and GPX concentrations were increased after 84g almonds compared to a 120 g pork diet after 4 weeks (94). Among 25

healthy adults, almond as 10/20% of fat in a 2000 kcal diet for 4 weeks led to a reduction in inflammatory markers, E-selectin and CRP but not IL-6 (Rajaram et al., 2010). On the other hand, among Korea adults with overweight and obesity, 56 g almonds increased the levels of vitamin E, reduced the levels of inflammatory markers, IL-6, and IL-1 beta but did not decrease protein carbonyls (implicated in lipid peroxidation) and MDA (Jung et al., 2018). These findings show that almonds may increase the levels of antioxidant enzymes while decreasing the levels of pro-inflammatory cytokines such as IL-6 majorly implicated in AI.

Aging-Related Cognitive Decline

Inflammation is a cause of anemia in hospitalized patients, individuals with critical illnesses and the elderly. It is commonly seen in diseases associated with persistent immune activation, autoimmune diseases, and cancer (Weiss et al., 2019). Anemia of chronic disease (ACD) or anemia of inflammation (AI) is a multifactorial malignancy from elevated concentrations of pro-inflammatory cytokines and hepcidin, iron retention and impaired erythropoiesis (Cheng et al., 2013; Weiss et al., 2019).

It is not fully known whether AI is solely a marker seen with the progression and increased severity of chronic diseases or a cause of disease outcomes (Weiss et al., 2019). This is because, red blood cells are susceptible to immune system damage accelerating its destruction which may trigger a pro-inflammatory response. The increased levels of TNF-alpha, IL-1B, IFN-gamma leads to erythrophagocytosis, and impaired erythroid progenitor regeneration. This response may lead to AI and is characterized by a non-regenerative and mild to moderate decrease in the number of red blood cells (RBCs). The consequent reduction in iron limits oxidation and free radical formation, and bacterial growth (Germolec et al., 2018). Some

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COVID-19

Viral infections, due to the high-grade fever and the inflammatory response, result in reduced availability of nutrients and increased metabolic state compromising nutritional status (Morais et al., 2020; Thurnham, 2015). Severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) originated in the city of Wuhan, Hubei Province, China (Sanche et al., 2020). Named on January 7, 2020, the SARS-COV-2 is one of the viruses attributed to the common cold and is genetically similar to the SARS-COV-1 (*Novel Coronavirus (2019-NCoV), Situation Report 1*, 2020). The virus multiplies within the host and its mutation increases its ability to cross-species increasing its infectivity. It is a single stranded RNA, 50-200 nm in diameter belonging to the coronavirus family. SARS-COV-2 is a spherical enveloped virus consisting of accessory and structural proteins (S-spike, M-membrane, E-envelope, N-nucleocapsid). Accessory proteins attack the host defense, non-structural proteins are responsible for vesicle formation while structural proteins are responsible for the morphology, and fusion into the host cell membrane (Durmaz et al., 2020). The S-protein is composed of a single mutation which increases its binding affinity and infectivity in the host. The transmembrane protease serine 2 (TMPSS2) cleaves the spike protein allowing for fusion of the viral envelope with the membrane of the host (Perico et al., 2021).

The SARS-COV-2 infection begins when the S protein, a 3-heterodimer spike glycoprotein binds to the angiotensin converting enzyme 2 (ACE2) receptor in the host. The S-

protein is responsible for binding affinity and efficiency of replication of the virus (Y. Huang et al., 2020). ACE2 is highly expressed in the heart and the pneumocytes of the lungs hence the invasion of the virus is responsible for the respiratory and CVD symptoms seen (Chambers et al., 2020). Binding of SARS-COV-2 to the ACE 2 receptor aggregates toll-like receptors and pattern recognition molecules resulting in a chemokine surge/storm with aggregation of neutrophils and inflammatory cytokines. The cytokine storm (hypercytokinemia) associated with SARS COV-2 may alter and prime the microglia upregulating the inflammatory state (J. Shen et al., 2021). The associated destruction of the alveolar capillary wall also leads to fluid accumulation within the alveolar sacs (Xiu et al., 2020).

COVID-19 Symptomology

Human to human transmission of COVID-19 occurs via aerosols and respiratory droplets produced during coughing or sneezing (C. Huang et al., 2020). ACE2 receptors and TMRPSS2 are highly expressed in the GI, nasal mucosa, tongue, and mouth (Sungnak et al., 2020). As a result, in some cases, diarrhea and vomiting have been observed after 33 days with COVID-19. Other GI symptoms include nausea and anorexia which may exist without associated respiratory symptoms (Galanopoulos et al., 2020). For 80 % of infected cases, COVID-19 is a mild condition characterized by dry cough, fatigue and fever that clears in 6-10 days and is restricted to the upper gastrointestinal tract (Ish et al., 2020; F. Li et al., 2021).

In the 20 % of severe cases, symptoms are exacerbated due to caspase-8 induced apoptosis which occurs mainly in lung epithelial cells (S. Li et al., 2020). Additionally, due to increased expression of ACE 2 receptors in the lungs, this is followed by an exaggerated pro-inflammatory response in the lungs seen with bilateral pneumonia (Ackermann et al., 2020). This can progress to acute respiratory distress which is characterized by a hyper-inflammatory state

and organ damage (Xu et al., 2020). Because severe cases of COVID-19 are also systemic (Bae & Kim, 2020), the virus may spread to the heart, eye, and kidney and can be observed with anosmia, ageusia, diarrhea, cardiac injury, endothelial damage, and coagulopathies (Patel et al., 2020; Perico et al., 2021). Anosmia and/or ageusia recently included in the list of COVID-19 symptoms by the Centers for Disease Control and Prevention (CDC), may be present in patients with or without fever or other accompanying symptoms (CDC, 2020b). Hypoxic injury in the central nervous system and basal forebrain neurons that project to the olfactory bulb drives the pro-inflammatory state of the microglia which results in the anosmia and/or ageusia (J. Shen et al., 2021). Hyper-inflammation during the cytokine storm with viral replication may also lead to severe complications such as acute respiratory distress syndrome, septic shock, and multiple organ dysfunction syndrome. In a meta-analysis, there was a 3 % prevalence of stroke in severe cases of COVID-19 compared to a 1 % prevalence in the non-severe group. Mechanisms described include the upregulation of vascular cell adhesion molecule 1 (VCAM-1) resulting in a proinflammatory process, hyper-coagulable state and increased thrombus formation (J. Shen et al., 2021).

COVID-19 and Nutrient Status

Efforts remain to address the underlying hyper-inflammation in COVID-19. There is growing evidence regarding the effect of bioactive food components on the immune system and reducing viral load. For example, imbalances in the intake ratios of omega 3 & 6, amount and type of fat may increase viral replication in human studies (Iddir et al., 2020). Quercetin may inhibit 3C-like protease that functions in the cell replication of glycoproteins responsible for viral replication of the SARS-COV-2 virus (Colunga Biancatelli et al., 2020).

With a known link between anti-inflammatory diets and viral infections, there is a need to focus on antioxidant nutrients. Hence, additional considerations for improved nutritional status from adequacy of minerals (Se, iron, Zn), polyphenols, vitamins B_6, and B_{12}, vitamins A, and C are needed. It is important to maintain these levels within recommendations due to the possibility of an exacerbated anti-inflammatory response blunting the immune response (Iddir et al., 2020). This is also the case with Vitamin D, which may be protective against acute respiratory disease particularly among Vitamin D inadequate (<75 nmol/L) individuals. Vitamin D may also enhance the activity of cathelicidins and defensins against viral replication among these individuals (Grant et al., 2020). 25-hydroxy vitamin D_3 ($25(OH)D_{3)}$ may modulate the innate immune system and increase the chemotactic and phagocytic function of immune cells (Sassi et al., 2018). A low mean concentration of Vitamin D was associated with high cases of mortality from COVID-19 while Li et al. (Y. Li et al., 2021) showed that low levels of Vitamin D were not associated with SARS-COV-2 seropositivity. Vitamin A may also be protective against a destruction in the mucosal barrier caused by COVID-19 (Ali, 2020). Selenium deficiency may increase viral replication and decrease T-lymphocyte replication (Bae & Kim, 2020). A review by Bermano et al. showed that suboptimal Se status may be associated with increased RNA viral replication for respiratory viruses and may impair the immune response to the SARS-COV-2 virus (Bermano et al., 2021).

Homeostatic control of Zn is responsible for modulating the expression of IFN-gamma and the cytotoxicity of invading pathogens. Zinc deficiency is associated with reduced non-specific immunity and decreased antibody production with supplementation associated with a decrease in TNF-alpha and markers of oxidative stress (Gammoh & Rink, 2017). Hence, Zn supplementation in Zn deficient individuals may be beneficial in viral infections (Wessels et al.,

2017). However, among newly diagnosed COVID-19 patients, there was no significant difference in the number of days until 50% reduction in symptoms in those receiving standard care, 50mg Zn gluconate/day, 8000mg ascorbic acid/day or both Zn and ascorbic acid therapies (Thomas et al., 2021).

CHAPTER III: PHYTATE INTAKE IS ASSOCIATED WITH COGNITION IN OLDER ADULTS, NHANES 2013-2014

This chapter is a modified version of a paper, "*Estimated Phytate Intake is Associated with Improved Cognitive Function in the Elderly, NHANES 2013 – 2014"* submitted to *Antioxidants.*

Abstract

Phytate, an antioxidant, may improve cognition by inhibiting iron catalyzed hydroxyl radical formation. Particularly in the elderly, this provides a potential dietary approach for mitigating age-related brain neuronal dysfunction and loss. In this study, we investigated the relationship between phytate intake and cognitive function in the elderly. We used data from the 2013-2014 National Health and Nutrition Examination Survey (NHANES) and the corresponding Food Patterns Equivalents Database (FPED). Phytate content of food groups from published data were merged with the appropriate FPED data to estimate the total phytate intake for each subject. Principal component analysis was used to develop a composite score from four cognitive function scores in NHANES data, and regression analysis was used to determine the relationship between this score and phytate intake. Median phytate intake was 0.65 (0.61, 0.71) g/day. It was low among females, non-Hispanic blacks, and people with a history of at least one chronic disease ($P < 0.05$). In regression analysis adjusted for confounders, phytate intake was positively associated with cognitive function [β (95% CI) = 1.90 (0.73-3.07); $P = 0.015$]. These results suggest that phytate may be associated with improved cognition, hence the need to consider including phytate-rich foods in the diet among the elderly.

Introduction

Cognition encompasses a spectrum of higher order cerebral function from normal to subjective complaints to evidence of decline in cerebral function to dementia (Cohen et al., 2016). Older age is a known risk factor for dementia which affects approximately 10 million people worldwide yearly with 150 million people estimated to be living with the condition by 2050 (Prince et al., 2013; WHO, 2017). Aging and age-related disorders accelerate brain neuronal dysfunction and loss resulting in decline in processing speed, attention and executive function (Cohen et al., 2016; Murman, 2015).

Cognitive decline in aging is multifaceted (Murman, 2015) and is attributed to factors such as impaired calcium homeostasis, mitochondrial dysfunction, oxidative damage and inflammation and increased susceptibility to stress leading to epigenetic modifications that affect learning, memory and synaptic processes in the brain (Harman & Martín, 2020). Inadequate intake of certain food groups including fruits, vegetables, cereals and grains, as well as nutrients such as zinc, selenium, copper, fiber and some vitamins have also been linked with cognitive decline (Bruins et al., 2019; S. Li et al., 2019). In addition to nutrients and food groups, phytochemicals such as phytates and some polyphenols are potential agents for improving cognitive health in aging due to their antioxidant and anti-inflammatory properties (8).

Phytate (myo-inositol hexakisphosphate), a salt of phytic acid with 6 phosphate groups and an inositol ring is a reservoir for phosphorus needed for germination of plants and seeds (Raboy, 2003). It is known that the ability of phytate to chelate iron, zinc, copper and magnesium may decrease the bioavailability and absorption of these minerals from the diet (Fardet, 2010). However, the body of research suggests that this chelation property is dependent on the ratio of phytate to metal and hence consuming a healthy and balanced diet with adequate proportions of

these trace metals can minimize this chelation property and not result in mineral deficiency (Graf et al., 1987; Reddy & Sathe, 2001). As an antioxidant, phytic acid accelerates the oxidation of Fe2+ to Fe3+, thus minimizing availability of Fe2+ for iron catalyzed hydroxyl radical formation via the Fenton reaction (Anekonda et al., 2011a; Graf et al., 1987; Silva & Bracarense, 2016). In this way, phytate may prevent lipid peroxidation and hence acts to mitigate inflammation and neurodegenerative diseases (Anekonda et al., 2011a; Fulcher et al., 1981; O'Dell et al., 1972; Reddy & Sathe, 2001). In mice models of Alzheimer's disease - the commonest age-related neurodegenerative disease - a 2% phytic diet, resulted in upregulation of cyclooxygenase (ensures mitochondrial integrity) and down regulation of malondialdehyde (MDA) in treatment group compared to the wild type controls. MDA is a by-product of oxidative stress, lipid peroxidation and cell membrane damage (Talarowska et al., 2012). A body of evidence shows that increased MDA may be observed in brain regions of Alzheimer's disease patients and in aged rats showing cognitive deficits (González-Fraguela et al., 2018; Zabel et al., 2018). In MC65 human neuroblastoma cells, 100μM phytic acid in the presence of tetracycline resulted in reduced concentrations of hydrogen peroxide and increased concentrations of proteins responsible for autophagy of the neuroblastoma cells (beclin-1 and SIRT1) (Anekonda et al., 2011a). These findings are supported by results from a Parkinson's disease rat cell line where phytic acid also decreased apoptosis and DNA fragmentation compared to non-treated cells in both normal and iron excess conditions (Q. Xu et al., 2011).

Despite the protective effect of phytic acid seen in rodent and cell studies, no human study has investigated the relationship between phytate consumption and cognitive function, especially in the elderly who are vulnerable to age-related cognitive decline. The aim of this study was to investigate this relationship using data from the NHANES 2013-2014 cycle. We

hypothesized that phytate intake will be associated with improved cognitive function among the elderly.

Materials and Methods

Data Source and Study Population

Data for older adults 60 years or more from the 2013-2014 National Health and Nutrition Examination Survey (NHANES) and the corresponding Food Pyramid Equivalents Database (FPED version 2013-2014) were used for this study. NHANES is a complex multi-stage survey collected in 2-year cycles to assess the health and nutritional status of non-institutionalized children and adults in the US (CDC, 2013). Data collected includes interviews for demographic and dietary data, laboratory tests and physical examination. FPED is used to evaluate whether the food and beverage intake of Americans meets the recommendations of the dietary guidelines for Americans. It converts food and beverages from What We Eat in America (WWEIA), NHANES into the 37 food pattern components. Since phytate intake data is not available from the NHANES survey data, the estimated phytate content of different food groups (dark green vegetables, potatoes, other starchy vegetables, legumes, whole grains, refined grains, soy products, and nuts and seeds) were obtained from a publication by the International Zinc Nutrition Consultative Group (IZiNCG) (International Zinc Nutrition Consultative Group (IZiNCG) et al., 2004). This data was merged with the appropriate FPED data for the 2013/2014 survey cycle of WWEIA to estimate phytate intake of NHANES participants (Armah, 2019; Larvie & Armah, 2021). Informed consent was obtained from all individual participants and all procedures performed involving human subjects in NHANES were in accordance with the Declaration of Helsinki (IRB number 2011-17).

Cognitive Assessments

Cognitive functioning is measured periodically in the NHANES survey as part of the household survey or in the Mobile Examination Center among individuals aged 60 years or more. Assessments include; 1) word learning and recall modules based on the Consortium to Establish Registry for Alzheimer's disease (CERAD), 2) Animal fluency and 3) Digit Symbol Substitution Test. The CERAD Word List Learning and Recall tests are used to ascertain immediate and delayed learning of new verbal information. In the CERAD Word List Learning, participants are required to read aloud 10 words in three trials with the order of the words in each trial altered. In both the word learning and recall modules, the highest points equal to 10. The CERAD Recall testing occurs after the Animal fluency and Digit Symbol Substitution Tests. The Animal Fluency is used to measure categorical verbal fluency; participants are asked to name animals in a 1 min duration; where scores obtained equal the number of animals named correctly. The Digit Symbol Substitution (DSST) test is obtained from the Weschler Intelligence Scale and is used to assess processing speed, working memory and sustained attention. This is a paper-based test with a key at the top containing nine numbers and their corresponding symbols. Participants have 2 minutes to draw these corresponding symbols into 133 boxes containing their respective numbers. The scores represent the number of symbols correctly drawn. In all cognitive function tests, higher scores indicate higher cognitive function (CDC, 2013; H. Li et al., 2018).

Covariates

A variety of covariates known to be related with cognitive function and phytate intake were included in analyses. These were race/ethnicity (Mexican-American, non-Hispanic White, non-Hispanic Black and non-Hispanic Asian), tobacco smoking, alcohol consumption, poverty to

income ratio, marital status, education, and medical condition history (H. Li et al., 2018). Smoking was categorized as follows: 1) Individuals who reported smoking at least 100 cigarettes in lifetime and smoke every day or some days were classified as current smokers; 2) those who have smoked at least 100 cigarettes in lifetime and now do not smoke were classified as former smokers; 3) those that have not smoked 100 cigarettes in their lifetime were classified as never smokers. Alcohol consumption was classified as: 1) Moderate drinkers (<8 drinks/week); 2) heavy drinkers (8 drinks or more/week). Ratio of family income to poverty guidelines was classified as: 1) Low family income to poverty ratio (≤0.99); 2) High family income to poverty ratio (≥1.00). Marital status was classified as: 1) Married/living with partner; 2) widowed/divorced/separated; 3) never married. Educational status was classified as: 1) Less than high school; 2) high school; 3) college. Subjects were considered to have a medical condition history if they reported at least one of these conditions, stroke, diabetes, coronary heart disease, coronary heart failure, heart attack, hypertension. Otherwise, they were considered to have no medical condition history. Age group was classified as: 1) Old adults from 60 to 70 years; 2) older adults from 71 to 80 years.

Statistical Analysis

Data were analyzed using R Studio version 1.2.5001. Analysis was performed using the "survey" package accounting for sampling weight, strata, and primary sampling unit in the survey design. The four cognitive scores (CERAD (Consortium to Establish Registry for Alzheimer's disease) word learning and recall scores, animal fluency and Digit Symbol Substitution Test (DSST)) were used in principal component analysis for data reduction. Descriptive statistics used for the study population were median (95% confidence intervals) and percentages. Statistical significance was set at $p \leq 0.05$. Mood's median test was used to compare

medians (95% CI) between groups and Benjamini Hochberg correction was used to adjust for false discovery rate. Stepwise multiple linear regression with backward elimination was used to determine the association between cognitive function and phytate intake. Principal component analysis was used on cognitive function test scores to generate the different principal components. We used the first principal component scores (containing largest variance) and DSST. This is because the DSST score is a good measure for evaluating frontal lobe-related functions including visuospatial skills, sustained attention, and motor speed-of-processing (Rosano et al., 2005; Tsai et al., 2016). In both models the following covariates were adjusted for: age, sex, daily fiber intake, educational status, medical condition history, and poverty to income ratio. Since phytate and fiber are mostly from the same foods and some studies have reported an association between fiber intake and cognitive function, we controlled for fiber as a potential confounder in all regression analysis.

Results

In Table 1, data for phytate intake was available for 1567 study participants. While Non-Hispanic Whites (77%) formed a majority of the sample population, Non-Hispanic Asians (4%) and Mexican American (4%) were the fewest ethnic groups. Half (50%) of the study participants had never smoked, and a majority (70%) had a history of at least one medical condition.

Table 1. Background Characteristics of Older Adults 60 Years and Above, NHANES 2013 – 2014

Demographic characteristics	N (Unadjusted)	Percentage (Adjusted)[1]
Total	1841	100
Sex		
Male	967	54
Female	874	46

Ethnicity[2]		
Non-Hispanic White	896	77
Non-Hispanic Black	388	9
Non-Hispanic Asian	168	4
Mexican American	210	4
Smoking status		
Never	917	50
Current user	232	10
Former user	690	40
Medical history[3]		
None	507	30
At least one	1334	70

[1]Adjusted for sampling weight, strata, and primary sampling unit; [2] estimates of "other" race not reported but included in analysis; [3]medical history includes stroke, coronary heart disease, coronary heart failure, diabetes, heart attack and hypertension.

In Table 2, refined grains contributed over half of the daily phytate intake. This was followed by whole grains (21%) while potatoes, legumes, soy, other starchy vegetables, and dark green leafy vegetables made up less than 5% of the daily phytate intake from the NHANES 2013 – 2014 cycle.

Table 2. Percentage Contribution of Different Food Groups to Daily Phytate Intake of Adults 60 Years and Above, NHANES 2013 – 2014

Food Group	% Adjusted Contribution
Refined grains	52
Whole grains	21
Nuts and seeds	12
Potatoes	4
Other vegetables	4
Legumes	3

Soy	1
Other starchy vegetables	1
Dark green leafy vegetables	1

From Table 3, the daily median phytate intake was significantly higher for males [0.74 (0.68, 0.79)] g/day than females [0.6 (0.54, 0.65)] g/day, p = 0.0002. Similarly, fiber intake was higher in males (17 (15.8, 18.3) g/day) compared to females (13.7 (13, 14.5)) g/day, p<0.0001. Non-Hispanic Whites also consumed significantly more phytate [0.67 (0.62, 0.73) g/day] than their Non-Hispanic Black counterparts [0.5 (0.45, 0.59) g/day]. Phytate intake was significantly higher among those who had never smoked [0.67 (0.61, 0.75) g/day] compared to current smokers [0.5 (0.45, 0.6) g/day]. DSST scores for females [54 (53, 56)] were significantly higher than for males [51 (49, 53)]; p = 0.0001. Compared to those with no medical condition history, DSST scores for study participants with at least one medical condition history were significantly lower [57 (54, 59)] versus 51 (49, 53); p = 0.0003. The trend in DSST scores for medical condition history were confirmed with the first principal component scores as those with at least one medical condition had lower scores (1.18 (0.89, 1.41)) compared to those without a medical condition history (1.7 (1.38, 1.99)), p = 0.0004. Also, individuals who had never smoked also had significantly higher DSST scores compared to current smokers.

Table 3. Phytate Intake, Fiber Intake, Cognitive Function, and Principal Component Scores (from Cognitive Function) of Adults, 60 Years and Above, NHANES 2013 – 2014[1]

Demographic data	Phytate intake (g/day)	Fiber intake (g/day)	Digit symbol substitution score	Animal fluency score	CERAD recall score	CERAD learning score	Principal component score
Total	0.65 (0.61, 0.71)	15 (14.4, 15.8)	53 (51, 54)	18 (17, 18)	7 (6,7)	7 (6.7, 7)	1.35 (1.07, 1.66)
Sex							
Women	0.6 (0.54, 0.65) [a]	13.7 (13, 14.5) [a]	54 (53, 56) [a]	18 (17, 18) [a]	7 (7, 7) [a]	7.3 (7, 7.3) [a]	1.35 (1.07, 1.62) [a]
Men	0.74 (0.68, 0.79) [b]	17 (15.8, 18.3) [b]	51 (49, 53) [b]	18 (17, 19) [a]	6 (6, 7) [b]	6.7 (6.3,7) [b]	1.33 (0.97, 1.7) [a]
Ethnicity[2]							
Non-Hispanic White	0.67 (0.62, 0.73) [a]	15 (14.4, 16) [a]	54 (53, 56) [a]	18 (18, 19) [a,b]	7 (7, 7) [b]	7 (7, 7.3) [b]	1.85 (1.68, 2) [a]
Non-Hispanic Black	0.5 (0.45, 0.59) [b]	13 (12, 14.1) [b]	40 (36, 44) [b]	14 (13, 15) [b]	6 (6, 7) [a]	6.7 (6.3, 7) [a]	-1.2 (-1.62, -0.87) [b]
Non-Hispanic Asian	0.8 (0.67, 0.91) [a]	17.2 (15.2, 18.8) [a]	53 (52, 55) [a,b]	14 (13, 15) [b]	7 (7, 8) [b]	7 (6.7, 7) [b]	-0.31 (-0.64, -0.11) [a,b]
Mexican American	0.69 (0.59, 0.84) [a]	17.6 (14.5, 20.4) [a,b]	41 (37, 45) [b]	17 (16, 17) [a]	6 (5, 7) [a]	6.3 (5.7, 6.7) [a,b]	-1.5 (-1.96, -1.18) [b]
Smoking status							
Never	0.67 (0.61, 0.75) [a]	15.6 (14.7, 16.1) [a]	54 (52, 56) [a]	18 (17, 19) [a]	7 (7, 7) [a]	7 (6.7, 7.3) [a]	1.33 (1.03, 1.68) [a]
Current user	0.5 (0.45, 0.6) [b]	10.8 (9.4, 12.3) [b]	49 (44, 53) [b]	16 (15, 18) [b]	7 (6, 7) [b]	7 (6.7, 7) [b]	0.79 (0.25, 1.53) [b]
Former user	0.66 (0.61, 0.73) [a]	15.6 (14.7, 16.7) [a]	51 (48, 54) [a]	18 (17, 19) [a]	7 (6, 7) [b]	7 (6.7, 7) [b]	1.48 (1.07, 1.81) [a]
Medical history[3]							
None	0.75 (0.71, 0.8) [a]	16.3 (15, 17.4) [a]	57 (54, 59) [a]	19 (18, 20) [a]	7 (7, 7) [a]	7.3 (7, 7.3) [a]	1.7 (1.38, 1.99) [a]
At least one	0.62 (0.58, 0.65) [b]	14.4 (13.3, 15.2) [b]	51 (49, 53) [b]	17 (17, 18) [b]	7 (6, 7) [b]	6.7 (6.7, 7) [b]	1.18 (0.89, 1.41) [b]

Note. Values with dissimilar superscripts differ significantly ($p \leq 0.05$, Benjamini Hochberg correction for multiple comparisons)
[1]Values are median (95% CI), [2] estimates of "other" race not reported but included in analysis; [3]medical history includes stroke, coronary heart disease, coronary heart failure, diabetes, heart attack and hypertension.

In Table 4, when regression analysis was used to determine the association between phytate intake and cognitive function adjusting for potential confounders, phytate intake showed a significant positive association with the first principal component score [1.9 (0.73, 3.07); P = 0.015] and DSST [0.23(0.13, 0.33); P= 0.003]. This finding was independent of sex, age group, fiber intake, medical condition history, income to poverty ratio and education status.

Table 4. Association between Phytate Intake and Cognitive Function (Digit Symbol Substitution Test and First Principal Component Score) among Adults, 60 Years and Above[1]

Demographic/Nutrient intake	**Digit Symbol Substitution Score (n=1353)**			**Principal Component Score (n=1340)**		
Predictors	*β*	*CI*	*p*	*β*	*CI*	*p*
(Intercept)	35.33	27.14, 43.52	**<0.001**	-1.12	-1.66, -0.58	**0.005**
Phytate intake (g/day) [2]	1.90	0.73, 3.07	**0.015**	0.23	0.13, 0.33	**0.003**
Sex						
Male	RG			RG		
Female	5.16	3.48, 6.83	**0.001**	0.26	0.12, 0.40	**0.009**
Fiber intake (g/day) [2]	1.10	-0.51, 2.71	0.222	-0.04	-0.21, 0.13	0.675
Age group						
Old adult (60-70 years)	RG			RG		
Older adult (71-80 years)	-9.83	-11.40, -8.26	**<0.001**	-0.77	-0.92, -0.62	**<0.001**
Medical condition history [3]						

None	RG			RG		
At least one	-2.74	-4.52, -0.96	**0.020**	-0.21	-0.35, -0.06	**0.025**
Income to poverty ratio						
≤ 0.99	RG			RG		
≥ 1.00	8.44	6.02, 10.87	**<0.001**	1.24	1.01, 1.48	**<0.001**
Educational status						
College educated	RG			RG		
High school	-8.02	-9.99, -6.05	**<0.001**	-0.63	-0.82, -0.45	**<0.001**
Less than high school	-22.08	-26.71, -17.45	**<0.001**	-1.67	-2.32, -1.03	**0.001**
R^2	**0.322**			**0.277**		

Note. RG, Reference group
[1] p-values are based on multiple regression analysis; [2] Values were log-transformed before analysis; [3] Medical history includes stroke, coronary heart disease, coronary heart failure, diabetes, heart attack and hypertension.

Discussion

In this study among adults 60 years and above, daily phytate intake was positively associated with cognitive function after controlling for potential confounders. Phytate is a naturally occurring phosphorus compound found in high fiber food sources with global estimates for daily intake ranging from 0.18 to 4.57g/day, depending on type of diet and preparation methods used (Buades Fuster et al., 2017; Reddy & Sathe, 2001; Schlemmer et al., 2009). For example, the median phytate intake for countries with Western diets such as the UK is 0.81 g/day and 0.75 g/day in the US while 2.2g is reported in countries with a pre-dominant plant-based diet as seen in Nigeria (Amirabdollahian & Ash, 2010; Harland & Peterson, 1978) and South Korea (1.68 g/day) with major contributions from rice (Kwun & Kwon, 2000). The median phytate

intake in this study was 0.65g/day similar to 0.6 g/day reported among adults 20 years and older (Armah, 2019).

Aging is associated with cognitive decline due to accelerated brain neuronal dysfunction and loss resulting in decline in processing speed, attention and executive function (Cohen et al., 2016; Murman, 2015). Among adults, neurodegenerative diseases such as Parkinson's Disease and Alzheimer's Disease results in motor function abnormalities, dementia, sleep disturbances, memory problems, and death (Obisesan & Gillum, 2009). Studies also show that age-related conditions such as stroke, diabetes, hypertension and tobacco smoking may be associated with cognitive impairment (Lo Coco et al., 2016) and as seen in our study, having at least one of these medical conditions was associated with cognitive decline measured by DSST scores. Studies show that low physical activity, a reduced involvement in activities of daily living, an increased risk of cardiovascular disease, high blood pressure and poor glycemic control among older adults may hasten cognitive decline (Landau et al., 2012; Rovner et al., 2016). Additionally, the contribution of some dietary patterns in slowing cognitive decline in the elderly have been studied. Among adults over 65 years with a previous history of stroke, being in the highest tertile of a Mediterranean and DASH diet score was associated with a slower rate of cognitive decline compared to those in the lowest tertile (Cherian et al., 2019). Phytochemicals such as phytates are potential agents for improving cognitive health in aging as suggested by the findings of our study due to their antioxidant and anti-inflammatory properties (S. Li et al., 2019).

Phytic acid is an antioxidant and may prevent iron-related free radical generation to protect against neurodegeneration hence mitigating neuronal damage and loss (Rahmati et al., 2015; Q. Xu et al., 2011). Brain tissue is particularly susceptible to oxidative stress due to the high levels of polyunsaturated fatty acids, low antioxidant concentrations (superoxide dismutase

and catalase lower than in liver tissue) and the high oxidative stress environment (Q. Xu et al., 2011). Grases et al. (Grases et al., 2001) have shown that phytic acid can cross the blood brain barrier by demonstrating a ten-fold increase in rat brain concentrations of phytic acid compared to other tissues after a 10g phytic acid purified diet.

While there is a dearth of data showing the association between phytate intake and cognitive function in the elderly, our study showed that phytate intake was positively associated with cognitive function among adults 60 years and older. This finding concurs with a study among infants six to sixty months old where the mean phytate intake from complementary foods was associated with higher scoring trajectories in cognitive function based on the Bayley-III scale and the Wechscler Preschool and Primary Scale of Intelligence (McCormick et al., 2019).

The role of phytate in cognition has also been reported in several animal studies. In female mouse models of Alzheimer's disease, phytic acid improved mitochondrial integrity while reducing the proteins responsible for autophagy hence protecting against aggregation of amyloid plaques and tangled bundles of fiber/tau tangles seen in Alzheimer's disease (Anekonda et al., 2011a). This mechanism was also observed in Parkinson's disease model of male Wistar rats fed 100mg/kg phytic acid. There was a reduction in apormorphine-induced rotations which are indicators of nigrostriatal dopamine deletion compared to Parkinson's disease induced models only (Rahmati et al., 2015). These findings are also supported in Parkinson's disease rat immortalized mesencephalic dopaminergic neuronal cell lines where treatment with phytic acid decreased apoptosis by reducing caspase activity and DNA fragmentation compared to cells treated with hydroxydopamine to induce Parkinson's disease in both normal and iron excess conditions (Q. Xu et al., 2011). On the other hand, our study, showed that females had higher

DSST scores - despite the lower phytate intake - than their male counterparts., suggesting that sex differences may be evident in the relationship between phytate and cognition.

To determine the food sources of phytate in the US diet, we estimated the percent contribution of the different food groups to daily phytate intake. Refined grains contributed the highest (52 %) to the daily phytate intake followed by whole grains (21 %) and nuts and seeds (12 %). Although refined grains contain the least amount of phytate/100 g (197 mg) among the three food groups, its repeated consumption in the US diet may be the reason it contributed over half of the daily phytate intake. Dark green leafy vegetables and other starchy vegetables each contributed the least (1%) to the daily phytate intake. This is not surprising given their low phytate content (42 mg/100 g).

The strengths of this study include the large sample size, the use of a majority of covariates known to influence cognitive function and data reduction of cognitive function scores using principal component analysis to ensure the variability of each of the cognitive function scores were well accounted for. However, the use of principal component scores also limits an explanation to the effect sizes observed. Additionally, although an improved cognition is ascribed to phytate intake in this study, other bioactive components beyond fiber such as polyphenols may be associated with improved cognition. Future studies using pure phytic acid are warranted to determine the independent role of phytate in cognitive function. Another limitation of our study is its cross-sectional nature which does not support causal inference.

In conclusion, we have demonstrated a positive association between phytate intake and cognitive function among adults 60 years or older in this study. Future longitudinal studies as well as mechanistic studies are recommended to further understand the relationship between phytate intake and cognition in the elderly.

CHAPTER IV: COVID-19 IS ASSOCIATED WITH POOR SELENIUM INTAKE AMONG YOUNG ADULTS IN NORTH CAROLINA

Abstract

The coronavirus disease 2019 (COVID-19) remains a global public health emergency, due to the ensuing economic burden and death. With robust research into vaccines, antibody treatments, and antiviral drugs for COVID-19, there is still a dearth of evidence on the role of an individual's nutritional status. This study aimed to investigate the association between selenium (Se) and zinc (Zn) status and COVID-19 severity in North Carolina among individuals diagnosed with COVID-19. Subjects (n= 106) were recruited remotely as part of the Nutrition and COVID-19 in North Carolina (NC-NC) study and filled out online screening questionnaires and dietary surveys and provided toenail samples to be analyzed for Zn and Se concentrations. To assess the severity of COVID-19, subjects were asked about the presence and duration of 10 commonly reported symptoms. These responses were used to calculate a COVID-19 Severity Index (CSI). The relationship between Zn and Se status (intake and toe-nail concentrations) and CSI was explored using regression analysis. Our results showed that, the median (25, 75th percentiles) dietary Se and Zn intake from selected food sources were 65.2 µg (43.2, 112.9) and 4.3 mg (1.8, 8) respectively. Headache, cough, loss of smell or taste, and fever were reported by at least half of participants. In stepwise regression analysis, among individuals with low Se and Zn intake (below the median), Se but not Zn intake was inversely associated with CSI (β (95 % CI) = -0.66 (-1.21, -0.11); p=0.02). Findings from this study support a potential benefit of dietary Se among individuals with low intake, to mitigate COVID-19 severity.

Introduction

The coronavirus disease 2019 caused by the SARS-CoV-2 (severe acute respiratory coronavirus 2) virus remains one of the largest public health emergencies globally, due to the ensuing economic burden and death since its discovery in 2019 (CDC, 2020a). As of August 2022, over 88,000 new cases have been recorded in the US, compared to approximately 22,0000 newly reported cases in North Carolina (CDC, 2020c).

The COVID-19 infection leads to a dysregulated innate immune response resulting in a hyperinflammatory state/cytokine storm (Gustine & Jones, 2021). For instance, higher plasma levels of pro-inflammatory cytokines were observed in ICU compared to non-ICU admitted COVID-19 patients (C. Huang et al., 2020). This inflammatory state is linked with multiorgan damage to kidney, lungs, brain, and skin seen with severe COVID-19 infection (CDC, 2020a). Infection with the SARS-CoV-2 virus can also present with mild symptoms of fever, diarrhea, changes in smell or taste, cough or shortness of breath (CDC, 2020a). In light of this, current therapies such as corticosteroids and IL-6 antagonists have focused on mitigating/modulating the underlying dysregulated immune response among individuals infected with COVID-19 (Gustine & Jones, 2021).

There is increasing evidence of a link between nutritional status and the risk and progression of COVID-19 via immune function (James et al., 2021). A poor eating pattern or unbalanced diet can lead to a chronic low-grade inflammatory state and antioxidants such as selenium and zinc may reduce the cytokine storm seen with COVID-19 infection (James et al., 2021; Morais et al., 2020). In support of these findings, a study by Razeghi Jahromi and colleagues (Razeghi Jahromi et al., 2021) showed that high Se and Zn concentration were associated with low serum CRP (C-reactive protein) levels among COVID-19 patients. Selenium

deficiency may increase the progression and severity of COVID-19 (Fakhrolmobasheri et al., 2021; Khatiwada & Subedi, 2021). However, the evidence to support the role Zn deficiency in COVID-19 is somewhat conflicting. For example, among individuals at different levels of COVID-19 severity (determined by respiratory distress, pneumonia, ventilator use or death), 67% of patients showed Se deficiency without Zn deficiency (Im et al., 2020). With robust research into therapeutics such as vaccines, antibody treatments, antiviral drugs (Majumder & Minko, 2021), there is still a dearth of evidence highlighting the implications for dietary intake and nutritional status in mitigating the pro-inflammatory and oxidative stress states in COVID-19. This study aimed to investigate the association between Se and Zn intake and status and COVID-19 severity among individuals diagnosed with COVID-19 in North Carolina.

Materials and Methods

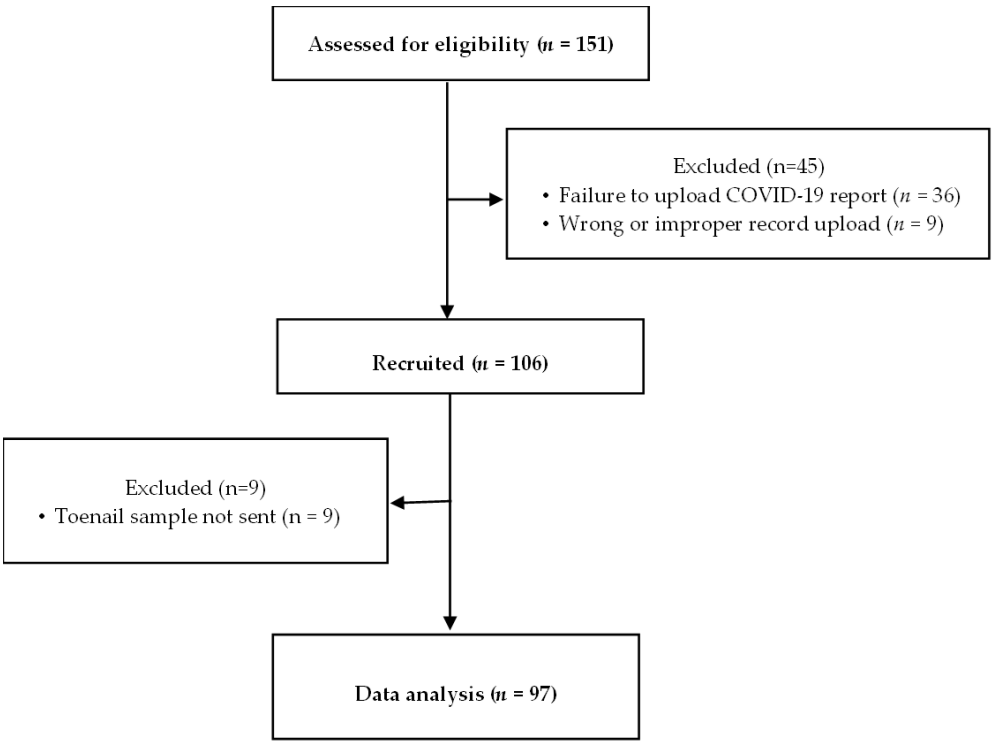
Study Participants and Recruitment

Subjects were recruited remotely via mass emails, social media, COVID-19 testing sites such as local pharmacies and directly through health professionals at Moses Cone Hospital as part of the Nutrition and COVID-19 in North Carolina (NC-NC) study. Potential subjects filled out an online screening questionnaire that included a copy of the informed consent form to read and electronically sign if they agreed to participate in the study. The informed consent contained information on the study, and benefits and risks of participating in the study. The screening form was an 86-item questionnaire to determine demographics, general health history and COVID-19 severity. Subjects were eligible for the study if they were 18 years and above, not pregnant or lactating, a resident of North Carolina, and previously diagnosed with COVID-19. Subjects who qualified based on the inclusion criteria were invited via email to participate in the study by uploading a copy of a positive diagnosis with COVID-19 followed by a brief online dietary

assessment. All surveys were developed using Research Electronic Data Capture (REDCap), an electronic data collection tool (Patridge & Bardyn, 2018).

It was estimated that 103 subjects will be needed to determine a significant association between COVID-19 severity and selenium and zinc intake and status in a regression model assuming an r-squared 0.5 for regression model with up to 7 predictors at an alpha level of 0.05 and with a statistical power of 0.8. To meet this sample size, we recruited a total of 106 subjects. Details on subject recruitment are shown in Figure 1. A total of 106 subjects were recruited while complete data from 97 subjects were analyzed. The protocol for this study was approved by the Institutional Review Board of University of North Carolina Greensboro (IRB # 21-0044) and Moses Cone Hospital, Greensboro, NC (IRB # 1654979-2).

Figure 1. Study Recruitment



Study Design

This study was a cross-sectional study conducted remotely to obtain information on COVID-19 severity and dietary intake data, and to collect toenail samples from subjects. There were five remote contacts with participants based on continued eligibility; 1) Invitation to participate in study and complete screening questionnaire, 2) Emailed to upload copy of COVID-19 diagnosis test result, 3) Emailed to complete dietary questionnaire, 4) Sent toenail collection kit and received toenail samples and, 5) Emailed electronic gift cards. Instructions for completing all the records were included in the online questionnaires.

Upon completing all questionnaires, participants were mailed a toenail collection kit containing a stainless-steel clipper, instruction sheet with toenail guide (to measure at least 50 mg sample), plastic specimen bags and a return envelope. Toenail samples were analyzed to determine zinc and Se concentration.

Assessment of COVID-19 Status

To assess the severity of COVID-19, in the screening questionnaire, subjects were asked about date of diagnosis, hospitalization, ventilator use if hospitalized, the presence and duration of 10 commonly reported COVID-19 symptoms (headache, fever, diarrhea, shortness of breath, taste and smell, vision, toe and finger problems, hemoptysis, body ache and cough) (C. Huang et al., 2020; Ish et al., 2020; Perico et al., 2021). Duration was categorized into Never, 1-3 days, 4-7 days, 8-13 days, and 14 days or more. All participants who had been diagnosed for less than two weeks before filling out the screening questionnaire were sent a link to a COVID-19 follow up form to provide information on any change in the symptoms reported in the initial screening questionnaire.

Zinc and Selenium Intake and Dietary Intake

To estimate Se and Zn intake prior to diagnosis with COVID-19, eligible subjects were required to fill an online semi quantitative food frequency questionnaire which obtained information on how often they consumed high Se and Zn source foods (≥ 20 % Daily Value) and the typical amount they consumed in the month prior to COVID-19 diagnosis. Se sources included seafoods, beef, turkey, egg, ham, halibut, and chicken while Zn sources included breakfast cereals, pumpkin seeds, pasta, bread, brown rice and oatmeal and grains and its sources and cheese.

Subjects were also asked to fill out a 26-item dietary screener questionnaire (DSQ) developed by the National Cancer Institute. The DSQ asks about the frequency of consumption of selected foods and drinks in the past month. The DSQ captures daily intake of fruits and vegetables, dairy, calcium, added sugars, whole grains, fiber, and frequency of consumption of red meat and processed meat. Each of the 26 items on the screener is based on its relationship to one or more dietary factors of interest in the dietary guidelines for Americans (National Cancer Institute, 2009).

Sample Analysis

Toenail samples from all toes were collected for the measurement of selenium and zinc concentrations. The samples (approximately equal parts of big and other toes) were weighed and washed twice each with acetone and rinsed with deionized water to remove any remaining nail polish and unwanted particles. The samples were digested with 1 ml concentrated trace mineral grade nitric acid (65-70 % by weight) and 1.5 ml hydrogen peroxide (30 %) in a hot block at approximately 110 degrees Celsius until a clear solution was obtained. The samples were then diluted with deionized water to 10 ml. Next, 0.5 mL and 4 mL of the previous solutions were

further diluted to 10 mL with deionized water for Zn and Se analysis, respectively. The digested toenail samples were taken to the Chemistry department at Wake Forest University (department of Chemistry) for the measurement of selenium and zinc concentrations using the 8800 Tandem ICP-MS/MS from Agilent Technologies (Santa Clara, CA).

Statistical Analysis

The primary outcome variable, COVID-19 severity index (CSI) was calculated from the duration of the 10 reported symptoms. Mean scores of 2, 5.5, 11 and 21 were allocated for 1-3 days, 4-7 days, 8-13 days, and 14 days or more respectively for duration of each reported symptom. The CSI score for each subject was the sum of the mean scores for the different symptoms they reported. As a result, this score may range from 0 (no symptoms) to 210 (10 symptoms for 14 days or more). The daily intakes of Zn and Se from the semi-quantitative food frequency questionnaire were calculated from the product of the reported frequencies converted to frequency/day (Never/less than monthly = 0/day, 1-3x/month = 0.067/day, 1-3x/week = 0.286/day, 4-6x/week = 0.714/day, Daily = 1/day), amount of Se or Zn in a serving of the food from the USDA FoodData Central (USDA Food and Nutrient Database for Dietary Studies, 2010) and the individual servings consumed.

To minimize within-cluster variance, ward hierarchical clustering was used to group similar data points into two groups by separating individuals with similar observations from those that were dissimilar into different clusters. Clustering was performed on the entire dataset using the variables, CSI, daily intake of fruits and vegetables, whole grains, fiber, calcium, sugar sweetened beverages, added sugars and frequency of intake of processed meat and red meat per day. In sub-analysis, clustering was then performed solely among individuals with low Se and Zn

intake (≤ median) using food group data representative of whole grains, fruits, vegetables and sugar sweetened beverages as clustering variables.

The concentration (Se and Zn) of toenail samples were adjusted for average sample weights (before and after drying). Means and standard error were reported for continuous variables with normal distributions. Median and interquartile ranges were reported for Se and Zn intake. Percentages were reported for categorical variables. Stepwise linear regression analysis using the "MASS" package and the AIC method (provides a means for linear model selection) to yield a best fit model was used to determine the associations among CSI, dietary patterns obtained from cluster analysis, Se and Zn intake and toenail concentration, adjusting for the potential confounders, namely age, gender, and ethnicity. Statistical significance was set at $p \leq 0.05$. The R-software for statistical computing was used for data analysis (R Core Team, Vienna, Austria, 1993).

Results

Our results (Table 5) showed that, most (57%) of the participants were Non-Hispanic Whites followed by Non-Hispanic Blacks (21%). Additionally, most participants were females (71%) who had never smoked (76%). Individuals with normal weight were approximately half of the sample with 87% reporting no medical condition while slightly over half of the participants reported some college education without a degree.

Table 5. Demographic Characteristics of Participants Diagnosed with COVID-19[1]

Demographic Data	n (N=106)
Age (years) [2]	26.8±0.99
Age group (years)	
Young adults (<30)	83 (78)
Middle adults (29-49)	16 (15)

Older adults (>49)	7 (7)
BMI (kg/m^2)	
Underweight	4 (4)
Normal	49 (46)
Overweight	27 (25)
Obese	26 (25)
Gender	
Female	75 (71)
Male	31 (29)
Ethnicity	
Non-Hispanic Black	22 (21)
Non-Hispanic White	61 (57)
Hispanic	7 (7)
Others [3]	16 (15)
Chronic disease	
No	92 (87)
Yes	14 (13)
Smoking status	
Never	81 (76)
Former smoker	14 (13)
Current smoker	11 (11)
Educational status	
Less than or high school	16 (15)
Some college but no degree	53 (50)
Bachelor’s or graduate degree	37 (35)
Marital status	
Married	17 (16)
Single	85 (80)

Divorced or separated	4 (4)
Income	
$1-49,999	88 (83)
$50,000-99,999	13 (12)
$100,000 and above	5 (5)

[1] Values are frequencies (percentages); [2] Values are means ±SE; [3] Others include Multiracial, Asian, Middle Eastern

From Table 6, the mean CSI was 29.4 ± 2.3 while the median dietary Se and Zn intake from high food sources were 65.2 (43.2, 112.9) µg and 4.3 (1.8, 8) mg respectively. Participants consumed approximately 2 cups/day of total fruits and vegetables (2.3 ± 0.08 cups eq/day). Added sugars from sugar sweetened beverages (7.2 ± 0.7 tsp) was almost half of the total added sugar intake (16.2 ± 0.8 tsp) consumed per day.

Table 6. Biochemical Dietary Intake Data of Participants Diagnosed with COVID-19[1]

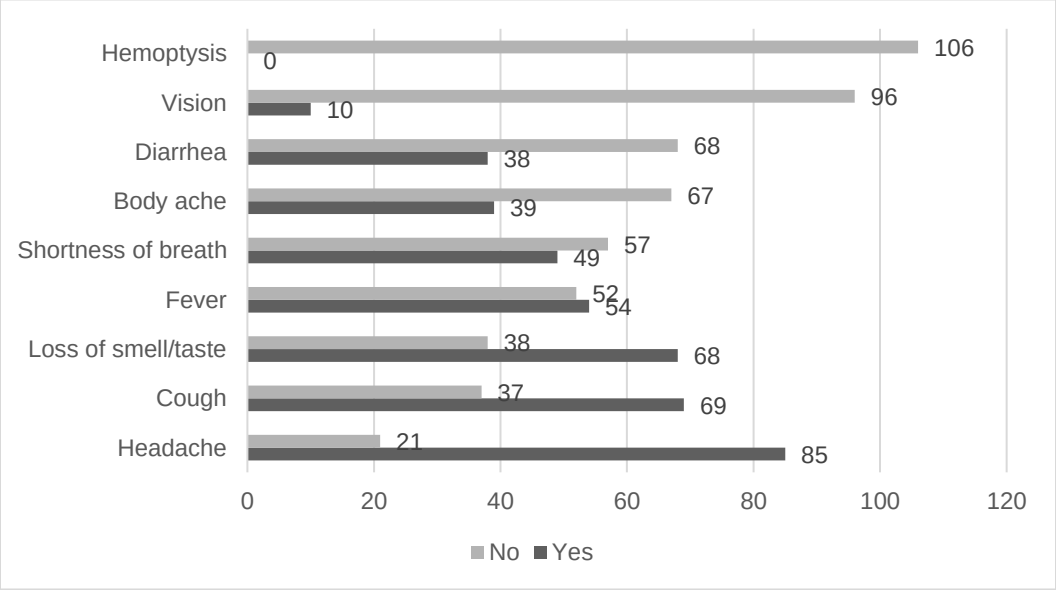
Biochemical and Dietary Intake Data	n (N=106)
CSI	29.4 ± 2.3
Selenium intake, µg [2]	65.2 (43.2, 112.9)
Zinc intake, mg [2]	4.3 (1.8, 8)
Calcium intake, mg	923 ± 20
Fiber intake, gm	16.4 ± 0.5
Whole grain, oz eq/day	0.94 ± 0.09
Dairy intake, cup eq/day	1.5 ± 0.04
Fruit intake, cup eq/day	0.95 ± 0.09
Fruit and vegetables, cup eq/day	2.3 ± 0.08
Vegetables, cup eq/day	1.4 ± 0.04
Added sugar, tsp eq/day	16.2 ± 0.8
Added sugars from sugar sweetened beverages, tsp eq/day	7.2 ± 0.7
Red meat, day	0.24 ± 0.02

Processed meat, day	0.19 ± 0.02
Toenail Se concentration, µg/g [2,3]	0.81 (0.73, 0.93)
Toenail Zn concentration, µg/g [2,3]	115.9 (100.2, 137.1)

[1]Values are means ± SE; [2] Values are median (25th, 75th percentile); [3] n = 97

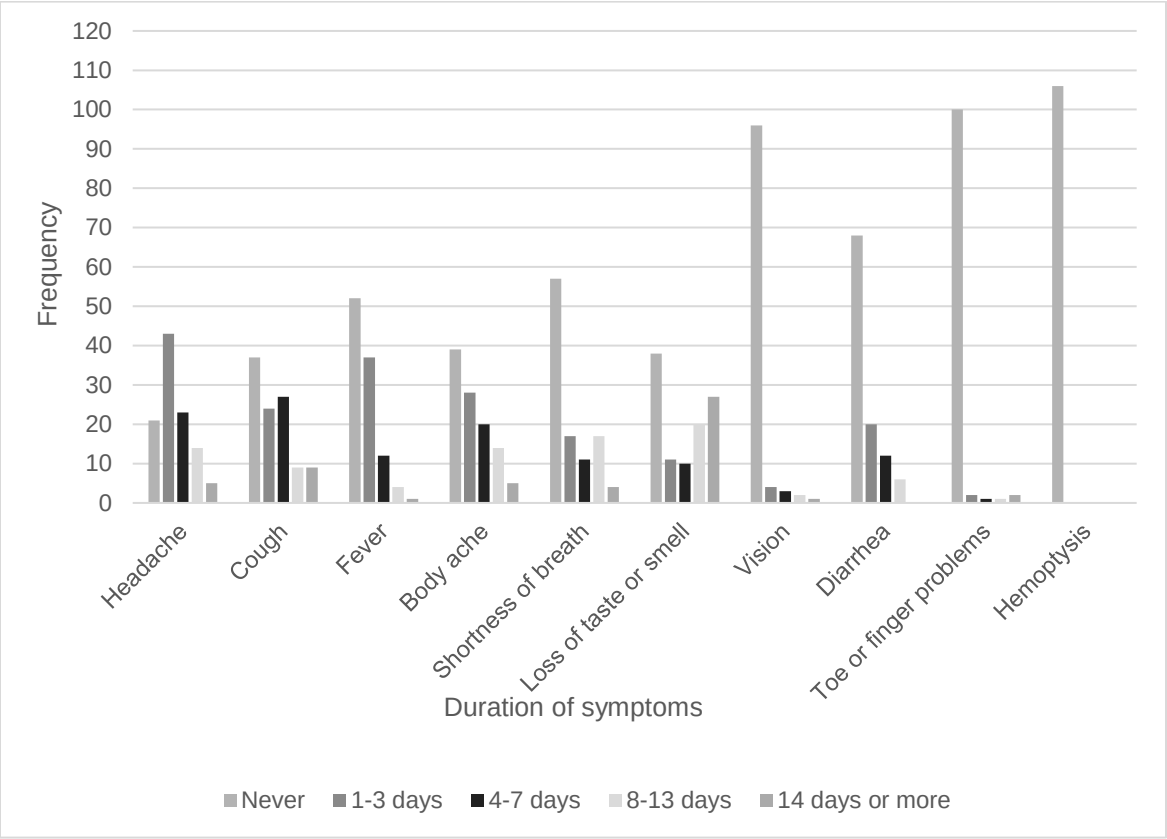
Four symptoms were reported by at least half of participants (Figure 2). These symptoms were headache, cough, loss of smell or taste, and fever. Most (80%) participants reported having a headache during diagnosis with COVID-19 while hemoptysis (coughing up blood) was not reported by any participant (Figure 2).

Figure 2. Number of Participants who Reported COVID-19 Symptoms



Most study participants reported that they had no symptom or symptoms lasted for 1-3 days (Figure 3). Among those who reported headaches, approximately, 43% reported symptoms lasted for 1-3 days. For symptoms that lasted the longest, among participants who reported a loss of smell or taste, 27% reported symptoms lasted for 14 days or more, followed by cough (9%), headache (5%), body ache (5%) and shortness of breath (4%).

Figure 3. Duration of Symptoms Reported by Participants



In Table 7, age, sex, and ethnicity were adjusted for in regression analysis predicting CSI. Age was positively associated with CSI while sex was inversely associated with CSI among males compared to females. In Model 1, when ethnicity and age were included as confounders for all participants, selenium status (intake and concentration) was not significant. This was similar to when a stepwise regression analysis was performed among all participants (Model 2). However, in Model 4, when a sub analysis was performed among individuals with selenium and zinc intake below the median, selenium intake was inversely correlated with CSI (β (95 % CI) = -0.66 (-1.21, -0.11); p=0.02).

Table 7. Association among CSI, Demographic Characteristics, Se and Zn Concentration and Intake

	Model 1 (n=97)			Model 2 (n=97)			Model 3 (n=34)			Model 4 (n=34)		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	14.64	-16.61 – 45.88	0.354	16.15	2.76 – 29.53	**0.019**	34.71	-43.14 – 112.55	0.367	59.14	34.55 – 83.73	**<0.001**
Age, years	0.61	0.13 – 1.09	**0.013**	0.61	0.16 – 1.06	**0.008**	0.69	-0.47 – 1.85	0.229			
Sex												
Female	RG			RG			RG			RG		
Male	-10.43	-21.42 – 0.55	0.062	-11.76	-22.00 – -1.53	**0.025**	-9.54	-31.33 – 12.26	0.376			
Ethnicity												
Non-Hispanic White	RG			RG			RG			RG		
Hispanic	16.09	-6.46 – 38.64	0.160				49.13	-5.88 – 104.15	0.078			
Non-Hispanic Black	0.20	-13.13 – 13.52	0.977				5.66	-27.06 – 38.37	0.724			
Others [1]	1.71	-12.51 – 15.94	0.811				2.31	-23.88 – 28.50	0.857			

Zn intake, mg	0.01	-0.53 – 0.55	0.974		1.13	-8.37 – 10.63	0.808			
Se intake, µg	-0.02	-0.07 – 0.03	0.448		-0.53	-1.31 – 0.26	0.182	-0.66	-1.21 – -0.11	**0.021**
Zn concentration, µg/g	0.03	-0.10 – 0.16	0.619		0.01	-0.46 – 0.48	0.955			
Se concentration, µg/g	-2.72	-28.63 – 23.20	0.836		-4.24	-79.76 – 71.29	0.909			
R^2		**0.145**		**0.116**		**0.327**			**0.156**	

Note. p-values are based on results from multiple regression analysis

In Figure 4, among the group classified as a healthy dietary pattern from the cluster analysis, participants had significantly higher Zn concentrations (128.3 vs 119.4, p=0.01) µg/g, and lower sugar sweetened beverages (5.9 vs 8, p=0.03) tsp eq/day compared to the unhealthy group. Dietary Se and Zn intake, fruit, and vegetables, added sugars, fiber intake and dairy intake were not significantly different between the two groups (p>0.05), Table 8.

Figure 4. Zinc Concentration between Healthy and Unhealthy Dietary Patterns (Groups) from Cluster Analysis

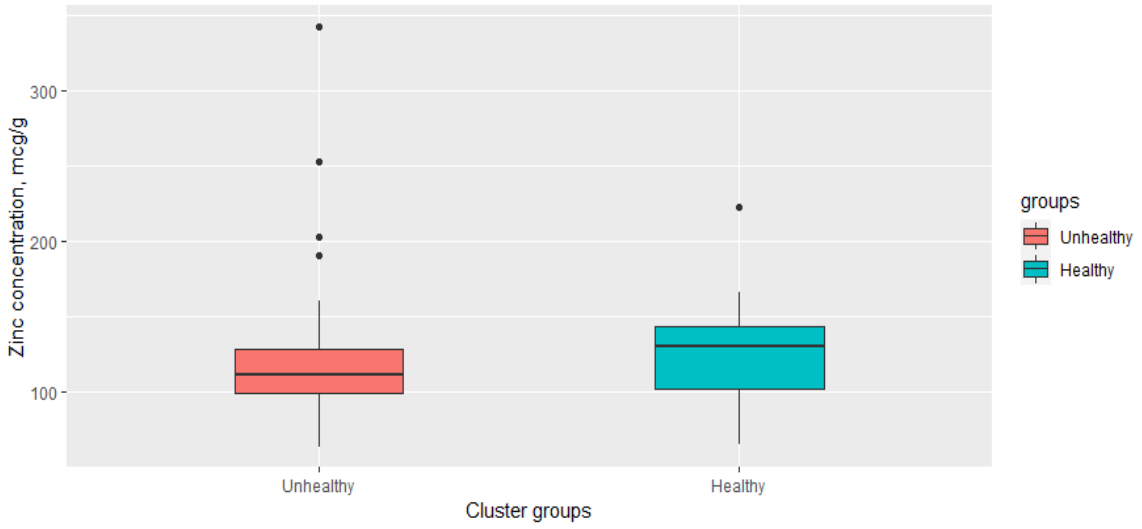


Table 8. Cluster Analysis comparing Dietary Intake and Toenail Concentrations of Se and Zn between Healthy and Unhealthy Dietary Patterns[1]

Dietary Intake and Toenail Concentrations	Group 1 (n= 68) Unhealthy pattern	Group 2 (n= 38) Healthy pattern	P-value
Covid Severity Index	28.3	31.4	0.27
Selenium intake, µg	117.8	72.9	0.54
Zinc intake, mg	8.1	5.3	0.74

Se concentration, µg/g	0.82	0.82	0.84
Zn concentration, µg/g	119.4	128.3	**0.01**
Added sugars, tsp eq/day	17.2	14.4	0.07
Dairy intake, cups eq/day	1.5	1.4	0.6
Whole grain, oz eq/day	0.9	1	0.7
Fiber, gm	16.7	15.9	0.44
Fruit intake, cup eq/day	1.1	0.8	0.07
Fruit and vegetables, cup eq/day	2.3	2.2	0.32
Vegetables, cup eq/day	0.4	0.4	0.77
Sugar sweetened beverages, tsp eq/day	8	5.9	**0.03**
Calcium, mg[1]	936	915	0.85
Red meat frequency, day	0.21	0.28	0.14
Processed meat frequency, day	0.17	0.2	0.37

[1]Values are based on mean CSI and dietary intake

In sub-analysis limited to individuals with low Se and Zn intake (≤ median), another cluster analysis (whole grains, fruits, and vegetables, added sugars as clustering variables) revealed that individuals in the healthy dietary pattern group consumed significantly higher calcium (934 vs 853) mg, Zn (2.4 vs 1.3) mg and Se (55.8 vs 28.8) µg as well as higher total added sugars (19.5 vs 13.3) tsp and added sugars from sugar sweetened beverages (9.9 vs 4.9) tsp daily compared to those with an unhealthy dietary pattern (Table 9).

Table 9. Cluster Analysis comparing Dietary Intake and Toenail Concentrations of Se and Zn between Healthy and Unhealthy Dietary Patterns among Individuals with Low Se and Zn (≤ median).

CSI and Food Group Intake	Group 1 (n= 22) Unhealthy dietary pattern	Group 2 (n= 14) Healthy dietary pattern	P-value
Covid Severity Index	35.6	21.3	0.18
Selenium intake, µg	28.8	55.8	**<0.001**
Zinc intake, mg	1.3	2.4	**0.007**
Se concentration, µg/g	0.8	0.9	**0.03**
Zn concentration, µg/g	116.3	117.8	0.88
Added sugars, tsp eq/day	13.3	19.5	**0.003**
Dairy intake, cups eq/day	1.3	1.5	**0.02**
Whole grain, oz eq/day	0.7	1.3	0.1
Fiber, gm	16	15.7	0.33
Fruit intake, cup eq/day	0.82	0.89	0.12
Fruit and vegetables, cup eq/day	2.3	2.3	0.4
Vegetables, cup eq/day	1.4	1.5	0.4
Added sugars from sugar sweetened beverages, tsp eq/day	4.9	9.9	0.03
Calcium, mg[1]	853	934	**0.04**
Red meat frequency, day	0.1	0.2	0.2
Processed meat frequency, day	0.1	0.2	0.21

[1]Values are based on mean CSI and dietary intake

In Table 10, age and gender were adjusted for in regression analysis predicting CSI. In Model 1, a healthy dietary pattern was not significantly correlated with CSI [(β (95 % CI) = -

12.91 27.92 – 2.09; p=0.09]. However, in Model 2, an inverse association which trended towards significance was observed in stepwise regression analysis between a healthy dietary pattern and CSI (using 6 symptoms commonly by participants) [(β (95 % CI) = -13.88 (-28.46 – 0.70); p=0.06)] among individuals with Se and Zn intake at or below the median.

Table 10. Association between CSI and Dietary Pattern among Individuals with Low Se and Zn Intake (≤ median)

	Model 1 (n=36)			**Model 2 (n=36)**		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	23.86	-2.95 – 50.67	0.079	31.71	22.30 – 41.13	**<0.001**
Dietary pattern						
Unhealthy dietary pattern	RG			RG		
Healthy dietary pattern	-12.91	-27.92 – 2.09	0.089	-13.88	-28.46 – 0.70	**0.061**
Gender						
Female	RG			RG		
Male	-5.77	-23.52 – 11.97	0.512			
Age	0.32	-0.58 – 1.23	0.472			
R^2	**0.124**			**0.099**		

Note. RG; Reference group, p-values are based on results from multiple regression analysis

Discussion

COVID-19 has resulted in over 6 million deaths globally as of September 2022 (WHO, 2020). Research shows that a cytokine storm plays a key role in COVID-19 pathology (C. Huang et al., 2020; Z. Xu et al., 2020). The cytokine storm results in oxidative stress and hyper inflammation leading to tissue fibrosis, pneumonia and lung injury (Karki & Bhandari, 2022). Consequently, COVID-19 can manifest a range of asymptomatic to fatal forms with variations in symptom duration (Sudre et al., 2021; Wiersinga et al., 2020). In our study, most participants were asymptomatic or reported mild symptoms (1-3 days) as shown by a mean CSI of 29.4 (range of 0-210). Participants commonly reported headaches (85%), followed by cough (69%), loss of smell or taste (68%), fever (54%), and shortness of breath (49%). Two other studies (Alimohamadi et al., 2020; Amin et al., 2021) reported fever followed by cough or fatigue as the most prevalent symptoms. Studies also show the evidence of other symptoms such as loss of taste, sore throat, and body ache (Amin et al., 2021; Subramanian et al., 2022). In our study, about one-third of individuals who reported a loss of smell or taste mentioned that symptoms continued for 14 days or more. This is similar to findings where anosmia (loss of smell), a prevalent symptom of long COVID may persist for 28 days (Sudre et al., 2021) or beyond 12 weeks (Subramanian et al., 2022).

Studies have proposed a link between nutritional deficiencies of Vitamin C, D, E, Zn, and Se to potentiate the severity of COVID-19 (Zabetakis et al., 2020). In our study, we found that among individuals with COVID-19 with low Se and Zn intake (below the median), Se intake was inversely associated with CSI. While this finding is supported by results from other studies (Kieliszek & Lipinski, 2020; Moghaddam et al., 2020; Razeghi Jahromi et al., 2021; Zhang, Taylor, et al., 2020) supporting a link between selenium deficiency and COVID-19 severity, a

majority of these studies sample older adults making our study of predominantly adults younger than 30 unique. It is established in research that low levels of serum Se may inhibit Se incorporation into selenoproteins, GPX and selenoprotein P (Moghaddam et al., 2020). Potential mechanisms through which Se via selenoproteins may act, may be through T-cell proliferation via the innate immune response, mitigating the pro-inflammatory response via IL-6, and increased DNA synthesis. Additionally, redox-active metabolites of dietary selenium beyond that needed for selenoprotein biosynthesis may also play a key role in minimizing COVID-19 severity (Zhang, Saad, et al., 2020). As has been reported by other studies, the association between Se and viral infections is observed among Se deficient individuals (Moghaddam et al., 2020; Zhang, Taylor, et al., 2020). This may be the reason we did not observe an association between selenium intake and CSI among all participants as the RDA of 55 µg (*Office of Dietary Supplements - Selenium*, n.d.) was exceeded in our study with a median Se intake of 65 µg. In a review, selenium intake at 55 µg/day and 105 µg/day corresponds to maximal GPX1 and Selenoprotein P activity respectively (Zhang, Saad, et al., 2020). Similar to our study findings, Zhang and colleagues (Zhang, Saad, et al., 2020) also showed that suboptimal intake below a mean Se intake of 65 µg/day may increase COVID-19 severity.

Nutrients such as Zn may also act against viral pathogenesis and a hyper-inflammatory state (Karki & Bhandari, 2022). Zinc functions as part of the innate immune system and in COVID-19, zinc stabilizes the host cell membrane preventing viral entry by inhibiting RNA synthesis and proteolytic processing (Kumar et al., 2020; Seyed Hosseini et al., 2020). Another supporting mechanism of action of Zn is that, ongoing inflammatory processes coupled with sub-optimal Zn intake, leads to a decrease in its redistribution and a consequent heightened susceptibility to infection (Wessels et al., 2020). However, the effect of zinc on infections or

diseases remain inconclusive (Ling & Zabetakis, 2021). Some studies show that Zn supplementation may be effective in decreasing the severity and progression of COVID-19 symptoms (Golabi et al., 2021; Jothimani et al., 2020). For instance, in a study by Gordon and Hardigan, oral zinc supplementation (10, 25 or 50 mg) resulted in 7 times decreased odds of developing symptomatic COVID-19 infection compared to the control group of COVID-19 symptomatic individuals receiving no supplementation. On the other hand, Sobczyk and Gaunt (Sobczyk & Gaunt, 2021) report contrasting findings where genetically predicted zinc concentration had no effect on SARS-CoV-2 infection, severity and hospitalization. In our study, among individuals with toenail Se and Zn concentration below the median, we observed an inverse association between Zn intake and CSI. However, this association only trended towards significance (p=0.06). The reason we may not have observed a significant inverse association between zinc intake and COVID-19 severity may have been because study participants were not zinc deficient. While the mean Zn intake (4.3 mg) in our study was well below the RDA for Zn, toenail Zn concentration was 116 µg/g which is similar to findings by Golabi and colleagues (Golabi et al., 2021) of plasma zinc concentration of 114 µg/dL among non-infected COVID-19 outpatients. It has been elucidated that SARS-COV-2 is able to utilize zinc for their own functions or to modify the function of ACE2 receptors, which may decrease the serum levels of zinc (Name et al., 2020).

Aside micronutrients, other anti-inflammatory dietary approaches have been elucidated in COVID-19 progression and severity. Plant foods such as fruits and vegetables may confer antiviral benefits and as such, reduce the risk and severity of COVID as they prevent viral replication, enhance antibody production against influenza virus, and improve T-cell function (Ling & Zabetakis, 2021; Merino et al., 2021). Based on the 2020-2025 Dietary Guidelines for

Americans, adults consuming a 2000 kcal diet are recommended to consume 2 cups/day of fruits and 2.5 cups/day of vegetables (*Dietary Guidelines for Americans, 2020-2025*, n.d.) which are higher than the mean fruit (1 cup/day) and vegetable (1.4 cups/day) intake observed in our study. Although fruit and vegetable intakes were low, among all participants, no significant differences were observed in fruit and vegetable intake between groups characterized as healthy vs unhealthy from cluster analysis (using food components from the DSQ). On the other hand, low intake of sugar-sweetened beverages was observed among individuals in the healthy compared to the unhealthy group while CSI, whole grain, fiber, and added sugar intake was the same between both groups. However, in sub-analysis, among individuals with low Se and Zn intake (≤ median), fruit intake was lower (1 vs 0.8 cup eq/day; p=0.04, results not shown) compared to individuals with Se and Zn intake above the median. Additionally, among participants with Se and Zn intake at or below the median, a healthy dietary pattern was characterized by higher dairy, calcium, Se, and Zn intake compared with an unhealthy dietary pattern and a trend towards an inverse association between the healthy dietary pattern and CSI.

A strength of our study is the sample population. It was predominantly made up of younger adults (mean age of 27 years) with no underlying chronic condition thereby adding to the existing literature on the nutritional status of individuals with COVID-19 living with no existing co-morbidities. Studies supporting similar findings from our study sample older adults, who due to existing co-morbidities may have a faster progression and increased severity of the COVID-19 infection. Also, several studies use serum or plasma concentrations of Zn and Se in assessing COVID-19 severity making our study one of the few studies which assessed toenail concentrations of Zn and Se as biomarkers of nutrient status. This is crucial because toenail concentrations of trace metals may reflect prior exposure of 3-12 months compared to blood

concentrations of trace metals which may be tightly regulated (Gutiérrez-González et al., 2019). Our findings are however limited by the observational study design precluding a cause-and-effect relationship.

There is minimal evidence to support a higher COVID-19 cure rate in selenium-adequate versus deficient individuals (Zhang, Saad, et al., 2020). As such, while Se-adequate individuals may not benefit from additional selenium intake mainly due to the narrow range from beneficial to adverse outcomes, findings from this study support a potential benefit from improved selenium intake among individuals consuming a low-selenium diet to mitigate COVID-19 severity.

CHAPTER V: ALMOND IMPROVED IRON STATUS IN AGING MICE FED AN ALMOND-SUPPLEMENTED DIET FOR 13 WEEKS

Abstract

Inflammation is the second most common cause of anemia, after iron deficiency. Almonds are a rich source of antioxidants and nutrients that can mitigate anemia of inflammation through reducing inflammation and improving the health of red blood cells (RBCs). In this study, we investigated the effect of almond intake on iron status and on the RBC antioxidant system of aged C57BL6 mice. Twenty C57BL/6 mice, all 20 months old (12 males and 8 females), were randomized to consume either a standard diet (SD) containing 15 % calories from fat, or a modified version of the SD diet with 15 % calories from almonds (SDA) for 16 weeks. Blood and tissue samples were analyzed for iron status and inflammatory markers, and activity of RBC antioxidant enzymes (SOD, CAT, GPX). Using intention to treat analysis, hemoglobin (13.3±0.8 *vs.* 10.6±0.9; p = 0.023), spleen iron (1252±329 µg/g *vs.* 407±62 µg/g, p=0.017), and liver iron (163±28 µg/g *vs.* 89±13 µg/g, p=0.017) were significantly higher in the SDA group compared to the SD group. However, plasma iron, and plasma hepcidin were not significantly different between groups (P>0.05). Among the antioxidant enzymes, CAT activity trended towards significance with higher activity in the SDA group (71±5 *vs.* 62±2 nmol/min/ml; p = 0.052). These findings suggest improved iron status in mice fed the SDA diet compared to the control group. Future studies are needed to investigate the underlying mechanisms responsible for these results.

Introduction

Anemia affects approximately one in three people around the world, contributing to 68.4 million years lived with disability (Kassebaum et al., 2014). It is caused by several factors, the main cause being iron deficiency, followed by inflammation (Nemeth & Ganz, 2014). Beyond these two, other nutrient deficiencies such as vitamin B_{12}, folate, vitamin B_6 or vitamin E can also lead to anemia. While there is currently no consensus among experts, a recent report suggested that the combination of inflammation and infection accounts for approximately 42% of the total anemia (Steinbicker & Muckenthaler, 2013b). Anemia of inflammation (AI) is commonly reported to be associated with chronic diseases, such as cancer and inflammatory bowel disease, and is attributed to the chronic inflammation in these conditions. This type of anemia is a concern given that more than 130 million adults in the US suffer from at least one chronic disease, many of which are associated with chronic inflammation (Dutta & Sengupta, 2016). Inflammation induces anemia by increasing the production of the anti-microbial peptide, hepcidin. Hepcidin plays a key role in iron metabolism through its regulation of ferroportin, the main iron exporter protein. By binding to and internalizing ferroportin, hepcidin prevents the export of iron from intestinal cells and macrophages, thus minimizing the availability of iron and impairing the production of red blood cells, leading to anemia. In addition, AI could be triggered by the activation of microphages during inflammation, resulting in phagocytosis and destruction of RBC. This is even more plausible in low antioxidant status because antioxidants are needed to quench reactive oxygen species released by myeloid cells, including macrophages, which can potentially damage RBC membranes and impair their deformability that can result in early aging. Thus anti-oxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) are important in preventing anemia of inflammation (Kuhn et al., 2017;

Mohanty et al., 2014). AI is common in aging due to aging-induced inflammation, oxidative stress, and aging-related chronic diseases. In the US, nearly 1 million older adults are affected by AI (Fraenkel, 2017). In the elderly, anemia is linked to several adverse health outcomes, namely depression, impaired cognition, increased recurrent falling and disability in daily living, increased hospitalization, and mortality (Penninx et al., 2005, 2006). With current data showing an increasing proportion of the US population with age ≥ 65 years old, (from 4.1% in 1900 to 12.9% in 2009) (Fowles & Saadia, 2010), there is a need to investigate sustainable approaches to addressing aging-related health problems, including AI. While there are experimental drugs that target hepcidin as a means of treating AI, their long-term adverse effects still must be evaluated. Almond, the most consumed nut in the United States, is a potential alternative for mitigating AI in the elderly due to its high content of vitamin E, phytic acid, polyphenols, and other antioxidant/anti-inflammatory nutrients. We propose that consumption of almonds can improve iron status by reducing the concentration of hepcidin and improving RBC antioxidant status. We hypothesize that iron status biomarkers such as hemoglobin and plasma iron, as well as antioxidant RBC components namely SOD, CAT and GPX activity, will be significantly improved in mice fed with an almond-supplemented diet compared to control mice.

Methods

Mice Models and Dietary Preparations

Twenty (n=10/group) C57BL/6 female and male mice (20 months old) were randomly assigned to consume either the control diet with 15% of total calories from fat or a modified version of the control diet with 15 % of calories from almond meal (almond diet). The sample size per group was based on the number of mice needed to determine a difference of 1.5 standard deviations in plasma iron concentration as statistically significant, with a power of 0.80 and at a

type I error rate of 0.05. This gives a sample size of 8 mice, which was increased to 10 mice per group to accommodate unexpected adverse events. The two diets were adjusted for their iron contents to eliminate any confounding effect of iron content on iron status biomarkers. All diets were produced by Research Diets Inc. To eliminate any confounding effect of iron content, the diets were adjusted to have the same iron contents. The composition of the two diets is shown in Table 11. The mice were kept in cages of 2 mice/cage under standard pathogen-free conditions, with food and water ad libitum, and under regular 12:12 light dark cycle. Food intake was measured twice a week and body weight of mice was measured weekly. After the 13-week intervention, all mice were euthanized due to poor survival rate. Blood and tissue samples were collected for measurement of hematological (plasma iron) markers, zinc concentration, antioxidant enzymes, and inflammatory (IL-6, hepcidin) markers. The study protocol was approved by the UNCG IACUC.

Table 11. Nutritional Composition (g/100g) of the Standard (SD) and Almond Supplemented (SDA) Diets.

Nutrient Composition	SD	SDA
Protein	17.16	17.2
Carbohydrate	64.75	64.7
Sugars	7.66	8.1
Fiber	4.85	4.8
Fat	6.42	6.4
SFA	1.54	0.8
MUFA	1.75	3.5
PUFA	2.79	1.8
Trans fat	0.01	0.0

Almond meal	0	10

Blood Sample Processing

Blood samples collected into EDTA tubes were transferred into 1.5 ml centrifuge tubes and spun for 15 minutes in a centrifuge at 1000xg at 4^0 C. The top yellow plasma layer was aliquoted from centrifuged EDTA tubes and stored in separate vials at -80 ^{0}C. The white buffy layer/leukocyte layer was carefully collected from the remains in the EDTA tube using a pipette and discarded. The erythrocytes were saved for the measurement of RBC antioxidant enzymes.

Plasma and Tissue Sample Processing and Analysis for Trace Metals

The concentrations of trace elements in plasma and tissue samples were determined at Dr. George Donati's Lab (Department of Chemistry, WFU) using an Agilent 8800 ICP-MS/MS (Agilent Technologies). Liver, spleen, and plasma samples will be digested for the analysis of iron and zinc with both HNO_3 (approximately 70%) and trace metal grade hydrogen peroxide (30 % v/v) to ensure complete digestion. The digested sample solutions will then be diluted with distilled-deionized water to a final concentration of approximately 3.5% v/v HNO_3 before analysis by ICP-MS/MS.

Measuring Hemoglobin, Hepcidin and IL-6 Concentrations

Hemoglobin was measured using the Hemocue America Hb201. Whole blood samples (~10 µL) were collected into the hemocue cuvette through capillary action. The hemocue cuvette was then wiped clean of any blood on the external surface before it was inserted into the hemocue for hemoglobin determination.

The collected plasma (100 µL after dilution) from whole blood was used in the analysis of hepcidin concentration using an ELISA kit (Intrinsic LifeSciences, CA). The experiment was

carried out per the instructions from the kit manufacturer, and molecular absorbance was read at 450 nm using the Epoch plate reader.

The IL-6 concentration in the plasma was also measured using an ELISA kit (Fischer Scientific, MA). The assay required 50 µL of sample/well and the experiment was carried out following the manufacturer's directions, and molecular absorbance was read at 450 nm using the Epoch plate reader.

Analysis of Erythrocytes for Antioxidant Enzymes

The activities of GPX, CAT and SOD in the RBC lysate were measured using ELISA kits from Cayman Chemical, MI and absorbances were read using the Epoch microplate reader. To prepare the lysate, 4 volumes of ice-cold water were added to the RBCs harvested after the plasma was collected from centrifuged blood. The resulting solution was further centrifuged at 10,000 x g for 15 min at 4 ^{o}C. The supernatant was collected and stored on ice for assaying using the ELISA kits.

To determine the GPX enzyme activity, 20 µL of the lysate was analyzed. The molecular absorbance was then read at 340 nm for at least 5 time points and the results used to calculate the GPX enzyme activity. Similar to the GPX assay, the CAT assay required 20 µL of sample, however molecular absorbance was read at 540 nm. The SOD assay required 10 µL of sample, and molecular absorbance was read at 440-460 nm.

Statistical Analysis

Data was analyzed using the R software for statistical computing (24). Data was transformed where necessary to approximate normality. Means for groups were compared using an independent t-test. To determine the effect of almond consumption on inflammatory markers and plasma trace elements in aging, the data between the aged group fed a normal diet and those

fed the almond-supplemented diet were compared. Using regression analysis, the association between inflammatory markers, as well as plasma, liver, and spleen trace element levels, with hematological markers were determined. Statistical significance was set at $p \leq 0.05$.

Results

A total of 20 mice begun the experiment. Out of this number, two mice died at weeks 9 and 10. Another pair of mice were euthanized around week 12 due to deteriorating health conditions. The remaining 16 mice were euthanized at the beginning of week 14 due to the previously observed low survival rate.

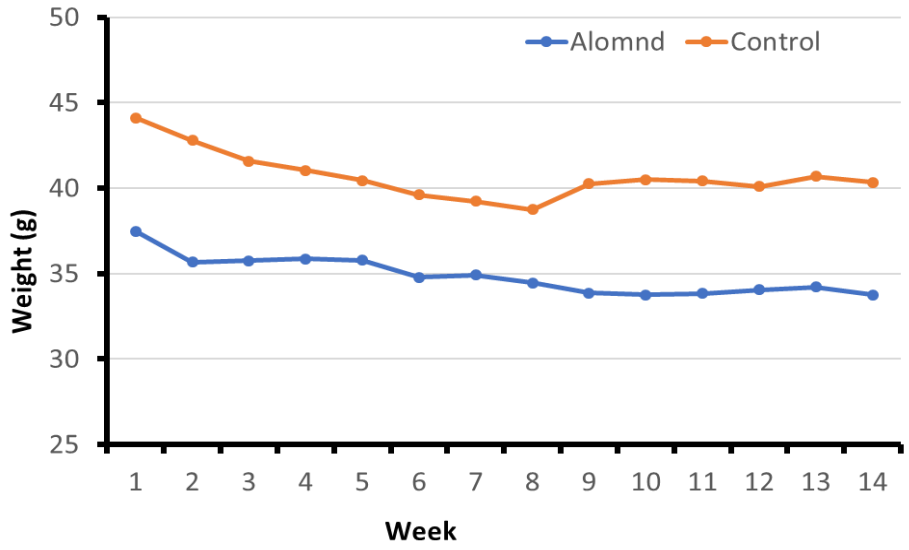
The mean weight of all mice at the baseline was 40.8±1.9 g, ranging from 26.8 g to 59.1 g. At the end of the intervention, the mean weight was 37.1±2.0 g, with a range between 25.7 g and 55.6 g (Table 12). Comparing almond and control groups, the baseline weight of mice was not significantly different between the two groups (37.5±3.0 g *vs.* 44.1±2.1 g; p= 0.09). Similarly, at 13 weeks, the weight difference between the two groups was not statistically significant (33.8± 2.0 g *vs.* 40.3 ± 3.3 g; p = 0.11). Within group, weight did not change significantly in both the almond group (36.4±3.3 g at baseline *vs.* 33.8±2.0 g at end line; p = 0.504) and the control group (45.1±2.3 g vs 40.3±3.3 g, p = 0.26). Similarly, there was no significant difference between the two groups in the mean change in weight (p = 0.86).

Table 12. Weight (g) of Mice in the Almond and Control Groups at Different Time Points[1]

Mice	Baseline	Endpoint	p-value
Almond	37.5±3.0	33.8± 2.0	0.50
Control	44.1±2.1	40.3 ± 3.3	0.26
All mice	40.8±1.9	37.1±2.0	0.23

[1]Values are means±SE

Figure 5. Weekly Weight (g) of Mice from Baseline to End of Intervention



The mean hemoglobin concentration for all mice at the end of the intervention was 12.0 ± 0.7g/dL. It was significantly higher in the almond group compared to the control group (13.3±0.8 *vs.* 10.6±0.9 g/dL; p = 0.023). Liver iron and spleen iron were also significantly higher in the almond group compared to the control group (P < 0.05 for all). Liver iron was over 80% higher in the almond group compared to the control group, while spleen iron was over 300% higher in the almond-supplemented group. However, plasma iron was not significantly different between the two groups (p=0.51) (Table 13).

Plasma IL-6 was below the detection limit for most mice, except for one mouse in each group (results not shown). Plasma hepcidin concentration was not significantly different between the two groups (p=0.37). In addition to iron status biomarkers, we also measured plasma and tissue levels of Se, copper, and Zn. Among these trace elements, none of them differed significantly between the two groups after the intervention (Table 13).

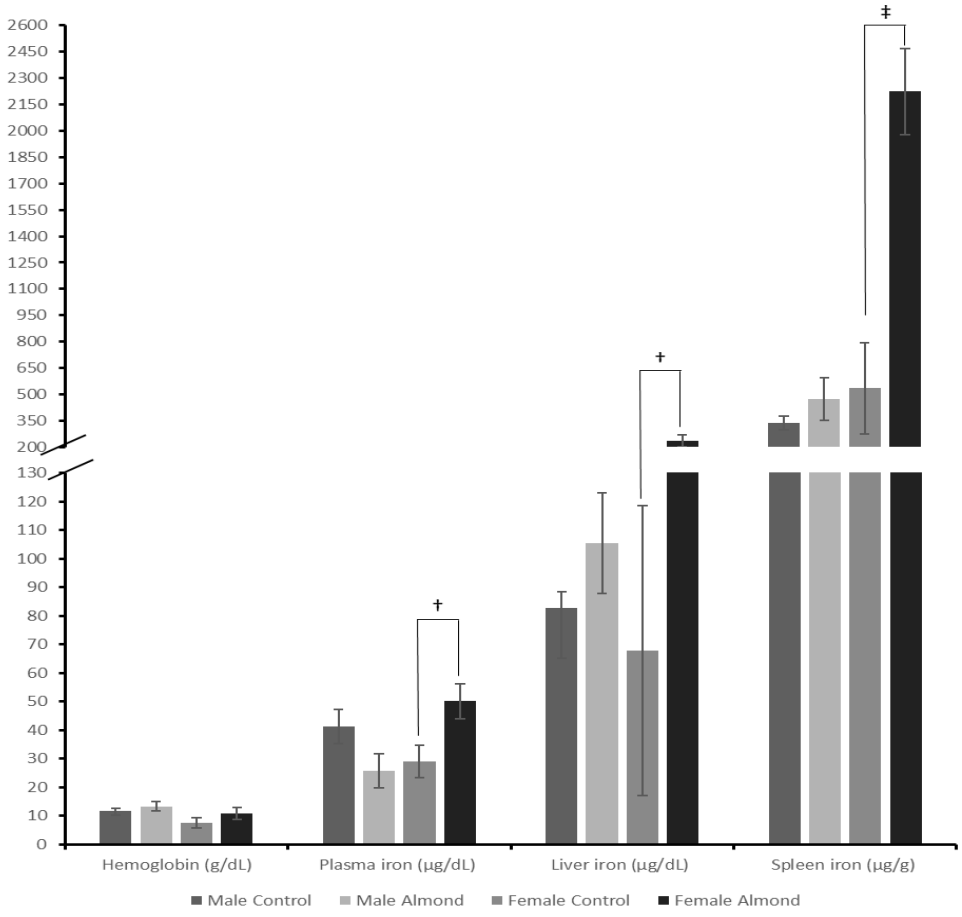
Table 13. Hematological Parameters, Antioxidant Enzyme Activity and Trace Mineral Concentrations after 13-week consumption of Almond-Supplemented Diet or Control Diet

Biochemical data	Almond	Control	p-value
Hemoglobin (g/dL)	13.3±0.8	10.6±0.9	**0.023**
Liver iron (µg/g)	163±28	89±13	**0.017**
Spleen iron (µg/g)	1252±329	407±62	**0.017**
Plasma iron (µg/mL)	3.8±0.6	3.8±0.5	0.514
Catalase nmol/min/mL	71±5	62±2	**0.052**
SOD (U/mL)	4.3±0.1	4.1±0.1	0.21
GPX (nmol/min/mL)	182±46	266±84	0.80
Hepcidin ng/mL	283±35	304±52	0.37
Plasma Cu(µg/L)	677±130	889±170	0.84
Liver Cu (µg/g)	2.96±0.11	3.41±0.44	0.83
Spleen Cu (µg/g)	0.85±0.06	0.97±0.10	0.85
Plasma Zn	2.02±1.3	2.46±0.5	0.62
Liver Zn (µg/g)	19.0±0.8	22.2±2.62	0.87
Spleen Zn (µg/g)	14.6±0.7	15.3±0.4	0.79
Plasma Se (µg/L)	411±22	513±32	0.99
Liver Se (µg/g)	1.29±0.07	1.36±0.12	0.67
Spleen Se (µg/g)	0.56±0.02	0.54±0.02	0.33

Note. p-values are based on results from independent sample t-test
[1]Values are means±SE

When the data was analyzed separately by sex, plasma iron (5.0±0.7 *vs.* 2.9±0.6 µg/mL; p= 0.04), liver iron (235 ± 33 *vs*. 100 ± 44 µg/g; p=0.034), and spleen iron (2222 ± 247 *vs.* 551 ± 151 µg/g; p= 0.001) in female mice were all significantly higher in the almond group compared to the control group. These significant findings were not observed in male mice (Figure 6).

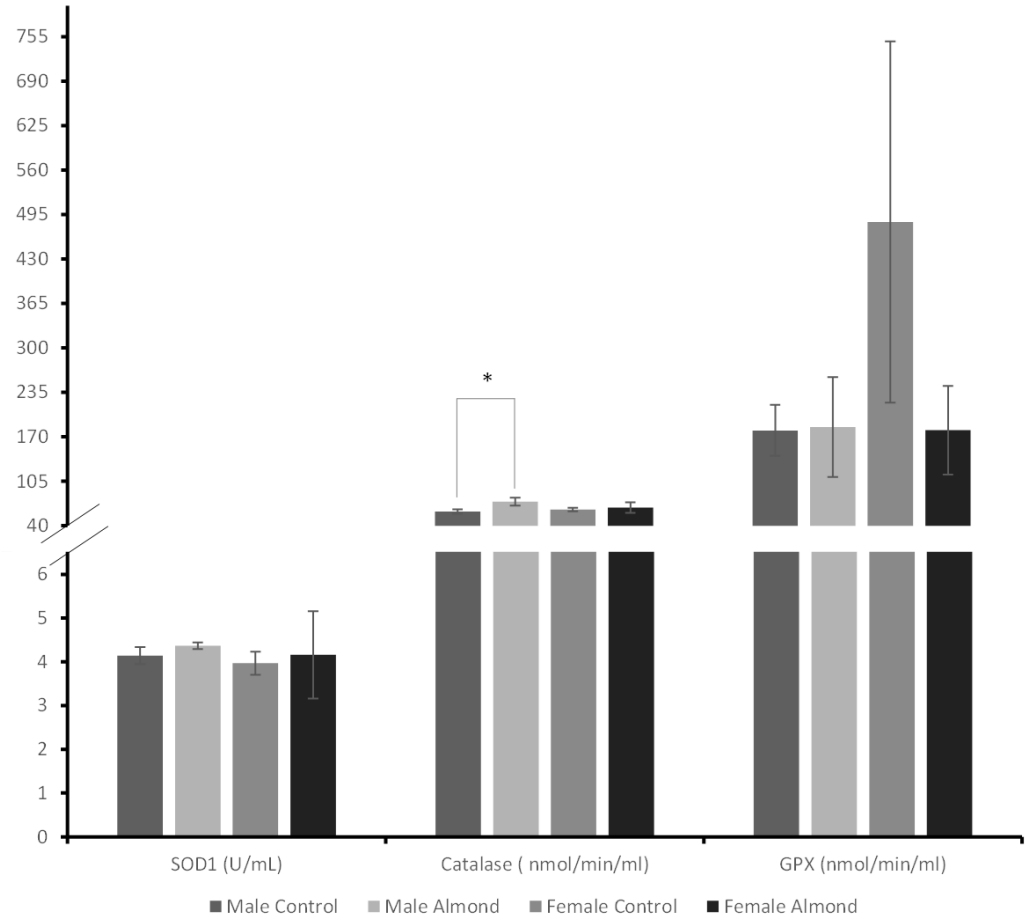
Figure 6. Concentration of Iron Biomarkers in Aging Mice after 13-week consumption of either Almond Supplemented Diet or Control Diet.



†p≤0.05, ‡p<0.01

Catalase trended towards significance among the antioxidant enzymes, with the almond group having higher activity compared to the control group (71±5 *vs.* 62±2 nmol/min/mL; p = 0.052), whereas GPX and SOD were not significant (p > 0.05) (Table 3). Once the data was split by sex, the CAT activity was significantly higher among male mice in the almond group compared to those in the control group (75±6 *vs.* 61±3.2 nmol/min/mL; p= 0.039). This difference was not observed among female mice (Figure 7).

Figure 7. RBC Antioxidants in Aging Mice after 13-week Consumption of Either Almond-Supplemented Diet or Control Diet.



*p≤0.05

Discussion

Aging is associated with inflammation and oxidative stress which together predisposes the elderly to various chronic diseases and AI. Anemia has been linked to several adverse outcomes in this age group, including recurrent falling, impaired cognition, and increased hospitalization. In a longitudinal study among 14-month-old rats, increased DNA damage and elevated levels of antioxidant enzymes, SOD, and CAT were observed compared to younger mice after 13 weeks (Achin et al., 2018). Aside chronic diseases, increased mortality with aging is associated with losses in lean and total body mass and declines in muscle mass (Alley et al., 2008). In our study, aging mice were fed an almond-supplemented diet for approximately 13 weeks instead of 16 weeks due to low survival rates. Additionally, while a total of 20 mice started the experiment, 4 mice were lost before the study was terminated. We also observed a decreasing trend in mice weight in both groups, however the decrease in weight was not statistically significant in both groups.

Age-related pro-inflammatory status is linked with ineffective erythropoiesis by inhibiting the proliferation and differentiation of erythroid progenitor cells with or without erythropoietin resistance (Ferrucci & Balducci, 2008). Other pathways include: 1) up-regulation of hepcidin production leading to a functional iron deficiency and 2) impaired RBC survival without compensation by erythropoiesis (Lang et al., 2005; Nemeth et al., 2004; Roy & Andrews, 2005). Almonds contain antioxidant and antiradical components which may decrease oxidative stress via an increase in RBC antioxidant enzyme activity (GPX, SOD, catalase) and a decrease in lipid peroxidation and inflammation (Jia et al., 2006; Ridha & Raheem, 2016; Zhou et al., 2021). In a study among mice, almond oil after 12 weeks, was beneficial against lipid peroxidation following UV-induced photoaging – which shares fundamental similarities with

cellular changes seen with normal aging (Sultana et al., 2007). In the present study using intention-to-treat analysis, key iron status biomarkers namely hemoglobin, liver and spleen iron were significantly higher in the almond group than in the control group, although plasma iron was not. The higher tissue iron observed in the present study are supported by findings from Arruda et al. (2013) among 15-month-old Wister rats compared to their 2-month old counterparts where liver and spleen iron were 170 and 1875 µg/g respectively (Arruda et al., 2013). In our study, the low hemoglobin concentration in the control group (10.6 g/dL) is very similar to the level characterized as anemia by McCranor et al. (2013) for 24-month-old retired breeder female mice (10.8 g/dL) and supports the assertion of anemia in aging even in the absence of any known chronic disease. On the other hand, the improved hemoglobin concentration (13.3 g/dL) in the almond group compares favorably to the value of 13.8 g/dL reported for younger mice aged 9-13 months old by the same authors, suggesting a beneficial effect of almond consumption in minimizing anemia risk.

Hormonal and genetic differences in sex exist in iron regulation in aging. For instance, among 16–18-month-old C57BL/6J mice, independent effects of sex and tissue iron accumulation were observed. Interestingly, while there was a higher level of iron in the liver among aging mice compared to young adult mice (2–6-month-old), the difference in liver iron accumulation was not observed by sex. This may have been because of the lower concentration of liver iron (66 µg/g) which is about 40 % less than what we observed in our study (Hahn et al., 2009). In our study, when we analyzed the data separately by sex, plasma, spleen, and liver iron concentrations were significantly higher in the almond group compared to the control group for female mice, whiles the difference in hemoglobin only trended towards significance ($p=0.052$). On the other hand, no significant difference in these variables was observed among male mice.

Overall, plasma hepcidin concentration was not significantly different between the two groups after the intervention. Arruda et al. (2013) also reported similar findings with no significant difference in *Hamp* gene expression among aged rats compared to young adult rats (Arruda et al., 2013). While we hypothesized that the almond-supplemented diet would result in reduced hepcidin and hence improved circulating iron and hemoglobin concentration, the lack of significant difference suggests the need for additional studies with multiple time points to understand the temporal changes in hepcidin concentration over the time of the intervention. It is possible that with improved iron status markers, hepcidin levels became elevated to regulate and prevent excess iron absorption and release from storage. Interestingly, the hepcidin concentration was lower among males in the almond group compared to their control group counterparts, suggesting potential a sex-dependent relationship between the dietary intervention and hepcidin concentration. This finding in addition to the higher iron stores in female mice compared to their male counterparts observed in our study concurs with evidence to support higher hepcidin expression and iron stores in the spleen and liver of female mice (Harrison-Findik, 2010). Conversely, among male mice, hepcidin expression does not predict tissue iron stores which is similar to findings from our study (McLachlan et al., 2017). Elevated iron stores increase hepcidin expression which may be one of the reasons for the increased hepcidin expression observed in female mice in our study. While the underlying mechanism is still under investigation, there is some evidence showing the role of estrogen in sex-specific regulation of hepcidin expression (Courselaud et al., 2004; Harrison-Findik, 2010). Given the small sample sizes after splitting the data by sex, additional studies with adequately powered sample size to further establish these findings are needed.

Among the antioxidant enzymes, while CAT activity trended towards significance, none of them was statistically significant. In a study by Al-Attar, in male albino rats, almond oil in the presence of lead-induced oxidative stress significantly increased SOD and glutathione but not CAT (Al-Attar, 2020). Antioxidant enzymes are important in maintaining the health of RBCs, as they prevent RBC membrane damage and early aging [4, 5]. The lack of statistical significance in catalase activity found in our study might be due to the small sample size, given that sample size was not based on catalase concentration. Once the data was split by sex, the CAT activity was significantly higher among male mice in the almond group compared to those in the control group. This difference was not observed among female mice. It is likely based on these findings that sex differences may contribute to the effect of almond consumption on both iron status and antioxidant status.

The current results provide preliminary data for additional studies with a larger number of male and female mice, which would allow for a more detailed investigation of sex-specific relations between almond supplementation in the diet and iron and antioxidant biomarker status.

Conclusion

Overall, the findings of this study show improved iron status in mice fed with the SDA diet compared to the control group. There is, however, a need for future studies with a larger sample size to investigate the underlying mechanism and the role of sex on the observed differences.

CHAPTER VI: EPILOGUE

In this study, we investigated the role of antioxidant food components and observed that dietary food components may improve inflammation-related outcomes. This study adds to the breadth of literature by focusing on three health conditions as proxies for inflammation to determine the interplay between the individual dietary components and as part of a food matrix. In the first study using the NHANES 2013 – 2014 cycle, we observed that increased phytate intake may be associated with improved cognition among the elderly, 60 years and above. To tease out the sole effect of phytate vs fiber, we adjusted for fiber intake in the regression analysis to minimize its confounding effects on the observed association. In the second study, among individuals diagnosed with COVID-19 with low Se and Zn intake, Se intake was inversely associated with CSI. We found no association between a healthy dietary pattern and CSI among all study participants. However, individuals in the unhealthy dietary pattern had lower Zn concentration and higher added sugar intake compared to the healthy group. Lastly, in an aging mice model, we observed that consumption of an almond meal resulted in increase in tissue iron and hemoglobin with elevated levels of CAT in male mice fed almonds compared to their male counterparts fed a control diet without almonds. Despite the observed inflammation in aging (Bektas et al., 2018), we observed no significant decrease in the concentration of plasma hepcidin among mice fed an almond diet compared to mice fed a control diet without almonds for 13 weeks. This may have been because of the length of the study (mice were euthanized due to a low survival rate) which may have been inadequate to markedly increase the concentration of pro-inflammatory markers. There is a need for further study to ascertain the mechanisms

underlying how dietary components mediate the oxidative stress state evident in inflammation-related outcomes.

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