A $^1$H NMR Metabolomic Exploration of Lifestyle Changes in Healthy Older Adults and The Ethics of Employing Nonhuman Animals in Empirical Research with an Emphasis on Aquatic Animals

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Abstract

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Lifestyle interventions such as improved physical activity and diet can help to prevent chronic diseases and enhance the quality of life in the aging population. This interdisciplinary dissertation aimed to employ NMR-based metabolomics to precisely detect and quantify the metabolic response to acute resistance exercise and n-3 PUFA dietary supplement at the molecular level in elderly individuals. Additionally, this dissertation explored the ethical ramifications of employing nonhuman animals for food and research. Chapter II examined the effects of healthy aging on peripheral blood metabolomic response to a single bout of resistance exercise. The results showed that human metabolic profiles and responses to RE are age dependent. Overall, the exercise-induced response to RE was considerably lower among the older group in several metabolites. Several factors including blunted anabolic response to exercise (Cuthbertson et al., 2005; Kumar et al., 2009), mitochondrial dysfunction (Short et al., 2005), and chronic inflammation (Lang et al., 2002; Toth et al., 2005) are potential contributors to this metabolic alteration in the elderly. These findings are substantial, especially in developing countermeasures to prevent age-related conditions. Chapter III investigate the effects of n3-PUFA supplementation on plasma lipoprotein subfractions in healthy older men and women without cardiovascular disease or dyslipidemia. This study showed that n-3 PUFA
supplementation slightly lowered the triglycerides and VLDL cholesterol levels and significantly changed the distribution and composition of HDL and LDL particles. It is still to be determined whether these changes in lipoprotein subclasses result in any meaningful benefit to healthy aging population. These findings should be considered alongside the ethical and environmental issues of the global consumption of marine-derived ω-3 fatty acid supplements. Chapter IV examined the scientific and ethical dilemmas in employing nonhuman animals in research, particularly marine-derived omega-3 fatty acids, the supplement used in this dissertation series. This ethical examination concluded that there is an urgent need for a paradigm shift in utilizing nonhuman animals in research. Consuming marine-derived omega-3 fatty acids entail ethical and environmental issues. It is necessary to eliminate the use of fishes in research and promote alternative omega-3 fatty acids to the public. Genetically modified plants, oleaginous microorganisms, and microalgae are promising alternatives for marine-derived omega-3 fatty acids.
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Acknowledgments

A large part of this doctoral dissertation was written during the 2022 emancipation movement of oppressed people in my home country, Iran. I want to use this space to declare my most profound solidarity with the progressive women and other marginalized people of Iran and to amplify their slogan, "Women, Life, Freedom," which has resonated worldwide. The revolutionary women in Iran have reminded the world once again that no one is free until we are all free.

I could not have completed this dissertation without the unwavering support of my mentors and their advice and assistance throughout my doctoral journey. It has been a privilege to receive guidance at every stage of writing my dissertation. My most profound appreciation is extended to Dr. Carol Ewing Garber and Dr. Ian Lanza, my doctoral advisors, for their endless encouragement and support, as well as their guidance and constructive feedback as I wrote this dissertation. I express my sincere gratitude to Dr. Francisco Javier Lopez Frias for giving me the courage to pursue research ethics and encouraging me throughout this learning journey. I also appreciate Drs. Megan Laverty and Bryan Keller, whose thoughtful input helped me strengthen my dissertation's qualitative and quantitative content.

This dissertation, unquestionably, would not have been possible without the love and support of my family and friends. My mother was my first role model as a strong, independent, and intelligent woman. She believed in me as no one else could. Because of her, I acknowledge the tremendous privilege I have had to pursue higher education. I want to remember my father, who passed away during my doctoral program. I felt disoriented for months and imagining my future without him was unthinkable. I am forever grateful to my brother, my best friend, for his everlasting love and patience, without which living in the diaspora would not have been possible.
Dedication

This dissertation is dedicated to all oppressed and voiceless individuals, particularly non-human animals, who endure suffering and injustice, and whose destiny we are committed to change.
CHAPTER I

Introduction

The word ‘metabolism’ is derived from the Greek word ‘Μεταβολισμός’ (Metabolismos), which means ‘change’ (Cresswell, 2021). Metabolism is vital in cell-fate decision-making, determining cell proliferation, differentiation, or dormancy (Shyh-Chang et al., 2013). In contrast, alterations in metabolite concentration or flux can significantly impact cellular and whole-body function, leading to acute and chronic human diseases. In the modern world, the availability of food and sedentary lifestyles combined with excess adiposity causes metabolic perturbations leading to a clustering of conditions (aka metabolic syndrome), including abdominal obesity, insulin resistance, dyslipidemia, and elevated blood pressure, which have been linked to a 5-fold greater risk of type 2 diabetes mellitus and an estimated doubling of cardiovascular disease risk (Cornier et al., 2008). Additionally, these age-related metabolic conditions are associated with comorbidities such as the hypercoagulation state, pro-inflammatory state, nonalcoholic fatty liver disease, reproductive tract abnormalities, and sleep disorders (Cornier et al., 2008). Because metabolic perturbations are at the root of most human diseases, strategies that modulate metabolism should be at the forefront of therapeutic and preventative measures.

Biological aging has been defined as a multi-faceted phenomenon characterized by a gradual loss of metabolic homeostasis and regenerative capacity leading to impaired function and increased mortality risk (López-Otín et al., 2013). Aging is accompanied by metabolic deregulation that includes insulin resistance (Fujita et al., 2007), mitochondrial dysfunction (Short et al., 2005), oxidative stress (Scicchitano et al., 2018), and altered protein metabolism (López-Otín et al., 2013). Aging diminishes muscle mass and function (Cruz-Jentoft et al.,
2019), and is a significant risk factor for cancer, and metabolic and cardiovascular diseases (Niccoli & Partridge, 2012).

Several countermeasures (e.g., exercise, and nutrition) have been developed to prevent age-related conditions and diseases. Physical exercise is a well-studied strategy that causes considerable alterations in energy metabolism, posing a significant challenge to whole-body homeostasis (Berton et al., 2017; Gorostiaga et al., 2012, 2014; Ishiguro et al., 2016; Valério et al., 2018). The depletion of metabolic substrates like phosphocreatine and glycogen and the accumulation of lactate are the immediate impacts of exercise on working muscles (Gorostiaga et al., 2012, 2014). Resistance exercise can reduce circulating blood glucose concentrations in healthy (Aguiar et al., 2018) and diabetic individuals (Ishiguro et al., 2016). Furthermore, resistance exercise is an effective method for increasing skeletal muscle mass and strength in healthy older adults and persons with chronic conditions and disabilities who want to improve their strength (Colberg et al., 2010; Guizelini et al., 2018). However, many older individuals demonstrate blunted anabolic response to exercise and nutrition, which leads to loss of muscle mass and function (Cuthbertson et al., 2005; Kumar et al., 2009). Therefore, a systematic assessment of metabolic response to acute RE at the molecular level is necessary to comprehend the aging-related metabolic dysregulation and response to exercise.

Dietary consumption of omega-3 polyunsaturated fatty acids (n-3 PUFA) is another preventive measure that has received much attention. n3-PUFAs, specifically, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have long been recognized for their ability to lower resting blood pressure and HR (Geleijnse et al., 2002) and reduce triglyceride levels in hyper-triglyceridemic individuals (Harris et al., 1983; von Loss onczy et al., 1978). Furthermore, n3-PUFAs have been linked to a variety of health benefits, including anti-inflammatory effects.
(Oh et al., 2010; Spencer et al., 2013), improved endothelial function, insulin sensitivity, and plaque stability (Din, 2004; Thies et al., 2003). The possibility that n3-PUFAs may alleviate insulin resistance and help prevent atherosclerotic cardiovascular disease (ASCVD) is particularly relevant to healthy aging, though these hypotheses are still being contested (Barry & Dixon, 2021; A. Lalia & Lanza, 2016). The potential protective and therapeutic effects of n3-PUFAs have been examined in healthy older adults in order to identify strategies to promote healthy aging (Herbst et al., 2014; A. Z. Lalia et al., 2017; Rodacki et al., 2012; Xyda et al., 2020). Despite some promising early evidence, there is insufficient data to support the hypothesis that n3-PUFA supplementation is beneficial to healthy older adults. Ethical and environmental issues, sustainability, cost, and potential adverse effects on human health are other critical factors to consider.

The human body can only produce a small quantity of EPA and DHA from alpha-linolenic acid (ALA) (Muskiet et al., 2004); therefore, these fatty acids must be consumed in the diet or supplemented to enhance overall body function. Currently, the primary source of n3-PUFAs for human consumption is marine fatty fishes\(^1\) such as salmon, mullet, and mackerel. Overfished stocks have been steadily growing since the 1950s onward (Worm et al., 2006), causing rapid and substantial alterations to marine ecosystems and biodiversity. On the other hand, aquaculture is rapidly growing, with yearly production increasing 50-fold between 1960 to 2015 (Ritchie & Roser, 2021). Although it is critical to question whether the global fishing industry is sustainable, the main issue in this dissertation is whether it is ethical to consume

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\(^1\) One way in which language denies other animals individuality and perpetuates their objectification is by using the same singular and plural forms to denote an individual or the entire species (DUNAYER, 2000). Such an approach minimizes the victims' sufferings and deaths. In this dissertation, 'fishes' is intentionally used as the plural form of 'fish' to emphasize that the use of identical terms for the singular and plural forms conceal the fact that the specie comprises unique individuals.
aquatic animals as sentient species with their rights, while cruelty-free alternatives are available to consumers such as n3-PUFAs sources from bacteria, fungi, plants, and microalgae.

**Significance**

Biological aging is an emerging phenotype caused by accumulation of molecular damage, leading to a steady decline in physiological capacities and the onset of various chronic illnesses (Tesch-Romer et al., 2021). The age-related conditions, including metabolic dysregulations and neurodegenerative and cardiovascular diseases (López-Otín et al., 2013; Niccoli & Partridge, 2012) can be prevented or delayed by holistic health-promoting interventions such as such as increased physical exercise and improved dietary habits (Tesch-Romer et al., 2021). These lifestyle change strategies promote healthier behaviors and improve the quality of life in the aging population. The effectiveness of these interventions cannot be restricted to a single or even a small number of biomarkers. Therefore, a global and systematic assessment of the metabolic fingerprint of exercise and dietary interventions is required to comprehend the aging process.

Metabolomics is a comprehensive analysis that involves quantifying the overall metabolic signature of a biological system using spectroscopy techniques such as quantitative proton nuclear magnetic resonance (1H-NMR) (Dunn et al., 2011). The human metabolome reflects the end-products of interactions between genes, proteins, and the cellular environment (Dunn et al., 2011). Therefore, investigating the impact of physical exercise and dietary interventions on a global level can provide novel insights into the underlying biochemistry of these approaches, possibly hinting at specific metabolic pathways related to the phenotypic response of the aging human organism.
In the recent decades, several studies have emerged utilizing metabolomics to examine the metabolic response to physical activity (Schranner et al., 2020); nevertheless, most of these studies have focused on metabolic alterations following endurance exercise in the healthy young populations. Given the current scarcity of research, portraying variations in metabolomic activities following resistance exercise and in aging adults remain essential. On the other hand, understanding the specific compositional changes in plasma caused by n3-PUFA supplementation in the aging population remains a challenge.

Therefore, this interdisciplinary dissertation will provide a foundation for understanding the metabolic responses to acute exercise and n3-PUFA supplementation at the molecular level in healthy older adults by employing a systemic and comprehensive approach to characterize plasma metabolites and lipoproteins and their responses to lifestyle change strategies. In addition, this work will shed light on the ethical considerations of viewing fishes as food sources, while cruelty-free alternatives are available to consumers such as n3-PUFAs sources from bacteria, fungi, plants, and microalgae.

Overview

The overarching objective of this interdisciplinary dissertation is to lay a foundation of empirical information to recognize the use of NMR spectroscopy to precisely identify and quantify the metabolic response to resistance exercise and n3-PUFA in the aging population. In addition, the goal of this dissertation is to examine the ethical considerations of employing nonhuman animals\(^2\) in research. The first study employs NMR-based metabolomics to

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\(^2\) Standard English perpetuates speciesism and promotes a false dichotomy between nonhuman and human animals (Dunayer, 2003). Misleading language legitimizes and conceals the institutionalized abuse of other animals. In this dissertation, the terms "other animals," "nonhuman animals," or "nonhumans" are used interchangeably instead of the term "animal" to highlight the importance of unbiased language in everyday life but specifically in science.
investigate the metabolic response to RE and describe a global picture of its impact on healthy older adults. The objective of the second study is to determine the effect of n3-PUFA supplementation on plasma lipoprotein subfractions in healthy older men and women in the absence of cardiovascular disease or hypertriglyceridemia. The fourth chapter provides a comprehensive review of the history of using nonhuman animals in experimental research and extensively examines scientific and ethical dilemmas in using nonhumans in empirical research.

The specific aims of this dissertation are to:

1) investigate the metabolic response to acute RE and describe a global picture of its impact on healthy older individuals in the absence of cardiovascular disease or hypertriglyceridemia using NMR-based metabolomics.

2) determine the effect of n3-PUFA supplementation on plasma lipoprotein subfractions in healthy older individuals in the absence of cardiovascular disease or hypertriglyceridemia using NMR-based metabolomics.

3) provide an ethical examination of using nonhuman animals in experimental research.

The hypotheses of this dissertation are:

1) the exercise-induced metabolic response to acute RE is different between healthy young and older individuals.

2) the use of n3-PUFA will shift the plasma concentrations of lipoprotein subclasses in healthy older men and women.

However, the author acknowledges that these terminologies (i.e., animals, nonhuman animals, and other animals) are not perfect alternatives as they are founded on anthropocentrism and human exceptionalism.
Dissertation Structure

This interdisciplinary dissertation specifically looks at metabolic responses to lifestyle changes in the aging population and the ethical challenges of using nonhuman animals in experimental research. In Chapters II, the NMR spectroscopy is employed to explore the metabolic response to RE. This chapter presents a global picture of RE's influence on the metabolites shift in healthy older adults within the first three hours of recovery. In Chapter III, the NMR-based metabolomics is used to identify and quantify plasma lipoproteins subclasses. This chapter investigates the effect of n3-PUFA supplementation on plasma lipoprotein subfractions in healthy older men and women who do not have cardiovascular disease or hypertriglyceridemia. The fourth chapter provides a thorough overview of the history of utilizing nonhuman animals in experimental research and a comprehensive investigation of the scientific and ethical issues surrounding the use of nonhumans as food sources. Finally, the fifth chapter summarizes the main findings of this series of dissertations. It brings back the main limitations of the studies and briefly introduces lingering questions for future research.
CHAPTER II

$^1$H NMR-Based Metabolic Investigation of Dynamic Response to Acute Resistance Exercise in Healthy Older Males and Females

Abstract

Metabolism plays a crucial role in human health, and intrinsic and extrinsic factors modulate it. Although exercise-induced adaptations are beneficial for the human body, the anabolic response to exercise and nutrition is often diminished in older adults due to a phenomenon known as "anabolic resistance." Research on the metabolic responses to resistance exercise in the aging population is scarce. Therefore, the objective of this study was to determine the effects of healthy aging on peripheral blood metabolomic response to a single bout of resistance exercise. Thirty young (20-35 years) and 49 older (65-85 years) men and women were studied. Participants completed a single bout of resistance exercise consisting of 8 sets of 10 repetitions of unilateral knee extension at 70% of the 1-repetition maximum. Blood samples were collected before exercise, immediately, and after 30-, 90-, and 180 minutes of recovery. Proton nuclear magnetic resonance spectroscopy was used to profile circulating metabolites at all time points. Our analysis revealed that one bout of resistance exercise elicits significant changes in the circulating metabolome of 18 out of 33 measured metabolites reflecting alterations in several metabolic pathways. The exercise-induced response in several metabolites was lower among the older adults. Furthermore, six analysts demonstrated significant interactions between the effects of exercise and age. Overall, this study indicated that human metabolic profiles and responses to RE are age dependent. The concentrations of several plasma metabolites were significantly different in older adults, and the exercise-induced response to RE was considerably lower among the older group. Therefore, the dysregulated metabolic fingerprint in older people must be given more consideration, especially in developing preventive strategies for age-related conditions.
Introduction

Aging is a time-dependent process in which the accumulation of molecular damage outpaces the organism's ability to mitigate it (López-Otín et al., 2013; Rattan, 2014; Tesch-Romer et al., 2021). The accumulation of cellular damage manifests as a gradual loss of physiological capacities and the onset of various chronic illnesses (Tesch-Romer et al., 2021).

Aging is accompanied by metabolic deregulation that includes insulin resistance (Fujita et al., 2007), mitochondrial dysfunction (Short et al., 2005), oxidative stress (Scicchitano et al., 2018), and altered protein metabolism (López-Otín et al., 2013). Aging diminishes muscle mass and function (Cruz-Jentoft et al., 2019), and is a significant risk factor for cancer, and metabolic and cardiovascular diseases (Niccoli & Partridge, 2012). Because metabolic perturbations are at the root of most human diseases, strategies that modulate metabolism should be at the forefront of therapeutic and preventative measures.

Physical exercise (e.g., aerobic, anaerobic) causes considerable alterations in energy metabolism, posing a significant challenge to whole-body homeostasis (Berton et al., 2017; Gorostiaga et al., 2012, 2014; Ishiguro et al., 2016; Valério et al., 2018). Due to the anaerobic metabolic pathways involved in resistance exercise (RE), various changes can be observed soon after one bout of RE. The depletion of metabolic substrates like phosphocreatine and glycogen and the accumulation of lactate are the immediate impacts of RE in muscle (Gorostiaga et al., 2012, 2014). In addition, studies have demonstrated that RE can reduce circulating blood glucose concentrations in healthy (Aguiar et al., 2018) and diabetic individuals (Ishiguro et al., 2016). Resistance exercise is an effective method for increasing skeletal muscle mass and strength in healthy older adults and persons with chronic conditions and disabilities who want to improve their strength (Colberg et al., 2010; Guizelini et al., 2018). However, many older individuals
demonstrate blunted anabolic response to exercise and nutrition, which leads to loss of muscle mass and function (Cuthbertson et al., 2005; Kumar et al., 2009). Despite prior studies on how aging affects adaptive responses to exercise, there is still a lack of understanding of the molecular and cellular signals that drive these adaptive responses (Sanford et al., 2020). Therefore, an extensive and systematic assessment of metabolic response to acute RE at the molecular level is required to comprehend the aging-related metabolic dysregulation and response to exercise.

Metabolomics is a comprehensive analysis that involves quantifying the overall metabolic signature of a biological system using spectroscopy techniques such as quantitative proton nuclear magnetic resonance (\(^1\)H-NMR) (Dunn et al., 2011). The human metabolome reflects the end-products of interactions between genes, proteins, and the cellular environment (Dunn et al., 2011). Therefore, investigating the impact of physical exercise on a global level can provide novel insights into the underlying biochemistry of exercise, possibly hinting at specific metabolic pathways related to the phenotypic response of the aging human organism. In recent decades, several studies have emerged utilizing metabolomics to examine the metabolic response to physical activity (Schranner et al., 2020); nevertheless, most of these studies have focused on metabolic alterations following endurance exercise in healthy young populations. Given the current scarcity of research, portraying variations in metabolomic activities following acute resistance exercise in the context of aging remains essential. Therefore, the present study aimed to determine the effects of healthy aging on peripheral blood metabolomic response to a single bout of resistance exercise.
Subjects and Methods

Participants and study design

All procedures were carried out according to the Declaration of Helsinki and were approved by the Mayo Foundation Institutional Review Board (IRB 17–004403, ClinicalTrials.gov identifier: NCT03350906). Thirty young (27.1 ± 4.11 years) men and women and 49 older (71.4 ± 4.53 years) men and women were recruited from the southeast Minnesota area. Inclusion criteria were age 20-35 years old for the young group and 65-85 years old for the older group. Participants were excluded if they reported diabetes or fasting plasma glucose greater than 126 mg/dL, anemia (hemoglobin less than 11 g/dL for females and less than 12 g/dL for males), active coronary artery disease or history of unstable macrovascular disease, renal failure (serum creatinine greater than 1.5 mg/dL), active liver disease (AST greater that 144 IU/L or ALT greater than 165 IU/L), history of blood clotting disorders, anticoagulant therapy, international normalized ratio (INR) greater than 2.0, substance abuse, untreated or uncontrolled hypothyroidism, pregnancy or breastfeeding. All participants provided written informed consent, followed by blood sampling for screening tests. The metabolomics analysis was not predeclared as a primary or secondary endpoint of the parent trial and should be regarded exploratory post-hoc analyses.

Outpatient muscle strength testing

Knee extensor muscle strength was determined from unilateral 1-repetition maximum (1-RM). Participants were familiarized with a pneumatic resistance leg extension machine (Keiser Air300, Keiser Corporation, Fresno, CA, USA), instructed in the proper range of motion, and allowed to perform a warm-up set of 10 repetitions at minimal resistance. Following the warm-
up set, participants performed 3 sets of 5–10 repetitions at progressively increasing resistance at
the discretion of the investigator and tailored based on participant's perceived exertion. Three
minutes of rest was provided between sets. Following habituation, 1-RM was determined from a
series of single attempts at incremental resistance with 3 min of rest between attempts (25-27). 1-
RM was defined as the maximum load that could be moved through the full range of motion with
proper form.

Inpatient acute exercise session

Within 3 months of completing outpatient testing, participants were admitted to the
Clinical Research and Trials Unit. In advance of this visit, participants were provided with
weight-maintaining meals (20% protein, ~50% carbohydrate, and ~30% fat) for 3 days according
to the Harris Benedict Equation (Mifflin et al., 1990). On the evening of admission to the
research unit, participants ate an evening meal at ~1800hrs and remained fasting except for water
until completion of the study the following morning. An intravenous catheter was placed
retrograde in the opposite hand, which was kept in a plexiglass box maintained at 55°C for
collection of arterialized venous blood samples. Participants completed 8 sets of 10 repetitions of
unilateral knee extensions at 70% of 1-RM. Blood samples were collected 30 minutes before
exercise, and immediately following completion of the final set, 30-, 90-, and 180-minutes post
exercise. Whole blood was placed on ice and processed immediately for plasma collection.
Plasma samples were stored at -80 until metabolomics analysis.
\textit{\textsuperscript{1}H-NMR spectroscopy and sample preparation.}

Plasma samples were analyzed using high-resolution proton nuclear magnetic resonance (\textit{\textsuperscript{1}H-NMR}) spectroscopy according to the Bruker B.I. QUANT-PS 2.0 standard platform as previously described (Xyda et al., 2020). Plasma samples were thawed on ice and mixed with Bruker VERBR plasma buffer (phosphate buffer pH 7.4 containing 0.1\% TSP-\textit{d}\textsubscript{4} (3-(trimethylsilyl)-2,2,3,3-tetradeutero propionic acid)) in 9:1 (v/v) ratio. 300 \textmu L of plasma was mixed with 300 \textmu L of phosphate buffer and transferred to an Eppendorf tube with 70 \textmu L of buffer subsequently added prior to vortexing the mixture for 20 s. The sample was then centrifuged at 5000 rpm for 10 minutes, and 600 \textmu L of the supernatant was transferred to a 5 mm NMR tube. The NMR spectra were collected using a Bruker 600 MHz Avance III HD spectrometer with a BBI room temperature probe head and SampleJet auto sampler (Bruker Biospin, Billerica, MA). Proton spectra were acquired using 1D NOESY pulse sequence with pre-saturation (noesygppr1d) over 32 total scans. Spectra were transferred to the Bruker Data Analysis server for automated remote analysis.

\textbf{Statistical analysis}

Descriptive and clinical characteristics of the study participants are presented as means and standard deviations with unpaired t-tests to compare variables in young versus older groups (Table 1). Since the plasma metabolite concentrations did not follow a normal distribution, raw data were log-transformed. The time variable was dummy coded. A mixed two-way repeated measures analysis of variance (ANOVA) was conducted to determine if there was a significant main effect for each independent variable by testing for between-subject effects (age), within-subjects effects (exercise), and an interaction effect (exercise*age) (Table 2). The exercise*age
interaction term was used to determine if changes in metabolite concentrations with acute exercise depended on participants’ age groups. In the absence of a significant exercise*age interaction, the main effects of exercise were used to determine if variables were different at any of the five sampling time points regardless of age. Likewise, the main effect of age was used to determine if metabolite concentrations differed by age group, regardless of exercise-induced responses to intervention. If there was a significant interaction, the Tukey’s Honest Significant Difference (HSD) test was used to compare the mean concentrations between the two groups at each sampling time point. P-values were compared to the level of statistical significance, which was set at $\alpha = 0.05$. All results were analyzed using RStudio, version 4.2.1.

Results

Participant characteristics

A total of 30 young and 49 older adults were included. Young and older adults had similar height, weight, and BMI. Older adults had significantly higher systolic blood pressure compared to young, but there was no difference in diastolic blood pressure (Table 1). Fasting plasma glucose was significantly elevated in older compared to younger adults with no significant difference in fasting insulin values between age groups (Table 1).

Pre-exercise plasma metabolomics

Plasma $^1$H-NMR metabolomics in baseline pre-exercise plasma samples (Table 2) revealed elevated plasma glucose ($P= 0.04$) concentration in older compared to younger adults. Glycine ($P<0.0001$), glutamine ($P=0.042$), tyrosine ($P<0.0001$), creatinine ($P=0.034$), ornithine ($P<0.0001$), trimethylamine N-oxide ($P=0.04$), and citrate ($P<0.0001$) were also significantly elevated in plasma from older compared to young adults.
Metabolomic response to acute resistance exercise

Of the 33 plasma metabolites detected and quantitated by $^1$H-NMR, 21 demonstrated time-dependent changes following acute resistance exercise (Table 2, Figures 1-3). The acute bout of single-leg RE immediately increased plasma organic acids (lactate, pyruvate) and decreased ketones (acetoacetate, acetate, 3-hydroxybutyrate) and several amino acids (methionine, valine, isoleucine, ornithine). In contrast, the amino acids glycine and alanine exhibited acute increases immediately following exercise. Other plasma metabolites demonstrated more latent changes following exercise that emerged in the later recovery period. These metabolites include succinate, 3-hydroxybutyrate, and acetoacetate, which were significantly elevated at 180 minutes of recovery.

Of the metabolites that were responsive to acute single-leg resistance exercise, 12 demonstrated significant exercise*age interactions, including pyruvate (P=0.009), lactate (P=0.012), alanine (P<0.039), acetoacetate (P<0.013), 3-hydroxybutyrate (P<0.001), lysine (P=0.032), ornithine (P=0.002), methionine (P=0.011), tyrosine (P=0.037), phenylalanine (P=0.033), glycerol (P=0.002), glucose (P=0.11), (Table 2, Figures 1-3). Notable trends that did not reach statistical significance included leucine (P=0.061), valine (P=0.095), creatinine (P=0.097), and isoleucine (P=0.098). Plasma lactate, pyruvate, alanine concentrations were acutely elevated immediately following exercise and returned to baseline at the 90-minute timepoint with older adults demonstrating smaller magnitude changes compared to young. Plasma ketones 3-hydroxybutyric acid and acetoacetic acid acutely decreased in young and older adults following exercise but with greater magnitude changes evident in young compared to older. The amino acids methionine, and isoleucine exhibited acute decreases immediately post-exercise that tended to be more apparent in young compared to older adults.
Discussion

This study quantitatively evaluates the global metabolic response to one bout of resistance exercise in healthy older adults. Irrespective of age, our analysis revealed that one bout of resistance exercise elicits significant changes in the circulating metabolome of 21 out of 33 measured metabolites reflecting alterations in several metabolic pathways (Table 2). Following RE, the glycolysis products (i.e., lactate, pyruvate) were upregulated, ketone bodies (acetoacetate, acetate, 3-hydroxybutyrate), and several other amino acids (leucine, isoleucine, valine, methionine, and ornithine) were downregulation. The exercise-induced response in several metabolites, including lactate, pyruvate, alanine, acetoacetate, 3-hydroxybutyrate, lysine, methionine, ornithine, tyrosine, phenylalanine, glycerol, and glucose was different between young and older groups (Figure 3). These key findings hint toward understanding the metabolic fingerprint of aging and provide insight in lifestyle interventions aimed at improving human health and disease prevention.

The pre-exercise baseline analysis revealed that older adults exhibited significantly elevated heart rates and systolic blood pressure. Plasma concentrations of glucose, citrate, and several amino acids, including glycine, glutamine, tyrosine, ornithine, and creatinine, were upregulated in older adults compared with the younger group (Table 2). Citrate is a TCA cycle intermediate reflecting mitochondrial function, while glycine is a critical inhibitory transmitter in the CNS (Hernandes & Troncone, 2009). Creatinine is an endogenous substrate produced primarily from creatine in muscle and is associated with muscular function (Patel et al., 2013). This study confirms previous findings that systolic blood pressure (Pinto, 2007), fasting blood glucose (Robert et al., 1982), and plasma creatinine levels (Salive et al., 1995) increase with age. Similarly, higher levels of citrate and several age-related amino acids, including tyrosine and
glutamine, have been reported in elderly adults (Darst et al., 2019). The plasma concentration of these metabolites serves as a metabolic fingerprint of aging and can hint toward understanding changes in several pathways involved in age-related conditions.

We compared the plasma metabolite concentrations at five different time points to assess the metabolic time-course response to one bout of RE. Our post-exercise analysis showed that the immediate increase in plasma glucose levels following RE was significantly greater among older adults compared to the young group. Plasma glucose concentrations slightly declined below the basal levels 90- and 180 minutes post-RE, although they did not reach the significance level. Previous studies reported changes in which the plasma concentrations of glucose were reduced following RE in healthy (Aguiar et al., 2018), and diabetic individuals (Ishiguro et al., 2016), and within 180 minutes of recovery (Borgenvik et al., 2012). This observation can be attributed to the enhanced glucose uptake by the muscles that continues for hours following exercise onset (Colberg et al., 2010). Due to the accelerated carbohydrate breakdown during RE, lactate, and pyruvate were remarkably accumulated soon after the cessation of exercise (Table 2, Figures 1). For many years, it was widely accepted that lactate is a byproduct of anaerobic glycolysis, and that aerobic glycolysis only occurs by pyruvate uptake in mitochondria. Contrary to this belief, there is sufficient evidence showing that lactate production occurs continuously at rest as well as during exercise in fully oxygenated conditions (Bendahan et al., 2017; Brooks, 2020; Rogatzki et al., 2015). The accumulation of pyruvate and lactate was significantly greater among younger individuals at 30 minutes post-RE. Pyruvate and lactate concentrations followed a steady decreasing trend and reached the basal levels within 90 minutes of recovery. Pyruvate significantly dropped below the baseline values at 180 minutes post-RE. Our observation is in
agreement with prior findings showing that blood pyruvate and lactate decline within an hour after RE (Berton et al., 2017; Borgenvik et al., 2012; Valério et al., 2018).

Many other metabolites exhibited changes in plasma concentrations after RE. It has been extensively demonstrated that TCA cycle intermediates are upregulated in the blood by both endurance and resistance exercise, especially in the first 30 minutes after exercise (Berton et al., 2017; Bowtell et al., 2007; Gibala et al., 1998; Gorostiaga et al., 2014; Morville et al., 2020; Schranner et al., 2020). In our study, the TCA cycle intermediate, citrate, increased 30 minutes after exercise and gradually returned to the basal levels 90 minutes into the recovery time in both young and older groups. Succinate concentrations fluctuated at all time points following RE and rose above the basal levels at 180 minutes following RE intervention. Prior studies reported increased plasma succinate concentrations directly post-exercise (Berton et al., 2017; Schranner et al., 2020; Valério et al., 2018). Succinate has been identified as a signal that mediates muscle adaptation and remodeling response to exercise (Reddy et al., 2020). Lastly, acetate concentrations significantly dropped immediately and 30 minutes post-RE and remained below the basal levels at 180 minutes following the intervention. Acetate is utilized by the TCA cycle to synthesize nicotinamide adenine nucleotide (NADH) and ATP (Bowtell et al., 2007); therefore, its levels decrease immediately after the onset of RE.

Furthermore, our results revealed that a single bout of RE considerably alters the content of amino acids in human blood. Branched-chain amino acids (BCAA, isoleucine, leucine, and valine) are a primary source of TCA cycle intermediates (Freund & Hanani, 2002; Newman & Verdin, 2014); and act as precursors for protein synthesis in the muscle (Freund & Hanani, 2002), thus, their concentrations are expected to attenuate during the recovery time following a
bout of RE (Berton et al., 2017; Borgenvik et al., 2012; Robert et al., 1982). Furthermore, BCAA leucine is a critical regulator of muscle protein anabolism by activating the mTOR pathway (Anthony et al., 2001). The current study revealed an immediate decreasing response in plasma concentrations of isoleucine, leucine, and valine in both older and young individuals. These concentrations remained below the basal levels 180 minutes post-RE. A prior study involving young participants reported that BCAAs decreased below basal levels as late as 60 minutes post-RE (Berton et al., 2017). We also detected a remarkable rise in alanine concentrations immediately and 30 minutes after RE which returned to basal levels 180 minutes into the recovery. This increase was more significant among the older adults compared to the young group. Multiple studies have demonstrated exercise-induced disruption of creatine synthesis and re-synthesis after different modes of exercises (Schranner et al., 2020). These observations vary from a gradual decrease of urinary creatine over the 2 hours of recovery time (Pechlivanis et al., 2015) to a rise in the concentration of creatine and creatinine after exhaustive exercise in healthy young individuals (Al Fazazi et al., 2019). In our study, creatine demonstrated a minor decrease throughout the recovery time, followed by a significant decrease 180 minutes after RE. Amino acids and their derivates response to exercise vary significantly between studies and protocols (Schranner et al., 2020). For example, the use of amino acids as a substrate in gluconeogenesis, ketogenesis (Evans et al., 2017), and protein synthesis (Newsholme et al., 2011) would reduce their concentrations, particularly after resistance exercise. On the other hand, pre-or post-exercise food intake can affect amino acid concentrations and other metabolites (Schranner et al., 2020). Carbohydrate consumption, for example, lowers gluconeogenesis, and ketogenesis (Evans et al., 2017); and the use of amino acids in these reactions leads to lower changes in amino acid concentrations. Exercise duration and intensity are other factors influencing the amino acid
concentrations after exercise. Amino acid concentrations drop following a moderate-intensity, long-duration exercise. In contrast, their values increase after a high-intensity and short-duration exercise (Schraner et al., 2020).

Several ketone bodies dropped post-RE immediately. Ketone bodies acetoacetate and 3-hydroxybutyrate showed an immediate decreasing response, followed by a remarkable accumulation higher than pre-exercise values at 180 minutes of recovery. Ketone bodies are used in the brain and muscle when carbohydrates are limited during fasting or prolonged exercise. In our study, participants were fasted a night before the RE intervention and during the three hours recovery. Our observation regarding acetoacetate response closely mirrored previously reported findings in which plasma concentrations of the ketone body acetoacetate dropped in response to acute RE (Goto et al., 2007; Schraner et al., 2020). Several studies demonstrated that the primary ketone molecules, 3-hydroxybutyrate, and acetoacetate, are released into the blood after exercise (Dohm et al., 1986; Evans et al., 2017; Schraner et al., 2020). Conversely, Berton et al. 2016 showed that 3-hydroxybutyrate and 2-hydroxybutyrate immediately increased after 15 or 30 min post-RE in young non-fasting individuals (Berton et al., 2017). Previous studies have demonstrated that 3-hydroxybutyrate can regulate adaptation mechanisms in skeletal muscle through positive effects on protein synthesis in human skeletal muscle (Evans et al., 2017; Nair et al., 1988).

Furthermore, our post-exercise analyses indicate that the metabolic response to RE differed between the elderly and young groups in several metabolites (Figure 3). The magnitude of exercise-induced response in metabolites associated with glycolysis and TCA cycle such as pyruvate, lactate, succinate, citrate, and acetate were lower among older adults compared with the younger group (Table 2, Figures 1). The drop in the TCA cycle intermediates suggests
changes in mitochondrial function. Alanine demonstrated similar trends since alanine synthesis and release into the bloodstream depend on the rate of pyruvate formation. Statistically significant exercise*age interactions were detected immediately and 30-minutes following the intervention in lactate, pyruvate, and alanine, respectively (Figure 3). Glucose concentrations significantly varied between the young and older adults immediately post RE. Similarly, valine, lysine, isoleucine, methionine, histidine, and trimethylamine-n-oxide (TMAO) demonstrated different responses to RE among the elderly compared with the young group (Figures 1-3).

Ornithine response in older adults was unique because its levels dropped soon after RE and remained below the basal level after 180 minutes of recovery. In contrast, ornithine concentrations among younger individuals increased immediately after exercise and remained above the baseline value throughout the three-hour recovery period. A significant age*exercise interaction was detected at 180-minutes into the recovery time (Figure 3). In addition, phenylalanine had a unique response to RE among older adults. The plasma concentration of phenylalanine immediately rose following the intervention in older adults and continued to increase beyond basal levels within 90 minutes post-intervention and remained elevated throughout the 180 minutes recovery time. In contrast, phenylalanine’s levels dropped immediately and 30-minute after RE in the younger group (Figure 3). Phenylalanine is an essential amino acid, and a building block of proteins. As a result, its use in the muscle is a measure of muscle protein synthesis, whereas its release is a measure of muscle protein breakdown (Volpi et al., 2001). Previous reports showed that the plasma (Borgenvik et al., 2012) and urinary (Pechlivanis et al., 2010) concentrations of phenylalanine were reduced 60 minutes after resistance and maximal exercise remained attenuated after that in healthy young individuals.
Among ketone bodies, 3-hydroxybutyrate and acetoacetate demonstrated different exercise responses between the young and older groups directly and 30 minutes post RE. Ketone body utilization after RE lasted longer in the younger group. Hepatic ketogenesis started soon after exercise in older adults, while this accumulation did not start as late as 30 minutes post-RE in the younger group. Ketones levels reached beyond the basal values 180 minutes post RE and this among was greater among older adults. (Figure 1). Beyond serving as a substitute fuel source, ketone bodies also have a variety of metabolic effects, such as reducing glucose uptake by peripheral tissues, inhibiting the lipolytic activity of adipose tissue, and possibly reducing the amount of protein breakdown in skeletal muscle (Robinson & Williamson, 1980). It has been shown that exercise-trained skeletal muscles have higher capacity to utilize ketones during exercise (Evans et al., 2017).

In conclusion, our study indicated that human metabolic responses to RE is age dependent. Several factors including blunted anabolic response to exercise (Cuthbertson et al., 2005; Kumar et al., 2009), mitochondrial dysfunction (Short et al., 2005), and chronic inflammation (Lang et al., 2002; Toth et al., 2005) are potential contributors to this metabolic alteration in the elderly. These findings are substantial, especially in developing countermeasures to prevent age-related conditions. This study has several limitations. First, this is a secondary analysis; therefore, the author had no control over data collection. Second, our small sample size may have limited the statistical power to detect a significant difference between the young and older groups and between pre-post exercise time points. Third, the difference between our sample population, healthy older adults, and the general aging population may affect our findings' external validity and generalization. Forth, the exercise protocol in terms of intensity, volume, and resting intervals may impact the analyte’s concentration variance. For example, RE programs with more
repetitions and brief resting intervals between sets elicit a higher energy demand and, therefore, higher increases in glycolysis products. Accordingly, the three-minute resting intervals in our study might have affected the production and utilization of various metabolites in the working muscle. Also, our results are limited to the metabolic platform, fasting status, and blood draw time points.

Tables and Figures

Table 1. Descriptive characteristics of young and older adults at baseline.

<table>
<thead>
<tr>
<th></th>
<th>Young (N = 30)</th>
<th>Old (N = 49)</th>
<th>Young vs Old</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range min-max</td>
<td>Mean ± SD</td>
<td>Range min-max</td>
</tr>
<tr>
<td>Sex, F/M¹</td>
<td>15F-15M</td>
<td>26F-23M</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>20–30</td>
<td>65-84</td>
<td>71.37 ± 4.53</td>
</tr>
<tr>
<td>Height, cm</td>
<td>154.3–185.5</td>
<td>149.6–192.05</td>
<td>168.74 ± 9.95</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>53.13–97.50</td>
<td>50.27–111.23</td>
<td>75.13 ± 13.45</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>19.90–28.95</td>
<td>21.45–37.20</td>
<td>26.28 ± 3.65</td>
</tr>
<tr>
<td>Heart rate, BPM</td>
<td>48–92</td>
<td>63 ± 8.50</td>
<td>63.50 ± 10.84</td>
</tr>
<tr>
<td>SBP, mm hg¹</td>
<td>97.50–139.50</td>
<td>107–170</td>
<td>129.70 ± 13.63</td>
</tr>
<tr>
<td>DBP, mm hg</td>
<td>47–86</td>
<td>53–93</td>
<td>72.95 ± 9.71</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>72–100</td>
<td>80–121</td>
<td>93.47 ± 8.31</td>
</tr>
<tr>
<td>Insulin, mIU/ml</td>
<td>2.9–16.4</td>
<td>2.4–29.7</td>
<td>7.90 ± 5.55</td>
</tr>
</tbody>
</table>

Note: M; Male, F; Female, BMI; body mass index, SBP; systolic blood pressure, DBP; diastolic blood pressure, *; statistically significant at the .05 level. Data are shown as mean ± SD.
Table 2. Plasma metabolites of young and older adults at baseline and after acute Resistance Exercise.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Young Pre-RE (N = 30)</th>
<th>Old Pre-RE (N = 49)</th>
<th>Young vs Old Pre-RE (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Carbohydrate Metabolites and TCA cycle intermediates</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactic acid</td>
<td>0.73 ± 0.19</td>
<td>0.81 ± 0.29</td>
<td>0.28</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>0.08 ± 0.02</td>
<td>0.09 ± 0.03</td>
<td>0.63</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.61 ± 0.38</td>
<td>5.85 ± 0.56</td>
<td>0.043*</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>0.005 ± 0.006</td>
<td>0.003 ± 0.01</td>
<td>0.37</td>
</tr>
<tr>
<td>Citric acid</td>
<td>0.15 ± 0.04</td>
<td>0.18 ± 0.03</td>
<td>0.03*</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0.08 ± 0.03</td>
<td>0.08 ± 0.03</td>
<td>0.63</td>
</tr>
<tr>
<td><strong>Ketone bodies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetoacetic acid</td>
<td>0.05 ± 0.07</td>
<td>0.05 ± 0.06</td>
<td>0.64</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.03 ± 0.02</td>
<td>0.03 ± 0.01</td>
<td>0.72</td>
</tr>
<tr>
<td>2-Hydroxybutyric acid</td>
<td>0.01 ± 0.03</td>
<td>0.02 ± 0.02</td>
<td>0.11</td>
</tr>
<tr>
<td>3-Hydroxybutyric acid</td>
<td>0.10 ± 0.09</td>
<td>0.08 ± 0.06</td>
<td>0.10</td>
</tr>
<tr>
<td><strong>Ketogenic amino acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>0.11 ± 0.02</td>
<td>0.11 ± 0.03</td>
<td>0.89</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.16 ± 0.06</td>
<td>0.19 ± 0.04</td>
<td>0.86</td>
</tr>
<tr>
<td><strong>Glucogenic amino acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>0.27 ± 0.05</td>
<td>0.28 ± 0.06</td>
<td>0.36</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.22 ± 0.04</td>
<td>0.27 ± 0.01</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.64 ± 0.11</td>
<td>0.68 ± 0.07</td>
<td>0.042*</td>
</tr>
<tr>
<td>Proline</td>
<td>0.06 ± 0.12</td>
<td>0.09 ± 0.14</td>
<td>0.29</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.08 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.13</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.05 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.23</td>
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<tr>
<td>Valine</td>
<td>0.27 ± 0.03</td>
<td>0.27 ± 0.04</td>
<td>0.79</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.04 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.05 ± 0.01</td>
<td>0.05 ± 0.02</td>
<td>0.59</td>
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<td>Phenylalanine</td>
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<td>0.04 ± 0.01</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Biogenic amines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatine</td>
<td>0.03 ± 0.02</td>
<td>0.04 ± 0.02</td>
<td>0.33</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.08 ± 0.02</td>
<td>0.09 ± 0.02</td>
<td>0.008*</td>
</tr>
<tr>
<td><strong>Other amino acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ornithine</td>
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<td>0.01 ± 0.01</td>
<td>&lt;0.0001*</td>
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<tr>
<td>Trimethylamine N-oxide</td>
<td>0.03 ± 0.02</td>
<td>0.03 ± 0.01</td>
<td>0.004*</td>
</tr>
<tr>
<td>Alpha-aminobutyric acid</td>
<td>0.02 ± 0.03</td>
<td>0.03 ± 0.03</td>
<td>0.29</td>
</tr>
</tbody>
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*Note: Data are shown as mean ± SD with asterisk (*) denoting where significant (p-value < 0.05) differences were observed.*
Table 2. Continued: plasma metabolites of young and older adults at baseline and after acute Resistance Exercise.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Overall RM-ANOVA (P-value)</th>
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<tr>
<td></td>
<td>Age</td>
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<tr>
<td>Carbohydrate Metabolites and TCA cycle intermediates</td>
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</tr>
<tr>
<td>Lactic acid</td>
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<td>Pyruvic acid</td>
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<tr>
<td>Glucose</td>
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<tr>
<td>Glycerol</td>
<td>0.852</td>
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<tr>
<td>Succinic acid</td>
<td>0.876</td>
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<tr>
<td>Citric acid</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Acetic acid</td>
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</tr>
<tr>
<td>Ketone bodies</td>
<td></td>
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<tr>
<td>Acetoacetic acid</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Acetone</td>
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</tr>
<tr>
<td>2-Hydroxybutyric acid</td>
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<tr>
<td>3-Hydroxybutyric acid</td>
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<tr>
<td>Ketogenic amino acids</td>
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<tr>
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<td>Lysine</td>
<td>0.017</td>
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<td>Glucogenic amino acids</td>
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<tr>
<td>Glycine</td>
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<td>Phenylalanine</td>
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<tr>
<td>Biogenic amines</td>
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<td>Creatine</td>
<td>&lt;0.0001</td>
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<tr>
<td>Creatinine</td>
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<td>Other amino acids</td>
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<tr>
<td>Ornithine</td>
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<tr>
<td>Trimethylamine N-oxide</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Alpha-amino butyric acid</td>
<td>0.023</td>
</tr>
</tbody>
</table>

Note: Data are shown as mean ± SD with asterisk (*) denoting where significant (p-value < 0.05) differences were observed.
Figure 1. Changes in carbohydrate, TCA cycle intermediates, and ketone body metabolites pre-post RE in young and older groups. Values are shown as mean ± SD.
Figure 2. Changes in Glucogenic and Biogenic amines pre-post RE in young and older groups.

Values are shown as mean ± SD.
Figure 3. Changes in plasma amino acids and metabolites pre-post RE in young and older groups, and pre-post-RE fold-change in young and older groups. Values are shown as mean ± SD.
CHAPTER III
A randomized trial of omega-3 fatty acid supplementation and circulating lipoprotein subclasses in healthy older adults

Abstract

Omega-3 polyunsaturated fatty acids (n3-PUFAs) have been shown to reduce triglycerides in individuals with dyslipidemia, but it is yet unknown if n3-PUFAs affect lipoprotein profiles in healthy older adults. This exploratory study aimed to examine the biological effects of dietary n3-PUFA supplementation on plasma lipoprotein subfractions in healthy, non-hypertriglyceridemic older individuals. Thirty young (20-35 years) and 54 older (65-85 years) men and women were enrolled in the study. Following baseline sample collection, 44 older adults were randomly assigned into placebo (n = 22) or treatment (n = 22) groups. They began a six-month supplementation with a placebo (corn oil) or n3-PUFAs (3.9 g/day, 675 mg EPA, 300 mg DHA). Following the intervention, older adults repeated the study procedures, with fasting blood samples collected following three days of a weight-maintaining diet and overnight stay in the hospital. In addition, 30 young participants were studied as a control group who did not receive an intervention. Pre- and post-intervention plasma samples were used for quantitative lipoprotein subclass analysis using high-resolution proton nuclear magnetic resonance (1H-NMR) spectroscopy. Post-intervention analysis revealed that six months of n3-PUFA supplementation decreased the number of large, least-dense LDL particles by 17-18% compared to placebo (<1% change, treatment*time p <0.01). The number of small, dense LDL particles increased 26-44% with n3-PUFA compared to placebo (~11% decrease, treatment*time p <0.01). The cholesterol content of large HDL particles increased by 32% with n3-PUFAs and by 2% in placebo (treatment*time p <0.01). The cholesterol content of small HDL particles.
decreased by 23% with n3-PUFAs and by 2% in placebo (treatment*time p <0.01). Despite an increase in the abundance of small, dense LDL particles associated with cardiovascular risk, n3-PUFAs reduced total triglycerides and systolic blood pressure. Furthermore, changes in the composition of HDL subclasses, particularly an increase in the cholesterol contents in the larger, less dense HDL particles and a decrease in the cholesterol and Apo-AI contents in the smaller, denser particles, may be associated with the atheroprotective function of HDL particle.

Everything considered, our findings suggest potential benefits of n3-PUFA supplementation to lipoprotein profiles in healthy older adults without dyslipidemia, which should be considered when evaluating the potential health benefits against the ethical and ecological aspects of the widespread use of n3-PUFA supplements.
Introduction

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) have been known for their triglyceride-lowering effect for decades (Harris et al., 1983; von Lossonczy et al., 1978). These bioactive lipids may prevent atherosclerotic cardiovascular disease (ASCVD) by reducing insulin resistance and through their anti-inflammatory properties (Oh et al., 2010; Spencer et al., 2013). These potential benefits of n-3 PUFAs are particularly relevant to healthy aging, although both are still under debate due to heterogeneous and inconsistent results from randomized controlled trials (Barry & Dixon, 2021; A. Lalia & Lanza, 2016). Dyslipidemia and inflammation are important factors in developing several age-related chronic illnesses. Hence, dietary n-3 PUFAs may be a part of a primary prevention strategy for older adults.

The potential protective and therapeutic effects of dietary n3-PUFAs have been examined in healthy older adults without hyperlipidemia or ASCVD. Published studies indicate that n3-PUFA supplementation in generally healthy older adults improves markers of insulin sensitivity (A. Z. Lalia et al., 2017), improves key aspects of mitochondrial physiology (Herbst et al., 2014; A. Z. Lalia et al., 2017), enhances skeletal muscle protein synthesis (A. Z. Lalia et al., 2017; Smith et al., 2011), and enhances strength gains with exercise training in older women (Rodacki et al., 2012). Despite some promising early evidence (Xyda et al., 2020), there still needs to be more data on whether n3-PUFA supplementation benefits healthy older adults without dyslipidemia or ASCVD. The environmental impact, cost, sustainability, and other adverse effects must be considered.

In this direction, the objective of this study was to perform a secondary analysis of a randomized, double-blind, placebo-controlled study to determine the effects of n3-PUFA supplementation on plasma lipoprotein subfractions in healthy older adults in the absence of
ASCVD or hypertriglyceridemia. The quantitative 1H-NMR spectroscopy may help understand the systemic compositional changes in plasma lipoprotein subclasses following n3-PUFA supplementation in healthy older adults.

Methods

Participants and study design

All study procedures were approved by the Mayo Foundation Institutional Review Board (IRB no. 17–004403, ClinicalTrials.gov Identifier: NCT03350906) and conformed to principles outlined in the Declaration of Helsinki. Thirty young (27.7 ± 4.1 years) men and women and 54 older (71.39 ± 4.40 years) men and women were recruited from the southeast Minnesota area. Inclusion criteria were age 20-35 years old for the young group and 65-85 years old for the older group. Participants were excluded if they reported regular use of omega-3 nutritional supplements, diabetes or fasting plasma glucose greater than 126 mg/dL, anemia (hemoglobin less than 11 g/dL for females and less than 12 g/dL for males), active coronary artery disease or history of unstable macrovascular disease, renal failure (serum creatinine greater than 1.5 mg/dL), active liver disease (AST greater than 144 IU/L or ALT greater than 165 IU/L), history of blood clotting disorders, anticoagulant therapy, international normalized ratio (INR) greater than 2.0, substance abuse, untreated or uncontrolled hypothyroidism, pregnancy or breastfeeding, or fish or shellfish allergy. Following written informed consent, participants in the older age group were randomly assigned to placebo or n3-PUFA based on a randomization table prepared by a statistician. Masking of investigators and participants to the group assignments was maintained by the Mayo Clinic Research Pharmacy and unblinding protocols implemented by the pharmacy staff and the Mayo Clinic IRB. The pharmacy staff handled all aspects of
medication procurement, storage, and dispensation to participants in accordance with the randomization table. A total of 25 older adults were randomized to receive omega-3 fatty acid ethyl esters, sold commercially as Ocean Blue® Professional Omega-3 2100™. Participants were instructed to swallow two soft gels with a morning meal and 2 soft gels with an evening meal. Each soft gel was formulated as 1000 mg liquid-filled capsules with approximately 675 mg of EPA and 300 mg of DHA for a total daily dosage of 3.9 g/day for 6 months. A total of 24 older adults were randomized to the placebo group and instructed to swallow 2 1000 mg soft gels containing corn oil twice per day with morning and evening meals for 6 months. A total of 5 participants dropped out after randomization (2 placebo, 3 n3-PUFA, supplemental figure 1). Placebo and n3-PUFA soft gels were identical in appearance, and both contained an orange flavoring. Participants received a 1-month supply of soft gels from the research pharmacy. Participants reported to the CRTU after an overnight fast every 4 weeks to obtain a prescription refill and for fasting blood samples to measure liver function and coagulation. Unused medication was returned to the research pharmacy to assess participant compliance. The participants in the young age group were studied as a comparator group and did not undergo the intervention. Fasting blood samples were collected from a peripheral vein prior to the intervention and again following 6 months of placebo or n3-PUFA. Whole blood was immediately processed to isolate plasma, which was stored at -80 until analysis. Additional whole blood specimens were used to isolate red blood cells (RBCs) for measurement of EPA and DHA concentrations. Following centrifugation, packed erythrocytes were resuspended in cold 0.9% NaCl and centrifuged again, and this step was repeated twice before freezing at -80. Concentrations of EPA and DHA were measured against a standard curve on a triple quadrupole mass spectrometer coupled with an Ultra Pressure Liquid Chromatography system (LC/MS) as
previously described (15). Plasma glucose and insulin were measured in the clinical chemistry lab by Cobas analyzer. Free living physical activity was measured by accelerometer (Actigraph wGT3X-BT) over a two-week period. A participant flow diagram is included as online supplemental material (supplemental figure 1). The lipoprotein particle analysis was not predeclared as a primary or secondary endpoint of the study and should be considered exploratory post-hoc analyses.

\(^1\)H-NMR spectroscopy and sample preparation

We used high-resolution proton nuclear magnetic resonance (\(^1\)H-NMR) spectroscopy for quantitative lipoprotein subclass analysis according to the Bruker B.I.-LISA lipoprotein platform standard operating procedure. Plasma samples were thawed on ice and mixed with Bruker VERBR plasma buffer (phosphate buffer pH 7.4 containing 0.1\% TSP-\(d_4\) (3-(trimethylsilyl)-2,2,3,3-tetradetero propionic acid)) in 9:1 (v/v) ratio. 300 \(\mu\)L of plasma was mixed with 300 \(\mu\)L of phosphate buffer and transferred to an Eppendorf tube, with 70 \(\mu\)L of buffer subsequently added prior to vortexing the mixture for 20 s. The sample was then centrifuged at 2400 x g for 10 minutes, and 600 \(\mu\)L of the supernatant was transferred to a 5 mm NMR tube. The NMR spectra were collected using a Bruker 600 MHz Avance III HD spectrometer with a BBI room temperature probe head and SampleJet auto sampler (Bruker Biospin, Billerica, MA). Proton spectra were acquired using 1D NOESY pulse sequence with presaturation (noesygppr1d), over 32 total scans. Spectra were transferred to the Bruker Data Analysis server for automated remote analysis.
Statistical analysis

Due to the exploratory nature of these secondary outcomes of the trial, there were no formal sample size estimates performed *a priori*. Unpaired *t* tests were used to evaluate the effect of ageing on lipoprotein concentrations between young adults and the pre-intervention samples from older adults in both arms of the trial. A one-way analysis of covariance (ANCOVA) was conducted to determine the effect of omega-3 supplement on lipoprotein concentrations of older adults, using baseline values as a covariate. In other words, the placebo and treatment groups were compared after adjusting for baseline values. ANCOVA is known to be superior in randomized pretest-posttest studies because it gives the largest power and the smallest confidence interval (Van Breukelen, 2006). Normality, homogeneity, and linearity checks were carried out and the assumptions met. The estimated marginal mean differences (adjusted mean differences) and confidence intervals are reported. All results were analyzed using RStudio, version 1.3.95.

Results

Participant characteristics

Participants' characteristics pre- and post-intervention are shown in Table 1. At baseline, prior to intervention, young and older adults were similar in height, weight, and body mass index. Systolic blood pressure was significantly higher in older adults compared to young with no difference in diastolic blood pressure (Table 1). Physical activity, assessed from average steps per day was similar in young and older adults at baseline, although there was a non-significant trend (*P*=0.089) for lower time spent in moderate to vigorous physical activity (MVPA) in older adults (Table 1). Average steps per day and MVPA decreased from baseline to follow-up in both
treatment groups. A total of 30 young and 54 older adults were included in these baseline comparisons. At the time of analysis, 44 of the older adults completed follow-up studies and were included in the analysis of placebo vs n3-PUFA. These participants were studied between April 2018 and August 2020. The placebo and n3-PUFA groups were similar in age, body mass index, and blood pressure at baseline. Of these variables, only systolic blood pressure showed a significant treatment-by-time interaction, decreasing in the n3-PUFA group but not in the placebo group. A total of 14 participants were on statin therapy (6 in placebo group, 8 in n3-PUFA group). Fasting glucose was significantly higher in older compared to young at baseline (Table 1), but insulin and HOMA-IR were similar in young and older adults. Glucose, insulin, and HOMA-IR were significantly lower at follow-up in both groups. The concentrations of EPA and DHA in red blood cells increased significantly in the n3-PUFA group but not in the placebo group.

**Main lipid parameters**

Prior to intervention older adults exhibited normal triglycerides and only modestly elevated total cholesterol, LDL cholesterol, HDL cholesterol, Apo-A1, and Apo-B100 compared to young that did not reach statistical significance and is consistent with the overall good health of this cohort of older adults (Table 2). Lipoprotein particle numbers did not differ significantly between young and older adults except for the large, least-dense LDL-1 which were more abundant in older adults compared to young. Total triglycerides decreased by approximately 24% in the n3-PUFA group and by about 6% on average in the placebo group for an overall significant effect of time. Total cholesterol and LDL cholesterol were unchanged in both groups, while HDL cholesterol increased in the n3-PUFA and placebo groups. The two most abundant protein components of HDL, Apo-A1 and Apo-A2, both decreased in the n3-PUFA group but
not in the placebo group. VLDL particle number decreased in n3-PUFA and placebo groups, with a nonsignificant (p=0.16) group-by-time interaction. The group treated with n3-PUFA exhibited a marked shift in the distribution of LDL particles. Compared with the placebo group, the n3-PUFA group significantly decreased the number of large, less-dense LDL particles (LDL-1, LDL-2) and increased the number of small, denser LDL particles (LDL-5, LDL-6).

Main lipoprotein fractions

Although total triglyceride was similar in young and older adults at baseline, older adults exhibited significantly higher triglyceride content of LDL and HDL (Table 3, Figure 1A). The cholesterol content of VLDL, IDL, LDL, and HDL were not remarkably different between young and old, except for HDL free cholesterol, which was significantly higher in older compared to young (Table 3, Figure 1B-C). Older adults also exhibited a nonsignificant trend (p=0.072) for lower Apo-A2 content of HDL (Table 3, Figure 1E). Triglyceride content of VLDL and IDL decreased in the n3-PUFA and placebo groups, although the magnitude of decrease in IDL tended (P=0.079) to be greater in the n3-PUFA group (Table 3, Figure 1A). LDL triglyceride content increased in the n3-PUFA group but not placebo group (Table 3, Figure 1A). Total and free cholesterol content of VLDL and IDL decreased in both groups, while HDL cholesterol content increased to a greater extent in n3-PUFA compared to placebo (Table 3, Figure 1B-C). The Apo-A1 and Apo-A2 content of HDL decreased in older adults following n3-PUFA supplementation, but not placebo (Table 3, Figure 1E).
VLDL subfraction

The composition of 5 different VLDL particles, numbered in order of increasing density and decreasing size, were analyzed by NMR. Prior to intervention, older adults exhibited lower triglyceride and phospholipid content of the large, least-dense VLDL-1 subfraction compared to young (Table 4, Figure 2A, C). In contrast, older adults exhibited higher triglyceride, esterified cholesterol, and phospholipid content of the small, less dense VLDL-4 and VLDL-5 particles compared to young (Table 4, Figure 2A, B, D). Several VLDL subfraction parameters were found to change similarly in both groups, including decreased triglyceride and cholesterol (free and esterified), and phospholipid content of VLDL particles (Table 4, Figure 2 A-D). Several VLDL particle parameters demonstrated notable treatment-by-time interactions including cholesterol content of the VLDL-1 and VLDL-5 particles, which decreased in n3-PUFA but not placebo group (Table 4, Figure 2 B, C).

LDL subfraction

A total of 6 LDL particles of differing density and size were evaluated. Triglyceride content of large LDL particles (LDL-1, LDL-2, LDL-3) was significantly higher in older compared to young adults, with no differences by age in the small, dense LDL particles (Table 5, Figure 3A). The cholesterol (esterified and free), phospholipid, and ApoB content were significantly higher in older compared to young for LDL-1 particles, but similar for LDL-2,3,4,5, and 6 (Table 5, Figure 3B, C, D, E). Following the intervention, older adults treated with n3-PUFAs exhibited decreased triglyceride content of large LDL-1 particles and increased triglyceride content of smaller LDL-3, LDL-4, LDL-5, and LDL-6 particles compared to placebo (Table 5, Figure 3B, C, D, E). A similar general pattern was observed for cholesterol,
phospholipid, and Apo-B content where the n3-PUFA group decreased the content in large less-dense LDL particles (LDL-1, LDL-2, LDL-3) and increased content in smaller, denser LDL-4, LDL-5, and LDL-6 particles compared to the placebo group (Table 5, Figure 3B, C, D, E).

**HDL subfraction**

Four distinct HDL particle subfractions were evaluated. Older adults exhibited higher triglyceride content in large, least-dense HDL-1 particles and a nonsignificant trend (p=0.084) for higher triglyceride in HDL-2 particles compared to young (Table 6, Figure 4A). Esterified and free cholesterol content of HDL subfractions was similar in young and older adults except for a trend (p=0.086) for higher cholesterol in the large HDL-1 particles (Table 6, Figure 4B). Phospholipids, Apo-A1, and Apo-A2 content were similar in young and older adults except for Apo-A2 content of HDL-3 and HDL-4, which were lower in older compared to young (Table 6, Figure 4F). When compared with the placebo group, older adults treated with n3-PUFAs exhibited significant decrease in triglyceride content of small, least dense HDL-4 particles (Table 6, Figure 4A). A consistent pattern was observed for the effect of n3-PUFA supplementation on the cholesterol, phospholipid, Apo-A1, and Apo-A2 content of HDL particles where the content was increased in the larger HDL-1 and HDL-2 particles but reduced in the smaller, denser HDL-3 and HDL-4 particles (Table 6, Figure 4C, D, E, F).

**Discussion**

The main finding of this study shows that six-months of n3-PUFA supplementation in healthy older adults leads to modest reductions in triglycerides and cholesterol content but remarkable shifts in the distribution and composition of LDL and HDL particles. Triglyceride, cholesterol, phospholipid, and Apo-B content in the large, least-dense LDL particles decreased in
older adults supplemented with n3-PUFA while the smaller, densest LDL particles increased in this group. Furthermore, the number of small dense LDL particles increased in the n3-PUFA group. The concentration of cholesterol, phospholipid, Apo-A1, and Apo-A2 in HDL particles was enhanced by n3-PUFAs in the larger particles but decreased in the smaller, densest HDL particles. Due to the atherogenic character of these particles, the rise in small, dense LDL particles caused by n3-PUFAs may be detrimental to older adults. On the other hand, the critical observation that n3-PUFAs enhance the lipid and protein content of large HDL particles and reduce small, dense HDL particles, increases the likelihood of cardioprotective effects in elderly people. These findings encourage further research into the potential advantages and disadvantages of n3-PUFA supplementation in healthy older adults without hypertriglyceridemia.

The triglyceride-lowering effects of n3-PUFAs are well documented in people with hypertriglyceridemia (Harris et al., 1983; von Lossonczy et al., 1978). In the present study, healthy older adults showed a 24% reduction in circulating triglyceride levels caused by n3-PUFAs; however, this reduction did not reach statistical significance. The reduction in triglyceride content of the largest, least dense VLDL-1, VLDL-2, and LDL-1 particles, which contain most triglycerides of all lipoprotein subfractions, appears to be the primary cause of the overall triglyceride-lowering effect of n3-PUFAs supplementation. Other lipoprotein particles, particularly the smaller, denser VLDL and LDL particles and larger HDL particles showed different alterations in triglyceride concentration. As previously seen in individuals with dyslipidemia, one of the main ways that n3-PUFAs are thought to lower circulating triglycerides is by decreasing hepatic VLDL synthesis (Chan et al., 2003; Nestel et al., 1984). We speculated that the observed decrease in triglycerides may be due to the clearance of apo-B containing
lipoprotein because neither the overall Apo-B100 concentration nor the particle number changed significantly with n3-PUFA supplementation in the current investigation. Despite the fact that we did not evaluate VLDL flux in the current investigation, the absence of any change in Apo-B content of LDL or IDL suggests that increased catabolism of Apo-B containing lipoproteins is an unlikely explanation. Although the drop in VLDL Apo-B levels shown after n3-PUFA supplementation was not statistically different from that seen in the placebo group, there was a trend for a more significant decrease (20%) with n3-PUFA compared to placebo (7%). It is possible that the mechanism of triglyceride reduction by n3-PUFAs is different in people with dyslipidemia compared to those with normal triglycerides (Nestel et al., 1984). In fact, individuals with hypertriglyceridemia decreased VLDL generation, whereas those with normal triglycerides enhanced clearance (Nestel et al., 1984).

Among the major circulating lipoproteins, HDL is of particular interest because of its negative association with heart disease and important anti-atherogenic effects through its role in scavenging cholesterol and transporting it to the liver for hepatobiliary secretion. In the current study we observed a significant increase in total HDL cholesterol that was similar in n3-PUFA, and placebo treated older adults. This observation is consistent with prior studies in mildly hyperlipidemic individuals (Mori et al., 2000; Padro et al., 2015) but in contrast to other studies where HDL cholesterol increased following n3-PUFA supplementation (Suzukawa et al., 1995; Thomas et al., 2004). The composition and distribution of the distinct HDL subclasses provides additional insight beyond total HDL cholesterol levels given that the largest HDL particles are, in particular, inversely associated with ASCVD (Asztalos et al., 2004; Johansson et al., 1991).

We observe a distinct shift in the composition of HDL subclasses with n3-PUFA supplementation that was highly dependent on the size and density of the HDL particle. n3-
PUFAs increased the triglyceride, cholesterol, phospholipid, and Apo-A content of the larger, less dense HDL-1 and HDL-2 particles and decreased these parameters in the smaller, denser HDL-3 and HDL-4 particles. This finding is consistent with an observational study of monozygotic twins where n3-PUFA intake was associated with higher proportions of larger HDL particles and lower proportion of smaller HDL particles (Bogl et al., 2011) and prospective studies of n3-PUFA supplementation in healthy and hyperlipidemic humans (Calabresi et al., 2004; Thomas et al., 2004). It is likely that the shift in composition of HDL particles is a reflection of the prevailing number of HDL particles in circulation, but in the absence of specific measurements of HDL particle number, we are unable to determine the extent to which HDL composition shifts independently of particle number. The new data from the present study support that n3-PUFA supplementation shifts the distribution of HDL particles toward a cardioprotective profile in healthy older adults without hyperlipidemia.

The small, dense LDL particles are thought to more easily penetrate the walls of the arteries and exhibit other atherogenic properties, including prolonged clearance from circulation, susceptibility to oxidation, and affinity for proteoglycans (Lamarche et al., 1997; Sancho-Rodríguez et al., 2011). A paradoxical increase in the small, dense LDL particles in a small cohort of healthy older adults after an open-label n3-PUFA supplementation has been reported (Xyda et al., 2020); which contrasted with earlier reports of decreased small dense LDL particles after n3-PUFA supplementation (Baumstark et al., 1992; Minihane et al., 2000; Wilkinson et al., 2005). The current study confirms the earlier finding in a larger independent cohort of older adults with a rigorous randomized, placebo-controlled, double-blind study for six months. Although the amount of free and esterified cholesterol in the larger LDL particles significantly decreased with n3-PUFA supplementation, the amount of free and esterified cholesterol in the
small, dense LDL particles significantly increased. Total LDL cholesterol remained unchanged in response to n3-PUFAs. In addition to the changes in the LDL-C concentration, there was a remarkable increase in small, dense LDL-5 and LDL-6 particle numbers and an elevation in the Apo-B content associated with higher LDL-5 and LDL-6 particle numbers in older adults following n3-PUFA supplementation. These findings, while at odds with some published data (Baumstark et al., 1992; Minihane et al., 2000; Wilkinson et al., 2005), are in line with other reports (Mostad et al., 2008; Sullivan et al., 1986; Xyda et al., 2020), and they highlight a significant lack of consensus in the literature that merits further analysis. The extent to which these changes in LDL particle distribution with n3-PUFAs might be viewed as harmful or advantageous is still an unanswered subject. Increased small, dense LDL levels have been associated with elevated Apo-B concentrations and reduced HDL-C levels, which are positive risk factors for ischemic heart disease (Tornvall et al., 1991) and myocardial infarction (Austin, 1988). Furthermore, increased levels of small LDL particles combined with elevated Apo-B concentration leads to a sixfold higher risk of developing ischemic heart disease(Lamarche et al., 1997). Uncertainty exists regarding the relationship between the observed increases in small, dense LDL particles and any relevant cardiovascular risk, particularly in the absence of any HDL or Apo-B changes or reductions.

In conclusion, this study revealed that n3-PUFA supplementation in healthy older adults without dyslipidemia leads to small reductions in triglycerides and VLDL cholesterol content and notable shifts in the distribution and composition of HDL and LDL particles. Although the observed increase in small, dense LDL particles could be interpreted as an undesirable risk factor for ASCVD, the absence of any increase in Apo-B, reduction in total triglycerides, maintenance of total HDL, HDL particle profile, and reduced systolic blood pressure altogether suggest a
potential cardioprotective benefit of n3-PUFA supplementation in healthy older adults. An important unanswered question is whether these changes in lipoprotein subclasses translate into any meaningful benefit for healthy older adults. This question is especially pertinent in light of the ethical and environmental dilemmas associated to the global use of supplements containing marine-derived omega-3 fatty acids.

Several limitations should be acknowledged when interpreting the findings of the current study. First, the main limitation of this study was its secondary analysis nature. Second, this was a cross-sectional study, which limits our ability to evaluate the effect of aging on plasma lipoprotein profile, as causation cannot be implied. Third, our small sample size may have limited the statistical power to detect a significant difference between the treatment and placebo groups. Fourth, the older adults who participated in this study were screened to rule out several age-related chronic conditions. Participants excluded from our study with the aging-related disease may yield different outcomes. Thus, the current research results may not be generalized to the overall elderly population in the U.S. Fifth, the corn oil used in this study may have affected some of the measured variables in the comparison group. Lastly, our results are limited to the metabolic platform and type and dose of the supplement used in this study.
**Table 1. Descriptive characteristics.**

<table>
<thead>
<tr>
<th></th>
<th>Young (N = 30)</th>
<th>Old (N = 54)</th>
<th>Young vs Old</th>
<th>Old Placebo (N = 22)</th>
<th>Old n3-PUFA (N = 22)</th>
<th>Old n3 vs Old Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>P-value</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>15F/15M</td>
<td>28F/26M</td>
<td>&lt;0.001*</td>
<td>70.96 ± 4.30</td>
<td>71.54 ± 4.90</td>
<td>72.32 ± 4.32</td>
</tr>
<tr>
<td>Age (years)</td>
<td>27.7 ± 4.11</td>
<td>71.39 ± 4.40</td>
<td>0.20</td>
<td>167.29 ± 10.82</td>
<td>167.07 ± 10.96</td>
<td>169.61 ± 10.25</td>
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<tr>
<td>Height (cm)</td>
<td>171.35 ± 8.56</td>
<td>168.64 ± 9.72</td>
<td>0.74</td>
<td>72.47 ± 13.50</td>
<td>72.45 ± 13.83</td>
<td>77.69 ± 13.19</td>
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<tr>
<td>Weight (kg)</td>
<td>73.77 ± 11.35</td>
<td>74.73 ± 13.43</td>
<td>0.10</td>
<td>25.83 ± 3.62</td>
<td>25.87 ± 3.72</td>
<td>26.72 ± 3.70</td>
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<tr>
<td>BMI (kg/m2)</td>
<td>24.91 ± 2.70</td>
<td>26.16 ± 3.59</td>
<td>0.03*</td>
<td>64.04 ± 9.85</td>
<td>66.92 ± 11.35</td>
<td>61.66 ± 6.92</td>
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<td>Heart rate (BPM)</td>
<td>67.52 ± 10.84</td>
<td>62.91 ± 8.17</td>
<td>0.03*</td>
<td>128.25 ± 15.70</td>
<td>131.75 ± 19.59</td>
<td>131.08 ± 11.45</td>
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<tr>
<td>SBP (mm hg)</td>
<td>116.03 ± 10.10</td>
<td>129.34 ± 13.47</td>
<td>&lt;0.001*</td>
<td>74.23 ± 9.62</td>
<td>75.54 ± 13.10</td>
<td>71.72 ± 9.83</td>
</tr>
<tr>
<td>DBP (mmhg)</td>
<td>72.08 ± 9.13</td>
<td>73.34 ± 9.95</td>
<td>0.57</td>
<td>74.23 ± 9.62</td>
<td>75.54 ± 13.10</td>
<td>71.72 ± 9.83</td>
</tr>
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*Note:* M; Male, F; Female, BMI; body mass index, SBP; systolic blood pressure, DBP; diastolic blood pressure, *; statistically significant at the .05 level. Data are shown as mean ± SD.
TABLE 2. Main plasma lipoprotein parameters and particle number of young and older adults at baseline and older adults before and after 6 months of placebo or n-3-PUFA supplementation

<table>
<thead>
<tr>
<th></th>
<th>Young (N = 30)</th>
<th>Old (N = 54)</th>
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<td></td>
<td>Baseline</td>
<td>Follow up</td>
<td>Baseline</td>
<td>Follow up</td>
<td>Treatment</td>
<td>Time</td>
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<td>Triglycerides</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
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<tr>
<td>HDL Cholesterol</td>
<td>98.7 ± 44.3</td>
<td>99.7 ± 41.9</td>
<td>101 ± 41.7</td>
<td>77.1 ± 20.9</td>
<td>100 ± 47.5</td>
<td>94.3 ± 22.2</td>
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<td>LDL Cholesterol</td>
<td>178 ± 32.2</td>
<td>190 ± 43.2</td>
<td>180 ± 53.1</td>
<td>183 ± 35.9</td>
<td>195 ± 54.9</td>
<td>195 ± 36.4</td>
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<td>LDL-1</td>
<td>96.4 ± 26.2</td>
<td>101 ± 28.8</td>
<td>99.5 ± 26.4</td>
<td>106 ± 29.8</td>
<td>104 ± 34.4</td>
<td>103 ± 27.7</td>
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<tr>
<td>HDL Cholesterol</td>
<td>71.8 ± 10.5</td>
<td>55.5 ± 14.2</td>
<td>56.4 ± 12.3</td>
<td>58.6 ± 13.5</td>
<td>57.1 ± 16.7</td>
<td>60 ± 13.9</td>
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<tr>
<td>Apo-A1</td>
<td>31.1 ± 4.3</td>
<td>27.5 ± 5.2</td>
<td>27.3 ± 3.9</td>
<td>23.9 ± 3.1</td>
<td>26.9 ± 6.9</td>
<td>27.5 ± 5.6</td>
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<tr>
<td>Apo-B100</td>
<td>72 ± 20.7</td>
<td>78.6 ± 19.4</td>
<td>78 ± 17.4</td>
<td>79.8 ± 20.5</td>
<td>80.3 ± 22.9</td>
<td>76.3 ± 15.9</td>
</tr>
</tbody>
</table>

Values are mean ± SD unless otherwise indicated. TG, triglyceride.
### TABLE 3. Main plasma lipoprotein subfractions of young and older adults at baseline and older adults before and after 6 months of placebo or n-3-PUFA supplementation

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<th>Young (N = 30)</th>
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<td>P-value</td>
<td>F-value</td>
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<td><strong>Triglycerides, VLDL</strong></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>61 ± 33.1</td>
<td>53.8 ± 34</td>
<td>56.9 ± 33.6</td>
<td>42.4 ± 17.5</td>
<td>1.132</td>
<td>0.365</td>
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<tr>
<td><strong>Triglycerides, IDL</strong></td>
<td>8.3 ± 7.4</td>
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<td><strong>Cholesterol, VLDL</strong></td>
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<td>15.4 ± 3.6</td>
<td>17.7 ± 5.1</td>
<td>0.852</td>
<td>0.019</td>
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<td><strong>Cholesterol, IDL</strong></td>
<td>8.8 ± 2.7</td>
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<td><strong>Cholesterol, HDL</strong></td>
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<td>27.6 ± 5.2</td>
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Values are mean ± SD unless otherwise indicated.
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<th>Follow up</th>
<th>Old n3-PUFA (N = 22)</th>
<th>Baseline</th>
<th>Follow up</th>
<th>Treatment</th>
<th>Time</th>
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<td>7.6 ± 4.5</td>
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<td>2.5 ± 1.6</td>
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<td>2.1 ± 1.8</td>
<td>0.03 ± 0.8</td>
<td>1.8 ± 1.4</td>
<td>1.6 ± 0.7</td>
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<td>0.48 ± 0.4</td>
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<td>0.47 ± 0.5</td>
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Values are mean ± SD unless otherwise indicated.
## TABLE 5. Plasma LDL subfractions of young and older adults at baseline and older adults before and after 6 months of placebo or n-3-PUFA supplementation

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<th>Young (N = 30)</th>
<th>Old (N = 38)</th>
<th>Old n3-PUFA (N = 22)</th>
<th>Old Placebo (N = 22)</th>
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<td>9.0 ± 4.4</td>
<td>9.6 ± 3.8</td>
<td>9.9 ± 3.7</td>
<td>9.2 ± 4.5</td>
<td>&lt;0.001</td>
<td>8.375</td>
</tr>
<tr>
<td>Apo-B, LDL-1</td>
<td>11.6 ± 2.2</td>
<td>13.9 ± 3.4</td>
<td>13.5 ± 3.4</td>
<td>10.9 ± 2.8</td>
<td>0.006</td>
<td>12.394</td>
</tr>
<tr>
<td></td>
<td>8.9 ± 2</td>
<td>9.7 ± 3.3</td>
<td>9.1 ± 3.1</td>
<td>7.6 ± 2.5</td>
<td>0.006</td>
<td>6.070</td>
</tr>
<tr>
<td></td>
<td>9.8 ± 2.4</td>
<td>9.8 ± 3.3</td>
<td>9.3 ± 3.1</td>
<td>8.8 ± 3</td>
<td>0.006</td>
<td>3.560</td>
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<tr>
<td></td>
<td>9.1 ± 3.5</td>
<td>8.5 ± 3.8</td>
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<tr>
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<td>13.9 ± 6.2</td>
<td>14.4 ± 6.1</td>
<td>18.1 ± 6.4</td>
<td>0.006</td>
<td>8.921</td>
</tr>
</tbody>
</table>

Values are mean ± SD unless otherwise indicated.
Figure 1: Cross-sectional comparisons of main plasma lipid parameters measured by NMR in young and older adults, and the effects of 6 months of placebo and n3-PUFA supplementation on lipoprotein particles.

Data are shown as mean ± SD with asterisk (*) denoting where significant (p-value < 0.05) differences between young and older adults were observed at baseline and omega (Ω) denoting where significant treatment-by-time interactions were found. Apo-A: apolipoprotein-A, Apo-B: apolipoprotein-B, HDL: high density lipoprotein, IDL: intermediate density lipoproteins, LDL: low density lipoproteins, VLDL: very low-density lipoproteins.
Figure 2: Cross-sectional comparisons of plasma VLDL subfractions measured by NMR in young and older adults, and the effects of 6 months of placebo and n3-PUFA supplementation on lipoprotein particles.

Data are shown as mean ± SD with asterisk (*) denoting where significant (p-value < 0.05) differences between young and older adults were observed at baseline and omega (Ω) denoting where significant treatment-by-time interactions were found. VLDL: very low-density lipoproteins.
Figure 3: Cross-sectional comparisons of plasma LDL subfractions measured by NMR in young and older adults, and the effects of 6 months of placebo and n3-PUFA supplementation on lipoprotein particles.

Data are shown as mean ± SD with asterisk (*) denoting where significant (p-value < 0.05) differences between young and older adults were observed at baseline and omega (Ω) denoting where significant treatment-by-time interactions were found. Apo-B: apolipoprotein-B, LDL: low density lipoproteins.
Figure 4: Cross-sectional comparisons of plasma HDL subfractions measured by NMR in young and older adults, and the effects of 6 months of placebo and n3-PUFA supplementation on lipoprotein particles.

Data are shown as mean ± SD with asterisk (*) denoting where significant (p-value < 0.05) differences between young and older adults were observed at baseline and omega (Ω) denoting where significant treatment-by-time interactions were found. Apo-A1: apolipoprotein-A1, Apo-A2: apolipoprotein-A2, HDL: high density lipoproteins.
Supplemental figure 1: Participant flow diagram

Assessed for eligibility (n=131)

Young group (n=59)

- Excluded (n=29)
  - Declined consent (n=10)
  - Ineligible (n=7)
  - Withdrew consent (n=4)
  - Admin/medical decision (n=3)
  - Lost contact (n=2)
  - Noncompliance (n=2)
  - Other (n=1)

Older group (n=72)

- Excluded (n=18)
  - Declined consent (n=8)
  - Ineligible (n=4)

Baseline blood collection (n=54)

- Randomized (n=49)
  - Allocated to placebo (n=24)
    - Discontinued (n=2)
  - Allocated to n3-PUFA (n=25)
    - Discontinued (n=3)

- Dropped prior to randomization (n=5)

Analyzed (n=30)

Analyzed (n=54)

Analyzed (n=22)

Analyzed (n=22)
CHAPTER IV
The Ethics of Employing Nonhuman Animals in Empirical Research with an Emphasis on Aquatic Animals

Introduction

Despite the growing political and ethical concerns and the increasing availability of alternative strategies, nonhuman animals\(^3\) continue to be exploited in the biomedical sector globally. According to an estimate in 2015, more than 192 million nonhuman animals—including mice, rats, frogs, dogs, cats, rabbits, hamsters, guinea pigs, monkeys, fishes, and birds— are employed for scientific purposes worldwide each year (Taylor & Alvarez, 2019). This figure may be underestimated due to several factors, including a lack of transparent statistics from the research institutes in the United States. The Animal Welfare Act (AWA) in the U.S. excludes laboratory rats and mice, birds, and fishes; therefore, the number of these nonhuman animals is only partially reported to government agencies.

On the other hand, the Guide for the Care and Use of Laboratory Animals (Council et al., 2010), and the current Public Health Service policy (Office for Protection from Research, 1986) all permit the conduct of "Category E" studies in which animals experience significant pain or distress that will be left untreated because painkillers are anticipated to interfere with the study's objectives (Carbone, 2011). An estimation suggests that over 44 million AWA-covered rodents are experiencing pain or distress in laboratories. This estimation is based on the only available self-reported pain classification data in the U.S. and only includes rats and mice; therefore, the number of painful studies may be higher than indicated above (Carbone, 2011, 2021).

\(^3\) See note 2 above.
Nonhuman animals’ sentience and capacity to experience pain are no longer disputed. In 2012, Cambridge Declaration on Consciousness declared the scientific consensus that “…humans are not unique in possessing the neurological substrates that generate consciousness. Non-human animals, including all mammals and birds, and many other creatures, including octopuses, also possess these neurological substrates.” (Low et al., 2012). This agreement paved the way for an ongoing discussion over the different forms of consciousness and the capacity for nuanced experience among other creatures, primarily fishes, invertebrates, and insects.

Nonhuman animals are exploited not only as test subjects in research but also as dietary sources. If there are solid ethical grounds against exploiting nonhuman animals in science, these could be extended to viewing nonhuman animals as nutritional sources. The primary sources of n3-PUFAs, the dietary supplement used in this dissertation, are marine fatty fishes. According to statistics, overfished stocks have been steadily growing from the 1950s onward (Worm et al., 2006), causing rapid and substantial alterations to marine ecosystems and biodiversity. On the other hand, global aquaculture is rapidly growing, with yearly production increasing 50-fold between 1960 to 2015 (Ritchie & Roser, 2021). Aquatic species have hundreds of subgroups that live in various settings. As a result, we are still learning about their distinct needs and moral status. However, we have substantial evidence supporting the claim that fishes are sentient and deserving of moral status (Michaelson & Reisner, 2018). Therefore, it is necessary to investigate the ethical issues of utilizing nonhuman animals, particularly aquatic animals, in the name of science.

This chapter considers whether it is ethically justified to utilize nonhuman animals, particularly aquatic animals, in empirical research. The chapter briefly reviews the history of employing nonhuman animals in experiments. Next, it summarizes some of the scientific flaws
of nonhuman animal experimentation. Then it explores the ethical issues of utilizing nonhuman animals in experiments as testing subjects and dietary sources. Lastly, this chapter discusses several alternative technologies to nonhuman animal testing and alternatives to dietary polyunsaturated fatty acid supplements.

Overall, this chapter provides a deontological argument in favor of animal rights and calls for abolishing nonhuman animal exploitation in research, the food industry, and so forth. According to the theory of positionality, the sociopolitical location of a researcher influences what they choose to investigate and how they conduct the research (Holmes, 2020). In this regard, the author acknowledges that their positionality as a vegan and animal rights activist influenced this chapter to some extent.

A brief history of nonhuman animals’ utilization in experimental research and the emergence of nonhuman animals research ethics in Western culture

Vivisection, that is, performing exploratory operations on living beings, has been practiced since the rise of anatomy and medicine. Using live nonhuman animals in experiments was common in ancient Greece because humans were considered closer to the divine and hence ranked higher in the hierarchy of beings (Franco, 2013). This perspective later heavily shaped the Judeo-Christian notion of human supremacy and dominance over all of nature (Franco, 2013). With the rise of Christianity in Europe, nonhuman animal experiments were mostly halted as people became less concerned with the scientific causes of diseases (Franco, 2013). In Europe, physiological experiments on nonhuman animals re-emerged during the Renaissance (fifteenth–sixteenth centuries), and carried on throughout the Age of Enlightenment (seventeenth century), an era conducive to scientific growth (Franco, 2013). During this period, the philosophical and
theological attitude toward nonhuman beings provided scientists with a way to justify the use of nonhuman animals for horrifying experiments when the anesthetics were unavailable. René Descartes (1596–1650), for instance, described nonhuman animals as “machine-like” bodies (Regan & Singer, 1989), lacking consciousness, and therefore, incapable of feeling pain (Steiner, 1998, 2005). Later, Francis Bacon (1561–1626), known as the founder of modern scientific methodology, reapproved and justified vivisection or, as he stated “the dissection of beasts alive” (Bacon & Montagu, 1857; Franco, 2013). Despite the skepticism raised in opposition to inducing suffering in nonhuman animals in the name of scientific experiments, physiologists defended and justified their works by accepting the animal-machine paradigm (Franco, 2013; McClure, 2006).

In the eighteenth century, experimenting on live nonhuman animals became common and led to several significant contributions. Stephen Hales (1677–1761), a clergyman without formal medical training, first measured blood pressure by studying a live mare (DeMello, 2021; Franco, 2013). Antoine Lavoisier (1742-1794) designed an ice calorimeter and nearly froze a guinea pig in its chamber to measure the heat produced by respiration. He concluded that combustion and respiration are the same, and every episode of respiration involves combustion (Karamanou & Androutsos, 2013). It was not until the nineteenth century, when Eduard Pflüger (1829–1910), first showed that respiration is an intracellular activity (Karamanou & Androutsos, 2013), that Albrecht von Haller (1708–1777) demonstrated that animals feel pain by measuring their reactions to various painful stimuli (Conrad et al., 1995; Franco, 2013). Public demonstrations of live nonhuman animals experiments, notably Boyle's infamous air pump experiment (Franco, 2013), heightened resistance to vivisection. The debate among philosophers and physiologists was no longer about whether other animals could feel pain and to what extent they could, but
rather whether vivisection could be justified based on the benefits to humans. Many philosophers, from Voltaire (1694–1778) to Jean-Jacques Rousseau (1712–1778), Jeremy Bentham (1748–1832), and Arthur Schopenhauer (1788–1860), began to challenge viewing nonhumans as mere "means to an end" (Bentham, 1781; Rousseau, 1755; Schopenhauer, 1998). By referring to nonhuman animals as sentient beings, these thinkers proposed a transition from an anthropocentric point of view to a view wherein humans have responsibilities towards other animals for the benefit of the nonhuman animals themselves (Franco, 2013).

At the beginning of the nineteenth century, medical practice underwent a revolutionary paradigm shift. With the construction of hospitals and academies, laboratory experimentation became the gold standard of academic medicine. Furthermore, the French Revolution laid the groundwork for a flourishing academic environment in which science merged into medicine. Ultimately, patient experiments were replaced by nonhuman animal testing as an easy solution to the moral issues of clinical studies (Franco, 2013). Amongst many other scientists, French physiologist, François Magendie (1783-1855), known as the founder of experimental physiology, was notorious for his morally dubious exploratory experiments on nonhuman animals. By way of illustration, in one of his experiments, he fed dogs a regimen of sugar and distilled water to study the function of nitrogen in the diet (Semba, 2012), which resulted in blindness and the death of dogs after one month (Semba, 2012). Magendie was also notorious for performing public vivisections. He dissected a dog's facial nerves at one of these presentations in England, while the animal was nailed down by each paw and left overnight for additional surgery the next day (Tubbs et al., 2008). It is said that Magendie alone vivisected 4000 dogs to prove the theory of motor and sensory functions of the nerves, and then 4000 more to disprove it (Davis, 1885). All these experiments were carried out without anesthesia. Following the discovery of anesthetics,
nonhuman animal experimentation became routine in the 1840s, eliciting less guilt among the public. However, Magendie and almost all his students continued avoiding the use of anesthetic agents because it was thought to make the experiments unreliable (Preece, 2011).

Magendie’s successor, Claude Bernard (1813–1878) introduced experimental epistemology and advocated for rigorous vivisection on the grounds that humans have an absolute right to conduct experiments on animals. His description of the ideal vivisector is a physiologist who “…does not hear the animals' cries of pain. He is blind to the blood that flows. He sees nothing but his idea, and organisms which conceal from him the secrets he is resolved to discover” (Preece, 2011). Bernard described one of his experiments in which he dissected conscious, but paralyzed dogs. He wrote: “A dog was first rendered helpless (by curare) and incapable of any movement, even of breathing, which function was performed by a machine blowing through a hole cut in its windpipe; but its intelligence, its sensitiveness, and its will remained intact, a condition which, under the operation, was accompanied by the most atrocious sufferings that the imagination of man can conceive” (Davis, 1885).

Although vivisection was widely perceived as inhumane, a few publications played an essential role in the sharp increase in the use of nonhuman animals in research during the 19th century. Darwin’s 1859 publication, the *Origin of Species*, provided a solid scientific justification for recognizing the close kinship between humans and other animals, which gave both physiologists and anti-vivisectionists a strong argument for their respective positions (Franco, 2013). For anti-vivisectionists, the close relationship with nonhumans meant that vivisection could not be justified, no matter the scientific gain in knowledge. On the other hand, the biological similarities between nonhuman and human animals led to the increased use of nonhumans as a perfect model for scientific experiments. Bernard’s book *Introduction to the*
Study of Experimental Medicine (1865) provided a utilitarian justification for vivisection and convinced most scientific observers that nonhuman animal models are beneficial for human health and the development of medical and physiological knowledge (Franco, 2013; Preece, 2011). After Bernard’s publication and John Burdon-Sanderson’s Handbook for the Physiological Laboratory (1873), utility concerns revoked moral considerations and eventually led to the expansion of nonhuman animal experimentations in the 1870s in Britain (Franco, 2013; Preece, 2011).

The rise of nonhuman animal testing in the second half of the nineteenth century in Britain escalated public anger toward animal cruelty in the name of science. For most people, nonhuman animal cruelty was seen as related to violence against humans; hence, putting an end to it was in the public’s interest (Bates, 2017). The “Cruel Treatment of Cattle Act” of 1822 (also known as Martin’s Act) was one of the first nonhuman animal protection laws in recorded history (Bates, 2017; Franco, 2013). However, vivisection was not included in Martin’s Act probably because its author was unaware of nonhuman experimentations until 1824, when Magendie demonstrated public vivisection in London (Bates, 2017). Many feminists and suffragists also argued against nonhuman animal exploitation by noting that men victimized women and nonhuman animals in similar ways (DeMello, 2021). In 1875, Frances Power Cobbe (1822-1904), the famous feminist and suffragist, founded the world’s first nonhuman animal protection and anti-vivisection society: the Victoria Street Society (VSS), also known as National Anti-Vivisection Society (NAVS) (Bates, 2017; DeMello, 2021; Franco, 2013). The Society’s goals included preventing extreme experiments and eventually abolishing experimentation on live nonhuman animals. Cobbe drafted the Henniker bill in 1875, which was counteroffered by a group of scientists with Charles Darwin being the most prominent of them.
Opposition to vivisection eventually resulted in the First Royal Commission on Vivisection in 1875. This group drafted the “Cruelty to Animal Act” of 1876 (Vivisection Act) to regulate (legalized) the use of nonhumans in experiments, including licensing and supervising laboratories (Bates, 2017). This legislation exterminated the public demonstrations of painful experiments but kept the private use of nonhuman animals for medical research and education possible; and eventually led to more experimentation than if vivisection had remained unlicensed (Bates, 2017). Even though a small number of physiologists were involved in nonhuman animal experimentation, the public feared that the newly licensed vivisectors would become so desensitized that experimentation would be extended to human. In fact, Robert Koch (1843–1910) did experiment on poor people. Louis Pasteur (1822–1895) proposed experimenting on prisoners, and Jonathan Hutchinson (1828–1913) delayed the treatment of a patient with a painful disease to demonstrate the signs to his students (Bates, 2017). Despite all the criticism by VSS, the Cruelty to Animal Act 1876 remained valid for 110 years, until it was replaced by the Animals Scientific Procedures Act 1986 (Bates, 2017).

In the United States the anti-vivisection movement initially emerged in the 1860s and 1870s, with Caroline Earle White founding the American Anti-Vivisection Society (AAVS) in Philadelphia in 1883 (DeMello, 2021). The AAVS was originally established to regulate the use of animals in scientific study, but it later changed its goal to abolish such research. Similar to the NAVS, the AAVS was founded by women also involved in other social movements, such as the fight for women's suffrage, child protection, and anti-slavery (DeMello, 2021). Although the Cruelty to Animals Act was introduced in England in 1876, the Animal Welfare Act (AWA) was not passed in the United States until almost a century later (DeMello, 2021). Unfortunately, neither is adequate to safeguard research on nonhuman animals. The AWA regulates the
transportation, housing, feeding, and veterinary care of “warm-blooded animals” in laboratories, as well as animals bred and transported for the pet industry, zoos, and circuses. Mice, rats, and birds are not included in the USDA's definition of "warm-blooded animals" (DeMello, 2021). This is significant because, in at least 16 large American universities, rats and mice make up nearly 99.3% of all animals (Carbone, 2021). According to estimates, the United States alone employed 111.5 million mice and rats annually in 2017–18 (Carbone, 2021).

The expansion of the antivivisection struggle in the late nineteenth century paralleled with the rise of public health advancements from nonhuman animal research. For instance, Louis Pasteur and Robert Koch performed nonhuman experiments on the relationship between germs and diseases, which led to profound benefits for public health (Franco, 2013). Pasteur’s experiments required the infection of various animals, including dogs, chickens, rabbits, rodents, pigs, cows, sheep, and nonhuman primates (Franco, 2013). Although nonhuman animal experimentation became more common in the nineteenth century, the number of nonhumans exploited by physiologists during the entire century is insignificant in comparison to the hundreds of animals used by Pasteur to develop vaccines (Guerrini, 2021).

The twentieth century witnessed significant advancements in biomedical disciplines such as toxicology, immunology, and surgical procedures, based on nonhuman animal experimentation combined with the dissection of dead humans. This, in combination with the use of anesthetics in nonhuman experiments, resulted in more people accepting the benefits of scientific medical knowledge derived from nonhuman animal research. On the other hand, the emergence of rodent species as a nonhuman animal model in research helped silence the criticism of nonhuman experimentation. To the public, rodents were seen as insidious creatures unworthy of moral considerations, which sequentially made their use in research more
acceptable (Franco, 2013). Furthermore, two world wars and a remarkable economic decline toned down the antivivisection struggle until its revival in the 1970s (Franco, 2013). Nonhuman experimentation increased dramatically in the twentieth century. While Pasteur employed hundreds of nonhumans in his experiments, Paul Ehrlich (1854–1915) exploited thousands of mice to develop his syphilis medication two decades later. Millions of primates were sacrificed to produce the Polio vaccines in the 1950s (Guerrini, 2021).

The history of medical development illuminates the reliance of biomedical research on other animals. However, nearly in any body of scholarship, nonhuman animals are marginal and invisible. They appear because of their implications for human health and their capacity for medical development. Nevertheless, the most significant health threats are not species-specific. When we talk about human health, we also talk about nonhuman animals. The COVID-19 pandemic has highlighted the crucial intersections between nonhuman and human animal diseases, transmission, and treatments. This critical insight demonstrates the importance of adopting a less anthropocentric approach toward medical studies and placing nonhuman animals besides humans in the center of the discourse. The twenty-first-century issues such as emerging diseases that transmit between nonhumans and humans, antibiotic resistance, food insecurity, and climate change can only be tackled effectively through multidisciplinary approaches in which the health of nonhuman animals is considered in relation to that of humans, and the environment (Woods et al., 2018).

**Current discourse on the exploitation of nonhuman animals in experimental research**

Practical and ethical issues characterize the discourse concerning exploiting other animals in experimental research. The former answers whether employing nonhumans is essential in
biomedical research. The latter responds to whether using nonhuman animals in experiments can be morally justified if such experiments are shown to be necessary from an empirical perspective. Gary Francione referred to these matters as the “necessity issue” and “justification issue,” respectively (Francione, 2007). This section focuses on the current discourse around employing nonhuman animals in empirical research from the necessity and the justification perspective.

The issues of necessity

In 1994, the U.S. Public Health Service claimed, without evidence, that animal research was responsible for every significant development in human medicine during the last century. Since then, this claim has been a prevalent argument among proponents of nonhuman animal experiments. However, it remains unclear whether using nonhuman animals in research is necessary or even reliable for medical advances (Matthews, 2008).

The claims of necessity are problematic in many ways. First, the knowledge and scientific advancement derived from using other animals in research have been greatly exaggerated. Since other animals are almost always utilized in experiments, no one can claim with certainty that medical discoveries attributed to nonhuman animals would not have been achieved without using them (Francione, 2007).

Second, as discussed more in-depth in the next section, nonhuman animal models are significantly unreliable. In-vivo research outcomes can vary depending on laboratories, methodologies, species, and strains used in experiments (Akhtar, 2015; Francione, 2007; Pippin, 2012). Despite efforts to improve nonhuman experimentation, the significant failure rate in drug testing and development suggests that these models are inadequate and, thus, implausible to yield
valuable information about human diseases (Akhtar, 2015; Pippin, 2012). Moreover, because humans and other animals differ biologically, extrapolating the findings of nonhuman animal experiments to human conditions is controversial (Akhtar, 2015; Pippin, 2012). Despite the implicit assumption that nonhuman animal experiments contribute positively to medical advancement and knowledge, numerous instances show that they have been counterproductive and misleading data from nonhuman animal studies can harm human health (Akhtar, 2015; Pippin, 2012).

Third, the assumptions that animal welfare laws protect laboratory animals and that the researchers who observe such laws take a morally responsible position regarding the treatment of other animals are overly optimistic (Francione, 2007). Most nonhuman animals employed in research, such as rats and mice, are not even covered under the Animal Welfare Act. Hence, there are no data available to support such claims. Some have argued that researchers inflict only the necessary amount of suffering, yet this claim is also questionable (Francione, 2007). Evidence suggests that researchers who believe that other animals feel pain still do not consider even invasive procedures painful to lab animals (Phillips, 1993).

Lastly, all necessity arguments build upon the premise that alternatives to answer human health problems are unavailable. One rebuttal to this assumption is that the outcome might be better if the funds devoted to nonhuman animal research were invested in other research methods (Akhtar, 2015; Francione, 2007). Several alternatives to in-vivo research will be discussed later in this chapter.

In the following pages, three main factors undermining the reliability of nonhuman animal experimentation to inform human health will be discussed: 1) the effects of the laboratory environment and research design on study outcomes. 2) physiological, anatomical, and genetic
differences between the species. 3) disparities between nonhuman models of disease and human diseases due to inherent differences.

The nonhuman animal studies are inherently flawed by design

Laboratory conditions and routine procedures cause significant fear, stress, and distress in nonhuman animals, which ultimately have substantial scientific and ethical implications. Keeping the animals in captivity, subjecting them to laboratory stress-inducing conditions, such as artificial lighting, human-produced noises, restricted housing environments, and any non-invasive handling procedure, including lifting a nonhuman animal, cleaning, or moving the cage, cause significant and prolonged elevations in stress-related responses, as well as an alteration in the immune system and abnormal behaviors among laboratory species (Akhtar, 2015; Balcombe et al., 2004, 2004; Morgan & Tromborg, 2007). Laboratory conditions alter nonhuman animals' neurochemistry, genetic expression, and nerve regeneration. A computational analysis of a large dataset showed that the researcher performing the test and numerous other laboratory variables, such as season/humidity, cage density, time of day, sex, and within-cage testing order, can have an impact on nociception (Chesler et al., 2002). Moreover, noise levels in laboratories have been shown to cause blood vessel damage in laboratory animals. Even the type of flooring can impact whether a medicine is effective in spinal cord injury trials in nonhuman animals (Akhtar et al., 2008). The blood sampling procedure is a stressful measure for nonhuman animals. It has been shown that serum cortisol levels rise in cynomolgus macaques watching other monkeys undergo blood draws (Flow & Jaques, 1997). Similarly, rats' blood pressure and pulse rates rise when they see fellow rats being decapitated (Balcombe et al., 2004). This is remarkable because it shows that nonhuman animals are not only sentients but also social beings who can feel each
other’s pain. To give another example, the handling, confinement, and electrical shock implemented in Treadmill-based forced-exercise procedures cause a significant increase in corticosterone and norepinephrine levels, a strong stress response in the biological system (Balcombe et al., 2004). Most laboratory research on nonhuman animals is intrinsically and significantly stressful, as well as ethically “inhumane” and scientifically “unreliable.” These stress-related physiological changes add variables that can confound data and obstruct human extrapolation.

**Interspecies differences in physiology and genetics**

Nonhuman animals share many biological and psychological features with humans, most notably the shared experiences of pain, fear, and suffering. In contrast, evolutilional distance in physiology, anatomy, genetics, and behavior between humans and other animals outweighs the similarities, invalidates the reliability of in-vivo studies, and hinders the ability to extrapolate data derived from nonhuman animals to humans.

In a review study of nonhuman animal models in spinal cord injury (SCI), Akhtar et al. (2008) showed that drug testing results vary across species and even different strains within a species (Akhtar et al., 2008). Surprisingly, test results appear to be altered even when the rodent models are from the same strain but from different suppliers (Akhtar, 2015). In addition, an earlier study of the heritability of nociception showed that responses to 12 different pain sensitivity assays - including thermal, chemical, and mechanical nociception - varied among 11 strains of mice (Mogil et al., 1999). The anatomical and biological mechanisms underlying the inability of nonhuman animal models of SCI to translate to subsequent human trials have been extensively reviewed (Akhtar et al., 2008).
A verity of nonhuman animals, including rats, mice, nonhuman primates, pigs, and dogs, is used to recreate spinal cord injuries. SCI models can be classified as contusion, compression, distraction, dislocation, transection, and chemical (Cheriyan et al., 2014). The first weight-drop contusion model of SCI was developed in 1911 (Allen, 1911). One year later, New York University developed a more advanced contusion device, which was later adopted for use in the Multicenter Animal Spinal Cord Injury Study (MASCIS), an NIH-funded multi-site preclinical drug trial conducted in the 1990s (Cheriyan et al., 2014). The injury infliction procedure involves a laminectomy at the targeted level and stabilization of the spinal cord. A rod of specific weight is dropped from a precise height above the surface of the cord to induce SCI. The study group recommends inflicting the injury 60 min after the administration of anesthesia, but strict adherence to these recommendations appear to be impractical (Cheriyan et al., 2014). It is worth mentioning that despite the enormous impact of MASCIS on the SCI field, the results of their treatments failed to produce precise positive results and, as a result, were never published. Until recently, Almeida et al. (2021) recovered and digitized the MASCIS Dark data (unpublished raw data) from 1125 rats (Almeida et al., 2021).

Further illustrating the physiological differences among species, in 2013, a sizeable systematic comparison of the genomic response between human inflammatory diseases and murine models reported that the inflammatory responses in mouse models poorly correlate with human conditions (Seok et al., 2013). They compared the gene expression patterns in human inflammatory diseases, including sepsis, burns, infection, and trauma, with the mouse models of these conditions. The analysis of about 5,000 genes showed no correlation between mouse and human conditions in the magnitude or direction of expression change. Furthermore, gene expression patterns required longer time to return to normal in humans than in mice (Seok et al.,
In another study, researchers discovered that, among the more than 4,000 genes in both humans and mice, 41 to 89 percent of the time, transcription factor binding sites varied between the two species (Gawrylewski, 2007).

Mice have been used in most of the genetic research, particularly those involving diseases, not only because their genomes are comparable to those of humans, but also because of their availability, convenience in handling, high reproductive rates, and low cost of use. Despite genomic similarities, most mice do not replicate the same genetic diseases as humans. Genetic modification to induce human disease in nonhumans is manly approached in two methods: non-directed method in which radiation and chemicals are used to cause mutations. Directed techniques include transgenesis, single-gene knock-outs and knock-ins, conditional gene modifications, and chromosomal rearrangements. The use of transgenic mice has dramatically increased in the past two decades, wherein the DNA sequences are artificially inserted into the mouse genome. However, one issue with transgenic models is that human genes will likely function differently when expressed in mice. In humans, for example, clathrin isoform CHC22 has a role in blood glucose clearance by sorting the GLUT4 glucose transporter to an insulin-responsive intracellular compartment in skeletal muscle and fat (Vassilopoulos et al., n.d.). However, this protein is absent in mice. When the human CHC22 gene was expressed in transgenic mice, it had the opposite effect from that observed in humans: the mice developed some diabetic symptoms, including high blood sugar and reduced responses to insulin (Vassilopoulos et al., n.d.).

The incompatibility between mice models and humans led some researchers to use nonhuman primates (NHPs), with the implicit bias that NHPs will better mimic human conditions. However, failures in translation have invalidated this assumption. For example,
misleading NHPs research results contributed to decades of inappropriate hormone replacement therapy (HRT) in millions of postmenopausal women to cure menopausal symptoms and prevent cardiovascular disease (Akhtar, 2015; Pippin, 2012). By the end of the twentieth century, more than sixty-five million menopausal hormone therapy (MHT) prescriptions were generated annually, and more than six million American women were taking an estrogen and progestin combination drug (Pippin, 2012; Spake, 2002). In 2002, The Women’s Health Initiatives reported that an average 5.2-year follow-up among healthy postmenopausal US women confirmed a 26% increased breast cancer risk, 29% increased risk of coronary heart disease, 41% increased risk of stroke, 107% for deep vein thrombosis, and 113% for pulmonary embolism (Rossouw et al., 2002).

A further example of notable failure in translating nonhuman animal experimentation is the HIV/AIDS vaccine research. Despite decades of time and enormous resources devoted to creating NHP models of HIV, all the vaccines that succeeded in NHPs failed in clinical trials to prevent or treat HIV infection in human animals (Bailey, 2008). In fact, most of the products that improved life expectancy and quality of life for those who live with HIV/AIDS have come from clinical trials with human beings (Bailey, 2008). HIV vaccine tests in other animals have not yet yielded accurate predictions of how the vaccines will work in humans. After six large-scale vaccine efficacy trials against HIV (Duerr et al., 2012), each at the cost of over US$100 million, no candidate vaccine has shown enough efficacy to be approved for clinical use. The first three of these trials failed to demonstrate any protection against the acquisition of HIV infection. In the third of these trials, the STEP study (Matthews, 2008) was stopped once the first interim data analysis confirmed that the vaccine did not prevent HIV infection or mitigate viral load (Matthews, 2008; Pippin, 2012). Furthermore, a four-year follow-up study of infected
participants documented a 40% statistically significant increased risk of HIV infection among vaccinated individuals compared to placebo recipients (Buchbinder et al., 2008).

**Disparities between nonhuman models of diseases and human diseases**

Human diseases are usually artificially induced in other animals; however, the immense challenge of replicating anything close to the complexity of human diseases in nonhuman animals limits the utility of the latter. Animal studies appear to overestimate the likelihood that a treatment will be therapeutic by roughly 30% because negative results are frequently unpublished (Sena et al., 2010). Even if the studies are designed and conducted appropriately, the results' translation to the clinic may fail due to differences between the nonhuman experimental model and the human condition. According to the FDA, 92% of medications that enter into clinical trials do not pass Phase I (Hackam & Redelmeier, 2006). According to a more recent analysis, despite efforts to improve the predictability of nonhuman testing, the failure rate has risen to nearly 96% (Pippin, 2012).

Stroke research is an essential example of the failure in modeling human diseases in nonhuman animals. Nonhuman animal research has been the default scientific approach to studying and developing stroke treatments for decades. However, systematic reviews and meta-analyses have proven the futility of nonhuman animal research in developing a treatment for human animal stroke. In one study, Macleod (2005) documented that all 150 human drug trials for stroke have failed to improve survival. He estimated that approximately 250,000 animals were used between 1985 and 2005 in these failed studies (Haynes & Mascola, 2017; Pippin, 2012). Despite efforts, such as the STAIR guideline, to standardize stroke study protocols, nonhuman animal stroke experiments fail to translate to human animals successfully. Most
nonhuman animals do not naturally develop significant atherosclerosis. Thus, researchers restrict the blood vessels or artificially insert blood clots to model atherosclerosis in nonhuman animals (Akhtar, 2015). These methodologies, however, cannot represent the underlying causes of atherosclerosis. The difficulty of mimicking the condition in other animals in a way comparable to human stroke has contributed to a high drug development failure rate (Akhtar, 2015).

Despite decades of research, nonhuman animal study has been unproductive in developing effective treatments for major motor neuron diseases, including amyotrophic lateral sclerosis (ALS), primary lateral sclerosis (PLS), and progressive and spinal muscular atrophy (Pippin, 2012). To give an example, Traynor et al. (2006) reported 113 ALS drugs derived from nonhuman animal studies, all of which failed to benefit human patients (Traynor et al., 2006). Currently, there is no cure for ALS. Riluzole is the only drug available for ALS treatment in the United States, which can only slow the disease's progress (Pippin, 2012).

For more than forty years, rodent models have been employed to study human cancer. Nevertheless, a 2011 study showed that 95% of the cancer drugs developed from nonhuman animal models failed in phase III clinical trials (Hutchinson & Kirk, 2011). The TGN1412 experiment is a prominent illustration of an effective nonhuman animal model that did not translate into clinical trials (Attarwala, 2010). To confirm TGN1412's safety and effectiveness in preclinical animal models, several nonhumans, including mice, were examined before undertaking human trials. These toxicity experiments showed that harmful effects were not induced at doses a hundred times higher than those given to humans. Despite being delivered at a dose 500 times lower than the level deemed acceptable in animal studies, TGN1412 fatally damaged patients' organ systems during the initial human clinical trials (Attarwala, 2010).

Several limitations have been identified for this significant failure in nonhuman animal research.
in the study of human cancer. For one thing, since rodent models are intentionally immune-deficient to accept unregulated tumor growth, they present an unrealistic biological environment (Pippin, 2012). Second, nonhuman models cannot mirror humans’ tumor architecture, growth rate, or drug response (Pippin, 2012).

Genetically modified mouse models have been employed in Alzheimer's disease (AD) research for over 20 years. Despite tremendous advances in transgene animal technology, all the amyloid-targeted drugs to reach phase III clinical trials have failed (Pippin, 2012). Some of the most evident obstacles to these failures include different pharmacological effects of the same substance on humans versus rodents, different neurotransmitter pathways in human and rodent brains, differences in drug and metabolism exposure; and limitations in modeling the complete spectrum of human pathology (Geerts, 2009).

The fundamentally utilitarian cost-benefit argument that human benefits justify the harms to animals in research is in jeopardy when there is a real risk of serious harm to humans, including lost opportunities, resource waste, and risks to human research subjects. An honest assessment of the costs, harms, and advantages of nonhuman research clearly show that very few of these studies are necessary or valuable. Hence, very few of them are ethically justifiable in utilitarian terms.

The justification issue: the moral justification for employing nonhuman animals in empirical research

The utilitarian viewpoint holds that a course of action is acceptable only when it achieves a better balance of harms and benefits. From a deontological perspective, however, even if a few instances of nonhuman animal research proved to be necessary for biomedical research, this
research would still be morally unjustifiable. Both nonhuman and human animals have an interest in not being used as means to someone else’s ends (Francione, 2007). Therefore, both types of beings are entitled to the same moral status and ought not to be exploited. The defining elements of morality are disputed; however, many interpretations hold that sentience and cognitive capacities play a significant role in moral agency (Jaworska & Tannenbaum, 2013). Humans’ right not to be exploited as non-consenting subjects in experiments is safeguarded by law and ethical considerations, regardless of whether the outcomes of utilizing them would benefit the rest of humanity.

The human-centric idea that nonhuman animals “were intended for human use” can be traced back to early Western history and still appears with surprising frequency in day-to-day views, philosophical debates, experimental studies, and even animal rights literature (Linzey & Linzey, 2017). Moral anthropocentrism or human exceptionalism is a hierarchical belief that humans are the most essential entities in the world, and thus, only humans possess intrinsic value. This view has been recorded in the work of Xenophon and Aristotle in classical Greece (Preece, 2011) and later manifested itself in the Judeo-Christian perspective of human superiority over all nature (Franco, 2013; Linzey & Linzey, 2017). Moral anthropocentrism overlooks the interests of other animals or, if it does accept nonhuman beings’ interests, dismisses the moral significance of these interests.

One of the most significant barriers to the progress of nonhuman animal ethics is Cartesianism. Its founder, Rene Descartes (1596-1650), believed that nonhuman animals are soulless machines created by God, and therefore, they are rendered senseless, incapable of feeling pain or pleasure (Regan & Singer, 1989). Although this animal-machine paradigm was
heavily criticized at the time, it reassured many physiologists that animals could be treated and experimented on without any ethical considerations (Franco, 2013).

By the end of the eighteenth century, Immanuel Kant (1724–1804) declared the idea that individuals should be treated “always as an end and never solely as a means.” (Preece, 2011). Kant rejected Cartesian mechanistic views and acknowledged other animals’ sentience; however, he believed that nonhuman animals were rationally inferior and denied them moral status (Linzey & Linzey, 2017; Preece, 2011). The key aspect of Kant's theory regarding the moral status of animals is the notion of will. According to his anthropocentric beliefs, other animals are incapable of having a good will, since they lack any kind of will, hence, they do not possess any inherent value. Therefore, humans’ duty to other animals is indirect. Humans may use other animals for their purposes but should treat them with care. This virtuous treatment is not for other animals’ sake; instead, it is humans’ moral self-duty to improve their dignity (Linzey & Linzey, 2017; Preece, 2011).

Many philosophers, including Voltaire (1694–1778), Jeremy Bentham (1748–1832), and Arthur Schopenhauer (1788–1860) advocated for a transition from our duty of compassion to nonhuman animals, to human obligations towards other animals for the sake of the animals themselves (Franco, 2013). Jeremy Bentham is widely considered the first Western philosopher to propose that nonhuman animals should be treated with the same moral respect as humans. He attributed moral standing to other animals by referring to sentience rather than intelligence in nonhumans. He famously stated: "the question is not, can they reason? Furthermore, are they able to communicate? However, can they Suffer?" (Bentham, 1781). However, he also approved of killing and using animals, provided pointless cruelty was avoided. According to Bentham’s utilitarian philosophy, which holds that moral behavior maximizes overall wellbeing for all
parties involved, he considered nonhuman research acceptable, acknowledging that humans had priority over nonhuman animals within the bounds of respect for other animals suffering (Franco, 2013).

This utilitarian point of view significantly impacted contemporary discourse regarding employing nonhuman animals in experiments, even when concern about the inflected suffering of other animals is present. A direct link can be traced from Bentham’s utilitarianism to Peter Singer’s book Animal Liberation (Singer, 1975). According to Singer, because other animals can experience pain and suffering, they have an interest in avoiding such suffering. Singer does not endorse an absolutist standpoint on nonhuman animals and their rights; instead, he condemns most nonhuman experiments by applying a principle of equal consideration of interests. In doing so, he claims that a great measure of "good," such as a treatment for an incurable disease, could justify a nonhuman animal experiment. He acknowledges that humans may be given "preference" over other species, since the former possess sophisticated linguistic abilities, self-consciousness, an awareness of others, as well as the capacity to plan for the future (Singer, 1975).

In the 1970s, Richard Ryder coined the term *speciesism* to explain human-centered prejudice against other beings, analogous to racism and other forms of irrational discrimination (Singer, 1975). Singer characterized the notion of speciesism as a widespread “prejudice or attitude of bias in favor of the interests of members of one’s species and against those of members of other species.” (Singer, 1975).

Animal rights advocates, however, have proposed one of the most influential arguments against using nonhumans in research. Tom Regan (2004), and Gary Francione (2008), for instance, adopted an abolitionist and deontological rights approach, which is the view that other
animals have inherent value (Regan, 2004), and an interest in not being used merely as means (Francione, 2007). Because of this inherent value, nonhuman animals are entitled to the same moral status as human beings and ought to be respected. According to Regan, moral status should be grounded on rights, not the utilitarian principle (Regan, 2004). When nonhuman animals are used in experiments to advance human research, they are merely being employed as means to humans’ ends. Similarly, nonhuman animals raised for food are not considered an end in themselves, regardless of how they are treated or slaughtered. This is particularly the case of fishes and other aquatic animals.

**Consciousness and sentence in Fishes**

Sentience, “the capacity to suffer or experience enjoyment or happiness” (Singer, 2011), is a crucial concept in animal ethics and animal rights policy discourses. For many philosophers (Bentham, 1781; Singer, 1975) and non-philosophers (Browning & Birch, 2022), the capacity for subjective experience is necessary and sufficient for having moral standing and deserving some form of formal protection.

In 2012, the Cambridge Declaration on Consciousness (Low et al., 2012) professed the scientific consensus that “… humans are not unique in possessing the neurological substrates that generate consciousness. Non-human animals, including all mammals and birds, and many other creatures, including octopuses, also possess these neurological substrates.” This consensus opened the floor for a new and evolving debate concerning variations of consciousness among other animals, mainly fishes, invertebrates, and insects.

Whether fishes are sentient has been the subject of discussion for decades. In 1980, the first concrete evidence to show that fish, like all vertebrate creatures, experience pain was
published (RSPCA, 1980). Subsequently, the U.K. government's Farm Animal Welfare Committee (1996) recognized that fish experience anxiety, stress, and pain when removed from the water and that their physiological mechanisms for feeling pain are remarkably similar to those of mammals (Lymbery, 1992).

Giving fishes the same level of protection as other vertebrates would have significant ramifications. Fishes are among the most widely used species of vertebrates by humans; they are caught from wild stocks as part of the worldwide fishing industry, raised under intensive aquaculture conditions, kept as companion animals, and employed in scientific studies, being the second-most commonly used species after mice (Brown, 2015). However, compared to warm-blooded nonhuman animals, fishes rarely receive the same amount of compassion. The fact that humans rarely interact with fishes in their natural habitat and the substantial discrepancy between public perceptions of fish intelligence and scientific reality contribute to the lack of attention given to fishes in ethical discussions.

The cumulative collection of evidence offers compelling justification for assuming that fishes experience pain: bony fishes, like rainbow trout, have nociceptors comparable to those found in mammals. Fishes are found to have nociceptors (Sneddon et al., 2003), which are receptors designed to detect noxious stimuli. Fishes exhibit pain-related physiological and behavioral changes, including increased gill ventilation rate and performance of aberrant behaviors, such as rubbing lips against the walls and floor of their tank after being injected with acetic acid (Sneddon et al., 2003). These pain-related reactions in rainbow trout are dramatically mitigated by the administration of morphine (Sneddon, 2003). Rainbow trout exposed to unpleasant stimuli also indicate a change in motivational state, feed less (Sneddon et al., 2003),
and exhibit less antipredator behavior (Ashley et al., 2009). This evidence shows that pain is more significant to them than fear or predator avoidance behavior (Sneddon, 2015).

In addition, there is a broad spectrum of evidence that cephalopods are sentient. It has been shown, for instance, that octopus and squid have nociceptors, which connect to the central nervous system (Bazarini & Crook, 2020; Browning & Birch, 2022; Crook, 2021). However, it is yet unknown whether these nociceptors connect to the large integrative vertical lobe of the brain. After being exposed to noxious stimuli, octopus and squid exhibit altered behavior, such as engaging in defensive behavior (Bazarini & Crook, 2020), being more receptive to threats (Oshima et al., 2016), and grooming wounded arms (Crook, 2021). Additionally, injured octopuses favor a chamber with analgesia (Crook, 2021).

Fish exhibit a wide variety of cognitive behaviors. For instance, they exhibit social intelligence, establish complex traditions, have good long-term memories, recognize each other and cooperate, and even utilize tools (Brown, 2015). Preference attachment in guppies (Heathcote et al., 2017), social motivation in cichlids (Galhardo et al., 2011), free-choice exploration (Graham et al., 2018), and increased shoaling behavior when kept in naturalistic environments in zebrafish are a few other examples of social cognitive behaviors and positive emotion in fishes (Franks, Graham, et al., 2018). Perhaps the most remarkable finding is evidence that giant manta rays passed the Mirror Self-Recognition test, often regarded as the gold standard for exhibiting self-awareness (Ari & D’Agostino, 2016).

The literature on whether and how fishes experience pleasure has grown increasingly. Fish leaping and somersaulting spontaneously, as well as the deliberate manipulation of non-functional objects and social interaction that meets the criteria for play, have all been the subject of studies going back to the early 20th century (Burghardt, 2006; Franks, Sebo, et al., 2018). Play
may be the best illustration of behavior frequently seen in the animal kingdom as naturally occurring. At least in humans, play is linked to delightful emotions like joy and amusement (Franks, Sebo, et al., 2018).

The absence of a neocortex, the brain region most closely linked to conscious experience in humans, is cited by opponents of the idea that fishes and invertebrates have sentience (Key, 2016). However, this argument can be countered by the multiple realization thesis in the philosophy of mind (Bechtel & Mundale, 1999), involving that the same mental property, state, or event can be realized by different physical properties, states, or events. In other words, there is no evidence to support the idea that the neocortex is required for the generation of pain or sentience in general, nor is there evidence to support the idea that similar subjective experiences cannot be produced by different mechanisms (Browning & Birch, 2022).

Humans are increasingly harming fishes at an alarming rate. At least 280 million tons of fishes were estimated to be captured and raised in aquaculture in 2018 (FAO, 2020). This number is much larger considering the illegal and unreported fishing activities. According to the Food and Agriculture Organization (FAO, 2020), Fisheries and Aquaculture Department, illegal fishing has caused losses estimated at US$23 billion per year. Along with the destruction of habitats and overfishing of wild populations, the use of fishes in science is also growing quickly.

If fishes are sentient, some techniques they are subjected to, such as destructive fishing techniques using dynamite or cyanides, live cutting, and boiling, are likely to cause significant suffering. Current behavioral and neurophysiological evidence leans toward the possibility that all fish species process information at the conscious level and are capable of experiencing psychological stressors in addition to physiological stressors (Sánchez-Suárez et al., 2020). Even
though the current body of literature does not definitively resolve the lingering questions about most nonhuman animal species, enough evidence warrants reconsidering current treatments of fishes.

The *precautionary principle* is the moral course one should take when faced with uncertainty, particularly given the asymmetry of risk and the significant amount of suffering humans inflict on fishes (Bradshaw, 1998; Elder, 2014). The precautionary principal aids in decision-making when faced with numerous unknowns. It states that the burden of proof that a course of action is not harmful rests with the individual taking it when clear consensus is absent. According to this principle, one should postpone taking any possibly harmful actions until the action have been proven harmless. Given the ambiguity of extant scientific evidence and lack of scientific consensus, the precautionary principle ensures that the least amount of suffering is inflicted on the fishes (Elder, 2014).

**Alternatives to nonhuman animal experimentations**

Science’s approaches to nonhuman animal research must undergo a paradigm shift (Taylor, 2019). One strategy is to use technology to solve some of the ethical issues the use of nonhuman animals raised. It is time to abandon the traditional method of providing results derived from nonhuman animal models in preclinical studies. Since the 1960s, efforts have been undertaken to develop advanced technologies to substitute for nonhuman animal testing (Taylor, 2019). These alternative methods encompass a wide range of procedures, from in-vitro cell-based models and in-silico computer-based systems to more advanced technologies such as micro-physiological systems (MPS, organ-on-a-chip) (Virumbrales-Muñoz & Ayuso, 2022).
Organ-on-a-chip is a promising microfluidic cell culture system that simulates several organ-specific microarchitecture and pathophysiology in vitro (Shrestha et al., 2019); including the lung (Benam et al., 2016; Shrestha et al., 2019), heart (Sakamiya et al., 2020), liver (Lauschke et al., 2019), kidney (Lee & Kim, 2018), and intestine (Bein et al., 2018).

Microfluidic organ-on-a-chip technology has the potential to revolutionize preclinical nonhuman toxicity testing by simulating organ-level pathophysiology and clinical responses. Heart-on-a-chip devices have lately emerged as a reliable and promising platform for cardio-related drug screening applications due to their ability to capture important biological and physiological parameters of cardiac tissue (Kitsara et al., 2019; Sakamiya et al., 2020). A sensor-integrated liver-on-a-chip device can track the dynamics of metabolic response to mitochondrial dysfunction in real time. It can identify chemical toxicity before detecting any effects on cell or tissue viability (Bavli et al., 2016). A human liver-on-a-chip can predict the safety of drugs with 87% sensitivity compared to less than 50% using existing nonhuman animal models (Ewart et al., 2021). Lung-on-a-chip technology has been utilized to investigate viral infections and to facilitate the development of vaccines and therapeutics for viral diseases (Afewerki et al., 2022; Tang et al., 2020). For instance, the models have been used to study how the influenza virus developed to become drug-resistant and to discover novel resistance mutations (Tang et al., 2020). Moreover, several organ-on-a-chip models, such as gut-on-a-chip (Shin & Kim, 2022), intestine-on-a-chip (Bein et al., 2021) and airway-on-a-chip (Gard et al., 2021), have been utilized as a potential model to study SARS-CoV-2 infections (Afewerki et al., 2022).

Most of the conversation regarding alternatives to nonhuman animal research continues to be focused on Russell and Bruch’s 3Rs principles. The ideas of replacement, reduction, and refinement (3Rs) to improve the condition of nonhumans while raising the standard of scientific
and medical experimentation were introduced in *The Principles of Humane Experimental Technique* in 1959 (Russell & Burch, 1959). Russell and Burch defined replacement as “the substitution for conscious living higher animals of insentient material” (Russell & Burch, 1959). Replacement is not defined in the Principles as using non-animal material instead of nonhuman animals. Replacement is the use of insentient material instead of sentient material. Russell and Burch did not define replacement as not using other animals because they classify the use of insentient animals as instances of replacement. The 3Rs principles fail to address the ethics of employing nonhuman animals in research. This is problematic and can have significant negative consequences.

One consequence of using ambiguous definitions for "replacement" is the widespread use and acceptance of nonhuman animals as "alternatives," including fishes (Zebrafish), invertebrates, animal tissues, embryos, sera, and animal parts, even though these nonhuman animals will suffer or be slaughtered (Redmond, 2019). As mentioned earlier in this chapter, many legislations presumed to protect laboratory animals do not protect all nonhuman animals. The AWA, for instance, covers fewer than 10% of nonhumans used in US laboratories because it excludes birds, rats and mice, and cold-blooded species, including fishes, reptiles, and amphibians, as well as farm animals used in agricultural research such as cows and pigs (Carbone, 2021; DeMello, 2021).

Over the past three decades, Zebrafish have become by far the most prominent species of fish used in animal research (Message & Greenhough, 2019). In the EU, the use of fishes in research has increased more than any other species (European Commission, 2013). Fishes are utilized frequently in the U.K., ranking second after mice (Hudson-Shore, 2016). Zebrafish currently make up about 50% of all fishes employed in U.K. laboratories because of their lower
cost than mammals, the accessibility of their eggs for manipulation, and their short generation time (Hudson-Shore, 2016; Vliet, 2011). Fishes in general, but zebrafish specifically, are frequently viewed as the “easier ethical option” and have become a “widely accepted relative replacement model” in basic and physiological research (Message & Greenhough, 2019; Redmond, 2019).

Alternatives to marine-derived omega-3 polyunsaturated fatty acids

In recent decades, long-chain polyunsaturated amino acids have become recognized as significant boosters of human health, leading to increased global demand for omega-3 lipid-rich foods and supplements. These lipids are essential components of the neural cell membranes in humans. It has been suggested that n-3 PUFA consumption, particularly EPA and DHA, can lower the risk of cardiovascular, neurological, and inflammatory conditions (Barry & Dixon, 2021; Oh et al., 2010; Spencer et al., 2013).

The traditional commercial source for n-3 PUFA production is fish oil. According to the estimations, the global intake requirement is approximately 1.3 million metric tons of EPA/DHA annually. While the total EPA/DHA available for human consumption (200 000 metric tons) represents only about 15% of the human requirement (Salem & Eggersdorfer, 2015). It is crucial to emphasize that omega-3 consumption is still far lower than the recommended 500 mg/day across most of the world, if not wholly absent (Finco et al., 2017).

Consuming fish oil entails ethical and environmental issues. As discussed earlier, there is convincing evidence to support the view that fishes are sentient beings, and there are not even enough of them to meet the demand in the human population. Therefore, it is necessary to search
for alternative sources, eliminate the use of marine-derived omega-3 fatty acids in research, and make alternative sources available to the public.

Polyunsaturated fatty acids are present in several sources. Plant-derived n3-PUFA, particularly flaxseed and walnut, are essential sources of ALA; however, they cannot neutrally produce EPA and DHA (Santos et al., 2020). Although the conversion of ALA into EPA and DHA is insufficient in humans, only 5% of ALA is converted to EPA, and less than 0.5% of ALA is converted to DHA, research suggests that plant sources of ALA are positively linked to favorable cardiometabolic (Santos et al., 2020) and brain function outcomes (Nguemeni et al., 2013). These effects may be driven by other plant components, such as fiber and minerals, working in concert with ALA (Santos et al., 2020).

Transgenic plant oils are terrestrial alternatives for marine-derived omega-3 fatty acids. Genetically modified plants accumulating EPA and DHA have long been considered a noble pursuit (Domergue et al., 2005). Both Arabidopsis and Camelina have been found to exhibit a range of EPA and DHA values (Napier et al., 2015). However, genetically engineered canola (Brassica napus) has been developed as one of the first land-based production systems for omega-3 fatty acids (Walsh & Metz, 2013), and its safety for human or nonhuman foods is well-established (MacIntosh et al., 2021). It has been reported that one hectare of a genetically modified Brassica napus crop producing 12% DHA oilseed would be equivalent to that produced from 10000 fish (Petrie et al., 2012). Recently, transgenic Camelina sativa has been shown to produce EPA and DHA at similar or greater levels than that found in Northern Hemisphere fish oils (Napier, 2021).

The use of single-cell organisms to produce oils for the food sector advanced in the middle of the 1970s (Finco et al., 2017). The oleaginous microorganisms, including fungi,
yeasts, bacteria, and microalgae, can produce up to 2000 kg/m³ of lipid annually, while other oleaginous sources, including soybean, peanut, rapeseed, palm, and coconut, may only produce 500–5000 kg/ha of lipids every year (Thevenieau & Nicaud, 2013). In addition to the benefits related to production capacity, microbial oils can be produced with minimal space requirements and can be extracted in any climate. Last but not least, when generated on a large scale, microbial oils have a smaller negative effect on the environment (Finco et al., 2017).

Microalgae and microalgae-like protists are the best natural resource of high-value molecules such as pure EPA/DHA (Spolaore et al., 2006). Many microalgae species, including Nannochloropsis, Phaeodactylum, Schizochytrium, and Thraustochytrium, have been studied for their high EPA/DHA levels (Adarme-Vega et al., 2014). Phaeodactylum tricornutum (Yongmanitchai & Ward, 1991) and Nannochloropsis sp. (Sukenik, 1991) were found to have an EPA content of up to 39% of total fatty acids when grown in an autotrophic environment. In a similar vein, Thraustochytrium and Schizochytrium limacinum were reported to contain DHA levels between 30 and 40% of total fatty acids, when grown in heterotrophic fermentative conditions (Adarme-Vega et al., 2012). Schizochytrium sp. is considered as an essential and suitable substitute for fish oil due to its rapid development, great purity, and little fishy scent (Liang et al., 2020). By optimizing the media composition and culture conditions, Schizochytrium sp. can accumulate lipids up to 50% of its dry weight, with DHA typically making up 40% or more of the total oils (Liang et al., 2020; Ren et al., 2014; Sun et al., 2014).

Currently, less than 2% of human EPA/DHA consumption is derived from algal oil. However, this source has increased significantly because of several socially desirable qualities, such as its environmental friendliness, the absence of ocean-borne contaminants, its vegetarian
nature, and the ability to manufacture under kosher or halal conditions (Salem & Eggersdorfer, 2015).

Conclusion

Nearly all the current regulatory frameworks in place for nonhuman research are utilitarian. These regulations favor the interest of the human species while exploiting members of other species. AWA is the only federal law in the U.S. governing how nonhuman animals are treated during research, exhibition, transportation, and by suppliers. However, AWA excludes birds, rats, and mice bred for research. AWA does not cover farm animals used for food or fiber, cold-blooded species, invertebrates, horses not used for research purposes, or fishes.

There must be a paradigm shift in how nonhuman animal research is conducted. Nonhuman animal research can change due to technological advancements ranging from in-vitro cell-based models and in-silico computer-based systems to more complex methods like micro-physiological systems. However, alternatives to nonhuman animal testing are not free of limitations. For example, the current practice of fetal calf serum (fetal bovine serum) harvesting is inhumane. Fetal calf serum is the most widely-used component of in vitro cell culture media (Jochems et al., 2002; Redmond, 2019). Bovine fetal blood is harvested by cardiac puncture, performed by inserting a needle directly into the heart of the unanesthetized fetus (Jochems et al., 2002; Redmond, 2019). Other supplies, including horse, goat, rabbit, porcine, and chicken serum, are sold as less expensive alternatives (Redmond, 2019). Every year, between 1-2 million bovine fetuses are used worldwide. Therefore, it is a mistake to think that cell culture procedures involving fetal bovine serum provide an alternative to the use of animals in research (Redmond, 2019). It is essential to go beyond the 3Rs principles, adopt an abolitionist strategy, and
contribute to advancing alternative technologies to ultimately liberate nonhuman animals from being exploited for scientific purposes.

Moreover, consuming marine-derived omega-3 fatty acids, the supplement used in this dissertation series entails ethical and environmental issues. Therefore, it is necessary to search for alternative sources, eliminate the use of fishes in research, and promote plant-derived omega-3 fatty acids to the public. As vegetarianism and veganism are rising in popularity for various reasons, including individual choices and environmental and ethical concerns, the introduction of ALA-rich foods is a cornerstone for people looking for omega-3 sources other than fish and fish oil. Transgenic plants, microalgae, and microalgae-like protists are promising alternatives for fish oil omega-3 fatty acids. Most alternative sources of n−3 PUFA discussed in this chapter are currently available as commercial formulations. However, there is a lack of research investigating the bioavailability of n-3 fatty acids and blood indicators in vegetarians and vegans (Lane et al., 2022). A few randomized control trials that have examined plant-based n-3 supplementation rarely used the proper vegan and vegetarian populations. As a result, there is limited advice regarding suitable plant-based n-3 substitutes for fish oil (Lane et al., 2022). Future research should determine the ideal EPA and DHA dosages and ratios for vegetarian and vegan populations. An official recommendation for plant-based alternatives is essential for vegetarian and vegan people.
CHAPTER V

Conclusion

This interdisciplinary dissertation series sought to establish a solid empirical foundation for employing NMR spectroscopy to identify and quantify the metabolic response to acute RE and n3-PUFA in the healthy aging population. This dissertation also aimed to investigate the ethical issues associated with employing nonhuman animals for research purposes. Accordingly, two NMR-based metabolomics research studies were conducted to 1) investigate the effect of acute RE on plasma metabolites in healthy older adults; and 2) determine the effect of n3-PUFA supplementation on plasma lipoproteins in healthy older adults. The third study examines ethical dilemmas in using nonhuman animals in science, emphasizing aquatic animals.

Study one revealed that the metabolic response to acute RE is age dependent. The concentrations of several plasma metabolites were significantly elevated in older adults at baseline. The exercise-induced response to RE was considerably weaker among the older group. These findings are important in developing effective countermeasures to prevent age-related conditions. The second study discovered that n-3 PUFA supplementation decreased total triglycerides and altered the distribution and composition of HDL and LDL particles. Despite the increase in small, dense LDL particles associated with a higher risk factor for ASCVD, the absence of any increase in Apo-B, reduction in total triglycerides, maintenance of total HDL, and reduced systolic blood pressure altogether suggest a potential cardioprotective benefit of n3-PUFA supplementation in healthy older adults. These findings should be considered alongside the environmental impact and ethical issues of prescribing marine-derived ω-3 fatty acid supplements. Moreover, this dissertation investigated the moral ramifications of using nonhuman
animals for food and research. Study three concluded that there is an urgent need for a paradigm shift in utilizing nonhuman animals in research. Consuming marine-derived omega-3 fatty acids, the supplement used in this dissertation series entails ethical and environmental issues. It is necessary to search for alternative sources, eliminate the use of fishes in research, and promote plant-derived omega-3 fatty acids to the public. As vegetarianism and veganism are rising in popularity for a variety of reasons, including individual choices, and environmental and ethical concerns, the introduction of ALA-rich foods is a cornerstone for people looking for ω-3 sources other than fish and fish oil. Genetically modified plants and oleaginous microorganisms, including fungi, yeasts, bacteria, and microalgae, are promising alternatives for fish-oil omega-3 fatty acids.

Significance

1H-NMR spectroscopy is a comprehensive method for the analysis of the overall metabolic signature of a biological system (Dunn et al., 2011). By employing 1H-NMR spectroscopy, we can investigate the global metabolic impacts of dietary and physical activity interventions and their underlying biochemistry and metabolic pathways in the human organism. The metabolic response to physical activity has been the subject of multiple research projects using metabolomics in recent years (Schranner et al., 2020); nevertheless, most of these studies have concentrated on metabolic changes following endurance exercise in young and healthy individuals. Therefore, the depiction of differences in metabolomic activity following resistance exercise among older people remains lacking. Moreover, it is still challenging to comprehend the precise plasma compositional changes caused by n3-PUFA supplementation in the healthy aging population. This may be particularly important to target because of the high
ethical and environmental costs associated with the widespread use of n3-PUFA supplements. Therefore, it remains crucial to determine whether consuming marine-derived omega-3 fatty acid supplements are beneficial and ethical for humans and nonhuman animals. The studies included in this interdisciplinary dissertation series fill these substantial gaps in the literature by investigating the metabolic responses to acute resistance exercise and dietary n3-PUFA supplementation at the molecular level in healthy older adults by employing 1H-NMR spectroscopy to characterize plasma metabolites and lipoproteins and their underlying metabolic pathways. In addition, this work will shed light on the ethical considerations of viewing fishes as food sources, while cruelty-free alternatives are available to consumers, such as n3-PUFAs sources from genetically modified plants, bacteria, fungi, yeast, and microalgae.

Overall, the findings from this dissertation series suggests that:

1. 1H-NMR spectroscopy is a holistic method to comprehend the aging-related metabolic dysregulation and response to physical activity and dietary interventions.

2. Metabolic response to acute RE is age dependent. The basal levels of several metabolites elevate in aging adults. The exercise-induced response to RE is weaker among healthy older adults.

3. n3-PUFA supplementation may potentially benefit lipoprotein profiles in healthy older adults without dyslipidemia. The potential health benefits of n3-PUFA supplementation for healthy older adults should be weighed against critical factors associated with its global utilization, including ethical and ecological issues, sustainability, cost, and possible adverse effects on human health.
4. It is necessary to eliminate the use of marine-derived n3-PUFA in research and promote alternative sources to the public. Genetically modified plants and oleaginous microorganisms, including fungi, yeasts, bacteria, and microalgae, are promising alternatives for fish-oil omega-3 fatty acids.

Limitations and Future Directions

Several limitations need to be taken into consideration when interpreting the results of the current dissertation series:

1. The cross-sectional and secondary nature of the first two studies, the small sample size, and the inclusion criteria of our sample population constrain the external validity and generalization of the outcomes.
2. The corn oil used in the second study may have affected some of the measured variables in the comparison group.
3. It is essential to consider the ratio of EPA and DHA in omega-3 dietary supplements when comparing the findings of this dissertation to those from other studies in the prior literature.
4. The exercise protocol used in this study in terms of intensity, volume, and the three-minute resting intervals might have affected the production and utilization of various metabolites in the working muscle.
5. These results are limited to the metabolic platform, fasting status, and blood draw time points.
There are many barriers to replacing nonhuman animal testing, including but not limited to economic, social, and political regulations, bureaucracy, and entrenchment in the scientific establishment. As omega-3 fatty acids are one of the most valuable products from microalgae, future research is needed to elucidate the benefits and efficacy of microbial EPA and DHA on CVD outcomes with long follow-up periods, especially in the general aging population. Additionally, future research should determine the ideal EPA and DHA dosages and offer an official recommendation for vegetarian and vegan populations.

The COVID-19 pandemic highlighted the intersections between nonhuman and human animal diseases, transmission, and treatments. This critical insight allows us to adopt a less anthropocentric approach toward medical studies and place nonhumans beside human animals in the center of the discourse. The twenty-first-century issues such as emerging diseases that transmit between nonhumans and humans, antibiotic resistance, food insecurity, and climate change can only be tackled effectively through multidisciplinary approaches in which the health of other animals is considered in relation to the health of humans and the environment (Woods et al., 2018).
Bibliography


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Appendix A
IRB Approvals

Mayo Clinic, IRB Approval

Subject: 17-004403 - IRB Application has been Approved
Date: Wednesday, July 19, 2017 at 8:48:57 AM Central Daylight Time
From: IRB
To: Lanza, Ian R., Ph.D.

Principal Investigator Notification:
From: Mayo Clinic IRB
To: Ian Lanza
CC: Hinnah Abid
Surendra Dasari
Corey Hart
Michael Jensen
Kenton Kaufman
Katherine Klaus
Ian Lanza
K Sreekumaran Nair
Eric Polley
John Port
Roberta Soderberg
Paul Takahashi
Adrian Vella
Re: IRB Application # 17-004403

Study Title: Enhancing Adaptations to Exercise

Please note that all correspondence (modifications, continuing reviews, reportable events) related to this application must be submitted electronically in the IRB system.

The following is an excerpt from the minutes of the Mayo Clinic Institutional Review Boards (IRB-C) meeting dated 7/14/2017:

DECISION: The Committee reviewed and approved the above referenced application and noted that all requirements for approval of research (21CFR56.111 and 45CFR46.111) were met. This approval is valid for one year unless during that time the IRB determines that it is appropriate to halt or suspend the study earlier. IRB approval will expire on July 13, 2018. The Committee approved the accrual of 144 male and female adult subjects from a screening population of 200. The Committee approved the following site to conduct this study: Mayo Clinic in Rochester, Minnesota.

REVIEW: The Committee noted receipt of the protocol. The Committee noted that the Data Monitoring Plan was appropriate for the study. The Committee reviewed the Conflict of Interest (COI) Review Board determinations related to Surendra Dasari, Michael Jensen, Kenton Kaufman, Sreekumaran Nair, Paul Takahashi and Adrian Vella. The Committee accepted the COI Review Board determination of no conflicts of interest. The Committee noted the IND# 115520 for the study drug, Omega-3 fatty acids ethyl esters, dated May 23, 2012. The Committee noted the Omega-3 fatty acids ethyl esters drug details. The Committee approved remuneration up to $2000.00.

CONTACT MATERIALS: The Committee approved the research study initial screening document and the Care Following Your Muscle Biopsy as submitted.
Teachers College, Columbia University, IRB Exemption Notification

Attachments:
- Exemption Notification - IRB ID: 22-344.pdf

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**Teachers College IRB**

**Exempt Study Approval**

To: Darya Moosavi  
From: Curt Naser, TC IRB Administrator  
Subject: IRB Approval: 22-344 Protocol  
Date: 06/29/2022

Thank you for submitting your study entitled, "A 1H NMR Metabolomic Exploration of Lifestyle Changes in Healthy Older Adults and The Ethics of Exploiting Nonhuman Animals in Empirical Research with an Emphasis on Aquatic Animals," the IRB has determined that your study is **Exempt** from committee review (Category 4) on 06/29/2022.

Please keep in mind that the IRB Committee must be contacted if there are any changes to your research protocol. The number assigned to your protocol is **22-344**. Feel free to contact the IRB Office by using the "Messages" option in the electronic Mentor IRB system if you have any questions about this protocol.

As the PI of record for this protocol, you are required to:
- Use current, up-to-date IRB approved documents
- Ensure all study staff and their CITI certifications are on record with the IRB
- Notify the IRB of any changes or modifications to your study procedures
- Alert the IRB of any adverse events

You are also required to respond if the IRB communicates with you directly about any aspect of your protocol. Failure to adhere to your responsibilities as a study PI can result in action by the IRB up to and including suspension of your approval and cessation of your research.

You can retrieve a PDF copy of this approval letter from Mentor IRB.

Best wishes for your research work.

Sincerely,
Curt Naser, Ph.D.
TC IRB Administrator
curtin@axon-mentor.com

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