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THE IMPACT OF AGE ON THE
POSTPRANDIAL METABOLOMOMIC AND
INFLAMMATORY RESPONSES TO A
BREAKFAST MEAL

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A thesis submitted in partial fulfilment of the requirements for the degree of
Doctor of Philosophy in Health Sciences
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ABSTRACT

With the elderly representing a growing segment of the population, their heightened morbidity and mortality are of great public health concern. Despite the rapid gains in medical knowledge and treatments, older adults are more likely to experience chronic illnesses that can decrease quality of life or accelerate mortality. Nutrition is a key modifiable lifestyle factor which greatly impacts chronic disease risk. With ageing, macronutrient processing and immune system function may alter both the digestive and absorptive responses to food and the elicited immune responses. These changes may be instrumental in the development of age-related metabolic diseases. Surprisingly, the digestive responses of older adults are not well characterised. Given the importance of digestive capacity and the impacts on metabolic and immune outcomes, this thesis aimed to examine the post-meal protein, lipid, and inflammatory responses to complex meals in older adults. The particular focus is to examine the impact ageing exerts on protein digestion, focusing on the appearance of amino acid in circulation. Secondly, the chylomicronaemic response is examined through the appearance of exogenous lipids in circulation. Thirdly, the inflammatory responses to high fat and low fat meals are examined in older adults.

Muscle loss in ageing (sarcopenia) contributes to morbidity and mortality in the elderly. This muscle loss in part stems from impaired protein synthetic responses to ingested proteins and amino acids. Protein digestion in response to whole meals in the elderly is inadequately described. In response to a mixed meal, older adults demonstrated no difference in total amino acid appearance in circulation. However, older adults had delays in serum total, essential, and branched-chain amino acid appearance after a high protein, low fat meal. The magnitude of age-related delay in amino acid appearance was reduced following a high protein, high fat meal, likely attributable to differences in digestibility and glycaemic responses evoked by the meal structure and composition. These findings demonstrate the impact of a mixed meal on protein digestion in older adults and implicate a role for meal composition and design in the nutritional support of muscle maintenance in older adults.

Cardiovascular disease affects older adult at increased rates, and is contributed to by postprandial lipaemia and inflammation. Detailed studies of the post-meal dynamics and composition of chylomicrons examined these aspects in response to mixed meals. The aims were to analyse the size and fatty acid differences in older adults’ chylomicrons. Older adults had exaggerated and prolonged postprandial lipaemia, but elevated triacylglycerols were not attributed to the chylomicron fraction; this was particularly apparent after a low fat meal which provoked endogenous TAG elevation in older adults. Older adults had smaller, more numerous chylomicrons containing greater proportions of MUFA and less PUFA in the TAG and PL fractions, although specific age differences in TAG fatty acids varied depending on meal composition. These data establish the altered dynamics and composition of older adults’ chylomicrons, and illustrate the importance of endogenous TAG contributions to elevated postprandial lipaemia in the elderly.

The systemic low-grade inflammation that contributes to chronic disease is fueled by post-meal inflammatory responses. The elderly have altered metabolic and immune function, which is known to contribute to this proinflammatory state in younger adults. Yet postprandial inflammation has not been described in older adults,
which was therefore an aim of the study. The activation of immune responses by ingested lipids, bacterial translocation, and oxidative stress were examined in older adults following high and low fat meals. Older adults demonstrated baseline inflammatory gene and protein expression indicative of age-typical cellular senescence and inflamming. Yet the immune activation by lipoproteins and translocated endotoxin was no different between older and younger adults. However, baseline and postprandial antioxidant capacity may differ in older adults, although this does not appear to transiently affect immune activation. A high fat meal triggered greater circulating concentrations of cell-free DNA in younger adults, detected for the first time as a potential transient marker of acute stress responses to feeding. These findings demonstrate that immunosenescence in older adults does not allow for detrimental postprandial immune activation in metabolically healthy individuals. This suggests that healthy ageing preserves appropriate immune responsiveness to feeding.

Importantly, this thesis demonstrates that older adults have preserved protein and lipid absorption, but that the kinetics of these responses differs from younger adults. Delayed appearance of amino acids may have implications for stimulations of the muscle protein synthetic response to feeding. Additionally, differences in chylomicron formation may contribute to altered lipaemic clearance following a meal, but endogenous lipoprotein production may be an equally important factor. Furthermore, this thesis shows that immune responses are preserved in the healthy elderly, and do not likely contribute to a heightened post-meal inflammatory response. Notably, both protein and lipid digestive responses may be responsive to modifications of meal composition and structure.
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To my William, for always economising and caring, and lending me his reason and accountability.
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LIST OF ABBREVIATIONS

I6SrRNA 16S ribosomal RNA
AA Amino acid
ABCA1 ATP-binding cassette transporter 1
ALT Alanine transaminase
Alu-81 Alu repeat sequence 81 base pairs
ANOVA Analysis of variance
apoB Apolipoprotein B
apoB48r Apolipoprotein B-48 receptor
AST Aspartate transaminase
bacDNA Bacterial DNA
BAK1 B-cell lymphoma 2-antagonist/killer 1
BCAA Branched chain amino acid
β-actin Beta actin
BMI Body mass index
BSA Bovine serum albumin
CCK Cholecystokinin
CD14 Cluster of differentiation 14
CD40LG Cluster of differentiation 40 ligand
cfDNA Cell-free DNA
Cmax Maximum concentration
COX-2 Cyclooxygenase 2
Cp Crossing point
CRP C-Reactive protein
CVD Cardiovascular disease
DAG Diacylglycerol
DEXA Dual-energy x-ray absorptiometry
DGAT Diacylglycerol acyltransferase
DHA Docosahexaenoic acid
DPA Docosapentanoic acid
EAA Essential amino acid
EDTA Ethylenediaminetetraacetic acid
EPA Eicosapentaenoic acid
FA Fatty acid
FAME Fatty acid methyl ester
GapDH Glyceraldehyde 3-phosphate dehydrogenase
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>GC-FID</td>
<td>Gas chromatography with flame ionisation detection</td>
</tr>
<tr>
<td>GIP</td>
<td>Glucose-dependent insulinotropic peptide</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide 1</td>
</tr>
<tr>
<td>GPX1</td>
<td>Glutathione peroxidase 1</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Glycated haemoglobin</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HF</td>
<td>High fat</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>Homeostatic model assessment of insulin resistance</td>
</tr>
<tr>
<td>iAUC</td>
<td>Incremental area under the curve</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate density lipoprotein</td>
</tr>
<tr>
<td>IGFBP3</td>
<td>Insulin growth factor 3 binding protein</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LAL</td>
<td>Limulus amebocyte lysate</td>
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<tr>
<td>LBP</td>
<td>Lipopolysaccharide binding protein</td>
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<td>LDL</td>
<td>Low density lipoprotein</td>
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<tr>
<td>LDLr</td>
<td>Low density lipoprotein receptor</td>
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<td>LF</td>
<td>Low fat</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRW</td>
<td>LAL reagent water</td>
</tr>
<tr>
<td>MAG</td>
<td>Monoacylglycerol</td>
</tr>
<tr>
<td>MAPCRU</td>
<td>Maurice and Agnes Paykel Clinical Research Unit</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein 1</td>
</tr>
<tr>
<td>MetS</td>
<td>Metabolic Syndrome</td>
</tr>
<tr>
<td>MGAT</td>
<td>Monoacylglycerol acyltransterase</td>
</tr>
<tr>
<td>MPS</td>
<td>Muscle protein synthesis</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acid</td>
</tr>
<tr>
<td>NEAA</td>
<td>Non-essential amino acid</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acid</td>
</tr>
<tr>
<td>NFW</td>
<td>Nuclease-free water</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
</tr>
<tr>
<td>NIP</td>
<td>Nutrient information panel</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NTC</td>
<td>Non-template control</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>-------------</td>
</tr>
<tr>
<td>OGGT</td>
<td>Oral glucose tolerance test</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PL</td>
<td>Phospholipid</td>
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<tr>
<td>PPC</td>
<td>Positive product control</td>
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<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
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<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<td>SEM</td>
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<td>SOD2</td>
<td>Superoxide dismutase 2</td>
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<td>STD</td>
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<td>T2DM</td>
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<td>TLR</td>
<td>Toll-like receptor</td>
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<td>T_{max}</td>
<td>Time of maximum concentration</td>
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<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
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<tr>
<td>TRL</td>
<td>Triacylglycerol-rich lipoprotein</td>
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<tr>
<td>UPLC</td>
<td>Ultra performance liquid chromatography</td>
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**Nature of contribution by PhD candidate:** Collected and interpreted data. Wrote paper.

**Extent of contribution by PhD candidate (%):** 75

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The undersigned hereby certify that:

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Older adults have an altered chylomicron response to a high fat meal

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Acute postprandial endotoxaemic and inflammatory responses to a high fat meal are not altered in the healthy elderly

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CHAPTER 1

INTRODUCTION
1.1. INTRODUCTION

As the global population ages, the increasing incidence of chronic disease will contribute to increased health care costs, morbidity, and mortality as well as decreased quality of life. Chronic disease affects a large proportion of the older population, and is greatly influenced by the nutritional environment. Older adults face changes to their physiological, behavioural, and overall health which may increase their risk for disease and negative health outcomes. There is a need for specialised nutritional recommendations for the elderly aimed at supporting health in older age and reducing chronic disease risk. This requires an understanding of the responses to macronutrient ingestion, regarding their nutritional contributions and also their influence on chronic disease risk. The purpose of this review is to examine the age-related factors that contribute to nutrition-related negative health outcomes in the elderly. Sections of this review have been published in Advances in Food and Nutrition Research (see Appendix 8.1 Milan et al., 2015).

1.2. DEMOGRAPHICS AND SOCIETAL IMPACTS OF POPULATION AGEING

New Zealand’s population, along with the populations of most advanced economies, is ageing (1). In the past 40 years, the population over 65 years doubled to half a million and is projected to be almost triple that by the middle of the 21st century (2). By 2051, this will represent 25% of the population, double the current proportion (Figure 1A) (3). Additionally, the increase in life expectancy (3) will contribute to a greater proportion of seniors being age 85 years and older (Figure 1B) (2). While the relative proportion of older adults is projected to increase more gradually in developing countries, it is important to note that by 2020, 67% of the world’s population over 65 years will be living in developing countries (4).

1.2.1. HEALTH BURDEN OF POPULATION AGEING

Increasing age is associated with lower health status and higher health care costs (5, 6), which, if combined with population ageing projections, necessitates rising government health care costs in the near future (Figure 1B). In New Zealand, adults’ health status generally declines with age, particularly physical health. More than 60% of older adults in New Zealand self-report having multiple chronic illnesses, with a greater number of chronic illnesses reported as age increases (7). Additionally, risk factors for chronic illness are at increased incidence in older adults, such as high blood pressure, high cholesterol and reduced physical activity; all of these increase the likelihood of chronic illness (8). These increases in chronic illness correlate with health care expenditure per capita in New Zealand, which increases exponentially after age 50 (9). Morbidity and the need for medical care is substantially higher after the age of 75 (10). Thus, with the trending increase in life expectancy, the number of years of life spent with chronic disease or disability will also rise (10, 11). The ageing population with declining health status is expected to increase the demand for health care services and expenditures in the future (12).
1.2.2. HETEROGENEITY IN AGEING

The ageing experience varies and chronic disease risk, although increased with age, is not necessarily coincident (6, 10). Ageing is associated with changes in immune, digestive and metabolic function; however, while such changes may often be described as ‘characteristic’ of ageing, the heterogeneity inherent in this population makes universal definitions or cutoffs problematic (13). Metabolic or basal health status may be assessed as altered (14, 15) or unchanged (16) in ageing, sometimes attributable to factors such as physical fitness, habitual diet (17), or living environment (18) of the subjects evaluated. Furthermore, the increased prevalence of co-morbidities in an ageing population make it challenging to evaluate the specific effects of ageing without the confounding influence of metabolic modulators such as increases in fat mass (19), fatty liver disease (20), or insulin resistance (21), to name only a few. As such, investigating the inherent impact of age on immune, digestive, and metabolic function is subject to many confounding factors which require consideration.

1.2.3. CHRONIC DISEASE CONCERNS IN AGEING

Chronic diseases have replaced communicable diseases as the leading cause of death in the developed world and in all but the very poorest developing countries (22). However, the impact of chronic diseases is not limited to mortality as they are by nature long-lasting health concerns causing often prolonged disability and
reduced quality of life (22, 23). The World Health Organization (WHO) (24) identifies cardiovascular disease (CVD), cancer, chronic respiratory diseases, and type 2 diabetes (T2DM) as the most prevalent and lists unhealthy diets, physical inactivity, and tobacco use as major common risk factors. Globally, the WHO estimates that cardiovascular disease accounts for nearly 30% of all deaths, representing half of all deaths attributed to non-communicable, or chronic, diseases (25). Similarly, circulatory diseases are the leading cause of death in New Zealand, accounting for 38% of all deaths in 2005 (26). Importantly, the mortality associated with chronic diseases affects the elderly to a greater extent; nearly 90% of deaths attributed to circulatory diseases in New Zealand were of adults over 65 years (26).

In addition to mortality from chronic disease, the negative impact of chronic disease on quality of life is a significant concern. Although diseases such as sarcopenia, osteoporosis or arthritis pose no immediate risk of death, they contribute greatly to a loss of functional capacity, decreased quality of life, and increased risk of complications and injury (27). Sarcopenia, the loss of muscle mass, is estimated to affect nearly a third of adults over 60 years and half of those over 80 years (28) (Figure 2), and is estimated to contribute to 1.5% of total annual health care costs in the United States at $18 billion (29). Worldwide, musculoskeletal conditions like osteoarthritis increase in prevalence with advancing age and decrease quality of life through persistent pain and disability (27). Similarly, osteoporosis prevalence and decreased bone mineral density increases with age, with morbidity presenting as fractures which contribute significantly to disability but also death (30).

![Figure 2: Prevalence of musculoskeletal diseases in adults over 65 years.](image)

Average prevalence (men and women) of osteoarthritis (light gray bars), sarcopenia (dark gray bars), osteoporosis (black bars), and low bone mineral density (BMD; white bars), in older adults in the United States based on National Health and Nutrition Examination Survey (NHANES) data. Osteoporosis and BMD data from NHANES 2005-2008 (31), remaining data from NHANES III (1988-1994) (32, 33). Note that proportionally, osteoporosis and osteoarthritis affect more women than men, and sarcopenia affects more men than women (not reflected as both sexes are pooled).
1.2.4. AETIOLOGY OF CHRONIC DISEASE IN THE ELDERLY

The origins of increased chronic disease in the elderly and the opportunities for intervention and prevention are of significant public health and economic concern. Although genetic factors likely contribute to chronic disease development in ageing, there is no doubt that environmental factors including lifestyle are major contributors (24). Understanding the mechanisms behind the progression of diseases such as CVD or sarcopenia is essential for the prevention and treatment of these diseases.

1.2.4.1. CARDIOVASCULAR DISEASE

Although older adults are at risk for a number of chronic illnesses, cardiovascular disease remains the most common affliction and cause of death. Coronary heart disease and cerebrovascular disease (stroke) are the most prevalent of the circulatory conditions that fall under the CVD umbrella (34), and represent the cumulation of metabolic and immune dysfunction (35), leading to mortality. While global increases in obesity and diabetes are prominent concerns, mortality associated with metabolic disturbances is closely linked with cardiovascular events. Although genetics is a significant risk factor for CVD, many factors associated with the development of heart disease are modifiable and can be attributed to lifestyle (34). Cardiovascular disease remains the leading cause of death for those with T2DM (36), which is unsurprising considering the common underlying pathologies of disturbed lipid metabolism and chronic low-grade inflammation (35, 37). Not surprisingly, the shared nature of common risk factors and incidence of other chronic diseases may be more than coincidence and emphasises the importance of understanding the underlying nutritional and immune-related mechanisms behind these diseases.

Cardiovascular disease is often characterised by changes in or damage to the arteries that impair blood flow, and may manifest as hardening or blocking of the arteries (34). Atherosclerosis, or the buildup of artery-blocking plaque, is identified as the most frequent underlying cause of CVD with comparable risk factors (38). Atherosclerosis progresses as the development of lesions in the endothelium of cardiac vessels, not dissimilar to the immune response following injury (35). Pro-atherogenic substrates, such as lipids or oxidised products, initiate cytokine production, which perpetuate inflammation through the recruitment of monocytes (39) and the stimulation of cytokine production and adhesion molecules (40). Although many of the cascading inflammatory actions of cytokines are mutually self-perpetuating, the introduction of atherogenic substrates into the vascular environment contributes greatly to the progression of plaque development. These substrates can provide additional stimulation of cytokine production or even act as building blocks to become part of the atherosclerotic plaque themselves.

Lipoproteins, as carriers of dietary lipid components, contribute to plaque build-up in atherosclerosis. LDL in particular is a potent substrate, especially if oxidised (41) as macrophage receptors for altered lipoproteins are abundant (42). Similarly, plaque formation is contributed to by remnant lipoproteins, the byproducts of triacylglycerol-rich lipoprotein (TRL) hydrolysis by lipoprotein lipase to release energy-rich triacylglycerols (TAG) to cells (43). Although quickly cleared from circulation in healthy individuals, these remnants easily penetrate
arterial tissue and become trapped in the subendothelial space (44), making cholesterol available to nearby macrophages. These remnants can then stimulate macrophage lipid-loading, an early sign of atherosclerosis (45, 46).

Atherosclerosis is progressed through immune activation, provoked by atherosclerotic substrates. In addition to the metabolic substrates linked to lipid metabolism, other proinflammatory substrates such as bacterial components have been identified in plaque and are known to elicit a potent proinflammatory response (47). Circulating lipopolysaccharide, or endotoxin, has been associated with chronic inflammation, obesity and dyslipidaemia (48). Furthermore, the development of atherosclerosis is associated with exposure to endotoxin by means of acceleration of atherosclerotic plaque development (49, 50). Endotoxin originating from periodontal plaque has been linked to increased risk of CVD (51) and endotoxin has been found incorporated in the developing atheroma (52).

Chronic low-grade inflammation is evident in individuals with CVD, who exhibit increased leukocyte counts, C-reactive protein (CRP) (53, 54), and proinflammatory cytokines (interleukin (IL)-6 (IL-6)) (55), monocyte chemotactic protein-1 (MCP-1) (56), IL-8 (57), and tumour necrosis factor-α (TNF-α) (58, 59)). Chronic low-grade inflammation may directly contribute to the development of metabolic and cardiovascular disease. TNF-α (60, 61) and IL-6 (62) have been shown to affect insulin sensitivity, and a chronic inflammatory status predicts the development of T2DM (37). Similarly, cytokines have a proposed role in the development of dyslipidaemia (63), as several studies have shown their ability to affect lipid metabolism by inducing elevated TAGs (64), reducing lipase activity (65), and increasing lipolysis (66). Moreover, elevated cytokine concentrations predict myocardial infarction risk, demonstrating a link between low-grade inflammatory status and adverse cardiovascular outcomes (67). Regardless of this proinflammatory state preceding or succeeding initial cardiovascular disease development, chronic low-grade inflammation perpetuates the progression of atherosclerosis (35).

1.2.4.2. SARCOPENIA

Musculoskeletal conditions are fueled by metabolic and immune dysfunction (68, 69), and represent a major cause of disability and decreased quality of life in the elderly (27). Conditions such as arthritis and sarcopenia are expected to affect a growing proportion of the population as life expectancy increases (27). Sarcopenia describes the progressive loss of skeletal muscle mass that occurs with older age, characterised by losses in muscle mass and strength, resulting in functional muscle decline (70). Ultimately, sarcopenia leads to impairment of skeletal muscle function, and can lead to loss of mobility and decreased quality of life (70). The maintenance of skeletal muscle mass depends on the balance between protein synthesis and degradation which is affected by factors such as chronic disease and metabolic protein demands, dietary protein intake, physical activity, insulin resistance, and inflammation.

At the root of sarcopenia is the decrease in muscle protein synthesis (MPS) responsible for the repair and generation of muscle tissue (71). In older adults, the stimulation of MPS is problematic, as muscles fail to respond adequately to amino acid availability in circulation for the synthesis of new muscle and the inhibition of protein degradation (72). Notably, the elderly display an anabolic resistance to protein ingestion, whereby protein supplied fails to stimulate MPS (73). The mechanisms for this are complex (70), and have been suggested to be caused by
inadequate muscle stimulation caused by physical inactivity (74) or hormonal changes (75, 76), neuro-muscular degeneration (71), inadequate protein digestion (77) and absorption (78), or inflammation (68).

Chronic low-grade inflammation has a catabolic effect on muscle tissue, suppressing protein synthesis and initiating protein degradation. Increased concentrations of inflammatory cytokines have been linked to low muscle mass and strength (79, 80), and correlate with increased disability (81). Cytokines like TNF-α, IL-1β, and IL-6 have demonstrated a role in protein catabolism (69). Indeed, recent research has suggested that blocking low-grade inflammation may help to limit muscle wasting in ageing rats by increasing anabolic activity (82). Chronic low-grade inflammation is often found in the elderly or with chronic disease states including obesity (83). The increase in adipose tissue associated with ageing and the loss of lean muscle mass may contribute to this proinflammatory state (84) and serve to further progress sarcopenic wasting (68).

1.2.4.3. LOW-GRADE INFLAMMATION AND IMMUNOSENESCENCE IN AGEING

Chronic low-grade inflammation is common to many chronic diseases, including cardiovascular (35), metabolic (83), and musculoskeletal diseases (68), as well as neurological degeneration (85, 86), depression (87, 88), and cancer (84, 89). Successful ageing is associated with a state of chronic low-grade inflammation which can be seen as a 2 to 4 fold increase in plasma concentrations of inflammatory markers, such as TNF-α, CRP, and IL-1 (90-92). It is thought that the deterioration of the immune system is a result of slow degradation caused by chronic and continuous challenge by antigens (93). Through this, there is a related failure to attenuate these challenges through anti-inflammatory mechanisms (94), termed as immunosenescence (95). Overall, this underlying state of inflammation may contribute to the increased incidence of chronic disease in the elderly.

It is difficult to identify whether the changes to these inflammatory markers are a result of ageing itself or are influenced by confounding factors and comorbidities (96). Increased inflammation is an indicator for deteriorating conditions such that raised levels of TNF-α, IL-6, IL-2, and CRP are predictive of all-cause mortality (97, 98). These markers could be influenced by factors such as increased abdominal adipose tissue, associated with increased IL-6 and TNF-α (99), decreased physical activity or sex-hormones (100), and altered gut microbiota (94). Additionally, chronic diseases such as diabetes show significant increases in inflammatory markers in older populations (94). Chronic disease states such as obesity and diabetes are linked to increased serum inflammatory markers and cytokines: TNF-α, IL-6, and CRP (101). This underlying low-grade inflammation is described as a part of the pathophysiology of cancers (84), obesity, and T2DM (37) in part by increasing the risk of insulin resistance and atherosclerosis (35).

The immune system of the elderly seems to be well preserved, although variations in responsiveness may exist. Similar activity to younger immune systems has been observed for monocyte activity, natural killer (NK) cells, and neutrophils as well as normal expression of surface receptors such as toll-like receptors, Fc receptors, and complement receptors (102, 103). However, there may still be differential expression of immune cells. In young adults, the CD14++CD16- variant of peripheral blood mononuclear cells (PBMC) represent <95% of monocyte expression. In contrast, the CD14+CD16+ variant is more highly expressed in ageing, and is associated with increased chemokine receptors, inflammatory activity and ability to interact with endothelial cells (104). An
underlying proinflammatory state in the elderly may be supported by increased concentrations of inflammatory markers, such as the increase of IL-6 at a rate of 0.016 pg/ml per year of life (105). Inhibitors of IL-1 are decreased with age while TNF-α receptors are more highly expressed (106). Recently, profiling of the senescent gene expression patterns found in the elderly has been reported for a variety of tissues (107, 108). While some patterns of up- and down-regulation may be interpreted in the context of metabolic dysfunction (e.g. increased IGFBP3 (108) or BAK1 expression (107)) or declines in cellular repair or proliferative abilities (e.g. decreased CD40LG expression (107)), the contributions of these phenotypes to the progression of age-related disease require further investigation (109). These changes may be a partial explanation for the prolonged and exaggerated inflammatory response observed in the elderly.

1.2.4.3.1. IMMUNE RESPONSIVENESS IN AGEING

In response to an immune challenge, such as lipopolysaccharide or endotoxin from bacteria, older adults may have altered immune activation. Immune response to infections such as pneumococcus are followed by greater magnitude and duration of inflammation in the elderly (110). Studies in aged mice show that endotoxin is poorly tolerated and results in greater and longer TNF-α, IL-1, IL-6 increases. These mice are more sensitive to lethality and although IL-10 concentrations were higher in older mice, this did not translate into a depression of inflammation (111). This increased lethality may be attributable to enhanced endotoxin activation of the immune system through increased expression of CD14 and toll-like receptor (TLR)4 following exposure in aged mice (112). In humans, endotoxin exposure leads to higher hypotension, excess epinephrine, more prolonged fever, TNF-α and CRP expression in older adults (113). This observation of higher inflammatory response to endotoxin in old versus young is well supported (92, 114, 115). This alteration in immune response corresponds with increased incidence of sepsis and related mortality in adults over 65 (106), particularly when endotoxin is detectable in plasma at onset (116). CVD risk may also be enhanced through endotoxin activated increases in fibrin deposition and impaired fibrinolysis, potentially contributing to the risk of cardiac events (112).

1.2.4.3.2. CELL-FREE DNA

Cellular stress and death result in the extra-cellular release of DNA, detectable in circulation as cell-free DNA (cfDNA). Concentrations of cfDNA are increased under conditions of insult or stress causing apoptosis or necrosis such as pregnancy, malignant tumours, transplantation, myocardial infarction or in burn victims.

Measurement of cfDNA is increasingly being investigated as a relevant and accessible biomarker for identification of injury and prognosis in conditions such as cancer (117), myocardial infarction (118), or pre-natal screening (119). While concentrations of cfDNA likely indicate the degree of cellular stress or damage experienced, they may also activate an immune response through TLR9 (120), acting as both a messenger and instigator of systemic inflammation. For example, higher circulating concentrations of cfDNA observed after myocardial infarction likely result from the injury site (121), and are correlated with poorer prognosis (118). In nonagenarians, higher total cfDNA concentrations have been associated with greater systemic inflammation (122), and are likewise correlated with cardio-metabolic risk factors such as insulin resistance and elevated inflammatory cytokines in circulation (123, 124).
1.3. NUTRITION, FOOD CONSUMPTION, AND HEALTH IN THE ELDERLY

1.3.1. RELATIONSHIP BETWEEN HABITUAL DIET AND HEALTH

Nutritional patterns are closely linked with chronic disease and health. Epidemiological data has provided strong evidence for specific eating patterns increasing the risk of diseases such as CVD or sarcopenia. Moreover, changes to habitual diet have been shown as capable of improving prognosis (125), reducing markers of disease progression (126, 127), and even reversing disease diagnosis (128-130). Cardiovascular disease has been linked to dietary patterns high in animal products and saturated fats, while diets featuring fish, vegetables, and legumes appear to be protective against CVD development (131). Overall, the adoption of a ‘Mediterranean’ style diet (e.g. fish, non-refined cereals, olive oil, vegetables (132)) as opposed to a ‘Western’ style diet (e.g. processed meat, refined grains, high-fat dairy products, sweets (133)) is suggested to be metabolically and cardio-protective (134). These associations have been investigated expensively at cohort levels and are supported by randomised control trials confirming the associations between specific dietary patterns and positive or negative health outcomes (135). Overall, these studies emphasise the link between food choices and patterns of consumption, the risk or development of chronic disease, and the resulting mortality.

Diet has an equal role to play in the progression or prevention of musculoskeletal decline. Habitual, adequate dietary protein is essential for the maintenance of muscle mass (136, 137), and low protein intakes are associated with greater loss of muscle mass in advancing age (138). In addition to protein, adequate vitamin D, carotenoids, selenium, vitamin E and C, and the long chain omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) may also be essential for muscle maintenance (139). Furthermore, adequate protein throughout the day has been proposed by some as essential, suggesting that simply meeting daily requirements may be insufficient to maximise MPS and that more continual supply and stimulus of MPS may be necessary (136).

Just as habitual dietary patterns may be pivotal determinants of health and disease, diet is also capable of improving health outcomes. For example, adding psyllium fibre to the diet for eight weeks can reduce elevated circulating cholesterol concentrations by 15% (126). The reduction of saturated fat intake has equally been effective for reducing LDL cholesterol in both young and elderly adults (127). Muscle wasting may be attenuated with adequate protein intake (138), and interventions increasing protein intake in the elderly may have merit (140). However, increasing dietary protein in older adults has not always been shown to be effective for improving muscle-related outcomes (139), although ensuring adequate nutrient quality and quantity has been demonstrated to produce better muscle-related outcomes in older adults following ‘healthy’ diets (141).

1.3.2. NUTRITION AND HEALTH IN THE ELDERLY

Within the literature, interventions which have shown to be effective at disease risk reduction in younger age groups have not produced the same expected beneficial outcomes when repeated in elderly populations. In fact,
some of these usual strategies may increase risk and mortality in this age group. For example, aggressive pharmaceutical glucose control has been linked to hypoglycaemia and a resulting increased mortality in older adults (142, 143). Similarly, the use of statins in adults over 80 years may be counterindicated as low total cholesterol is associated with higher all-cause mortality in this demographic (144). Lifestyle interventions inducing weight loss may not be advantageous to older adults, even if weight loss is likely to improve risk characteristics. Weight loss in the elderly often indicates decreased nutritional status and increased risk of death (145, 146), and malnutrition or progression of sarcopenia may be further unintended consequences (147). These findings highlight the need to assess interventions in elderly populations prior to prescribing interventions based on strategies evaluated in younger cohorts, and elevates the role of lifestyle management in the elderly as preferable to the aggressive pharmaceutical interventions that confer adverse outcomes.

Indeed, the majority of studies still show that management of cardiovascular risk factors in older adults improves prognosis (125). Nutritional intervention strategies are effective in the elderly for improving survival (148), reducing CVD risk (149), improving dementia (150) and bone mineral density (151), or promoting weight loss and reducing inflammation (152). Recently, a nutritional cointervention with exercise was shown to preserve muscle mass in older obese adults (153), however there is overwhelming evidence that nutritional strategies alone are likely insufficient to prevent sarcopenic wasting (72). Furthermore, older adults (65 years and older) are infrequently included as primary or largely represented populations in direct nutritional intervention studies for CVD or diabetes (154). This means that nutritional recommendations for this older age group are often extrapolated from data concerning young or middle-aged adults (155). This represents an area of discrepancy, since the nutritional requirements, concerns, and physiology of older adults differ from younger adults, yet remains incompletely understood. A better understanding of the digestive and metabolic differences in older adults is required to put nutritional recommendations into perspective.

1.3.3. CHANGES IN FOOD CONSUMPTION AND MACRONUTRIENT INTAKE IN THE ELDERLY

1.3.3.1. DECLINING TOTAL ENERGY INTAKE AND THE ‘ANOREXIA OF AGEING’

Food intake decreases 25% between 40 and 70 years of age (156) resulting in overall lower energy intake characterised by behaviours such as decreased snacking and reduced carbohydrate and fat intake (157). Older adults are reported to eat more slowly, feel less hungry and thirsty, eat smaller meals and snack less than younger adults (156). Overall protein intake, although sometimes reported to exceed the nutritional recommendations of 0.8g·kg⁻¹·day⁻¹ at 0.9g·kg⁻¹·day⁻¹ (158), is generally regarded as inadequate in older adults, particularly for the prevention of sarcopenia (136, 158, 159). It is estimated that one third of older adults do not even meet these recommendations, and that 10% of elderly women are likely to have daily protein intakes lower than 0.66g·kg⁻¹·day⁻¹ (138, 160). Furthermore, patterns of intake may be imbalanced throughout the day, with inadequate protein consumption reported at breakfast and dinner for older adults (158). This has been shown to be particularly true of elderly adults classified as frail or pre-frail (161) suggesting that such an eating pattern correlates to negative functional outcomes
in the elderly. Although older adults are reported to consume more fruits and vegetables than the general population, these levels of consumption still fail to meet dietary recommendations and may therefore remain inadequate. Furthermore, nutrient-dense fruit and vegetable intake may be insufficient, and total intake may be decreased depending on health or social factors (162).

The age-related decline in energy intake, or the ‘anorexia of ageing’, has been attributed to changes in eating habits, enjoyment, and physiological responses to foods. Eating habits may also be altered due to economic or social factors affecting the elderly, or limitations of abilities to prepare, ingest, or tolerate certain foods (163). Changes to the upper gastrointestinal tract, including loss of taste or smell perception or difficulties in chewing or swallowing (164) inevitably affect an older adult’s choices and consumption patterns. Although these preceding factors are likely to vary between individuals and may not be universally applicable, physiological changes documented in the elderly have also been shown to affect food intake. Early satiation is frequently reported (163), and appetite regulation appears to be impaired in older adults, with failure to compensate for energy intake imbalances consistently reported in elderly subjects (165), leading to over- or under-consumption (163). Declines in resting metabolic rate, physical activity, and appetite regulating hormones also likely impact total energy consumption (166).

1.3.3.2. GASTROINTESTINAL MODIFICATIONS IN AGEING

There remains conflicting evidence on whether ageing per se affects digestion (167). In the elderly, chewing capacity may be affected (168), and there is evidence of notable decreases in gastric emptying times (169), gastric acid secretions (170), lactose tolerance, and both stomach and small intestine motility (171), although transit time is no different between the young and the elderly (172-174). However, the rate of digestion may be affected by delayed gastric emptying, which has been reported in older adults for large but not small meals (166). Delayed gastric emptying of lipids, in addition to reductions in digestive secretions such as gastric acids (174), pancreatic lipases and bile salts (167) may contribute to potential differences in protein and fat digestion with increasing age (167). However, these findings have been reported inconsistently, and may depend largely on health status and the prevalence of co-morbidities, and may not be characteristic of ageing per se (175, 176).

A number of gastrointestinal complications are observed at significant and increased prevalence in the ageing population: these include dysphagia, gastro-oesophageal reflux disease, peptic ulcers, dyspepsia, constipation, diverticulitis, and diarrhoea (175). Some of these complications may be the result of physiological changes in ageing such as reduced colon transit time contributing to constipation (177). However, alterations to specific gastrointestinal cells and their function in ageing are reported inconsistently in ageing animals and humans, making it difficult to determine whether reports of altered intestinal epithelial architecture or integrity, cell proliferation, enzyme production, innervation or blood flow are characteristic of ageing (178). Furthermore, the influence of factors such as medication use have the potential to greatly affect gastrointestinal function, just as one complication may contribute to another. For example, reduced gastric acid secretions or overuse of non-steroidal anti-inflammatory drugs may contribute to H. pylori infections (175).
Overall, the change in eating patterns and digestive function apparent in older adults makes inadequate nutrition a risk, and secondarily infers increased chronic illness risk. The reduced variety and intake of food increases the risk of malnutrition and undernutrition in the elderly. The prevalence of undernutrition in elderly men is estimated at 14% for those in the community, 21% for those institutionalised, and 72% for those hospitalised (179, 180). Importantly, changes in eating patterns, nutrient absorption, and nutritional health status may greatly affect older adults’ susceptibility to and the projected outcomes of chronic disease. Certainly, changes or imbalances in nutrients are fundamental in the aetiology of lifestyle-related diseases, and understanding the age-related differences in requirements may be essential in directing appropriate preventative strategies.

There is a paucity of data on the digestive responses to meals in the elderly. Despite the knowledge that sarcopenic and cardiovascular disease pathophysiology are greatly influenced by postprandial phenomena (72, 181), as will be expanded on subsequently, age-specific responses have been poorly explored. There is adequate data to strongly support altered absorption and processing of fat in conditions of disturbed metabolism such as obesity or T2DM (182), which contributes to and progresses adverse cardiometabolic outcomes. Further, sarcopenic muscle wasting is controlled by inadequate muscle responses to protein feeding (73). Yet there remains an incomplete understanding of how the aged response to meals is inherently altered to contribute to these pathologies.

1.4. STUDYING POSTPRANDIAL RESPONSES TO MEALS

After a meal, there are many physiological changes that occur. As expected, meal-derived nutrients appear in the blood shortly after ingestion, depending on the time it takes for them to be digested and absorbed. These nutrients circulate in the body and act as the substrates for metabolic and biochemical reactions, some of which may trigger physiological pathways. For most individuals this postprandial period spans several hours after a meal, and translates into a non-fasting state for most of the day (183, 184). For this reason the postprandial effects of meal components have the potential to greatly impact long-term health outcomes (185). Studies of the postprandial state allow for the evaluation of the relative digestibility and absorbability of meal components including macro- and micronutrients. These may differ between individuals or be modifiable through meal design or eating patterns. Furthermore, these digestive differences affect the bioavailability of ingested nutrients, impacting their appearance in circulation (186-188).

1.4.1. MACRONUTRIENT RESPONSES

1.4.1.1. POSTPRANDIAL CARBOHYDRATE METABOLISM

Starch and simple or complex sugar molecules are broken down by salivary and pancreatic amylase, to be absorbed as monosaccharides in the small intestine (Figure 3). Carbohydrate absorption at the small intestinal brush border is achieved by active sodium-dependent transporters (i.e. glucose and galactose) and facilitated diffusion (i.e. fructose). Dietary fibre, most commonly present as cellulose, is indigestible, but may act as a substrate for microbial fermentation in the colon. Carbohydrates stimulate the release of insulin, as well as other hormones, responsible for the regulation of digestive factors such as gastric emptying. The majority of ingested glucose is then oxidised
taken up by splanchnic tissues (~22%), or stored in muscle glycogen (~50%) (190). During the metabolism of glucose, endogenous glucose production in the liver and fat oxidation are suppressed (191).

Under conditions of impaired glucose tolerance, fasting and postprandial glycaemic control are compromised leading to increased circulating concentrations of glucose. Various cohort studies have linked impaired glucose tolerance with increased cardiovascular risk factors in non-diabetic men (192). Some of the proposed mechanisms of hyperglycaemic contributions to CVD include protein glycation, particularly of lipoproteins, increasing their oxidative potential and reducing receptor and scavenging effectiveness; the production of free radicals and oxidative stress; or increased prothrombin release promoting thrombosis (193). As well as these potential direct mechanisms promoting atherosclerosis, impaired glucose tolerance signals a state where insulin responsiveness is impaired, with the potential for additional impairments of postprandial protein and lipid metabolism, as are indicated subsequently.

1.4.1.2. POSTPRANDIAL PROTEIN METABOLISM

Although the digestion of proteins occurs mainly in the stomach and small intestines via proteolytic enzymes, mechanical processing in the mouth and stomach also contribute to the breakdown of dietary proteins (Figure 3). In the stomach, gastric juices including HCl and HCl-activated pepsin act to denature proteins and hydrolyse oligopeptides into peptides. Inactive pancreatic proteases are activated within the small intestine to proteases including trypsin and chymotrypsin through the action of intestinal enterokinase and trypsin respectively, and act to hydrolyse large peptides into smaller peptides and individual amino acids. These peptides and amino acids are transported into the enterocyte by sodium-dependent transporters. Some larger food-derived peptides may be absorbed directly into the intestinal lymphatic system with the potential to have physiological effects (194); however, the probable concentration of these compounds remains very low and the extent of their potential activities requires further investigation (195). Overall amino acid digestibility varies between food products, affecting bioavailability (196).

Although the majority of digested dietary amino acids are almost entirely absorbed, these do not equally appear in circulation, affecting their overall bioavailability (197). This is attributed to the use of amino acids by the intestinal tissue: 50% of ingested threonine may be taken up by intestinal tissue and dietary glutamate and aspartate are almost exclusively used by the intestines and do not appear in circulation in substantial amounts (197). The fate of the remaining digested amino acids is to accommodate the metabolic amino acid demands and to replenish post-absorptive nitrogen losses (198).

The balance between postprandial protein degradation and synthesis are regulated mainly by insulin and amino acid supply, with marked suppression of proteolysis following meal ingestion (198). In healthy young adults, the amount of protein fed differently stimulates and inhibits protein degradation, synthesis, and oxidation, adjusting postprandial protein synthesis to match available leucine concentrations (199). Hence, at lower leucine intakes and circulating concentrations, protein synthesis is stimulated to a lower extent. Critically, proteolysis suppression accounts for most of the anabolic response to protein ingestion (198), an effect which is enhanced by ingestion of specific amino acids such as essential amino acids (200).
Digestive enzymes are secreted by the mouth, stomach, pancreas, and enterocytes of the small intestine to facilitate progressive breakdown of dietary components. Proteases are secreted in inactive forms to avoid self-hydrolysis and catabolism of the gastrointestinal cells, and require activation by co-enzyme factors, such as HCl. The monoacylglycerols (MAG) and non-esterified fatty acids (NEFA) resulting from hydrolysis of triacylglycerols (TAG) and diacylglycerols (DAG), are emulsified by bile salts from the gall bladder to form soluble micelles. At the brush border, micelles dissociate and MAG and NEFA are absorbed through passive diffusion and transport proteins. Carbohydrates and proteins are cleaved into monosaccharides and amino acids by brush border enzymes, and absorbed through active sodium transport proteins into the enterocyte.
Importantly for the maintenance and repair of skeletal muscle, this postprandial response to protein ingestion is crucial for MPS. This may be affected by altered rates of digestion, either originating from food composition or physiological differences (77), as well as the availability of circulating amino acids following meal ingestion (78). Feeding patterns may be used to manipulate the availability or rate of amino acid delivery to the muscle to facilitate the anabolic response to feeding. For example, a ‘spread’ feeding pattern, or a slowly digested protein like casein, enhances whole body protein balance and utilisation in healthy young adults (77).

1.4.1.3. POSTPRANDIAL LIPID METABOLISM

Lipoproteins are the carriers of triacylglycerols and cholesterol in circulation. Longer chain dietary lipids in the small intestine are packaged into chylomicrons which enter circulation where the TAGs they carry are used by cells for energy via the action of lipoprotein lipase (201). Lipoprotein lipase likewise catalyses the release of non-esterified fatty acids to be used as energy and in adipose tissue stimulates their storage. Chylomicrons partially depleted of TAG become chylomicron remnants which are taken up by the liver. The liver in turn produces very low density lipoproteins (VLDL), still triacylglycerol-rich, which become intermediate density lipoproteins (IDL) and eventually low density lipoproteins (LDL) as they become further depleted of TAGs (202). High density lipoproteins (HDL) are produced separately by the liver and have a very different structure and different functions than the other lipoproteins. While triacylglycerol-rich lipoproteins and LDL increase triacylglycerol and cholesterol available in circulation, HDL transports cholesterol back to the liver.

The post-meal increases in circulating concentrations of triacylglycerol-rich lipoproteins, described as postprandial lipaemia, is a risk factor for CVD (203), and has been described as a better predictor of risk than fasting TAGs (204-206). During the postprandial period, dietary lipids packaged into chylomicrons influx into circulation, increasing the total circulating triacylglycerol load. Endogenous triacylglycerols are also present in the form of other lipoproteins, namely VLDL (207, 208), and contribute to total TAG concentrations. Non-esterified fatty acids are actively suppressed by insulin, but gradually rise in concentrations as they are released through lipolytic clearance from lipoproteins and make their way to tissues or other lipoproteins.

The magnitude of postprandial lipaemia is dependent on habitual diet, the macronutrient and lipid composition of the ingested meal, and chylomicron formation and clearance, which in turn is subject to metabolic alteration depending on health status. These factors act through modulation of the triacylglycerol content of chylomicrons and VLDL, postprandial chylomicron and VLDL formation and clearance, and the kinetics of fatty acid and cholesterol metabolism, as outlined subsequently.

1.4.1.3.1. CHYLMICRON FORMATION AND CLEARANCE

Dietary lipids, predominantly in the form of triacylglycerols, are digested and metabolised mainly by enzymatic means, by the actions of various lipases (Figure 3). Dietary triacylglycerols are progressively hydrolysed by lingual, gastric, and pancreatic lipases before their appearance in the small intestine at their site of absorption. These non-polar TAG are solubilised in the aqueous chyme through emulsification by bile salts released by the gall bladder to form micelles. These micelles eventually dissociate in the unstirred water layer bordering the intestinal mucosa, enabling lipid absorption. These fatty acids, primarily non-esterified fatty acids and di- and mono-
acylglycerols (DAG and MAG) are absorbed passively or by active transporters at the brush border into the enterocyte. The enterocyte reassembles the non-esterified fatty acids, DAG, and MAG along with the occasional endogenous fatty acids into transient TRLs, chylomicrons, which are released into circulation via the lymph. Chylomicron formation begins at the onset of lipid ingestion (201), with the stimulation of chylomicron release at 30 to 60 minutes, containing mainly the previous meal's fatty acids. The appearance of the ingested meal's fatty acids (209, 210) typically peaks between three and four hours postprandially, followed by a gradual decline in chylomicron and total TAG concentrations as TRLs are cleared from circulation through the actions of lipoprotein and hepatic lipases (211, 212), and rapidly metabolised into chylomicron remnants (201). As such, chylomicrons are not normally present in fasted plasma. The rate at which chylomicrons are cleared from circulation may have implications in elevated CVD risk; in familial hypertriacylglycerolaemia, chylomicron remnant clearance is delayed (213). Overall, chylomicron appearance and clearance, and likely their importance in CVD risk, are influenced by their formation, composition, size, and concentration in circulation.

The formation of these chylomicrons occurs in the enterocyte, reliant on the availability of the apolipoprotein (apo)B-48 molecule and regulated by a complex network of factors including hormonal, genetic, dietary, and pharmaceutical influences (214). Any number of these factors is known to be influenced by states of metabolic dysfunction, contributing to differences in chylomicron secretion rates. For example, the transcription of apoB-48 is known to be upregulated under conditions of insulin resistance such as T2DM, possibly to facilitate adequate and efficient fatty acid absorption (215). In healthy individuals, chylomicrons appear to be released continuously, increasing in size with feeding (216), and represent 80% of the postprandial increase in TAGs (217). Chylomicron clearance by lipoprotein lipase is regulated by insulin, and is competitive between TRLs (211), therefore dependent on both chylomicron characteristics and the postprandial TRL environment.

In addition to chylomicron formation in the postprandial period, triacylglycerol-rich VLDLs contribute to postprandial hypertriacylglycerolaemia. Importantly, VLDL constitute over 90% of the particle contribution (218, 219), acting as a significant participant in late postprandial hypertriacylglycerolaemia (219). Small VLDL particles are continuously secreted by the liver, while the secretion of larger VLDL is regulated by TAG availability and the actions of insulin (220). TAG availability is increased by hepatic uptake of TRL remnants, elevated NEFAs released from adipose or inadequate up-take by muscle, or by de novo synthesis, as stimulated by carbohydrate overfeeding, all of which are regulated in one way or another by insulin (219). A failure to suppress large VLDL production in the postprandial period is characteristic of insulin resistance (221), and contributes to postprandial hypertriacylglycerolaemia in T2DM (222). As such, hepatic VLDL regulation has the potential to directly and indirectly affect postprandial lipaemia by contributing to the TRL and remnant pool and competing for lipolytic clearance.

1.4.1.3.2. Chylomicron composition

Chylomicrons, similar in composition to other TRLs, have an outer phospholipid monolayer which surrounds a central core of triacylglycerols and cholesterol esters. Triacylglycerol-rich lipoproteins possess a single non-transferrable apoB molecule per particle, which easily identify the lipoprotein's origins based on the apoB
length: chylomicrons of intestinal origin have a shorter variant of the usual apoB-100 molecule, apoB-48, which is not recognised the same way as apoB-100 on LDL receptors (223).

The postprandial composition of the chylomicron closely reflects the dietary lipids of an ingested meal (209, 210). This fatty acid composition governs the formation of the chylomicrons and their ultimate clearance. Habitual and acute differences in fatty acid ingestion have an impact on chylomicron composition and the resulting postprandial lipaemia. Saturated fatty acids (SFA) generally cause the greatest increase in postprandial TAGs followed by monounsaturated fatty acids (MUFA), omega-6 polyunsaturated fatty acids (PUFA) and omega-3 PUFA (224). Longer SFAs (at least 14-16 carbons long) increase circulating lipoproteins the most (201), especially LDL due to a resulting impaired rate of removal (14). However, lower increases in TAGs have been observed following a meal high in SFA compared with MUFA (225). Similarly, dairy fat (butter), although saturated, induces a lower TAG response after a meal compared with omega-6 PUFA due to a decreased accumulation of chylomicron particles (226). However, these results are inconsistent with previous studies demonstrating the opposite, where butter showed a higher chylomicron response compared to olive oil (227). Omega-3 PUFA induce the lowest postprandial lipaemia in comparison with other fats (224). Short term, diets high in omega-3 PUFA can decrease postprandial lipaemia and reduce TRL production (228-230).

Although the fatty acids in postprandial chylomicrons are mostly of dietary origin, there are also endogenous contributions to TRLs. The enterocyte itself may influence the fatty acid composition of chylomicrons. The enterocyte contains storage pools of fatty acids originating from de novo synthesis (231), the previous meal (232-234), and circulating non-esterified fatty acids (232), which are likely to appear in early postprandial chylomicrons. Triacylglycerol and cholesterol exchange between TRLs and LDL or HDL is a known phenomenon (219), revealing complex fluctuations in lipoprotein dynamics in the postprandial period. Indeed, dietary fatty acids have been reported as more prominently found in the VLDL of older adults rather than the chylomicron fraction (235), suggesting age-related differences in this rate of transfer, which may affect fatty acid composition.

Stereospecificity for specific fatty acid positions within TAGs are known to be different between varieties of lipases (lingual, gastric, pancreatic, lipoprotein, hepatic (236, 237)) and for intestinal monoacylglycerol acyltransferase (MGAT) and diacylglycerol acyltransferase (DGAT) involved in the re-esterification of diacylglycerol and monoacylglycerol to the triacylglycerols produced from absorbed fatty acids and found in chylomicrons (238). The TAG structure of fatty acids and their positioning along the glycerol backbone may impact postprandial lipaemia and lipoprotein metabolism, although this is not yet confirmed (239, 240). It is thought that the stereospecific nature of lipase catabolism of TAGs, in favouring the stereospecific number ($sn$)-1 and $sn$-3 positions, leaves the fatty acid in the $sn$-2 position intact, determining its fate within the chylomicron. Additionally, fatty acids are preferentially mobilised, based on the relative proportionality, saturation, and double bond positioning (241-243). Age or metabolic-related alterations to the relative availability of these lipases (175), or activity of MGAT or DGAT (244), could lead to shifts in relative non-esterified fatty acid proportions, impacting the rate at which specific fatty acids are digested, absorbed, incorporated into chylomicron TAGs, or cleared from TRLs. For example, PUFA rich chylomicrons are cleared more readily than SFA rich chylomicrons (245, 246), but omega-6 rich TRLs are catabolised more slowly in the elderly (247).
**1.4.1.3.3. Chylomicron size**

Chylomicron particle size, as a function of the number of particles produced and triacylglycerol content, is known to be affected by health status (248) and is responsive to both acute (249, 250) and long term (251-255) dietary influences (256). Postprandially, chylomicron size is larger with greater TAG content (216), and decreases after adequate clearance through lipolysis. The estimates of chylomicron particle size range from as low as 30nm to as high as 6μm (257, 258) with considerable discrepancy between sources. One reason for this may be the classification methods used to characterise lipoproteins and chylomicrons. Chylomicrons may be identified by a number of different methods ranging from flotation (259), density (258), protein electrophoretic mobility (260), weight or diameter (258, 261), leading to fluid lines of separation between chylomicrons and other lipoproteins. Differential densities (g/ml) and Svedberg flotations (Sf) correspond well (258), leading to relative ease in separating lipoproteins through methods such as density gradient ultracentrifugation (262). More recently, the structure of chylomicrons has been investigated by more sophisticated technologies. Chylomicron and lipoprotein particle size can now be measured quickly and easily by dynamic light scattering methods (261, 263-265), but to date has not been used for chylomicron analysis in a postprandial study.

There is a direct relationship between chylomicron particle size and its triacylglycerol content (258) which contributes to postprandial lipaemia and may be atherogenic by reducing insulin sensitivity, and increasing oxidative stress and inflammation (260). As chylomicrons are the product of dietary lipid ingestion, they respond differently to different meals; typically, greater lipid loads with longer and less saturated TAGs result in larger chylomicrons (253, 255). Chylomicrons consisting of SFA from meals like butter are smaller than MUFA/PUFA rich chylomicrons from meals of sunflower or olive oil (226, 266, 267). Chylomicron sizes remain similar between MUFA and PUFA meals (226, 249, 268). Additionally, evidence suggests increases in chylomicron remnant concentrations where SFAs are in the sn-2 triacylglycerol position (269). It is still unknown how chylomicron particle size may be affected by chronic disease states such as T2DM, the Metabolic Syndrome (MetS), or even ageing. These potential differences in chylomicron particle size may be of particular importance in determining standardised normal versus disease state dimensions and determining risk associated with CVD.

**1.4.1.3.4. Chylomicron remnant formation, clearance, and CVD risk**

The formation of chylomicron remnants and their respective rates of clearance from plasma may be a contributing factor to the association between postprandial lipaemia and CVD risk (181). Early research on chylomicron clearance suggested that large chylomicron particles are cleared from circulation more quickly than small particles, likely due to their relative susceptibility to lipoprotein lipase (270). More recent work showed that this is only the case when there is a larger relative number of large particles (261). When equal numbers of small and large particles are present in plasma, the smaller, more abundant particles are cleared first, indicating that particle number is a more important determinant of chylomicron particle clearance. This number-based competition for lipolytic clearance has the potential to result in delayed clearance where greater numbers are present, particularly of chylomicron remnants, as chylomicrons are the preferred lipolytic substrate over remnants (211, 271). Importantly, the size of chylomicrons, particularly their remnants, likely determines their relative
atherosclerotic risk as small lipoproteins have been shown to be better able to penetrate the arterial intima and contribute to atherosclerotic plaque formation (203).

**1.4.1.3.5. Postprandial Lipaemia in Chronic Disease and Impact on CVD**

Chronic disease states generally exhibit exaggerated postprandial lipaemia and more of the associated risks for atherosclerotic development (272). This is apparent in the differing circulating concentration of lipoproteins in individuals with chronic diseases who exhibit low HDL, and high fasting LDL and TAGs (204), directly impacting the relative risk of developing CVD. Postprandial lipaemia is exaggerated in obese individuals (273), or those with T2DM (182). Elevated concentrations of VLDL or amplified hepatic production are known to contribute to postprandial lipaemia, and are phenomena evident in fatty liver disease (274, 275), insulin resistance, and increased fat mass (276-278). Reductions in body weight can decrease postprandial lipaemia (279, 280). Similarly, exercise before high fat meals also reduces postprandial lipaemia (224) potentially due to increased TRL clearance by increased plasma lipoprotein lipase activity. As expected, sedentary behaviour has the opposite effect and impairs postprandial lipid metabolism in much the same way as obesity (281).

The mechanisms for altered postprandial lipid metabolism in chronic disease states may be linked to insulin resistance and the effects of insulin on lipid metabolism. Insulin is involved in the stimulation of lipoprotein lipase and NEFA suppression. Under conditions of insulin resistance, lipoprotein lipase is inhibited (282) leading to accumulation of TRLs such as VLDL (283). Insulin resistant and T2DM patients exhibit raised fasting lipoprotein and remnant lipoprotein concentrations (284), and impaired postprandial lipid metabolism which correlates well to the degree of insulin resistance (285, 286). However, the association between impaired postprandial lipoprotein metabolism and the development of insulin resistance has not yet been established (287), despite the correlation between elevated apoB-48 concentrations with obesity and insulin resistance (288). Notably, both intestinal (248) and hepatic (221) overproduction of TRL are apparent in insulin resistance and types of dyslipidaemia (289).

In the postprandial state, increases in triacylglycerol-rich lipoproteins reduce the availability of lipoprotein lipase through direct competition, leading to the accumulation of these atherogenic lipoproteins. Not only does the increase in circulating lipoprotein concentrations have atherosclerotic implications, but through the residual risk hypothesis, CVD risk is equally impacted by remnant lipoproteins and existing alterations in the arterial walls, providing the perfect atherogenic conditions (287). Clearance rates of lipoproteins are decreased in T2DM and CVD (204), contributing to elevated fasting TAGs and increased atherogenic risk (290). Overall, it is uncertain whether reducing postprandial lipaemia can positively impact CVD risk; increased TAGs may be markers of CVD or may indicate metabolic abnormalities (291).

Altered lipid profiles, increased fat mass, and changes in physical activity are commonly associated with the elderly, and while these elderly may still fall within a ‘healthy’ range, the underlying metabolic variations these may indicate can affect postprandial responses. The increased incidence of insulin resistance in the elderly, likely in part due to changes in body composition, has the potential to influence or confound age-related differences in lipaemia; however, the insulin sensitivity of older adults is infrequently accounted for during evaluations of age-related
changes in postprandial lipid metabolism. Overall, the healthy elderly are likely to exhibit similar metabolic traits absent of apparent dysfunction, but it is unknown whether these are inherent to, or concurrent with, ageing.

1.4.2. POSTPRANDIAL INFLAMMATION

The immune and metabolic pathways are highly integrated (292). Postprandial inflammation is a phenomenon activated by all macronutrients. Although the effect differs in magnitude between macronutrient types, the low-grade and transient inflammation that results is consistent. High calorie meals evoke a strong postprandial inflammatory response (293) while individual macronutrients elicit varying degrees of response. Protein induces the smallest postprandial inflammatory response of the macronutrients, followed by dietary fats and finally carbohydrates (294). However, aside from glucose, all of the most potent triggers of the postprandial inflammatory response are lipids, specifically TAGs, SFA, and cholesterol derived oxysterols (295, 296). In vitro studies show direct activation of PBMC inflammatory gene expression by non-esterified fatty acids, the products of triacylglycerol catabolism (297); similarly, these cells take up remnant lipoproteins (298). In the context of CVD, postprandial inflammation provides ample opportunity for the development of atherosclerotic plaque due to the continual inflammatory state combined with the availability of atherogenic substrates (183, 184, 294). Postprandial inflammation is also suspected to contribute greatly to the development of insulin resistance and MetS, although the exact mechanisms remain unknown (299, 300).

As with inflammation associated with CVD, the postprandial inflammatory response is in fact an adaptive response of the immune system to deal with the exogenous stress caused by the influx of nutrients and other dietary and metabolic components (294). Postprandial inflammation is caused by the activation of the immune system in several ways. Immunoresponsive cells such as adipocytes, monocytes, and macrophages respond directly to meal components such as TAGs and SFA (94). Lipids such as TAGs are additionally increased over the postprandial period peaking around 3 to 4 hours after a meal (301). This postprandial lipaemia correlates with an increased inflammatory state as well as reduced antioxidant capacity (302). Oxidised meal components similarly activate an oxidative or inflammatory response (94). More recently, the presence and translocation of endogenous bacterial components such as lipopolysaccharide and bacterial DNA have been identified as food-associated inflammation aggressors (94, 303).

1.4.2.1. THE ROLE OF POSTPRANDIAL LIPAEMIA AND CHYLOMICRONS

Postprandial lipaemia is known as a major contributing factor in postprandial inflammation. Non-fasting elevations in TAG have been established as an independent risk factor for CVD through several prospective epidemiological studies, hinging on the postprandial elevation of TRLs and the inflammation they perpetuate (208). The postprandial lipaemic-related mechanisms behind this immune activation can be linked to the type of fatty acids in circulation, the residence time of triacylglycerol-rich lipoproteins in circulation, and the additional actions of these lipoproteins while in circulation. Chylomicrons and TAGs adhere to (93) and activate monocytes (287, 298) eliciting this immune response. High fat meals, in comparison to high carbohydrate meals, increase proinflammatory cytokines (285) through activation of nuclear factor kappa B (NF-κB) in PBMCs (304) and
decrease insulin sensitivity (305). Correspondingly, a long-term low carbohydrate diet decreases postprandial inflammation more than a low fat diet (306).

Not all fatty acids elicit the same postprandial lipaemic or inflammatory responses. Compared with olive oil (MUFA) and walnut oil (PUFA), a breakfast containing butter (SFA) causes a greater increase in messenger RNA (mRNA) expression of TNF-α and IL-6 (304), although a subsequent study (307) found that this did not affect plasma concentrations of these inflammatory markers. SFAs may also increase postprandial intercellular adhesion molecule-1 (ICAM-1) (225), while palmitic acid specifically induces inflammation via the cyclooxygenase-2 (COX-2) pathway (308) increasing IL-6 expression (309, 310). Chronic SFA ingestion is associated with increased CVD risk (311, 312) which may in part be due to their postprandial inflammatory effects. Yet, the nature of inflammatory activation by fatty acids is not limited to fatty acid saturation, as dairy fat, although saturated, does not induce a proinflammatory response compared with other fats (313, 314). Appropriately, long term dairy consumption is inversely associated with CVD risk (226).

Unsaturated fatty acids, including MUFAs, omega-6 and omega-3 PUFAs, generally have a lesser impact on postprandial inflammation compared with SFAs (224). There is however conflicting evidence suggesting that the lines are not clear-cut; in normal weight men fed a high fat meal with differing ratios of saturated to unsaturated fat, no differences in postprandial inflammatory markers were detected (315). PBMC gene expression of inflammatory cytokines is still increased with a meal high in MUFA or PUFA (297) although the replacement of SFA with omega-6 PUFA results in decreased postprandial inflammation (316). Compared with SFA, high proportions of omega-6 PUFA in a mixed meal decreases plasma concentrations of IL-6, TNF-α, soluble TNF receptors I and II and soluble vascular adhesion molecule-1 (VCAM-1) in overweight men (316). However, PUFA have a much more complex role in postprandial inflammation. When compared with SFA or omega-3 PUFA, omega-6 PUFA induces higher expression of ICAM-1 and VCAM-1 \textit{in vitro} (317). The omega-6 PUFAs, linoleic and arachidonic acid, activate the COX pathway which synthesises proinflammatory prostaglandins; omega-3 PUFA acts to inhibit this activation (318). Despite this, no differences in postprandial inflammatory markers were found in men with MetS after a meal high in SFA with increased omega-3 PUFA content (319). Additionally, postprandial omega-3 PUFA ingestion was shown to enhance NF-κB activation more than SFA (225). Hence, it is clear that the quality of fat in a meal can influence TRL metabolism and postprandial inflammatory responses.

1.4.2.2. THE ROLE OF BACTERIAL AND GENOMIC MATERIAL

Emerging evidence is defining the link between chylomicron formation and metabolism with postprandial inflammation. While the mechanisms behind the role of dietary fatty acids on postprandial inflammation are better understood, recently the role of chylomicrons in introducing additional proinflammatory substrates into circulation has been investigated. The gut has an integral role in innate immunity and the breakdown of its barrier function may also be a key factor in the proinflammatory state associated with high fat meals.

There is increasing evidence that postprandial inflammation after high fat meals is contributed to by bacterial translocation as observed by the presence of bacterial endotoxin, a known inducer of inflammation, in the plasma after a meal (49). The mechanisms allowing the transport of bacteria or their structural components across
the gut barrier are multifaceted, while the inflammation these components induce depends upon the complex symbiotic relationship between bacteria and host.

The permeability of the gut is an important component in the function of the immune system. Gut associated lymphatic tissue acts as a barrier to the external environment. Deterioration of the intestinal barrier can have implications ranging from infection to changes in adipose tissue (320). Gut permeability may be altered by disease states such as alcoholic liver disease (321) or T2DM through changes to the mucosal barrier (48), or accompanying diseases of gut inflammation and damage such as Crohn's and ulcerative colitis. Intestinal inflammation in Crohn's disease is associated with a higher ratio of intra-abdominal fat to total abdominal fat (322). In healthy women, increases in intestinal permeability are associated with increased visceral adiposity, a hallmark of metabolic dysfunction (323). Different dietary fatty acid types have the potential to increase gut permeability; this has been observed with palm oil (324) and appears to be increased in omega-6 PUFA over SFA (325).

Bacterial translocation of endotoxin into plasma is associated with high fat diets. High calorie or high fat diets increase the concentration of circulating endotoxin (326, 327) and contribute to increased risk of insulin resistance and atherosclerosis (328-330). The ability to increase circulating LPS and lipopolysaccharide binding protein seems to be high-fat/high-carbohydrate meal specific as these changes do not occur after 'healthy' meals (331). Lipid metabolism is also indicated as a factor in endotoxaemia with increases in LPS concentration positively correlated to serum apoB concentrations, suggesting a relationship with triacylglycerol rich lipoproteins (48). Recent work has recognised the role of endotoxin in postprandial inflammation further supporting a link between bacterial translocation, endotoxin and dynamic changes in lipid metabolism. Concentrations of plasma LPS have been reported to peak around 60 minutes following a high fat meal (49). Subsequent studies have shown similar findings and have demonstrated that postprandial increases in endotoxaemia are enhanced by emulsified lipids (324), long chain fatty acids (332), palm oil (333), and that LPS is associated with chylomicron-rich plasma fractions (324, 332).

1.4.2.2.1. THE ROLE OF BACTERIAL ANTIGENS IN METABOLIC AND CARDIOVASCULAR HEALTH

Circulating endotoxin, derived from the cell wall of gram negative bacteria, is believed to be mainly of gut bacteria origin rather than exogenous sources (332), is detectable in healthy individuals, but is raised under abnormal conditions (50). The immune response to endotoxin is similar to that of atherosclerosis and can lead to sepsis in compromised individuals. Endotoxin and other bacterial surface molecules (Figure 4) interact mainly with TLRs (334), facilitated by complement factors (CD14, CD11, CD18) for recognition by monocytes (335) which are recruited by MCP-1 expression and excretion following endotoxin exposure (336). The initiated cytokine response (335, 337) contributes to systemic low-grade inflammation. LPS initiates LDL oxidation, contributing to increased availability of atherogenic substrates (338). When bound to lipoproteins such as LDL, LPS can interact with LDL receptors to facilitate cellular uptake of LPS in the artery wall by smooth muscle cells or macrophages (339).

Bacterial DNA (bacDNA) is another proinflammatory bacterial component that triggers macrophage TNF-α, IL-6, and IL-12 expression (340) (see Figure 4). At high concentrations, the magnitude of a TNF-α response is comparable to that of LPS and can trigger toxic shock (341). Bacterial DNA is proinflammatory due to the recognition of unmethylated CpG sequences by TLR9, as mammalian DNA is 70 to 80% methylated (342,
As with LPS, the proinflammatory stimulation is dependent on the bacterial species from which CpG sequences originate (344). Additionally, bacDNA is present in plasma of patients with ascites and cirrhosis (345), alcoholic liver disease (346), Crohn's disease and ulcerative colitis, but not healthy subjects (347). Many of these conditions are associated with a breakdown of the gut barrier integrity (347) and changes in gut microbiota profiles (346). Patients with irritable bowel disease showed the presence of bacDNA from several species including *E. coli* and *Staphylococcus aureus*, while those with Crohn's disease additionally showed DNA from *Klebsiella* spp and *Streptococcus pneumoniae*. Furthermore, this bacDNA was detected in the absence of infection, and exhibit greater concentrations if the condition is active (347). CD14 -/- mice showed no such translocation of bacDNA, suggesting a role for CD14 in the bacterial translocation and subsequent induction of inflammation of bacDNA (303). The translocation of bacDNA has been further shown with its detection in atherosclerotic plaque in quantities relative to leukocyte content. Additionally, the species contributing to bacDNA in plaque were identified as commonly found in the gut and mouth, such as *Chryseomonas*, *Veillonella* and *Streptococcus* (47).

**Figure 4: Postprandial inflammation activation.**

Lipopolysaccharide (LPS) and bacterial DNA (bacDNA) initiate an inflammatory response through toll-like receptor (TLR) activation. The CpG sequences present in bacDNA activate TLR9 within the cell to stimulate the production of cytokines. Triacylglycerol-rich lipoproteins (TRL) can be taken up by the monocyte through apolipoprotein B-48 receptor (apoB48r) for chylomicrons or low density lipoprotein receptor (LDLr) for very low density lipoproteins (VLDL) also containing apolipoprotein B-100. TRL similarly initiate an inflammatory response.
Similarly to LPS, bacDNA may initiate inflammation after a high fat meal. Recent work has shown the presence of bacDNA in the plasma, adipose tissue, and lymph nodes of mice fed high fat diets prior to the onset of diet-induced obesity (303). However, no studies have acutely examined bacDNA translocation during the postprandial period, or in ageing, nor explored a relationship to lipid metabolism. These states are likely to favour translocation due to increased gut permeability, impaired lipid metabolism, and exaggerated immune responses.

1.4.2.2.2. Mechanisms of bacterial translocation and immune activation

The mechanisms of LPS inflammatory activation are complex and not yet fully understood. Membrane CD14 is the favoured LPS receptor, mediating LPS activity in immune cells. To facilitate this action, lipopolysaccharide binding protein (LBP) binds to LPS to form a stable complex recognised by CD14 to activate TLR4 (348). Alternatively, cells without CD14 expression, such as endothelial cells, can be activated by LPS bound to soluble CD14 (sCD14) (349, 350). Lipoproteins, particularly TRL, have a high affinity for LPS-LBP complexes through apoB (351), which facilitates endotoxin transport. These intermediates (lipoproteins, LBP, CD14) all facilitate monocyte immune activation, but also act to sequester and neutralise LPS (352, 353), suppressing an inflammatory response. For this reason, greater abundance of these mediators may signify both greater immune activation by LPS (354, 355), or a more capable systemic avoidance of sepsis (339, 356) (see Figure 5).

Lipoprotein and LPS interactions appear to be particularly complex, with a dual role of TRL for translocation and neutralisation of LPS. Postprandial increases in plasma LPS rely on chylomicron formation, and intestinally absorbed LPS associates with chylomicron remnants (332). A subsequent study visualised LPS bound to chylomicron surfaces following a high fat meal, and reported greater LPS concentrations in the chylomicron fraction relative to the chylomicron-poor fraction of plasma (324). Not only do these studies describe the role of chylomicrons in gut translocation of LPS, but a resulting inflammatory state has also been reported, including elevations in cytokine expression of TNF-α (332) and IL-6 (324). However, lipoprotein bound LPS has been shown to induce cytokine expression of IL-1 and IL-6 at rates of 20 to 1000 fold less when compared with unbound LPS (357). Indeed, in bacterial sepsis, hyperlipidaemia is protective against death due to LPS neutralisation through binding to lipoproteins (339). Lipoproteins, in particular HDL, have the capacity to remove LPS bound to monocytes, neutralising an immune response (358). Chylomicrons similarly demonstrate LPS neutralisation capacity (359), complicating their role as instigator and attenuator of endotoxaemia.

The interaction of LPS with lipoproteins has implications regarding lipid profiles and lipaemic responses. First, increases in circulating lipoproteins may be effective in neutralising LPS immune activation, as observed in cases of sepsis (360, 361). High serum HDL, with its high affinity for and neutralisation capacity of LPS, has been shown to be protective against death from LPS sepsis (362), with HDL infusions capable of improving survival (363). For populations with altered lipid profiles, such as MetS, T2DM, or the elderly, this may influence LPS responses. Additionally, high fat meal induced lipaemia facilitates LPS translocation (332). Concurrently, the endotoxin neutralisation capacity after high fat meal induced lipaemia is increased (49). Therefore, the immune response to LPS relies on a variety of factors, balancing immune activation and LPS neutralisation. As such, the magnitude hypertriacylglycerolaemia alone may not fully predict the concurrent endotoxaemic response.
Figure 5: Lipopolysaccharide translocation, immune activation, and neutralisation.

(1) Endogenous lipopolysaccharide (LPS) may be transported from the gut by chylomicrons. (2) Once in circulation, LPS can bind to intermediate factors, including CD14 and lipopolysaccharide binding protein (LBP) to be transported in the blood stream, able to form stable complexes with lipoproteins. (3) LBP and soluble (s) or membrane bound CD14 facilitate monocyte immune activation by LPS. Lipoprotein-LPS complexes are likewise associated with inflammatory activation. (4) Lipoproteins, particularly high density lipoprotein (HDL) are able to scavenge LPS to neutralise an immune response.
In summary, high fat diets can modulate postprandial endotoxaemia in conjunction with lipaemia through LPS translocation by chylomicrons, contributing to inflammation. Paradoxically, chylomicrons and other lipoproteins are integral in neutralisation of LPS, attenuating the resulting immune response, although this may only be the case under conditions of particularly high circulating endotoxin concentrations. It is still unknown how this dynamic relationship between lipid metabolism, LPS and postprandial inflammation interacts in older adults.

1.4.2.3. EMERGING BIOMARKERS OF POSTPRANDIAL STRESS: CELL-FREE DNA

Although not related to microbiome or gut integrity alterations, cell-free DNA is a potential transitory inflammatory substrate just as bDNA, acting along a similar mechanistic pathway. Recently, acute cfDNA fluctuations have been identified after exercise, an acute stress event. cfDNA has been used to identify overtraining induced inflammation in chronic resistance exercise (364), but has recently been reported as acutely increased after endurance exercise, returning to basal concentrations between 30 minutes (365) to 2 hours (366) post-exercise. Currently, the origins and implications of cfDNA are poorly understood, and investigation into confounding factors or the daily conditions acutely affecting circulating cfDNA are lacking (367). High fat meals are known to trigger an inflammatory response, in part through the oxidative pathways suspected to lead to DNA damage in post-exercise inflammation (366), and could lead to postprandial changes in circulating cfDNA.

1.4.2.4. POSTPRANDIAL INFLAMMATION AND ENDOOTOXAEMIA IN CHRONIC DISEASE

Elevated postprandial lipaemia and inflammation both contribute to increased CVD risk (368). Postprandially induced hypertriacylglycerolaemia is accompanied by increased glucose, proinflammatory cytokines, and markers of oxidative stress (369). The systemic stress caused by repeated exposure to high fat and energy meals stimulates the development of atherosclerosis and may contribute to acute cardiac events (370, 371).

Additionally, the metabolic alterations associated with chronic disease states may amplify the inflammatory meal response. Hypertriacylglycerolaemic subjects show marked leukocyte activation after postprandial glycaemia and lipaemia (298, 372). Patients with CVD show higher fasting concentrations of remnant lipoproteins (373), leukocyte lipid content (374), and postprandial IL-6 (375). Obesity is likewise associated with greater fasting and postprandial inflammatory markers (376). Obese women fed a carbohydrate meal with or without fat show higher serum IL-6 than lean women (377). Visceral adipose tissue may also contribute to postprandial inflammation with a response similar to monocytes, activating the NF-κB pathway (294, 378). Postprandial inflammation may be attenuated by body weight reduction (379, 380), although exercise prior to a high fat meal shows no impact (381).

Endotoxin responses are different in subjects presenting different concentrations of immune modulators, potentially due to altered disease and metabolic states. Circulating LPS, lipopolysaccharide binding protein and sCD14 are increased in alcoholic liver disease, possibly due to an associated impairment of the gut barrier integrity (321). Additionally, serum CD14 concentrations are increased in T2DM, especially those who are obese (382). High fat meals increase sCD14 concentrations in healthy subjects in conjunction with observed increases in LPS and IL-6, linking sCD14 availability after a high fat meal to immune activation of LPS (324).
Gastrointestinal tract diseases such as Crohn’s or colitis show increased circulating LPS concentrations, related to increased circulating LBP and sCD14, both immune modulators of LPS activity (383). This is associated with increased proinflammatory cytokines which are returned to normal after treatment for intestinal flare-ups, alluding to the importance of the gut barrier integrity (383). Dyslipidaemia, insulin resistance, obesity, and chronic inflammation (all indicators of MetS) are linked to high circulating endotoxin concentrations; in diabetics and non-diabetics, serum LPS is inversely correlated with insulin sensitivity (48).

Not only is LPS found in high concentrations in states of metabolic dysfunction, but introduction of endotoxin is sufficient to induce metabolic abnormalities. Animals infused with LPS quickly develop dyslipidaemia, insulin resistance or fasting hyperglycaemia, obesity and elevated proinflammatory indices, outlining the link between exposure to LPS and the development of metabolic dysfunction (326, 327).

Similarly to postprandial lipaemia, the risk factors for elevated postprandial immune responses are closely linked to underlying metabolic dysfunction. Indeed, postprandial lipaemic responses themselves are closely linked with a related inflammatory response, and as such the two mechanisms jointly contribute to a postprandial proinflammatory, pro-atherosclerotic environment. The changes in body composition and metabolic and digestive function that co-occur with ageing would predict similarities in postprandial inflammatory responses between the elderly and those with chronic conditions. Yet the inherent impact of advancing age on postprandial inflammation and the related mechanisms has not been fully explored.

1.5. DIGESTIVE RESPONSES IN THE ELDERLY

1.5.1. CHANGES IN CARBOHYDRATE METABOLISM IN AGEING

Carbohydrate absorption is typically preserved in the elderly, although it has been argued to be slightly impaired (384). Evaluation of glucose absorption with D-xylose has shown that absorptive capacity is unimpaired in the elderly when renal function is considered (385). The absorptive capacity of complex carbohydrates has been evaluated through hydrogen breath excretion, indicating that some, but not all, elderly subjects have greater complex carbohydrate malabsorption, exacerbated by larger carbohydrate loads (i.e. 200g) (386).

Glucose homeostasis is controlled mainly by insulin, and requires a balance between glucose synthesis de novo and glucose disposal by oxidation and storage (387). Evidence of dysregulation in the elderly has been demonstrated by age-associated decreases in glucose tolerance, measured as fasting glucose and glucose tolerance tests (388). Long-term glucose control, evaluated by HbA1c, also tends to gradually increase with older age (389, 390); however, strong differences in these age-related markers of impairment are not consistently reported (391, 392), and frequently wane when normalised over lean mass (393, 394). Importantly, hepatic suppression of glucose synthesis does not seem to be impaired in the elderly (395, 396), and is in fact activated more rapidly (397). Similarly, the secretion of insulin does not seem to be impaired in the healthy elderly (398); together, these factors indicate that impairment of glucose homeostasis is more likely the result of deficits in glucose disposal, exacerbated by tissue insulin resistance (396).
Muscle is the predominant tissue for insulin dependent glucose uptake (190). Ageing is associated with decreased peripheral insulin sensitivity, although this relationship was not significant after adjustment for BMI, after which the relationship only applied to lean women (393). These researchers (393) and others (399) have suggested waist-to-hip ratio and intraabdominal fat mass as the likely culprits influencing insulin sensitivity. Additionally, other confounding factors such as physical fitness (400) and habitual diet (401, 402) have strong influences on insulin sensitivity in the elderly, questioning the impact of age per se on peripheral insulin sensitivity (387, 398).

1.5.2. CHANGES IN PROTEIN METABOLISM IN AGEING

Changes to the digestive tract prevalent in ageing include chewing impairment (168), decreased stomach acid secretion (170), delayed gastric emptying (169), and morphological changes to the small intestine (403). Although it may be assumed that these factors have the potential to reduce digestive and absorptive capacity in the elderly, this is not overwhelmingly supported (404). There is minimal literature regarding the effects of ageing on protein digestion and absorption (404-406). Generally, there is little evidence of impaired protein digestion with advancing age; however, most data are compiled from dated animal studies (407-412) and few human subjects (413-415). Any absorptive impairment described in vitro and in vivo in animals, typically describes decreased absorption rates with ageing; however, there are many inconsistencies in the literature (Table 1), suggested to arise from methodological variation (416). Furthermore, the biological significance of absorptive capacity is questionable since these observations generally represent the intestinal affinity of the amino acid and are presented per weight of intestinal tissue. The overall length of the small intestine may compensate for any age-related differences based on per-segment assessments, since unabsorbed amino acids at a proximal segment may be absorbed further down the intestine (173). Indeed, excretion of unabsorbed dietary protein does not appear to differ in the elderly at moderate intakes (414). Additionally, investigations of protein digestion in elderly humans show conflicting results. At low (0.4g·kg⁻¹·day⁻¹) or moderate (0.8g·kg⁻¹·day⁻¹) protein intakes, no age differences in digestibility were observed, with enhanced digestibility reported at high intakes (1.6g·kg⁻¹·day⁻¹) (415). However, another study found that faecal protein excretion was increased in some, but not all, elderly subjects with higher protein intakes (1.5g·kg⁻¹·day⁻¹) (414).

Protein homeostasis in older adults depends on the balance between protein demand and protein supply. Although there are conflicting reports of whole body protein turnover, older adults appear to have unchanged whole body protein turnover when normalised to lean mass, (387, 417). However, metabolic demand is decreased in the healthy elderly, while postprandial protein utilisation, or efficiency of protein use, is unchanged, leading to apparently decreased protein requirements (418, 419). However, it remains controversial and without consensus whether protein requirements are changed with advancing age (420). Part of the reason for this is the discrepancy between protein requirements, efficiency of use, and functional outcomes such as the maintenance of muscle mass and strength, stemming from additional, complex mechanisms governing protein metabolism (72). Additionally, metabolic protein demands are likely to be affected by individual health status, particularly since co-morbidities like illness, injury, infection, or inflammation, not uncommon in the elderly, increase these requirements (421).
Table 1: Rate of amino acid absorption in aged animals relative to young animals.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Absorption</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>↑</td>
<td>(407)</td>
</tr>
<tr>
<td></td>
<td>↓&lt;sup&gt;3&lt;/sup&gt;</td>
<td>(422)</td>
</tr>
<tr>
<td>Arginine</td>
<td>Same</td>
<td>(408, 409)</td>
</tr>
<tr>
<td></td>
<td>↓</td>
<td>(410)</td>
</tr>
<tr>
<td>Aspartate</td>
<td>↓</td>
<td>(410)</td>
</tr>
<tr>
<td></td>
<td>↓&lt;sup&gt;3&lt;/sup&gt;</td>
<td>(422)</td>
</tr>
<tr>
<td>Glycine</td>
<td>↓</td>
<td>(407)</td>
</tr>
<tr>
<td>Histidline</td>
<td>↓</td>
<td>(423)</td>
</tr>
<tr>
<td>Leucine</td>
<td>↑</td>
<td>(407)</td>
</tr>
<tr>
<td></td>
<td>↓&lt;sup&gt;3&lt;/sup&gt;</td>
<td>(422)</td>
</tr>
<tr>
<td>Lysine</td>
<td>Same</td>
<td>(409)</td>
</tr>
<tr>
<td></td>
<td>↓&lt;sup&gt;3&lt;/sup&gt;</td>
<td>(422)</td>
</tr>
<tr>
<td>Methionine</td>
<td>↑</td>
<td>(424)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>↓</td>
<td>(411, 412)</td>
</tr>
<tr>
<td>Proline</td>
<td>↓</td>
<td>(411)</td>
</tr>
<tr>
<td></td>
<td>↓&lt;sup&gt;3&lt;/sup&gt;</td>
<td>(422)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Same</td>
<td>(411)</td>
</tr>
<tr>
<td></td>
<td>↓</td>
<td>(412)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>↓</td>
<td>(410, 412, 425)</td>
</tr>
</tbody>
</table>

<sup>1</sup>Absorption is rate of uptake in old animals relative to young animals.
<sup>2</sup>All studies were performed in vitro or in vivo.
<sup>3</sup>Not significant.
As fasting protein breakdown and MPS rates are not thought to be particularly impaired in the elderly, altered postprandial protein metabolism is likely the main contributor to age-related defects in protein homeostasis that influence muscle wasting in older adults (72). First-pass protein absorption is known to be influenced by splanchnic tissues, with certain dietary amino acids being predominantly consumed by the gut (426). Older adults have increased splanchnic uptake of leucine (427, 428), phenylalanine (429), and glutamate (430). This splanchnic sequestering has the potential to influence postprandial amino acid appearance and availability for MPS (427). However, despite the age-related increase in first-pass splanchnic use of amino acids, delivery to the muscle (431) and stimulation of MPS was found to remain intact in older adults (429).

The rate of protein digestion is known to affect postprandial protein retention (77); however, this effect is altered in elderly subjects (432). In young healthy adults, protein balance is optimal after a slowly digested protein, like casein, when compared to a fast protein, like whey (77). Yet, in older adults, the opposite is true, with better protein retention observed after whey ingestion (432, 433) or with pulse feeding of a large protein bolus (434). However, protein digestion rates may not translate to differences in MPS. Regardless of age, fast proteins and bolus feeding patterns result in greater MPS (435-438), indicating that this type of availability may be essential for optimal postprandial muscle outcomes. Indeed, it has been hypothesised that a ‘threshold’ concentration of leucine is necessary to activate MPS (439).

Possibly the most important contributing factor to altered protein metabolism in ageing is the inadequate stimulation of MPS in response to feeding, termed anabolic resistance (73). There is evidence that in the elderly MPS may be resistant to postprandial insulin increases (440). However, it is more likely that inadequate amino acid stimulation, possibly contributed to by upstream digestive factors as discussed, are the main cause. The stimulation of MPS requires adequate amino acids, but in particular essential amino acids, namely leucine (441). In the elderly, increasing the leucine content of a meal may help to overcome inadequate leucine concentrations (442), required to ‘trigger’ (73) MPS. To aggravate this anabolic resistance of the muscle to feeding, older adults are likely to have insufficient protein intake (443), unevenly distributed throughout the day (158), concurrent with alterations in digestive function (175) and insulin responsiveness (387) which all impact amino acid supply and ultimately MPS.

1.5.3. CHANGES IN LIPID METABOLISM IN AGEING

Lipid profiles are known to be altered with ageing; the typically elevated total cholesterol levels (201) and rampant use of cholesterol lowering medications (444) attest to the increased incidence of altered lipid metabolism in older adults (445). The origins of changed lipid metabolism in ageing are difficult to identify due to the co-occurrence of co-morbidities affecting metabolism. Changes to the digestive tract altering lipid digestion and absorption may provide a possible explanation, although hormonal and metabolic mechanisms involved in the clearance and storage of ingested lipids are likely key determinants of age-related differences in lipid metabolism.

Differences in lipid metabolism in older adults are readily apparent through routine blood chemistry analysis. Most circulating lipids increase with age including plasma cholesterol, TAG, and LDL, although HDL does not change as markedly (201). This trend shifts after age 70 when total cholesterol, LDL, and TAG decrease in
relation to age, a decline observed later in women than in men (201). Increased plasma LDL concentrations are further observed by the increased concentration of apoB-100 in the elderly (235). Ultimately, basal lipid metabolism and status is a factor in determining CVD risk but also in metabolic function in the short term.

Numerous studies of postprandial lipaemia in older adults have demonstrated prolonged and exaggerated circulating TAG responses to high fat loads (14, 15, 235, 446-448). This postprandial lipaemia is often characterised by greater TAG iAUC (15, 235), and greater chylomicron triacylglycerol concentrations (188, 446). Postprandial TAG concentrations are increased in the elderly (235), and ingested lipids contribute more to postprandial plasma non-esterified fatty acids and small (S=20-100) triacylglycerol-rich lipoproteins (14, 235). In adults over 41 years, postprandial lipaemia is higher and remains increased after 6 hours (449, 450), compared with adults less than 40 years whose serum TAG concentrations decrease between 2 & 6 hours postprandially (449). Through examination of carotenoid bioavailability in the elderly, the chylomicron triacylglycerol response in older subjects was found to be 27% higher than young subjects, attributed to delayed clearance and reduced lipoprotein lipase activity (188). However, TRL clearance rates have been shown to be equal in lean and MetS middle-aged men, and hypertriacylglycerolaemia was rather attributed to increased production rates of VLDL and chylomicrons (215). Moreover, many of the factors known to contribute to increased VLDL production, such as increased fat mass, fatty liver disease or insulin resistance (276), are at a high prevalence in older populations (19-21). Indeed, higher VLDL concentrations are typical in older adults (235). It is clear that the elderly have an altered postprandial lipaemic response; however the contributing age-related differences in dietary lipid absorption, postprandial TRL formation and contribution, or TRL and fatty acid clearance have yet to be fully described.

1.5.3.1. ALTERED FAT AVAILABILITY

Age-related differences in lipid metabolism may be impacted by changes to the digestive tract in the elderly. The possibilities in the elderly of reduced gastric emptying, pancreatic secretions, possible pancreatic lipase insufficiency, reduced bile acid secretion (175), changes to the unstirred water layer affecting intestinal permeability of nutrients (451) seen in ageing, highlight the myriad potential variations contributing to altered lipid absorption. Lipase production and activity depends on the integrity of the mouth (lingual lipase), stomach (gastric lipase), and pancreas (pancreatic lipase). Adequate bile acid secretion is essential for lipid emulsification to facilitate absorption, and loss of gall bladder function or the actions of cholesterol lowering medications could impact bile salt availability. Changes in lipase availability also have the potential to influence fatty acid absorption through their stereospecific actions, and through fatty acid catabolism and lipoprotein clearance from circulation.

1.5.3.2. ALTERED FAT ABSORPTION

Determinations of age differences in fat absorption have focused on indirect measures, namely faecal fat content and the absorption of fat-soluble vitamins. In a study examining the faecal fat content in subjects aged 19 to 91 years old, no differences in fat absorption were reported based on evidence that age-related differences in the D-xylose absorption test were attributable to renal function decline (201). Contrarily, another study showed that faecal fat content increased in the elderly, hospitalised and malnourished subjects, suggesting a decreased ability to absorb fat (172). Despite this increase in faecal fat content, subjects were still capable of absorbing 329g of fat daily.
This is consistent with reports that fat absorption is unchanged in older adults (172, 173). In contrast, the elderly are reported to have greater faecal fat excretion with intakes of 115-120g·day⁻¹ (414).

Research on fat soluble vitamins as indicators of fat absorption offers more consistent conclusions. For example, vitamin E is transported exclusively by lipoproteins and requires chylomicrons to be brought into circulation from the gut making it an indicator of the gut’s ability to absorb fat (447). In the elderly, the relative content of vitamin E in chylomicrons was lower while the content in plasma was higher but proportional to the plasma content in younger adults. This imbalance suggests that ageing causes a possible decrease in intestinal absorption of vitamin E in the elderly, and affects the transport of dietary vitamin E through lipoprotein metabolism (447). Vitamin A rich chylomicrons are cleared half as quickly in older adults compared with younger adults, indicating chylomicron clearance may be delayed with ageing due to age-related decreases in lipoprotein lipase (188, 446, 452). The closely related lycopene, while not a vitamin, is similarly transported by lipoproteins and has reported decreased bioavailability in the elderly (188). This finding correlated with a higher, although not significant increase in chylomicron triacylglycerol response, indicating differences in fat metabolism between old and young adults (188). Finally, although research suggests differences in vitamin D and K absorption in the elderly, these differences are more likely due to endogenous vitamin production and vitamin specific gut receptors rather than fat metabolism (453). The differences in chylomicron clearance of vitamin E and A suggest possible differences in fat absorption and probable differences in lipoprotein metabolism.

Aside from studies of lipid soluble vitamin appearance, differences in postprandial lipid absorption pertaining to specific lipid structures has not been investigated in older adults. Evidence from animal models suggests that absorption or uptake of certain fatty acids may be enhanced in older age (451, 454-459); increased uptake of oleic acid (455), linoleic acid (457), and saturated fats (403) have all been shown in the intestines of aged rats compared with younger animals. The origin of these differences is unknown, and is typically attributed to differences in intestinal integrity (403, 456), the passive permeability of unstirred water layers (451, 456, 457), or chylomicron formation (460). Such differences in fatty acid absorption may be reflected in longer-term fatty acid depots, as small differences in absorption amplified over the course of years have the potential to become biologically significant (455). Cross-sectional studies in the elderly have found differences in fatty acid compositions in circulation (461-463) or adipose tissue (464), which could originate from absorption differences as suggested by animal models. Decreased SFA (465) and linoleic acid (464) and increased MUFA (465) content of adipose tissue has been reported in the elderly. Serum triacylglycerols of older men contained greater proportions of arachidonic acid and omega-6 fatty acids but lower overall PUFA (461). However, health status and habitual diet may confound these findings as other research on institutionalised elderly subjects conversely showed lower circulating triacylglycerol arachidonic acid (463). Similarly, previous research has shown lower phospholipid proportions of linoleic acid (461) and arachidonic acid (463) in the elderly, although non-institutionalised elderly were reported as having greater proportions of arachidonic acid (461). Additionally, older adults have been reported as having relatively more phospholipid EPA and DHA (462). However, the metabolic mechanisms behind these differences have yet to be investigated and may depend on habitual diet and health status more than lipid absorption.
1.5.3.3. ALTERED TRIACYLGLYCEROL-RICH LIPOPROTEIN FORMATION

Postprandial lipaemia can be further described by chylomicron dynamics and composition after meal ingestion. Chylomicron composition and size is reported to fluctuate differently in the elderly. Prior to a fat load, TAGs measured in the TRL of older adults were more abundant in the large particles (S=175-400) while younger adults showed higher concentrations of TAGs in small particles (S=20-100) (450). After a meal, the concentrations of TAGs in TRL trended towards an increase in less dense, larger particles (Sf>400), mainly represented by chylomicrons (450). However, size measurements used in postprandial studies in the elderly have been inconsistent and are often proxy measures, such as fat soluble vitamin appearance (188, 446-448), or apoB-48 to TAG ratios (254), offering little certainty over the true postprandial changes in size.

1.5.3.4. ALTERED TRIACYLGLYCEROL-RICH LIPOPROTEIN CLEARANCE

There is strong evidence to suggest that older adults have impaired lipoprotein, and specifically triacylglycerol-rich lipoprotein, clearance. Impaired lipoprotein clearance is often attributed to reduced lipoprotein lipase activity in older adults (282, 447), impairing not only TRL clearance (446) but also LDL clearance (201). However, a more recent study found that chylomicron lipolysis was not impaired in elderly adults, but more specifically, that chylomicron remnant clearance appears to be impaired 2 fold (466). Impaired remnant clearance has been associated with decreased hepatic LDL receptor expression in aged rats (467). Additionally, older adults are reported to have slower catabolism of omega-6 rich TRLs which require adequate lipoprotein lipase for effective clearance (247). However, since chylomicron remnant clearance depends more on hepatic lipase, found to be unchanged in older adults (282), clearance differences in ageing may rely on factors other than lipase activity and could be explained by TRL clearance competition. VLDL and chylomicron clearance is competitive for lipoprotein lipase, and greater proportions of VLDL particles in older adults (235, 468) could impair chylomicron clearance. Furthermore, greater total apoB concentrations are thought to directly limit lipolytic capacity (271).

Furthermore, whole body lipid metabolism may impact postprandial lipid dynamics. Fatty acid oxidation has been found to be impaired in older women, which could indicate a susceptibility to store dietary fatty acids in adipose tissue rather than oxidising them for energy (469, 470). Catabolised fatty acids from TRLs show up in circulation as non-esterified fatty acids, normally suppressed in the early postprandial period. Impaired postprandial non-esterified fatty acid suppression is evident in obesity (471) and has been reported in the elderly (235). As fat oxidation is impaired in older women (470) and dietary fat clearance of chylomicron triacylglycerols is impaired with insulin resistance and obesity (472), compensatory tissue uptake of dietary non-esterified fatty acids in older adults is less likely, resulting in elevated postprandial non-esterified fatty acid due to insufficient fatty acid clearance. This non-esterified fatty acid spillover of lipolysed chylomicron TAGs has been reported as increased by 40% in elderly adults (235); however, this was reported in elderly adults that were not matched for body mass index (BMI) or fat mass, and insufficient adipose or muscle uptake of non-esterified fatty acids may have contributed to a so-called age-related difference (235, 473). Additionally, the authors showed in a subsequent study (15) that addition of an L-arginine supplement to a pure fat load attenuates the postprandial accumulation of endogenous non-esterified
fatty acids, allowing for adequate tissue uptake of meal derived non-esterified fatty acid, and attributed this to the greater insulin response triggered by the ingestion of protein.

Additionally, older adults, as a heterogeneous population, are more likely to exhibit additional risk factors for altered postprandial lipaemia. Basal lipid status, insulin sensitivity, body composition, habitual diet, and health status, are all known to influence post-meal lipid metabolism (224). Similarly, increased body weight and adipose tissue are correlated with increased plasma TAGs, a likely circumstance in elderly populations (446). Muscle wasting, or sarcopenia, may contribute to postprandial lipaemia in the elderly (474) since skeletal muscle is important in the removal of circulating TAGs (475). Loss of skeletal muscle may also explain differences in fat oxidation (476). In summary, ageing is associated with increased postprandial lipaemia that may or may not be independent of the many other changes that occur with increasing age.

1.5.4. GUT IMMUNE RESPONSES IN AGEING

1.5.4.1. THE GUT AND BARRIER FUNCTION IN AGEING

Changes in digestion, metabolism, and gut microbiota that occur in ageing may impact the interactions of the gut with the food environment. Intestinal diseases such as small intestinal bacterial overgrowth are more frequent in the elderly; this is associated with steatorrhoea, likely caused by deconjugation of bile salts by pathogenic bacteria (172). Reduced bile salts in the gut, whether from bacterial interference or decreased production, increase the possibility of gut barrier failure and bacterial translocation by allowing bacterial colonisation (477, 478) and decreasing macrophage activation (479, 480). Indeed, the immune function of the gut may be altered with age (481). The intestinal epithelial cells in the ageing gut display upregulation and increased secretion of inflammatory cytokines (482). Furthermore, alterations in gut morphology, including cell proliferation, mucus secretions, and intestinal permeability may contribute to local gut changes in junction protein expression, anti-microbial peptides, or bacteria adherence affecting interactions with the bacterial environment (481). However, there are limited definitive data available on alterations of these factors in the elderly, discussed in brief below, to enable determination of their effects on gut immunity in the elderly (Table 2).

There have been indications of age-related alterations in intestinal morphology; however these are frequently reported from animal models and provide conflicting results when compared with human evidence (Table 2). In general, broader (483, 484) and shorter (484-486) villi have been frequently reported with ageing, alongside possible alterations in mucosal properties (403, 487), although an absence of morphological differences are also reported (488-490). Yet, it is argued that even if such morphological changes are characteristic of ageing, it cannot be determined whether these can be expected to affect permeability or nutrient absorption since no clear associations have been made (384).
Table 2: Age-related changes in intestinal morphology and function.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Change</th>
<th>Reference model</th>
<th>Animal</th>
<th>Human</th>
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<td></td>
</tr>
<tr>
<td>Size</td>
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<td></td>
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<tr>
<td>Number</td>
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<td>(491)</td>
<td></td>
</tr>
<tr>
<td>Ratio (to villi)</td>
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<td></td>
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<td>(488)</td>
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<tr>
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<td>Paneth cell secretion</td>
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<td></td>
<td>(493,494)</td>
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<tr>
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<td>(495)</td>
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<td></td>
<td>(453,498-500)</td>
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¹Evaluated in comparison with young counterparts.
²Assessed after gamma irradiation injury.
In ageing rats, intestinal permeability has been shown to be compromised, reducing the ability of the small intestine to exclude large (496) and medium sized molecules (497). Nonetheless, the integrity of gut barrier function in the elderly may not necessarily be compromised. Lactulose mannitol tests suggest that small intestinal permeability is maintained in older age (453, 499, 500). More recently, this was confirmed in a large cohort of 215 healthy elderly which also found no increase in intestinal permeability with older age (498). However, the researchers noted that the presence of insulin resistance or low-grade inflammation were more strongly associated with compromised gut barrier function. Importantly, they concluded that intestinal permeability is likely affected by, rather than contributing to, these co-morbidities. Furthermore, high fat diets, and the resulting changes to gut microbiota have been shown to increase gut permeability to lipopolysaccharide in mice (501), reiterating the impact that changes in dietary patterns or the digestive environment can have on postprandial events. This may provide insight into the apparent link between metabolic dysfunction and increased intestinal permeability to a high fat meal. Whether the elderly have increased susceptibility to high fat meal induced endotoxaemia is thus far unknown.

1.5.4.2. CHANGING MICROBIOME IN AGEING

The bacteria that populate the gut are diverse, innate (502) and so important they are often described as an organ in their own right (503, 504). Emerging evidence has revealed the role of the gut microbiota in chronic conditions such as obesity, micronutrient and functional metabolite production, and overall gut health. The composition of the gut microbiota responds to environmental pressures and is found to be different between subjects with different health status or even habitual diet. It remains to be identified whether such differences are causative to the phenotypes they associate with or whether they are a phenotype in and of themselves.

The bacteria in the gut can directly affect intestinal epithelial cell differentiation and proliferation (504, 505), and through their own proliferation alter the demand for uptake of nutrients such as glucose (506). Additionally they can increase nutrient absorption by increasing mucosal vascularisation and blood flow (506, 507). Not surprisingly, it has been suggested that the composition of gut microbiota is related to energy balance (508). This effect could be related to the ability of the gut flora to metabolise indigestible nutrients such as certain polysaccharides, converting them to short-chain fatty acids and monosaccharides (506).

The activities of these gut microbiota and their related health outcomes vary depending on the particular species residing in the gastrointestinal tract. The relative ratio of the Firmicutes and Bacteroidetes examined in recent studies has shown to be altered in certain disease states (509-511) and can be influenced by changes to the diet (508, 512-514). The Firmicutes, representing the largest phylum and including genera such as Lactobacillus, Mycoplasma, Bacillus, and Clostridium (515), are more dominant than Bacteroidetes in obese subjects compared with lean (509, 516, 517), although others report opposite findings (510), while no differences have also been reported (511). An increase in Firmicutes has also been shown in animal studies on ob/ob mice fed a polysaccharide rich diet; this effect was due to a 50% decrease in Bacteroidetes (518). Decreases in Bacteroidetes seen with a Western diet have been shown to be caused by the opportunistic growth of other bacterial classes such as Mollicutes, in turn allowing for an increase of Firmicutes; this effect can be reversed with a return to a normal diet (512, 513). A Western-type diet is able to induce changes to the gut microbiota even in RELM-B KO mice that are resistant to
high-fat induced obesity, demonstrating that separate from obesity, diet alone is sufficient to alter the intestinal environment (514, 519).

The gut microbiome is different in obese individuals (509-511) and can change with the onset of obesity (518) or with changes to the diet (514). High fat diets influence gut microbiota consumption of choline, leading to changes in VLDL assembly and secretion and potential alterations of bile acid conjugation, inducing metabolic abnormalities such as insulin resistance, and hepatic lipid storage (520, 521). Further research has shown that transplanting the bacterial flora of obese mice to lean (508, 512) or germ-free mice will induce obesity, showing that the microbiota profile can influence chronic disease (512, 516).

Environment (522), diet (508, 512-514), and health status (509-511) are known to affect the gut microbiome. Similarly, ageing has been associated with characteristic differences in microbiota. The physiological changes to the gastrointestinal tract in ageing are likely to affect the composition of the gut microbiota. The elderly are reported to have higher proportions of the phylum Bacteroidetes while younger adults have higher proportions of Firmicutes (523). Centenarians appear to have less microbial diversity, with a relatively greater proportion of Proteobacteria (524). Other age-related differences, such as greater proportions of the genus Akkermansia, have been reported by some (524) but not others (525), demonstrating the sustained importance of individual variation on microbiota determination. As with many so-called age-related differences, the impact of living-environment, including health status and habitual diet may be a more important determinant of gut diversity that age per se. In a recent study, individuals living in long-term care facilities had higher proportions of Bacteroidetes and less diverse microbial populations, while community dwelling subjects showed higher proportions of Firmicutes (522). Importantly, these less diverse populations have been more highly associated with increased frailty, decreased diet diversity, and increased inflammation. Indeed, changes over the lifespan are reported to be slight within individuals despite vast differences in inter-individual variation (522), emphasising the complex influence of nutritional, environmental, and additional factors on the microbiome.

1.5.5. POSTPRANDIAL INFLAMMATORY RESPONSES IN AGEING

Although the postprandial lipaemic effects of a high-fat meal have been described in older adults (14, 15, 188, 235, 446-448), the associated inflammatory response has not yet been investigated. Postprandial lipaemia and inflammation are already documented as elevated in metabolically compromised adults, such as those displaying insulin resistance or T2DM (272, 377, 526, 527). As these conditions are more prevalent in the elderly (21, 528), and ageing is associated with a state of chronic low-grade inflammation (95), it is likely that the inflammatory response to a meal is exaggerated in older adults. However, age-related differences in postprandial inflammation have not been described relative to younger controls, yet the similarity of postprandial response between healthy elderly and centrally obese or MetS elderly (529) suggests that elevated postprandial inflammation may be inherent to ageing itself.
1.5.5.1. POSTPRANDIAL OXIDATIVE STRESS RESPONSE IN AGEING

Oxidative stress, the accumulation of free radicals, is increased in obesity and ageing, and has been linked to the development of chronic diseases through mechanisms ranging from protein to cellular to genomic damage contributing to loss of function, cellular death and inflammation (530). Postprandial inflammation is in part initiated by a meal-related increase in oxidative stress, resulting in increased immune activation and production of cytokines (531). The postprandial lipaemic response is a potent activator of this oxidative response, which is likewise elevated in metabolic dysfunction (182). Despite these associations, the postprandial oxidative response in the elderly has only been studied in relation to the impacts of long-term dietary interventions, without comparison to healthy younger adults (532, 533).

1.5.5.2. POSTPRANDIAL ENDOXOAEMIA IN AGEING

Conditions of metabolic dysfunction have been associated with increased concentrations of circulating endotoxin. Dyslipidaemia, insulin resistance, obesity and chronic inflammation all show elevated endotoxaemia; in diabetics and non-diabetics, serum LPS is inversely correlated with insulin sensitivity (48). In a recent study, elevated plasma LPS concentrations were found in healthy elderly subjects (≥65 years) in comparison with healthy younger adults (≤45 years) (534). This suggests that older adults, particularly those with reduced insulin sensitivity or co-morbidities, may be more likely to have elevated background LPS concentrations, which may predict postprandial endotoxin responses in the elderly.

As lipid metabolism (324, 535, 536) and intestinal permeability (537) are integral in the translocation of endotoxin after a high fat meal (324, 535, 536), it is not surprising that conditions of dyslipidaemia, such as obesity or T2DM (538), are associated with exaggerated postprandial endotoxaemic responses. The elderly have the combined potential circumstances of increased basal LPS, an increased and prolonged immune response to LPS (113), altered postprandial lipoprotein metabolism (173, 201, 235, 450), delayed TRL clearance and variations in intestinal integrity (173) or microbiota composition (178). All or any of these conditions make an altered postprandial endotoxaemic response in the elderly probable.

1.6. THE USE OF mixed meals IN POSTPRANDIAL RESEARCH

Postprandial research, by definition, studies the physiological responses post ingestion. While it may be assumed that the purpose of this research is to understand the body’s response to meals, many studies choose to examine the isolated effects of individual components by administering isolated foods or even macronutrients independently. In particular, the postprandial responses of the elderly to individual food components have the potential to be drastically different from the responses to whole foods. These isolated responses are unlikely to appropriately predict responses to realistic food consumption, but are highly likely to erroneously predict usual postprandial digestion and metabolism and inform nutritional recommendations.

‘Meal’ challenges have been widely diverse in the literature and range from the ingestion of isolated macronutrients (i.e. amino acids (429, 539-541)), to purified food components (i.e. hydrolysed protein (435, 542),
casein (433), or test oil in soup form (250)), to individual foods (i.e. cream (235), milk (543) or steak (544)), to mixed isolated macronutrients (i.e. nutritional supplements (427, 545-547)) or mixed whole foods (448). Furthermore, the size of a test bolus may be isocaloric (526), tailored to the individual's body weight (235, 446), or may be supplied in far larger quantities than typically consumed in a normal setting, a feeding pattern known to induce greater postprandial endotoxaemia (548). While these types of studies may facilitate investigations of the unique physiological effects of specific food components, they contribute to a body of literature which does not describe the postprandial response expected from a typical meal. As such, it is inherently difficult to determine the translatability of this research.

1.6.1. IMPACT OF MACRONUTRIENTS ON POSTPRANDIAL RESPONSES

The composition and quantity of macronutrients, micronutrients and other meal components influence the magnitude and duration of the physiological alterations associated with the postprandial state. Macronutrients, when ingested separately (549), exert differing effects on postprandial glucose, protein and lipid metabolism, in part through the differences experienced in gastrointestinal transit times, and enzymatic and hormonal stimulation. These effects are altered when macronutrients are ingested together (549, 550). It is not surprising that the study of postprandial responses to meals may be highly influenced by the relative composition and structure of the foods ingested.

Carbohydrates, when added to a high fat meal (551), can delay (256, 552) and reduce postprandial lipaemia (224, 553). This effect depends on the quantity (554) and quality of carbohydrate in the meal, and is particularly evident when dietary fibre is included (553). Carbohydrate co-ingestion with protein also delays the postprandial appearance of amino acids (545), potentially negatively impacting on downstream postprandial phenomena such as MPS (545, 546). These alterations to the postprandial response may originate from the effects of carbohydrate ingestion on insulin secretion or the gastrointestinal transit time of starch or fibre gelatinisation (195). Regardless of the mechanisms, the impact of carbohydrate co-ingestion on the postprandial responses to other macronutrients ultimately influences the findings reported in literature as well as the translatability of responses in a real world context.

Protein likewise has attenuating effects on postprandial lipaemia (555), particularly in older adults where postprandial lipaemia is already exaggerated (15). The type of protein affects the magnitude of postprandial inflammation resulting from a high fat meal: whey protein is a less effective postprandial inflammatory suppressant than cod or gluten protein (556), demonstrating the importance of protein type on the resulting post-meal responses.

Just as protein or carbohydrate quality affects postprandial responses, the type of fat ingested greatly influences postprandial lipaemia and inflammation. Fat type is known to affect postprandial lipaemia (224) and inflammation (333), but further, not all types of fat are created equal. Although saturated fats are typically associated with greater lipaemia and inflammation, butter elicits a lower inflammatory response than olive oil, indicating that the individual fatty acids are the true determinants of the predicted postprandial response. Indeed, epidemiological
evidence has hinted at the long-term cardiovascular protective nature of dairy products (557), and emerging evidence of dairy-specific saturated fatty acids present at greater quantities in circulation of individuals with lower CVD risk suggests that individual fatty acids may have a role to play (558). The physiological effects of triacylglycerol interesterification have demonstrated that altering the frequency of specific fatty acids in the sn-2 triacylglycerol position can increase or decrease the resulting lipaemia (559). These effects are thought to rely on the physical properties of these fats resulting from their chemical structure (560), further emphasizing the complexity of examining the effects of food on physiological responses.

Postprandial lipaemia and the associated oxidative stress and inflammatory response are reduced after meals which include micronutrients such as the antioxidants or polyphenols from foods like orange juice (331, 536, 561), tomatoes (562), strawberries (563) and green tea (564). When designing a postprandial challenge or considering the postprandial effects of specific foods, macro- and micronutrient components are indeed important considerations.

1.6.2. IMPACT OF FOOD STRUCTURE ON POSTPRANDIAL RESPONSES

In addition to the macronutrient selection and composition of a meal, the structure of the foods, including factors such as emulsification or microstructure, are known to, and indeed manipulated to, impact food digestion (565, 566). Digestion of more intact physical structures such as milk (casein and whey) (543) or steak (544), is delayed compared to food matrices where amino acids are more easily accessible such as whey or minced beef.

Even the gold standard Oral Glucose Tolerance Test (OGTT) provides a carbohydrate load in the form of simple monosaccharides, which typically make up merely 2-3% of a food carbohydrate load and are therefore poorly applicable to normal physiological responses (567). The differential effects of carbohydrates with varying glycaemic indices also makes the OGTT weakly comparable to typical food, or even carbohydrate, ingestion, as criticised by others (567, 568).

Fat digestion and metabolism are impacted by the molecular structure of individual triacylglycerols, the supramolecular structure of TAG, and the macromolecular structure of the food matrix (569). Molecular structure has the potential to affect absorbability of fats as well as their lipaemic effects. Greater postprandial lipaemia is observed when fats with SFA in the sn-2 position are ingested (570), which may be due to the relatively greater absorbability of sn-2 saturated fatty acids (571). The positioning of MUFA in the sn-2 position of milk fat may be one factor responsible for the beneficial properties of milk despite being a highly saturated fat source (572). This may further be influenced by the tendency for long-chain fatty acids to saponify if in the sn-1 or sn-3 as they interact with calcium, decreasing absorption and increasing the faecal fat content (573). Furthermore, the physical properties imbued by sn-positioning of fatty acids may be a significant factor in the magnitude of postprandial lipaemia. Fats with greater solid fat content at body temperature result in reduced postprandial lipaemia (560). Additionally, supramolecular structures, such as emulsions and the size of lipid droplets may affect fat digestion. TAG are metabolised more slowly when presented as small fat droplets in emulsion (511, 574), in part due to the effects of droplet size on delaying gastric emptying (572). Furthermore, lipase activity can be mediated by the
phospholipid/sphingomyelin or protein composition of the lipid-water interface in an emulsion, factors which vary widely between food products and impact absorption (569). Macromolecular matrix effects on lipid digestion can be exemplified by almonds, whose fat remains inaccessible during digestion due to unruptured cell walls (575), resulting in notable faecal fat excretion and lower than expected energy content (576), and can affect lipaemia (577). Dairy products may also have differing effects on postprandial lipaemia depending on their varied physical structures; studies in rats have shown greater fatty acid absorption after cream compared with butter or cream cheese (578), while milk fat added to milk accelerates lipaemia compared with milk fat in the form of cream (574). In humans, butter ingestion results in delayed TAG appearance compared to milk or cheese in T2DM (579); however, this may not result in differences in total TAG appearance (579, 580).

1.6.3. DISCREPANCIES IN POSTPRANDIAL RESEARCH ATTRIBUTABLE TO MEAL VARIATION

Despite the wealth of research available in the field of food science on the impact of macronutrient interactions and food structures on digestion (195, 565, 566, 569), physiological research has not adequately addressed representative food composition as a consideration for predicting an individual's response to a meal. While the varying effects of different meal formulations or components have been investigated in the postprandial literature in healthy subjects (545, 550, 581), these interactions are more rarely examined in the context of individuals with altered postprandial responses such as in ageing populations or those with metabolic dysfunction. Although varying meal challenges may have been studied in obese, insulin resistant, or elderly populations, these findings are often reported without healthy controls or typical meal controls, making the conclusions stemming from these studies difficult to place in the context of the expected physiological responses real world meals.

Indeed, studies evaluating the effects of fat type (i.e. butter vs. oil) (226) without controlling for fat structure (i.e. water-in-oil emulsion vs. oil-in-water emulsion) have been criticised as structure impacts digestibility and bioavailability (572). Postprandial phenomena such as endotoxaemia may be confoundingly influenced by meal structure, as many studies comparing high fat meals with lower fat meals ultimately vary in lipid supra- and macromolecular structure. For example, emulsified lipids elicit greater endotoxaemia than do free lipids (324), a factor not controlled for in other studies of the endotoxaemic (582) or inflammatory effects of meals (377, 583, 584).

1.6.4. MEAL STANDARDISATION AND MIXED MEAL USE IN POSTPRANDIAL RESEARCH

Although test boluses used to evaluate postprandial lipaemia generally attempt to approximate a mixed meal, these formulations inevitably vary widely from study to study. There have been suggestions to standardise fat tolerance tests (208, 585-587), attesting to the importance of studying postprandial lipaemic responses to fat loads that are physiologically relevant, and representative of typical mixed meal composition (208). While some research laboratories have successfully employed their own standard test meal (560, 588, 589), or have drawn on standardised high carbohydrate, high-fat mixed meals representative of the Western diet (536, 561, 582), there still
remains wide variation between studies. Unfortunately, research on protein metabolism falls even further behind with studies using non-representative test ingredients (429, 432, 433, 435, 539-542, 544, 590, 591) at similar frequencies to those attempting ‘food-like’ boluses (427, 543, 545-547, 592, 593).

1.7. CONCLUSION

In summary, nutrition is an essential and malleable factor in the maintenance of health and progression of chronic disease. Alterations in macronutrient supply through dietary, digestive, or metabolic means has the potential to impact long-term health. The elderly, a growing segment of the population, face social and biological changes that increase their risk of negative health outcomes, many of which may be influenced by nutrition. The progression of CVD and sarcopenia is affected by the postprandial protein, lipid, and inflammatory responses elicited by macronutrients, which is in turn affected by their quality, quantity, proportions, and physical structures.

Research is needed to bridge the gap between the effects of individual macronutrients, and the effects of real foods on postprandial responses. Further, the distinction between the metabolic phenotypes of ageing per se or confounding co-morbidities may clarify the specific health and nutrition concerns in healthy ageing. Understanding the differences in the acute responses to foods in the elderly is necessary for shaping appropriate nutritional recommendations for the ageing population for the prevention of disease and maintenance of lifelong health.

1.8. PERSPECTIVES

As metabolic and cardiovascular morbidities rates rise concurrently with the ageing population, the importance of understanding age-related pathophysiology and developing interventional strategies grows. There is still incomplete knowledge of changing digestive responses in the elderly, despite the increasingly apparent postprandial contribution to muscle maintenance and cardiovascular disease. As the population ages, strategies to temper the negative metabolic effects of certain foods while increasing the effectiveness of the beneficial nutrients may be essential in developing nutritional interventions and recommendations to prolong life by conserving and supporting health in older age. Future research must focus on understanding the mechanisms driving age-related differences in postprandial metabolism to assist in developing nutritional strategies for the elderly.

There is still incomplete knowledge of the differences in postprandial macronutrient metabolism experienced by older adults. Specifically, amino acid absorption in older adults has been investigated in the context of individual foods or macronutrients, but has not been studied in the context of whole food meals in comparison with younger adults. Secondly, postprandial lipoaemia is known to be altered in older adults, but there is poor understanding of age-related differences in fatty acid absorption, chylomicron dynamics, and TRL clearance in the postprandial period. Thirdly, the acute postprandial inflammatory responses to meals have not been investigated in older adults in comparison with younger adults. Despite the known participation of TRL and elevated lipoaemia in acute inflammatory activation by lipoproteins, fatty acids, endotoxin, and oxidative products, these have not been considered in older adults in comparison with younger adults.
1.9. AIMS

The aims of this thesis were to investigate the metabolic and digestive changes that are associated with ageing and to examine the postprandial inflammatory consequences of these differences after the ingestion of a meal to ultimately provide insight into the nutrition-related mechanisms driving chronic disease risk. Specifically, this thesis aimed to characterise the differences in protein digestion in older adults which may contribute to sarcopenia in the elderly by exploring postprandial protein absorption in older adults following mixed high protein meals. Secondly, we aimed to detail the differences in chylomicron dynamics and composition which may drive the postprandial lipaemic contribution to CVD risk. We also aimed to account for the impact of representative whole meals on these lipaemic responses, to provide physiological relevant findings for older adults. Thirdly, we aimed to determine whether ageing per se is associated with increased postprandial inflammation, and to identify the contributions of lipid, immune, and gut related mechanisms involved.

To do this, a randomised cross-over acute meal intervention including younger and older adults was designed to examine the postprandial responses to a high fat, high carbohydrate, high protein mixed meal and a low fat, high carbohydrate, high protein mixed meal. The details of this study design are described in Chapter 2. In Chapter 3, the aminoacidic responses to high protein mixed meals in older adults are presented. In Chapter 4, the chylomicron dynamic and lipidomic responses of older adults to a high fat meal are presented, while Chapter 5 explores these responses to a low fat meal. In Chapter 6, the immune responses to a high and low fat meal in older adults are presented. Overall conclusions and future directions are proposed in Chapter 7.

1.10. HYPOTHESES

It was hypothesised that older adults would exhibit deficits in postprandial macronutrient absorption, alterations of postprandial metabolism, and increased immune responses related to impaired gut barrier and immune function following mixed meals. Specifically, older adults were hypothesised to have (i) reduced or delayed amino acid appearance, (ii) elevated triacylglycerolaemia characterised by larger chylomicrons with greater TAG concentrations, altered chylomicron TAG and phospholipid fatty acid profiles, and (iii) increased endotoxaemia, inflammatory gene response, and inflammatory marker appearance.
CHAPTER 2

METHODOLOGY
2.1. RESEARCH DESIGN AND MAIN OUTCOMES

This study was designed to address the main aims of the research using the study population of older adults examining the metabolic and inflammatory effects of meal ingestion, using an acute cross-over design. The main areas of analysis were protein metabolism, lipid metabolism, and immune responses. Protein metabolism was assessed through amino acid metabolomics by UPLC. Lipid metabolism was characterised by plasma lipid profiles including circulating TAG and NEFA, with detailed chylomicron analysis including particle size analysis by dynamic light scattering, and TAG and phospholipid composition analysis by GC-FID. Immune responses were assessed by peripheral blood mononuclear cell gene expression by qPCR inflammatory cytokine expression, oxidative stress, lipid metabolism, senescence, as well as protein expression of inflammatory cytokines. The gut related mechanisms of inflammatory responses were assessed through measurement of endotoxin, bacterial DNA and cell-free DNA in circulation.

2.2. ETHICAL CONSIDERATIONS AND FUNDING

Ethics was applied for through the University of Auckland Human Participants Ethics Committee (UAHPEC) and was obtained on May 3, 2012, Ref# 8026. The trial was registered prospectively at Australia New Zealand Clinical Trials Registry (ANZCTR) ID: ACTRN12612000515897. The Liggins Institute Trust provided funding through University of Auckland Foundation grant #3701462, “Precise strategies to measure digestion in infancy to old age”. All subjects gave written informed consent prior to study participation.

2.3. STUDY DESIGN

2.3.1. PARTICIPANT RECRUITMENT

Community-dwelling participants were recruited through university internal email and newspaper advertising. Potential participants were provided study information and completed an oral or written screening questionnaire to establish eligibility.

2.3.2. INCLUSION AND EXCLUSION CRITERIA

Eligible participants were required to be able to consume both meals and were excluded if they were unable or unwilling to do so. Subjects between the ages of 20-25 years and 70-75 year were initially recruited to participate; the age range for the older group was lowered to 60 years on March 13, 2013 through ethical approval, and was changed due to difficulty in recruiting eligible participants in the age range over 70 years. Eligible subjects were required to have a body mass index (BMI) between 18 and 30kg/m².

Exclusion criteria were established to eliminate the recruitment of participants with abnormal metabolism, lipid metabolism, heart disease risk, or who may be taking medications that could influence these factors or any
other primary outcomes. Participants were required to have a reported BMI of less than 30kg/m². Eligible participants were required to have no current or past history of diabetes or heart disease; however, hypertension was an allowable condition depending on the current treatment regime. Current use of medications interfering with lipaemia or inflammatory outcomes deemed potential subjects excluded. The list of included and excluded medications is found in Table 3; in general, antihypertensives, diuretics, supplements, and antidepressants were allowed while receptor agonists, non-steroidal anti-inflammatory drugs, antihyperglycaemics, or statins were not. Several potential participants were excluded for history of thyroid conditions or current use of thyroid regulating hormones. Further, one potential participant was excluded for a recent history of diverticulitis, as this was deemed to be a potential confounding factor for the investigation of gut barrier function and bacterial translocation as a study outcome.

2.3.3. DESIGN AND TREATMENTS

Subjects consumed one of two treatment test meals: high fat breakfast (HF) or a low fat breakfast (LF) provided in a randomly selected sequence generated using www.random.org (Figure 6).

2.3.4. MEALS AND PREPARATION

A standard high fat test meal was used as described in previous studies (582) (Table 11 in Chapter 4, section “4.2.3.2. Study procedures”). The low fat breakfast was protein and carbohydrate matched to the high fat breakfast based on standardly available nutritional information (Table 7 in Chapter 3, section “3.2.3.2. Study procedures”). Further, the composition of the low fat meal was designed to follow the Australian Guide to Healthy Eating. The macronutrient composition of both meals is presented in Table 17, Chapter 6, section “6.2.3.2. Study design and treatments” while ingredient information is presented in Table 4.

The HF breakfast was purchased from McDonald’s Restaurant locations in Auckland Central. The low fat breakfast was prepared onsite. The HF meal was weighed and recorded prior to subject ingestion. Wet and dry ingredients for the LF meal were measured twice each using a kitchen scale (Breville, Sydney, Australia) the day prior to the trial, and stored in sealed containers. Food items were labelled with participant codes and stored at 4°C. The morning of the trial, the bread was toasted, and the oats were prepared with milk. Approximately 1/3 of the milk volume allocated for oats was added to the dry oats, stirred, covered, and microwaved on high for 20 seconds. Oats were stirred and an additional ~1/3 of the milk was added to the oats, stirred, covered, and microwaved on high for 20 seconds. Oats were stirred, covered and microwaved on high for a final 20 seconds. The remaining milk was added and oats were served hot.
Table 3: Included and excluded medications for participant recruitment.

<table>
<thead>
<tr>
<th>Allowed</th>
<th>Not allowed</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE inhibitor</td>
<td>Alpha-adrenergic blocker</td>
</tr>
<tr>
<td>Angiotensin II receptor antagonist (ARB)</td>
<td>Antihyperglycaemic</td>
</tr>
<tr>
<td>Antidepressant SSRI</td>
<td>Aspirin</td>
</tr>
<tr>
<td>Antidepressant tricyclic</td>
<td>Beta-blocker</td>
</tr>
<tr>
<td>Diuretics</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>Calcium channel blocker</td>
<td>Statin</td>
</tr>
<tr>
<td>Calcium supplement</td>
<td>Thyroid medication</td>
</tr>
<tr>
<td>Thiazide diuretic</td>
<td></td>
</tr>
<tr>
<td>Vitamin D supplement</td>
<td></td>
</tr>
<tr>
<td>Xanthine oxidase inhibitor</td>
<td></td>
</tr>
</tbody>
</table>

Figure 6: Participant recruitment and randomisation.

Participants were recruited and screened based on eligibility criteria. 30 participants were randomised and completed the study. Each participant served as his/her control and completed a 14 day wash-out period between treatment arms.
Table 4: Ingredient information of food products in low and high fat meals.

<table>
<thead>
<tr>
<th>Item</th>
<th>Ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High fat meal</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Sausage Egg McMuffin</strong></td>
<td>Wheat Flour, Water, Yeast, Sugar, Canola Oil (Food Add (330)), Wheat Gluten, Iodised Salt (Anti-caking Agent (535), Potassium Iodate), Flour Treatment Agent (Soy Flour, Maize Starch, Emulsifiers (481, 472e), Mineral Salt (170), Acidity Regulator (297), Antioxidant (300), Enzymes (1100, 1101, 1104)), Corn Grits, Preservatives (262, 282, 202), Acidity Regulator (341).</td>
</tr>
<tr>
<td><strong>English Muffin</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Egg</strong></td>
<td>Fresh, Shelled Egg</td>
</tr>
<tr>
<td><strong>Sausage Patty</strong></td>
<td>Beef, Seasoning [Potato Starch, Salt, Maltodextrin, Dextrose, Herbs, Dehydrated Vegetables, Spice, Maize Oil, Antioxidants (321, 320, 310), Spice Extract (contains Soy), Acidity Regulator (330)]. [321]</td>
</tr>
<tr>
<td><strong>Cheese Slice</strong></td>
<td>Cheese [Milk, Salt, Starter Cultures, Enzyme (Animal or Microbial Rennet)], Water, Milk Solids, Non Fat Milk Solids, Emulsifiers (331, 332), Flavour, Salt, Acidity Regulator (260, 330), Soy Leithin, Colours (160b, 160c), Preservative (200).</td>
</tr>
<tr>
<td><strong>Cooking Oil</strong></td>
<td>Canola Oil, Emulsifiers (Soy Leithin), Flavour, Colour (160a), Acidity Regulator (330).</td>
</tr>
<tr>
<td><strong>Hash Brown</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Hash Brown</strong></td>
<td>Potatoes, Canola Oil, Seasoning (Black Pepper, Salt, Modified Corn Starch)</td>
</tr>
<tr>
<td><strong>Cooking Oil</strong></td>
<td>McDonald’s Vegetable Oil Blend [High Oleic Sunflower Oil, Canola Oil]</td>
</tr>
<tr>
<td><strong>Low fat meal</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Harraways Rolled Oats</strong></td>
<td>100% Natural Oats</td>
</tr>
<tr>
<td><strong>Tararua Lite Cottage Cheese</strong></td>
<td>Skim Milk, Milk Solids, Cream, Salt, Thickeners (410, 407), Preservative (202), Calcium (341), Dextrose, Culture, Rennilase (Vegetarian Rennet).</td>
</tr>
<tr>
<td><strong>Kraft Reduced Fat Peanut Butter</strong></td>
<td>Roasted peanuts (65%), Maltodextrin (from Tapioca), Sugar, Vegetable Oil (Peanut Oil, and &lt;10% Palm Oil) (Antioxidant (320)), Salt, Emulsifier (471)</td>
</tr>
<tr>
<td><strong>Fresh Peach</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Homebrand Trim Milk</strong></td>
<td>Skim Milk</td>
</tr>
</tbody>
</table>

*Ingredients ordered by decreasing content by weight, obtained from nutrition information available from manufacturers.*
2.3.5. **Clinical Procedures, Sample Collection, and Analyses**

Subjects were asked to abstain from vigorous physical activity, high-fat foods, alcohol, and anti-inflammatory medications and supplements the day prior to their visit. Subjects arrived fasted on two separate occasions a minimum of 14 days apart. Anthropometric data of height and weight were collected using a stadiometer and scale before a catheter was inserted into an antecubital vein and a baseline sample (time 0) was taken followed by consumption of the test breakfast within 15 minutes. Blood samples (16 ml) were collected post-meal into serum and EDTA containing blood collection tubes (Becton Dickinson, NJ, USA) hourly for 5 hours. Bloods for plasma analysis were centrifuged at 1500 × g for 15 minutes at 4°C for plasma separation and were processed under laminar flow using pyrogen-free consumables. Peripheral blood mononuclear cells were collected from whole blood as described in Chapter 6. An aliquot of plasma was kept at 4°C for chylomicron separation within 6 hours and lipoprotein separation within 36 hours and the remaining plasma was collected in pyrogen-free microtubes and stored at -20°C. All analytical techniques are described along with their respective results in Chapters 3 through 6.

2.4. **Protocol Development**

Several protocols were developed over the course of this research. Details of method development follow, with protocols described in their respective chapters. Methods for chylomicron and lipoprotein fraction separation (p. 49), lipoprotein particle size analysis (p. 53), apoB detection by Western blotting (p. 53), endotoxin detection (p. 56), cell-free DNA extraction, and identification of bacterial DNA in plasma (p. 142) were all developed. Analysis of serum free amino acid concentrations were developed at the Liggins Institute Laboratory by Eric Thorstensen. Fatty acid analysis methods including fatty acid extraction, methylation and gas chromatography of meal and chylomicron samples were performed according to protocols described previously (594-596) at the University of Turku under the supervision of Adjunct Professor Kaisa Linderborg.

2.4.1. **Lipoprotein and Chylomicron Isolation**

Lipoproteins were separated from fresh plasma, held at 4°C. Chylomicrons were processed within 6 hours while VLDL, LDL, and HDL were removed within 36 hours. The chylomicron-free fraction, VLDL, LDL and HDL fractions were respectively separated for the endotoxin analysis of each fraction, pending results from plasma and chylomicron fractions.

Lipoprotein separation was developed based on the methods of from Havel et al. (262) and adapted for the equipment available at the Liggins Institute laboratory based on methods by from Oikawa et al. (597). Protocol optimisation and fraction purity was assessed to ensure cohesive size separation between fractions by using dynamic light scattering particle size analysis (263) described in section “4.2.3.4. Chylomicron-rich fraction particle size analysis” (see Figure 7). Storage conditions were tested to ensure optimal recovery of the chylomicron fraction.
Since the density of chylomicrons and VLDL particles are extremely similar, it is difficult to ensure complete elimination of VLDL contamination in the chylomicron fraction. For this reason, some (264, 598) but not all researchers (262, 599-603), employ an additional separation step of the chylomicron fraction, to remove any possible contamination. The methods detailed here do not include such a step to prevent unnecessary endotoxin contamination, and as such, particle size analysis demonstrated that this method of single separation yielded some other lipoprotein contamination. However, subsequent Apolipoprotein B analysis of the chylomicron fraction confirmed minimal VLDL presence.

Chylomicron separation is described in full in Chapter 4. In brief, chylomicrons were separated by layering 3.5ml plasma in an OptiSeal tube (Beckman Coulter, CA, USA) with 1.4ml of saline solution (d=1.006g/ml), centrifuging for 10 minutes at 117 000 × g in a TLA-110 rotor for an Optima MAX-XP ultracentrifuge (Beckman Coulter). Chylomicrons were aspirated from the top layer and stored in microtubes. After chylomicron aspiration and removal, the chylomicron-free fraction was removed by piercing the OptiSeal tube and drawing out the lower phase. Lipoproteins were further separated (n=10 subjects) from the lower phase by first overlaying 2.35ml of chylomicron-free plasma with 2.35ml of saline density gradient (d=1.018g/ml) in OptiSeal tubes to achieve a final density of 1.012g/ml. After centrifuging at 657 000 × g for 2 hours 55 minutes, VLDLs were aspirated from the top half of the upper phase (~1.175ml). LDLs were separated by removing the lower phase using a syringe and overlaying 2.35ml of this lower fraction with 2.35ml of density gradient solution (d=1.114g/ml) to achieve a final density of 1.063g/ml. After centrifuging at 657 000 × g for 4 hours 20 minutes, LDLs were aspirated from the top half of the upper phase (~1.175ml). The HDL containing lower layer was removed using a syringe and transferred into pyrogen-free microtubes. This process is visualised in Figure 8.
Figure 7: Particle size analysis of density gradient separated lipoprotein fractions.

Particle size intensity frequencies of four representative lipoprotein fractions separated by density gradient ultracentrifugation of density d<1.063g/ml (white bars), d=1.063g/ml (light gray bars) d=1.012g/ml (dark gray bars) d<1.006g/ml (black bars) respectively. Values are presented as the intensity obtained by dynamic light scattering as a frequency of size category as provided by the NanoZetasizer. Dashed lines represent literature estimates of lipoproteins approximating high density lipoproteins (HDL; 5-12nm (257)), low density lipoproteins (LDL; 21-25nm (258)), very low density lipoproteins (VLDL; 28-75nm (258)), and chylomicrons (75-1200nm (261)). Note that literature values for VLDL and chylomicron size range varies (257, 258, 261) and that size differentiation between remnant chylomicrons and VLDL is poorly demarked. The low relative frequency of d<1.006g/ml particles below 43nm may represent VLDL contamination or remnant chylomicron particles.
Figure 8: Chylomicron and lipoprotein separation process.

Chylomicron, very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) fractions were separated by sequential ultracentrifugation according to the schematic. Chylomicron free plasma was used for lipoprotein separations. After ultracentrifugation at the indicated force and time, the top lipoprotein layer was aspirated and the bottom layer removed with a syringe for sequential lipoprotein separation.
2.4.2. PARTICLE SIZE ANALYSIS OF LIPOPROTEINS

Particle size analysis was developed based on the methods of Ruf and Gould (265) and Sakurai et al. (263), and adapted for optimisation for a NanoZetasizer S (Malvern Instruments, United Kingdom). The NanoZetasizer measures particle size by dynamic light scattering, which assesses the intensity of light scattered off of particles obtained after a laser beam is shone through a liquid sample (604) (see Figure 7 for representative intensity data). This technique allows for size characterisation of colloid systems (mixture of solid and liquid), and is able to determine the size and number of particles in solution based on the light scattering. The size of the particles (i.e. chylomicrons) is calculated from the Stokes-Einstein equation based on the speed of the particle movement in suspension and the fluctuations this creates in light intensity. Based on Brownian motion, larger particles move slowly while smaller particles move more quickly; faster moving (i.e. smaller) particles will fluctuate light more quickly. The NanoZetasizer uses the fluctuation in light scatter to determine size distribution of particles. These distributions are presented as intensities, from which number and volume parameters can be calculated. However, reliable determination of these other parameters becomes more complicated with heterogeneous particles distributions since the calculations converting intensity to number rely on exponential sphere volume equations (i.e. $V = \frac{4}{3} \pi r^3$) (604). That is, when equal volumes of particles are present but the size differential is larger, the smaller particles become the only detectable number on a distribution frequency scale. This complicates particle size evaluation of very heterogeneous emulsions, like plasma. This also means that minimal contamination of smaller particles, when present in a number quantity greater than the less numerous larger particles (i.e. LDL vs. chylomicrons) results in number readings skewed in percentage towards the smaller particles which makes relative number quantification of the larger particles impossible. For this reason, only size values as intensity readings were compared between samples and formula derived particle numbers were not quantified using the NanoZetasizer.

Samples were diluted for analysis using 10µl chylomicron fraction in 1500µl phosphate buffered saline (PBS). Although the chosen dilution provided reliable results for the majority of samples with ample lipoproteins, samples such as fasting chylomicron samples with low concentrations were more difficult to measure, as the NanoZetasizer requires a threshold of particle counts to reliably determine intensity fluctuations. The standard protocol was to re-measure samples if errors during reading were obtained. The range of data available from dynamic light scattering measurements allowed for identification of particle number, volume, and size. This was obtainable as mean/mode peak values as well as distribution data, all available as a %distribution of arbitrary categories of particle sizes/volumes/numbers.

2.4.3. APOLIPROTEIN B WESTERN BLOTTING

The apoB measurements obtained from the autoanalyser can only provide an indication of the total apoB content of the entire chylomicron sample. Although this fraction is likely to contain a majority of chylomicrons, as mentioned above in “2.4.1. Lipoprotein and chylomicron isolation”, some VLDL/LDL contamination in the chylomicron fraction is likely, which would provide contamination with apoB-100 molecules. Additionally, fasting measures of chylomicron TAG and particle size indicated values greater than zero, implying the presence of
triacylglycerol-rich lipoproteins. These TRLs may have been chylomicron remnants remaining in circulation from
the previous meal, but were more likely representative of VLDL/LDL contamination of the chylomicron fraction.
This contamination was at unknown concentrations since number determination with the NanoZetasizer was not
possible. As described above, any contamination of smaller lipoprotein particles is exponentially amplified through
dynamic light scattering calculations.

Therefore, to identify the lipoprotein content of the chylomicron fraction, Western Blot analysis was
developed to attempt to separate both apoB-48 and apoB-100 through SDS-PAGE and to provide a relative
measure of both particles for each sample. A standard curve was employed to allow for relative quantification
between samples and blots, and to allow for gel loading of identical plasma-relative volumes of chylomicron
samples for accurate comparison between samples.

A number of variables were optimised to obtain the cleanest SDS-PAGE separation and quantification of
apoB-48 and apoB-100, proteins of 256 and 549kDa respectively (605). Separation of the two bands was optimised
by extending run times and lowering acrylamide content of the gels. Pre-cast gradient gels by NuPAGE (NuPAGE
Novex Life Technologies, CA, USA) designed for high molecular proteins were also attempted as well as custom
made Tris-acetate gels (606). Wet transfer run times and transfer buffer composition (addition of SDS) were tested
and additional steps to enhance gel permeability or membrane fixing were attempted. Band identification by size
discrimination was made less reliable due to the specificity of the high molecular weight NuPAGE ladder (HiMark
Pre-stained protein standard, NuPAGE) for the NuPAGE Tris-acetate pre-cast gels causing shifts in the ladder run
not perfectly matching with expected sample molecular weights, so a commercial apoB standard was run alongside
samples to assist with band identification.

The following protocol was used for direct apolipoprotein B quantification from isolated chylomicron
fractions. Chylomicron samples were corrected to an equivalent volume of plasma based on the dilution factor
described above in normal saline (d=1.006g/ml) and denatured in Laemmli’s buffer. Serial dilutions of human apoB
standard (Abcam, Cambridge, UK) were prepared to 0.05µg/µL, 0.025µg/µL, and 0.0125µg/µL. Chylomicron
samples and standards (10µL each) were separated by SDS-PAGE, adapted from the methods described by Smith et
al. (607). Gels were wet transferred onto polyvinylidene fluoride membranes (BioRad, Hercules, CA, USA). After
transfer, membranes were fixed with 10% acetic acid, rinsed with MilliQ, and air dried. Membranes were washed in
TBST (0.1% v/v Tween-20 in Tris buffered saline, Sigma-Aldrich) and blocked at room temperature using 5%
bovine serum albumin (BSA; Sigma-Aldrich) in TBST. Membranes were incubated overnight with Human apoB
rabbit polyclonal antibody (1:1000, Abnova, Jhongli, Taiwan) in 5% BSA at 4°C, subsequently washed then
incubated with Goat Anti Rabbit IgG (H & L) Peroxidase Conjugated secondary antibody (1:10000) for 1 hour at
room temperature. Membranes were washed with TBST and proteins visualised using enhanced chemiluminescence
(ECL. Select kit, GE HealthCare) on a ChemiDoc MP system (BioRad). Band density was quantified using Image J
software (NIH, Bethesda, USA, version 1.48).

Challenges with imaging of apoB electrophoresis have been reported by others. Overloading of apoB can
create non-linear absorbance of bands, affecting concentration analysis; this is particularly apparent with LDL.
samples (608). The band density was very variable between apoB-100 and apoB-48 bands, making it difficult to quantify both apoB-100 and apoB-48 with the same exposure. These bands, if present, were quantified with separate exposures. Others have shown that optimal loading concentration varies between lipoprotein fractions (608), a factor not easily accounted for if loading single samples. The bright apoB band was ultimately quantified; however, detection of the apoB band was unreliable due to protein degradation of samples, particularly evident with the LF chylomicron samples (Figure 9). Ultimately, statistical analysis showed no treatment, time, or age differences in apoB. Due to the poor reliability of sample integrity and the discordance of these data with apoB data obtained from autoanalyser, these results were not presented or included in discussions of the lipaemic effects of mixed meals.

![Figure 9: Representative apolipoprotein B-100 Western Blot after high fat meal.](image)

A representative polyvinylidene fluoride membrane (BioRad, Hercules, CA, USA) visualised using enhanced chemiluminescence (ECL Select kit, GE HealthCare) on a ChemiDoc MP system (BioRad) showing apolipoprotein B (apoB)-100 protein in chylomicron samples. Lanes show high molecular weight ladder (HiMark NuPAGE Novex Life Technologies, CA, USA) showing 500kD, 279kD, and 251kD: standards (STD) 1 to 3 (0.05, 0.025, and 0.0125µg/µL respectively) of human apoB standard (Abcam, Cambridge, UK) followed by individual baseline (T0) and hourly (T1 to 5) chylomicron samples.
2.4.4 ENDOTOXIN DETECTION

Although endotoxin detection in biological samples has been reported extensively in the literature and a variety of commercial kits are available, the detection in human plasma and chylomicron samples is not always straight-forward. This is evident with the extensive supplementary application and technical notes available from common suppliers of endotoxin detection reagents and kits (609, 610). Measurement of endotoxin in biological samples such as human plasma, are subject to inhibition of the limulus assay through plasma proteins (359), lipids (611) and other factors such as EDTA which can interfere with various aspects of the reaction cascade or absorbance detection (612). Optimisation of endotoxin kits requires adequate recovery of a positive product control to ensure there is no inhibition of endotoxin detection inferred by the sample. Unfortunately, achieving an acceptable positive product control (PPC) recovery rate was difficult for preliminary test samples as well as subject samples. A variety of optimisation protocols were employed to attempt to improve recovery rates (Table 5). The heat treatment protocol of Laugerette et al. (324) combined with the acidification protocol of Ketchum and Novitsky (613) provided the best positive product recovery results.

2.5. DATA HANDLING AND STATISTICAL ANALYSES

2.5.1. DATA MANAGEMENT

Although the study was designed as cross-over with repeated treatment measures to study the effects of meal ingestion over time in distinct subject groups, data analysis was not always performed using this three-factor study design. Test meal composition variation and the inevitable interaction of macronutrient components made a three-way statistical design impractical or redundant in one treatment versus the other, or made comparison of treatment responses dubious. For these reasons, lipid and amino acid data analyses were carried out separately, and are reported separately as cross-sectional studies.

2.5.2. OUTLIER MANAGEMENT

The general practice for outlier removal was to first identify if a particular value appeared to be an error of the assay, the assay was repeated, or else the value was removed. There was a not a standard identification practice (i.e. > 2 SD) since different biological variation may be expected depending on the assay, and relative accuracy of assays differed; therefore, the identification of outliers was chosen specific to each assay and attempted to remove ‘noise’ in the data while eliminating as few data points as possible (Table 6).
Table 5: Optimisation of endotoxin detection in plasma.

<table>
<thead>
<tr>
<th>Challenge</th>
<th>Protocol</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kit design</td>
<td>LAL Kinetic-QCL assay(^2)</td>
<td>Similar low PPC(^1)</td>
</tr>
<tr>
<td></td>
<td>LAL PyroGene Recombinant Factor C assay(^2)</td>
<td>Similar low PPC</td>
</tr>
<tr>
<td></td>
<td>LAL PYROGENT-5000 assay(^2)</td>
<td>Similar low PPC</td>
</tr>
<tr>
<td></td>
<td>Kinetic Chromogenic LAL assay(^3)</td>
<td>Similar low PPC</td>
</tr>
<tr>
<td></td>
<td>EndoLISA(^4)</td>
<td>Not attempted due to cost</td>
</tr>
<tr>
<td>Low PPC(^1) recovery</td>
<td>Dilution with LRW (609, 610)</td>
<td>Endotoxin not detected in samples. Dilution of &gt;1:200 necessary to achieve acceptable PPC lowered detectable range below expected outcome</td>
</tr>
<tr>
<td>EDTA inhibition</td>
<td>Magnesium Chloride buffer to eliminate chelation (609, 610)</td>
<td>Minimal improvement in PPC, still poor detection</td>
</tr>
<tr>
<td></td>
<td>PYROSPERSE dispersing agent(^2) to eliminate aggregation (609)</td>
<td>Minimal improvement in PPC, still poor detection</td>
</tr>
<tr>
<td>Aggregation</td>
<td>Sonication (324)</td>
<td>No improvement</td>
</tr>
<tr>
<td>Lipid inhibition (binding of LPS to phospholipids (611))</td>
<td>Phospholipid removal with Phree(^5) solid phase columns</td>
<td>Endotoxin not detected in samples. PPC improved, but LPS likely removed from samples (bound to lipids/phospholipids)</td>
</tr>
<tr>
<td>Plasma protein interference</td>
<td>Dilution with LRW (609, 610)</td>
<td>See above</td>
</tr>
<tr>
<td></td>
<td>Heat treatment (324)</td>
<td>Improved PPC</td>
</tr>
<tr>
<td></td>
<td>Acidification (613)</td>
<td>Improved PPC</td>
</tr>
</tbody>
</table>

\(^1\)PPC: positive product control recovery  
\(^2\)Lonza, TN, USA  
\(^3\)Charles River, VA, USA  
\(^4\)Hyglos GmbH, Bernried, Germany  
\(^5\)Phenomenex, CA, USA
Table 6: Data clean up protocols and criteria.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicron apoB (Hitachi autoanalyser)</td>
<td>&gt;3 SD</td>
</tr>
<tr>
<td>Amino acid (UPLC)</td>
<td>&gt;3 SD</td>
</tr>
<tr>
<td>Glucose (Hitachi autoanalyser)</td>
<td>None</td>
</tr>
<tr>
<td>Insulin (Elecsys autoanalyser)</td>
<td>&gt; 2 SD above mean</td>
</tr>
<tr>
<td>Particle size (Zetasizer)</td>
<td>Exclusion was made during the data cleaning process (see 2.5.3. Particle size data management)</td>
</tr>
<tr>
<td>Lipidomics (GC-FID)</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>For comparisons between meal and chylomicrons: if fatty acids were not found in appreciable amounts in either the meal TAG or chylomicron TAG, these were not analysed</td>
</tr>
<tr>
<td>PBMC gene expression and cell-free DNA detection (qPCR)</td>
<td>Anything below the detectable limit (or NTC) was regarded as blank (see 2.5.4. qPCR missing values)</td>
</tr>
<tr>
<td>Chylomicron apoB (Western blotting)</td>
<td>&gt;2 SD</td>
</tr>
<tr>
<td>C-reactive protein (Hitachi autoanalyser)</td>
<td>&gt;2 SD</td>
</tr>
</tbody>
</table>

1Abbreviations: apoB: apolipoprotein B; UPLC: ultra performance liquid chromatography; GC-FID: gas chromatography with flame ionisation detection; PBMC: peripheral blood mononuclear cell; qPCR: quantitative polymerase chain reaction; SD: standard deviation; TAG: triacylglycerol; NTC: non-template control.
2.5.3. PARTICLE SIZE DATA MANAGEMENT

Manual data cleaning was necessary for particle size data. In addition to the multiple entries obtained when errors were recorded, samples were screened to ensure that the appropriate size particle peak was included in data analysis. Duplicate samples were removed where a technical error existed, or else kept for mean pooling. Peaks were sorted by size, as this was not provided automatically by the Zetasizer software. Approximate lipoprotein classes were identified for each recorded peak based on available size data in the literature. Samples without appropriate particle sizes (not within the range of chylomicrons or VLDL) were removed and the data point left blank; this was particularly necessary for baseline samples and LF samples as these samples likely contained few or no chylomicron particles. If two peaks provided particles within the appropriate range, the larger peak was kept for analysis.

2.5.4. QPCR MISSING VALUES

For SOD2, three-way ANOVA analysis on SPSS automatically removed any subjects with a missing data point, resulting in a greatly reduced included n-value and calculated means drastically different from mean values for all available data points. Therefore, the mean value for each age and timepoint was inserted into each missing data point for three-way ANOVA analysis to obtain a representative result. Samples which gave either high duplicate variation in housekeeping genes, or crossing point (Cp) values for housekeeping genes not reliably above the NTC, were eliminated from data analysis.

2.5.5. STATISTICAL PACKAGES AND MEASURES

Microsoft Excel 2010 (Microsoft, version 14.0.7143.5000) was used for general data handling. SPSS (IBM, version 21) was used for all statistical analyses while GraphPad Prism (GraphPad Software, version 6.0) was used for graph creation. R (R Development Core Team, version 2.15.2) was used for the production of heat maps.

Data are presented as means ± SEM. Data are presented to the significant figures or decimal points of the assay on which they were performed. Statistical analyses were performed using appropriate factor analysis of variance (ANOVA) with repeated-measures when necessary. Single factors comparisons were compared using Student's t test. Statistical measures are indicated with results where applicable. Where Mauchly's sphericity test failed, the Huynh-Feldt correction was applied. Sidak post hoc tests were used for all multiple comparisons between different groups. Alpha was set at P<0.05.
CHAPTER 3

OLDER ADULTS HAVE PRESERVED AMINO ACID ABSORPTION AFTER HIGH PROTEIN MIXED MEALS
3.1. PREFACE

This chapter presents work investigating the aminoacidaemic responses to high protein meals in older adults. Age-related changes in protein metabolism contribute to the progressive loss of skeletal muscle that accompanies ageing; however, the digestive and metabolic differences following a meal present with ageing are not well described.

Protein digestion was investigated following two compositionally distinct mixed meals. These meals differed in their amino acid content, physical protein and food structures (i.e. readily available casein and whey vs. animal muscle and egg protein), and macronutrient quality and quantities. As these differences are likely to affect postprandial amino acid responses beyond protein absorption and metabolism (e.g. gastric emptying, glycaemic and lipaemic response), it would have been inappropriate to compare these data using a three-factor model. Therefore, three-way repeated-measures ANOVA analysis of the data was not performed, but rather the data from each meal were analysed separately. As the aim of this research was to focus on the age-related differences to a high protein mixed meal, data describing only the amino acid appearance to a low fat meal were compiled in a manuscript submitted for publication. Nevertheless, data from both meals showed similar patterns of response. Section 3.3. begins with a comparison of the metabolic responses to both meal types, followed by discussion of the data from the high fat meal, and provides information regarding amino acid responses to an alternate food form, allowing for further discussion regarding the impact of food structures on digestive responses in the elderly.

The following section contains a reproduction of the article “Older adults have delayed amino acid absorption after a high protein mixed breakfast meal”, co-authored by Amber M. Milan, Shikha Pundir, Chantal A. Pileggi, Eric B. Thorstensen, Matthew P. G. Barnett, James F. Markworth, David Cameron-Smith, and Cameron J. Mitchell (see Appendix 8.1 Milan et al., 2015 for the unaltered reproduction of the article). This article was published in the Journal of Nutrition, Health & Aging, Volume 19, Issue 8, p. 839-845. The Journal of Nutrition, Health & Aging is published by Springer Science+Business Media with a 2014 impact factor of 2.66 and a five year impact factor of 2.83. The final publication is available at link.springer.com/article/10.1007/s12603-015-0500-5.
3.2. MANUSCRIPT:

OLDER ADULTS HAVE DELAYED AMINO ACID ABSORPTION AFTER A HIGH PROTEIN MIXED BREAKFAST MEAL

3.2.1. ABSTRACT

Objectives: To measure the postprandial plasma amino acid appearance in younger and older adults following a high protein mixed meal. Design: Cross-sectional study. Setting: Clinical research setting. Participants: Healthy men and women aged 60-75 (n=15) years, and young controls aged 20-25 years (n=15) matched for body mass index and insulin sensitivity based on the homeostatic model assessment of insulin resistance. Intervention: High protein mixed meal of complete food products. Measurements: Circulating amino acid concentrations were determined hourly before and for 5 hours after meal ingestion. Results: There was no difference between cohorts in postprandial appearance of non-essential amino acids, or area under the curve of any individual amino acid or amino acid class. However, older adults had higher baseline concentrations of aspartic acid, glutamic acid, glycine, ornithine, threonine and tyrosine and lower baseline concentrations of hydroxyproline, isoleucine, leucine, methionine and valine compared to younger adults. Younger adults showed peak essential (EAA) and branched-chain amino acid (BCAA) concentrations at 1 hour post-meal while older adults’ peak EAA and BCAA concentration was at 3 hours. Similarly, peak total amino acid concentrations were at 3 hours in older adults. Conclusion: Older adults digested and absorbed the protein within a mixed meal more slowly than younger adults. Delayed absorption of AA following a mixed meal of complete food products may suppress or delay protein synthesis in senescent muscle.

3.2.2. INTRODUCTION

The ageing of the world’s population, most notably within developed economies, presents many health challenges. Ageing affects body composition, with a characteristic loss of lean muscle mass, ultimately resulting in impaired skeletal muscle function and the onset of sarcopenia (72). The aetiology of sarcopenia is complex; however, lifestyle factors including the habitual diet are a significant determinant (136). Dietary protein supports the maintenance of muscle mass (137), and daily intake may be insufficient in older adults (443). Beyond the changes in habitual diet, the digestive responses and metabolic fate of ingested protein differ with advancing age. It has been shown that protein digestion rate (77) and circulating amino acid (AA) availability (78) contribute to the muscle protein synthetic response to feeding, a response known to be impaired with ageing (73). Older adults may have altered protein digestion which could contribute to changes in postprandial amino acid availability.

Age-related differences in digestive factors have been previously reported. Older adults may have decreased chewing capacity (168), gastric acid secretion (170), reduced gastric peristalsis (171) or delayed gastric emptying (169), all of which could impair older adults’ ability to digest and absorb protein (175). Older adults have lower efficiency of protein utilisation (418) and show reduced protein synthesis in the fed state, possibly due to differences in protein absorption and splanchnic use (590). Ageing is associated with higher rates of splanchnic
uptake and use of AAs such as leucine (427, 428), phenylalanine (429) and glutamate (430). Increased intestinal demands for AAs (614) likely result in reduced AA appearance (427) and availability of AA for protein synthesis in ageing (590).

Older adults also have different metabolic responses to protein ingestion compared to younger adults. While in young adults, slowly digested proteins, such as casein (77), and ‘spread’ feeding patterns (protein fed throughout the day) enhance whole body protein balance and utilisation, the opposite is seen in elderly subjects. Fast proteins, i.e. whey, induce better postprandial whole body leucine balance in older subjects (432, 433) and elderly women experience better protein retention after pulse feeding (434). This shows that the kinetics of AA availability differs between young and old subjects and impacts postprandial muscle protein synthesis. However, at the level of the muscle, fast protein and bolus feeding patterns result in greater muscle protein synthesis in both young and older adults (435-438).

Protein digestion rates influence postprandial AA availability and may differ with meal composition; intact proteins such as those found in milk (casein and whey) (543) or steak (544) are more slowly digested when in these food matrices than whey alone or minced beef respectively. Additionally, carbohydrate ingested with protein delays postprandial AA appearance (545). These studies suggest that just as meal composition and food physical structure affect digestion, absorption, and subsequent appearance of carbohydrates (565) and fats (566), protein digestion is equally dependent on meal structure. As such, meal structure and composition may be important considerations for assessing amino acid availability and ultimately muscle protein synthesis in response to protein ingestion. Surprisingly, no studies have yet reported age-related differences in amino acid appearance in the context of a whole food mixed meal.

Therefore, we aimed to demonstrate the effects of ageing on protein digestion in the context of a whole food mixed meal; we examined the postprandial AA appearance in healthy older and younger adults after ingestion of a high protein mixed meal consisting of whole food products. We hypothesised that older adults would have impaired digestion of dietary protein in a mixed meal.

### 3.2.3. METHODS

#### 3.2.3.1. SUBJECTS

Thirty healthy, community-dwelling subjects (n=7 young women, n=8 young men, n=9 older women, n=6 older men) from the Auckland region were recruited through newspaper advertisements and from the university community to participate in the study. Eligible subjects were required to have a body mass index (BMI) between 18 and 30kg/m² and be between the ages of 20-25 years or 60-75 years. Individuals with a history of cardiovascular or metabolic disease/conditions, or taking medications that may interfere with study endpoints (i.e. anti-inflammatory drugs, statin drugs) were not eligible for participation. All subjects gave written informed consent and the study was approved by the University of Auckland Human Participants and Ethics Committee (Ref # 8026). This study was registered prospectively at Australian New Zealand Clinical Trials Registry at anzctr.org.au (ID: ACTRN12612000515897).
3.2.3.2. STUDY PROCEDURES

This cross-sectional trial was conducted at the Maurice and Agnes Paykel Clinical Research Unit (MAPCRU) at the Liggins Institute, University of Auckland, Auckland, New Zealand. The mixed breakfast meal (Table 7) was formulated to follow the Australian Guide to Healthy Eating, while maintaining a low fat load (16.6g), and high protein (49.8g) and carbohydrate (77.4g) loads with a total energy content of 2790kJ. The meal was prepared onsite at the MAPCRU.

Subjects were required to abstain from vigorous physical activity, high fat foods, anti-inflammatory medications, and dietary supplements the day prior to their visit. Subjects arrived fasted at the MAPCRU; anthropometric data were collected before a catheter was inserted into an antecubital vein and a baseline sample (time 0) was taken followed by consumption of the breakfast. Blood samples were collected hourly up to 5 hours post-meal from resting subjects into blood collection tubes (BD, Mt Wellington, New Zealand) for serum and EDTA plasma. Serum tubes were allowed to clot for 15 minutes at room temperature before serum and plasma tubes were centrifuged at 1500 × g for 15 minutes at 4°C and the supernatants collected in pyrogen-free microtubes and stored at -20°C until analysis.

Table 7: Macronutrient composition of low fat breakfast meal (Table 1 in article).

<table>
<thead>
<tr>
<th>Item name</th>
<th>Weight (g)</th>
<th>Carbohydrates (g)</th>
<th>Fat (g)</th>
<th>Protein (g)</th>
<th>Energy (kJ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rolled oats</td>
<td>37.0</td>
<td>20.8</td>
<td>1.9</td>
<td>5.0</td>
<td>495</td>
</tr>
<tr>
<td>1% cottage cheese</td>
<td>167.0</td>
<td>4.5</td>
<td>1.0</td>
<td>19.7</td>
<td>450</td>
</tr>
<tr>
<td>Mixed grain bread</td>
<td>41.5</td>
<td>11.2</td>
<td>2.2</td>
<td>5.1</td>
<td>390</td>
</tr>
<tr>
<td>Reduced fat peanut butter, smooth</td>
<td>25.0</td>
<td>8.4</td>
<td>9.4</td>
<td>4.4</td>
<td>575</td>
</tr>
<tr>
<td>Fresh peach</td>
<td>154.0</td>
<td>14.6</td>
<td>0.3</td>
<td>1.4</td>
<td>250</td>
</tr>
<tr>
<td>Trim milk</td>
<td>365.0</td>
<td>17.9</td>
<td>1.8</td>
<td>14.2</td>
<td>630</td>
</tr>
<tr>
<td>Total</td>
<td>77.4</td>
<td>16.6</td>
<td>49.8</td>
<td>2790</td>
<td></td>
</tr>
</tbody>
</table>

Values presented are based on available nutrient panel data for individual products.
3.2.3.3. SERUM FREE AMINO ACID CONCENTRATIONS AND PLASMA MEASURES

Free amino acids were assayed from 20µl of serum with 15µM L-Nor-Valine as internal standard extracted with 20µl 10% sodium tungstate and 160µl of 0.04M sulphuric acid. The mixture was incubated on ice for 3 minutes then centrifuged at 12000 x g for 10 minutes at 4°C. 70µl of 0.2M borate buffer (pH 8.8) was added to the supernatant before adding 10µl of AccQ-tag reagent (2.8mg/ml in acetonitrile). In a sealed vial, the mixture was heated at 55°C for 10 minutes before being subjected to ultra performance liquid chromatography (UPLC). The UPLC system used a Thermo Scientific Dionex Ultimate 3000 pump, autosampler (maintained at 10°C), column oven and fluorescence detector (set at Ex 250 nm, Em 395nm) (Thermo Scientific, Dornierstrasse, Germany), and a Kinetex 1.7µm C18 100A 100 x 2.1mm column, preceded by a Krudkatcher inline filter (Phenomenex, Auckland, New Zealand) at 45°C. Mobile phase buffer, (80mM sodium acetate, 3mM triethylamine, 2.67µM disodium calcium ethylenediaminetetraacetic acid) at pH 6.43 (obtained by addition of orthophosphoric acid), run with a complex gradient of acetonitrile from 2 to 17% (balance, water) over 24 minutes. Data was directly captured by computer with Chromeleneon 7.1 software (Thermo Scientific). AA concentrations were calculated from standard curves generated for each AA from the standard injections. The internal standard (L-Nor-Valine) signal in each chromatogram was used for data normalisation for analyte recovery and quantification.

Plasma glucose was measured using a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan) by enzymatic colorimetric assay (Roche, Mannheim, Germany). Plasma insulin was measured using an Abbott AxSYM system (Abbott Laboratories, Abbott Park, USA) by microparticle enzyme immunoassay.

3.2.3.4. CALCULATIONS

Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated from fasting glucose and insulin concentrations using the equation from Matthews et al. (615). Serum free amino acids were pooled for mathematical analysis into total amino acids (all proteogenic amino acids), essential amino acid (EAA), branched-chain amino acids (BCAA) and non-essential amino acids (NEAA). Although arginine, proline, glutamine, glycine, taurine and tyrosine are considered conditionally essential, these were considered nonessential as the study criteria excluded persons with critical illness or malnutrition.

3.2.3.5. STATISTICAL ANALYSES

Statistical analyses were conducted with SPSS. Data are represented as means ± SEMs. Incremental area under the curve (iAUC) was calculated after subtracting fasting values. Baseline subject characteristics, amino acids, iAUC, and maximum peak concentrations were compared using Student’s t test. Two-factor (age and time) repeated-measure ANOVA followed by Sidak post hoc test was used for all multiple comparisons between different groups. Alpha was set at P<0.05. The heatmap representation of postprandial amino acids concentration as a percent change relative to younger fasting values was created with R software.
3.2.4. RESULTS

3.2.4.1. SUBJECT CHARACTERISTICS

A total of 30 subjects completed the study. Subject characteristics are shown in Table 8. There were no age differences in BMI, fasting measurements of glucose, or HOMA-IR. Glucose and insulin response to the meal was not different between age groups (Figure 10).

3.2.4.2. OLDER ADULTS HAVE ALTERED BASAL AMINO ACID PROFILES

Baseline glutamine concentrations were 15% lower in older adults compared to younger adults (P=0.042) while glutamic acid was 193% greater (P<0.001, Table 8). Older adults also had higher baseline concentrations of aspartic acid, glycine, ornithine, threonine, and tyrosine than younger adults, and lower concentrations of hydroxyproline, isoleucine, leucine, methionine, and valine (Table 8).

3.2.4.3. OLDER ADULTS HAVE DELAYED POSTPRANDIAL APPEARANCE OF AMINO ACIDS

Older adults showed a delayed increase in serum postprandial concentrations of TAAs, BCAAs, and EAAs (Figure 11). No such differences were apparent in NEAA appearance (Figure 11D). Younger adults had higher concentrations of BCAA (Figure 11B) and EAA (Figure 11C) at 1 hour after the meal when compared to older adults. In older adults, serum concentrations of TAAs, BCAAs, and EAAs peaked at 3 hours post-meal, at which time circulating concentrations were significantly higher compare to younger adults. The iAUC for isoleucine tended to be greater in older adults compared to younger adults (P=0.067). iAUC did not differ between any other individual AA or class of AA. Maximal AA concentration did not differ between younger and older adults for AAs that did not show previous baseline differences, maintaining that total postprandial plasma availability was not different between age groups. A heatmap displaying mean percentage change in all detected individual serum amino acids from fasting serum concentrations in younger adult is presented (Figure 12).
Table 8: Baseline subject characteristics and serum amino acid profile of older and younger adults before low fat meal (Table 2 in article).

<table>
<thead>
<tr>
<th>Subject characteristics:</th>
<th>Younger adults (n=15; 8 men, 7 women)</th>
<th>Older adults (n=15; 6 men, 9 women)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>22.7 ± 0.4</td>
<td>67.3 ± 1.5***</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.8 ± 0.8</td>
<td>24.4 ± 1.0</td>
</tr>
<tr>
<td>Fasting Plasma Glucose (mmol/l)</td>
<td>5.1 ± 0.1</td>
<td>5.2 ± 0.1</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.2 ± 0.3</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>Branched-Chain Amino Acids:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>84.7 ± 6.5</td>
<td>65.3 ± 3.0**</td>
</tr>
<tr>
<td>Leucine</td>
<td>146.5 ± 8.5</td>
<td>123.8 ± 5.0*</td>
</tr>
<tr>
<td>Valine</td>
<td>269.2 ± 17.8</td>
<td>226.9 ± 9.7*</td>
</tr>
<tr>
<td>All Other Essential Amino Acids:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>78.8 ± 5.2</td>
<td>74.4 ± 3.7</td>
</tr>
<tr>
<td>Lysine</td>
<td>158.5 ± 12.0</td>
<td>150.6 ± 8.0</td>
</tr>
<tr>
<td>Methionine</td>
<td>46.9 ± 3.1</td>
<td>38.2 ± 1.9*</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>72.7 ± 4.5</td>
<td>73.6 ± 3.5</td>
</tr>
<tr>
<td>Threonine</td>
<td>58.6 ± 2.5</td>
<td>84.9 ± 5.4***</td>
</tr>
<tr>
<td>Non-Essential Amino Acids:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>401.0 ± 24.3</td>
<td>411.8 ± 21.3</td>
</tr>
<tr>
<td>Arginine</td>
<td>76.2 ± 6.0</td>
<td>84.0 ± 4.9</td>
</tr>
<tr>
<td>Asparagine</td>
<td>52.0 ± 4.7</td>
<td>44.2 ± 2.1</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>6.7 ± 1.2</td>
<td>12.4 ± 1.3**</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>71.8 ± 16.6</td>
<td>210.4 ± 17.0***</td>
</tr>
<tr>
<td>Glutamine</td>
<td>556.7 ± 49.2</td>
<td>475.2 ± 30.2*</td>
</tr>
<tr>
<td>Glycine</td>
<td>236.6 ± 15.2</td>
<td>311.6 ± 26.4*</td>
</tr>
<tr>
<td>Proline</td>
<td>192.6 ± 11.9</td>
<td>189.4 ± 11.5</td>
</tr>
<tr>
<td>Serine</td>
<td>120.6 ± 8.3</td>
<td>125.3 ± 21.6</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>80.9 ± 7.5</td>
<td>88.4 ± 20.5*</td>
</tr>
<tr>
<td>Non-proteogenic Amino Acids:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Methylhistidine</td>
<td>11.2 ± 2.0</td>
<td>7.1 ± 1.2</td>
</tr>
<tr>
<td>Citrulline</td>
<td>66.3 ± 4.4</td>
<td>64.6 ± 1.9</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>15.6 ± 1.9</td>
<td>10.2 ± 0.8*</td>
</tr>
<tr>
<td>Ornithine</td>
<td>52.6 ± 3.4</td>
<td>65.8 ± 5.7*</td>
</tr>
<tr>
<td>Taurine</td>
<td>109.7 ± 10.8</td>
<td>107.3 ± 8.3</td>
</tr>
</tbody>
</table>

Values represent means ± SEM. Amino acid values measured in µmol/l. HOMA-IR: Homeostatic model assessment of insulin resistance. Significance was determined by Student’s t-test. *P<0.05, **P<0.01, ***P<0.001 compared with younger adults.
Figure 10: Postprandial plasma glucose and insulin concentrations after low fat meal (Figure 1 in article).

Postprandial plasma glucose (A) and insulin (B) concentrations in older (●) and younger (○) adults after low fat meal. Values represent means ± SEM in mmol/l and µU/ml respectively. No main effects of age or time were identified by two-factor repeated-measures ANOVA.
Figure 11: Postprandial serum concentrations of amino acid groupings after low fat meal (Figure 2 in article).

Postprandial serum concentrations of total amino acids (TAA) (A), BCAA (B), BCAA iAUC (B inset), EAA (C), and NEAA (D). Values represent means ± SEM in µmol/l. iAUC presented as arbitrary units. There were significant differences over time in the TAA, BCAA and EAA responses between older (●) and younger (○) adults (age × time interactions of P<0.05, P<0.001, and P<0.01 respectively, two-factor repeated-measures ANOVA). There were no differences in NEAA response or BCAA iAUC between older and younger adults. *P<0.05, **P<0.01 older vs. younger at a given time-point (Sidak).
Figure 12: Heatmap of postprandial changes in amino acid concentrations of older and younger adults after low fat meal (Figure 3 in article in black and white).

Values are presented as mean percent changes relative to younger adult time 0 concentration for each amino acid (i.e. white represents a 0% change from younger baseline). Green hues represent negative percentage changes; red hues represent positive percentage changes up to 100%; blue hues represent positive percentage changes above 100%. BCAA: branched-chain amino acids; EAA: essential amino acids; NEAA: non-essential amino acids.
3.2.5. DISCUSSION

For the first time we have shown that older adults digest and absorb amino acids more slowly total than younger adults after a mixed meal high in protein. Older adults had similar overall protein appearance by iAUC after a mixed meal, but delayed appearance of EAA and BCAA.

Previous postprandial protein kinetic research in older adults has generally investigated the response to a variety of independent macronutrient components such as individual amino acid supplements (429, 539-541), protein fractions (hydrolysed (435, 542) or intact (432, 433, 590)), and protein (544, 591) or carbohydrate (616) independently. A few studies have tried to emulate the macronutrient composition of whole foods through formulations with protein isolates, carbohydrate in the form of dextrin, and fats such as vegetable oils (427, 543, 545-547, 592, 593). Our study is the first to look at the differential aminoacidaemic response in older adults to protein ingestion in the physiological context of feeding a real food mixed meal.

Our study population was well matched for BMI, insulin sensitivity, and fasting plasma glucose. Nevertheless, baseline amino acid profiles appeared to differ between older and younger adults. Older adults displayed lower fasting concentrations of serum BCAAs as has been reported in some (617, 618) but not all (619) previous studies. Furthermore, significant baseline differences in other individual plasma EAAs, NEAAs, and non-proteogenic amino acids were observed. Importantly, we found that higher baseline concentrations for some amino acids in older adults only impacted postprandial peak concentrations, not overall total serum amino acid response as determined by postprandial AUC. Nevertheless, differences in basal concentrations of certain AAs may contribute to absolute postprandial AA concentrations in older adults.

There is already clear evidence that in older adults, slower availability of amino acids after protein feeding in isolation results in poorer postprandial protein balance, in contrast with young adults. Slowly digested proteins like casein (432) or spread feeding patterns (434) result in lower postprandial leucine balance and nitrogen balance respectively in older adults. In the present study, we have shown that in older adults, ingesting protein in the form of a mixed meal results in slower amino acid appearance when compared to younger adults. Although not measured in the current study, this would potentially result in suboptimal postprandial protein balance and an impaired muscle protein synthesis.

Meal composition and structure are known to impact digestion and absorption in older adults; minced beef enhanced protein digestion and absorption in elderly men when compared to beef steak (544). Similarly, casein in a hydrolysed state accelerated protein digestion in elderly men when compared to intact casein (542), showing that older adults have slower protein digestion and absorption with more intact ingested protein. This supports our finding that combined and intact casein and whey were more slowly digested and absorbed by older adults when studied in the context of a mixed meal. As previous studies have shown no impact of age on casein (590) or whey (435) digestion when ingested independently, our data highlight the importance of assessing protein metabolism in the context of intact proteins in representative whole ingested foods.
The anabolic resistance to food in older adults (73) is demonstrated through reduced muscle protein synthetic responses to EAA ingestion (540) which may reflect delayed or prolonged postprandial availability of EAAs and BCAAs as suggested by Condino et al. (539). It is conceivable that delayed availability of EAAs in older adults may contribute to reduced postprandial muscle protein synthesis and anabolic resistance in ageing. Adequate circulating concentrations of EAAs are primarily responsible for the stimulation of muscle protein synthesis (78), particularly in healthy elderly adults (541), while NEAAs seem to be less important (620). Furthermore, the leucine trigger hypothesis suggests that a threshold concentration of leucine is required to adequately stimulate muscle protein synthesis (439), a threshold that may not necessarily be reached following delayed protein absorption. It appears that adequate EAAs must be present in circulation at the same time as the leucine peak (621), suggesting that a lag in total AA availability caused by delayed digestion and absorption in ageing could impact on muscle protein synthesis. This is supported by evidence that older adults show no difference in muscle protein synthesis rate when studied throughout the later postprandial period, which may suggest a delay in early feeding induced muscle protein synthesis activation (622), possibly attributable to delayed EAA digestion and absorption.

A possible explanation for the delayed postprandial appearance of EAAs in older adults is increased intestinal use of many EAAs. Increased splanchnic leucine extraction in elderly men has already been demonstrated (427); however, Koopman et al. (590) showed no significant effect of age on splanchnic extraction of dietary phenylalanine. As our data do not show decreased postprandial iAUC of any EAAs in older adults, splanchnic use of EAAs is not a likely explanation for delayed EAA appearance after a mixed meal. Furthermore, our data show no different postprandial changes in dietary glutamine, glutamic acid or aspartic acid, which are nearly completely used by the intestines (426), implying no difference in postprandial intestinal use of dietary amino acids between older and younger subjects.

Co-ingestion of carbohydrates with protein in elderly adults results in slower amino acid plasma appearance just as in younger adults (545), but this may in fact accelerate dietary amino acid incorporation into elderly muscle, although this effect may not be sustained in the later postprandial period (546). Previous studies in older adults have shown that ingestion of isolated EAAs results in greater postprandial aminoacidaemia (539) and that casein ingestion elicits a greater iAUC appearance of BCAAs (590) compared to younger adults. Our data show no iAUC differences in EAAs or BCAAs suggesting that in a mixed meal, intact protein ingested with carbohydrates may negate a greater iAUC protein appearance in older adults.

Despite delayed amino acid appearance, carbohydrate co-ingestion does not impact on muscle protein synthesis in young (616, 623) or elderly (545) adults. In young adults, the slower appearance of casein from mixed dairy ingestion resulted in more sustained systemic amino acid delivery to the skeletal muscle (543). Our data suggest that this delay in amino acid appearance after mixed dairy ingestion may be even further delayed in older adults; however this delay may not predict an effect on skeletal muscle delivery.
3.2.6. Conclusions

This study shows that older adults digest and absorb proteins from a mixed meal more slowly than younger adults. Furthermore, protein ingestion in a mixed meal is a relevant concern when assessing amino acid appearance and use after a meal since these age-related delays in protein availability have not been previously reported. This slower rate of protein digestion and absorption in ageing after a mixed meal may provide some explanation for the observed decreases in postprandial muscle protein synthesis reported previously, and should be an important consideration when applying protein ingestion strategies for nutritional recommendations in older adults. As such, future investigations into dietary effects on muscle protein synthesis in ageing should explore these questions in the context of real foods, representative of a normal and realistic diet.
3.3. Amino Acid Appearance After a High Fat Mixed Meal in Older Adults

The differences in glycaemic responses between the high fat and low fat meals was analysed to provide context for the differing effects of meal structure on postprandial protein responses. Protein structures, amino acid content, and food matrices differed between the two meals, although total protein and carbohydrate content was similar. This had the potential to affect digestion through means such as gastric emptying or glycaemic response, influencing digestive responses beyond simple amino acid digestion. For this reason, the meals were not compared in a three-factor analysis, to avoid introducing confounding variable. Therefore, age comparisons of the aminoacidaemic response to the high fat meal are presented separately, and have been discussed against the findings presented in the preceding manuscript. Furthermore, a discussion of the potential significance of baseline amino acid profiles in older adults follows, as well as comments on the influence of sex on protein digestion responses.

3.3.1. METHODS

The low fat meal intervention was conducted as described in Chapter 2, section “2.3. Study design”. Analysis was performed as described earlier in this chapter, sections “3.2.3.1. Subjects”, and “3.2.3.5. Statistical analyses”. Glucose and insulin iAUC, maximum peak times (Tmax), and concentrations (Cmax) and total amino acid iAUC between the two meals were compared using two-way repeated-measures ANOVA (age & treatment). Postprandial glucose and insulin responses were compared using three-way repeated-measures ANOVA (age, time, & treatment). Baseline sex-related differences were compared two-way ANOVA with Sidak corrected post hoc analysis was used for all multiple comparisons and alpha was set at P<0.05.

3.3.2. RESULTS

3.3.2.1. DIFFERENCES IN GLUCOSE AND INSULIN RESPONSE BETWEEN HIGH AND LOW FAT MEALS

Postprandial glucose and insulin, although not different between age groups, were different between the high and low fat meals, when compared by three-way repeated-measures ANOVA. Glucose and insulin were both higher after the HF meal, and insulin tended to be more different at specific time points (time × treatment interaction of P=0.054 by three-way repeated-measures ANOVA). After the HF meal, insulin concentrations tended to be greater at 3, 4, & 5 hours (P=0.014, P=0.002, and P=0.024 respectively). These differences did not result in any differences in glucose or insulin iAUC or Tmax (Figure 13), but Cmax for glucose was greater after the HF meal, while insulin Cmax did not differ. Notably, these differences in postprandial glucose and insulin response were not dependent on age.
Figure 13: Postprandial glucose and insulin iAUC, $C_{\text{max}}$, and $T_{\text{max}}$ in older and younger adults after a high and low fat meal.

Postprandial glucose (left panel) and insulin (right panel) incremental area under the curve (iAUC) (A & B), maximum concentration ($C_{\text{max}}$) (C & D), and time of maximum concentration ($T_{\text{max}}$) (E & F) in older (■) and younger (□) adults after a high (HF) and low fat (LF) meal. Values are presented as mean ± SEM for iAUC in arbitrary units (AU), $C_{\text{max}}$ in mmol/l of glucose and µU/ml of insulin respectively, and $T_{\text{max}}$ in minutes. There was a significant difference between the high fat and low fat meals in glucose $C_{\text{max}}$ (treatment effect of $P=0.009$; two-factor repeated-measures ANOVA). ** $P<0.01$ older vs. younger (Sidak).
3.3.2.2. POSTPRANDIAL AMINO ACID ABSORPTION AFTER A HIGH PROTEIN HIGH FAT MIXED MEAL

Baseline age differences in amino acid profile before the high fat meal were consistent with the findings before the low fat meal. Like the baseline concentrations before the low fat meal, older adults had higher aspartic acid, threonine, and tyrosine than younger adults, and lower concentrations of hydroxyproline and isoleucine (Table 9). Age differences in leucine, valine, and ornithine were similar, but failed to reach statistical significance (P=0.062, P=0.058, and P=0.097 respectively). Unlike baseline profiles before the LF meal, older adults had lower concentrations of asparagine than younger adults before the HF meal (P=0.026) and showed no difference in glycine or methionine concentrations.

Overall iAUC of postprandial amino acid appearance after the high fat meal was lower than after the low fat meal (P=0.003; Figure 14), despite the matched protein content. Older adults tended to show a delayed increase in serum postprandial concentrations of BCAAs (P=0.053), but unlike after the LF meal, TAAs and EAAs did not show statistical differences in appearance just as with NEAAs (Figure 15). There were no age differences in iAUC, C\text{max}, or T\text{max} for any AA groupings. Although isoleucine appearance was delayed in older adults just as after the LF meal, the leucine and valine responses were less pronounced (time × age interaction of P=0.026, P=0.055, and P=0.092 respectively). Older adults tended to have lower isoleucine at 2 hours (P=0.058) arising from no increase in postprandial concentrations between 1 & 2 hours while younger adults isoleucine concentrations continued to rise at these times (P=0.353 vs. P=0.013).

Just as after the low fat meal, serum AA concentrations after the high fat meal for individual AAs varied; these are presented in a heatmap displaying mean percentage change in all detected individual serum AAs from fasting serum concentrations in younger adults (Figure 16). Similarly to LF meal postprandial responses, older adults had consistently greater concentrations of glutamic and aspartic acids, threonine, and ornithine, while glutamine was lower (P<0.001, P=0.021, P=0.014, P=0.019, and P=0.031 respectively). In contrast to the LF meal data, older adults showed notably lower concentrations of asparagine throughout the postprandial period with higher concentrations of arginine (P=0.025 and P=0.05 respectively). Meanwhile, tyrosine showed a greater age difference being statistically higher in older adults after the HF meal (P=0.005).

3.3.2.3. SEX DIFFERENCES IN AMINO ACID ABSORPTION BEFORE AND AFTER A LOW FAT MEAL

As sex can affect metabolism, particularly in older adults where women are most likely postmenopausal, the potential effects of sex on basal and postprandial differences in AA metabolism were investigated. These analyses revealed that age-related basal differences in glycine, isoleucine, leucine, ornithine, serine, and taurine were highly influenced by sex (P>0.05, age × sex interaction; Table 10). No main sex differences were observed in postprandial AA appearance. Arginine concentrations were higher in males at 2 hours after the meal compared with females (P=0.01 time × sex interaction). Postprandial taurine appearance was higher in younger males and older females while younger females and older males had similarly lower concentrations (P=0.041 age × sex interaction). Ornithine tended to be greater after the LF meal in older females (P=0.073 age × sex interaction).
Table 9: Serum amino acid profile of older and younger adults before high fat meal.

<table>
<thead>
<tr>
<th></th>
<th>Younger adults (n=15; 8 men, 7 women)</th>
<th>Older adults (n=15; 6 men, 9 women)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Branched-Chain Amino Acids:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>82.4 ± 5.4</td>
<td>65.9 ± 3.3*</td>
</tr>
<tr>
<td>Leucine</td>
<td>142.1 ± 8.3</td>
<td>122.8 ± 4.8 (P=0.062)</td>
</tr>
<tr>
<td>Valine</td>
<td>267.6 ± 15.1</td>
<td>231.6 ± 9.0 (P=0.058)</td>
</tr>
<tr>
<td>All Other Essential Amino Acids:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>82.1 ± 4.7</td>
<td>75.5 ± 4.8</td>
</tr>
<tr>
<td>Lysine</td>
<td>150.8 ± 7.6</td>
<td>143.0 ± 6.9</td>
</tr>
<tr>
<td>Methionine</td>
<td>43.5 ± 3.1</td>
<td>38.7 ± 2.4</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>71.5 ± 3.1</td>
<td>73.1 ± 3.6</td>
</tr>
<tr>
<td>Threonine</td>
<td>64.4 ± 4.1</td>
<td>79.7 ± 5.0*</td>
</tr>
<tr>
<td>Non-Essential Amino Acids:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>414.3 ± 23.4</td>
<td>381.0 ± 19.3</td>
</tr>
<tr>
<td>Arginine</td>
<td>75.2 ± 4.8</td>
<td>83.8 ± 6.8</td>
</tr>
<tr>
<td>Asparagine</td>
<td>54.0 ± 4</td>
<td>41.7 ± 3.0*</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>7.6 ± 1.6</td>
<td>12.0 ± 1.1*</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>86.8 ± 18.5</td>
<td>214.2 ± 16.4***</td>
</tr>
<tr>
<td>Glutamine</td>
<td>567.2 ± 35.4</td>
<td>444.0 ± 36.3*</td>
</tr>
<tr>
<td>Glycine</td>
<td>279.2 ± 27.2</td>
<td>298.0 ± 27.1</td>
</tr>
<tr>
<td>Proline</td>
<td>203.0 ± 16.6</td>
<td>183.0 ± 12.4</td>
</tr>
<tr>
<td>Serine</td>
<td>129.8 ± 6.4</td>
<td>117.5 ± 5.2</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>72.5 ± 3.8</td>
<td>90.3 ± 4.8**</td>
</tr>
<tr>
<td>Non-proteogenic Amino Acids:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Methylhistidine</td>
<td>14.6 ± 2.7</td>
<td>11.4 ± 3.4</td>
</tr>
<tr>
<td>Citrulline</td>
<td>71.9 ± 3.7</td>
<td>63.8 ± 3.3</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>18.7 ± 3.4</td>
<td>10.5 ± 0.8*</td>
</tr>
<tr>
<td>Ornithine</td>
<td>46.4 ± 4.5</td>
<td>56.7 ± 3.7 (P=0.097)</td>
</tr>
<tr>
<td>Taurine</td>
<td>115.0 ± 11.3</td>
<td>99.2 ± 6.4</td>
</tr>
</tbody>
</table>

Values represent means ± SEM. Amino acid values measured in µmol/l. Significance was determined by Student’s t-test. *P<0.05, **P<0.01, ***P<0.001 compared with younger adults.
Figure 14: Serum amino acid area under the curve after high and low fat meals in older and younger adults.

Values represent means ± SEM in arbitrary units of total amino acids (TAA). There was a significant difference in the incremental area under the curve (iAUC) between the high (HF) and low fat (LF) meals in both older (■) and younger (□) adults (treatment effect of **P<0.01, two-factor repeated-measures ANOVA).
Figure 15: Postprandial serum concentrations of TAA, BCAA, EAA, and NEAA in older and younger adults after a high fat meal.

Postprandial plasma concentrations of total amino acids (TAA) (A), BCAA (B), EAA (C), and NEAA (D). Values represent means ± SEM in µmol/l. There were no significant differences over time in the TAA, BCAA and EAA responses between older (●) and younger (○) adults.
Figure 16: Heatmap of postprandial changes in amino acid concentrations of older and younger adults after the high fat meal.

Values are presented as mean percent changes relative to younger adult time 0 concentration for each amino acid (i.e. white represents a 0% change from younger baseline). Green hues represent negative percentage changes; red hues represent positive percentage changes up to 100%; blue hues represent positive percentage changes above 100%. BCAA: branched-chain amino acids; EAA: essential amino acids; NEAA: non-essential amino acids.
Table 10: Sex dependent baseline amino acid differences before low fat meal.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Younger</td>
<td>Older</td>
</tr>
<tr>
<td>Asparagine</td>
<td>11.1 ± 1.1</td>
<td>7.6 ± 1.2*</td>
</tr>
<tr>
<td>Glycine</td>
<td>283.3 ± 21.2</td>
<td>252.3 ± 21.8*</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>67.1 ± 6.4</td>
<td>64.3 ± 5.3</td>
</tr>
<tr>
<td>Leucine</td>
<td>120.3 ± 8.4</td>
<td>118.7 ± 6.8</td>
</tr>
<tr>
<td>Ornithine</td>
<td>48.5 ± 7.3</td>
<td>76.7 ± 5.5*</td>
</tr>
<tr>
<td>Serine</td>
<td>104.2 ± 10.1*</td>
<td>131.9 ± 8.2</td>
</tr>
<tr>
<td>Taurine</td>
<td>103.6 ± 13.7</td>
<td>125.1 ± 11.2</td>
</tr>
<tr>
<td>Valine</td>
<td>222.7 ± 18.9</td>
<td>220.7 ± 15.4</td>
</tr>
</tbody>
</table>

Values presented as means ± SEM. Significance determined by two-way ANOVA with Sidak corrected post hoc analysis. There were significant interactions between age and sex for all amino acids shown except asparagine and glycine. Only main effects for sex are presented for asparagine and glycine. For all other amino acids, significance is noted for a single age and sex group (e.g. older females); significance indicates post hoc comparisons between age and sex independently. *P>0.05; **P>0.01; *P>0.001.

Main age effect found for asparagine (P>0.01).
3.4. GENERAL DISCUSSION

This study shows that amino acid absorption in the elderly is preserved, but delayed after mixed meals, and that the magnitude of the age-related difference is affected by food composition and matrix. This highlights the importance of meal and food structure on the digestive responses to protein in older adults and may have implications for the effects of different foods on interfering with or facilitating the anabolic response to feeding in the elderly.

3.4.1. MEAL EFFECT ON POSTPRANDIAL GLYCAEMIA AND INSULINAEMIA

Although postprandial glucose and insulin responses were not different between age groups, the overall glycaemia and insulinaemia in all subjects were greater after the high fat meal. This was likely due to the differences in ingredients, their relative food matrices, and their cumulative effects on digestion rates, as factors such as food structure, starch type, and food preparation, among other factors, are known to affect glycaemic responses (624). Dietary fibre is known to influence protein digestion, possibly due to delayed gastric emptying (625), a factor known to influence glycaemic responses (624). Likewise, protein metabolism is influenced by postprandial insulinaemia (626) and may account for differences in macronutrient metabolism observed between meals.

3.4.2. BASELINE AND SEX-RELATED AMINO ACID DIFFERENCES

There was some variation in age-related baseline amino acid profiles between the two treatment arms. Lower baseline leucine and valine were not statistically lower in older participants before the HF meal, although all BCAAs were lower before the LF meal. Additionally, glycine and ornithine were only significantly higher in older subjects before the LF meal. As the order of treatments was randomised and a washout period employed, it is unlikely that either treatment affected the basal readings of the other. Hence, this points to notable variation in individual fasting AA profiles, highlighting a shortcoming of using amino acid profiling as a tool to evaluate health status, as done by others (617, 627-630). A better understanding of the factors contributing to individual variation and using pre-trial control measures to limit variation may be necessary.

Furthermore, some of these baseline profile differences were more strongly contributed to by men rather than women. Younger men had higher BCAA concentrations, which has also been reported elsewhere (619), driving age-related differences, while older men had lower taurine than all other subjects. However, women also contributed to variation with younger women influencing age differences in serine and older women determining greater ornithine concentrations in older subjects. The sex specific differences described here in basal and postprandial AA concentrations highlight the difficulties in comparing heterogeneous populations and the necessity of considering sex impacts on protein digestion and metabolism in ageing. Men have greater prevalence and risk of sarcopenia in older age than women (28, 631-633), which suggests there may indeed be sex-specific differences in AA metabolism between sexes. Indeed, many studies choose to eliminate this confounding influence by limiting investigations to men (427, 430, 431, 592, 617) or women (434); however, this practice may introduce additional bias by not considering 50% of the population, while also limiting research applicability.
Although this study aimed to include comparable numbers of men and women, 50% more older women were studied. Women, regardless of age, had lower concentrations of BCAAs, a finding consistent with previous reports of lower leucine concentrations in women versus men (547). Yet older men had BCAA concentrations similar to women of all ages, which were lower than younger men. This suggests that older men may have impaired metabolism of BCAA compared to their younger counterparts, which may influence data from men-only (427, 430, 431, 592, 617) investigations, particularly since it was observed that basal profiles influence postprandial AA responses. On the other hand, these findings indicate that baseline AA profiles are insufficient to evaluate protein intake or chronic metabolism as younger females’ baseline BCAA profile matched that of older adults. This also suggests that younger men may have inherently different AA metabolism to younger women, which may amplify any so-called age-related differences observed in protein metabolism studies. Investigations of factors contributing to the development of sarcopenia may benefit from comparisons with less muscular younger females rather than more muscular younger males (547, 634). Indeed, the relatively greater loss of muscle mass reported in older men versus women has been suggested to be an artefact of men having more muscle mass to lose initially (547). Regardless, we have exposed potentially relevant differences in AA profiles between older men and women which may have implications for relative sarcopenic risk.

3.4.3. POSTPRANDIAL AMINO ACID RESPONSES TO A HIGH FAT MEAL

The matched protein content of the two meals allowed for appropriate comparison of the age-related amino acid appearance to alternate mixed high protein meal. Older subjects had equal total AA appearance after the high fat meal, with no significant differences in postprandial response patterns. Notably, overall AA appearance was lower after the HF meal, indicating that although protein content was matched, overall digestibility was lower for both age groups. Although the HF data do not statistically support the previous finding of delayed TAA, EAA, and BCAA absorption in older subjects as shown after a LF mixed meal, similar trends were observed, particularly in the BCAAs. Older participants failed to maintain postprandial increases in isoleucine in the early postprandial period, which may have contributed to this. However, there were no other postprandial age differences observed that did not involve baseline differences, and overall AA appearance as indicated by iAUC, was not impaired in older subjects. These data support the main LF meal findings, displaying similar patterns of response and age-related amino acid differences, yet highlight the integral role of meal composition and structure on postprandial responses in the elderly.

Although no major postprandial differences in circulating amino acid groupings were apparent after the high fat meal, some individual AA responses differed between age groups. All of the proteogenic AA showing greater postprandial concentrations in older subjects (i.e. glutamic and aspartic acids, threonine, and glutamine), are known to be completely consumed in first-pass splanchnic uptake, aside from threonine (426). Only 50% of dietary threonine is typically consumed in first-pass uptake (197), which may implicate age differences in splanchnic threonine consumption. Additionally, de novo splanchnic synthesis of arginine, tyrosine, alanine, and proline is known to contribute to elevated postprandial concentrations (426). Older participants had greater HF meal
postprandial concentrations of arginine and tyrosine, again supporting altered postprandial splanchnic metabolism in older adults.

Sex specific differences in postprandial amino acid appearance, as observed for arginine, taurine, and ornithine, likely indicate differences in postprandial liver metabolism, since these AAs serve as precursors or products of hepatic amino acid turnover (635, 636). Postprandial increases in ornithine were highest in older women, matching their basal trends and suggesting a link between postprandial alterations and basal AA profiles. This correlation between basal and postprandial differences was similarly observed in sex and age differences for taurine in older men. These sex-specific differences could indicate that metabolic handling or production of taurine and ornithine differs between older men and women, and could have implications on the relative sex-specific risk of chronic illnesses. However, further investigation is required to establish the significance of these differences in metabolic dysfunction and disease. Regardless, these differences highlight the importance of accounting for sex-related differences in AA metabolism for research in the elderly.

### 3.4.4. IMPLICATIONS OF AGE DIFFERENCES IN PROTEIN METABOLISM

#### 3.4.4.1. ORIGINS AND IMPLICATIONS OF ALTERED AMINO ACID PROFILES

It was found that age-related differences in basal amino acid profiles typically persisted into the postprandial period after the less aminoacidaemic high fat meal. This indicates that older adults' postprandial AA concentrations may be highly dependent on basal AA profiles, implying the importance of basal status in determining postprandial protein responsiveness. Basal AA age differences may have resulted from differences in habitual protein uptake (637), but more likely reflect differences in utilisation, and chronic protein turnover (630, 638). Patients with anorexia nervosa display altered AA profiles, yet these differ from typical malnutrition profiles (627), exemplifying the complexity of AA profiles in response to nutritional intake and metabolic alteration. Lower concentrations of AAs such as BCAAs were observed in these older subjects, consistent with AA profiles reported elsewhere (617, 639). Furthermore, lower BCAA concentrations are consistently found in conditions of protein energy malnutrition (628), or decreased liver function (639). Interestingly, the lower BCAA concentrations observed in these older subjects conflicts with typical BCAA profiles of obese, insulin resistant, or T2DM subjects (640). Furthermore, no age differences were observed in circulating liver function enzymes (Chapter 4). This is significant as BCAA metabolism is known to be affected by disturbances in insulin sensitivity (641), which is not indicated in these older adults by the presented amino acid profiles.

The altered amino acid profile of older subjects presented in the current study, indicates differences in inter-related AAs, shown to be modifiable by supplementation, as is frequently done in trauma patients (642, 643). For example, BCAA supplementation has been shown to normalise glutamine and taurine in septic patients (642), while arginine supplementation increases circulating ornithine and glycine (644). For this reason, the age differences in AA profiles are potentially telling of global metabolic disturbances in protein homeostasis.

The profile observed in older subjects of low glutamine and higher glutamic acid has been previously associated with ageing (428). This may indicate increased metabolic demand or skeletal muscle catabolism, as this
profile is seen in cancer cachexia and HIV patients (645) as well as in untreated diabetes mellitus (646). Postprandial protein utilisation is an unlikely explanation for these differences, as dietary glutamine is almost entirely consumed by the splanchnic tissue (647). Age-related metabolic differences in glutamine to glutamate conversion in the liver may explain elevated glutamic acid and decreased glutamine, since hepatic glutaminase activity was identified as the likely factor responsible for this same profile in aged rats (428). Starvation is known to increase hepatic glutamine conversion to glutamate, but so has diabetes, where profiles of greater glutamic acid to glutamine proportions have been observed (648). The mechanisms contributing to glutamine and glutamic acid variation in these older subjects may have implications for both skeletal muscle maintenance and overall chronic disease risk.

Baseline profile differences in older adults may indicate alterations in amino acid metabolism and homeostasis which could impact BCAA availability for skeletal muscle. Both BCAA and glutamine were lower in these older subjects, and are important regulators and indicators of whole body protein turnover. Under periods of catabolic stress, glutamine is mobilised and synthesised de novo from skeletal BCAAs, which are also used to synthesise glutamic acid (643, 649). Under stress-induced protein catabolism, plasma glutamine depletion results in increased leucine oxidation, decreasing circulating BCAAs and potentially worsening catabolic wasting (650). Indeed, leucine infusions have been shown to increase circulating glutamine, while glutamine infusions lead to decreased leucine oxidation, increasing circulating BCAA concentrations (643). Although this does not match the lower BCAA profile of these older subjects, altered glutamine may still suggest increased metabolic pressure on skeletal muscle BCAAs, having the potential to impair muscle maintenance in older adults.

Glutamine changes may influence differences in other AA concentrations, and may have implications for chronic disease risk in the elderly. Glutamine is crucial for the functionality of immune cells, cardiomyocytes, gut barrier function, and production of the antioxidant glutathione (651). The older participants' taurine production (652) could be reduced by insufficient glutamine, explaining the lower taurine concentrations observed in these older men. Trauma, sepsis, and critical illness are known to contribute to low taurine, which has been shown to respond positively to BCAA supplementation (642). Low taurine is also correlated with increased ischemic heart disease and stroke risk, suggesting that adequate circulating taurine may be necessary for lowering CVD risk (653).

Overall, these findings suggest that altered AA profiles in these older subjects may result from several factors, and may be signify underlying metabolic disease. Additionally, these altered profiles, arising from chronic, or even temporary alterations in protein turnover, have the potential to impact absolute postprandial responses to meals. Furthermore, older adults may benefit from targeted supplementation, or enhanced availability in food, of specific amino acids (i.e. glutamine) as this has been shown to be effective at ‘normalising’ amino acid profiles in other populations (642-644).

### 3.4.4.2. IMPLICATIONS FOR PROTEIN INGESTION TIMING

Although the timing of protein ingestion has been suggested as irrelevant for elderly adults who consume adequate dietary protein (654), the current evidence that older adults have delayed EAA and BCAA appearance indicates that protein ingestion timing may in fact be a valid consideration for optimising muscle wasting countermeasures. Indeed, it has been suggested that the lack of effect of nutritional interventions aimed at
maximising post-exercise gains may signify the importance of protein ingestion timing (72). It is recognised that exercise in older adults is effective in promoting MPS and opposing the muscle wasting associated with sarcopenia (655, 656). In young females, BCAA utilisation after light resistance exercise was facilitated when a high protein snack was ingested prior to exercise (657). Similarly, in elderly subjects, protein ingestion immediately following exercise was more effective in promoting muscle hypertrophy than delayed ingestion (658). The delayed appearance of BCAA after a mixed meal in older adults suggests that earlier protein ingestion, prior to exercise, may be required to ensure adequate amino acid availability for post-exercise MPS. Indeed, recent recommendations prescribe exercise following ingestion of ‘intact’ proteins (421), supporting guidelines aimed at accounting for delays in protein digestion.

3.4.4.3. IMPLICATIONS FOR MUSCLE PROTEIN SYNTHESIS

Importantly, the current data highlight the preservation of postprandial protein digestion in older adults, which despite delayed appearance reaches postprandial concentrations equal to younger adults. This signifies that the anabolic resistance of older adults which contributes to sarcopenic wasting in the elderly is a deficit of mechanisms at the level of the muscle rather than deficits of postprandial amino acid availability. Aside from strict deficits in availability, some possible culprits are the threshold of amino acid stimulation, signalling deficits, possibly driven by external influences such as physical activity and inflammation (655).

Stimulation of MPS has been suggested to rely on adequate thresholds of BCAAs, or specifically leucine, rather than total amino acid availability (73, 439). Yet these findings indicate no differences in BCAA or leucine iAUC or peak concentrations between age groups, further incriminating deficits of muscle responsiveness. This supports an increased protein (659), or leucine (655), threshold in older adults. Therefore, despite ‘normal’ amino acid availability post-meal, older adults may still experience inadequate aminoacidaemia for their requirements, necessitating larger protein boluses for similar responses.

The elderly are suggested to have anabolic signalling deficits (72), in part characterised by inadequate activation of mammalian target of rapamycin (mTOR) (73) and S6 kinase phosphorylation (660). Additionally, the elderly demonstrate resistant insulin activation of MPS (440), blocking carbohydrate co-ingestion from enhancing MPS (626). Not only does this imply anabolic influence of peripheral insulin resistance in the elderly, but also suggests a role for glycaemic and insulinaemic actions of a meal. Additionally, inflammation may contribute to anabolic signalling deficits (655). Low-grade inflammation impairs the muscle protein synthetic response to feeding in aged rats (661). Cytokines, such as TNF-α, may directly inhibit MPS (662), and countering systemic inflammation has been shown to restore muscle anabolism after feeding in aged rats (82). While nutritional intervention may be unable to acutely improve resistant signalling mechanisms, longer term interventions aimed at improving insulin resistance or reducing low-grade inflammation may be beneficial for promoting MPS activation in the elderly.

3.4.4.4. IMPLICATIONS FOR FOOD COMPOSITION

Overall, the individual amino acid findings from after the high fat meal were consistent with the findings from the low fat meal, but age-related differences were not as strong. Although protein and carbohydrate loads
were matched, the physical structure of the foods and protein differed. While some of the meal inconsistencies in amino acid differences may be attributable to the unique AA profile of each meal, protein and food structure were likely greater determinants. The greater glycaemic potential of the HF meal indicates relatively faster gastric emptying (663), potentially attributable to differences in protein content, dietary fibre and carbohydrate quality between the meals.

It has already been shown that protein fed in a less processed form, such as steak, when compared to a more processed form, such as minced meat, results in delayed amino acid appearance in the postprandial period (544). Furthermore, the whey from milk is reputed to be a ‘fast’ protein (433), while the structure of egg and beef as in the HF meal are more likely to have greater gastric transit times for both ages. The current study showed that more easily digested proteins were more differently digested in older subjects, suggesting that protein type and meal structure more greatly impacts older adults’ digestion. This implies that in the elderly, rapidly digested proteins like whey cannot be expected to appear in circulation as quickly, and slowly digested proteins such as meat products may be incapable of eliciting rapid amino acid peaks. Strategies to overcome these deficits, such as improved protein digestibility through foods designed for the elderly, may assist in maximising the anabolic response to feeding.

Additionally, digestive responses were likely affected by meal differences in carbohydrate and fat content and structure. Indeed, delayed gastric emptying caused by carbohydrate co-ingestion has previously been shown to delay protein digestion in older adults (545). Variation in meal composition such as dietary fibre (625), fat (664), and certain physical properties of food (e.g. emulsion size, viscosity) (569) may have also affected gastric emptying. However, these meal specific factors affecting glycaemic response and gastric emptying were presented equally to older and younger subjects. This suggests protein digestion kinetics are more sensitive to alteration by the carbohydrate content and quality of a meal in the elderly. Therefore, the selection of carbohydrate sources and the design of structured meals for older adults may be effective in (i) evoking desirable glycaemic and insulinaemic responses, (ii) controlling the rate of gastric emptying, and (iii) controlling the rate of protein digestion.

3.5. CONCLUSIONS

Older adults have preserved AA appearance after ingestion, which is delayed after a low fat mixed meal with less striking differences after a high fat mixed meal. Basal amino acid profiles may influence postprandial amino acid appearance differently in older adults, and may be indicative of underlying chronic metabolic differences in protein turnover. Furthermore, sex specific differences in basal AA profiles and postprandial responses may influence protein digestion differently in men and women. The combination of macronutrients and their relative effects on digestion are an important determinant of postprandial AA appearance in older adults. Careful selection of whole food meals may ameliorate any intrinsic age-related differences in AA appearance following a meal and should be further investigated to develop appropriate dietary recommendations for the prevention of muscle wasting and maintenance of overall health in older adults.
CHAPTER 4

OLDER ADULTS HAVE AN ALTERED LIPAEMIC RESPONSE TO A HIGH FAT MEAL
4.1. Preface

This chapter presents work investigating the lipaemic responses to a high fat meal in older adults. Older adults experience an exaggerated lipaemic response to lipid ingestion, which has been characterised by alterations in chylomicron triacylglycerol content and clearance. Despite this knowledge, the postprandial chylomicron dynamics of older adults has not been fully described in terms of chylomicron size and content differences. To investigate these differences in chylomicron dynamics and composition between older and younger adults, we provided older and younger adults with a standard high fat breakfast meal, used in previous literature.

This study was done in collaboration with Adjunct Professor Kaisa Linderborg from Food Chemistry and Food Development, Department of Biochemistry, University of Turku, Turku, Finland, who developed the methods of lipidomic analysis of chylomicron samples used in this chapter. I visited the University of Turku in June 2014 to conduct these analyses.

The data included in the publication describe the lipaemia findings from the high fat breakfast meal only. As expected, the low fat meal did not elicit a marked postprandial lipaemia, and age-related differences were less pronounced. Furthermore, the differing lipid compositions and food structures between the two meals meant that lipaemic and lipidomic comparisons between the two meals would be subject to a variety of confounding influences. For this reason, data from the low fat meal were not included in the statistical analyses of age-related differences in lipid metabolism. These data were analysed separately, since meal-specific differences in lipid metabolism in older versus younger adults were observed. These findings are presented separately in Chapter 5.

The following section contains the manuscript “Older adults have an altered chylomicron response to a high fat meal”, co-authored by Amber M. Milan, Anu Nuora, Shikha Pundir, Chantal A. Pileggi, James F. Markworth, Kaisa M. Linderborg, and David Cameron-Smith. This article has been accepted for publication in the British Journal of Nutrition. The British Journal of Nutrition is published by Cambridge University Press with a 2013/2014 impact factor of 3.34 and a five year impact factor of 3.60. The final publication will be available at journals.cambridge.org.
4.2. MANUSCRIPT:

OLDER ADULTS HAVE AN ALTERED CHYLOMICRON RESPONSE TO A HIGH FAT MEAL

4.2.1. ABSTRACT

Aging is associated with a prolonged and exaggerated postprandial lipaemia, but the impact of aging on chylomicron synthesis, size and composition is unclear. Healthy older (60-75 years; n=15) and younger (20-25 years; n=15) subjects consumed a high fat breakfast. Following the meal, chylomicron dynamics and fatty acid composition was analysed for 5 hours. Plasma triacylglycerol concentrations were elevated following the meal in the older subjects (P=0.007). For the older subjects, circulating chylomicrons at all postprandial time points were smaller (P=0.011), with greater apoB abundance (P=0.035). However, total chylomicron triacylglycerol concentration between the groups was unaltered post-meal. The older subjects exhibited a greater proportion of oleic acid in the triacylglycerol and phospholipid fraction (P=0.033 and P=0.028), plus lower proportions of linoleic acid in triacylglycerol fraction of the chylomicrons (P=0.003). Thus, following the ingestion of a high fat meal, older individuals demonstrate both smaller, more numerous chylomicrons, with a greater total MUFA and lower PUFA content. This study demonstrates that the increased postprandial hypertriacylglycerolaemia of ageing, in healthy individuals, is not due to increased plasma chylomicron triacylglycerol. Rather aging is associated with changes in chylomicron size, apoB content and fatty acid composition of the chylomicron triacylglycerol and phospholipid fractions which may have implications for cardiovascular disease risk.

4.2.2. INTRODUCTION

Postprandial lipaemia, characterised by increased concentrations of triacylglycerol-rich chylomicron and remnant particles, exerts a significant and often unappreciated impact on cardiovascular disease risk (203). Chylomicrons are synthesised within the small intestinal enterocyte, with a lipid composition highly reflective of the prior meal ingestion (209, 210). Chylomicrons tend to increase in size with increasing quantities of ingested lipids, but also larger when composed of proportionally more longer chain and less saturated triacylglycerols (TAG) (253, 255). Although larger chylomicrons are cleared more slowly than smaller particles, the total number of particles competing for removal has been shown to be the rate limiting factor in postprandial chylomicron and remnant clearance (261) with chylomicrons being the preferential lipolytic substrate over remnants (211, 271).

Older individuals experience a greater postprandial lipaemia (14, 15, 188, 235, 446-448). The current evidence suggests that ageing is associated with an increased TAG content of chylomicrons (188, 446). Further, measurements of postprandial chylomicron dynamics in older adults have been inconsistent and are frequently reliant on proxy measures, such as fat soluble vitamin appearance (188, 446-448), or apoB-48 to TAG ratios (254). Direct measurement of chylomicron particle size in ageing is lacking and this can be quantified by dynamic light scattering methods (265). As chylomicron size dictates lipolytic clearance, age-related differences in chylomicron
composition and size could contribute to the reported impaired chylomicron remnant clearance and hypertriacylglycerolaemia in elderly adults (446).

Fat meal loads used in previous studies to induce postprandial lipaemia have varied widely and have often been limited to simple fat boluses which are poorly representative of a typical meal (587). As macronutrients exert differential metabolic responses when ingested alone (549) or in unison (549, 550) it is of considerable interest to understand the metabolic response to representative mixed meals. High-fat and high-carbohydrate meals representative of a typical Western diet have increasingly been used to study postprandial lipaemia in healthy (536, 561, 582) and metabolically compromised populations (250, 665). Thus in this study direct measurement of the effect of age on chylomicron dynamics and composition following a high fat (and high carbohydrate) meal, representative of a Western diet, was conducted. Analysis was performed to examine chylomicron particle size, apoB concentration and fatty acid composition of both the chylomicron TAG and phospholipid (PL) fractions, in a healthy younger and older adult population. We hypothesised that an exaggerated postprandial lipaemic response in older adults would be due to the formation of larger chylomicrons containing greater TAG that would delay clearance. We further aimed to describe the fatty acid composition of both the chylomicron TAG and phospholipid fraction in older adults.

4.2.3. METHODS

4.2.3.1. SUBJECT SELECTION

Thirty healthy, community-dwelling subjects (n=7 young females, n=8 young males, n=9 older females, n=6 older males) from the Auckland region were recruited through newspaper advertisements and from the university community to participate in the study. Eligible subjects were required to have a BMI between 18 and 30 kg/m² and be between the ages of 20-25 years and 60-75 years. Individuals with a history of cardiovascular or metabolic disease/conditions, or who used medications that may interfere with study endpoints (i.e. anti-inflammatory drugs, Statin drugs) were not eligible for participation. This study was conducted according to the guidelines laid down by the Declaration of Helsinki and all procedures involving human subjects were approved by the University of Auckland Human Participants and Ethics Committee (Ref # 8026). Written informed consent was obtained from all subjects. This study was registered prospectively at Australian New Zealand Clinical Trials Registry at anzctr.org.au (ID: ACTRN12612000515897).

4.2.3.2. STUDY PROCEDURES

This study was a cross-sectional, postprandial trial using a high fat breakfast, chosen as a standard test meal with a high fat (62.2 g) and protein load used previously (582) (Table 11). Subjects were asked to abstain from vigorous physical activity, high fat foods, anti-inflammatory medications, and nutritional supplements the day prior to their visit. Subjects arrived fasted on the morning of their visit; anthropometric data were collected before a catheter was inserted into an antecubital vein and a baseline sample (time 0) was taken followed by consumption of the test breakfast. Blood samples were collected post-meal into EDTA containing and serum blood collection tubes (Becton Dickinson, NJ, USA) hourly for 5 hours. Serum tubes were left to clot at room temperature for 15 minutes
before all tubes were centrifuged at 1500 × g for 15 minutes at 4°C for serum and plasma separation. An aliquot of plasma was kept at 4°C for chylomicron-rich fraction (CMRF) separation within 6 h and the remaining plasma was collected in pyrogen-free microtubes and stored at -20°C until analysis.

4.2.3.3. CHYLOMICRON-RICH FRACTION ISOLATION

The chylomicron-rich fraction, containing chylomicrons and their large remnants, was separated in 4.7ml OptiSeal tubes (Beckman Coulter, CA, USA) in an Optima MAX-XP ultracentrifuge using a TLA-110 rotor with methods adapted from Oikawa et al. (597). Density gradient saline solutions were prepared with NaCl and 0.005% EDTA (Sigma-Aldrich, MO, USA) according to Naito (599) and separation protocols were based on those of Kupke and Wörz-Zeugner (600). In brief, 3.5ml plasma was overlaid with 1.2ml saline solution (d=1.006g/ml) and centrifuging at 117 000 × g for 10 minutes. The visible top layer was aspirated into microtubes and corrected to a final collection volume of 1.4ml using saline solution. This provided a standardised dilution factor of the collected CMRF volume relative to initial plasma volume. The CMRF was stored in pyrogen-free microtubes at -80°C until use.

4.2.3.4. CHYLOMICRON-RICH FRACTION PARTICLE SIZE ANALYSIS

CMRF particle size was measured using a NanoZetasizer S (Malvern Instruments, Malvern, Worcestershire, UK) by dynamic light scattering. If more than one size peak was observed, the mean peak data for the peak within the range of large triacylglycerol-rich lipoproteins were used (>70nm) based on the lower range of mean diameters of small chylomicrons (S: 400-1000) reported by Fraser (259).

4.2.3.5. BIOCHEMICAL ANALYSIS

Plasma glucose, cholesterol, LDL, HDL, TAG, NEFA, and CMRF TAG, apoB, and serum alanine transaminase (ALT) and aspartate transaminase (AST) were measured using a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan) by enzymatic colorimetric assay (Roche, Mannheim, Germany). Plasma insulin was measured using an Abbott AxSYM system (Abbott Laboratories, Abbott Park, USA) by microparticle enzyme immunoassay.

4.2.3.7. FATTY ACID COMPOSITION ANALYSIS OF MEALS AND CHYLOMICRON-RICH FRACTION TRIACYLGLYCEROLS AND PHOSPHOLIPIDS

Meals were homogenised using a domestic blender with 600g water. Samples were aliquoted into 50ml centrifuge tubes, frozen, and stored at -80°C until use. Fatty acid extraction, methylation and gas chromatography of meal and CMRF samples were performed according to protocols described previously (594-596). In short, an internal standard mixture of triheptadecanoin (Sigma-Aldrich) and dinonadecanoylphosphatidylcholine (Sigma-Aldrich) was added to the isolated CMRFs. Then 1.5ml methanol, 3ml chloroform, and 0.8ml 0.88% KCl in water were added and the blend was thoroughly vortexed after each addition. Samples were centrifuged at 2000 × g for 3 minutes to separate the layers, and the lower chloroform rich layer was removed, and the upper layer extracted again (594). Food lipids were extracted just as CMRFs after further homogenisation with an Ultra Turrax (IKA,
CMRF TAGs and PLs were isolated from the extracted lipid mixture with solid phase extraction based on Sep-Pak® Vac 1cc- silica columns (Waters, MA, USA) (595).

Fatty acid methyl esters (FAME) were prepared with a sodium methoxide method. In short, the lipids were suspended to 1ml dry diethylether; then 25μl methylacetate and 25μl sodium methoxide were added and the blend was incubated for 5 minutes while shaken at times. The reaction was stopped with 6μl acetic acid. Samples were centrifuged at 2000 × g for 5 minutes, after which the supernatant was removed and gently evaporated to dryness. The resulting FAME were transferred to 100μl inserts in hexane (596). The FAME were analysed with gas chromatography (Shimadzu GC-2010) equipped with AOC-20i auto injector, flame ionisation detector (Shimadzu corporation, Kyoto, Japan) and wall coated open tubular column DB-23 (60m x 0.25mm i.d., liquid film 0.25μm, Agilent Technologies, J.W. Scientific, Santa Clara, CA, USA). Splitless/split injection was used and the split was opened after 1 minute. Supelco 37 Component FAME Mix (Supelco, St. Louis, MO, USA), 68D (Nu-Check-Prep, Elysian, MN, USA) and GLC-490 (Nu-Check-Prep) were used as external standards.

4.2.3.8. CALCULATIONS

Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated from fasting glucose and insulin concentrations using the equation from Matthews et al. (615). Plasma fatty acids were pooled for mathematical analysis into SFA, MUFA, PUFA, and dairy derived odd-chain fatty acids.

4.2.3.9. STATISTICAL ANALYSES

Statistical analyses were conducted with Statistical Package for the Social Sciences version 21 (SPSS, IBM Corporation, Armonk, NY, USA). Data are presented as means ± SEM. Incremental AUC (iAUC) was calculated after subtracting fasting values. Baseline subject characteristics, iAUC, and maximum peak times (T_{max}) and concentrations (C_{max}) were compared using unpaired Student’s t test. Two-factor repeated-measures analysis of variance (ANOVA, time and age) followed by Sidak adjusted post hoc test was used for all multiple comparisons between different groups. Where Mauchly’s sphericity test failed, the Huynh-Feldt correction was applied. Alpha was set at P<0.05. Heat maps were created using R software version 2.15.2 (R Development Core Team, Vienna, Austria).
Table 11: Macronutrient and fatty acid composition of high fat breakfast meal (Table 1 in article).

<table>
<thead>
<tr>
<th>Meal Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macronutrients</strong></td>
<td></td>
</tr>
<tr>
<td>Fat (g)</td>
<td>62.2</td>
</tr>
<tr>
<td>Carbohydrates (g)</td>
<td>77.4</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>49.8</td>
</tr>
<tr>
<td>Energy (kJ)</td>
<td>4530</td>
</tr>
<tr>
<td><strong>Fatty Acid Mass Proportion (g/100g)</strong></td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td>2.9 ± 0.13</td>
</tr>
<tr>
<td>16:0</td>
<td>18.1 ± 0.60</td>
</tr>
<tr>
<td>16:1 n-7</td>
<td>1.8 ± 0.10</td>
</tr>
<tr>
<td>18:0</td>
<td>9.6 ± 0.40</td>
</tr>
<tr>
<td>18:1 n-7</td>
<td>1.7 ± 0.07</td>
</tr>
<tr>
<td>18:1 n-9</td>
<td>50.0 ± 1.70</td>
</tr>
<tr>
<td>18:1 n-9\text{trans}</td>
<td>1.3 ± 0.90</td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>8.7 ± 0.30</td>
</tr>
<tr>
<td>18:3 n-3</td>
<td>2.5 ± 0.10</td>
</tr>
<tr>
<td>Others</td>
<td>3.35 ± 0.50</td>
</tr>
</tbody>
</table>

1Meal consisting of 2 sausage egg muffin sandwiches containing English muffin (wheat), egg, sausage patty (beef), cheese slice, and rapeseed oil, with 2 hash browns containing potatoes and canola oil. Values presented are based on nutrient panel data obtained from the website of the fast food restaurant.

2\text{n}=3, values as means ± SEM.

3Fatty acids with a mass proportion less than 0.5g/100g are pooled to others category.
4.2.4. RESULTS

4.2.4.1. SUBJECT CHARACTERISTICS

A total of thirty subjects completed the study (n=15 younger, n=15 older). There were no significant differences between the two age groups for BMI or fasting measurements of plasma glucose, plasma insulin, HOMA-IR or plasma and CMRF TAGs (Table 12). Fasting plasma lipid profiles were within a normal range for both age groups, although total, LDL, and HDL cholesterol were all higher in older subjects (P<0.001, P=0.002, and P<0.001 respectively). Liver function measured by serum ALT and AST were within the normal range for all participants; however, the older participants displayed lower concentrations for each (P=0.004 and P=0.001 respectively).

Table 12: Baseline subject characteristics and fasting plasma, serum, and chylomicron-rich fraction lipid profile of older and younger adults after high fat meal (Table 2 in article).

<table>
<thead>
<tr>
<th>Measure</th>
<th>Units</th>
<th>Younger adults(^1) (n=15)</th>
<th>Older adults(^2, 3) (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>years</td>
<td>22.7 ± 0.4</td>
<td>67.3 ± 1.5**</td>
</tr>
<tr>
<td>BMI</td>
<td>kg/m²</td>
<td>23.7 ± 0.8</td>
<td>24.4 ± 1.0</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td></td>
<td>2.0 ± 0.2</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>mmol/l</td>
<td>5.1 ± 0.1</td>
<td>5.2 ± 0.1</td>
</tr>
<tr>
<td>Total Cholesterol</td>
<td>mmol/l</td>
<td>4.0 ± 0.2</td>
<td>5.0 ± 0.2***</td>
</tr>
<tr>
<td>LDL</td>
<td>mmol/l</td>
<td>2.4 ± 0.2</td>
<td>3.0 ± 0.2**</td>
</tr>
<tr>
<td>HDL</td>
<td>mmol/l</td>
<td>1.4 ± 0.1</td>
<td>1.8 ± 0.1***</td>
</tr>
<tr>
<td>TAG</td>
<td>mmol/l</td>
<td>0.82 ± 0.07</td>
<td>0.91 ± 0.07</td>
</tr>
<tr>
<td>NEFA</td>
<td>mmol/l</td>
<td>0.66 ± 0.08</td>
<td>0.57 ± 0.09</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALT</td>
<td>U/l</td>
<td>10.0 ± 2.2</td>
<td>1.9 ± 0.8**</td>
</tr>
<tr>
<td>AST</td>
<td>U/l</td>
<td>19.1 ± 1.7</td>
<td>12.1 ± 0.7**</td>
</tr>
<tr>
<td>Chylomicron-rich fraction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAG</td>
<td>mmol/l of plasma</td>
<td>0.07 ± 0.02</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>Size</td>
<td>nm</td>
<td>191 ± 20</td>
<td>140 ± 24</td>
</tr>
<tr>
<td>apoB</td>
<td>mmol/l of plasma</td>
<td>2.3 ± 0.5</td>
<td>3.4 ± 0.5</td>
</tr>
</tbody>
</table>

\(^1\)HOMA-IR: homeostatic model assessment of insulin resistance.
\(^2\)Values represent means ± SEM.
\(^3\)Significance was determined by unpaired Student’s t test; ***P<0.001, **P<0.01 compared with younger adults.
4.2.4.2. PLASMA GLUCOSE, INSULIN, AND LIPID RESPONSES

Postprandial glucose (Figure 17A) and insulin (Figure 17B) responses to the meal did not differ between younger and older subjects. The older group had a delayed time to achieve peak plasma TAG concentrations ($T_{max}$ 3.4 ± 0.2 vs. 2.1 ± 0.2 hours respectively, $P<0.001$), reaching peak TAG concentration ($C_{max}$) after ~3 hours ($P<0.001$ vs. baseline). The younger subjects reached maximum peak concentration at ~2 hours ($P=0.02$ vs. baseline). The plasma TAG in the younger subjects also returned to baseline by 4 hours; however, in the older group plasma TAG remained significantly elevated at 5 hours post-meal (Figure 17C). As such, we observed a prolonged TAG response in older subjects. This pattern of response did not impact on the $iAUC$ (0-5 hours) response between younger and older groups (103.6 ± 21.6 vs. 102.5 ± 19.1mmol/l respectively; $P=0.97$; data not shown). Marked suppression of circulating plasma NEFA was observed following the meal, with no difference between younger and older subjects (Figure 17D). Postprandial total plasma cholesterol decreased in the older but not younger participants (Table 13; time-age interaction, $P<0.001$).

4.2.4.3. CHYLOMICRON-RICH FRACTION DYNAMICS

CMRF particle size increased after the meal ($P=0.01$; Figure 18A) and was smaller for older compared to younger participants ($P=0.011$, main effect of age). Due to variation in time to peak size between individuals, maximum peak CMRF particle size and $T_{max}$ were compared. Overall mean peak time was just under 3 hours post-meal ($T_{max}$ 175 ± 20 vs. 160 ± 25 minutes, younger vs. older respectively; $P=0.506$). Maximum CMRF particle size tended to be smaller in the older subjects (321 ± 29 and 390 ± 22nm, older vs. younger respectively; $P=0.072$). Since CMRF particle size was not different at baseline between age groups, post hoc analysis was conducted to determine age group postprandial changes in size, despite no time-age interaction. The older group’s CMRFs remained constant in size after the meal, while the younger group’s CMRF size increased, resulting in an age difference in size at 2 & 3 hours ($P=0.022$ and 0.007 respectively). Postprandial CMRF TAG concentration was not different between older and younger subjects ($P=0.894$; Figure 18B).

Changes in CMRF particles numbers were assessed by apoB concentrations. Older subjects had higher apoB concentrations than younger (mean postprandial concentration 0-5 hours of 5.3 ± 0.4 vs. 4.2 ± 0.4 × 10^{-2}g/l respectively; $P=0.035$; Figure 18C). Overall, apoB concentrations peaked at 2 hours post-meal ($P=0.001$) returning to comparable baseline concentrations by 5 hours.
Postprandial plasma concentrations of glucose (A), insulin (B), triacylglycerol (TAG) (C) and NEFA (D) in older (●) and younger (○) adults. Values represent means ± SEM (n=15/group). There were no differences in glucose, insulin, or NEFA responses between older and younger adults. There were significant differences over time in the TAG response between older (●) and younger (○) adults (age × time interaction of $P<0.01$, two-factor repeated measures ANOVA). *** $P<0.001$ change from baseline; $\alpha$ $P<0.01$, change from baseline older; $\beta$ $P<0.01$ change from baseline younger; $\gamma$ $P<0.01$ change from 5 hours (Sidak corrected post hocs).

Table 13: Postprandial total cholesterol in older and younger subjects after the high fat meal (Table 3 in article).

<table>
<thead>
<tr>
<th></th>
<th>Baseline (mmol/l)</th>
<th>1h</th>
<th>2h</th>
<th>3h</th>
<th>4h</th>
<th>5h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Younger adults</td>
<td>3.99 ± 0.18</td>
<td>-1.5</td>
<td>-1.1</td>
<td>-3.2</td>
<td>-1.4</td>
<td>-0.6</td>
</tr>
<tr>
<td>(n=15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Older adults</td>
<td>5.04 ± 0.22</td>
<td>-2.3</td>
<td>-4.0**</td>
<td>-5.6**</td>
<td>-5.7***</td>
<td>$\delta$</td>
</tr>
<tr>
<td>(n=15)</td>
<td></td>
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</tbody>
</table>

% change in total cholesterol within each age group from baseline to each time point was calculated. Significance was determined by two-factor repeated measure ANOVA with Sidak corrected post hoc analysis. **$P<0.01$; ***$P<0.001$ compared to baseline; $\delta$ $P<0.01$ compared to 1h. All time point comparisons between younger and older adults were significant to $P<0.01$. 

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Figure 18: Postprandial chylomicron-rich fraction dynamics and lipaemia in older and younger adults after high fat meal (Figure 2 in article).

Postprandial chylomicron-rich fraction (CMRF) size (A), triacylglycerol (TAG) concentration (B), Apolipoprotein B (apoB) concentration (C) in older (●) and younger (○) adults. Values represent means ± SEM (n=15/group) in nm for size and in mmol/l of plasma for TAG, and g/l for apoB. There were no differences in CMRF TAG responses or apoB-100 concentrations between older and younger adults. There were significant main age differences in the size and total apoB responses between older and younger adults (age effect marked (a) of P<0.05 each, two-factor repeated measures ANOVA). * P<0.05 change from baseline; ** P<0.01 change from baseline (Sidak corrected post hocs).
4.2.4.4. CHYLOMICRON-RICH FRACTION TRIACYLGLYCEROL AND PHOSPHOLIPID FATTY ACID COMPOSITION

4.2.4.4.1. COMPARISON OF CHYLOMICRON-RICH FRACTION TRIACYLGLYCEROL COMPOSITION BETWEEN AGES

Older subjects had greater proportions of MUFAs in their CMRF TAGs (P=0.014; Figure 19). Also, 4 hours after the meal, older subjects had a lower proportion of PUFA in CMRF TAGs (P=0.004). Older participants’ CMRF TAGs tended to have lower proportions of SFAs at 2 hours (P=0.079), a trend that was evened out after 4 hours.

These differences in MUFA and PUFA CMRF TAG composition were mainly attributable to changes in major fatty acid components, oleic and linoleic acid. The older group’s CMRF TAGs had relatively more oleic acid (18:1n-9, P=0.033; Figure 20A). Although 18:2n-6 (linoleic acid) was found in equal proportions in both groups’ CMRF TAGs at 2 hours, by 4 hours the older subjects had a lower proportions (P=0.003). The older group had lower proportions of 18:0 (stearic acid) at 2 hours compared to younger participants (P=0.009) while these age differences were not apparent after 4 hours. Older subjects had lower proportions of 15:1 at 2 hours than the younger group (P=0.037).

Pooling of the dairy derived odd-chain fatty acids (15:0, 15:1, 17:0, 17:1) showed higher proportions in the CMRF TAGs of older subjects (P<0.001). In both groups, CMRF TAG composition changed over the postprandial period. From 2 hours to 4 hours after the meal, proportions of CMRF TAG 20:4n-6 (arachidonic acid) and 22:6n-3 (DHA) increased (P=0.002 and P=0.042 respectively).

4.2.4.4.2. COMPARISON OF CHYLOMICRON-RICH FRACTION PHOSPHOLIPID COMPOSITION BETWEEN AGES

CMRF PL composition changed over the postprandial period. At 4 hours, CMRF PLs contained relatively more PUFAs (P=0.034), less SFAs (P=0.042) and tended to contain more MUFAs (P=0.052) than at 2 hours (Figure 19). This difference in the later postprandial period was characterised by relatively more linoleic acid (18:2n-6, P=0.023) than at 2 hours (Figure 20B).

The CMRF PLs of the older subjects contained relatively more MUFAs (P=0.038), less PUFAs (P=0.032), and tended to contain less SFAs (P=0.085; Figure 19). Arachidonic acid was found in lower concentrations in older subjects (P=0.018).

Just as in the chylomicron-rich TAG fraction, the chylomicron-rich PL fraction in the older group contained greater proportions of oleic acid (18:1n-9, P=0.028). Elaidic acid (18:1n-9trans at 2 hours, P=0.001) and 18:2n-6trans (P=0.012) were more abundant in the chylomicron-rich PL fraction of older participants. This was accompanied by relatively more of the dairy derived odd-chain fatty acids (P=0.001) particularly 15:0, and 15:1 (P=0.018, and P=0.003 respectively). Furthermore, the older subjects had greater proportions of the PUFA docosapentaenoic acid (DPA, 22:5n-3, P=0.013), tended to have relatively more EPA (P=0.058).
Figure 19: Heatmap of distribution changes of chylomicron-rich fraction SFA, MUFA, and PUFA after the meal of older and younger adults after high fat meal (Figure 3 in article).

Values are presented as mean percent changes relative to younger adult 2 hour mass proportion for each saturation class (i.e. white represents a 0% change from younger 2 hour). Green hues represent negative percentage changes; red hues represent positive percentage changes.
Figure 20: Major chylomicron triacylglycerol and phospholipid composition of younger and older adults after high fat meal (Figure 4 in article).

Major chylomicron-rich fraction (CMRF) triacylglycerol (A) and phospholipid (B) fatty acid composition of younger adults 2 hour (white bars) and 4 hour (light gray bars) and older adults 2 hour (dark gray bars) and 4 hour (black bars) as mass proportions. Data marked (a) differ with age, data marked (t) differ with time; \( P<0.05 \) by two-factor repeated measures ANOVA. Age × time interaction post hocs (Sidak corrected within each fatty acid) are indicated as *\( P<0.05 \), **\( P<0.01 \).
4.2.5. DISCUSSION

Following a high fat meal, the older participants of the current study had smaller chylomicron particles with a greater proportion of oleic acid and a lower PUFA content over a 5 hour postprandial period. This study highlights that there are subtle changes in the dynamics governing lipid absorption, which occur along with differences in relative absorption and/or clearance of specific fatty acids, predominately oleic acid. Furthermore, our data do not support either altered appearance or delayed chylomicron clearance as a major regulator of the exaggerated lipaemia typically present in older individuals. Our data suggest that the major contributor to the heightened postprandial triacylglycerol response is in the non-chylomicron-rich fraction and includes other triacylglycerol-rich particles, including VLDL and chylomicron remnants (235).

Consistent with previous reports, our data demonstrates prolonged postprandial triacylglycerolaemia in older adults (14, 15, 188, 235, 446-448). Yet within this literature, few studies have addressed the dynamics and role of chylomicron structure and composition in this hypertriacylglycerolaemic response. In the present study, the concentration of chylomicron-rich fraction TAG was not different between older and younger participants. Previous studies reporting differences in chylomicron TAG (188, 446, 448), have shown greater TAG content in large, rather than small, TRLs in the elderly (447), plus a greater postprandial NEFA spillover from exogenous TRL (235). Neither mechanism was demonstrated in the current study. Rather, our data implicate alterations of small TRL (endogenous VLDL and small chylomicron remnants) production and clearance as a major determinant of the sustained postprandial hypertriacylglycerolaemia in the elderly. It must be noted that there were no age differences in BMI, HOMA-IR, fasting TAG and NEFA, or indications of hepatic dysfunction in this present study. Previously, these factors have been shown to adversely affect postprandial chylomicron dynamics (235). Furthermore, our findings are presented in the context of a mixed breakfast meal rather than in response to the ingestion of isolated lipid loads.

In this study the older participants had a maximum chylomicron size on average 18% smaller and 27% more numerous than the younger group. Importantly, chylomicron sizes were measured directly through dynamic light scattering, a technique used previously for chylomicron and lipoprotein size analyses (263, 265), although rarely in the context of postprandial dynamics (666). These smaller and more numerous triacylglycerol-rich lipoproteins are a reported feature of insulin resistance (248) and certain types of dyslipidemia (289). Smaller and more abundant TRLs may be due to intestinal overproduction of apoB-48 (667, 668), and/or failed suppression of postprandial large VLDL production (221). Yet in this study no age difference in fasting insulin sensitivity, as measured by HOMA-IR, were observed. Thus, the mechanisms contributing to the increased concentrations of smaller and more numerous chylomicron particles was not determined in the current study.

Differences in chylomicron size and number may have important implications for chylomicron clearance and potentially atherosclerotic risk in these older subjects. Smaller chylomicron particle size has been shown to retard clearance rate (270); however, more recent investigations have shown that this may be an artefact of unmatched number of particles (261), indicating that larger numbers, often corresponding with smaller size, are cleared more slowly. Smaller and greater circulating numbers of chylomicron remnants, due to their ability to
penetrate the arterial intima, have been shown to increase atherosclerotic risk (203). Yet, our results suggest that the large TRL fraction, although containing relatively smaller particles, is cleared efficiently, while the small TRL fraction may persist.

In addition to the alterations in the size and number of circulating chylomicrons, differences in the lipid composition of both the triacylglycerol and phospholipid fractions were measured. The older individuals had greater proportions of MUFA, particularly oleic acid, in chylomicron TAGs, with a corresponding lowering of the proportion of PUFA. These compositional differences were also present in the PL fraction. Our findings correspond with reports of age-related differences in adipose and circulating TAG composition with age-related declines in SFA (465), linoleic acid (18:2\(n-6\)) (464), and total PUFA content (461), along with increases in MUFA content (465). Similarly, our findings of lower chylomicron PL PUFAs are similar to what has been previously demonstrated in elderly populations (461-463). Habitual diet or health status has been suggested to impact these age-related compositional differences (463, 464). However, as our postprandial findings match longer-term evaluations of circulating fatty acids in elderly populations, metabolic handling of fatty acids may contribute to age-related fatty acid differences in the elderly.

There are likely to be several mechanisms exerting influence on the differences in chylomicron FA composition between the younger and older subjects following the consumption of an identical meal, including possible differences in fatty acid absorption and clearance from TRL. Enterocyte FA metabolism may be involved as chylomicron incorporation of fat-soluble vitamins has suggested age-related differences in postprandial absorption (188, 447). The greater proportions of MUFAs observed in the older group may indicate enhanced intestinal absorption of oleic acid. This is supported by animal models indicating that uptake of oleic acid (455), linoleic acid (457), and saturated fats (403) are increased in aged rats, fitting with our data. Additionally, chylomicron TAG are known to contain proportions of FA originating from enterocyte storage pools, containing FA from the previous meal (232, 233) as well as early postprandial contributions from NEFA (232). It has been suggested that in insulin resistant states, the intestines may store greater quantities of fat, contributing to aberrant apoB production (276). It is unknown whether differences in intestinal enterocyte storage pools between age groups may have contributed to postprandial fatty acid compositional differences. However, it is probable that the predominant mediator is exchange with either circulating free or lipoprotein derived PLs and TAGs, or the presence of endogenous large TRL. Indeed, the postprandial reductions in total cholesterol we observed in the older group suggests increased inter-lipoprotein transfer; however, measure of this activity such as cholesterol ester transfer activity were not assessed. Furthermore, the origins of altered proportions of fatty acids in any endogenous lipoproteins, such as greater MUFA content, is remains unknown.

The postprandial age-related differences in older participants’ chylomicron composition have metabolic implications outside of transient effects on postprandial lipaemia. Since older people incorporate ingested TAGs at a greater rate into small TRL rather than chylomicrons (235), any differences in fatty acid uptake will be amplified with the increased residence time of these remnants compared to chylomicrons (14, 446). Additionally, the increased surface area to volume ratio of chylomicrons in the older group implies proportionally more PLs, magnifying the impact of these compositional differences. Specifically, enhanced uptake of oleic acid could have
implications for uptake of other fatty acids, as long-chain fatty acid uptake into the enterocyte is competitive (669), and could explain the lower proportions of PUFAs found in older subjects’ chylomicron TAGs. Furthermore, the fatty acid composition differences reported in these older adults have been identified as features in individuals with metabolic dysfunction; lower serum linoleic acid has been associated with an increased risk of developing impaired fasting glucose or type 2 diabetes mellitus in middle aged men (670). Additionally, as we show that some age-related compositional differences are dependent on the time of postprandial sampling, differences in rates of uptake or clearance may contribute, highlighting a downfall of compositional profile analysis from a single time-point when temporal metabolic differences exist between populations.

4.2.6. CONCLUSIONS

The exaggerated postprandial lipaemia typical of older individuals is not characterised by elevated chylomicron TAG. The current study demonstrates that older healthy cohort have no impairment large chylomicron clearance. Despite preservation of total chylomicron lipid transport, chylomicrons are smaller and more numerous in the elderly, containing greater proportions of MUFAs and lower proportions of PUFAs. The implications of these fundamental differences in chylomicron dynamics and composition are unknown in the context of cardiovascular disease risk and warrant further investigation.
4.3. GENERAL LIMITATIONS AND DISCUSSION

4.3.1. ENDOGENOUS LIPID CONTRIBUTIONS TO LIPAEMIA

4.3.1.1. DISTINCTION BETWEEN ENDOGENOUS AND EXOGENOUS TRIACYLGlycerol-RICH LIPOPROTEINS

Density gradient separation of lipoproteins relies on the distinct densities of different classes. However, since the density of a lipoprotein relates to the relative TAG content it contains, this density will ultimately change as a lipoprotein becomes more deplete of TAG. As the metabolic fate of the TRL is to lose TAG through lipolysis, the relative density and size of these particles decreases and becomes inherently heterogeneous (219). Furthermore, the ability to distinguish between intestinally and hepatically derived TRL particles through size or density becomes diminished during a state of lipid catabolism. Ultimately, it is unlikely that definitive separation or identification can be made without employing multiple methods, such as apoB-48 or -100 identification, apoE concentrations, or gel permeation high performance liquid chromatography, for instance (671).

It should be noted that in the current study, no additional methods were employed to enable distinction between nascent, remnant, intestinal, or endogenous TRL apart from density gradient separation methods. Therefore, it is difficult to guarantee complete removal of VLDL from the chylomicron fraction which was obtained, as it is similarly problematic to know the fate of remnant particles. However, it should also be noted that density gradient separation is still largely used in the literature (15, 603, 666), and that the use of multiple means of definitive identification has been infrequent (14, 15, 188, 446-448) in literature of postprandial lipaemia in the elderly (235). Hence, the fraction colloquially termed ‘chylomicron’ in some past literature may in fact more accurately describe a TRL fraction with an indeterminate quantity of endogenous lipoproteins. However, the postprandial contributions of VLDL to lipaemia are becoming better appreciated, as VLDL production has been shown to account for 80% of the particle increase in postprandial lipoproteins (671). Furthermore, the relevance of the distinction between hepatic and intestinal origins of TRL has been challenged by some, who argue that the metabolic activities of these lipoproteins during the postprandial state are indeed a greater concern (450, 672). These ‘large TRLs’, whether consisting of chylomicrons or VLDL, are responsible for elevations in postprandial lipaemia. Furthermore, these larger TRL drive the production of highly atherogenic remnant particles, creating competition for clearance. Hence, while more standard identification and reporting of the TRL fraction in the future may assist in identifying the players in postprandial lipaemia, the relevancy of the d<1.006g/l TRL fraction to metabolic and cardiovascular health remain.

4.3.1.2. POSTPRANDIAL TRIACYLGlycerol, NON-ESTERIFIED FATTY ACID, AND CHOLESTEROL TRANSFER

Older and younger subjects had similar NEFA responses to the high fat meal. This finding was unexpected as impaired postprandial NEFA suppression is evident in obesity (471) and has been reported in the elderly (473), indicating inadequate removal of lipolysed chylomicron TAGs (235). It is possible similarities in BMI between age
groups could account for less prominent differences in body composition and hence similarities in fat mass, known to influence NEFA appearance (673). Furthermore, the high quantity of protein in the HF meal may have acted to attenuate NEFA spillover, since protein has been shown to effectively attenuate postprandial lypaemia in the elderly, likely by triggering a greater insulin response (15). The high protein load provided in this study may have ameliorated the NEFA response of older participants, providing better NEFA suppression that a low protein meal may have. Furthermore, insulin secretion may have influenced the ability of the older individuals to maintain NEFA suppression. Although fat alone stimulates insulin secretion (549), the insulin response to carbohydrate (550) or a mixed meal (549) is much greater. This suggests that postprandial NEFA spillover in older adults may well depend on insufficient insulin secretion in response to ingestion of fat alone, and that postprandial lipid dynamics in older adults may be altered in the context of a mixed meal.

In addition to TAG dynamics involving NEFA, dietary TAG may be transferred directly between chylomicrons and other lipoproteins. These data suggest indirect evidence of the postprandial transfer of dietary TAG to other circulating lipoproteins in older adults as indicated by age differences in postprandial total cholesterol changes. Overall, older adults’ postprandial cholesterol decreased from baseline, while younger adults’ cholesterol remained stable, or even increased in the last hour of measurement. Changes in postprandial total cholesterol have been previously reported after fat ingestion, and although also measured in subjects up to 79 years (14), no age comparison was made, and it was reported that changes in cholesterol were not significantly correlated with age. More recently, decreased non-fasting LDL cholesterol has been reported in T2DM (674), and has been reported as more pronounced in men with MetS compared with healthy controls (675).

Changes in total cholesterol in the non-fasted state are attributable to cholesterol transfer between lipoproteins, and decreases are likely caused by decreases in LDL cholesterol (14, 239, 676). Reduced postprandial LDL concentrations may be caused by a reduced conversion of LDL to VLDL due to increased apoB particles (212) and suggesting increased VLDL remnant uptake (676) or increased LDL uptake (676, 677). Indeed, lower non-fasting LDL concentrations have been associated with concurrent decreases in apoB concentrations (675) suggesting a reduction in total particle numbers and supporting enhanced LDL uptake, or alternatively as the authors suggest (675), increased cholesterol uptake in the vascular endothelia. Additionally, postprandial transfer of TRL TAG to LDL co-occurs with reciprocal cholesterol transfer (678). This transfer via cholesterol ester transfer protein activity (679) has been suggested as a cause of reduced postprandial LDL cholesterol concentrations (676). Therefore, older adults’ greater basal LDL and greater postprandial TRL numbers likely enhanced the opportunity for TAG/cholesterol exchange. This transfer of TAG to LDL in the later postprandial period may have further contributed to exaggerated postprandial lypaemia in older adults. Furthermore, this may shed some light on the origins of postprandial differences in chylomicron fatty acid composition; if a proportion of the TAG measured in older adults’ chylomicrons is of VLDL origin rather than dietary origins, greater differences in composition would be expected. Indeed, older adults have been reported to have greater proportions of dietary fatty acids incorporated in small TRL, more likely VLDL, attributed to fatty acids passing from chylomicrons to de novo VLDL via NEFA pools (235). However, this study did not trace fatty acids nor measure chylomicron cholesterol or cholesterol ester
transfer protein activity, making it difficult to assess how TAG and cholesterol transfer may have affected chylomicron TAG concentrations or composition.

4.3.2. POTENTIAL CONFOUNDING VARIABLES IMPACTING POSTPRANDIAL LIPAEMIA

4.3.2.1. HABITUAL DIET AND POSTPRANDIAL LIPAEMIA

Habitual diet is known to affect both chylomicron size and composition. Apart from metabolic age differences, habitual diet may have contributed to age differences in chylomicron size in the current study, as older adults’ habitual dietary intake often differs from the young (680-682) and diet is known to affect chylomicron size (251-255). Although background diets were not recorded in this population, differences in PL composition may provide clues regarding habitual intakes, as this is often used as an indication of long-term diet (683). The greater chylomicron PL MUFA proportion in these older subjects could suggest greater MUFA intake; however, previous studies have linked higher habitual intakes of MUFAs (252, 254) and PUFAs (251, 684) to larger chylomicron sizes as determined by apoB and chylomicron TAG ratios. As the current data contradict these particle size predictions, it is less likely that habitual diet explains age-related size differences. Furthermore, our TAG compositional findings correspond with profiles described in the elderly for adipose tissue (464, 465), and serum TAG (461), and suggest that age-related differences are metabolically rather than diet derived.

4.3.2.2. INSULIN SENSITIVITY AND POSTPRANDIAL LIPAEMIA

Our subjects were well matched for BMI and insulin sensitivity (by HOMA-IR) and older adults showed basal lipid profiles characteristic of their demographic (201). Although body composition was not measured, it is likely that older adults had greater relative fat mass (19, 685-687) contributing to elevated lipaemia (235). Importantly, peripheral insulin resistance, not measured or accurately assessed by basal insulin and glucose concentrations, is known to influence postprandial NEFA and VLDL dynamics (387), and could be a silent influence on the postprandial lipaemic responses of these older adults. More complete assessment of insulin resistance and the influence of related fluctuations in postprandial fatty acid pools in older adults are required to fully explain the age-related differences in endogenous postprandial lipaemia.

4.4. CONCLUSIONS

Our data present differences in the dynamics and composition of older adults’ chylomicrons, and imply that chylomicrons do not contribute more greatly to postprandial lipaemic responses in the elderly than in the young. The influence on lipaemia of endogenous lipid dynamics in the postprandial period may contribute to age-related differences in composition observed here, but as yet remain incompletely explored in the elderly. Regardless, we have uncovered distinct postprandial differences in chylomicron size and TAG and PL composition that may correlate with metabolic health in the elderly. The origins and implications of these possible differences in dietary fatty acid absorption and/or clearance require further investigation in older adults.
CHAPTER 5

OLDER ADULTS HAVE AN ALTERED LIPAEMIC RESPONSE TO A LOW FAT MEAL
5.1. Preface

This chapter presents work investigating the lipaemic responses to a low fat meal in older adults. The postprandial lipaemic response is known to be exaggerated in the elderly following a high fat meal, but few studies have investigated the lipaemic responses to low fat loads in either younger or older populations. The current dietary recommendations encourage limiting dietary fat intake to promote cardiovascular and metabolic health; however, the postprandial implications of this type of meal are unknown in older adults. To investigate the effect of a low fat load on postprandial lipaemia in the elderly, we provided older and younger subjects with a representative low fat breakfast meal, with similar ingredients to meals used in previous literature (582, 665).

This analysis was done in collaboration with Adjunct Professor Kaisa Linderborg from Food Chemistry and Food Development, Department of Biochemistry, University of Turku, Turku, Finland, who developed the methods of lipidomic analysis of chylomicron samples used in this chapter.

The following section contains an analytical comparison of the compositional differences between the high fat and low fat meals, followed by analysis of the findings for the postprandial lipaemic effects of a low fat meal in older adults.
5.2. METHODS

5.2.1. LOW FAT MEAL STUDY DESIGN AND ANALYSIS

The intervention was conducted as described in Chapter 3, sections “3.2.3.1. Subjects” and “3.2.3.2. Study procedures”. Analytical and statistical methods were as described in Chapter 4, section “4.2.3. Methods” sections 4.2.3.3. through 4.2.3.9.

5.2.2. FOOD COMPOSITIONAL ANALYSIS

Food samples for nutrient analysis were homogenised using a commercial blender (Waring, NJ, USA) in triplicate. The HF meal was purchased fresh, and the LF meal was prepared as described in “2.3.4. Meals and preparation”. Distilled water (600g) was added to each meal preparation to facilitate homogenisation. Samples were aliquoted into microtubes and 50ml falcon tubes and stored at -20°C. Meal composition was analysed by Eurofins Scientific Auckland. Meal fatty acid analysis was performed as described in Chapter 4, section “4.2.3.7. Fatty acid composition analysis of meals and chylomicron-rich fraction triacylglycerols and phospholipids”.

5.2.3. STATISTICAL ANALYSES

Data are presented as means ± SEM. iAUC was calculated after subtracting fasting values. Fatty acid mass proportions, iAUC, and maximum peak times (Tmax) and concentrations (Cmax) between meals were compared using unpaired Student’s t test. Three-factor repeated-measures ANOVA (time, treatment, and age) was used for comparisons of apoB and cholesterol. Two-factor repeated-measures ANOVA (treatment and age) was used for iAUC, and Tmax and Cmax between meals and for all other measures (time and age). Sidak adjusted post hoc tests were used for all multiple comparisons. Where Mauchly’s sphericity test failed, the Huynh-Feldt correction was applied. Alpha was set at P<0.05. Heat maps were created using R software.

5.3. RESULTS

5.3.1. COMPOSITION DIFFERENCES BETWEEN HIGH AND LOW FAT MEALS

Although meal composition as analysed by Eurofins closely matched the nutrient panel information available, there were some differences, particularly for the HF meal (Table 14). Both meals measured as having more carbohydrates than anticipated; while protein closely matched the expected values for the low fat meal (50.9g) it measured as lower in the high fat meal (33.9g) than expected. Further, the high fat meal measured as having roughly half the quantity of total fat (32.8g) indicated in the nutrient panel information (62.2g).

The low and high fat meals differed in fatty acid composition in all measured fatty acids (P<0.001). The LF meal had greater proportions of oleic acid, linoleic acid and other lesser components as indicated in Table 15. All other fatty acids were found in lesser proportions in the LF meal.
Table 14: High and low fat meal food analysis per 100g and per serving.

<table>
<thead>
<tr>
<th>Measure</th>
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<th>Low fat</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Food analysis</td>
<td>NIP</td>
<td>Food analysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Per 100g</td>
<td>Per serve</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Fat</td>
<td>g/100g</td>
<td>3.23</td>
<td>32.8</td>
<td>1.18</td>
</tr>
<tr>
<td>Protein</td>
<td>g/100g</td>
<td>3.34</td>
<td>33.9</td>
<td>49.8</td>
</tr>
<tr>
<td>Sodium</td>
<td>mg/100g</td>
<td>192</td>
<td>1950</td>
<td>71.3</td>
</tr>
<tr>
<td>Ash</td>
<td>%m/m</td>
<td>0.87</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>g/100g</td>
<td>11.1</td>
<td>112.8</td>
<td>77.4</td>
</tr>
<tr>
<td>Sugar Profile</td>
<td>%m/m</td>
<td>0.07</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td></td>
<td>0.1</td>
<td>&lt;0.05</td>
<td>0.94</td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td>&lt;0.05</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>Lactose</td>
<td></td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Maltose</td>
<td></td>
<td>&lt;0.05</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td></td>
<td>&lt;0.05</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>Total Sugars</td>
<td></td>
<td>0.17</td>
<td>1.53</td>
<td></td>
</tr>
<tr>
<td>Fatty Acid Profile</td>
<td>g/100g</td>
<td>1.11</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>Saturated fat</td>
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<td>2.12</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>Unsaturated fat</td>
<td></td>
<td>1.77</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>Monounsaturated fat</td>
<td></td>
<td>0.35</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>Polyunsaturated fat</td>
<td></td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td></td>
</tr>
<tr>
<td>trans fat</td>
<td></td>
<td>0.6</td>
<td>&lt;0.5</td>
<td></td>
</tr>
<tr>
<td>16:0 (Palmitic acid)</td>
<td></td>
<td>1.6</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>18:1n−9 (cis-Oleic acid)</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unidentified fatty acids</td>
<td>&lt;0.5</td>
<td>0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Omega 3</td>
<td></td>
<td>8.4</td>
<td>12.4</td>
<td></td>
</tr>
<tr>
<td>Omega 6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1Meal weight including 600g distilled water.
2Food analysis as described in “5.2.2. Food compositional analysis”; NIP: nutrient information panel.
3Fatty acids not listed but with numerical names other than 16:0 and 18:1(n−9) were detected in quantities less than 0.5g/100g in either meal.
Table 15: Relative mass proportions of fatty acids in the low fat meal.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Mass Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>2.1 ± 0.02</td>
</tr>
<tr>
<td>15:0</td>
<td>0.2 ± 0.00</td>
</tr>
<tr>
<td>15:1</td>
<td>0.04 ± 0.00</td>
</tr>
<tr>
<td>16:0</td>
<td>15.6 ± 0.06</td>
</tr>
<tr>
<td>17:1</td>
<td>0.1 ± 0.00</td>
</tr>
<tr>
<td>18:0</td>
<td>4.1 ± 0.02</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>58.4 ± 0.1 †</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>10.8 ± 0.04 †</td>
</tr>
<tr>
<td>20:1n-9</td>
<td>1.5 ± 0.01 †</td>
</tr>
<tr>
<td>22:0</td>
<td>1.5 ± 0.01 †</td>
</tr>
<tr>
<td>24:0</td>
<td>1.0 ± 0.00 †</td>
</tr>
<tr>
<td>Others²</td>
<td>4.9 ± 0.23</td>
</tr>
</tbody>
</table>

¹Values represent mean % (g/100g) ± SEM, n=3. †: greater proportions in the low fat meal compared to the high fat meal compared with Student’s t test; P<0.05.
²Fatty acids with a mass proportion less than 1.0% are pooled to others category except for dairy derived fatty acids.

5.3.2. DIFFERENCES BETWEEN HIGH AND LOW FAT MEAL RESPONSES

A comparison of the glucose and insulin responses to the high and low fat meals is included in Chapter 3, section “3.3.2.1. Differences in glucose and insulin response between high and low fat meals”.

Comparison of the plasma TAG response between the two meals showed that the plasma TAG iAUC and T_max was greater after the HF meal than the LF meal (Figure 21A & C), and older adults had a greater T_max after either meal, indicating a later peak in older subjects’ plasma TAG regardless of meal fat content. Although the interaction of treatment and age for C_max was not significant (P=0.178), overall plasma TAG analysis of the LF meal indicated that older adults had higher concentrations in the later postprandial period (Figure 22A). Therefore, post hoc analysis of the C_max were performed which indicated that younger participants had lower C_max after the LF meal compared to the HF meal (P=0.030) while older participants had no meal differences in C_max (P=0.741).

Apolipoprotein B concentration was higher at baseline before the LF meal (treatment × time interaction of P=0.013; three-factor repeated-measures ANOVA, P=0.007 post hoc, data not shown), and lower at 4 & 5 hours (P=0.024 and P=0.012 respectively). While apoB concentrations increased at 2 hours, decreasing back to baseline values by 5 hours (P=0.003 and P=0.868 respectively), there were no significant changes over time after the LF meal except for a decrease from 3 to 5 hours (P=0.001).
Figure 21: Postprandial plasma triacylglycerol iAUC, C_{max}, and T_{max} in older and younger subjects after a high and low fat meal.

Postprandial plasma triacylglycerol (TAG) incremental area under the curve (iAUC) (A), TAG maximum concentration (C_{max}) (B), and TAG time of maximum concentration (T_{max}) (C) in older (■) and younger (□) subjects after a high (HF) and low fat (LF) meal. Values are presented as mean ± SEM for plasma TAG iAUC in arbitrary units (AU), TAG C_{max} in mmol/l, and TAG T_{max} in minutes. There were significant differences between the high fat and low fat meals in plasma TAG iAUC and T_{max} (treatment effect of P=0.013 and P=0.035 respectively; two-factor repeated-measures ANOVA) and between older and younger subjects in T_{max} (P<0.001). * P<0.05; *** P<0.001; #P>0.05.
The older subjects’ total cholesterol decreased from baseline throughout the postprandial period of each meal while younger subjects’ cholesterol remained stable (time × age interaction; P<0.001), slightly increasing between 4 & 5 hours (P=0.007; Table 13 in Chapter 4 & Table 16). Changes in postprandial cholesterol differed between meals (time × treatment interaction; P=0.037). While after the HF meal cholesterol changed between baseline and 3 & 4 hours (P=0.001 and P=0.002 respectively), after the LF meal changes occurred between baseline and 2 & 4 hours (P=0.001 and P<0.001 respectively). Additionally, the LF meal showed a time difference between 4 & 5 hours (P<0.001), attributable to an increase in the younger group’s cholesterol concentrations (P=0.007).

5.3.3. POSTPRANDIAL LIPAEMIA AFTER A LOW FAT MEAL IN OLDER ADULTS

Baseline subject characteristics, glycaemia and insulinaemia are included in Chapter 3, section “3.2.4.1. Subject characteristics”.

5.3.3.1. PLASMA AND CHYLOMICRON LIPID RESPONSES AFTER A LOW FAT MEAL

The older subjects reached peak plasma TAG concentration after 3 hours and TAGs remained elevated at 5 hours (P<0.001). The younger group reached peak TAG concentration by 3 hours (P=0.007), then declining back to baseline concentrations by 4 hours (P<0.001 time-age interaction, Figure 22A). Although the older subjects’ TAG elevation was prolonged, there was no difference in TAG iAUC between age groups. While chylomicron TAG response was similar to the plasma TAG response, this was not different groups (P=0.1; Figure 22B). Older participants tended to have smaller chylomicrons; however, neither chylomicron size nor apoB concentrations differed significantly between groups (P=0.097 and P=0.29 respectively; data not shown).

Younger subjects had lower NEFA concentrations after the LF meal (P=0.024, Figure 22C), characterised by continued NEFA suppression. Since baseline differences were absent, post hoc analysis was conducted to determine group differences, despite no time-age interaction. NEFA remained suppressed in the younger group (P>0.05). After initial suppression, the older group experienced increased NEFA concentrations between 1 & 3 hours (P=0.012) up to 5 hours (P<0.001), resulting in greater NEFA at 3 hours than younger subjects (P=0.04).

Table 16: Postprandial total cholesterol after low fat meal in older and younger subjects.

<table>
<thead>
<tr>
<th></th>
<th>Baseline (mmol/l)</th>
<th>% change from baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1h</td>
<td>2h</td>
</tr>
<tr>
<td>Younger subjects</td>
<td>4.0 ± 0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>(n=15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Older subjects</td>
<td>5.1 ± 0.2 ψ</td>
<td>-3.3</td>
</tr>
<tr>
<td>(n=15)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1Mean ± SEM total cholesterol.
2% change in total cholesterol within each group from baseline to each time point was calculated. Significance was determined by two-factor repeated-measures ANOVA with Sidak corrected post hoc analysis. Older subjects had lower cholesterol concentrations compared to baseline at all postprandial time points (ψ; P<0.01 compared with all other time points). All time point comparisons between age groups were significant to P<0.01.
Figure 22: Postprandial plasma and chylomicron lipid dynamics in younger and older subjects after the low fat meal.

Postprandial concentrations of plasma TAG (A), chylomicron TAG (B), and plasma NEFA (C) in older (●) and younger (○) subjects. Values represent means ± SEM (n=15/group). There were no significant differences in NEFA or chylomicron TAG responses between older and younger subjects. There were significant differences over time in the TAG response between older and younger subjects (age × time interaction of P<0.001, two-factor repeated-measures ANOVA). There was a significant main age difference in NEFA response between older and younger subjects (age effect marked (a) of P<0.05). *** P<0.001 change from baseline; ** P<0.01 change from baseline; α P<0.01 change from baseline older; β P<0.01 change from baseline younger; γ P<0.05 change from 5 hours younger; θ P<0.01 age difference at specified time point (Sidak corrected post hocs).
5.3.3.2. CHYLOMICRON TRIACYLGLYCEROL AND PHOSPHOLIPID FATTY ACID COMPOSITION AFTER A LOW FAT MEAL

5.3.3.2.1. CHYLOMICRON TRIACYLGLYCEROL COMPOSITION

Older participants had a lower proportion of PUFA chylomicron TAGs (P=0.014; Figure 23), characterised by lower proportions of linoleic acid (18:2n-6; P=0.002; Figure 24A). Dairy derived odd-chain fatty acids were proportionally higher in the older group’s chylomicron TAGs (P=0.01) particularly 17:1, 15:0 (P=0.009 and P=0.012 respectively; Figure 25A).

Regardless of time following the meal, the older group’s chylomicron TAGs had relatively more 22:1n-9 (P=0.011) and less 20:0, 20:1n-9, and 22:0 than the younger group’s (P=0.026, P<0.001, and P=0.02 respectively), with similar trends of lower proportions of 18:3n-3 and 20:4n-6 (P=0.078 and P=0.065 respectively). In both groups, the proportion of MUFA chylomicron TAGs was greater at 4 hours than at 2 hours (P=0.049), accompanied by increased proportions of 24:0, 22:0, and 22:1n-9 (P=0.041, P=0.024, and P=0.019 respectively). At 4 hours, chylomicron TAGs contained decreased proportions of 18:2n-6, 16:1n-7, 22:5, 20:3n-6 and (P=0.049, P=0.004, P=0.024, and P=0.046 respectively).

5.3.3.2.2. CHYLOMICRON PHOSPHOLIPID COMPOSITION

The older group’s chylomicron phospholipids contained greater proportions of MUFA (P=0.001; Figure 23B), particularly oleic acid (P=0.001). This was accompanied by proportionally lower proportions of arachidonic acid (20:4n-6; P=0.029; Figure 24B). Similarly to chylomicron TAGs, older subjects’ chylomicron phospholipids tended to contain relatively more dairy derived odd-chain fatty acids (P=0.055), specifically of 15:0 and 17:1 (P=0.001 and P=0.036 respectively; Figure 25B). The older group had greater proportions of the PUFA DPA (22:5; P=0.003) and lower proportions of 22:3 (P=0.024) than the younger group. In both groups, the SFA content of chylomicron phospholipids decreased at 4 hours (P<0.001).
The distribution changes of SFA, MUFA, and PUFA after the low fat meal in older and younger subjects. Values are presented as mean percent changes relative to younger subject 2 hour mass proportion for each saturation class (i.e. white represents a 0% change from younger 2 hour). Green hues represent negative percentage changes; red hues represent positive percentage changes.
Figure 24: Major chylomicron triacylglycerol and phospholipid composition of younger and older subjects after the low fat meal.

Major chylomicron triacylglycerol (A) and phospholipid (B) composition of younger subjects 2 hour (white bars) and 4 hour (light gray bars) and older subjects 2 hour (dark gray bars) and 4 hour (black bars) as mass proportions. Data marked (a) differ with age, data marked (t) differ with time; P<0.05 by two-factor repeated-measures ANOVA.
Figure 25: Dairy derived odd-chain fatty acid chylomicron triacylglycerol and phospholipid composition of younger and older subjects after the low fat meal.

Dairy derived odd-chain fatty acid chylomicron triacylglycerol (A) and phospholipid (B) composition of younger subjects 2 hour (white bars) and 4 hour (light gray bars) and older subjects 2 hour (dark gray bars) and 4 hour (black bars) as mass proportions. Data marked (a) differ with age; P<0.05 by two-factor repeated-measures ANOVA.
5.4. DISCUSSION

5.4.1. POSTPRANDIAL LIPAEMIC EFFECTS OF A LOW FAT MEAL IN OLDER ADULTS

Following a mixed low fat meal, older adults experience an exaggerated lipaemia with impaired NEFA suppression and chylomicrons containing lower PUFA and greater dairy derived odd-chain fatty acid proportions over a 5 hour postprandial period. These data highlight the impairment of endogenous lipid control in the postprandial period, largely independent of chylomicronaemia, as age-related differences in chylomicron rates of appearance or clearance were not apparent. Further, these data indicate that older adults may have differences in lipid absorption and/or clearance that depend on the relative proportions of fatty acids in a meal and their lipid structure.

Postprandial lipaemia has been well described after large fat doses, and results in exaggerated chylomicron and triacylglycerol responses in older adults after a fat challenge (14, 15, 188, 235, 446-448). However, few studies have measured the dose response to fat or examined lipaemia after a low fat load (688-692), despite the evidence that the magnitude of postprandial lipaemia is dose dependent (688). Additionally, altering the macronutrient content of a meal, such as the addition of protein to a fat load, has been shown to attenuate postprandial lipaemia in the elderly (15). As such, the lipaemic response to a low fat meal may well be subject to macronutrient influences on postprandial lipaemia, particularly for a meal where fat is not the primary constituent. Importantly, it remains unknown whether the lipaemic response to a low fat load is conserved in older adults, or what effect the current dietary recommendations towards reduced dietary fat intake (693) may have on acute lipid metabolism.

We have already confirmed that after a high fat meal, older adults have a greater postprandial TAG response, consistent with other reports (14, 15, 188, 235, 446-448). These data show that even with a low fat meal, older adults have an exaggerated and prolonged TAG response. In older adults, a low fat meal resulted in a comparable plasma TAG to that expected after a high fat meal, a phenomenon not observed in younger adults. Indeed, others have shown that meal fat loads of 15g or less did not result in postprandial lipaemia in healthy adults (692). However, our data suggests that older adults may still be susceptible to exaggerated postprandial lipaemia even after a low fat load.

Consistent with our findings after a high fat meal, these older adults did not experience a greater chylomicron TAG response, again indicating that chylomicrons are not the main contributors of postprandial hypertriglycerolaemia in older adults. Additionally, chylomicron size differences were less apparent than previously observed after a high fat meal, not surprising given the much lower fat content of the meal. However, animal studies have shown that chylomicron production rates remain constant, even in the fasted state (216), suggesting that apoB concentrations should be comparable between high and low fat meals. Yet, in the current study the same pattern of apoB variation was observed in both groups between the two meals, suggesting these
observations are not abnormal. Overall, chylomicronaemia did not differ with age after a low fat meal, and does not likely contribute to elevated postprandial lipoaemic responses in older adults.

Elevated lipoaemia, without chylomicronaemia, and inadequate suppression of NEFA, collectively implicate VLDL as a major contributor to exaggerated postprandial lipoaemia in older adults after a low fat meal. Inadequate suppression and spillover of NEFA have been described as part of the postprandial lipoaemic response in the elderly (235), and would contribute to impaired suppression of VLDL and chylomicron production (694). Indeed, postprandial triacylglycerolaemia in men with MetS has been characterised by higher postprandial production rates of both VLDL in addition to chylomicrons (215). Previously reported age-related differences in fat mass (19), fatty liver disease (20), or insulin resistance (21) could contribute to a similar amplified production of hepatically derived VLDL (276, 283) in these older adults. However, the older adults studied here showed no apparent signs of fatty liver disease or insulin insufficiency, although only indirect measures were employed. Furthermore, competition for apoB particle clearance (212) may contribute to older adults’ prolonged lipoaemia, originating from greater numbers of postprandial VLDL, but also increased basal LDL concentrations. Indeed, the postprandial decrease in total cholesterol we observed likely indicates enhanced LDL uptake (676), suggested postprandial removal of LDL rather than TRL in older adults. Notably, the younger subjects alternately displayed increases in postprandial total cholesterol. Additionally, greater decreases in non-fasting LDL has been observed in both T2DM (674) and MetS, with suggestions for a relationship to cardiometabolic dysfunction (675).

The mechanisms driving endogenous lipoprotein production after a low fat meal likely originate from age-related differences in insulin activity. Although insulin sensitivity was not different between age groups as measured by HOMA-IR, peripheral insulin resistance was not assessed. Insulin is known to inhibit both intestinal and hepatic TRL production (283, 694). Likewise, insulin is required for NEFA tissue uptake in older adults, and increase insulinemia has been proposed as the mechanism by which protein co-ingestion attenuates postprandial lipoaemia in the elderly (15). Hepatic and skeletal muscle insulin resistance would result in the increases in TRL production and elevated NEFA observed in these older subjects. Importantly, these findings suggest that insulin sensitivity may be a more important factor in controlling the postprandial lipoaemic response in older adults. This is particularly evident in the context of a low fat meal, where endogenous lipoprotein production appears to fuel elevated lipoaemia in the elderly.

Older adults had less chylomicron TAG PUFAs and more PL MUFAs than younger adults. These findings are consistent with our previous findings after a high fat meal, and match other reports of longer-term compositional differences in the elderly (461, 464, 465). However, some compositional differences we previously reported were not evident after a low fat meal, notably greater oleic acid in chylomicron TAGs despite a greater oleic acid content of the low fat meal. This supports a transient postprandial nature of age-related compositional differences, and implicating the participation of additional factors in determining chylomicron fatty acid content. While this may be explained by meal composition including the stereospecific positioning (603) and fat physical properties of dietary lipids, other factors such as endogenous lipid pools, or alterations in clearance times may also participate.
5.4.2. **ODD-CHAIN FATTY ACID VARIATION SIGNIFICANCE**

These data show that older adults have greater proportions of dairy derived odd-chain fatty acids in chylomicron TAGs, which tends to be conserved in the PL fraction. These findings mirror our observations after a high fat meal, and may be more strongly supported after the low fat meal due to the relatively greater contribution of dairy products to the low fat meal; however, the measured proportions of the dairy derived fatty acids in the low fat meal indicated these were in fact less abundant in the low fat meal compared to the high fat meal. This suggests that differences other than relative meal availability of these fatty acids, and may point to endogenous origin of these fatty acids rather than dietary, which would further support their prevalence in chylomicron phospholipids. Regardless of their origin, greater proportions of these fatty acids in circulation have been inversely associated with metabolic and cardiovascular disease, however the exact relationship between these factors has not been fully elucidated (695).

The greater proportion of odd-chain fatty acids in these older adults may result from age-related differences in exposure to these fatty acids in the diet and bioaccumulation. Odd-chain fatty acids are typically synthesised by bacteria (696) or yeast (697). Dairy and beef products contain these odd-chain fatty acids as a by-product of their gut microbiota (696). Although these fatty acids in human circulation or adipose are good indicators of dairy consumption implying their origins are mainly from dairy products (698), their relative proportions do not typically match up (699), suggesting other food products (700) or endogenous production (695) may be relevant sources of these fatty acids. Species of candida are used as protein sources in animal feeds (700, 701), and contain substantially high proportions of odd-chain fatty acids (e.g. 17:1 represents 27% of fatty acids in *Candida lipolytica* (697)). Bioaccumulation of candida derived odd-chain fatty acids occurs in animals and animal products raised on these products (e.g. poultry and eggs) (700), and has been reported in a dose-wise fashion in rats (697). Adipose tissue from rats fed for 3 months on diets consisting of 15% or 50% *Candida lipolytica* showed proportions of 17:1 of 1.5 and 5.8% respectively, compared to 0.3% in controls (697). Furthermore, odd-chain fatty acids may be less preferentially oxidised compared with even numbers fatty acids (702), contributing to their bioaccumulation; however this is inconsistently reported (697). Therefore, older adults may have greater lifetime exposure to these fatty acids from dairy, other animal products containing odd-chain fatty acids, or *de novo* synthesis.

A recent study reported that odd-chain fatty acids appearing in NEFA are highly correlated with the odd-chain fatty acid content of visceral and subcutaneous adipose tissue (703). The greater postprandial concentrations of NEFA in older adults (particularly at 4 hours) could offer some explanation of age-related differences in chylomicron odd-chain fatty acid content. If older adults have greater proportions of odd-chain fatty acids in adipose, these may appear at higher concentrations postprandially in NEFA, and be transferred to chylomicrons or appear in endogenous TRL. Although the current study did not evaluate such a phenomenon, the age differences in these potentially bioaccumulated fatty acids further supports an elevated role of endogenous TRL production in older adults, and after lower postprandial availability of exogenous fatty acids.

Importantly, these compositional differences in circulating fatty acids, whether absorbed in chylomicrons, stored in adipose, or released postprandially by VLDL secretion, may have metabolic implications. Greater
circulating proportions of dairy derived odd-chain fatty acids (17:1), as observed in our older adults, could be protective against the development of T2DM in this population. Adipose tissue accumulation of odd-chain fatty acids (particularly 15:0 & 17:0) are indicative of habitual dairy intakes (698), and higher dairy consumption is linked to decreased T2DM risk (557). Furthermore, higher circulating proportions of 15:0 and 17:0 have been found in non-diabetic subjects when compared to diabetic subjects (558). Greater uptake or storage of these fatty acids may be a protective adaptation towards decreased T2DM risk of older adults, or more specifically, of the healthy, non-metabolically compromised older adults included in this trial.

5.4.3. COMPARISON OF MEAL COMPOSITION AND LIPAEMIC RESPONSES BETWEEN HIGH AND LOW FAT MEALS

While the nutrient panel information taken from all food products was not expected to be fully accurate, the macronutrient breakdown of the HF meal was surprisingly very different from expected values. With more than 45% more carbohydrate, and 32% less protein than expected, the HF meal appears to not have been particularly well protein-carbohydrate matched to the LF meal. Although variation between restaurant meal preparations is expected and could explain the source macronutrient discrepancies between the measured proportions and the nutrient panel proportions, these findings were still surprising given the level of standardisation in fast food chain meal preparation. The total fat content of the HF meal was measured as only 50% of the nutrient panel values; although this could have decreased the total lipaemia experienced by subjects after eating the meal, it is unlikely that the differences from expected amount of fat would account for the age differences in lipaemia observed here.

Macronutrient differences between meals as well as variation in food structure likely contributed to differences in lipaemia, and may have differently affected younger and older subjects. Carbohydrate (256, 552) is known to delay the postprandial lipaemic response while protein (15, 555, 704) and fibre (552, 553) attenuate postprandial lipaemia. While meals were designed to be protein and carbohydrate matched, food analysis suggests that the HF meal had less fat and protein, and more carbohydrate than predicted. While meal differences in fat content undoubtedly contributed to differences postprandial lipaemia, the dissimilar macronutrient composition and food structures could have caused relatively greater glycaemic and insulinaemic responses to the HF meal, altered gastric transit, and impaired attenuation of lipaemia by protein co-ingestion.

The glycaemic index or carbohydrate composition of a meal has been shown to affect TRL production. Chylomicron production is greater after a higher glycaemic index meal (705), which may have contributed to greater chylomicron production after the HF compared to the LF meal. Meal differences in the proportions of monosaccharides may have influenced TRL production, since monosaccharides show differential effects on TRL formation. Fructose, but not glucose, stimulates apoB-100 formation, while both glucose and fructose stimulate apoB-48 production (706). This is likely due to the inability of fructose to stimulate insulin secretion, as this mechanism is responsible for glucose suppression of hepatic apoB production (707). Therefore, differences in carbohydrate quality may have influenced both exogenous and endogenous TRL production; however, as these were not measured directly, age differences in endogenous TRL production are unknown. Furthermore, given that
subjects all consumed identical meals, the current data suggest that older adults are more sensitive to the glycaemic and insulinaemic effects of meal composition and structure.

Older subjects consistently demonstrated postprandial decreases in total cholesterol regardless of meal composition, in contrast to younger adults. These decreases seem to be indicative of preferential LDL uptake (676) and postprandial lipoprotein TAG transfer (678), and may be characteristic of metabolic dysfunction (674, 675). In young healthy adults, variations in meal cholesterol content have been shown to impact changes in non-fasted cholesterol concentrations (708), which could explain why these younger adults showed no decrease in total cholesterol following the lower cholesterol low fat meal. Furthermore, this suggests that older adults are less responsive to changes in meal composition with regards to postprandial lipoprotein dynamics, and further implicates resistance of responsiveness to endogenous lipid metabolism regulation.

Furthermore, any meal effects on gastric emptying may have impacted timing comparisons between the two meals. This has implications for the comparison of chylomicron fatty acid composition, and inferences surrounding production or clearance of TRL. Meal differences in transit time could be a possible explanation for the lack of significant oleic acid age differences after the LF meal. Above all, these findings suggest that older adults’ postprandial lipaemic response is more sensitive to alterations in meal composition and structure.

5.5. CONCLUSIONS

We have shown that older adults display disturbed postprandial lipaemia, even after a low fat meal, characterised by altered TAG and PL composition and overproduction of endogenous TRL causing exaggerated and prolonged lipaemia. As fat was a minor component of this low fat mixed meal, other macronutrients, namely carbohydrate, were the probable culprits instigating endogenous dyslipidaemia. Importantly this suggests that adjusting the fat content of a meal may be a poor strategy for controlling exaggerated postprandial lipaemia in the elderly. Future studies need to carefully determine the contributions of endogenous lipoproteins to the postprandial lipaemic response, and identify ways of attenuating this response. Furthermore, the health implications of reduced linoleic acid and increases dairy odd-chain fatty acid proportions in the TRL of older adults require explanation in the context of well-being in the elderly.
CHAPTER 6

OLDER ADULTS DO NOT HAVE A GREATER POSTPRANDIAL INFLAMMATORY RESPONSE
6.1. PREFACE

This chapter presents the work done to investigate the postprandial immune responses to a high fat meal in older adults. There is evidence that high fat meals, in addition to initiating a state of lipaemia, also provoke a post-meal inflammatory response, particularly after meals high in saturated fat. Moreover, this postprandial inflammation does not occur after a carbohydrate-only bolus, indicating the integral role of lipaemia in the inflammatory pathways. Recently, the postprandial inflammatory role of the transient postprandial lipoprotein, the chylomicron, has been elucidated: chylomicron formation/secretion is essential to the bacterial translocation of the endotoxin lipopolysaccharide, which crosses the gut barrier after a high fat meal and directly initiates an immune response.

Despite evidence that postprandial lipaemia is exaggerated in older adults and the known connection between lipaemia and postprandial inflammation, this response has surprisingly not been investigated previously in older adults. Furthermore, the prevalence of metabolic disturbances associated with ageing (i.e. insulin resistance, lipaemia, chronic low-grade inflammation, etc.), provoke questions about whether the aggravated postprandial inflammation observed in metabolically compromised individuals may also be characteristic of ageing.

The data included in the publication describe the findings for the endotoxaemic, inflammatory, and oxidative stress responses to a high fat breakfast meal. Exploratory data investigating other potential contributors to the postprandial inflammatory response are included and discussed separately in this chapter.

The following section contains the manuscript “Acute postprandial endotoxaemic and inflammatory responses to a high fat meal are not altered in the healthy elderly”, co-authored by Amber M. Milan, Shikha Pundir, Chantal A. Pileggi, James F. Markworth, and David Cameron-Smith. We plan to submit this article to the journal Rejuvenation Research. Rejuvenation Research is published by Mary Ann Liebert with a 2013/2014 impact factor of 3.931 and a five year impact factor of 2.57.
6.2. MANUSCRIPT:

ACUTE POSTPRANDIAL ENDOTOXAEMIC AND INFLAMMATORY RESPONSES TO A HIGH FAT MEAL ARE NOT ALTERED IN THE HEALTHY ELDERLY

6.2.1. ABSTRACT

Postprandial inflammation and endotoxaemia are determinants of cardiovascular and metabolic disease risk and are amplified by high fat meals. We aimed to examine determinants of postprandial inflammation and endotoxaemia in older and younger adults following a high fat mixed meal. In a randomised cross-over trial, healthy participants aged 20-25 and 60-75 years (n=15/group) consumed a high fat breakfast and a low fat breakfast. Plasma taken at baseline and post-meal for 5 hours was analysed for circulating endotoxin, cytokines (MCP-1, IL-1β, IL-6, and TNF-α), lipopolysaccharide binding protein (LBP), and expression of inflammatory genes in peripheral blood mononuclear cells (PBMC). Older subjects had lower baseline PBMC expression of GPX-1 but greater IGFBP3 and circulating MCP-1 compared to younger subjects. After either meal, there were no age differences in plasma or chylomicron endotoxin or plasma LBP concentrations, nor in inflammatory cytokine gene and protein expression (MCP-1, IL-1β, and TNF-α). Unlike younger participants, the older group had decreased SOD-2 expression after the meals. After a high fat meal, older adults have no increased inflammatory or endotoxin response, but an altered oxidative stress response in comparison with younger adults. Healthy older adults, without apparent metabolic dysfunction, have a comparable postprandial inflammatory and endotoxaemia response to younger adults.

6.2.2. INTRODUCTION

Ageing is associated with dysfunction of the maintenance of cardiovascular and metabolic health, likely aggravated by the current Western lifestyle resulting in declining health and increased chronic disease risk. Low-grade inflammation is associated with ageing and contributes to morbidity and mortality (709). The immunosenescence associated with ageing (95) manifests as an elevated immune status (90) along with insufficient immune activation after a challenge (710). Evidence is mounting in support of chronic low-grade inflammation as a factor responsible in the development of insulin resistance and Type 2 Diabetes Mellitus (T2DM) (37), along with other chronic illnesses such as cardiovascular disease or cancers (84) and overall as detrimental to healthy ageing (711). Moreover, oxidative stress and inflammation contribute directly to the cellular senescence that helps perpetuate the condition of immune decline in ageing (83, 530).

Acute inflammatory responses can be initiated by meals, particularly those high in fat. The mechanisms of these postprandial inflammatory responses are highly reliant on the lipaemia caused by increased chylomicron formation and triacylglycerol (TAG) content. Chylomicron and TAG adherence to (93) and activation of
monocytes (287) provokes an acute immune response. Additionally, bacterial translocation across the gut, facilitated by fat absorption and chylomicron formation, triggers an immune response, manifesting as postprandial endotoxaemia (327). The corresponding postprandial inflammatory state may further contribute to a systemic low-grade inflammation (94), already typically prevalent in ageing populations (83).

These postprandial lipaemic and inflammatory responses are already documented as elevated in metabolically compromised adults, such as those displaying insulin resistance or T2DM (272, 377, 526, 527), conditions found more prevalently in older adults (21, 528). Postprandial endotoxaemia is reliant on the formation of triacylglycerol-rich lipoproteins (TRL) (324, 535, 536), is evidently elevated in T2DM subjects (538), and is associated with increased intestinal permeability (537). Ageing has sometimes been associated with digestive differences (175, 176), changes to the gut microbiota (178), or gut barrier function (173) which could therefore contribute to elevated postprandial endotoxaemia after a high fat meal (501, 712). Furthermore, the exaggerated lipaemic response to high fat meals seen in older adults (14, 15, 188, 235, 446-448), including prolonged appearance (188, 446) and overproduction of TRL, would likely contribute to greater postprandial endotoxaemia and inflammation in this population. Despite this evidence, studies examining the postprandial inflammatory responses of healthy older adults have yet to be conducted. Therefore, the aim of this study was to investigate the postprandial inflammatory response to a high fat mixed meal in older adults compared to younger adults. It was hypothesised that older adults would have greater postprandial endotoxaemia and inflammation, characterised by greater TRL transport of endotoxin and an exaggerated immune and oxidative stress response.

6.2.3. METHODS

6.2.3.1. SUBJECT SELECTION

Thirty healthy, community dwelling subjects (n=7 young females, n=8 young males, n=9 older females, n=6 older males) from the Auckland region were recruited to participate. Eligible subjects were required to have a body mass index (BMI) between 18 and 30kg/m² and be between the ages of 20-25 years and 60-75 years. Individuals with a history of cardiovascular or metabolic disease/conditions, or who used medications that may interfere with study endpoints (i.e. anti-inflammatory drugs, statin drugs) were not eligible for participation.

All subjects gave written informed consent and the study was approved by the University of Auckland Human Participants and Ethics Committee (Ref # 8026). This study was registered with the Australian New Zealand Clinical Trials Registry (ID: ACTRN12612000515897).

6.2.3.2. STUDY DESIGN AND TREATMENTS

In a randomised cross-over design, subjects received two test meals in a random sequence and served as his/her own control. The high fat meal (HF) was chosen as a standard test meal with a high fat and protein load used previously to induce a postprandial inflammatory response (582) and was purchased from McDonald’s Restaurants in Auckland Central. The low fat meal (LF) was protein and carbohydrate matched to the HF meal.
based on standardly available nutritional information (Table 17) and was designed to follow the Australian Guide to Healthy Eating, while maintaining a low fat load.

6.2.3.3. STUDY PROCEDURES

Subjects were asked to abstain from vigorous physical activity, high fat foods, and anti-inflammatory medications and supplements the day prior to their visit. Subjects arrived fasted on two separate occasions a minimum of 14 days apart. Anthropometric data were collected before a catheter was inserted into an antecubital vein and a baseline sample (time 0) was taken followed by consumption of the test breakfast. Blood samples were collected post-meal blood collection tubes (Becton Dickinson, NJ, USA) hourly for 5 hours for serum and EDTA plasma. Serum tubes were allowed to clot for 15 minutes at room temperature before serum and plasma tubes were centrifuged at 1500 × g for 15 minutes at 4°C and the supernatants collected in pyrogen-free microtubes and stored at -20°C until analysis. Plasma supernatants were processed under laminar flow using pyrogen-free consumables. An aliquot of plasma was kept at 4°C for chylomicron separation within 6 hours and the remaining plasma was collected in pyrogen-free microtubes and stored at -20°C.

6.2.3.4. CHYLOMICRON ISOLATION

Chylomicron and lipoprotein separations were performed using gamma irradiated 4.7ml OptiSeal tubes (Beckman Coulter, CA, USA) in an Optima MAX-XP ultracentrifuge using a TLA-110 rotor with methods adapted from Oikawa et al. (597). Density gradient solutions were prepared with 0.005% EDTA using pyrogen-free water according to Naito (599) and separation protocols were based on those of Kupke and Wörz-Zeugner (600). Chylomicrons were separated by overlaying 3.5ml plasma with 1.2ml saline solution (d=1.006g/ml) and centrifuging at 117 000 × g for 10 minutes. The visible chylomicron top layer was aspirated into pyrogen-free microtubes and corrected to a final collection volume of 1.4ml using pyrogen-free saline solution. This provided a standardised dilution factor of the collected chylomicron layer volume relative to initial plasma volume. Chylomicron fractions were stored at -80°C.

6.2.3.5. PBMC ISOLATION AND RNA EXTRACTION

2ml whole blood, collected from EDTA blood collection tubes (Becton Dickinson) at 0, 2, & 4 hours, was layered over 2 ml of Histopaque solution (Sigma-Aldrich, MO, USA) and centrifuged for 30 minutes at 400 × g at room temperature. PBMCs were removed from the interface and washed twice with PBS. The PBMC pellet was lysed with 600µl of RNA extraction lysis buffer (Purelink RNA Mini Kit) and frozen at -80°C. RNA was isolated as per manufacturer’s protocol using a Purelink RNA Mini Kit (Life Technologies, CA, USA). Briefly, DNA contamination was eliminated by DNase digestion from the cell lysate prior to RNA isolation using the RNA Mini Kit spin columns. RNA concentration was measured using a NanoDrop (ND-1000 Spectrophotometer, Thermo Scientific, MA, USA). 125-250ng RNA was reverse transcribed using High Capacity RNA-to-cDNA Kit (Life Technologies) as per manufacturer’s instructions.
Table 17: Macronutrient composition of high and low fat breakfasts (Table 1 in article).

<table>
<thead>
<tr>
<th>Item name</th>
<th>Weight (g)</th>
<th>Carbohydrates (g)</th>
<th>Fat (g)</th>
<th>Protein (g)</th>
<th>Energy (kJ)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High fat breakfast</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sausage and Egg Muffin Sandwich (×2)</td>
<td>162</td>
<td>25.2</td>
<td>21</td>
<td>23.4</td>
<td>1630</td>
</tr>
<tr>
<td>Hash Brown (×2)</td>
<td>56</td>
<td>13.5</td>
<td>10.1</td>
<td>1.5</td>
<td>630</td>
</tr>
<tr>
<td>Total</td>
<td>77.4</td>
<td>62.2</td>
<td>49.8</td>
<td>4520</td>
<td></td>
</tr>
<tr>
<td><strong>Low fat breakfast</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rolled Oats</td>
<td>37</td>
<td>20.8</td>
<td>1.9</td>
<td>5.0</td>
<td>500</td>
</tr>
<tr>
<td>1% Cottage Cheese</td>
<td>167</td>
<td>4.5</td>
<td>1.0</td>
<td>19.7</td>
<td>460</td>
</tr>
<tr>
<td>Mixed Grain Bread</td>
<td>42</td>
<td>11.2</td>
<td>2.2</td>
<td>5.1</td>
<td>380</td>
</tr>
<tr>
<td>Reduced Fat Peanut Butter, Smooth</td>
<td>25</td>
<td>8.4</td>
<td>9.4</td>
<td>4.4</td>
<td>580</td>
</tr>
<tr>
<td>Fresh Peach</td>
<td>154</td>
<td>14.6</td>
<td>0.3</td>
<td>1.4</td>
<td>250</td>
</tr>
<tr>
<td>Trim Milk</td>
<td>365</td>
<td>17.9</td>
<td>1.8</td>
<td>14.2</td>
<td>630</td>
</tr>
<tr>
<td>Total</td>
<td>77.4</td>
<td>16.6</td>
<td>49.8</td>
<td>2800</td>
<td></td>
</tr>
</tbody>
</table>

*Values presented are based on nutrient panel data obtained from the website of the fast food restaurant and the low fat breakfast items.*
6.2.3.6. **QUANTITATIVE REAL-TIME REVERSE-TRANScriPTASE POLYMERASE CHAIN REACTION ANALYSIS**

Quantitative polymerase chain reaction was performed using a LightCycler 480 (Roche Applied Science, Penzberg, Germany) using SYBR Green I DNA-binding dye. The geometric mean of human β-Actin, GAPDH, and RNA18S genes was used as an endogenous control. Primers (Table 19) were obtained from Invitrogen (Life Technologies). Samples were run in duplicate 10µl reaction volumes with 1.25ng cDNA per reaction. Results obtained as crossing point (Cp) values were used to calculate absolute gene expression using the $2^{-\Delta\Delta\text{Cp}}$ method (713).

6.2.3.7. **BIOCHEMICAL ANALYSIS**

Biochemical measures of baseline plasma cholesterol, total LDL and HDL, and postprandial triacylglycerols, glucose, and serum C-reactive protein (CRP) were carried out using a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan) by enzymatic colorimetric assay (Roche, Mannheim, Germany). Postprandial plasma insulin was measured using an Abbott AxSYM system (Abbott Laboratories, Abbott Park, USA) by microparticle enzyme immunoassay. Plasma lipopolysaccharide binding protein (LBP) was assessed using a commercially available ELISA kit for quantification of human LBP (Abnova, Jhongli, Taiwan) at 0, 1, 2, & 3 hours only, based on the postprandial responses reported by others (582). Plasma inflammatory markers (n=7 younger, n=6 older) including tumour necrosis factor-alpha (TNF-α), monocyte chemotactic protein-1 (MCP-1), interleukin-1β (IL-1β), and interleukin-6 (IL-6) were analysed using a flow cytometric multiplex array (Milliplex MAP Kit Human Cytokine Magnetic Bead Panel Assay, Millipore, Mo, USA) after the HF breakfast only, at 0, 2, & 4 hour time points only matching with postprandial RNA analysis time points.

6.2.3.8. **ENDOTOXIN ANALYSIS**

Plasma and chylomicron endotoxin concentrations were determined (n=12/group) using the Kinetic-QCL chromogenic Limulus Amebocyte Lysate (LAL) assay (Lonza, TN, USA) as per manufacturer’s instructions. Chylomicron samples were diluted 1:100 with LAL reagent water (LRW) and heat inactivated at 70°C for 10 minutes. Plasma samples were acid treated to remove inhibitory plasma proteins as described by Ketchum and Novitsky (613) in reagents prepared in LRW. In brief, samples were treated with 1.32N nitric acid, heat treated at 37°C for 5 minutes and centrifuged at 1500 × g for 5 minutes. The supernatant was removed and neutralised with 0.55N NaOH before dilution to 1:100 with LRW. The endotoxin concentration was expressed as endotoxin units (EU)/ml.

6.2.3.9. **STATISTICAL ANALYSES**

Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated from fasting glucose and insulin concentrations using the equation from Matthews et al. (615). Statistical analyses were conducted with SPSS (IBM, version 21). Data are presented as means ± SEMs. Baseline concentrations were compared using two-way analysis of variance (ANOVA). CRP and inflammatory cytokine protein expression were compared using two-factor repeated-measures ANOVA while three-factor repeated-measures ANOVA was used for all other analyses.
Sidak post hoc tests were used for all multiple comparisons between groups. Where Mauchly’s sphericity test failed, the Huynh-Feldt correction was applied. Alpha was set at $P<0.05$.

6.2.4. RESULTS

6.2.4.1. SUBJECT CHARACTERISTICS

There were no main age differences for fasting measurements of BMI, plasma glucose, insulin, triacylglycerols or HOMA-IR. Older subjects had higher fasting total cholesterol, LDL and HDL ($P<0.001$, $P=0.008$, and $P<0.001$ respectively; Table 18). Glucose concentrations changed similarly between age groups after meal ingestion, and were more elevated from 2 hours onward after the HF breakfast than the LF ($P=0.043$) despite the equal carbohydrate content of the two meals. Differences in postprandial lipaemia are reported elsewhere (Chapter 4). In brief, older participants showed exaggerated and prolonged postprandial elevations of TAG.

6.2.4.2. POSTPRANDIAL ENDOTOXAEARIA

Plasma endotoxin concentration did not differ in the postprandial period (Figure 26A). Postprandial endotoxaemia tended to be greater after the HF breakfast ($P=0.156$), with no evident impact of age ($P=0.84$). Although endotoxaemia did not differ with age, older subjects tended to have prolonged endotoxaemia after 3 hours ($P=0.059$) while the younger group did not. Chylomicron endotoxin concentration tended to differ between breakfast treatments ($P=0.061$) and treatment differences tended to depend on time ($P=0.097$; Figure 26B). Chylomicron endotoxaemia after the HF breakfast varied more greatly between subjects, and post hoc analysis revealed a tendency towards greater differences at baseline between study visits, and at 1 & 4 hours after the meal ($P=0.064$, $P=0.052$, and $P=0.133$ respectively). Changes in LBP concentration differed between treatment days and age groups ($P=0.013$, Figure 26C). Older subjects had greater LBP concentrations at baseline and 1 hour during the LF challenge than during the HF breakfast ($P=0.008$ and $0.036$ respectively). This corresponded with higher LBP concentrations in the older group at baseline and 1 hour during the LF challenge ($P=0.017$ and $P=0.024$ respectively). With the higher baseline concentration in older participants before the LF meal, the older group experienced a significant decrease in LBP concentration by 2 hours post-meal (baseline vs. 2 hours; $P=0.016$). The younger group had higher concentrations of LBP at 3 hours after the LF than after the HF breakfast.

6.2.4.3. PBMC SENESCENCE RNA EXPRESSION

IGFBP3 expression was greater in the older group ($P=0.001$) and tended to be higher before the LF meal ($P=0.073$) causing a significant time-treatment interaction ($P=0.005$; Figure 27A). BAK1 expression was no different between groups and did not differ with feeding (Figure 27B). Both groups had increased CD40LG expression at 4 hours after both meals ($P=0.002$; Figure 27C).
Table 18: Baseline subject characteristics before high and low fat meals (Table 2 in article).

<table>
<thead>
<tr>
<th>Measure</th>
<th>unit</th>
<th>Younger subjects (n=15)</th>
<th>Older subjects (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>years</td>
<td>22.7 ± 0.4</td>
<td>67.3 ± 1.5***</td>
</tr>
<tr>
<td>BMI</td>
<td>kg/m²</td>
<td>23.7 ± 0.8</td>
<td>24.4 ± 1.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>mmol/l</td>
<td>5.1 ± 0.1</td>
<td>5.2 ± 0.1</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td></td>
<td>2.1 ± 0.2</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>mmol/l</td>
<td>4.0 ± 0.1</td>
<td>5.0 ± 0.1***</td>
</tr>
<tr>
<td>LDL</td>
<td>mmol/l</td>
<td>2.5 ± 0.1</td>
<td>3.0 ± 0.1**</td>
</tr>
<tr>
<td>HDL</td>
<td>mmol/l</td>
<td>1.3 ± 0.0</td>
<td>1.8 ± 0.1***</td>
</tr>
<tr>
<td>TAG</td>
<td>mmol/l</td>
<td>0.8 ± 0.0</td>
<td>0.9 ± 0.0</td>
</tr>
<tr>
<td>Insulin</td>
<td>µU/ml</td>
<td>9.2 ± 0.8</td>
<td>8.7 ± 1.2</td>
</tr>
</tbody>
</table>

1BMI: body mass index; HOMA-IR: homeostatic model assessment of insulin resistance; TAG: triacylglycerol.
2Values presented as means ± SEM over both treatments.
3Main effects and interactions analysed by two-factor repeated-measures ANOVA (treatment and age). There were no differences between group baseline values between treatment days; ***P<0.001, **P<0.01 compared with younger subjects.
Older high fat (filled square) older low fat (open square) younger high fat (filled circle) younger low fat (open circle). Values represent mean ± SEM in EU/ml for plasma endotoxin (A; n=12/group) and chylomicron endotoxin (B; n=12/group) and in µg/ml for LBP (C; n=15/group). There were no differences in plasma or chylomicron endotoxin responses between older and younger subjects. There were significant differences in the LBP response over time dependent on age and treatment (age × time × treatment interaction of P<0.05, three-factor repeated-measures ANOVA). ε P<0.05 age difference after LF; § P<0.05 treatment difference in older subjects; ϕ P<0.05 treatment difference in younger subjects; † P<0.05 time difference after LF in older subjects (Sidak corrected post hoc).
6.2.4.4. PBMC RNA EXPRESSION OF ENDOTOXAEMIC ACTIVATION

Younger subjects had decreased CD14 expression at 4 hours compared to 2 hours (P=0.005; Figure 27D) while older subjects showed no changes after meal ingestion. TLR2 response was not different between groups after either meal but was higher at 2 hours than 4 hours (P=0.002; Figure 27E). TLR9 expression tended to be lower overall in older participants (P=0.055; Figure 27F).

6.2.4.5. PBMC RNA EXPRESSION OF ACTIVATION BY LIPOPROTEINS

No change in ABCA-1 expression was observed after either breakfast meal in either age group (Figure 27G). ApoB48r expression tended to be higher in older participants (P=0.111), higher after the LF meal (P=0.087) and higher at baseline (P=0.15; Figure 27H). The older group had lower LDLr expression (P=0.038; Figure 27I).

6.2.4.6. PBMC INFLAMMATORY CYTOKINE GENE EXPRESSION

IL-1β expression changed after meal ingestion (P=0.03) and tended to decrease compared to baseline (P=0.107). MCP-1 expression did not change after meal ingestion and the response was similar between age groups (Figure 28A & B). TNF-α expression increased over time (P=0.033; Figure 28C), but was not different between breakfasts or age groups.

6.2.4.7. PBMC RNA EXPRESSION OF OXIDATIVE STRESS RESPONSE

Younger subjects had higher GPX-1 expression (P=0.014; Figure 28D) however this did not change after either meal. SOD-2 expression decreased at 2 & 4 hours after meal ingestion in the older group, but not the younger group (P<0.001; Figure 28E). For this reason, older subjects had lower SOD-2 expression at 2 & 4 hours compared to younger subjects (P=0.044 and P=0.004 respectively).

6.2.4.8. CYTOKINE PROTEIN EXPRESSION

There were no differences between age groups in postprandial concentrations of CRP (P=0.84, Figure 29A), IL-1β (data not shown), IL-6, and TNF-α (Figure 29B & D; P=0.111 and P=0.112 respectively), although IL-6 and TNF-α tended to be higher in older participants. Cytokine concentrations measured for IL-1β and IL-6 were very low with reference to the standard curve, and IL-6 concentrations increased between baseline and 4 hours in both age groups (P=0.007). MCP-1 concentration was higher in the older group, and unchanged with meal ingestion (P=0.028, Figure 29C).
Figure 27: Relative senescence-related, endotoxaemia-related and lipid receptor peripheral blood mononuclear cells (PBMC) gene expression responses to high fat (HF) and low fat (LF) breakfasts in older and younger subjects (Figure 2 in article).

Older high fat (filled squares), older low fat (open squares), younger high fat (filled circles), younger low fat (open circles). Values represent mean ± SEM (n=15/group) in relative fluorescent units (RFU) for IGFBP3 (A), BAK1 (B), CD40LG (C), CD14 (D), TLR2 (E), TLR9 (F), ABCA-1 (G), ApoB48r (H), and LDLr (I) respectively. There were no differences in BAK1 TLR2, TLR9, ABCA-1, or ApoB48r responses between older and younger subjects. There were significant age differences in LDLr gene expression (age effect of P<0.05) and changes over time for CD40LG and TLR2 (time effect of P<0.05 and P<0.01 respectively). There were significant age differences and time differences dependent on treatment in the IGFBP3 response (time × treatment interaction of P<0.01, age effect of P<0.01, three-factor repeated-measures ANOVA). CD14 expression was significantly different between older and younger subjects over time (time × age interaction of P<0.05), a P<0.05 main age difference; ** P<0.01 change from baseline; λ P<0.05 time difference in younger subjects; γ P<0.01 time difference (Sidak corrected post hocs).
Figure 28: Relative inflammatory cytokine and oxidative stress peripheral blood mononuclear cells (PBMC) gene expression responses to high fat (HF) and low fat (LF) breakfasts in older and younger subjects (Figure 3 in article).

Older high fat (filled squares), older low fat (open squares), younger high fat (filled circles), younger low fat (open circles). Values represent mean ± SEM (n=15/group) in relative fluorescent units (RFU) for IL-1β (A), MCP-1 (B), TNF-α (C), GPX-1 (D), and SOD-2 (E) respectively. There were no differences in IL-1β, MCP-1, or TNF-α, responses between older and younger subjects. There were significant age differences in GPX-1 gene expression (age effect of P<0.05) and changes over time for IL-1β and TNF-α (time effect of P<0.05 each respectively). The SOD-2 response differed over time dependent on age and treatment (age × time × treatment interaction of P<0.05, three-factor repeated-measures ANOVA). * P<0.05 main age difference; ε P<0.05 change from baseline; § P<0.05 age difference after LF; ¶ P<0.05 treatment difference in older subjects; ‡ P<0.05 time difference after HF in older subjects (Sidak corrected post hocs).
Figure 29: C-reactive protein and cytokine protein expression in older and younger subjects after the high fat breakfast only (Figure 4 in article).

C-reactive protein (CRP, n=15/group) and cytokine protein expression in older (filled squares, n=7) and younger subjects (filled circles, n=6) respectively. Values represent mean ± SEM for CRP (A) in mg/l, IL-6 (B) in pg/ml, MCP-1 (C) in pg/ml, and TNF-α (D) in pg/ml. There were no differences in CRP, IL-6, and TNF-α responses between older and younger subjects. There were significant age differences in MCP-1 protein expression (age effect of P<0.05) and changes over time for IL-6 (time effect of P<0.01, two-factor repeated-measures ANOVA). * P<0.05 change from baseline; γ P<0.01 time difference (Sidak corrected post hocs).
6.2.5. DISCUSSION

This study demonstrates that in an otherwise healthy older cohort, postprandial inflammation is not exaggerated compared to younger adults. Our results indicate that baseline differences in immune status may be present in healthy older adults, but that any such differences are not associated with differences in the postprandial immune response to a high fat meal. The similarities in immune response between older and younger adults are in spite of exaggerated postprandial lipaemia in these older subjects (see Chapter 4) and suggest that the inflammatory response to a high fat meal is not exacerbated in older age or with age-related differences in basal immune profile, but is in fact maintained.

Previous studies have shown that a high fat meal induces postprandial inflammation; however, the magnitude and the specific measurable markers of this inflammation vary between studies (208), and are reliant on the precise meal components included (331, 562, 714, 715) as well as the health status of subjects (526). Inflammatory gene expression (526), plasma cytokines (377), and endotoxaemia (538) are reported to be particularly elevated after a high fat meal in metabolically compromised adults, such as those with T2DM and the Metabolic Syndrome. These acute meal responses may contribute to a state of chronic low-grade inflammation, accelerating the development of insulin resistance and cardiovascular disease through progression of atherosclerosis (716, 717). To our surprise, no marked difference in postprandial endotoxaemia or inflammation was seen between younger and older subjects in the current study, suggesting that older adults do not display a greater acute inflammatory response to ingestion of a single high fat meal.

Postprandial inflammation is elevated after a high fat meal in part due to monocyte activation by circulating lipoproteins resulting from postprandial lipaemia (718). Although older adults have increased numbers of circulating TRLs, we found that this did not correspond with higher postprandial gene expression of monocyte lipoprotein receptors, apoB48r and LDLr, suggesting no greater postprandial potential for monocyte activation by lipoproteins in older adults. Metabolically, greater insulin resistance is associated with greater postprandial endotoxaemia (330, 538); as HOMA-IR was not different between older and younger participants, this supports the idea that the two conditions may be linked, and may explain the absence of postprandial endotoxaemia in these older subjects. Our results suggest that despite the higher incidence of insulin resistance or hyperlipidaemia typical in older adults, and the greater postprandial inflammatory effects of these conditions (526, 538), older individuals free from metabolic dysfunction do not experience greater postprandial inflammation.

Along with metabolic effects on postprandial inflammation, gut barrier function may have a crucial role in regulating the immune response to a high fat meal. Postprandial endotoxaemia is increased after a high fat meal (49) corresponding with elevated lipaemia (536, 582), a finding recently linked to direct chylomicron translocation of endotoxin (324, 327, 535, 582). Although the absorptive and immune function of the gut has been suggested to be impaired with ageing (482, 496), our finding of equal postprandial plasma and chylomicron endotoxaemia between older and younger adults contradicts this notion, and supports the concept that ageing itself does not conclusively impair gut barrier function (175, 176). Furthermore, this suggests that older adults have maintained gut barrier function in spite of exaggerated chylomicronaemia.
The postprandial immune response to endotoxin is initiated by acute LBP production (582) which also facilitates the endotoxaemic immune response by activation of NF-κB through CD14 and TLR4 while other bacterial components act through TLR2 (719) and TLR9. Corresponding with no postprandial age differences in endotoxaemia, we saw no greater postprandial LBP concentration or PBMC genes involved in bacterial immune defence in older participants after a high fat meal. Furthermore, the typical (720) basal elevation of LBP in older subjects did not contribute to a greater endotoxaemic response, indicating that an elevated immune status in these individuals does not increase the magnitude of postprandial endotoxaemia. Similarly, postprandial cytokine gene and protein expression was not different between age groups indicating no deficit or elevation in postprandial immune response associated with ageing.

Oxidative stress is also suggested to contribute to the postprandial inflammatory response through cytokine production (665, 721). Although IL-6 concentrations were elevated after meal ingestion, this change was equal between age groups. Despite the age similarity in cytokine response, older participants had decreased and lower SOD-2 expression after the high fat meal while younger subjects maintained expression levels equal to baseline, similar to other reports (561). This suggests that the postprandial antioxidant response may be inadequate in older adults. Increased postprandial GPX-1 expression has been reported following a meal high in saturated fat as a response to increased reactive oxygen species production (533). As we also reported lower baseline GPX-1 expression that was maintained after the ingestion of either meal, these older subjects may have lower antioxidant capacity, which suggests that basal immune status may contribute to postprandial antioxidant capacity but not necessarily immune response in older individuals.

While the lack of elevated immune response to a high fat meal in older participants likely indicates no impairment of acute activation, we investigated the possibility that immunosenescence could also elicit such a moderate acute immune responsiveness, particularly since oxidative stress has been associated with cellular immunosenescence (83, 530). Analysis of expression of genes associated with immunosenescence such as impaired apoptosis regulation (BAK1) and cellular immune activation (CD40LG) (107) indicated no obvious impairment of the immune response in these older subjects. The elevated expression of IGFBP3 that we observed is typical of the senescence profile reported in older adults (108), and suggests that despite characteristic cellular senescent changes in these older adults, postprandial immune activation remains unaffected.

6.2.6. CONCLUSIONS

Our results highlight that despite typically elevated postprandial lipaemia otherwise metabolically healthy older adults do not have an exaggerated postprandial immune or endotoxaemic response to a standard high fat mixed meal. These findings provide the first description of the comparative evaluation of postprandial immune responses in older adults and indicate that the basal health and metabolic status of older adults should be considered as a confounding factor in the study of postprandial immune responses in an ageing population, since these healthy elderly show no impairment of postprandial response. Furthermore, this suggests that maintaining health status into older age, or the factors that allow the maintenance of health in old age, may be protective against
the detrimental effects of acute high fat meal ingestion, in the sense that older adults are at no greater risk than younger adults for chronic low-grade inflammation initiated by fat ingestion.

### 6.2.7. SUPPLEMENTAL TABLE

Table 19: Primer sequences for targeted PBMC gene expression (Table S1 in article).

<table>
<thead>
<tr>
<th>Primer set/</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin²</td>
<td>TGGCACCCAGCACAATGAA</td>
<td>CTAAGTCATAGTCCGCCTAGAAGCA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGCAGTGAAAGGGAGCATTCT</td>
<td>GCTGCTTTAACATAGGCCAGGT</td>
</tr>
<tr>
<td>RNA18S</td>
<td>GATGGTAGTCCGCCTGCC</td>
<td>GCCTGCTGCCTTTCTTGG</td>
</tr>
<tr>
<td>ABCA1</td>
<td>GGCCTTTTCTCTTCTTCTT</td>
<td>CCGTGCTGGATCAATTACGT</td>
</tr>
<tr>
<td>apoB48r³</td>
<td>GCCAGTCACCTCTTCCTCT</td>
<td>AATGAGGGCAATAGGCTGGCTCT</td>
</tr>
<tr>
<td>BAK1</td>
<td>CGGCAGAGAATGCCTATAGG</td>
<td>AGTCAGGGCCATGCTGGTAGA</td>
</tr>
<tr>
<td>CD14</td>
<td>AGAGGGTTCGGAGACTTTATCG</td>
<td>TCGGAAAAAGTTCAGAGACGC</td>
</tr>
<tr>
<td>CD40LG</td>
<td>AATCCTCAAATGCGGCACA</td>
<td>TTAACCGGTCAAGCTGGTCCCA</td>
</tr>
<tr>
<td>GPX-1</td>
<td>TATCGAGAATGTGGCGTCCC</td>
<td>TCTTGGGCTTCTCTGATGC</td>
</tr>
<tr>
<td>IGFBP3</td>
<td>TCAACGCTAGTGGCCAGTCAG</td>
<td>AATGGAGGGGGTGGAACTTG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>TATCGAGAATGTGGCGTCCC</td>
<td>TCTTGGGCTTCTCTGATGC</td>
</tr>
<tr>
<td>LDLr</td>
<td>ACCACAGAGGATGAGGATCCA</td>
<td>TGACCATCTGTCAGGGGG</td>
</tr>
<tr>
<td>MCP-1</td>
<td>GCAATCAATGCCCCAGTCAC</td>
<td>CTTCAGATCACAGCTCTTTTG</td>
</tr>
<tr>
<td>SOD-2</td>
<td>GGCTACTAGTGAACACCTGA</td>
<td>TGGGCTGAAATCTCTCGCTTG</td>
</tr>
<tr>
<td>TLR2²</td>
<td>ATCTCTCAATCAAGGCTCTCT</td>
<td>ACACCTCTGTAGGGTACGTG</td>
</tr>
<tr>
<td>TLR9²</td>
<td>GTGACAGATCAAGGCTGAAGT</td>
<td>CTTCCTCTACAAATGCACTACT</td>
</tr>
<tr>
<td>TNF-α²</td>
<td>AGCCCATGTTGAGCAACC</td>
<td>TGGGTACAGGGCCCTCTGAT</td>
</tr>
</tbody>
</table>

¹Unless otherwise indicated, primers were designed using Primer-BLAST (NCBI).
²Common sequence.
³Primers taken from Varela et al. (301).
6.3. POSTPRANDIAL CIRCULATING CELL-FREE DNA

To further investigate potential postprandial bacterial translocation, cell-free DNA was isolated from plasma to look for the presence of bacterial genomic fragments in the postprandial period. The isolation and identification of the bacterial ribosomal gene sequence 16SrRNA in circulation has been demonstrated in the literature in patients with ascites (345), liver dysfunction (cirrhosis) (722), inflammatory bowel disease (347), gastric cancer (723), and more recently in mice fed a long-term high fat diet (303). The presence or quantity of bacterial DNA (bacDNA) in plasma samples has not been investigated in older adults nor in the postprandial period.

During these experiments, quantification of the low concentrations of cell-free DNA (cfDNA) recovered by conventional DNA extraction from plasma samples proved problematic. Therefore, a novel quantification method was attempted, using qPCR amplification of a ubiquitous human gene as described by Park et al. (724). Through this, we made the serendipitous discovery that this quantification procedure might be useful in itself as an analyte for the stress response to a meal.

6.3.1. METHODS

6.3.1.1. STUDY DESIGN AND PROCEDURES

The intervention conducted was as described earlier in this chapter, sections “6.2.3.1. Subject selection” “6.2.3.2. Study design and treatments” “6.2.3.3. Study procedures”.

6.3.1.2. CELL-FREE DNA EXTRACTION

DNA was extracted from 200ml plasma using a DNeasy Blood & Tissue Kit (Qiagen, Venlo, Netherlands) under laminar air flow as per manufacturer's instructions with a final eluent volume of 50µl, twice eluted. DNA concentration was measured using NanoDrop. Cell-free DNA extraction concentrations were not normalised after extraction to allow for direct comparison of cell-free DNA concentration between samples per 200µl of plasma. Cell-free DNA concentrations are reported as concentration per ml of plasma.

6.3.1.3. QUANTITATIVE REAL-TIME REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION ANALYSIS

Quantitative polymerase chain reaction (qPCR) was performed using a LightCycler 480 (Roche Applied Science) using SYBR Green I DNA-binding dye. Primers (Table 20) were taken from literature and were obtained from Invitrogen (Life Technologies) as lyophilised powder which were resuspended in nuclease free water to a concentration of 100µM and stored at -20°C. Primers designed by Park, Kim (724) for an 81-bp fragment of the human Alu-repeat sequence were used to quantify the relative cell-free human genomic material. A standard for the Alu-81 sequence was created by amplifying a pooled sample of human cell-free DNA and extracting the qPCR product using a QIAquick PCR Purification Kit (Qiagen). A standard curve was created following Alu-81 quantification with a NanoDrop. Samples were run in duplicate 10µl reaction volumes. Template concentrations of Alu-81 were variable as cell-free DNA concentrations were not normalised after DNA extraction, rather, 4µl of
DNA extract was loaded per reaction volume. Results obtained as Cp values were used to calculate gene expression in relative and absolute terms using the $2^{-\Delta C_{p}}$ method for absolute units of change.

### 6.3.1.4. BACTERIAL DNA ANALYSIS

Bacterial DNA, as an inflammatory pyrogen, can be detected with the ubiquitous 16SrRNA gene found in the genome of all prokaryotic cells. The detection of bacterial DNA in circulation has been demonstrated by others (345, 347, 722, 725, 726) and begins with DNA extraction from plasma or serum as described in “6.3.1.2. Cell-free DNA extraction”. Methods to detect postprandial changes in bacterial DNA concentrations in plasma were developed as follows.

Primers for the detection of the 16SrRNA gene were taken from literature and obtained from Invitrogen (Table 20). Early method optimisation revealed low concentrations of amplified products (high Cp values) and amplification of products within the no-template control (NTC) indicating genomic contamination of reagents. A series of investigations were undertaken to identify the origins of contamination (Table 21).

Primers designed by Amar et al. (303) for the 16SrRNA gene were obtained from Invitrogen. Primers were reconstituted with unopened NFW under laminar air flow using pyrogen-free consumables. A standard for the 16SrRNA sequence was created by amplifying a sample of DNA isolated from *E. coli* and extracting the qPCR product using a QIAquick PCR Purification Kit (Qiagen). A standard curve was created following 16S rRNA quantification with a NanoDrop. qPCR was performed as described in section “6.2.3.6. Quantitative real-time reverse-transcriptase polymerase chain reaction analysis” using unopened SYBR Green 1 DNA binding dye and a loading template volume of 4μl: the final DNA concentration loaded was unknown and variable due to the nature of the DNA quantification method used and to allow for relative comparison of plasma content of bacterial DNA between samples.

### 6.3.1.5. STATISTICAL ANALYSES

Data are presented as means ± SEMs. Cell-free DNA concentrations were compared using three-factor repeated-measures ANOVA. Sidak post hoc tests were used for all multiple comparisons between groups. Where Mauchly’s sphericity test failed, the Huynh-Feldt correction was applied. Alpha was set at P<0.05.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alu-81$^1$</td>
<td>CCTGAGGTCAGGAGGCTCTGAG</td>
<td>GCCCGGGCTAATTTTGTAT</td>
</tr>
<tr>
<td>16SrRNA$^2$</td>
<td>TCCTACGGGAGGCAGCAGT</td>
<td>GGACTACAGGCTATCTAATCCTGTT</td>
</tr>
</tbody>
</table>

$^1$Primer sequence from Park et al. (724).
$^2$Primer sequence from Amar et al. (303).
### Table 21: Optimisation of bacterial DNA detection.

<table>
<thead>
<tr>
<th>Challenge</th>
<th>Protocol</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTC contamination:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NFW contamination</td>
<td>Use unopened NFW</td>
<td>Contamination persists</td>
</tr>
<tr>
<td>Primer contamination (from NFW)</td>
<td>Order new primers, reconstitute with unopened NFW</td>
<td>Contamination persists</td>
</tr>
<tr>
<td></td>
<td>DNase treatment of primers</td>
<td>Primers likely destroyed</td>
</tr>
<tr>
<td>SYBR Green contamination</td>
<td>Use unopened SYBR Green</td>
<td>Contamination persists</td>
</tr>
<tr>
<td></td>
<td>DNase treatment of SYBR Green (727)</td>
<td>Contamination reduced</td>
</tr>
<tr>
<td>Detected concentration in sample below NTC</td>
<td>Increase sample volume: the largest feasible volume was used (limited by master qPCR mix to template ratios and available sample quantities)</td>
<td>4µl was the maximum practical volume</td>
</tr>
<tr>
<td></td>
<td>Attempt fresh primers: after initial contamination, new primers were ordered and reconstituted using unopened NFW</td>
<td>Contamination reduced</td>
</tr>
</tbody>
</table>

NTC: non-template control; NFW: nuclease-free water
6.3.2. RESULTS

6.3.2.1. POSTPRANDIAL CEL-FREE DNA IN POSTPRANDIAL PLASMA

There were no differences at baseline in detected Alu-81 sequence numbers. Ingestion of the low fat meal did not result in any detectable postprandial changes in Alu-81 concentration in either age group. High fat meal ingestion resulted in greater Alu-81 sequence concentration in younger but not older subjects (P=0.045; Figure 30). This tended to be characterised by an age difference at 2 hours (P=0.065) and an increase in Alu-81 concentrations from baseline in younger participants (P=0.038) which was not sustained at 4 hours (P>0.05).

![Figure 30: Postprandial cell-free DNA content of plasma.](image)

Older high fat (filled squares), younger high fat (filled circles), older low fat (open squares), younger low fat (open circles). Values represent mean ± SEM (n=15/group) in ng of Alu-81 repeat sequences per ml of plasma. There was a significantly greater concentration of Alu-81 repeats after the high fat meal in younger subjects (treatment × age interaction of P=0.045; three-factor repeated-measures ANOVA).
6.3.2.2. BACTERIAL DNA IN POSTPRANDIAL PLASMA

Preliminary qPCR of postprandial samples targeted for the Amar16S primer sequence revealed NTC contamination along with several positive hits for Amar16S primer amplification in human samples. However, these hits were not at reliably greater concentrations than the NTC, suggesting false positive amplification in these samples. Subsequent attempts revealed that primer and SYBR Green contamination was also highly probable. After eliminating sources of potential bacterial DNA contamination, a standard curve of known DNA concentration was run along with postprandial samples. The logistic regression of the standard curve showed diminished accuracy beyond 40 Cp, estimating quantities less than $2.7 \times 10^{-9} \text{ng/µl}$ (Figure 31). Nevertheless, qPCR was run up to 50 Cp to allow for possible amplification of low concentrations of 16SrRNA. In 154 of 180 samples the Cp was not reached before 50 cycles. The remaining 26 samples were not statistically different from the NTC ($P=0.29$). Of the 26 sample with Cp values before 50, 17 were from the same 8 participants; 5 of these 8 had multiple hits on samples taken from the same treatment day. Furthermore, the melting temperatures obtained from the standards (85.8 ± 0.05°C) differed from the melting temperature of the human samples (76.7 ± 0.33 °C; $P<0.001$), indicating a product differing from that of the *E. coli* 16SrRNA sequence.

![Figure 31: Standard curve of *E. coli* sequences of the Amar16S ubiquitous primer.](image)

Logistic regression standard curve of serial dilutions of *E. coli*-based amplified genomic fragments of the Amar16S primer sequence for the bacterial 16SrRNA gene ranged from $0.016$ to $6.55 \times 10^{-11} \text{ng/µl}$ (log -2 to log -11). The equation of the logistic regression (*solid line*) was $y = -16.555x + 18.118$ with an $R^2=0.9772$. Crossing point (Cp) end cycles are represented by the broken lines: regular end cycle (*dashed line*) and extended cycle (*dotted line*).
6.3.3. DISCUSSION

6.3.3.1. CELL-FREE DNA IN POSTPRANDIAL PLASMA

We have shown that younger adults have postprandial increases in cell-free DNA concentrations while older adults show no such elevation. While no effect of age on cell-free DNA concentrations has been previously reported (724), this is in contrast to others showing elevated cell-free DNA in older adults (123). As a proxy measure of cellular damage, whether by apoptosis or necrosis, long-term increased concentrations of cell-free DNA have been linked to cancer (724, 728), myocardial infarction (121, 729), and inflammation (364, 730), and may have the most usefulness in prognosis (367, 731). It has also been observed that in nonagenarians, increased concentrations of cell-free DNA have been associated with cardiometabolic risk factors, increased inflammation (124), and increased mortality (732). Our data suggest that the older subjects in the current study may not have an increased risk of these age-related negative health outcomes, and further, that the younger participants here studied may inversely be exhibiting a poorer prognostic plasma profile of cell-free DNA concentrations. However, due to the nature of study designs investigating cell-free DNA, there has not been an established causality link between cell-free DNA concentrations and negative outcomes (733). These data may instead have more direct implications for possible age-related differences in circulating DNase activity (365) which may be more relevant.

Importantly, we have shown postprandial changes in cell-free DNA, demonstrating the transient nature of fluctuations in cell-free DNA. Further, the negative associations of increased concentrations of cell-free DNA may additionally be associated with high fat meals. Transient changes in cell-free DNA concentrations have been recently documented. Short-term decreases in cell-free DNA have been shown in women undergoing stress reduction therapy during in vitro fertilisation treatment (734). However, other transient states, such as menstruation, have shown no effect (735). More acutely, exercise is able to induce increases in circulating cell-free DNA (364, 736-738), which may even be limited to within 1 hour post-exercise (739). Thus, we have identified that a high fat meal also has the capacity to elicit transient increases in cell-free DNA. The related health implications of this are unknown in the context of chronic illness and deserve further investigation regarding the acute and chronic dietary effects and correlation to specific health outcomes. Furthermore, the implications of meal-related changes in cell-free DNA to the use of cell-free as a biomarker are significant. Awareness of conditions that may increase cell-free DNA may be essential in establishing sampling conditions (e.g. exercise, diet, stress) likely to affect analysis or produce false positives, particularly since cell-free DNA is increasingly being debated as a diagnostic and prognostic biomarker (367, 731).

6.3.3.2. BACTERIAL DNA IN POSTPRANDIAL PLASMA

The detection of bacterial DNA in human plasma presented many challenges as the 16SrRNA gene is ubiquitous (hence its usefulness as a genomic target) and the isolation and amplification of low concentrations of genomic matter is difficult. The Cp values obtained from the human samples probed for the 16SrRNA gene were as low as the NTC and effectively below any detection capacity of the LightCycler. This, in addition to the different melting temperatures, greatly suggests that any amplified products detected in the human samples represented
either contamination, or more probably, eventual primer-dimer products. However, as DNA gels were not run to confirm this, there remains the possibility that these products represent true 16SrRNA amplified sequences. This is also plausible since different species of bacteria will produce different amplified products from the same primer pair (740) as the exact intervening sequence may contain different frequencies of or total numbers of base pairs (741). If these products do in fact represent 16SrRNA products, the frequency at which they occur in samples from the same participants and participants from the same day of sampling suggests that individual variation or day-to-day variation more appropriately predicts the presence of bacDNA in human plasma than age or an acute response to a meal. Indeed, the presence of bacDNA in human plasma has been strongly linked to the individual's health status, with positive correlations to factors like increased postprandial glucose or waist to hip ratio (742). Moreover, bacDNA has been reported in healthy human blood at frequencies around 10% (742), suggesting that our sample size may have been inadequate to detect notable age-related differences in bacDNA if individual status is more important than the postprandial period. Regardless, further investigation using more robust extraction and analytical methods (743) may be warranted to determine the potential postprandial impact of fat ingestion on circulating bacDNA.

6.3.4. CONCLUSIONS

We have shown that endogenous cell-free DNA is present in postprandial circulation, and is increased after a high fat mixed meal in younger adults but not older adults. This novel biomarker of acute stress demonstrates transient meal-specific changes, which are furthermore subject to age-related differences in response. Although the origins and implications of increased cell-free DNA after a high fat meal are as yet unknown, the future identification of their significance may reveal aspects of age-specific differences in postprandial responses and chronic disease risk. Additional research is required to examine the effects of other nutrients and meals on cell-free DNA responses and to describe the specific repercussions these may have on postprandial inflammation.

6.4. GENERAL LIMITATIONS AND DISCUSSION

6.4.1. MINIMAL POSTPRANDIAL INFLAMMATION AFTER A HIGH FAT MEAL

Surprisingly, we report no marked difference in inflammatory markers between the two test meals, despite the use of a standardised high-fat meal known to induce postprandial inflammation (582). Changes to meal formulation or differences in regional ingredients may explain this discrepancy as specific meal components are known to affect postprandial inflammation (331, 562, 714, 715). For example, changing to higher MUFA oils in the HF meal, as McDonald’s has during efforts to reduce trans and saturated fats in its products (744-746), could reduce postprandial inflammation in all subjects, as high MUFA meals cause relatively little postprandial inflammation (714), oxidative stress (715), and endotoxaemia (537) compared to high SFA meals. Additionally, a mixed meal stimulates a greater insulin response when compared to a fat only challenge (549) which may alter the postprandial endotoxaemic response (536), and the addition of food components such as orange juice (331) or tomato (562) are known to suppress the inflammatory response to a high-fat meal.
6.4.2. LIMITATIONS OF ENDOTOXIN DETECTION

The exaggerated lipaemic response of older adults may have inhibited effective detection of endotoxin. Lipaemia is known to affect assays by interfering with absorbance through light scattering (612), and this could have reduced the measured concentrations of endotoxin in older subjects. Furthermore, as LPS is known to bind to chylomicrons (324) with a high affinity to apoB (351) as well as plasma proteins such as CD14 (350), LBP (359) or albumin (335), differences in concentrations of these substrates which may accompany ageing (747, 748) may have limited the availability of the LAL assay to detect LPS (749).

Although bacterial translocation of endotoxin has been associated with the TRL fraction (324, 332), we observed a trend towards decreased rather than increased TRL endotoxin concentrations during the period of peak chylomicron and TAG appearance. This trend towards minimal TRL endotoxaemia at the point of maximal TRL appearance and plasma endotoxaemia may point to a dynamic relationship between TRL classes and lipopolysaccharide that may change over the course of the postprandial period. The neutralisation activity of lipoproteins may provide an explanation for such a response. The high affinity of LPS to LBP and lipoproteins has been proven instrumental in the prevention of death during sepsis by lipid infusions (360, 750). Postprandial fluxes in the availability of neutralisation mediators or their relative activity may affect the concentrations of measurable LPS while changing the inflammatory capacity; however, these relationships have not been adequately described. Although differences in the neutralisation capacity between older and younger participants was not assessed as an endpoint in this study, despite the availability of such experimental methods (49), our data do not suggest that older adults have differences in their response to endotoxin, whether this includes neutralisation response or not.

6.5. CONCLUSIONS

These data suggest that, remarkably, ageing per se is not associated with greater postprandial inflammation, despite elevated lypaemia. Healthy ageing does not seem to be associated with impaired postprandial immune or gut integrity, although our lack of positive findings may indicate that more direct measures of immune responsiveness or gut permeability are required. Future studies should aim to explain why and/or how healthy older adults maintain a tempered immune response to a high fat challenge, as this may be a key feature or adaptation of the healthy elderly. Furthermore, the significance of postprandial increases in cell-free DNA, a novel biomarker of acute stress, may help shed light on these age-related differences.
CHAPTER 7

CONCLUSIONS
7.1. INTRODUCTION

This thesis set out to explore how the digestive responses to realistic meals are altered in the elderly, with focus on the macronutrient and immune responses integral to chronic disease development. This work sought to understand the metabolomic differences in macronutrient appearance in older adults in relation to the postprandial responses to protein and fat, known to be disturbed in the elderly. Furthermore, the differences in postprandial immune response between old and young were analysed. Thus, this thesis aimed to characterise older adults' acute digestive responses to a mixed meal to further our knowledge of the role of digestion on nutrient mediated health in older age.

7.2. SUMMARY OF MAJOR FINDINGS

It was demonstrated that older adults have delayed amino acid appearance following a mixed meal, exaggerated lipaemic responses to meals including differences in chylomicron size and fatty acid composition, and no greater inflammatory or endotoxaemic responses to a high fat meal when compared with younger adults.

Specifically, it was demonstrated that compared with younger adults, older adults have delayed but preserved amino acid appearance. This is particularly evident for BCAAs and EAAs following a low fat mixed meal. Additionally, the ingestion of a high fat meal of differing composition may not exert the same effect.

Through chylomicron and lipidomic characterisation of the postprandial lipaemic responses to mixed meals, older adults exhibited a prolonged and exaggerated lipaemia following both a high and low fat meal which is not characterised by greater chylomicron TAG content. Furthermore, older adults produce smaller and more numerous chylomicrons following a high fat meal which carry greater proportions of oleic acid and lesser proportions of PUFAs in both TAG and PL fractions. Moreover, it was demonstrated that a low fat meal stimulates the production of comparable numbers of chylomicrons in older adults compared to younger adults, without differences in the oleic acid content of chylomicron TAGs. However, consistent with our observations after a high fat meal, older adults' chylomicrons have decreased PUFAs in TAG and PL with raised PL oleic acid compared with younger adults'. Furthermore, compared to younger adults, older adults consistently exhibit enhanced absorption/retention/appearance of dairy derived odd-chain fatty acids in chylomicron TAGs and PLs following low and high fat meals.

This research has shown that metabolically healthy older adults do not exhibit signs of increased postprandial endotoxaemia or PBMC inflammatory response following a high or low fat meal despite typical monocyte expression phenotypes and exaggerated lipaemia, but may have an exaggerated oxidative stress response to a high fat meal.

Overall, the macronutrient content and structural composition of a mixed meal is instrumental in determining postprandial responses. Older adults may be particularly prone to the impact of mixed meal dynamics as the digestive rate and relative stimulation of hormonal processes essential for the efficient processing of
individual macronutrients may be more susceptible to alterations due to digestive, hormonal, and basal health status differences typical of ageing.

7.3. IMPLICATIONS AND FUTURE DIRECTIONS

Digestive responses are only recently becoming a targeted and more appreciated aspect in research of the relationship between food and health outcomes (751). The elderly have altered digestive and metabolic responses, and as a growing segment of the population, the resulting health implications of these differences are of great public health concern. There is a need to build an understanding of the impact of food and meal structures on altered digestive responses and to employ this knowledge to manipulate dietary patterns and food design to promote optimal digestive responses in the elderly for the promotion of metabolic health.

The findings of this thesis evoke questions about (i) how food structure affects digestion in the diverse elderly population, (ii) what the implications of these digestive differences are for metabolic health, and (iii) how food structure may be manipulated to elicit specific health outcomes.

7.3.1. PHENOTYPES: FACTORS AFFECTING DIGESTIVE RESPONSES

The elderly are a heterogeneous population, with variation in digestive, metabolic, and physical health, as well as differences in eating and activity behaviours. Importantly, this work was investigated in a particularly healthy cohort, and a small one, poorly accounting for the myriad variations in protein digestion in the elderly accompanying sex differences or health status alterations. While this thesis indicates the healthy elderly have delayed protein digestion, exaggerated and prolonged lipaemia, and a maintained immune response to mixed meals, these digestive and metabolic responses may be further changed by differences in lifestyle or declining health status. For instance, habitual diet or regular physical activity likely influence body composition, insulin resistance, and lipid or amino acid profiles, and as such should be controlled as confounding factors or investigated as possible means of improving digestive responses to meals. Furthermore, the digestive differences accompanying gastrointestinal dysfunction and disease common in the elderly (175, 177) may further alter the digestive responses to mixed meals. Malnutrition, also common in the elderly, has the potential for both altered digestion and specific nutritional requirements (147), with comprehension of both being important for directing nutritional interventions.

The effects of subsequent meals on digestive responses are relevant to the design and efficacy of nutritional intervention strategies to improve health by modulating digestive responses. The digestive response to subsequent meals has been shown to be primed by the previous meal, such that a greater lipaemic response may be elicited when the previous meal is high in fat (234), with a similar relationship for sequential carbohydrate loads (752). Additionally, insulin sensitivity can be primed; for example, a breakfast meal high in SFA reduces lunchtime insulin sensitivity compared to a MUFA or PUFA–rich breakfast (753). This has significant implications for the development of daily eating pattern recommendations, particularly if this priming response is amplified or suppressed in the aged. Protein recommendations for even distribution throughout the day in the elderly (136) may operate on incomplete descriptions of postprandial responses by not accounting for the digestive responses to
subsequent meals. The diurnal variation in digestive response needs to be understood in order to be accounted for, or even to ameliorate, digestive responses through food design and dietary patterns in the elderly. These aspects of digestive and metabolic function in the elderly warrant further investigation.

7.3.2. MECHANISMS: IMPLICATIONS OF ALTERED RESPONSES FOR METABOLIC HEALTH

While this thesis demonstrated that older adults have specific digestive differences in response to mixed meals, the implications of these differences to metabolic health remain unclear. More information is required to link specific response differences to health outcomes, and these relationships require additional mechanistic explanation to determine their implications and origins.

Our research uncovered metabolic profiles, both fasting and postprandial, which may have implications as factors responsible for, indicative of, or resulting in, metabolic status differences. This correlation-causation-implication puzzle requires untangling, since the digestive and metabolic differences that has been uncovered are suggestive of both impairment and protection in these elderly subjects. For example, older adults displayed marked differences in fatty acid profiles, but it is unclear whether differences such as increased proportions of odd-chain fatty acids simply indicate increased habitual intake (754, 755) or reveal fundamental metabolic differences that allow the maintenance of health. Similar profiles have been reported elsewhere such as decreased proportions of odd-chain fatty acids in diabetics (558); however, no directional relationship has been established. Additional studies are required to explain both the significance of these digestive differences between old and young, and the mechanistic origins of these differences.

7.3.2.1. MECHANISTIC SIGNIFICANCE AND IMPLICATIONS OF DIGESTIVE RESPONSES

Research which measures specific health related outcomes is needed to establish the link between digestive responses and long term health in the elderly. While postprandial lipaemia itself is regarded as a significant risk factor and causative mechanism for negative cardiovascular health (181), the health implications of more subtle lipaemic differences such as fatty acid composition, remain elusive. It is known that inadequate protein supply, whether by intake or due to increased needs, is associated with negative health outcomes in the elderly, contributing to increased morbidity and mortality through sarcopenia, weight loss, and an associated frailty (68). However, the relationship between digestive differences and the impact on outcomes like muscle protein synthesis remain unclear, and were unexplored in the current study. Furthermore, even the measurement of specific outcomes such as muscle protein synthesis has garnered criticism for ineffectively describing the direct functional consequences of nutritionally related outcomes (421). Therefore, in order to better establish the relationship between digestive responses and health outcomes in the elderly, future studies need to include measures indicative of metabolic health and disease related outcomes, and should aim to include measures of functional health, such as slow walking speed (161), as well. This will be essential in illustrating the links between acute digestive responses and long-term health outcomes.
Although these findings have identified differences in older adults’ digestive responses following mixed meals, they have not fully described the direct health consequences of these differences. Delayed protein digestion in the elderly has implications for stimulation of muscle protein synthesis after a meal. However, this research did not clearly establish such a link since muscle protein synthesis was not directly measured. Future studies should include muscle protein synthesis as a measured outcome to more directly signify the impact of these digestive differences on sarcopenic risk, while long-term studies should include functional outcomes such as muscle strength.

Similarly, the resulting implications of altered lipid metabolism including compositional differences, regardless of their origins, may be important to the health of older adults, but were not measured directly in the current study. The detrimental effects of prolonged lipaemia have been established, but other age-related differences observed in this thesis such as phospholipid composition are less well understood in the context of human health. Changes in phospholipid fatty acid content are likely to impact membrane fluidity (756), and incidentally enzymatic activity (757), which could contribute to differences in lipase activity, whether directly or by altering the local lipid environment (758). The implications of compositional differences, including membrane fluidity, lipase activity, and impact on the downstream receivers of TRL phospholipids such as HDL (759), should be investigated in the context of ageing and the pathophysiology of cardiovascular and metabolic disease.

7.3.2.2. MECHANISTIC ORIGINS OF DIGESTIVE RESPONSES

More challenging will be to identify whether differences in metabolic profiles precede or succeed healthy ageing. For example, the equivalent immune response observed in these older adults may well have implications for continued postprandial health in older age, but a very relevant question is whether this type of response is instrumental in the maintenance of health in older age. Are these differences in metabolic and immune responses to mixed meals demonstrative of ageing, or do these older adults have a specific adaptive response apparent in older age that promotes well-being in a metabolically healthy population? To answer these questions and unravel the related mechanisms will require a more complete understanding of the digestive responses of diverse ageing populations. However, it may be possible to commence with characterisation of intermediate players likely to be affected by, or even responsible for, aspects of the digestive and metabolic differences in ageing as will be presented subsequently. The current data have provided clues about the metabolomic and lipidomic outcomes of these differences which invite further mechanistic investigation.

This thesis incompletely described the postprandial fate of individual amino acids and fatty acids after digestion in the elderly. The metabolic turnover of amino acids may be important in identifying the underlying causes of age-related differences in amino acid appearance following a meal, and may aid in identifying nutritional areas of intervention. For example, fundamental differences in glutamine metabolism might contribute to altered BCAA metabolism (643) in the elderly, influencing the postprandial amino acid and anabolic response. Similarly, without tracing of dietary fatty acids, this study was unable to determine whether the differences observed in relative proportions of fatty acids between older and younger adults were due to absorption, clearance, or time-delays in metabolism. The inclusion of traceable isotopes has been used in investigations of lipoprotein kinetics (760), and has even been developed for intact proteins in milk (761) and beef products (762). By employing similar
techniques, future studies may map a more complete picture of the postprandial disappearance, appearance, and whole-body kinetics of specific amino and fatty acids may be established.

The mechanisms underlying age differences in fatty acid composition could stem from altered metabolic handling of meal fatty acids through endogenous contributions or stereospecific handling, or from differences in bioactive lipids. These features of lipid metabolism were not fully investigated in the current study, but may be essential in identifying targets for nutritional interventions aimed at improving postprandial lipaemia in the elderly.

The potential importance of endogenous VLDL in the postprandial lipaemic response in the elderly was described, although their involvement was not fully investigate in the current study. The current research indicates that VLDL may in fact be the greater contributor to postprandial lipaemia, supported by the finding of elevated lipaemia in older adults even with a low fat load. Evidence from populations with metabolic dysfunction suggests that postprandial overproduction of VLDL is a major cause of postprandial lipaemia (763). Furthermore, 80% of postprandial remnant particles stem from large VLDL rather than chylomicrons (671). As such, postprandial VLDL and remnant dynamics require targeted investigation in the elderly. Improved methods of chylomicron, remnant, and VLDL separation have been proposed by antibody and size differentiation techniques (671). These techniques may enable the careful distinction between nascent and remnant lipoproteins, and may facilitate the identification of the gut and liver contributions to the postprandial TRL pool. Additionally, the transfer of TAG, cholesterol, and phospholipids between these TRL and other lipoproteins, and the resulting implications on postprandial lipaemia requires better understanding in the general population as well as the elderly. Future studies should aim to accurately qualify the TRL measured, and identify the endogenous lipoprotein kinetics instrumental to this altered postprandial lipaemia to provide evidence for appropriate targets for reduction of CVD risk.

The compositional fatty acid differences observed here pave the way for more complete lipidomic profiling in older adults, with the potential to explain the origins of age-related proportional differences, and possibly to identify key players in the maintenance or deterioration of health in the elderly. Age-related enzymatic differences in the enterocyte membrane or in the re-assembly of chylomicron TAGs, could account for compositional differences, but may also explain time-delays in clearance in older adults. Analysis of the stereospecific positioning of chylomicron TAGs in relation to meal TAGs will provide the necessary evidence to support such differences. Additionally, if age-related differences in the stereospecific handling of TAG do exist, this may an adjustable aspect of meal TAGs capable of attenuating postprandial lipaemia in the elderly. Secondly, differences in fatty acid composition may affect downstream lipid biosynthesis, in the form of diacylglycerols, ceramides and sphingolipids, cellular signalling molecules increasingly implicated in the development of chronic disease. There is already evidence that the cellular signalling properties of DAG are affected by fatty acid composition (764), and alterations in signalling has been implicated in the development of chronic diseases such as cancer (765). Likewise, ceramides and sphingolipids have been implicated in the pathogenesis of cardiovascular and metabolic disease (766), with ceramide quantities increased in T2DM patients, possibly linked to insulin resistance (767). These bioactive lipids could have direct mechanistic links to alterations in lipid metabolism in ageing; for example, sphingolipid derived sphingomyelin may directly inhibit lipoprotein lipase activity, affecting lipoprotein catabolism (768). Nutritional interventions are capable of influencing these lipids, as compositional differences in DAG have been reported in
sedentary mice relative to exercise or diet controlled mice (769). These mechanistic candidates, who may participate in the relationships between postprandial profiles and disease outcomes, or be modifiable targets for nutritional intervention, should be included as measurable outcomes in future research.

The complex mechanisms involved in the relationships between digestion and health are unlikely to be easily deciphered, but the simultaneous impact of dietary interventions and related health outcomes may assist in illustrating this relationship.

### 7.3.3. STRATEGIES: INTERVENTION INTERACTIONS ON PROTEIN, LIPID, AND INFLAMMATORY MEAL RESPONSES

Nutritional strategies need to be developed to address deficits in protein digestion and impairments in lipid metabolism that are likely to affect the maintenance of good health in the elderly. Although strategies may be developed to target single aspects of dysfunctional postprandial responses, such as the addition of protein to a fat load to attenuate lipaemia (15), such tactics may not address, or may even aggravate, other responses. For instance, an attempt to reverse age-related delays in amino acid appearance by feeding high glycaemic index carbohydrates to speed gastric emptying, may unintentionally elevate lipaemia (705). Additionally, lower protein interventions have shown beneficial effects at reducing low-grade inflammation (770), complicating the advocacy of particularly high protein recommendations for the elderly. This speaks to the need to consider the chronic role of underlying mechanisms, such as insulin sensitivity or low-grade inflammation, in the design of interventional strategies. Furthermore, including these mechanisms as targets for intervention alongside the forefront issues of delayed protein digestion and exaggerated endogenous lipaemia, may amplify interventional efficacy.

The findings of this thesis suggest that both insulin secretion and resistance may contribute to the altered amino acid and lipaemic responses to mixed meals in older adults. In this sense, controlling acute and chronic glycaemia and insulinaemia may have beneficial outcomes for both altered lipid and protein responses in the elderly. While glycaemic control may be achieved through manipulation of the macronutrients, such as the addition of protein to a meal to temper glycaemia (770) (or lipaemia (15, 771)), the carbohydrate quality or quantity may also modify the glycaemic response (772). Indeed, lower glycaemic index diets have been shown to promote long-term glycaemic control and improve insulin sensitivity (773), while similarly demonstrating the ability to lower total cholesterol (774), possibly triacylglycerols (775), and even low-grade inflammation (770). Furthermore, the quantity of carbohydrate ingested may additionally be important, as insulin sensitivity has been shown to improve more robustly with interventions reducing total carbohydrate intake rather than manipulating glycaemic index; similar benefits were reported for total cholesterol, fasting triacylglycerols (775). This effect may relate to reduced stimulation of carbohydrate induced VLDL production (219), supporting a role for controlling carbohydrate quantity, quality, and digestibility beyond glycaemic control towards lipaemic control.

Similarly, effective postprandial amino acid responses may benefit from manipulating the glycaemic and insulinaemic effects of the carbohydrate in a meal. Indeed, a postprandial hyperinsulinaemic state initiated by carbohydrate, has been shown to interfere with hyperaminoacidaemia, impairing the muscle protein synthetic
response in the elderly (626). This integral role of the postprandial insulinaemia may explain the lack of benefit observed in muscle protein synthesis stimulation after leucine supplementation or carbohydrate co-ingestion. Indeed, these interventions have shown positive effects in younger adults (616, 776) with uncertain benefits in the elderly (546, 777, 778). The implication that carbohydrates may negatively impact the protein digestive response beyond gastric emptying effects suggests that selection of less hyperinsulinaemic carbohydrates should be considered for the elderly. In this sense, lower carbohydrate loads, or carbohydrate loads designed to attenuate glycaemic responses may be optimal in the elderly.

Additionally, countering low-grade inflammation, although not evidently an exacerbated postprandial concern in the healthy elderly may be a logical area for nutritional intervention due to its central involvement in chronic illness and its contributions to the pathophysiology of dysfunctional protein, lipid, and carbohydrate metabolism. Anti-inflammatory or antioxidant nutrients may be easy additions to dietary plans, but crucially, these are by their nature closely linked with whole food structure. As an example, the anti-inflammatory and anti-oxidant components of fruits and vegetables, anthocyanins and phenolic acids, may be inextricably bound to plant cellular structures, impairing bioavailability in normal adults (779). The bioavailability of bioactive compounds is not well understood in the elderly (188, 447), particularly in the context of food and meal structure. Could it not be that increased postprandial oxidative stress in the elderly stems from an impaired and inadequate digestion of meal antioxidants, brought on by altered digestion to mixed meals? Obviously this is an oversimplified conjecture; yet remarkably the age-related digestive differences in plant bioactive bioavailability have been poorly considered in literature despite the exuberant proposals of their preventative roles in age-related chronic diseases.

7.3.4. FOOD DESIGN: EFFECTIVE FOODS FOR HEALTH MAINTENANCE AND IMPROVEMENT IN THE ELDERLY

The following goals are proposed for future food design, most likely to optimise digestive responses to whole foods in the healthy elderly while aiming to improve health status. First, foods must be designed for increased amino acid availability, requiring minimal digestion, with enhanced availability of leucine (ideally >2.2g per serving (780)), arginine (ideally >11g per serving (15, 704)) and glutamine (possibly 20% of daily protein intake (781)). This may require selection of high-quality animal proteins or ‘fast’ proteins when taken intact from whole foods, mechanical processing of protein sources to increase their rate of digestion (i.e. pre-digestion), or fortification with isolated amino acids. Secondly, the rate of gastric emptying may be controlled by the composition and quality of macronutrients, with the aim of not necessarily correcting possible delays experienced by the elderly, but of reducing prolongment of gastric emptying; while this thesis describes delayed amino acid absorption suggesting involvement of delayed gastric emptying, the maximal threshold of circulating concentrations has been hypothesised as more important with regards to muscle protein synthesis (439). Microencapsulation with polysaccharides, as is employed for targeted protein drug delivery (782, 783), may be a feasible option to control the release of amino acids, or a single amino acid (i.e. leucine), in a more controlled manner to promote synchronised appearance in circulation. This technique may also enhance delivery of other functional food ingredients such as plant polyphenols or long-chain PUFAs which may have reduced bioavailability in older adults. Thirdly, different
carbohydrate structures may require optimisation for specific digestive outcomes; insulin stimulation may be beneficial for muscle and endogenous lipid control, however hyperglycaemia is likely detrimental. Therefore, there is likely a careful balance of glycaemic index, carbohydrate intake, and stimulated rate of digestion that is optimal in the elderly. Once this has been elucidated, permutations of fibre, resistant starches, and carbohydrate sources may be optimised in specific food matrices.

7.4. PROPOSED RESEARCH OPPORTUNITIES

Overall these data highlight the impact of meal composition on digestive responses in the elderly, which may contribute to chronic disease and deteriorating health with advancing age. Ultimately, how can diet and food be manipulated to address and correct age related differences in digestion, which may result in metabolic differences, and how can these be used to improve health in the elderly? Future studies should be designed employing food-based interventions, measuring potential mechanistic biomarkers, as well as disease specific variables aimed at evaluating the long-term health benefits of these interventions. To do this, the following research should be undertaken to investigate, explain, and improve the digestive responses of older adults to complex food structures in the context of health maintenance.

7.4.1. MEAL MANIPULATION TO ATTENUATE ENDOGENOUS POSTPRANDIAL LIPAEMIA

Postprandial endogenous lipaemia significantly contributes to exaggerated postprandial lipaemia in healthy older adults. Peripheral and hepatic insulin resistance contribute to this endogenous response, and modifying the insulinaemic response to a meal may attenuate endogenous TRL production. Strategies aimed at acutely improving postprandial insulin and glycaemic responses may be beneficial in tempering endogenous postprandial lipaemia. Interventions aimed at priming metabolic responses may be beneficial in reducing the endogenous responses to meals in the elderly. For instance, exercise the night before a lipid challenge tempers the postprandial lipaemic response in postmenopausal women, concurrent with lower insulinaemia (784). Long term high carbohydrate, low fat diets result in greater postprandial insulinaemia and greater triacylglycerolaemia after an acute challenge in subjects with T2DM (785), exemplifying the importance of postprandial insulin for lipaemic responses. A lower glycaemic index breakfast has been shown to induce lower insulin and TAG responses during a subsequent lunch (786), further demonstrating the priming effects of insulinaemic control on attenuating lipaemic responses. Meal frequency has been shown to affect postprandial responses, with greater rises in insulin found after less frequent meal consumption, resulting in lower total insulinaemia and triacylglycerolaemia over the course of a day (787). Therefore, while reducing the insulinaemic response to a single meal may be beneficial for reducing postprandial lipaemia long term (785), reducing the total daily increases in insulin may have a similar effect (787).

The following tables outline proposed study designs for the acute dietary control of endogenous lipaemia through acute meal manipulation (Table 22) and through priming of insulin responses by dietary means (Table 23).
Table 22: The effects of carbohydrate quality and quantity on endogenous lipaemia in the elderly.

<table>
<thead>
<tr>
<th>Background: Reduced insulinaemic responses elicited by lower carbohydrate diets (785) or manipulation of dietary patterns are associated with lower postprandial triacylglycerolaemia (787).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Question: Does controlling the insulin response to a single meal by dietary manipulation reduce the acute endogenous lipid responses to a meal?</td>
</tr>
<tr>
<td>Aim: To reduce the postprandial increase in endogenous lipaemia by reducing the total increase in postprandial insulin.</td>
</tr>
<tr>
<td>Design: Randomised cross-over breakfast challenge in healthy elderly.</td>
</tr>
<tr>
<td>Intervention: Breakfast meal: moderate fat and protein. Based on a 2500kJ meal: 20g fat (750kJ from fat, 30%), 25g protein (420kJ, 17%), plus 80g carbohydrate (1340kJ, 53%) in the control meal.</td>
</tr>
<tr>
<td>a) Control: high carbohydrate (80g, 48% of energy), high glycaemic index.</td>
</tr>
<tr>
<td>b) Low carbohydrate (40g, 36% of energy).</td>
</tr>
<tr>
<td>c) Low glycaemic index (80g, 48% of energy).</td>
</tr>
<tr>
<td>Measures: Plasma at baseline and hourly for 5 hours.</td>
</tr>
<tr>
<td>Glycaemic and insulinaemic responses.</td>
</tr>
<tr>
<td>Endogenous lipoprotein contributions to lipaemia: separation of chylomicron, large VLDL, small VLDL and TRL remnant species; analysis of lipoprotein TAG, cholesterol, and apoB.</td>
</tr>
<tr>
<td>Lipidomic analysis of TAG in TRL species.</td>
</tr>
<tr>
<td>Outcomes: Treatment effect: low carbohydrate ≥ low glycaemic index &gt; control.</td>
</tr>
<tr>
<td>Reduced:</td>
</tr>
<tr>
<td>• Glucose, insulin, and TAG iAUC.</td>
</tr>
<tr>
<td>• Postprandial large VLDL numbers and TAG.</td>
</tr>
<tr>
<td>• Total plasma cholesterol reduction, and less enrichment in VLDL.</td>
</tr>
<tr>
<td>Increased:</td>
</tr>
<tr>
<td>• TRL and remnant clearance.</td>
</tr>
<tr>
<td>• Meal versus endogenous lipidomic contribution to TRL TAG and PL species.</td>
</tr>
<tr>
<td>The findings will contribute an understanding of the role of carbohydrate quality and quantity on the endogenous lipaemic response in the elderly. This may provide opportunities for tailoring carbohydrate recommendations and products to reduce lipaemia in older adults.</td>
</tr>
</tbody>
</table>

1Abbreviations: TRL: triacylglycerol-rich lipoprotein; TAG: triacylglycerols; apoB: apolipoprotein B; iAUC: incremental area under the curve.
Table 23: Does the lipaemic response to breakfast start with dinner?

Background: Long-term metabolic priming with lower carbohydrate diets are associated with lower postprandial insulinaemic and triacylglycerolaemia responses (785). An acute bout of aerobic exercise is effective at eliciting a similar priming effect, reducing triacylglycerolaemia (784) and VLDL production (788).

Question: Can endogenous lipaemia be attenuated by priming a reduced insulinaemic response, and can this be achieved through dietary manipulation?

Aim: To reduce the postprandial increase in endogenous lipaemia by priming for a reduced insulinaemic response by dietary manipulation.

Design: Randomised cross-over breakfast challenge in healthy elderly.

Intervention: Night before: moderate fat, carbohydrate, and protein. Based on a 2800kJ meal: 15g fat (560kJ from fat, 22% of energy), 35g protein (590kJ, 22% of energy), and 90g carbohydrate (1500kJ, 56% of energy).

a) Control: no exercise, “non-priming dinner” high glycaemic index
b) Exercise: “non-priming dinner” high glycaemic index; exercise between lunch and dinner, moderate intensity walking for 90 minutes.
c) No exercise, “insulin priming dinner” low glycaemic index, high fibre, resistant starch.

Breakfast meal: Standard high-fat meal challenge (3800kJ with 90g carbohydrate, 25g protein, 50g fat; 40%, 11%, and 49% of energy respectively).

Measures: Plasma at baseline and hourly for 5 hours.
Glycaemic and insulinaemic responses.
Endogenous lipoprotein contributions to lipaemia: separation of chylomicron, large VLDL, small VLDL and TRL remnant species; analysis of lipoprotein TAG, and apoB.

Outcomes: Treatment effect: exercise ≥“insulin priming dinner” > control.
Reduced:
• Glucose, insulin, and TAG peaks and iAUC.
• Postprandial large VLDL numbers and TAG.
Increased:
• TRL and remnant clearance.
• Meal versus endogenous lipidomic contribution to TRL TAG and PL species.

These findings will contribute to understanding the ways in which daily eating patterns and physical activity influence postprandial lipaemia in the elderly. Such findings may open opportunities for developing acute lifestyle strategies to temper the exaggerated lipaemic response for the elderly.

Abbreviations: TRL: triacylglycerol-rich lipoprotein; TAG: triacylglycerols.
7.4.2. MEAL MANIPULATION TO FACILITATE RAPID AMINO ACID ABSORPTION AND ADEQUATE THRESHOLDS

Delayed gastric emptying or intestinal transit may be a major cause of delayed amino acid appearance. This delayed protein response may reduce the peak amino acid concentrations of BCAA achieved, which may be inadequate to stimulate muscle protein synthesis in the elderly, particularly since the elderly may have a higher necessary threshold (655, 659). While in the case of the elderly delayed gastric emptying could be due to reduced gastric acid secretions (170, 175) requiring additional time for adequate digestion (789), the composition and structure of a meal may be modified to normalise this. Greater quantities of fat and fibre in a mixed meal, as well as the solid content are likely to delay gastric emptying (790). In the elderly, large meals are also associated with delayed gastric emptying while small meals are not (164). Therefore, meal size and ingredients may be optimised to allow for more rapid gastric emptying. Similarly, greater viscosity of the digesta may hamper intestinal motility (789); again, inadequate gastric (791) of hormonal (790) secretions in the elderly (170, 175) may be a root cause, but meal composition may be altered to facilitate intestinal motility.

Increasing gastric emptying may seem counter-productive, since this may suggest the recommendation to remove soluble and insoluble fibre and replace these with high glycaemic index or highly processed foods. These foods, and the increase in gastric emptying, are likely to stimulate exaggerated insulin and endogenous triacylglycerol responses, potentially aggravating dyslipidaemia (774). Conversely to traditional fibres, resistant starches are non-viscous (792) and do not impair gastric emptying or protein digestion (793), but they do similarly dampen insulin and glycaemic responses (794). Hence, resistant starches may be a means to hasten gastric emptying without impairing protein digestion or contributing to hyperinsulinaemia.

The selection of digestible proteins may also facilitate amino acid appearance. Muscle protein synthesis in the elderly responds better to ‘fast’ proteins like whey (435). Paradoxically, whey protein delays gastric emptying in spite of more rapid amino acid appearance in circulation compared with casein, cod or gluten (795). This may speak to the importance of the failure of so-called ‘slow’ proteins like casein to elicit the postprandial EAA or leucine thresholds necessary for postprandial protein accretion. Indeed, supplementing protein meals in the elderly with additional leucine improves postprandial muscle protein synthesis (591, 778), which may due to improved accessibility. However, food sources may also be adaptable to improve digestibility in the elderly and to elicit rapid peaks in EAAAs and leucine. Animal protein is typically more easily digested than plant proteins (796). Indeed, improving the digestibility of meat protein by serving it as mince facilitates amino acid availability in the elderly (544). Heated milk proteins may reduce protease accessibility and impair digestibility, while enzymatically pre-digested proteins such as fermented or ripened dairy products may have enhanced digestibility (195). Therefore, a ‘slow’ protein’s digestibility may be increased for the elderly by appropriate mechanical or enzymatic processing, and the avoidance of milk heat treatment initiating the Maillard reactions that hinder protein digestibility.

Based on these principles, the following study design is proposed, as outlined in Table 24, to improve digestibility of protein, elicit rapid and enhance peak appearance, and to control hyperinsulinaemia.
Table 24: Enhanced proteins for enhanced muscles in the elderly.

Background: Easily digestible proteins (435), individual amino acid supplementation, and liquid meals have been shown to result in better postprandial protein balance in the elderly. The aminoacidaemic effects of whole food protein sources may be improved through food tailoring and structuring to improving protein digestibility and facilitate gastric emptying.

Question: Can delayed aminoacidaemia and inadequate amino acid peak concentrations be attenuated by improving meal amino acid bioavailability?

Aim: To facilitate rapid amino acid absorption and adequate peak amino acid thresholds by increasing amino acid availability through meal design.

Design: Randomised cross-over breakfast challenge in healthy elderly.

Intervention: Breakfast meal: moderate carbohydrate and fat. Based on a 2500kJ meal: 20g fat (750kJ from fat, 30%), 25g protein (420kJ, 17%, including >2.2g leucine, 11g arginine, 5g glutamine) plus 80g carbohydrate (1340kJ, 53%) in the control meal.

a) ‘Control’: moderate carbohydrate, medium glycaemic index, 20g fat, moderately digestible protein source. E.g. un-ripened cheese (mozzarella); unfermented milk; unfermented sausage; maize starch.

b) ‘Optimised’: isocaloric and macronutrient matched, including tailored food design. E.g. ripened cheese (Swiss), mechanically processed (grated); fermented milk (buttermilk); fermented sausage (salami) (195); resistant starch as high amylose maize starch (797).

c) ‘Supplement’: isocaloric and macronutrient matched, in the form of a liquid meal containing isolated amino acids/hydrolysed proteins, supplemented with desired amino acids.

Measures: Plasma at baseline and hourly for 5 hours.

Amino acid profile – UPLC.

Incretin effects of the meal and hormonal regulation of gastrointestinal motility: glucose, insulin, TAG, GLP-1, GIP, CCK.

MPS – intrinsically labelled dairy and meat with continuous infusion and muscle biopsy (baseline and 5 hours) (762).

Outcomes: Treatment effect: supplement ≥ optimised > control.

Reduced:

- Glucose, insulin, and TAG iAUC and peak concentrations.

Increased:

- Amino acid appearance rate, iAUC and peak concentration.
- Gastric emptying (GLP-1, GIP, CCK).
- MPS.

These findings will provide proof of concept that food designed to improve protein digestibility may be as effective as isolated supplementation at stimulating the muscle protein synthetic response to feeding in the elderly. This will provide opportunities for food product development aimed at enhancing the protein digestibility of whole foods for the elderly.

Abbreviations: UPLC: ultra performance liquid chromatography; TAG: triacylglycerols; GLP: glucagon-like peptide 1; GIP: glucose-dependent insulinotropic peptide; CCK: cholecystokinin; MPS: muscle protein synthesis; iAUC: incremental area under the curve.
7.4.3. Long Term Interventions for Improving Health Outcomes

The ability of an acute meal challenge to improve metabolic outcomes may be limited if the underlying mechanisms (i.e. insulin resistance or inadequate basal amino acids) are unaffected acutely. Therefore, it may be difficult to produce health improvements through acute dietary interventions, and longer-term timeframes may be required to elicit metabolic changes. Hence, the long-term efficacy of dietary interventions aimed at improving insulin sensitivity, minimising lipaemia, and maximising postprandial amino acid thresholds may be a more appropriate approach for improving postprandial outcomes in the elderly.

Additionally, longer-term interventions face complications of attrition and compliance (798); therefore simple interventions are an equally important consideration for designing interventions likely to prove successful in real world scenarios. Once tailored foods have been designed, these may be tested long-term as dietary interventions. Since older adults are likely to have the lowest protein intake at breakfast (421), and metabolic activity is likely to be impacted by the breakfast meal during the day, a breakfast intervention of tailor made foods followed by an exercise regime may be an easy way to promote protein and lipid digestion. Furthermore, isolated dietary interventions have proven less effective at promoting muscle health in older adults compared with interventions including exercise regimes (421). Exercise prior to muscle amino acid exposure has proven to be an effective means of promoting the anabolic response to protein (799), while lipaemia may be reduced with exercise 12-18 hours prior to a fat load (800).

The following study design, outlined in Table 25, is proposed to evaluate a long term intervention strategy to normalise basal metabolic indices, including insulin sensitivity, amino acid and lipid profiles, and to promote improvements in postprandial aminoacidaemic and lipaemic responses.
Background: Postprandial responses are highly dependent on long-term dietary intake.

Question: Can postprandial metabolic responses and health in the elderly be improved by improving insulin sensitivity and increasing the bioavailability of essential nutrients?

Aim: To improve insulin sensitivity, reduce endogenous lipoprotein production, and improve muscle strength and mass through dietary intervention aimed at reducing postprandial hyperinsulinaemia, triacylglycerolaemia, and delayed aminoaddaemia.

Design: Cohort intervention in elderly. Acute meal challenge at baseline and after 5 week intervention.

Intervention: Intervention: provision of daily breakfast meal, formulated from control breakfast or optimised (matched macronutrients, tailored food design).

Control breakfast meal: for intervention period and acute challenge. Moderate carbohydrate and fat. Based on a 2500kJ meal: 20g fat (750kJ from fat, 30%), 25g protein (420kJ, 17%, including >2.2g leucine, 11g arginine, 5g glutamine) plus 80g carbohydrate (1340kJ, 53%) in the control meal.

a) Control diet: no adjustments to macronutrient digestibility
b) Optimised diet: tailored food design for improved protein digestibility, reduced glycaemic index, high PUFA content (omega-3), in easily accessible form.

Measures: Baseline fasting and postprandial measures, repeated after 5 weeks. Hyperinsulinaemic-euglycaemic clamp assessment on separate day at baseline and follow-up.

Compliance and habitual intake - 3 day food diary.

Fasting measures:
- Insulin sensitivity and glycaemic control – HOMA-IR, HbA1C.
- Body composition – DEXA.
- Muscle strength – Isokinetic strength testing and walking gait.
- Blood lipid profile - total cholesterol, LDL, HDL, fasting TAG.

Postprandial measures (hourly for 5 hours):
- Postprandial triacylglycerolaemia; assessment of chylomicron and VLDL TAG.
- Lipidomic assessment of chylomicron TAG and PL; differences of downstream lipids: sphingolipids, ceramides, and DAGs (120 minutes only).
- Amino acid profile – UPLC.
- Protein and mRNA inflammatory cytokine expression.

Outcomes: Treatment effect: Optimised diet + exercise > optimised diet = exercise > control.

Reduced:
- Insulin sensitivity, HOMA-IR, HbA1C, and postprandial insulinaemia.
- Android body fat.
- Fasting total and LDL cholesterol, and TAG. Postprandial TAG iAUC.
- Postprandial VLDL triacylglycerolaemia (peak and iAUC).
- Fasting and postprandial iAUC inflammatory markers.

Increased:
- Muscle mass, isokinetic strength, walking speed.
- Fasting BCAA, EAA, glutamine, PUFA (omega-6 (306)).
- Postprandial rate of appearance of amino acids, iAUC.

These findings will demonstrate the ability to improve and normalise long-term metabolic health (endogenous lipaemia and muscle maintenance) in the elderly, by providing a link between dietary interventions and functional outcomes in older adults.

Abbreviations: HOMA-IR: homeostatic model assessment of insulin resistance; HbA1C: glycated haemoglobin; DEXA: dual energy x-ray absorptiometry; TAG: triacylglycerol; PL: phospholipid; DAG: diacylglycerol; UPLC: ultra performance liquid chromatography; iAUC: incremental area under the curve; BCAA: branched chain amino acid; EAA: essential amino acid; PUFA: polyunsaturated fatty acid.
7.5. CONCLUSION

Ageing is an inevitable process, and eating a continuous daily event, with a major impact on overall health. This thesis highlights that the ordinary eating practices of older adults (i.e. whole food as meals) greatly impact digestive responses but are somehow not typically evaluated in literature. This research emphasises the importance of considering the ‘meal’ as a standard means of evaluating digestion and metabolism, since our findings deviate from previous literature, yet still indicate that older adults have altered digestive responses. These data present new evidence of metabolomic variation between the old and young in response to representative meals, bridging research gaps between individual macronutrient responses and expected responses in typical daily living. These metabolic age differences present challenges for the maintenance of health in the elderly, and the provision of age-appropriate nutritional recommendations. Yet, alongside these challenges arise future opportunities to gain an understanding of the features and implications of age-related metabolic variation, and to address these dissimilarities with advances in food development and nutritional strategies.
CHAPTER 8

APPENDIX
8.1. Milan et al., 2015


Chapter 3 of this thesis contains material which has been published. The following section contains an unaltered reproduction of the article “Older adults have delayed amino acid absorption after a high protein mixed breakfast meal”, published in the Journal of Nutrition, Health & Aging, Volume 19, Issue 8, p.839-845. The final publication is available at link.springer.com/article/10.1007/s12603-015-0500-5.
OLDER ADULTS HAVE DELAYED AMINO ACID ABSORPTION AFTER A HIGH PROTEIN MIXED BREAKFAST MEAL


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Abstract: Objectives: To measure the postprandial plasma amino acid appearance in younger and older adults following a high protein mixed meal. Design: Cross-sectional study. Setting: Clinical research setting. Participants: Healthy men and women aged 60-75 (n=15) years, and young controls aged 20-25 years (n=15) matched for body mass index and insulin sensitivity based on the homeostatic model assessment of insulin resistance. Intervention: High protein mixed meal of complete food products. Measurements: Circulating amino acid concentrations were determined hourly before and for 5 hours after meal ingestion. Results: There was no difference between cohorts in postprandial appearance of non-essential amino acids or area under the curve of any individual amino acid or amino acid chain. However, older adults had higher baseline concentrations of aspartic acid, glutamic acid, glycine, ornithine, threonine, and tyrosine and lower baseline concentrations of hydroxyproline, isoleucine, leucine, methionine, and valine compared to younger adults. Younger adults showed peak essential (EAA) and branched-chain amino acid (BCAA) concentrations at 1 hour post meal while older adults’ peak EAA and BCAA concentration was at 3 hours. Similarly, peak total amino acid concentrations were at 3 hours in older adults. Conclusion: Older adults digested and absorbed the protein within a mixed meal more slowly than younger adults. Delayed absorption of AA following a mixed meal of complete food products may suppress or delay protein synthesis in senescent muscle.

Keywords: Ageing, sarcopenia, protein digestion, mixed meal.

Introduction

The aging of the world’s population, most notably within developed economies, presents many health challenges. Ageing affects body composition, with a characteristic loss of lean muscle mass, ultimately resulting in impaired skeletal muscle function and the onset of sarcopenia (1). The etiology of sarcopenia is complex; however, lifestyle factors including the habitual diet are a significant determinant (2). Dietary protein supports the maintenance of muscle mass (3), and daily intake may be insufficient in older adults (4). Beyond the changes in habitual diet, the digestive responses and metabolic fate of ingested protein differ with advancing age. It has been shown that protein digestion rate (5) and circulating amino acid (AA) availability (6) contribute to the muscle protein synthetic response to feeding, a response known to be impaired with ageing (7). Older adults may have altered protein digestion which could contribute to changes in postprandial amino acid availability.

Age-related differences in digestive factors have been previously reported. Older adults may have decreased chewing capacity (8), gastric acid secretion (9), reduced gastric peristalsis (10) or delayed gastric emptying (11), all of which could impair older adults’ ability to digest and absorb protein (12). Older adults have lower efficiency of protein utilisation (13) and show reduced protein synthesis in the fed state, possibly due to differences in protein absorption and splanchnic use (14). Ageing is associated with higher rates of splanchnic uptake and use of AAs such as leucine (15, 16), phenylalanine (17) and glutamate (18). Increased intestinal demands for AAs (19) likely result in reduced AA appearance (15) and availability of AA for protein synthesis in ageing (14).

Older adults also have different metabolic responses to protein ingestion compared to younger adults. While in young adults, slowly digested proteins, such as casein (5), and ‘spread’ feeding patterns (protein fed throughout the day) enhance whole body protein balance and utilisation, the opposite is seen in elderly subjects. Fast proteins, i.e. whey, induce better postprandial whole body leucine balance in older subjects (20, 21) and elderly women experience better protein retention after pulse feeding (22). This shows that the kinetics of AA availability differs between young and old subjects and impacts postprandial muscle protein synthesis. However, at the level of the muscle, fast protein and bolus feeding patterns result in greater muscle protein synthesis in both young and older adults (23-26).

Protein digestion rates influence postprandial AA availability and may differ with meal composition; intact proteins such as those found in milk (casein and whey) (27) or steak (28) are more slowly digested when in these food matrices than whey alone or minced beef respectively. Additionally, carbohydrate ingested with protein delays postprandial AA appearance (29). These studies suggest that just as meal composition and food physical structure affect digestion, absorption, and subsequent appearance of carbohydrates (30) and fats (31), protein digestion is equally...
OLDER ADULTS HAVE DELAYED AMINO ACID ABSORPTION AFTER A HIGH PROTEIN MIXED BREAKFAST MEAL

dependent on meal structure. As such, meal structure and composition may be important considerations for assessing amino acid availability and ultimately muscle protein synthesis in response to protein ingestion. Surprisingly, no studies have yet reported age-related differences in amino acid appearance in the context of a whole food mixed meal.

Therefore, we aimed to demonstrate the effects of ageing on protein digestion in the context of a whole food mixed meal: we examined the postprandial AA appearance in healthy older and younger adults after ingestion of a high protein mixed meal consisting of whole food products. We hypothesised that older adults would have impaired digestion of dietary protein in a mixed meal.

Methods

Subjects

Thirty healthy, community-dwelling subjects (n=7 young women, n=8 young men, n=9 older women, n=6 older men) from the Auckland region were recruited through newspaper advertisements and from the university community to participate in the study. Eligible subjects were required to have a body mass index (BMI) between 18 and 30 kg/m² and be between the ages of 20-25 years or 60-75 years. Individuals with a history of cardiovascular or metabolic disease/conditions, or taking medications that may interfere with study endpoints (i.e. anti-inflammatory drugs, statin drugs) were not eligible for participation. All subjects gave written informed consent and the study was approved by the University of Auckland Human Participants and Ethics Committee (Ref # 8026). This study was registered prospectively at Australian New Zealand Clinical Trials Registry at anzctr.org.au (ID: ACTRN12612005158977).

Study Procedures

This cross-sectional trial was conducted at the Maurice and Agnes Paykel Clinical Research Unit (MAPCRU) at the Liggins Institute, University of Auckland, Auckland, New Zealand. The mixed breakfast meal (Table 1) was formulated to follow the Australian Guide to Healthy Eating, while maintaining a low fat load (16.6 g), and high protein (49.8 g) and carbohydrate (77.4 g) loads with a total energy content of 2790 kJ. The meal was prepared onsite at the MAPCRU.

Subjects were required to abstain from vigorous physical activity, high fat foods, anti-inflammatory medications, and dietary supplements the day prior to their visit. Subjects arrived fasted at the MAPCRU; anthropometric data were collected before a catheter was inserted into an antecubital vein and a baseline sample (time 0) was taken followed by consumption of the breakfast. Blood samples were collected hourly up to 5 hours post meal from resting subjects into blood collection tubes (BD, Mt Wellington, New Zealand) for serum and EDTA plasma. Serum tubes were allowed to clot for 15 minutes at room temperature before serum and plasma tubes were centrifuged at 1500 × g for 15 minutes at 4°C and the supernatants collected in pyrogen-free microtubes and stored at -20°C until analysis.

Serum free amino acid concentrations and plasma measures

Free amino acids were assayed from 20µl of serum with 15µM L-Nor-Valine as an internal standard extracted with 20µl 10% sodium tungstate and 160µl of 0.44M sulphuric acid. The mixture was incubated on ice for 3 minutes then centrifuged at 12000 × g for 10 minutes at 4°C. 70µl of 0.2M borate buffer (pH 8.8) was added to the supernatant before adding 10µl of AceQ-tag reagent (2.8 µg/ml in acetonitrile). In a sealed vial, the mixture was heated at 55°C for 10 minutes before being subjected to ultra performance liquid chromatography (UPLC). The UPLC system used a Thermo Scientific Dionex Ultimate 3000 pump autosampler (maintained at 10°C), column oven and fluorescence detector (set at Ex 250 nm, Em 395 nm) (Thermo Scientific, Dornierstrasse, Germany), and a Kinetex 1.7µm C18 100A 100 x 2.1 mm column, preceded by a Knudkette inline filter (Phenomenex, Auckland, New Zealand) at 45°C. Mobile phase buffer, (80 mM sodium acetate, 3 mM triethylamine, 2.67µM disodium calcium ethylenediaminetetraacetic acid) at pH 6.43 (obtained by addition of orthophosphoric acid), run with a complex gradient of acetonitrile from 2% to 17% (balance, water) over 24 minutes. Data was directly captured by computer with Chromelion 7.1 software (Thermo Scientific). AA concentrations were calculated from standard curves generated for each AA from the standard injections. The internal standard (L-Nor-Valine) signal in each chromatogram was used for data normalisation for analyte recovery and quantification.

Plasma glucose was measured using a Hitachi 902 autoanalyzer (Hitachi High Technologies Corporation, Tokyo, Japan) by enzymatic colorimetric assay (Roche, Mannheim, Germany). Plasma insulin was measured using an Abbott AxSYM system (Abbott Laboratories, Abbott Park, USA) by microparticle enzyme immunoassay.

Calculations

Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated from fasting glucose and insulin concentrations using the equation from Matthews, et al. (1985, 32). Serum free amino acids were pooled for mathematical analysis into total amino acids (all proteogenic amino acids), essential amino acid (EAA), branched-chain amino acids (BCAA) and non-essential amino acids (NEAA). Although arginine, proline, glutamine, glycine, taurine and tyrosine are considered conditionally essential, these were considered nonessential as the study criteria excluded persons with critical illness or malnutrition.
Table 1

Macronutrient composition of breakfast meal. Values presented are based on available nutrient panel data for individual products

<table>
<thead>
<tr>
<th>Item name</th>
<th>Weight (g)</th>
<th>Carbohydrates (g)</th>
<th>Fat (g)</th>
<th>Protein (g)</th>
<th>Energy (kJ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rolled oats</td>
<td>37.0</td>
<td>20.8</td>
<td>1.9</td>
<td>5.0</td>
<td>495</td>
</tr>
<tr>
<td>1% cottage cheese</td>
<td>167.0</td>
<td>4.5</td>
<td>1.0</td>
<td>19.7</td>
<td>450</td>
</tr>
<tr>
<td>Mixed grain bread</td>
<td>41.5</td>
<td>11.2</td>
<td>2.2</td>
<td>5.1</td>
<td>390</td>
</tr>
<tr>
<td>Reduced fat peanut butter, smooth</td>
<td>25.0</td>
<td>8.4</td>
<td>9.4</td>
<td>4.4</td>
<td>575</td>
</tr>
<tr>
<td>Fresh peach</td>
<td>154.0</td>
<td>14.6</td>
<td>0.3</td>
<td>1.4</td>
<td>250</td>
</tr>
<tr>
<td>Trim milk</td>
<td>365.0</td>
<td>17.9</td>
<td>1.8</td>
<td>14.2</td>
<td>630</td>
</tr>
<tr>
<td>Total</td>
<td>773.4</td>
<td>166.6</td>
<td>16.6</td>
<td>49.8</td>
<td>2760</td>
</tr>
</tbody>
</table>

Statistical analyses
Statistical analyses were conducted with SPSS. Data are represented as means ± SEMs. Incremental area under the curve (AUC) was calculated after subtracting fasting values. Baseline subject characteristics, amino acids, AUC, and maximum peak concentrations were compared using Student’s t test. Two-factor (age and time) repeated-measure ANOVA followed by Sidak post hoc test was used for all multiple comparisons between different groups. Alpha was set at P<0.05. The heatmap representation of postprandial amino acids concentration as a percent change relative to younger fasting values was created with R software.

Figure 1
Postprandial plasma glucose (A) and insulin (B) concentrations in older (●) and younger (○) adults

Values represent mean ± SEM in nmol/L and μU/ml, respectively. No main effects of age or time were identified by two-factor repeated-measures ANOVA.

Results

Subject characteristics
A total of 30 subjects completed the study. Subject characteristics are shown in Table 2. There were no age differences in BMI, fasting measurements of glucose, or HOMA-IR. Glucose and insulin response to the meal was not different between age groups (Figure 1).

Figure 2

Postprandial plasma concentrations of total amino acids (TAA) (A), BCAA (B), BCAA AUC (B inset), EAA (C), and NEAA (D)

Values represent mean ± SEM in μmol/L. AUC presented as arbitrary units. There were significant differences were time in the TAA, BCAA, and EAA responses between older (●) and younger (○) adults (t-tests; p<0.001, p<0.001, and p<0.001 respectively, two-factor repeated-measures ANOVA). There were no differences in NEAA responses at BCAA AUC between older and younger adults. *p<0.05, **p<0.01 older vs. younger at age-related point (Sidak)

Older adults have altered basal amino acid profiles
Baseline glutamine concentrations were 15% lower in older adults compared to younger adults (P=0.042) while glutaminic acid was 193% greater (P<0.001, Table 2). Older adults also had higher baseline concentrations of aspartic acid, glycine, ornithine, threonine, and tyrosine than younger adults, and lower concentrations of hydroxyproline, isoleucine, leucine, methionine, and valine (Table 2).
Table 2
Baseline subject characteristics and serum amino acid profile of older and younger adults

<table>
<thead>
<tr>
<th>Subject characteristics</th>
<th>Younger adults (n=15; 8 men, 7 women)</th>
<th>Older adults (n=15; 6 men, 9 women)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>22.7 ± 4.0</td>
<td>67.3 ± 1.5***</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.8 ± 0.8</td>
<td>24.4 ± 1.0</td>
</tr>
<tr>
<td>Fasting Plasma Glucose (mmol/L)</td>
<td>5.1 ± 0.1</td>
<td>5.2 ± 0.1</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.2 ± 0.5</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>Branched-Chain Amino Acids:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>84.7 ± 6.5</td>
<td>65.3 ± 2**</td>
</tr>
<tr>
<td>Leucine</td>
<td>146.9 ± 8.5</td>
<td>125.8 ± 5*</td>
</tr>
<tr>
<td>Valine</td>
<td>269.2 ± 17.8</td>
<td>226.9 ± 9.7*</td>
</tr>
<tr>
<td>All Other Essential Amino Acids:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>77.8 ± 5.2</td>
<td>74.4 ± 3.7</td>
</tr>
<tr>
<td>Lysine</td>
<td>158.5 ± 12</td>
<td>150.6 ± 8</td>
</tr>
<tr>
<td>Methionine</td>
<td>46.9 ± 3.1</td>
<td>38.2 ± 1.9*</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>72.7 ± 4.5</td>
<td>73.6 ± 3.5</td>
</tr>
<tr>
<td>Threonine</td>
<td>58.6 ± 2.5</td>
<td>84.9 ± 5.4*</td>
</tr>
<tr>
<td>Non-Essential Amino Acids:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>401.0 ± 24.3</td>
<td>411.8 ± 21.3</td>
</tr>
<tr>
<td>Arginine</td>
<td>76.2 ± 6.6</td>
<td>84.4 ± 4.9</td>
</tr>
<tr>
<td>Asparagine</td>
<td>52 ± 4.7</td>
<td>-44 ± 2.1</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>67.7 ± 1.2</td>
<td>12.4 ± 1.3*</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>71.8 ± 16.6</td>
<td>210.4 ± 17**</td>
</tr>
<tr>
<td>Glutamine</td>
<td>556.7 ± 49.2</td>
<td>475.2 ± 30.2*</td>
</tr>
<tr>
<td>Glycine</td>
<td>256.6 ± 15.2</td>
<td>311.6 ± 26.4*</td>
</tr>
<tr>
<td>Proline</td>
<td>192.6 ± 11.9</td>
<td>189.4 ± 11.5</td>
</tr>
<tr>
<td>Serine</td>
<td>120.6 ± 8.3</td>
<td>125.3 ± 21.6</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>80.9 ± 7.5</td>
<td>88.4 ± 20.5*</td>
</tr>
<tr>
<td>Non-proteinogenic Amino Acids:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Methylhistidine</td>
<td>11.2 ± 2</td>
<td>7.1 ± 1.2</td>
</tr>
<tr>
<td>Citrulline</td>
<td>66.3 ± 4.4</td>
<td>64.6 ± 1.9</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>15.6 ± 1.9</td>
<td>10.2 ± 0.8*</td>
</tr>
<tr>
<td>Ornithine</td>
<td>52.6 ± 3.4</td>
<td>65.8 ± 5.7*</td>
</tr>
<tr>
<td>Taurine</td>
<td>109.7 ± 10.8</td>
<td>107.3 ± 8.3</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM. Amino acid values measured in mmol/L. HOMA-IR: Homeostatic model assessment of insulin resistance. Significance was determined by Student's t-test. ***P<0.001, **P<0.01, *P<0.05 compared with younger adults.

Older adults have delayed postprandial appearance of amino acids
Older adults showed a delayed increase in serum postprandial concentrations of TAA's, BCAA's, and EAAs (Figure 2). No such differences were apparent in NEAA appearance (Figure 2). Younger adults had higher concentrations of BCAA (Figure 2 B) and EAA (Figure 2 C) at 1 hour after the meal when compared to older adults. In older adults, serum concentrations of TAA's, BCAA's, and EAAs peaked at 3 hours post meal, at which time circulating concentrations were significantly higher compared to younger adults. The AUC for Isoleucine tended to be greater in older adults compared to younger adults (P=0.067). AUC did not differ between any other individual AA or class of AA. Maximal AA concentration did not differ between younger and older adults for AAs that did not show previous baseline differences, maintaining that total postprandial plasma availability was not different between age groups. A heatmap displaying mean percentage change in all detected individual serum amino acids from fasting serum concentrations in younger adult is presented (Figure 3).

Figure 3
Heatmap of postprandial changes in amino acid concentrations of older and younger adults

Values are presented as mean percent changes relative to younger adult time 0 concentration for each amino acid (i.e., the lightest grey represents a 0% change from younger baseline). White represents negative percentage changes, darker shades represent positive percentage changes up to 150%; BCAA: branched-chain amino acids; EAA: essential amino acids; NEAA: non-essential amino acids.

Discussion
For the first time we have shown that older adults digest and absorb amino acids more slowly than younger adults after a mixed meal high in protein. Older adults had similar overall
protein appearance by AUC after a mixed meal, but delayed appearance of EAA and BCAA.

Previous postprandial protein kinetic research in older adults has generally investigated the response to a variety of independent macronutrient components such as individual amino acid supplements (17, 33-35), protein fractions (hydrolysed (36, 37) or intact (14, 20, 21)), and protein (28, 38) or carbohydrate (39) independently. A few studies have tried to emulate the macronutrient composition of whole foods through formulations with protein isolates, carbohydrate in the form of dextin, and fats such as vegetable oils (15, 27, 29, 40-43). Our study is the first to look at the differential aminoaemic response in older adults to protein ingestion in the physiological context of feeding a real food mixed meal.

Our study population was well matched for BMI, insulin sensitivity, and fasting plasma glucose. Nevertheless, baseline amino acid profiles appeared to differ between older and younger adults. Older adults displayed lower fasting concentrations of serum BCAAs as has been reported in some (44, 45) but not all (46) previous studies. Furthermore, significant baseline differences in other individual plasma EAA, NEAAs, and non-proteogenic amino acids were observed. Importantly, we found that higher baseline concentrations for some amino acids in older adults only impacted postprandial peak concentrations, not overall total serum amino acid response as determined by postprandial AUC. Nevertheless, differences in basal concentrations of certain AAs may contribute to absolute postprandial AA concentrations in older adults.

There is already clear evidence that in older adults, slower availability of amino acids after protein feeding in isolation results in poorer postprandial protein balance, in contrast with young adults. Slowly digested proteins like casein (20) or spread feeding patterns (19) result in lower postprandial leucine balance and nitrogen balance respectively in older adults. In the present study, we have shown that in older adults, ingesting protein in the form of a mixed meal results in slower amino acid appearance when compared to younger adults. Although not measured in the current study, this would potentially result in suboptimal postprandial protein balance and an impaired muscle protein synthesis.

Meal composition and structure are known to impact digestion and absorption in older adults; mixed beef enhanced protein digestion and absorption in elderly men when compared to beef steak (28). Similarly, casein in a hydrolysed state accelerated protein digestion in elderly men when compared to intact casein (36), showing that older adults have slower protein digestion and absorption with more intact ingested protein. This supports our finding that combined and intact casein and whey were more slowly digested and absorbed by older adults when studied in the context of a mixed meal. As previous studies have shown no impact of age on casein (14) or whey (37) digestion when ingested independently, our data highlight the importance of assessing protein metabolism in the context of intact proteins in representative whole ingested foods.

The anabolic resistance to food in older adults (47) is demonstrated through reduced muscle protein synthetic responses to EAA ingestion (34) which may reflect delayed or prolonged postprandial availability of EAA and BCAAs as suggested by Condino, et al. (2013). It is conceivable that delayed availability of EAA in older adults may contribute to reduced postprandial muscle protein synthesis and anabolic resistance in ageing. Adequate circulating concentrations of EAA is primarily responsible for the stimulation of muscle protein synthesis (50), particularly in healthy elderly adults (35), while NEAAs seem to be less important (48). Furthermore, the leucine trigger hypothesis suggests that a threshold concentration of leucine is required to adequately stimulate muscle protein synthesis (49), a threshold that may not necessarily be reached following delayed protein absorption. It appears that adequate EAA must be present in circulation at the same time as the leucine peak (50), suggesting that a lag in total AA availability caused by delayed digestion and absorption in ageing could impact on muscle protein synthesis. This is supported by evidence that older adults show no difference in muscle protein synthesis rate when studied throughout the later postprandial period, which may suggest a delay in early feeding induced muscle protein synthesis activation (51), possibly attributable to delayed EAA digestion and absorption.

A possible explanation for the delayed postprandial appearance of EAA in older adults is increased intestinal use of many EAA. Increased splanchic leucine extraction in elderly men has already been demonstrated (15); however, Koopman, et al. (2008) showed no significant effect of age on splanchic extraction of dietary phenylalanine. As our data do not show decreased postprandial AUC of any EAA in older adults, splanchic use of EAA is not a likely explanation for delayed EAA appearance after a mixed meal. Furthermore, our data show no different postprandial changes in dietary glutamine, glutamic acid or aspartic acid, which are nearly completely used by the intestines (22), implying no difference in postprandial intestinal use of dietary amino acids between older and younger subjects.

Co-ingestion of carbohydrates with protein in elderly adults results in slower amino acid plasma appearance just as in younger adults (29), but this may in fact accelerate dietary amino acid incorporation into elderly muscle, although this effect may not be sustained in the later postprandial period (41). Previous studies in older adults have shown that ingestion of isolated EAA results in greater postprandial aminoacidemia (33) and that casein ingestion elicits a greater AUC appearance of BCAAs (14) compared to younger adults. Our data show no AUC differences in EAA or BCAAs suggesting that in a mixed meal, intact protein ingested with carbohydrates may negate a greater AUC protein appearance in older adults.

Despite delayed amino acid appearance, carbohydrate co-ingestion does not impact on muscle protein synthesis
OLDER ADULTS HAVE DELAYED AMINO ACID ABSORPTION AFTER A HIGH PROTEIN MIXED BREAKFAST MEAL

in young (39, 53) or elderly (29) adults. In young adults, the slower appearance of casein from mixed dairy ingestion resulted in more sustained systemic amino acid delivery to the skeletal muscle (27). Our data suggest that this delay in amino acid appearance after mixed dairy ingestion may be even further delayed in older adults, however, this delay may not predict an effect on skeletal muscle delivery.

Conclusions

This study shows that older adults digest and absorb proteins from a mixed meal more slowly than younger adults. Furthermore, protein ingestion in a mixed meal is a relevant concern when assessing amino acid availability and use after a meal since these age-related delays in protein availability have not been previously reported. This slower rate of protein digestion and absorption in aging after a mixed meal may provide some explanation for the observed decreases in postprandial muscle protein synthesis reported previously, and should be an important consideration when applying protein ingestion strategies for nutritional recommendations in older adults. As such, future investigations into dietary effects on muscle protein synthesis in aging should explore these questions in the context of real foods, representative of a normal and realistic diet.

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Ethical Standards: The experiments in this study comply with the current laws of the country in which they were performed.

Conflict of interest: AMD, RPD, SP, CAR, MPGB, JFM, DCS and CIJ have no conflicts of interest to declare.

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