The action of oleic acid, oleoylethanolamide and allied genetic variants in influencing body composition

by

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Abstract

N-acylethanolamines (NAEs) are lipid-derived signalling mediators synthesized from fatty acids (FA) that regulate numerous pathophysiological functions. Pharmacological research has explored the area of NAEs, while only limited nutritional studies have been conducted to understand the role of diet FA composition on NAE levels and the actions of NAEs, especially, oleoylethanolamide (OEA) on food intake-energy expenditure (EE) regulation and body composition (BC). The objective of this research was therefore to investigate plasma and tissue/organ NAE concentrations in hamsters in response to consumption of various dietary oils with different FA composition to understand the actions on EE and BC. A further objective was to characterize the effect of diets varying in monounsaturated fatty acid (MUFA) composition on plasma NAE concentrations and BC variables in humans. A human clinical trial using a cross-over design and energy-controlled paradigm was performed to elucidate the role of diet × genetic polymorphism interactions on NAEs and their influence on BC parameters. Results from both animal and human trials reveal that dietary FA composition influenced both plasma and tissue FA and NAE levels, with marked increases observed in plasma C18:1n9 and corresponding OEA levels after C18:1n9-enriched treatments. Animal data showed significant shifts in intestinal-brain OEA concentrations post consumption of C18:1n9-

enriched diets. A negative correlation was observed between gut-brain OEA concentrations and body weight. No differences were observed across olive oil, high oleic canola oil (HOCO), and HOCO diets with omega-3 FA blends in terms of EE and overall BC. The human trial data demonstrated an inverse relationship between the intake of C18:1n9 and alterations in body weight as a function of *LEPR* rs1137101-AA vs -GG polymorphism. The diet-genotype pattern interactions also showed elevated concentrations of plasma OEA in participants possessing the *GRP40* rs1573611-T allele post consumption of high-MUFA, but not low-MUFA diets. The findings demonstrate that plasma OEA concentrations reflect the dietary pattern of C18:1n9 intake and may be influenced by *GRP40* rs1573611 polymorphism. Humans possessing *GPR40* rs1573611-T and *LEPR* rs1137101-AA polymorphisms could benefit more from ingestion of C18:1n9-enriched dietary oils. Henceforth, we summarize that the endogenously synthesized increased OEA levels in the tissues enable the gut-brain-interrelationship. It can be speculated that the brain transmits anorexic properties mediated via neuronal signalling influenced by genetic variations; which may contribute to the maintenance of healthy body weight, consequently affecting the overall BC and wellness. Thus, the benefits of OEA can be enhanced by the inclusion of C18:1n9-enriched diets, pointing to the possible nutritional use of this naturally occurring bioactive lipid-amide in the management of obesity.

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Dedication

I dedicate my thesis to my late father whom I lost at a very tender age

and

my mother

Thanks for always being there…

Contents

List of Tables

List of Figures

Abbreviations

2-AG	2-Arachidonoylglycerol
ACD	Acyl-CoA-dehydrogenase
AEA	Arachidonoylethanolamide
AHA/NHLBI	American Heart Association/National Heart, Lung, and Blood Institute
ALEA	α-Linolenoylethanolamide
AM	Android mass
AMDR	Acceptable Macronutrient Distribution Range
AOX	Acyl-CoA-oxidase
AP	Area postrema
ARC or ARH	Arcuate nucleus of the hypothalamus
ATB	Adipose tissue brown
ATW	Adipose tissue white
BAT	Brown adipose tissue
BBB	Blood-brain barrier
BLA	Basolateral complex of the amygdala

BSS	Behavioural satiety sequence
BW	Body weight
C+S	25:75 corn oil:n9 safflower oil
cAMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin
CD36	Cluster of differentiation 36
CNR1	Cannabinoid receptors
CNS	Central nervous system
CO	Control oil
CRP	C-reactive protein
DFA	Dietary fatty acids
DHEA	Docosahexaenoylethanolamide
DIO	Diet-induced obesity
DIT	Diet-induced thermogenesis
DMH or DMN	Dorsomedial hypothalamic nucleus
DRD2	Dopamine receptor D2
DRN	Dorsal raphe nuclei
DS	Dorsal striatum
DXA	Dual energy X-ray absorptiometry
EE	Energy expenditure
EPEA	Eicosapentaenoylethanolamide
F+S	25:75 flaxseed oil:n6 safflower oil

FA	Fatty acid
FAAH	Fatty-acid amide hydrolase
FAEs	Fatty acid ethanolamides
FAs	Fatty acids
FAT	Fatty acid translocase
fMRI	Functional magnetic resonance imaging
FXCO	High oleic canola oil blended with flaxseed oil
GLP-1	Glucagon-like polypeptide-1
GPCRs	G protein-coupled receptors
GPR40	G protein-coupled receptors 40
H+DHA	85:15 high oleic canola oil:docosahexaenoic acid
H+EPA	85:15 high oleic canola oil:eicosapentaenoic acid
HDC	Histidine decarboxylase
HDL-C	High density lipoprotein cholesterol
HFD	High fat diet
HOCO	High oleic canola oil
HPA or HTPA axis	Hypothalamus–pituitary–adrenal axis
HR	Histamine receptors
I-D	Intestine-duodenum
I-J	Intestine-jejunum
i.c.v.	Intracerebroventricularly
i.p.	Intraperitoneally

IDF	International Diabetes Federation
IL-6	Interleukin-6
IL-8	Interleukin-8
JAK2/STAT3	Janus kinase 2-signal transducers and activators of transcription 3
LC	Liquid chromatography
LCFA	Long chain fatty acids
LDL-C	Low density lipoprotein cholesterol
LEA	Linoleoylethanolamide
LEPR	Leptin receptors
LF-HSD	Low fat-high sucrose diet
LFHS	Low fat high sucrose
LH	Lateral hypothalamus
MC4R	Melanocortin-4 receptor
MCFA	Medium chain fatty acids
MEA	Myristoylethanolamide
MetS	Metabolic syndrome
MUFA	Monounsaturated fatty acids
NAAA	*N*-acylethanolamine-hydrolyzing acid amidase
nAChRs	Nicotinic acetylcholine receptor
NAcS	Nucleus accumbens shell
NAEs	*N*-acylethanolamines

NAPE-PLD	*N*-acyl phosphatidylethanolamine phospholipase D
NAPEs	*N*-acyl phosphatidyl ethanolamines
NAT	*N*-acyl transferase
NO	Nitric oxide
NOPE	*N*-oleoyl-phosphatidylethanolamine
NST	Nucleus of the solitary tract
OEA	Oleoylethanolamide
OO	Olive oil
PE	Phosphatidylethanolamine
PEA	Palmitoylethanolamide
PGC1-α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PKA	Protein kinase A activity
PKC	Protein kinase C
PPAR-α	Peroxisome proliferator-activated receptor type-alpha
PPARs	Peroxisome proliferator-activated receptors
PUFA	Polyunsaturated fatty acids
PVN	Para-ventricular nucleus
PYY	Peptide YY
RBC	Red blood cells
RC	Regular canola oil
RCO	Regular canola oil

RER	Respiratory exchange ratio
RMR	Resting metabolic rates
RVLM	Rostral ventrolateral medulla
SAR	Subcutaneous to android fat mass ratio
SAT	Subcutaneous adipose tissue
SATM	Subcutaneous adipose tissue mass
SDA	Subdiaphragmatic vagal deafferentation
SEA	Stearoylethanolamide
SFA	Saturated fatty acids
SIRT1-PGC1-α	Sirtuin 1-Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
SNPs	Single nucleotide polymorphisms
SNS	Sympathetic nervous system
SON	Supraoptic nuclei
SVR	Subcutaneous to visceral fat mass ratio
TEF	Thermic effect of food
TG	Triglycerides
TRANS	Trans fatty acids
TRPV1	Transient receptor potential vanilloid 1
TVX	Subdiaphragmatic vagotomy
UCP-1	Uncoupling protein-1
VAT	Visceral adipose tissue

VATM	Visceral adipose tissue mass
VMH or VMN	Ventromedial hypothalamic nucleus
WBC	White blood cells
WD	Western diet
α-FMH	α-fluoromethylhistidine

Chapter 1

Overall Introduction

1.1 Introduction

The prevalence of obesity has almost tripled worldwide since 1975. In 2016, more than 1.9 billion adults, 18 years and older, were overweight; of these, over 650 million were obese (1). One of the primary drivers of the obesity epidemic is the consumption of an energy-dense high-fat diet which promotes hyperphagia secondary to attenuation of the gut-brain signalling mechanisms involved in the control of food intake (2). Moreover, decreased levels of physical activity are significant elements in the ongoing obesity epidemic leading to metabolic disorders. Therefore, to combat the challenges associated with obesity, research and knowledge on appetite regulation have increased in the past decade.

Appetite regulation is multifactorial, wherein appetite and energy homeostasis are regulated via stimulatory (orexigenic) or inhibitory (anorexigenic) signalling pathways (3). These cascades are modulated by the central nervous system (CNS) in concert with the gastrointestinal system, and adipose tissue, activating various energy expenditure

regulating hormones such as leptin (4). Furthermore, the regulation of appetite and food intake is partly governed by lipid mediators termed fatty acid ethanolamides (FAEs), in particular, the lipid amide, oleoylethanolamide (OEA) (5). OEA is endogenously synthesised post-consumption of monounsaturated fatty acids (MUFA), especially C18:1n9 but not the polyunsaturated fatty acids (PUFA) (3, 6, 7). Moreover, the controversies regarding the health effects of high-MUFA vs low-MUFA, i.e. either high-saturated fatty acids (SFA) or high-PUFA have remained a debatable subject. An increasing amount of scientific data show that the high-MUFA associates with shifts in energy balance resulting in higher caloric expenditure and reduced appetite (3).

OEA acts peripherally and causes a state of satiety accompanied by prolonged inter-meal intervals, reduced size of feedings, and increased fatty acid uptake. These key actions enlisted act via interaction with the peroxisome proliferator-activated receptor α (PPAR-α) and the transient receptor potential cation channel vanilloid-1 (TRPV1), which stimulate the vagal nerve (8), thereby indirectly signalling satiety to the hypothalamic nuclei (9). OEA has shown stronger appetite-reducing effects in animal models when compared to other FAEs that include myristoylethanolamide, stearoylethanolamide, palmitoylethanolamide, linoleoylethanolamide, α-linoleoylethanolamide, eicosapentaenoylethanolamide, and docosahexaenoylethanolamide (10).

Moreover, the effect of the quality of dietary fat on the obesity phenotype and obesity-related genes has been demonstrated (11). To date, limited data exist revealing interactions between genetic variants involved in the synthesis and degradation of FAEs

and resulting circulating concentrations of FAEs (12, 13). However, to the best of our knowledge, no research has looked into common variants within genes that stimulate the satiating activity of FAEs. Accordingly, this project represents a novel investigation of health benefits of various dietary oil blends and poses a comparison between high MUFA vs low MUFA diets through proposed FAE-related mechanisms of energy balance regulation, as impacted by an individual's genetic makeup. In addition, the impact of C18:1n9-enriched dietary oils on improving body composition requires further investigation. Therefore, a nutritional feeding experiment entailing an animal trial was selected to demonstrate which organs are involved in initiating an anorexic signalling, inducing satiety through the metabolic regulators such as FAEs. Additionally, a robust human trial with a crossover format was selected to investigate whether high-MUFA consumption assists in regulating overall body composition, leading to better health and wellness. Hence, the findings will provide a fundamental step towards an era of personalised nutrition to control appetite in obesity.

1.2 Rationale

Obesity is a contributing factor to most chronic diseases and a burden on human health throughout the world. The ability to limit or even reduce excess weight gain through modification of fatty acid intake would be beneficial in combating the obesity epidemic. Pharmacological evidence suggests that OEA and endocannabinoids act as key fat-dependent regulators of hunger and satiety. OEA is a lipid amide that is released by enterocytes upon absorption of dietary fat, especially C18:1n9 and may engage in the

initiation of satiety. Henceforth, providing a rationale that dietary C18:1n9 may elicit its action in weight control through mechanisms that involve OEA-mediated anorexigenic signalling, modulating energy metabolism. Despite considerable literature on the subject, science has failed to prevent the catastrophe of obesity and hence, the treatment of obesity continues to be far from satisfactory. To date, the available treatment options have significant limitations due to low efficacy, compliance issues, and associated side effects. Therefore, a need exists to explore the family of FAEs which are likely to improve body composition with no adverse side effects.

The voluminous pharmacological literature on rodent models demonstrates the anti-obesity properties of OEA. However, failure to translate promising effectiveness of OEA from animal models to human beings warrants further investigation of consumption of C18:1n9-enriched dietary oils on body composition parameters. Furthermore, the effect of novel combinations and supplementation of various n9-enriched dietary fats on different FAEs, body composition, and energy expenditure within different organs in an animal trial has never been studied, since the tissue-specific effects are difficult to measure in humans due to the difficulty in sampling specific tissue. Therefore, for applicable purposes, two robust clinical trials were designed with our primary goal to examine the effects of C18:1n9 on body composition. Moreover, the impact of variations in candidate genes involved in fat taste receptors and FAE synthesis as well as degradation will also be investigated to understand whether common genetic variants/single nucleotide polymorphisms (SNPs) influence the response on body composition and FAE levels.

Overall, the work performed by pioneers, presented and discussed in the detailed review by Sihag and Jones (3) (Chapter 2, Manuscript 1) assisted in identifying the current application/knowledge gap, and henceforth, the present study will advance our understanding of the effect of FAEs on weight management. Additionally, this research is postulated to assess the modulatory effect of candidate SNPs on the response of body weight to n9-enriched high oleic canola oil (HOCO) consumption and hence, will characterise sub-groups of individuals who may benefit more from HOCO consumption than other sub-populations.

Altogether, the rationale can be described below:

1. FAEs are formed from fatty acids, so different dietary fatty acids may affect the quantity as well as the distribution of specific endogenous FAE concentrations that may impact various organs, improving body composition parameters to maintain overall health.

2. Human physiology is complex and may be influenced by fat taste receptors involved in fatty acid perception that may alter body composition.

3. Human genetic variants involved in synthesis and degradation of FAEs, as well as the SNPs stimulating the action of FAEs, may affect body composition impacting FAE concentrations and vice versa.

1.3 Objectives

The overarching aim of the present research program is to identify whether the dietary consumption of C18:1n9-enriched oils, stimulating endogenous OEA signalling, in particular, is essential in mediating critical components of body weight regulation. Furthermore, the objective of the present reseach is to explore the effect of a combination of candidate SNPs involved in fat taste receptors as well as FAEs on body composition in response to an enhanced dietary C18:1n9 consumption. The long-term goal is to predetermine who will benefit more to n9-enriched nutritional intake as a weight management nutritional therapy.

Specific objectives include:

1. To understand the role of FAEs on satiety and energy expenditure in hamsters.

2. To investigate the effect of different dietary oils on FAE concentrations in various organs of hamsters that may influence overall body composition.

3. To explore the effect of high-monounsaturated vs low-monounsaturated diet and genotype pattern on plasma FAE concentrations.

4. To elucidate the impact of high-monounsaturated and low-monounsaturated dietary oils in overweight adult humans and demonstrate the interactions among SNPs of fatty acid taste receptors and body composition.

1.4 Hypotheses

The hypotheses to be tested include:

1. Different dietary oils to be investigated will impact the plasma and tissue FAE concentrations. Dietary fatty acids will act as precursors for FAEs.

2. The level of OEA generated through different dietary interventions will associate with the extent of reduction in body weight. Elevated levels of OEA post-consumption of C18:1n9-enriched dietary oils will demonstrate an inverse relationship with body weight.

3. Genetic variants/SNPs in humans related to FAE metabolism will affect FAE concentrations. The heterogeneity in candidate genes involved in FAE metabolism will influence the degree of circulating FAE response, following the consumption of dietary oils.

4. SNPs involved in fatty acid receptors and a hypothalamic regulatory marker will influence the body composition in humans. The ingestion of C18:1n9-enriched dietary oils in individuals with sensitivity to the fatty acid receptors as well as a hypothalamic regulatory marker will help to improve the body composition variables.

1.5 Organization of thesis

The research work presented in the thesis comprises two trials:

1. **Animal trial:**

The trial was conducted to investigate the 1st and 2nd objectives.

2. **Human trial:**

The trial was held to investigate the 3rd and 4th objectives.

Altogether, the thesis is drafted in 'Sandwich Style'. It includes five manuscripts. Manuscripts 1, 2, and 3 (Chapters 2, 3, and 4), respectively, have been 'Published'. Manuscripts 4 and 5 (Chapters 5 and 6) are 'In Preparation'. The thesis incorporates a bridge in between manuscripts to describe the interconnectivity between chapters.

The thesis commences with a general introduction (Chapter 1) and the related work is discussed in the next chapter (Chapter 2). The first manuscript (Chapter 2) summarises the literature surrounding the pharmacological as well as dietary action of OEA in animals and humans. The literature review is followed by four research manuscripts focussing each objective precisely. The second manuscript (Chapter 3) entails the animal work using golden Syrian hamster model that addresses the impact of various dietary oil blends on plasma fatty acid composition and plasma FAE levels, influencing energy expenditure and body composition. The third manuscript (Chapter 4) extends the animal work concentrating on the impact of the dietary fatty acid profile on circulating and tissue FAE concentrations. The fourth manuscript (Chapter 5) involves a human trial, addressing the effect of high-monounsaturated and low-monounsaturated diet as well as genotype pattern on plasma FAE concentrations. The fifth manuscript (Chapter 6) expands the work conducted in human trial describing the effect of high-monounsaturated vs low-monounsaturated dietary oils on plasma fatty acids and body

composition in overweight adults. Chapter 7 concludes the thesis and outlines the future work.

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Bridge to Chapter 2

Chapter 2 provides a cohesive and comprehensive narrative review of scientific evidence established in the field of lipid amide modulators, FAEs, especially OEA. The literature review cited demonstrates the pharmacological as well as limited nutritional data contributing to the anorexic signalling triggered by OEA. Therefore, the following chapter reveals how the consumption of C18:1n9 stimulates energy expenditure and induces satiety via the activation of OEA. Moreover, the findings reported served as the foundation of research projects presented in Chapters 3, 4, 5, and 6.

Chapter 2

Literature Review

Manuscript 1

This manuscript has been published in the Obesity Reviews: An official journal of the International Association for the study of obesity 2018; **19**: 178–197.

Oleoylethanolamide: The role of bioactive lipid amide in modulating eating behaviour

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2.1 Abstract

Fatty acid ethanolamides are lipid mediators that regulate a plethora of physiological functions. One such bioactive lipid mediator, oleoylethanolamide (OEA), is a potent agonist of the peroxisome proliferator-activated receptor-alpha (PPAR-α), which modulates increased expression of the fatty acid translocase CD36 that enables the regulation of feeding behaviour. Consumption of dietary fat rich in oleic acid activates taste receptors in the gut activating specific enzymes that lead to the formation of OEA. OEA further combines with PPAR-α to enable fat oxidation in the liver, resulting in enhanced energy production. Evidence suggests that sustained ingestion of a high-fat diet abolishes the anorexic signal of OEA. Additionally, malfunction of the enterocyte that transforms oleic acid produced during fat digestion into OEA might be responsible for reduced satiety and hyperphagia, resulting in overweight and obesity. Thus, OEA anorectic signalling may be an essential element of the physiology and metabolic system regulating dietary fat intake and obesity. The evidence reviewed in this article indicates that intake of oleic acid, and thereby the resulting OEA imparting anorexic properties, is dependent on CD36, PPAR-α, enterocyte fat sensory receptors, histamine, oxytocin and dopamine; leading to increased fat oxidation and enhanced energy expenditure to induce satiety and increase feeding latency; and that a disruption in any of these systems will cease/curb fat-induced satiety.

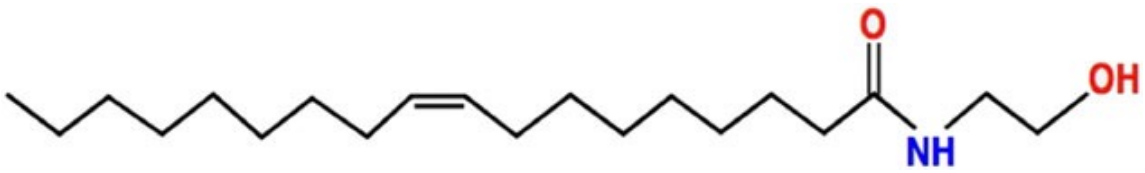
Keywords: Obesity, oleic acid, oleoylethanolamide, satiety.

2.2 Introduction

Obesity is a complex disorder that has reached epidemic proportions, particularly in industrialized/developed countries, and has been linked to an increased risk of non-communicable diseases. Despite the high prevalence of obesity, limited nutritional and pharmacological therapies are currently available. Therefore, the development of efficacious and innocuous anti-obesity approaches is of primary importance for both patients and health systems. In this context, recent data have explored and generated interest in a class of *N*-acylethanolamides, also termed as fatty acid ethanolamides (FAEs), which are arachidonoylethanolamide and 2-arachidonoylglycerol analogues but appear to operate through independent mechanisms involving cannabinoid receptor type 1 (CB1) receptors (1). Other ethanolamides include a subclass of saturated, monounsaturated and polyunsaturated FAEs such as myristoylethanolamide, stearoylethanolamide, palmitoylethanolamide, linoleoylethanolamide, α-linoleoylethanolamide, eicosapentaenoylethanolamide and docosahexaenoylethanolamide. This family of FAEs also includes the monounsaturated fatty acid (MUFA) species oleoylethanolamide (OEA) (Figure 2.1) (2).

Furthermore, DiPatrizio and Piomelli (1) have demonstrated that OEA and endocannabinoids act as key fat-dependent regulators of hunger as well as satiety. However, despite structural similarities, these substances interact with distinct molecular targets and elicit widely different biological responses. For example, OEA attenuates food intake by activating homeostatic brain circuits. On the other hand, endocannabinoids

Figure 2.1 Structure of *N*-oleoylethanolamide.



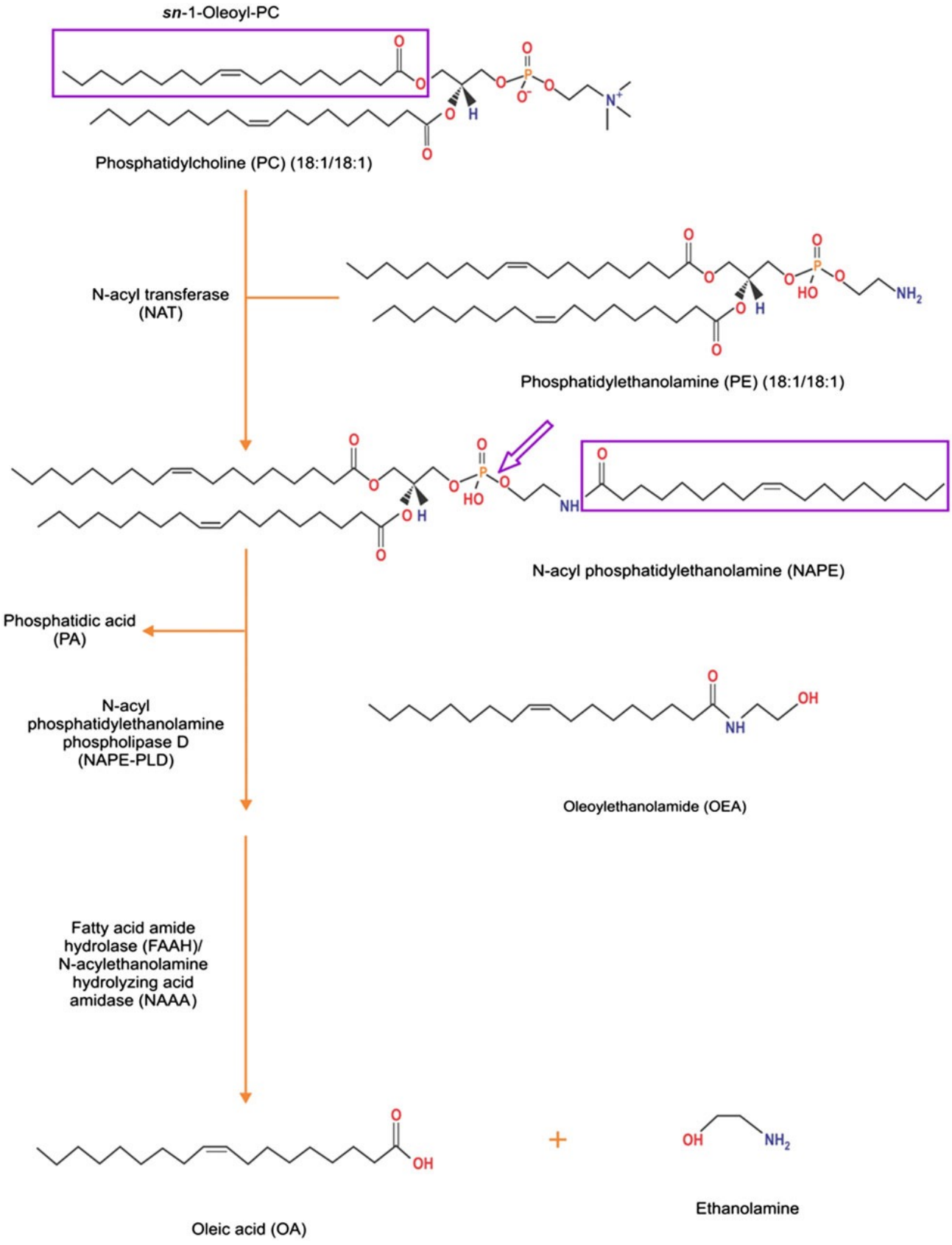
activate the hedonic cannabinoid receptors especially CB1. Stimulation and activation of CB1 lead to increased food intake (3).

OEA is a lipid amide that is released by enterocytes upon absorption of dietary fat and may engage in the initiation of satiety (4). Involvement of OEA in the process of food consumption and satiety has fuelled a new interest in the amide of fatty acids (FAs), particularly OEA. Therefore, the objective of the present review was to probe the effects of oral supplementation of OEA, either through the diet or supplements, on weight management by elucidating the physiological role of these lipid-signalling molecules in the modulation of food intake and energy expenditure (EE).

2.3 Metabolism of oleoylethanolamide

The pathway at a molecular level accounting for the anabolism and catabolism of OEA in mammalian cells involves a particular group of phospholipids, *N*-acylethanolamine phospholipids, termed *N*-acyl phosphatidylethanolamines (NAPEs), which have an additional FA bound to the amine group of phosphatidylethanolamine (PE) (5). These FAEs, including OEA, can be produced from NAPEs via two pathways that involve enzymatic activity: (i) *N*-acyl transferase and (ii) *N*-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) (Figure 2.2). All FAEs emerge from the analogous NAPEs. Therefore, NAPE species consisting of oleic acid at the amine end (*N*-oleoyl-PE) produce OEA at the time of hydrolysis. The primary action of the principal enzymatic pathway is to exchange FA between two membrane phospholipids, with the transfer of an acyl

Figure 2.2 Metabolism of oleoylethanolamide (OEA) in mammalian cells (adapted) (9, 27).



group from the donor stereospecific numbering-1 (*sn*-1) position of phosphatidylcholine to the amine group of PE (6, 7). Enterocytes thereby appropriate the diet-derived oleic acid, and synthesis of the membrane phospholipid, *N*-oleoyl-phosphatidylethanolamine, occurs utilizing it as a substrate. The reaction further leads to secondary activity by inducing the cleavage of NAPE by an NAPE-PLD to biosynthesize OEA (8–11). The hydrolysis of OEA terminates the physiological functions of this lipid mediator, resolving it into oleic acid and ethanolamine. Two intracellular amidases channel this reaction: fatty-acid amide hydrolase (FAAH), an intracellular membrane-bound serine hydrolase (12, 13), and *N*-acylethanolamine-hydrolysing acid amidase, a lysosomal cysteine hydrolase (14). Higher intensities of FAAH expression are present in the liver, small intestine and central nervous system (CNS) (15). By contrast, *N*-acylethanolamine-hydrolyzing acid amidase activity in rats is highest in the lungs, whereas in humans, it is highest in the liver; thus, cross-species variability is observed in the selective activity of the enzyme (14).

2.4 Anorexic properties of oleoylethanolamide/food intake regulation by oleoylethanolamide

Anorectic potency of OEA has been proposed by Fu and colleagues (16) in an experiment performed in rats. They showed a transitory decrease in the overexpression of NAPE-PLD that further resulted in attenuated food ingestion, concomitant with elevated concentrations of intestinal OEA in adult male Wistar rats. The hypophagic action of OEA accompanies the action of proliferator-activated receptor type-alpha (PPAR-α) (9, 10,

17–19), to which OEA binds with high affinity; as in PPAR-α-null mice, controlled feeding behaviour was observed to be ablated (17, 19). Additionally, in free-feeding mice or rats, systemic administration of OEA before dark escalated the feeding latency via the lipid-derived signal in dose dependent manner without affecting the meal size, whereas in food-deprived animals, OEA administration not only delayed feeding onset but also reduced the meal size (20), which is distinctive of satiety. To further substantiate its anorexic characteristics, OEA administration to mice or rats was observed to generate a time as well as dose-dependent effect on meal consumption, leading to reduced food intake at higher exogenous doses of OEA (20 $mg.kg^{-1}$, intraperitoneally [i.p.]) over the 24-h test period of OEA administration (17, 19–26).

Previous studies have shown that the administration of OEA to fasting rats and mice either i.p. or by gavage curtails food consumption (10, 18–20, 22, 24, 25). Free-feeding rats fed standard chow pellets (Prolab RMH 2500) also exhibit unusual effects by persistently inhibiting food consumption, but only at selective doses of OEA administered i.p. at 5 $mg.kg^{-1}$ i.p. and being most effective at 20 $mg.kg^{-1}$ i.p. Therefore, the findings suggest that systemic administration of OEA to free-feeding rats leads to a dose-dependent delay in eating onset, which is not associated with changes in meal size or the postmeal interval (20, 23). Altogether, OEA reduces meal frequency in free-feeding rats; however, OEA decreases both the rates of occurrence as well as the meal size in food-deprived rats (20). Additionally, fasting reduces and refeeding intensifies the OEA concentrations in the jejunum (25, 27, 28). To further confirm that OEA plays an important role in restricting the meal frequency, Fu and colleagues (27) observed NAPE-

PLD activity in free-feeding and 24-h food deprived rats and concluded that the activity of NAPE-PLD remained low during fasting but increased in intensity promptly upon refeeding, comparable to the NAPE precursors for OEA (28). Overall, the study results suggest that the synthesis of NAPE precursors and NAPE-PLD activity monitors the intestinal alterations in tissue OEA concentrations. By contrast, the OEA concentrations are not at all affected by the fluctuations involved in FAEs catabolism (9, 16, 27, 28). Therefore, the research studies discussed above substantiate that the function of the NAPE-generating enzyme *N*-acyl transferase may provide a pivotal contribution to the modulation of the intestinal concentration of anorectic *N*-acylethanolamides. Furthermore, the studies assert that exogenously administered OEA has a highly effective anorectic impact (18, 25).

Additionally, regarding its anorexiant properties, when administered subchronically to lean (25) or obese rats (29) and mice (19), OEA decreased body weight gain in normal animals but not in PPAR-α-null mutants (19). Similar results were obtained in a clinical trial conducted by Jones and colleagues (30); during this human feeding trial, participants were provided with diets enriched with high-oleic canola oil (HOCO), HOCO blended with flaxseed oil or a Western diet for 29 d. Findings showed a negative correlation between the plasma OEA levels and body fat percentage. Another human trial conducted by Pu and colleagues (31) showed that dietary oleic acid enriched HOCO resulted in elevated plasma OEA levels that affected the regional and total fat mass, suggesting that the effect might be caused via lipid-signalling channels. Associations between OEA levels and improved body composition results are in

agreement with another human trial performed with morbid obese patients (32). Barbour and colleagues (33) also conducted a human trial to examine energy intake; during the study, volunteers were offered high-oleic (oleic acid ~75% of total FAs) peanuts and regular peanuts (oleic acid ~50% and higher in polyunsaturated fatty acids [PUFAs]). The total energy intake was shown to be lower following the consumption of high-oleic and regular peanuts, suggesting that peanuts could be beneficial for maintaining a healthy weight. Moreover, a significant difference in energy intake was observed for the high-oleic peanuts with high levels of monounsaturated fat when compared with regular peanuts because of the high oleic acid content, which was readily oxidized and provided a more satiating effect. Recently, another human trial demonstrating the efficacy of oleic acid and circulating OEA levels leading to reduced energy intake was conducted by Mennella and colleagues (34). In this study, 30 mL of high levels of oleic sunflower oil and olive oil were offered to participants in a glass together with 30 g of white bread to be consumed within 15 min on different occasions as per the randomization in a bolus dose. The high oleic acid content of the oils increased the postprandial response of circulating OEA, resulting in diminished energy intake over 24 h following the experimental meal. Overall, the data from animal and human trials provide evidence of the practical involvement of the lipid mediator, OEA, in obesity.

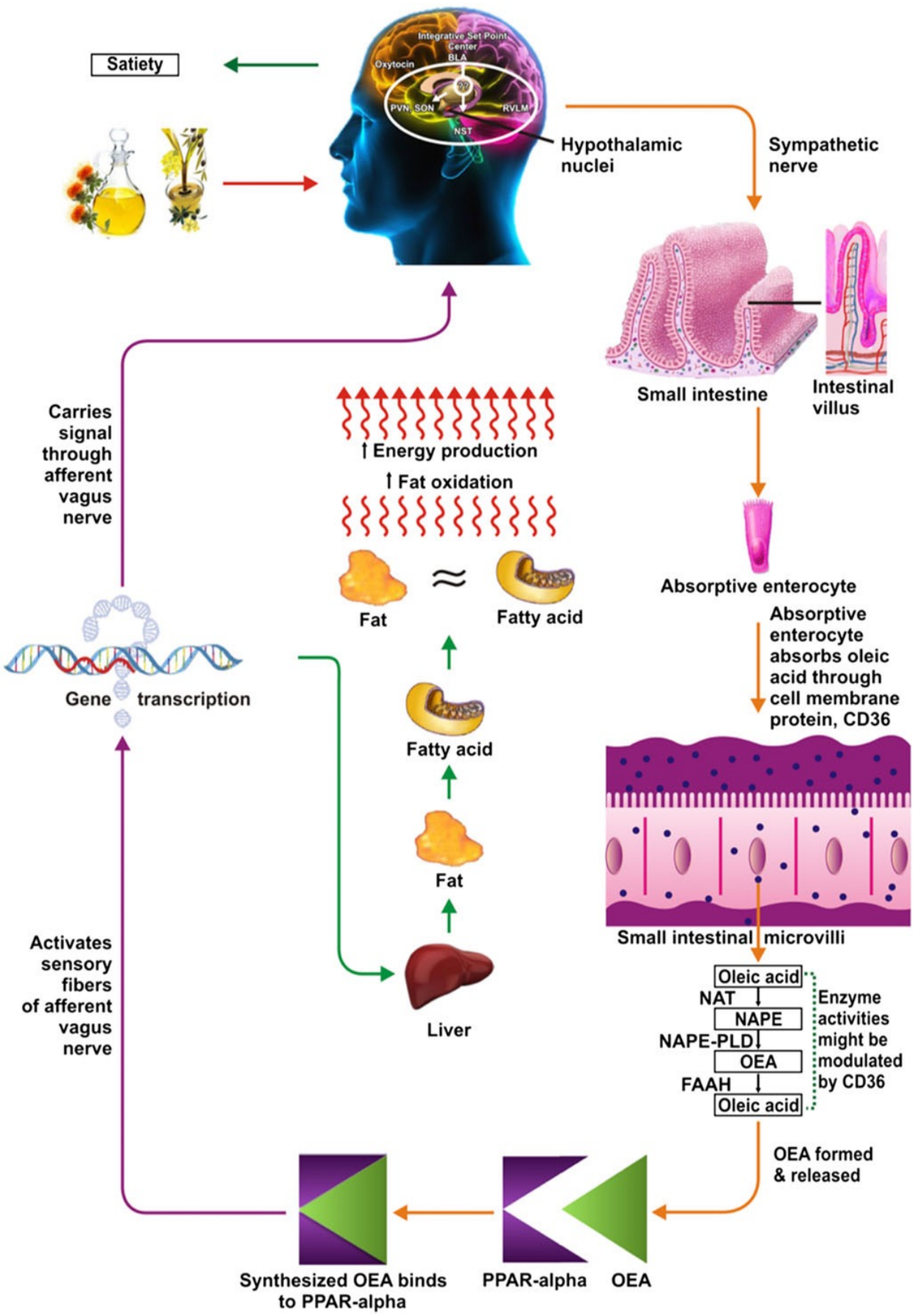
2.5 Association between dietary oleic acid and oleoylethanolamide mobilization via molecular targets

The ingestion of food, particularly dietary fat that is high in oleic acid, triggers the formation of OEA, leading to satiety, enhancing lipid absorption via PPAR-α and consecutively promoting lipolysis, thereby helping to reduce body weight (35). The anorexic effects provided by OEA involve a series of actions that include (i) stimulation of the local nuclear receptor, PPAR-α; (ii) activation of afferent sensory nerve fibres, conceivably the vagus nerve; (iii) networking of the appetite-regulating circuits that recruit histamine and oxytocin as neurotransmitters in the brain; and (iv) restoration of dopamine release (19, 36–38), the key neurotransmitter involved in the mediation of the reinforcing effects of foods and other reward-generating systems (Figures 2.3 and 2.4) (39–43). Overall, studies indicate a stimulation of the 'food reward system' that triggers anorexic signalling. Two key molecular targets imparting anorexic signalling to OEA are PPAR-α and CD36.

2.5.1 Action of peroxisome proliferator activated receptor-alpha in imparting anorexic signalling

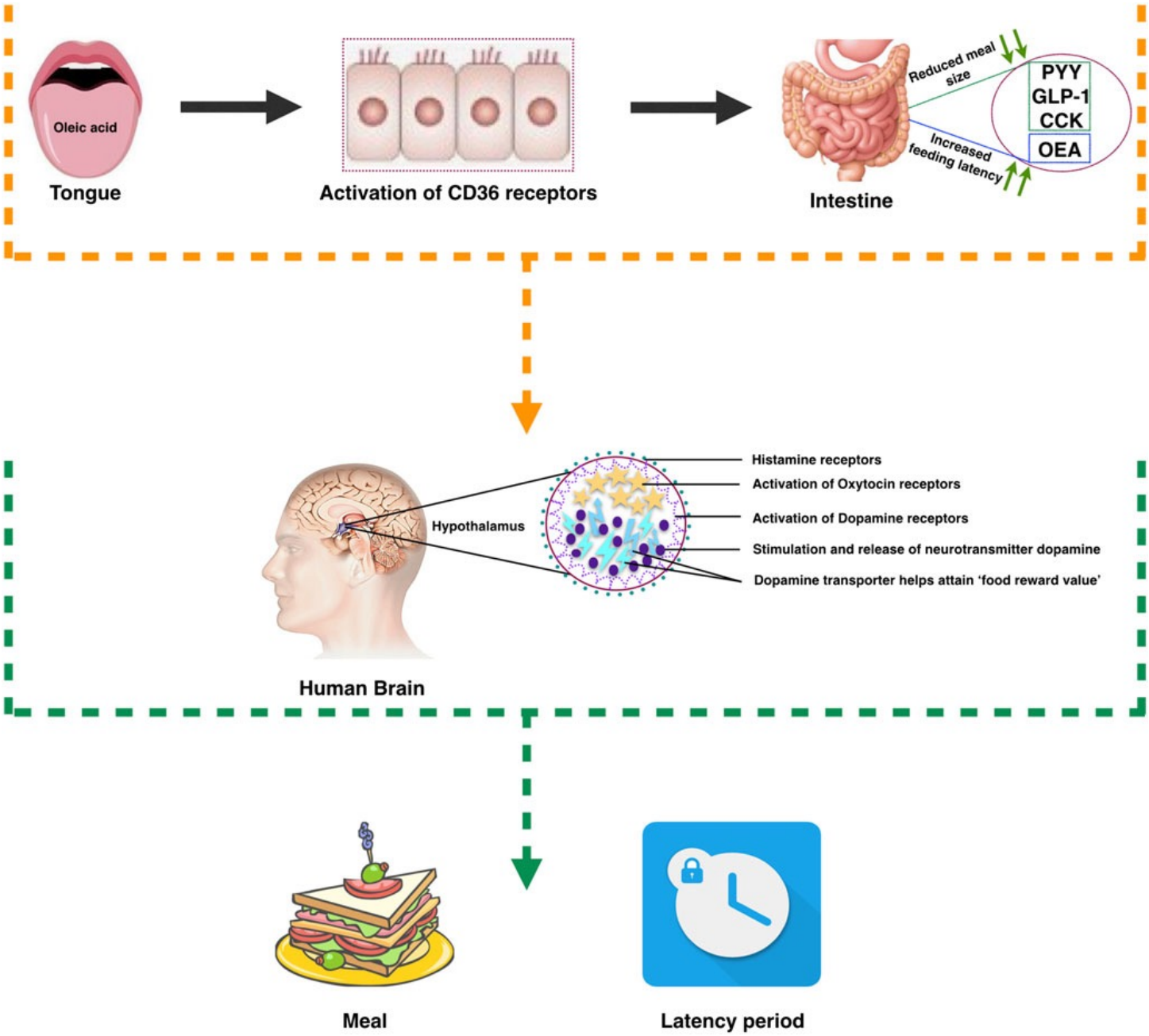
PPAR-α receptors, discovered in 1992, belong to a family of ligand-activated transcription factors (44). PPAR-α is a nuclear/transcription receptor/factor that regulates lipid and glucose metabolism (45). Stimulation of PPAR-α triggers uptake, utilization and catabolism of FAs via the up-regulation of genes associated with FA transport and peroxisomal and mitochondrial FA β-oxidation (44, 45). Thus, provided it is associated with the modulation of FA-oxidation, PPAR-α may play a significant role in the regulation of obesity, specifically central obesity, which is associated with insulin

Figure 2.3 Gut and brain interrelationship and regulation of feeding behaviour (partly adapted) (9, 27).



Note: BLA, basolateral complex of the amygdala; FAAH, fatty-acid amide hydrolase; NAPE, *N*-acyl phosphatidylethanolamine; NAPE-PLD, *N*-acyl phosphatidylethanolamine phospholipase D; NAT, *N*-acyl transferase; NST, nucleus of the solitary tract; OEA, oleoylethanolamide; PPAR, peroxisome proliferator activated receptor; PVN, para-ventricular nucleus; RVLM, rostral ventrolateral medulla; SON, supraoptic nuclei.

Figure 2.4 Consumption of oleic acid and action of neurotransmitters in the regulation of appetite and eating.



Note: PYY, peptide YY; GLP-1, glucagon-like peptide-1; CCK, cholecystokinin; OEA, oleoylethanolamide.

resistance syndrome (46).

The PPAR family consists of additional members; however, all do not carry appetite-suppressing effects. Fu and colleagues conducted a study in rats to investigate overall PPAR family and confirmed that only PPAR-α is associated with a weight-reducing effect; PPAR-β and PPAR-δ agonists (GW501516) and a PPAR-γ agonist (ciglitazone) were inefficient for reducing food intake in rats (19, 47). However, work performed by Wang and colleagues (48) verified that PPAR-δ has similar properties to PPAR-α and activates adipose tissue utilization and prevents diet-influenced obesity in animals. In general, PPAR-α is considered a metabolic sensor of dietary FAs (49, 50), and its key function is to sense FA flux into cells. This leads to the assumption that all the FAs communicate with PPAR-α via a direct route and henceforth, all FAs will exert anorexic effects similar to OEA by activating PPAR-α in tissues containing this receptor. However, work done by Akbiyik and colleagues (51) demonstrated that the stimulation of PPAR-α by OEA is structurally discriminating against homologous FAEs, including myristoylethanolamide (14:0), stearoylethanolamide (18:0) and anandamide (20:4n6), because they have no impact on this receptor (35). Conversely, similar concentrations were observed of two downstream targets of PPAR-α, acyl-CoA-dehydrogenase, the rate-restricting enzyme of peroxisomal β-oxidation, and acyl-CoA-oxidase, a mitochondrial β-oxidation enzyme. This finding implies that abundant FAs in the liver, such as palmitic acid, oleic acid, linoleic acid and arachidonic acid, do not change the function of PPAR-α (51). However, these fatty acids do activate the expression of PPAR-α, which leads to FA-

oxidation in the liver to regulate the increased FA load. This effect appears to be dependent on the function of the described FAs as endogenous ligands for PPAR-α (49, 52–55). Similar studies performed by Schwartz and colleagues (10) showed that amongst various fats, duodenal infusion of only oleic acid prompted OEA synthesis and initiated satiety, leading to increased PPAR-α expression in liver.

Furthermore, the expression of PPAR-α is not only restricted to the liver, organs such as the brain also contains high levels of PPAR-α in selected locations/selected lobes that have limited ability to generate FAs (56). Additionally, the sequence of the molecular chain linking PPAR-α activation to vagal sensory fibre stimulation yielding to satiating properties remains to be elucidated. The message is assumed to be transmitted through nitric oxide (NO) production. Enterocytes release high quantities of this gaseous transmitter, which may function as a peripheral food craving/hunger-activating signal (57, 58). Furthermore, PPAR-α suppresses the expression of enzymes responsible for NO intestinal release, including intestinal NO synthetase (19, 59). Hence, the suppression of intestinal NO synthetase expression by PPAR-α may play an influential role in inducing the prolonged satiating actions of OEA, which extend for numerous hours after the injection of this compound (19, 20), suggesting that NO can stimulate appetite (60) that can be suppressed by PPAR-α. Similarly, PPAR-α activation mediating the anorexic/anorexiant effects of OEA is yet to be accurately elucidated. Nevertheless, the finding that eating modulates OEA concentrations in the duodenum and jejunum (19, 25) suggests that lipid-derived anorexic signalling potentially acts on the PPAR-α that remains confined within cells of the small intestine. Thus, OEA is known to be a PPAR-α

agonist, which accounts for its appetite suppressing as well as its energy-enhancing effects.

2.5.2 Action of fatty acid translocase/CD36 in imparting anorexic signalling

CD36 is also known as fatty acid translocase because it binds to long-chain free FAs and facilitates their transport into cells (61). CD36 also binds to long-chain fatty acids and acts as a conveyor or modulator of FA carriage into the small intestine (62–64).

The apical membranes of taste bud cells in the tongue express CD36 (65–67), which contribute to the taste recognition of fats and to the initiation of the cephalic phase of digestion (66, 68). These events of taste perception regulated by signalling pathways are prompted by long-chain fatty acids bound to CD36. Several in vivo studies, conducted in both rodents and humans, have documented that CD36 plays an essential function in FA up-regulation, eventually resulting in FA-oxidation. Furthermore, numerous trials performed in CD-36 deficient mice (69, 70) and humans (71–73) have demonstrated a malfunction in tissue FA uptake and regulation and therefore abnormalities in FA metabolism. Another study performed by Martin and colleagues (74) in mice heterozygous for CD36 deficiency, a 50% reduction in CD36 expression was observed that was associated with a significant decrease in fat perception, suggesting that the findings of Martin and colleagues (74) may be applicable in humans. Henceforth, the studies performed in humans (75–77) indicate that *CD36* gene polymorphisms correlate to lipid level variations in plasma (78–80) due to diminished responsiveness to the taste perception of oleic acid (81). Therefore, human carriers of

CD36 gene polymorphisms are unable to synthesize OEA and are more prone to developing metabolic syndrome such as obesity (82, 83).

2.6 Dietary oleic acid and its association with oleoylethanolamide

The chemical composition of the ingested food is paramount in OEA formation. Schwartz and colleagues (10) showed that the infusion of glucose or proteins into the duodenum did not have any effect, whereas amongst several fats, only oleic acid elicited OEA production in animals. In humans, Joosten and colleagues (84) found that fasting and non-fasting plasma concentrations of OEA were positively correlated with both serum total free FAs and their particular FA precursor oleic acid. In fact, oleic acid may act as the precursor for OEA formation in the intestine, as previously demonstrated in animals (27, 28, 85), and engender some physiological mechanisms regulating its specific release from intestinal membrane phospholipids; thereby increasing the capacity to oxidize fatty acids by fat oxidation resulting in enhanced energy expenditure by means of utilizing oleic acid as precursor and chief FA.

2.6.1 Dietary oleic acid and the effects of oleoylethanolamide on fat oxidation

Fat distribution is directed by the composition of the diet (86–89), age or life stage (90–93) and the genetics of the individual (94–101), for either energy or storage (86, 102–105). The degree of dietary fat unsaturation also plays a critical role in whether the fat will be stored or oxidized. Long-chain fatty acids unsaturation has been suggested to affect the regulation of dietary fat in the direction of either oxidation or storage.

Numerous human and animal trials have provided evidence that the saturation of FAs affects rates of oxidation, with unsaturated FAs being more readily oxidized (106–108) and therefore potentially exerting improved body composition than saturated fatty acids (SFAs) (109) through the activation of uncoupling protein 1 mediated by sympathetic nervous system (SNS) (110). Furthermore, some animal studies (111–114), but not all (115, 116), propose that oleic acid is taken up more swiftly for utilizable energy compared with linoleic acid. Likewise, some human studies (117–119), excluding one (120), have demonstrated an elevated oxidation rate of oleic acid compared with linoleic or linolenic acids.

Furthermore, studies using the labelled FA approach have demonstrated that oleic acid and other unsaturated FAs are oxidized promptly when compared with SFAs (103, 112, 119, 121, 122). However, the isotope tracer data do not indicate whether altering the arrangement of dietary FAs would influence the total FA-oxidation. Moreover, a few studies conducted in humans and animals have shown that the consumption of diets rich in PUFAs and MUFAs results in elevated total FA-oxidation, EE or both compared with diets enriched with higher levels of SFAs (86, 116, 123–125). Additionally, in another human study, canola and peanut oil muffins, which are rich in oleic acid- MUFAs, resulted in greater fullness, with reduced hunger ratings after 30, 60 and 120 min (126). Regarding fat oxidation, Kien and allies (104) have also reported differences between MUFAs and SFAs enriched diets. The researchers performed a study in humans showing that augmenting the ratio of MUFAs rich in oleic acid (78.4%) to SFAs in the diet escalated fat oxidation. Additionally, there was no apparent reduction in fasting FA-

oxidation, which is consistent with similar findings reported by Jones and colleagues (86) as well as by Piers and colleagues (127). Furthermore, French and colleagues (128) performed another human trial with lean participants that showed that less food consumption and almost identical appetite ratings were noticed after infusion of an oil emulsion rich in linoleic acid, in contrast to an infusion rich in oleic acid and stearic acid. Therefore, due to inconsistent results, further randomized clinical trials are required to confirm the effect of different FAs on fat oxidation. However, the findings by Kien and colleagues (104) can be justified by the observation that oleic acid is an integral component of stored FAs in human physiology compared with palmitic acid (129) and support a high degree of oxidation similar to PUFAs due to the carbon–carbon double bond (86, 130). Similar findings have also been reported by another human trial performed by Alves and partners (131), where high oleic peanut consumption increased fat oxidation and reduced body fat in overweight and obese men. These findings were presumably due to the effect of oleic acid, which stimulates the cyclic adenosine monophosphate/protein kinase A pathway, further activating the sirtuin 1-peroxisome proliferator-activated receptor gamma coactivator 1-α transcriptional complex to regulate the rates of FA-oxidation (132). In summary, these data indicate that following intake of MUFA enriched diet, specifically oleic acid, gets utilized readily for energy production than being stored. Also, the relationship between oleic acid and OEA levels can be established from the fact that amount of circulating concentrations of oleic acid will correspond to the concentrations of OEA synthesized in the body (30, 31) leading to higher fat oxidation rates following consumption of oleic acid (35).

2.6.2 Dietary oleic acid, diet-induced thermogenesis and effects on energy expenditure

The dietary proportion of unsaturated to saturated fat alters EE in humans due to the high PUFA:SFA ratio, resulting in elevated resting metabolic rates and increased fat oxidation compared with a low PUFA:SFA fat ratio (86, 119, 130, 133). Additionally, one more component termed as diet-induced thermogenesis (DIT) plays a critical role in increasing the resting metabolic rates. Takeuchi and companions (116) conducted a study in rats showing that FAs have various effects on the thermic effect of food (TEF), also termed DIT. Furthermore, the postprandial data from the study performed by Jones *et al.* (130), illustrate that lean individuals oxidized fat more rapidly than their obese equivalents when fed a low PUFA:SFA diet because obese individuals were observed to have reduced fat oxidation towards TEF compared with their lean counterparts. Moreover, the trial demonstrated that postprandially, overweight individuals contribute less dietary saturated fat for oxidation compared with individuals with a normal body weight because in obese participants, malfunctioning gene transcription associated with PPAR-α renders FA transport and peroxisomal and mitochondrial FA β-oxidation ineffective, leading to blunted TEF and EE.

Evidence of MUFA-enriched diets on DIT has been supported by Piers and colleagues (127); the researchers conducted a human study including participants with a high waist circumference and observed that olive oil enriched in MUFAs considerably intensified postprandial thermogenesis as well as the rate of fat oxidation compared with

the administration of a cream rich in SFAs. Moreover, the total daily EE was significantly higher in individuals fed a high oleic acid diet (104). These results have been confirmed by studies performed in rats demonstrating that different fatty acids have a different thermic potency of food (116).

The findings of reduced EE after SFA diets feeding are supported by several studies (116, 123, 124), excluding one (134) performed in rodents fed varying dietary FA compositions, that suggest that the fractional elevation in SFAs in the diet increases susceptibility to reduced EE. Mechanistic explanations leading to this outcome could involve a diminished thermogenic response in brown adipose tissue (116, 123). Numerous studies (135–139) indicate that dietary and endogenous FAs along with their genes, chiefly PPAR-α, monitor FA-oxidation and energy uptake pathways and hence enable energy utilization. Therefore, the various oxidation rates of dietary FAs via peroxisomal β-oxidation or by enhanced activation of PPAR-α mechanisms could also lead to alterations in daily EE (140, 141), also enhancing utilization of oleic acid. These results imply that PPAR-α has a centrally coordinated role in the regulation of FA-oxidation (46). Henceforth, the upregulated oleic acid acts as precursor to OEA, activating PPAR-α and yields higher fat oxidation rates, thereby improving total energy expenditure. The action of OEA leading to enhanced EE is also supported by a pharmacological animal trial conducted by Suárez and affiliates (142); the study showed that co-administration of OEA (5 $mg.kg^{-1}$) and CL316243 (1 $mg.kg^{-1}$), a β3-adrenergic agonist, i.p. for 6 d, amplified both the reduction of food intake and body weight gain; with increase in EE and reduction in the respiratory quotient (VCO2/VO2). Overall, the

data demonstrate that the vital component for the maintenance of weight over the long term is the quality as well as the quantity of fat consumed that further activates the lipid transport pathways.

In summary, the evidence indicates that lipid transport appears to be the eventual effect of oral OEA to reduce adiposity that is also supported by data from the trial performed by Thabuis *et al.* (143) in mice via a minimum of seven different pathways including (i) lipid transport; (ii) energy intake; (iii) regulation of EE; (iv) endocannabinoid signalling; (v) lipogenesis; (vi) glucose metabolism; and (vii) faecal fat excretion. Consequently, oleic acid may function in triggering a negative feedback signal to handle an overflow of FAs and thus maintain lipid homeostasis. Moreover, the findings suggest that diets rich in oleic acid derived from MUFAs result in the synthesis of OEA, which may offer increased oxidation that translates into increased EE in the presence of CD36 and PPAR-α. Bowen *et al.* (144) have proposed a potential mechanism for the physiological effects of oleic acid-derived OEA on lipid metabolism in humans. Altogether, these findings indicate that oleic acid resulting in OEA may have a prospective relevance and clinical utility in the prevention of obesity.

2.7 Relationship between oleoylethanolamide and feeding regulating hormones

A complex interaction of central neurotransmitter systems and peripheral stimuli manages eating behaviour, including hunger and satiety. Satiation is fundamentally regulated by the hypothalamus, which is a key site for receiving different signals from the organs engaged in energy metabolism, including the mouth, duodenum, jejunum and

ileum. Furthermore, various channels modify feeding behaviour either by (i) pharmacological intervention with anorexigenic drugs or (ii) food-regulating hormones. However, the action of OEA imparting anorexic properties is different from other satiety-inducing hormones.

2.7.1 Difference between satiety effect exerted by oleoylethanolamide and feeding regulating hormones

In response to nutrient intake, the gastrointestinal tract plays a vital role and monitors energy homeostasis by releasing appetite-regulating lipid mediators and peptides. Energy homeostasis is regulated downstream by producing signals that can either be hormonal or neuronal, neuronal via the vagus nerve or hormonal by producing hunger and satiety-inducing peptide hormones such as ghrelin, cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) (145, 146). Together with these peptides, foods rich in oleic acid boost the synthesis of the lipid-derived mediator, OEA, which induces satiety, intensifies lipid absorption, ameliorates lipolysis and attenuates body weight, consequently imparting anorexic results (9, 25). These anorexiant properties are disparate from those elicited by traditional satiety regulators, as observed for CCK, which decreases the meal size without affecting the latency period between meals (147). By contrast, OEA reduces the meal frequency, thereby mediating the well-known satiety effect (148). Furthermore, the hypophagic functions of OEA also vary from those implemented by GLP-1 (149) and corticotropin-producing factors (150). Therefore, the best property of OEA in increasing the feeding latency in contrast to reducing the meal

size makes it a potential novel compound to control appetite.

2.7.2 Similarities between oleoylethanolamide and the feeding regulating hormones in imparting satiety

Although the above results demonstrate that the satiating action of OEA is distinct from the regular peptide hormones, some similarities exist between both as well. Mobilization of OEA is initiated by fat intake, specifically by oleic acid-enriched fat intake. However, animal data illustrate that prolonged subjection to a diet enriched in fat reduces intestinal concentrations of OEA in rodents (18, 38, 85, 151), consistent with the attenuated action of the gut peptide hormone GLP-1 (152, 153). Similarly, the reaction of GLP-1 receptor agonists was also altered in obesity leading to increased appetite (154). Additionally, when obese individuals and rodents were provided with moderate to high-fat diets, this peptide signalling is impaired along with diminished postprandial levels of CCK and PYY (155–158). Moreover, CCK, PYY and GLP-1 expression levels were attenuated in the jejunum in diet-induced obese rats, leading to reduced satiation due to the lower levels of endogenous satiety peptides (155); similar effect in reduced intestinal OEA levels after high-fat diet (HFD) fed animals has been reported by Igarashi *et al.* (151). Additionally, GLP-1 is classified as a potent insulin-releasing and satiety-inducing gut hormone (159). The activation of G protein-coupled receptors, especially GPR119 stimulates the release of GLP-1 from the intestine (160). Furthermore, Overton *et al.* (161) illustrated that OEA acts as an agonist of GPR119 enabling satiating effects. On the contrary, another animal study performed by Lan and co-workers (162)

demonstrated that OEA when administered to GPR119 knock-out mice-induced satiety, explaining that the role of GPR119 is important for insulin secretion but not for appetite suppression. Therefore, the clinical utility of activation of GPR119, stimulating the release of GLP-1 and hypophagic interactions with OEA, is yet to be explored. Henceforth, further investigation regarding molecular pharmacology of GPR119 is warranted in understanding its role in metabolic homeostasis.

Overall, from the cumulative evidence in humans and rodents and work done by Tinoco and colleagues (163), it is worth noting that a strong association between OEA and other feeding regulators exists imparting the satiating/anorexic properties. Therefore, future studies are required to clarify the satiating efficacy of OEA alone by performing studies using knock-out animal models for specific receptors involved in inducing satiation.

2.8 Impact of high fat diets on levels of oleoylethanolamide and satiety

Fat enriched in oleic acid is required to synthesize OEA, meaning that the higher the oleic acid content in fat, the higher is the OEA level inducing satiety mechanisms. This may lead to consumption of more dietary fat, hypothetically leading to the assumption of generating increased OEA content. However, increased fat intake may suppress the OEA mobilization leading to obesity. Furthermore, Diep and colleagues (164) conducted a study in mice in which animals administered a HFD showed decreased jejunal levels of OEA. Recent work by Igarashi and colleagues (151) suggests that in the gut of obese rodents, feeding-dependent OEA regulation is suppressed, revealing that short-term

exposure to a HFD, as well as a low-fat high-sucrose diet, may also contribute to hyperphagia, thereby leading to reduced satiety. However, research conducted by Tellez and colleagues (38) in mice subjected to a HFD, which rendered the mice obese, yielded data demonstrating the restoration of suppressed OEA levels in the gut by treating obese mice with exogenous OEA. Taken together, these processes could all be components of various molecular pathways advancing the renowned 'obesogenic sequel' of a HFD (83, 165, 166), impacting the satiating potency of OEA as well as peptide hormones involved in regulating energy homeostasis. Moreover, in an experimental arrangement in which animals were trained to lick a dry spout to self-administer gastric infusions (167) of fat emulsions, lack of motivation to consume food via the gastric route was observed after OEA infusion in low-fat fed mice (38). Additionally, the OEA injections showed anorectic effects in both low-fat and HFD fed mice during oral intake of a high-calorie emulsion; however, OEA administration was observed to increase the 'reward value' of the lower-calorie emulsion by stimulating low-fat intake during oral tests in HFD fed mice. These findings could be due to a restoration of 'gut-stimulated dopaminergic activity', which enhanced the 'reward value' of low-calorie foods. By contrast, HFD fed rodents demonstrated reduced oral acceptance of low-calorie fats without OEA (168–170). These results could also be a consequence of an increased detection threshold for fat in obesity (171).

Altogether, these studies suggest that in addition to contributing to the regulation of the quality of dietary fat for consumption, OEA may also function as a homeostatic intestinal stimulus that involves hedonic components (172) that has also been suggested

in one human trial (173). However, future research is needed by conducting acute as well as long term full feeding trials. Additionally, trials performed with free living participants enabling participants to opt for self-selected dietary fat may shed more light on the impact of fat quality and quantity. Addressing these knowledge gaps will further enhance the understanding on the impact of desirable quantity of fat required to generate anorexic efficacy of OEA; along with thorough investigation of feeding regulating hormones on appetite, satiety by visual analogue scale, fat oxidation, energy expenditure and overall body composition to ascertain the safe efficacy and usage of OEA in curbing obesity.

2.9 The gut lipid messenger oleoylethanolamide recruits the food reward system to regulate feeding behaviour in the brain

The preceding segment provides evidence that OEA induces satiety. Overall, OEA is a nanomolar agonist of PPAR-α, a key element of the large superfamily of nuclear receptors (9, 19). The biological actions of OEA are predominantly modulated by PPAR-α, including its ability to restrict food consumption (19, 25, 174), increase FA absorption in small intestinal enterocytes (19, 175) and intensify lipid lipolysis and oxidation in adipocytes, hepatocytes and skeletal myocytes (29, 142). However, this significant modulation of meal patterns in rats administered OEA is not observed in mice lacking PPAR-α. This effect has been attributed to the high affinity binding of OEA to PPAR-α and also its imitation by synthetic exogenous PPAR-α agonists, implying that the nuclear receptor is both vital as well as adequate for OEA-evoked hypophagia (17, 19).

Research has shown that various intestinal sensory receptors detect the amount of dietary fat in the lumen; the absence of these fat sensory receptors leads to reduced intestinal OEA concentrations and contributes to a hyperphagic prolongation of dietary fat ingestion (18, 159, 176). Based on the above findings, there is a large interest in obtaining a better understanding of how OEA signalling in the gut initiates a feedback reaction that initiates satiety via the food reward system to modulate feeding behaviour.

2.9.1 Impact of oleoylethanolamide on satiety inducing targets

Following the consumption of dietary fat, particularly oleic acid, OEA levels increase in the duodenum and jejunum but not in the bloodstream (27). Possibly OEA is produced in various peripheral tissues and the CNS (177). The presence of OEA in the CNS suggests that the anorexic properties of OEA are mediated in an analogous fashion to CCK by paracrine stimulation of vagal afferent nerve fibres (178). This theory is supported by three discoveries. First, animals treated with capsaicin, which deprives them of peripheral vagal and non-vagal sensory fibres, show abolished hypophagic activity of OEA (25). Second, OEA administered at 10 $mg.kg^{-1}$ i.p. does not penetrate the brain and, hence, has been found to instantly stimulate the transcription of the c-Fos gene, a marker of neuronal activation, in the brainstem nucleus of the solitary tract (NST) (19, 25, 36, 174). Third, surgical resection of the vagus nerve or blockage of NST activity either by infusion of the local anaesthetic lidocaine into the NST or the β-adrenergic antagonist propranolol into the basolateral complex of the amygdala impedes various functions of OEA, including strengthening memory retention (179) and

activation of dopamine production (38). However, a recent animal study demonstrated that total subdiaphragmatic vagotomy, termed subdiaphragmatic vagal deafferentation, a type of surgery that removes all abdominal vagal afferents, leaving roughly half of the efferents (180, 181), does not block OEA-induced hypophagia (182). The surgical side effects and/or small procedural differences might have contributed to the antagonism of the eating-inhibitory effect of OEA. In summary, evidence indicates that association of gut and brain interrelationship leads to the hypophagic actions of OEA as several signals generated in the gut activate vagal afferent nerves to promote meal termination.

The key involvement of vagal afferent nerves as well as SNS in promotion of meal termination and satiety induction is supported by another animal study conducted in rats. Sclafani and associates (183) revealed that gut vagal afferents and splanchnic nerves are not responsible for flavour-nutrient-liking adaptability, but both vagal afferents and splanchnic nerves are accountable for carbohydrate as well as fat-induced satiation following consumption of oleic acid, precursor for OEA. The group performed an experiment in which celiac-superior mesenteric ganglia were removed, following which the anorexic effects exerted by intraduodenal fat infusion were immediately extinguished (183). Similar findings have also been reported by Fu and colleagues (184) that proposed the potential mechanism for the results of work performed by Sclafani and coworkers (183). In addition, Fu and associates (184) demonstrated that the surgical resection of the celiac-superior mesenteric ganglia abolished biosynthesis of OEA in fasting-refeeding rats. These finding demonstrates that the SNS emerges equally to play a critical role along with the vagus nerve in the induction of oleic acid-generated

satiation signalling through OEA.

Furthermore, Sabatier *et al.* (185) demonstrated that gastrointestinal vago-vagal reflex modulates the feeding behaviour via the activation of parvocellular neurons of the paraventricular nucleus. Conversely, oxytocin released by magnocellular neurons diffuses to the hypothalamus targets involved in satiety after OEA release (185). Additionally, Romano and colleagues (174) demonstrated that OEA triggered an intense signal in the area postrema and NST, sites involved in regulating food intake. Interestingly, within the central part of the NST, c-Fos mRNA expression was highly apparent at the most rostral level, where this nucleus is in greater proximity to the area postrema. In summary, research findings suggest that magnocellular neurons of the paraventricular and supraoptic nuclei of the hypothalamus are closely intertwined to regulate feeding and energy homeostasis inducing satiety by the action of OEA (185, 186). Therefore, these studies suggest a direct effect of OEA in the CNS by extension of circumventricular organs via the bloodstream.

2.9.2 Functional interaction between oleoylethanolamide and neurotransmitter histamine

OEA imparts satiety through combined action of gastrointestinal tract and brain interrelationship. Additionally, OEA requires the integrity of the brain histamine system to fully exert its hypophagic effect (37). Evidence illustrate that OEA affects the CNS by the activation and release of neurotransmitters and hormones at various 'food rewarding sites'. To further confirm the effects of OEA on the CNS, many researchers have

conducted numerous experiments and robust trials. Different neurotransmitters together with OEA have also been shown to play important roles. One such neurotransmitter is histamine, which modulates fundamental homeostasis and vital functions in the brain, including feeding patterns, cognition, stimulation and circadian rhythms (187, 188). However, little is known about the interactions between OEA and histamine. To elucidate the association, Provensi and colleagues (37) recently reported that the anorectic effects of OEA were reduced after intracerebroventricular infusion of a histamine-synthesizing enzyme inhibitor. The histamine-synthesizing enzyme 'histidine decarboxylase inhibitor', α-fluoromethylhistidine, either affects the histamine-producing enzyme histidine decarboxylase or dramatically deprives the brain of histamine. Additionally, the administration of OEA and ABT-239, the H_3R antagonist, obstructs both auto-receptors and hetero-receptors in the CNS and also aids in increasing transitory histamine production (189). Furthermore, Masaki *et al.* (190) also support the notion that histamine influences the anorexic potency of OEA; the co-administration of OEA and ABT-239 led to significantly increased brain histamine levels that boosted OEA-initiated anorexic signalling, further triggering a reduced sensation of food ingestion. Conversely, histamine receptor antagonists or pharmacological manipulation of H_1R markedly affect food intake in various mammalian species (190, 191). H_3R antagonists and OEA have also been observed to modulate the release of several other neurotransmitters other than histamine (192, 193), thereby contributing to their hypophagic but independent actions.

Moreover, Torrealba and colleagues (194) demonstrated that histamine is distinctively associated with two-key features of eating behaviour: (i) appetitive phase

(searching for food) and (ii) consummatory phase. Valdés and colleagues (188, 195) have also indicated that histamine is more vital for the temptation to eat. Therefore, histamine contributes to varying functions in the brain concerning feeding behaviour (196). On the whole, these reports suggest that functional interactions occur between peripherally functioning hypophagic stimuli, such as OEA and brain histamine neurotransmission. Additionally, these studies also propose that in addition to affecting the homeostatic systems that modulate hunger and satiety in the brain, the regulation of food consumption induced by OEA also affects hedonic as well as non-homeostatic domains that modulate hunger and satiety.

2.9.3 Functional interaction between oleoylethanolamide and oxytocin receptors

The central effects of OEA, influenced by brain histamine, activate the oxytocin neuron-rich nuclei, the hypothalamic area involved in inducing satiety (37). Intestinal OEA, which reduces feeding by activating the vagus nerve (197), has also been observed to stimulate oxytocin mRNA expression in the paraventricular and supraoptic nuclei of the hypothalamus (25, 36). Moreover, Gaetani *et al.* (36) illustrated the use of the synthetic blockade of central oxytocin receptors that instantly hampered the anorexiant efficacy of OEA. In another study, rats fed extra virgin olive oil showed increased levels of hypothalamic oxytocin mRNA expression, indicating that oxytocin may be modulated by dietary lipids, especially oleic acid (198). Additionally, previous studies have documented that OEA's capabilities to enhance the expression of the neuropeptide oxytocin were obstructed when the oxytocin receptor antagonist L-368,899 was

administered intracerebroventricularly, which further prevented its ability to decrease food consumption (36, 199). Serrano *et al.* (193), in compliance with this observation, reported that when administered to rats peripherally (5–20 $mg.kg^{-1}$), OEA caused an increase in noradrenaline levels in the hypothalamus in a dose-dependent manner. This demonstrates the crucial involvement of noradrenaline which enhances production and release of the neurotransmitter, histamine. Histamine then exerts its hypophagic actions to completely inhibit food intake (37), via facilitated increased oxytocin mRNA expression.

2.9.4 Functional interaction between oleoylethanolamide and brain dopamine

The ingestion of dietary fat is recognized to have a hedonic impact, triggering dopamine stimulation in the reward regions of the brain to activate the 'reward circuit' (200, 201). However, Tellez *et al.* (38) demonstrated that excessive intake of dietary fats leads to diminished brain dopaminergic function leading to overeating and eventually obesity. Dopamine release in the dorsal striatum (DS) of mice is also evident after gastric infusions of a fat emulsion (38, 167). Another finding reported that intra-gastric infusions of a low-calorie diluted fat emulsion (7.5% and 15% IntraLipid®) triggered dopamine release (38). By contrast, intra-gastric infusions of a high-calorie concentrated fat emulsion (30% IntraLipid®) failed to induce any effect (38). Additionally, Tellez and colleagues (38) performed an animal experiment showing that in HFD-fed mice, in which the diet comprised 60 kcal% fat, 20 kcal% protein and 20 kcal% carbohydrate, intestinal infusion of a concentrated triacylglycerol emulsion comprising 30% soybean

oil, 1.2% egg yolk phospholipids, 1.7% glycerine and water decreased the dopamine response in mice, confirming a blunted 'reward response' making mice obese. The researchers also demonstrated, in the same rats, that before intra-gastric infusion of the concentrated fat emulsion, injected OEA i.p. instantly restored the weak dopamine response in the brain, whereas this re-established response was altered following injection of the PPAR-α-specific antagonist (GW6471) (38). These findings suggest that improved dopamine responses in the mice brain after intestinal OEA injection stimulated the 'reward circuit' because OEA injection improved the dopamine response in response to diluted fat emulsions in both controls and HFD fed mice (38) that further triggered the 'reward value' of food that induced satiety and less food consumption. Furthermore, the study also reported the ability of OEA to potentiate dopamine efflux was abolished in sub-diaphragmatic bilaterally vagotomised mice compared with unilaterally vagotomised mice (38). This finding confirms that OEA participates in modulating the hedonic actions of dietary fat via vagal afferent nerves suggesting the role of jejunum in sensing fatty acid oxidation sensors that can influence eating (202).

Moreover, in another experiment conducted by Murillo-Rodríguez and colleagues in rats (203), local administration of the FAAH inhibitor URB597, which increases the levels of OEA in the brain and OEA in the lateral hypothalamus and the dorsal raphe nuclei showed increased levels of dopamine in nucleus accumbens shell (NAcS). Subsequent investigations in the rat midbrain demonstrated a modulation of dopaminergic function due to the peculiar capability of OEA to regulate nicotinic receptors containing β2 subunits, a nicotinic acetylcholine receptor symbolized as β2*-

nAChRs expressed by dopamine neurons that enhance the reward system from a brain stimulus (204). Furthermore, the limbic forebrain comprising of the endocannabinoids CB1 and CB2, (205) is also known to regulate and modulate the reward properties of food as well as drugs and these endocannabinoids are impacted by OEA (206–217). The anorexic properties of OEA are also facilitated via the blockade of CB1 receptors, which, when administered systemically in combined therapies with the cannabinoid CB1 receptor antagonist Rimonabant and the PPAR-α agonists OEA, reduces food intake and body weight to supply a synergistic effect (218). Furthermore, evidence based on microdialysis techniques illustrate that gut endocannabinoids also act as hunger signals. During these experiments, rats, when subjected to fat (1% corn oil and 1% linoleic acid), showed a significant stimulation of dopamine release in the NAcS despite a very low calorie content (219, 220). Moreover, the opioidergic system in NAcS is considered a predominant mediator of the hedonic sensation triggered by food (221), thereby validating that NAcS is a central controller of value learning (222–226) that enables animals to retain memory and ingest a low caloric emulsion.

In summary, these findings confirm that vital function of OEA in the regulation of reward actions occurs via activated release of dopamine. Tellez and colleagues (38) successfully demonstrated that exogenous OEA administration in subchronic treatments restored a normal and functional reward system in obese rats wherein obesity was generated by chronic exposure to a diet rich in fat. After the OEA administration, dopamine diffusion was activated in the DS, a brain section that connects and combines hedonic responses to habit learning (227); enabling mice to consume low fat emulsions.

The findings are supported by Ferreira and colleagues (167) by demonstrating a similar increase in striatal dopamine flux after gastric fat infusions in lean mice and hence, regulating fat intake.

Additionally, L'hirondel and colleagues (228) reported that oleic acid did not affect dopamine release. By contrast, Heller and colleagues (229) ruled out this possibility by demonstrating that oleic acid affected dopaminergic function in primary neurons of mesencephalic origin and, therefore, increased the dopamine content. Henceforth, because of inconsistencies amongst the reported findings, further investigation is warranted to understand the potential ability of oleic acid-OEA to increase dopamine levels to regulate feeding behaviour. Therefore, human trials focussing exclusively on dopaminergic system after bolus doses of oleic acid may enhance the understanding of modulating feeding behaviour that may carry therapeutic relevance and may contribute to the development of efficient strategies for treating obesity.

2.9.5 Summary of gut and brain interrelationship and regulation of feeding behaviour

The hypothalamus plays a pivotal role in the modulation of nutrient segregation, energy metabolism and feeding behaviours. The functions of the hypothalamus–pituitary–adrenal axis and the gastrointestinal tract have deep-seated interconnections via the following: (i) the stimulation and release of peptides; (ii) neuroendocrine hormones; and (iii) anorexigenic (appetite suppressing) or orexigenic (appetite stimulating) signals

through endogenous compounds from the gut. The previous section demonstrates evidence that combined action of feeding regulating hormones, histamine, oxytocin and dopamine modulates feeding behaviour and regulates appetite inducing satiety. Oxytocin is one such hormone released from the hypothalamus that plays a crucial role in inducing the satiating properties of OEA via inducing oxytocin neurotransmission in the CNS. In addition, OEA triggers the dopamine stimulation in the reward regions of the brain post consumption of dietary fat enriched in oleic acid, to activate the 'reward circuit' in the brain via gut generated lipid signalling; enhancing sensitivity and motivation towards less palatable, yet healthier, foods, that will reduce increasing obesity.

Furthermore, regulation of the appetite is fundamentally oriented in the three core nuclei of the hypothalamus and the brain stem located mainly in the tuberal medial area: (i) the arcuate nucleus of the hypothalamus; (ii) the dorsomedial hypothalamic nucleus; and (iii) the ventromedial hypothalamic nucleus. These nuclei are predominantly associated with feeding behaviours and satiety signals. Salient features of the arcuate nucleus of the hypothalamus, along with the regulation of feeding behaviour, involve the release of various pituitary hormones. Dorsomedial hypothalamic nucleus functions to stimulate gastrointestinal activity, and ventromedial hypothalamic nucleus primarily participates in inducing satiety. The rostral ventrolateral medulla in the medulla is another site in the brain that functions as a key regulator of the SNS. Orexinergic and anorexic neurons from the lateral hypothalamus open up in the rostral ventrolateral medulla. This site further activates the β-adrenergic receptor signals carried

by sympathetic nerve fibres to the gut, enabling OEA production (184). Furthermore, to commence meal termination, satiating signals from the liver and gastrointestinal tract are initiated through the vagus nerve to the NST. These signals are integrated and assessed by the hypothalamus together with the NST to determine the feeding response.

The preceding section suggests that the sympathetic cascade exerts fat-induced OEA satiety signalling to the small intestine either by (i) modulation of expression or (ii) management of enzymes in the OEA synthesis cascade. Although a complete understanding of OEA-induced satiety signalling remains to be elucidated, this detailed review enables the investigation and provides a summary showing that the consumption of fat-enriched food items particularly in oleic acid and the biosynthesis of OEA requires a synchronized association between the parasympathetic and sympathetic nervous systems. Therefore, synchronization between these two systems influences feeding as well as feeding-stimulated satiety signals by regulating the vagal-nigro-striatal pathway.

2.10 Oleoylethanolamide as safe anti-obesity alternative to drugs

Numerous anti-obesity pharmacological drugs have reached clinical use; however, these drugs still lack safety and efficacy because most of these drugs are centrally acting drugs which bear the adverse-effects. Therefore, it is vital to find an effective yet safe alternative to drugs that can induce satiety without burden of side effects. OEA has been observed to be a safe satiety-inducing compound, as demonstrated by Romano and associates (230), who monitored the behavioural satiety sequence involving the eating, grooming, rearing, locomotion and resting over the course of the initiation of satiety in

mice. This study substantiated the hypothesis that OEA, a functional antagonist of anandamide, suppresses appetite by stimulating satiety without altering total motor activity. By contrast, mice treated with the CB1 antagonist rimonabant, in addition to demonstrating decreased eating activity, showed an apparent increase in time spent grooming and reduced horizontal motor activity (230). Therefore, the reported alterations might be indicative of aversive non-motivational effects on feeding. These findings are analogous to a recent innovative study performed by Provensi and colleagues (37), thus providing a basis for the safe and efficient usage of OEA as an anti-obesity treatment. In summary, from nutritional, behavioural and psychological perspective, the anorexiant properties of OEA do not include any signs of anxiety or any other after-effects with changes in circulating corticosterone concentrations, which is a crucial biochemical indicator that regulates the overall energy balance (24, 25), proving it to be a potentially safe anti-obesity alternative.

2.11 Future directions

Overall, to understand the safe and efficient therapeutic usage of OEA in humans, future Phases I and II clinical trials are required with careful insight on the data provided by these trials obtained from neurobiologists, nutritionists, pharmacologists, physiologists, and psychiatrists. Trial parameters could include neuroimaging by functional magnetic resonance imaging as well as motor, cognition, and behaviour assessment. Additionally, single nucleotide polymorphisms in genes may influence an individual's response to a specific nutritional intervention. Therefore, research is needed to confirm the therapeutic

potential of OEA in suppressing food intake to curb obesity by conducting human clinical trials wherein participants should be recruited based on selected single nucleotide polymorphism-related genotypes. These trials should investigate the short as well as long-term effects of oleic acid consumption and their resulting impact on food intake, food regulating hormones, satiety, energy expenditure, fat distribution and body composition. The inclusion of invasive techniques such as fat pad biopsy of the abdominal fat pad and brown adipose tissue should enable a deeper mechanistic understanding of OEA stimulating gene expression. Addressing these points will further clarify the role for OEA in the context of the outcomes discussed. Henceforth, an amalgamated approach of conducting human clinical trials wherein motor, cognitive, behavioural, and regulation of appetite, as well as eating, will be monitored in comparison with the existing anti-obesity drugs would merit OEA in developing as an effective anti-obesity approach.

2.12 Summary and conclusion

A suggested mechanism for the uptake of OEA by dietary fat has been proposed in a study demonstrating that the up-regulation of oleic acid from dietary fat via the membrane protein CD36 results in higher levels of *N*-oleoyl-PE in enterocytes (9, 27). The resultant *N*-oleoyl-PE is further broken down by NAPE-PLD to produce OEA. Newly generated OEA activates PPAR-α, which initiates the anorexic signalling through the afferent vagal fibre (9). Although the evidence regarding anorectic properties of OEA exist, a knowledge gap exists as well regarding both the conversion of FAs to their

respective FAEs, e.g., the progression following the ingestion of oleic acid to the biosynthesis of OEA, as well as the factors that contribute to the rate of transformation/conversion. The evidence reviewed in this article indicates that intake of oleic acid, and thereby the resulting OEA, is dependent on CD36, PPAR-α, enterocyte fat sensory receptors, histamine, oxytocin, and dopamine; leading to increased fat oxidation and enhanced energy expenditure to induce satiety and increase feeding latency; and that a disruption in any of these systems will cease/curb fat-induced satiety.

In conclusion, the evidence reveals that small intestinal enterocytes synthesize OEA during the digestion of fat-containing foods, rich in oleic acid that leads to satiety and involves a series of molecular events in the paracrine PPAR-α mediated pathway that also necessitate the involvement of afferent sensory nerve fibres. Conversely, HFD induces gastrointestinal dysfunction attenuating dopamine levels and hampering the 'reward sensitivity circuit.' This deficiency of dopamine exacerbates obesity by provoking hyperphagia to restore the 'food reward value.' Although the mechanism of OEA's anorexic signalling to induce satiety remains the same in every individual, in vivo evidence conducted in animals and humans demonstrates an immense variability in FA intake perception by individuals due to the distinct activity of receptors in each individual's gut, which plays a critical role in food consumption and obesity (231). Therefore, amongst obese subjects, significant amounts of fat ingestion for prolonged durations could result in a decreased sensitivity to FAs, encouraging excessive fat ingestion to attain an adequate taste perception and thus lead to obesity. Furthermore, prolonged exposure to HFDs may induce a feedback mechanism that ultimately

attenuates OEA levels due to diminished brain dopaminergic function (148). Hence, future studies should clarify the overall molecular cascade by extending knowledge to understand the molecular mechanisms involved in the ingestion of FAs and their further perception and conversion to FAEs, which eventually leads to fat oxidation and EE. Research is needed to understand the in-depth mechanisms carrying out intestinal anorexic OEA signalling in the obese gut. In particular, identification of FA intake receptors will enable the elucidation of how fat perception works from a molecular standpoint, leading to a greater understanding of the influence of fat perception in humans. Future studies should also address the association between genetic polymorphisms associated with *CD36* and consumption of oleic acid.

The studies reviewed in this article show that appetite regulation is multifactorial, and therefore, sophisticated clinical approaches must be developed. Overall, the studies reviewed propose that the lipid-amide OEA acts as a fat sensor that is regulated by the synchronization between two divisions of the autonomic nervous system. First, is the SNS, by triggering the activation of OEA through efferent nerve fibres in the gut. Second, the parasympathetic nervous system, which conveys the anorectic signalling through the afferent fibres to the brain. The evidence reviewed herein also indicates that oleic acid increases EE, but whether this effect can be developed into a fruitful weight maintenance strategy will require further research. Therefore, future robust human randomized clinical trials are required focussing on the consumption of oleic acid, leading to the synthesis of OEA and associated satiety signals in gut and brain receptors along with the positron emission tomography technique to successfully capture the neuronal activities in

humans. Results from such trials will facilitate the development of apposite nutritional and pharmacological strategies to check appetite in obesity.

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2.14 Conflict of interest statement

No conflict of interest was declared.

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Bridge to Chapter 3

Chapter 2 is comprised of a manuscript which presented a retrospective analysis of previously completed pharmacological and nutritional intervention trials involving OEA. These data helped design the nutritional feeding trial presented in Chapters 3 and 4. The data in Chapter 3 show that consumption of C18:1n9-enriched dietary oils increases the energy expenditure and attenuates the feed intake improving body composition.

Chapter 3

Research Article

Manuscript 2

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Dietary fatty acid composition impacts plasma fatty acid ethanolamide levels and body composition in golden Syrian hamsters

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3.1 Abstract

Fatty acid ethanolamides (FAEs) are a class of lipid amides that regulate numerous pathophysiological functions. To date, pharmacological research in this area has focused on the endocannabinoid system, metabolic pathways, and biological significance of FAEs; however, limited nutritional studies have been conducted to understand the actions of FAEs on food intake and their role on overall body composition. Therefore, the present study was designed with the hypothesis that high C18:1n9 will attenuate food consumption in golden Syrian male hamsters ($n = 105$). Moreover, the long-term (two months) effects of feeding hamsters various dietary oil blends, namely, C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; and RC, regular canola oil, on the plasma levels of seven different FAEs and fatty acids (FAs) composition were investigated. A further objective was to characterize the actions of these diets on energy expenditure and overall body composition to determine if dietary fatty acid (DFA) composition affects diet-induced obesity (DIO). The results show that DFA directly influenced plasma FA and FAE levels, with marked increases ($P < 0.05$) observed in plasma C18:1n9 levels after HOCO and OO treatments. Correspondingly, the most elevated plasma oleoylethanolamide (OEA) levels were observed with HOCO and OO treatments, which also decreased ($P < 0.05$) food intake by ~8% when compared with H+EPA dietary treatment when measured at the endpoint. Diminished food intake

subsequent to HOCO and OO feeding may have resulted from increased OEA concentrations, demonstrating the anorexic properties of the high C18:1n9 dietary components. No differences were observed across OO, HOCO, and HOCO diets with omega-3 FA blends in terms of body composition, energy expenditure, plasma C18:1n9 levels, or OEA concentrations. Based on these findings, we conclude that the addition of HOCO to diets aids in the reduction of food intake, which may contribute to the maintenance of healthy body weight.

Keywords: Fatty acid ethanolamides, food intake, energy expenditure, body composition.

3.2 Introduction

Obesity has become pandemic, leading to increased metabolic syndrome prevalence worldwide. In Canada, obesity is a serious health epidemic that affects 25% of adults and 10% of children, with six million Canadians living with clinical obesity and requiring support in managing and controlling excess weight (1). Interactions between fat intake and obesity have generated interest in elucidating the physiological signals governing satiety. Pharmacological studies have focused on the effects of fatty acid ethanolamides (FAEs) which exert potential anti-obesity roles (2, 3). FAEs exist as a class of lipid amides that regulate numerous pathophysiological functions (4–7). For instance, the FAEs palmitoylethanolamide (PEA), oleoylethanolamine (OEA), and linoleoylethanolamide (LEA) possess a variety of physiological activities via PPAR-α activation (6, 8, 9), whereas arachidonoylethanolamide (AEA, also known as anandamide) shows a high affinity for cannabinoid receptor activation (10–12). Moreover, evidence suggests that OEA administration in rodents causes a persistent reduction in food intake and body mass (13, 14). In addition, docosahexaenoic acid (DHA) intake elevates plasma docosahexaenoylethanolamide (DHEA) levels, which was found to be associated with lowered plasma OEA levels in humans when high oleic canola oil (HOCO) oil was supplemented with DHA @ 15% and henceforth was observed to increase the gynoid fat mass subsequently (15). However, the precise physiological actions of α-linolenoylethanolamide (ALEA) and eicosapentaenoylethanolamide (EPEA) remain unknown.

Despite the identification of several potent FAEs, the association between food-derived fatty acids (FAs) and resulting plasma FAEs through long-term nutritional feeding underscores the need to determine their respective effects on food intake and overall body composition. Consequently, a clear understanding of the link between various oil blends, food intake, energy expenditure, and overall body fat composition is critical to elucidate the optimal consumable oil blends capable of inducing weight loss and thus facilitating improved health outcomes. To date, research in this field has specifically focused on the pharmacological importance of FAEs in various tissues (16); however, the impact of dietary blends with varying fatty acid (FA) compositions through long-term nutritional feeding on diet-induced obesity (DIO) has yet to be studied. Thus, understanding the efficacy of these endogenous ligands altogether has yet to be elucidated through long-term nutritional feeding.

Moreover, previous reports demonstrated that short-term exposure to a high-fat diet (HFD) decreases OEA levels in the rodent small intestine (17, 18). As such, the present study sought to investigate the long-term effects of different dietary oils varying in their FA profile on plasma FAs and FAEs composition in hamsters. Therefore, to achieve this objective, a range of n-6, n-9, and n-3 enriched oils were selected and incorporated into the diets fed to hamsters. The trial was performed hypothesizing that n-9 enriched oil blends will reduce food consumption and enhance energy expenditure, subsequently improving the overall body composition. In addition, we examined the synthesis of FAEs from varying dietary fatty acid (DFA) with the goal of identifying the best blend of oils to improve overall health over the long term.

To achieve our objectives, a model diet was prepared to induce dietary obesity (Tables 3.1 and 3.2). The diet comprises 36% sucrose and 10% fat, which will lead to weight gain over an extended feeding period (60 days).

3.3 Materials and methods

3.3.1 Chemicals

Heptadecanoic acid (C17:0) internal standard was obtained from Sigma-Aldrich (St Louis, MO, USA) and the GLC 461 standards mix was purchased from Nu-Chek Prep Inc. (Elysian, MN, USA). FAE standards—including PEA, OEA, LEA, ALEA, AEA, DHEA, and EPEA—and deuterium-containing internal FAE standards—[$^2H_4$] PEA, [$^2H_4$] OEA, [$^2H_4$] LEA, [$^2H_4$] ALEA, [$^2H_8$] AEA, [$^2H_4$] DHEA, and [$^2H_4$] EPEA—were obtained from Cayman Chemical (Ann Arbor, MI, USA). Liquid chromatography (LC)-grade solvents were purchased from Sigma-Aldrich. All other chemicals and reagents were purchased from Sigma-Aldrich or Fisher Scientific (Waltham, MA, USA).

3.3.2 Animals

All experimental procedures were approved by the Animal Ethics Board, University of Manitoba, Canada. All experiments were conducted according to the Canadian Animal Care guidelines. Four-week-old male golden Syrian hamsters (n = 105) were purchased from Charles River Laboratories (Montreal, Quebec, Canada) and allowed to acclimatize for two weeks in the animal facility (temperature, 22 °C; humidity, 30–60%) with controlled 12 h light/dark cycles (daylight from 6:30 to 18:30). During acclimatization,

Table 3.1 Ingredients composition of experimental diets provided to hamsters.

Diet (%, w/w)	C+S	F+S	H+DHA	H+EPA	HOCO	OO	RC
Casein[a]	20	20	20	20	20	20	20
DL-methionine[b]	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Corn starch	23.70	23.70	23.70	23.70	23.70	23.70	23.70
Sucrose	36	36	36	36	36	36	36
Cellulose[a]	5	5	5	5	5	5	5
Mineral mix[a]	3.50	3.50	3.50	3.50	3.50	3.50	3.50
Vitamin mix[a]	1	1	1	1	1	1	1
Choline bitartrate[a]	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Ethoxyquin	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014
Cholesterol	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Beef tallow	4	4	4	4	4	4	4
Blend of C+S	6	-	-	-	-	-	-
Blend of F+S	-	6	-	-	-	-	-
H+DHA	-	-	6	-	-	-	-
H+EPA	-	-	-	6	-	-	-
HOCO	-	-	-	-	6	-	-
OO	-	-	-	-	-	6	-
RC	-	-	-	-	-	-	6

[a] Based on AIN-93G recommendations. [b] Based on AIN-76A recommendations.

Note: C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil.

Table 3.2 Overall composition of all experimental diets provided to hamsters.

Composition	g%	kcal%
Protein	20	19.51
Carbohydrate	60	58.54
Fat	10	21.95
Total	-	100
Kcal/g	4.10	-

the hamsters were provided access to water and a standard pelleted rodent chow diet (Prolab RMH 3000 Diet) *ad libitum*. After the acclimatization period, the hamsters were transferred to individual cages.

3.3.3 Diets

The hamsters were fed a low fat high sucrose (LFHS) diet (36 kcal% sucrose, 10 kcal% fat, 4.10 kcal g^{-1}) for 8 weeks. The diets were supplemented with various fats comprising 4% of the total dietary composition as follows: (1) C+S, 25:75 corn oil:n9 safflower oil; (2) F+S, 25:75 flaxseed oil:n6 safflower oil; (3) H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; (4) H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; (5) HOCO, (6) OO, olive oil; and (7) RC, regular canola oil.

All dietary treatments were prepared in the animal facility at the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN), University of Manitoba (Winnipeg, MB, Canada). Dietary ingredients were purchased from Dyets Inc. (Bethlehem, PA, USA). The n9 safflower oil was purchased from Vitahealth (Winnipeg, MB, Canada). HOCO was obtained from Richardson Oilseed (Winnipeg, MB, Canada) and the DHA and EPA oils were obtained from Croda Inc. (Edison, NJ, USA). The experimental diets and their FA composition are shown in Tables 3.1–3.3 and Figure 3.1. All animals were healthy throughout the observation period.

3.3.4 Experimental design

3.3.4.1 Study protocol

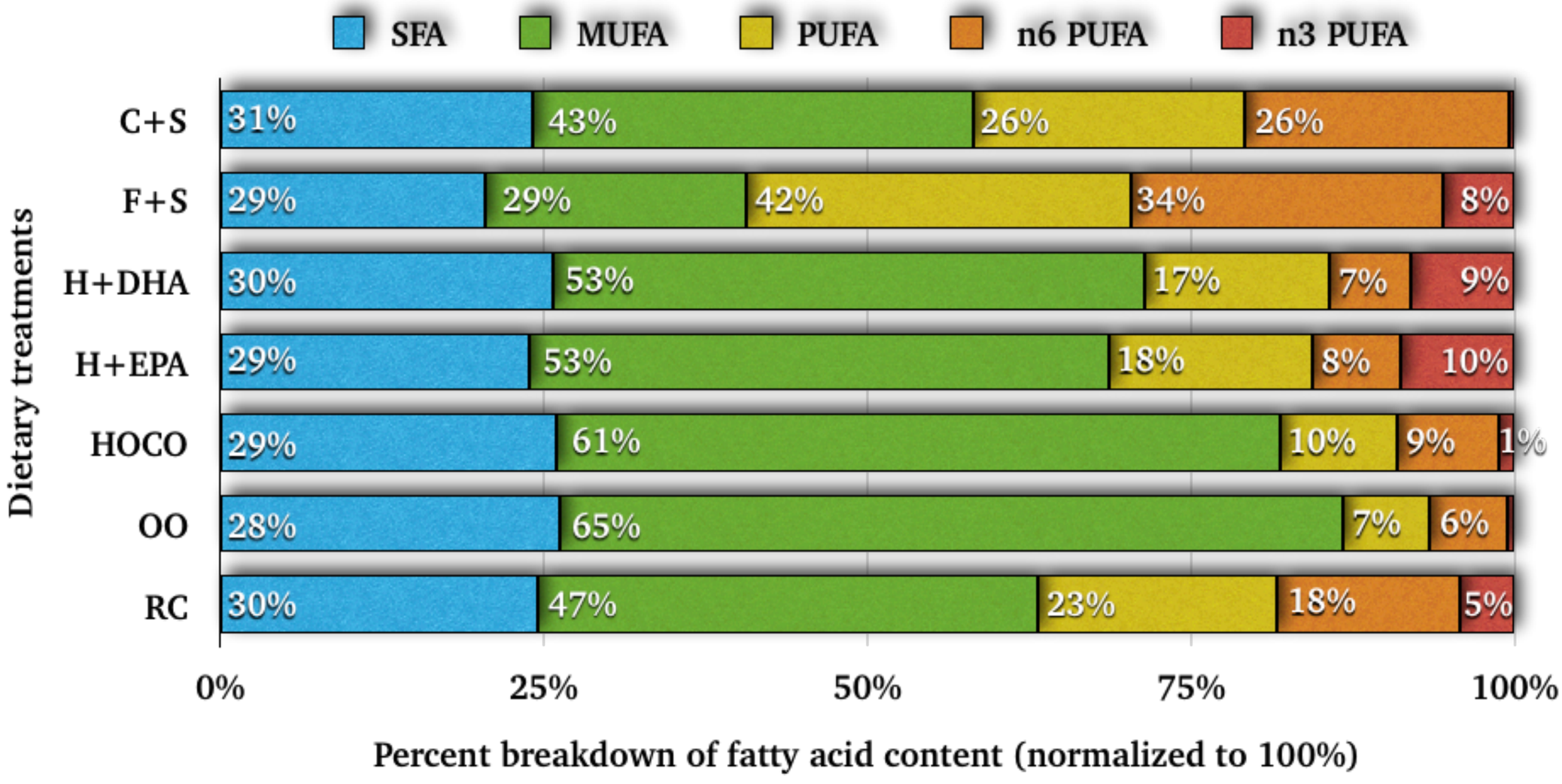
Table 3.3 Fatty acid composition of the diets (g/100g)*.

Fatty acid	C+S	F+S	H+DHA	H+EPA	HOCO	OO	RC
C8:0	0.01	0.01	0.01	0.01	0.01	0.01	0.01
C10:0	0.04	0.04	0.04	0.04	0.04	0.02	0.04
C12:0	7.17	6.29	6.78	6.30	7.20	5.24	7.29
C14:0	1.39	1.38	1.42	1.43	1.36	1.16	1.39
C14:1n9	0.35	0.34	0.39	0.36	0.38	0.29	0.35
C15:0	0.33	0.31	0.30	0.34	0.33	0.30	0.35
C16:0	14.23	13.80	12.33	12.41	12.35	15.14	13.54
C16:1n9	1.33	1.31	1.54	1.54	1.43	1.79	1.38
C18:0	6.98	7.09	7.26	6.96	6.92	6.00	7.09
C18:1n9	40.72	26.44	50.25	50.17	58.54	62.36	44.67
C18:2n6	25.75	34.25	6.62	6.85	8.31	6.19	17.06
C18:3n6	0.02	0.02	0.05	0.22	0.15	0.15	0.14
C18:3n3	0.34	7.59	0.86	1.04	0.97	0.41	4.76
C20:0	0.20	0.21	0.34	0.28	0.35	0.20	0.29
C20:1n9	0.21	0.22	0.78	0.61	0.72	0.24	0.62
C20:2n6	0.02	0.04	0.10	0.05	0.03	0.02	0.05
C20:3n6	0.03	0.04	0.07	0.10	0.04	0.03	0.04
C20:4n6	0.01	0.02	0.40	0.64	0.02	0.02	0.03
C20:3n3	ND	0.01	0.06	0.04	0.02	0.03	0.03
C22:0	0.05	0.06	1.47	0.47	0.02	ND	0.03

C22:1n9	0.29	0.17	0.19	0.26	0.25	0.10	0.23
C20:5n3	0.24	0.23	0.33	7.20	0.33	0.22	0.30
C22:2n6	ND	ND	0.01	0.06	ND	ND	ND
C22:4n6	0.05	0.06	0.09	0.16	0.08	ND	0.09
C22:5n3	0.01	ND	0.41	0.38	0.04	0.02	0.07
C22:6n3	ND	ND	7.72	1.80	ND	ND	0.03
C24:0	0.17	0.05	0.06	0.05	0.07	0.03	0.06
C24:1n9	0.05	0.03	0.14	0.04	0.05	0.02	0.07
Total SFA	30.57	29.23	30.00	28.28	28.65	28.10	30.09
Total MUFA	42.95	28.52	53.28	52.98	61.36	64.80	47.33
Total PUFA	26.48	42.25	16.72	18.54	9.99	7.10	22.59
Total n6PUFA	25.89	34.42	7.34	8.09	8.64	6.42	17.41
Total n3PUFA	0.59	7.83	9.38	10.45	1.35	0.68	5.18
n6:n3	43.85	4.39	0.78	0.77	6.37	9.49	3.36

Note: *The values are % abundance of each fatty acid to total fatty acids. C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil; ND, not detected; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

Figure 3.1 Major fatty acid composition of the diets (g/100g).



Note: C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

Following acclimatization, the hamsters were systematically randomized into study groups to ensure an even distribution of starting body weights across groups ($n = 15$ per treatment group). Blocks of hamsters were initiated into the study protocol every six days. Figure 3.2 outlines the graphical representation of the protocol for the animal trial.

3.3.4.2 Measurement of food intake and body weight

Food consumption was assessed every other day by subtracting the amount of food remaining in the feeder from that recorded the previous day. Food spillage was taken into account to ensure accuracy. Body weights were monitored every fourth day throughout the treatment period at the same time at 10:00 h.

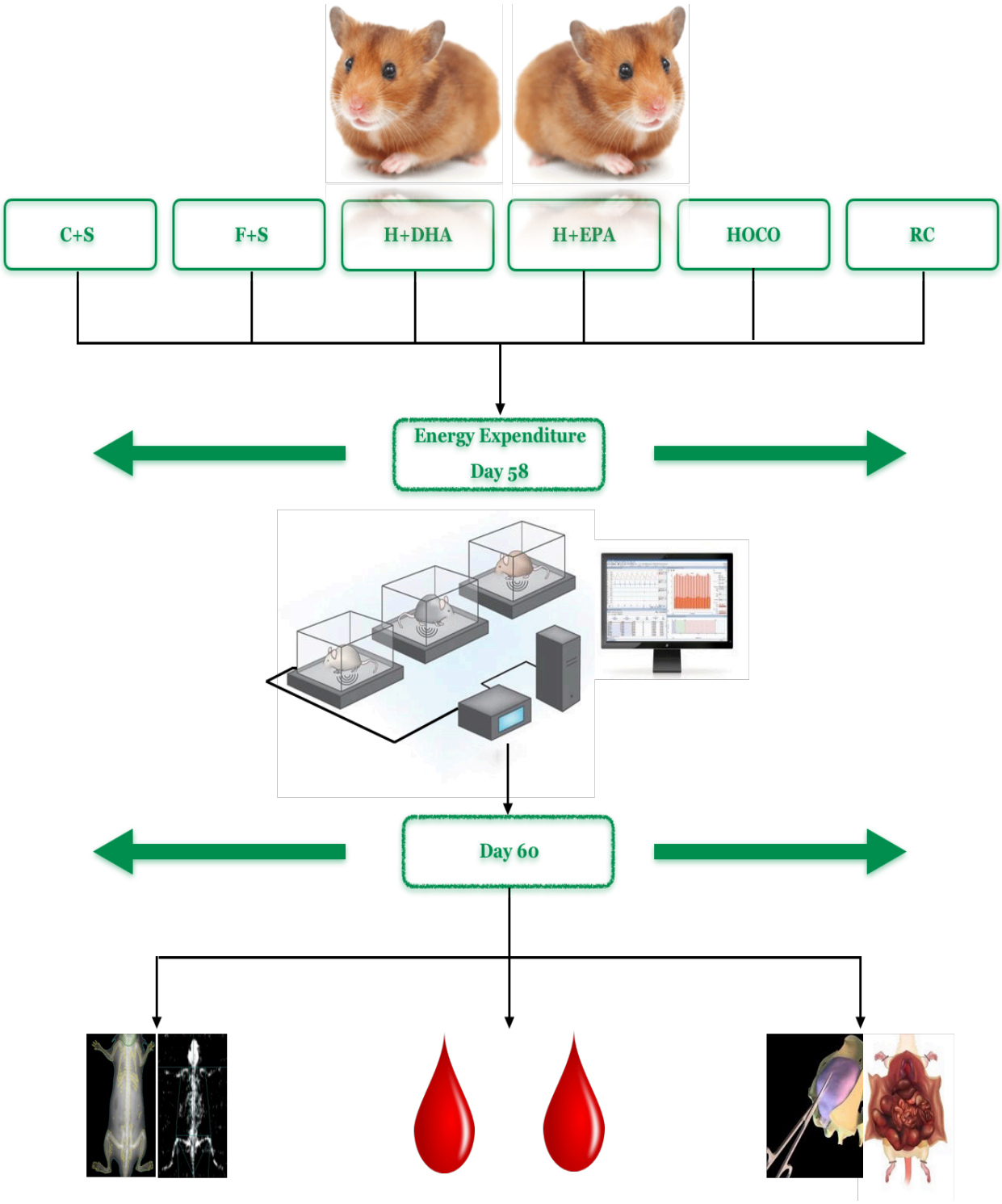
3.3.4.3 Energy expenditure

At the end of the animal trial, on day 58, energy expenditure was assessed using a respiratory gas exchange system for rodents (MM-100 CWE Inc., Pennsylvania, PA, USA). The animals were housed in individual metabolic chambers, and oxygen consumption was measured at 1 min intervals over 8 h. The percentage of total oxygen consumed by carbohydrate and fat was calculated with the Lusk formula as follows:

$$\text{carbohydrate oxidation (\%)} = \frac{\text{RER} - 0.707}{0.293} \times 100$$

$$\text{fat oxidation (\%)} = \frac{1 - \text{RER}}{0.293} \times 100$$

Figure 3.2 Study design and methods.



Note: C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil.

where RER is the respiratory exchange ratio (19).

3.3.4.4 Feeding restriction and sample collection

After feeding on day 59, the animals were starved for 12 h (21:00 to 9:00) before euthanasia by isoflurane inhalation (2.5%). Blood samples were collected by cardiac puncture and centrifuged at 1500g at 4 °C for 20 min. Plasma was then collected and immediately stored at − 80 °C until analysis.

3.3.4.5 Dual energy X-ray absorptiometry (DXA)

Body composition, including fat mass, lean mass, and fat-free mass, was assessed by DXA (Lunar Prodigy Advance, GE Healthcare, Madison, WI, USA). The data were evaluated with the Encore 2005 software (v. 9.30.044; GE Healthcare, Little Chalfont, UK).

3.3.5 Fatty acid analysis

FA profiles in the blood plasma were analyzed via direct transesterification followed by gas chromatography (20). For this, 2 mL of methanol:toluene (4:1 vol/vol) was added to the sample with heptadecanoic acid (17:0) as an internal standard. Acetyl chloride (200 µL) was added while vortexing and heated for 1 h at 80 °C. The samples were cooled and then 5 mL of K_2CO_3 was added before centrifuging at 2500 rpm for 5 min at 4 °C. The upper toluene phase containing FA methyl esters was collected and stored in a gas chromatograph vial at − 80 °C until further analysis.

Methylated FA samples were analyzed by gas chromatography using a fixed capillary column (30 m × 0.25 mm; film thickness, 0.25 µm, BPX70; SGE Analytical Science, Canada, Inc.) on a Varian 430 gas chromatograph equipped with a flame ionization detector. The injector and detector ports were set at 260 °C and 290 °C, respectively. The oven temperature was set to 70 °C for 2 min and then increased to 155 °C (30 °C min^{-1}) and subsequently 180 °C (5 °C min^{-1}), where it remained constant for 3 min. The temperature was then increased to 210 °C (4 °C min^{-1}), followed by 260 °C (50 °C min^{-1}), which was maintained for a total run time of 27 min. A split ratio of 40:1 and an injection volume of 1 µL were used. A known FA mixture was compared with the samples to identify the retention peaks in the Galaxie software (Varian Inc.). The level of each FA was then calculated according to the corresponding peak area relative to that of all FAs of interest to yield the relative percentage of total FA.

3.3.6 Fatty acid ethanolamide analysis

Plasma FAEs were extracted using a solid-phase extraction method (21, 22) with minor modifications. Synthetic deuterium-labeled FAEs served as internal standards to ice-cold plasma samples. The plasma samples were subjected to pre-activated Oasis HLB 1cc, 30 mg cartridge (Waters Corporation, Milford, MA, USA) and filtered at a ~1 mL min^{-1} flow rate with a vacuum manifold (Agilent Technologies, ON, Canada). The extract on the cartridge was washed, and compounds of interest were eluted with acetonitrile. FAE levels were further analyzed using a Waters Acquity UPLC system coupled to a Micromass Quattro *micro* API mass spectrometer (Waters, Milford, MA) equipped with

an atmospheric pressure ionization (API) probe and electrospray ionization interface (ESI). FAEs were separated on a Kinetex XB-C18 column (2.1 × 100 mm, 1.7 µm, Phenomenex Inc.) using an acetonitrile gradient. Solvent A consisted of Milli-Q water (18.2 MΩ) containing 0.1% formic acid, and Solvent B consisted of acetonitrile containing 0.1% formic acid. The solvent gradient profile was as follows: 0 min, 70% B; 0–1 min, 72% B; 1–2 min, 74% B; 2–6.10 min, 98% B linear gradient; 6.10–10 min, 70% B. Samples were processed at 4 °C with a 0.2 mL min^{-1} flow rate and the column was maintained at 40 °C. The ESI was set in positive ionization mode (+ESI) with a 4 kV capillary voltage. Cone voltage was set to 32 V for OEA and PEA, and 22 V for all other FAEs. The optimized MS/MS conditions for all FAEs were as follows: 135 °C source temperature, 500 °C desolvation temperature, 50.5 L h^{-1} cone gas, and 1000 L h^{-1} desolvation gas. The multiple reaction monitoring (MRM) mode was used to monitor the precursor to product ion transition. The data were acquired and processed using Masslynx version 4.1 (Waters, Milford, MA, USA) and the absolute amounts of FAEs were quantified with a calibration curve.

3.3.7 Statistical analyses

The results are expressed as mean ± SEM. The data were analyzed using SAS 9.3 (IBM Software, Armonk, NJ, USA). The statistical significance was evaluated with one-way analysis of variance (ANOVA) and Tukey's *post-hoc* testing when applicable. Pearson's correlation analyses were performed to examine the associations between plasma FAs and FAEs. Statistical significance was defined as $P < 0.05$.

3.4 Results

3.4.1 Effect of diet treatment on food consumption, energy intake, and percentage body weight change

The animals fed the HOCO, and OO diet consumed ~8% less food per day compared with those fed the H+EPA diet ($P < 0.05$); also, no significant differences were observed in food intake between HOCO and OO treatments. Furthermore, no significant differences were observed between the percentage of body weight changes across any dietary treatments (Table 3.4).

3.4.2 Effect of diet on energy expenditure and body composition

Elevated fat oxidation was observed in the hamsters fed OO (51% increase; $P < 0.05$), followed by H+EPA, H+DHA, F+S, and HOCO when compared to C+S. Similarly, the highest ($P < 0.05$) carbohydrate oxidation was observed in the C+S fed hamsters when compared to all other dietary treatments except RC; however, increased ($P < 0.05$) fat oxidation was only observed with C+S treatment when the n9 safflower oil was administered by gavage ($n = 10$; data not shown). Body composition analysis revealed that C+S treatment resulted in the highest ($P < 0.05$) fat mass retention when compared with the F+S and OO treatments. No changes in fat mass and lean mass were observed between HOCO and OO treatments, while the RC hamsters exhibited the higher ($P < 0.05$) lean mass when compared to the C+S dietary treatment (Table 3.5).

3.4.3 Effect of diet on plasma fatty acid levels

Table 3.4 Food consumption, energy intake and percentage of body weight change.

Treatments	Food consumption (g.day^{-1})	Energy intake (kcal.day^{-1})	Body weight change (%)
C+S	7.28 ± 0.14^{ab}	29.85 ± 0.55^{ab}	17.96 ± 2.24
F+S	7.05 ± 0.12^{ab}	28.91 ± 0.50^{ab}	16.02 ± 3.08
H+DHA	7.26 ± 0.15^{ab}	29.77 ± 0.58^{ab}	20.71 ± 2.91
H+EPA	7.57 ± 0.28^{a}	31.04 ± 1.10^{a}	18.48 ± 3.12
HOCO	6.96 ± 0.11^{b}	28.54 ± 0.46^{b}	15.00 ± 2.71
OO	6.81 ± 0.17^{b}	27.92 ± 0.67^{b}	16.23 ± 2.87
RC	7.26 ± 0.16^{ab}	29.77 ± 0.63^{ab}	17.74 ± 2.35

Note: The results are expressed as mean ± SEM (n = 15). The values with different superscript letters in the same column are significantly different from each other ($P < 0.05$). C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil: eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil.

Table 3.5 Energy expenditure and body composition.

Treatments	Energy expenditure		Body composition	
	Carbohydrate oxidation (%)	Fat oxidation (%)	Fat mass (g)	Lean mass (g)
C+S	52.80 ± 5.26[a]	47.20 ± 5.26[b]	70.42 ± 2.94[a]	64.74 ± 2.49[b]
F+S	33.06 ± 6.87[b]	66.94 ± 6.87[a]	59.87 ± 2.73[b]	68.86 ± 2.91[ab]
H+DHA	31.29 ± 10.52[b]	68.71 ± 10.52[a]	63.46 ± 3.71[ab]	72.28 ± 3.06[ab]
H+EPA	25.56 ± 7.75[b]	74.44 ± 7.75[a]	64.06 ± 4.66[ab]	70.61 ± 1.92[ab]
HOCO	35.77 ± 5.08[b]	64.23 ± 5.08[a]	62.33 ± 3.19[ab]	67.09 ± 3.03[ab]
OO	18.84 ± 5.54[b]	81.16 ± 5.49[a]	60.33 ± 1.89[b]	67.96 ± 2.15[ab]
RC	40.68 ± 4.72[ab]	59.32 ± 4.72[ab]	62.02 ± 3.18[ab]	73.56 ± 2.24[a]

Note: The results are expressed as mean ± SEM; energy expenditure (n = 5–12) and body composition (n = 9–12). The values with different superscript letters in the same column are significantly different from each other ($P < 0.05$). C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil.

The effects of dietary treatment on plasma FA levels are shown in Table 3.6. No detectable changes in medium chain FA levels were observed; however, notable alterations were found in long chain FA levels. Plasma C16:0 content was lower ($P < 0.05$) with the feeding of the C+S, RC, and HOCO diets when compared to F+S, H+DHA, H+EPA, and OO. An increase ($P < 0.05$) in the levels of plasma C18:1n9 was observed in the animals fed HOCO, OO, H+DHA, and H+EPA compared to those fed C+S, F+S and RC. Also, comparable contents of C18:1n9 were observed in the plasma of the hamsters fed HOCO and OO treatments. The overall monounsaturated fatty acid (MUFA) composition was also observed to be comparable when the animals were fed HOCO and OO diets. Linoleic acid, C18:2n6, content was increased ($P < 0.05$) after feeding the F+S diet; however, the low content of C18:3n3 with F+S reflected the higher ($P < 0.05$) concentration of C18:2n6 in the hamsters' plasma when compared to C18:3n3. C20:4n6 was the lowest ($P < 0.05$) in H+DHA and H+EPA dietary treatments, and C22:1n9 was not detected in the plasma of any animal. Moreover, the H+EPA-fed animals had the highest ($P < 0.05$) content of C20:5n3, while increased ($P < 0.05$) C22:6n3 was observed after the consumption of the H+DHA diet when compared to all other diets ($P < 0.05$). Furthermore, the F+S diet, followed by C+S and RC treatments, resulted in higher ($P < 0.05$) plasma polyunsaturated fatty acid (PUFA) levels, notably C18:2n6; which is indicative of the higher C18:2n6 levels present in these diets. Overall, the n6:n3 ratio of all dietary treatments directly corresponded to the n6:n3 ratios observed in the blood plasma.

Table 3.6 Plasma fatty acids (g/100g)* in response to dietary interventions.

Fatty acid	C+S	F+S	H+DHA	H+EPA	HOCO	OO	RC
C8:0	ND	ND	ND	ND	ND	ND	ND
C10:0	0.17 ± 0.01	0.17 ± 0.01	0.19 ± 0.02	0.20 ± 0.02	0.20 ± 0.02	0.16 ± 0.03	0.18 ± 0.01
C12:0	0.03 ± 0.01^{ab}	0.02 ± 0.01^{b}	0.04 ± 0.01^{a}	0.03 ± 0.01^{ab}	0.02 ± 0.01^{b}	0.03 ± 0.01^{ab}	0.02 ± 0.01^{b}
C14:0	0.35 ± 0.02^{ab}	0.31 ± 0.02^{b}	0.35 ± 0.01^{ab}	0.34 ± 0.02^{ab}	0.35 ± 0.02^{ab}	0.40 ± 0.02^{a}	0.33 ± 0.02^{b}
C14:1n9	0.01 ± 0.01^{b}	0.01 ± 0.01^{ab}	0.03 ± 0.01^{a}	0.01 ± 0.01^{ab}	0.01 ± 0.01^{ab}	ND	0.01 ± 0.01^{b}
C15:0	0.27 ± 0.01^{bc}	0.25 ± 0.01^{c}	0.32 ± 0.01^{a}	0.30 ± 0.01^{ab}	0.29 ± 0.02^{abc}	0.28 ± 0.01^{bc}	0.29 ± 0.01^{abc}
C16:0	18.35 ± 0.24^{abc}	17.01 ± 0.27^{d}	19.06 ± 0.20^{a}	19.13 ± 0.41^{a}	17.98 ± 0.35^{bc}	18.83 ± 0.42^{ab}	17.79 ± 0.29^{cd}
C16:1n9	1.50 ± 0.05	1.47 ± 0.04	1.48 ± 0.05	1.54 ± 0.09	1.42 ± 0.03	1.54 ± 0.03	1.44 ± 0.04
C18:0	10.87 ± 0.28^{ab}	11.25 ± 0.30^{a}	9.69 ± 0.54^{b}	10.70 ± 0.78^{ab}	10.68 ± 0.40^{ab}	10.52 ± 0.52^{ab}	10.35 ± 0.23^{ab}
C18:1n9	29.99 ± 0.62^{b}	25.16 ± 0.95^{c}	36.68 ± 1.37^{a}	36.41 ± 1.92^{a}	37.52 ± 1.26^{a}	39.28 ± 1.54^{a}	32.03 ± 0.84^{b}
C18:2n6	25.52 ± 0.26^{b}	31.81 ± 0.50^{a}	16.74 ± 0.56^{d}	16.28 ± 0.58^{d}	18.22 ± 0.38^{c}	16.82 ± 0.43^{d}	24.64 ± 0.35^{b}
C18:3n6	0.24 ± 0.01^{a}	0.16 ± 0.01^{c}	0.02 ± 0.01^{d}	0.03 ± 0.01^{d}	0.21 ± 0.01^{ab}	0.21 ± 0.02^{ab}	0.17 ± 0.01^{bc}
C18:3n3	0.13 ± 0.01^{d}	0.89 ± 0.07^{a}	0.30 ± 0.01^{c}	0.30 ± 0.02^{c}	0.17 ± 0.01^{d}	0.13 ± 0.01^{d}	0.58 ± 0.02^{b}
C20:0	0.07 ± 0.01^{b}	0.08 ± 0.01^{ab}	0.09 ± 0.01^{a}	0.08 ± 0.01^{ab}	0.07 ± 0.01^{ab}	0.07 ± 0.01^{b}	0.07 ± 0.01^{b}
C20:1n9	0.31 ± 0.01^{bc}	0.28 ± 0.02^{cd}	0.24 ± 0.01^{d}	0.25 ± 0.01^{d}	0.37 ± 0.02^{a}	0.34 ± 0.02^{ab}	0.31 ± 0.01^{bc}
C20:2n6	0.24 ± 0.01^{b}	0.30 ± 0.01^{a}	0.09 ± 0.01^{d}	0.09 ± 0.01^{d}	0.18 ± 0.01^{c}	0.15 ± 0.01^{c}	0.22 ± 0.01^{b}
C20:3n6	1.24 ± 0.04^{a}	0.97 ± 0.04^{b}	0.39 ± 0.02^{d}	0.55 ± 0.03^{c}	1.28 ± 0.04^{a}	1.24 ± 0.07^{a}	1.06 ± 0.03^{b}
C20:4n6	7.92 ± 0.26^{a}	5.60 ± 0.17^{d}	3.86 ± 0.20^{e}	4.02 ± 0.22^{e}	7.00 ± 0.40^{ab}	6.66 ± 0.57^{bc}	5.73 ± 0.22^{cd}
C20:3n3	0.03 ± 0.01^{abc}	0.06 ± 0.01^{ab}	0.01 ± 0.01^{c}	0.03 ± 0.01^{abc}	0.06 ± 0.02^{a}	0.05 ± 0.01^{abc}	0.02 ± 0.01^{bc}
C22:0	0.10 ± 0.01^{a}	0.08 ± 0.01^{ab}	0.09 ± 0.01^{a}	0.08 ± 0.01^{ab}	0.09 ± 0.01^{a}	0.07 ± 0.01^{b}	0.09 ± 0.01^{a}

Fatty acid	C+S	F+S	H+DHA	H+EPA	HOCO	OO	RC
C22:1n9	ND	ND	ND	ND	0.01 ± 0.01	ND	ND
C20:5n3	ND	0.08 ± 0.01^{c}	1.42 ± 0.10^{b}	3.46 ± 0.44^{a}	0.03 ± 0.01^{c}	0.02 ± 0.01^{c}	0.10 ± 0.01^{c}
C22:2n6	ND	0.01 ± 0.01	ND	ND	ND	ND	ND
C22:4n6	0.17 ± 0.01^{a}	0.11 ± 0.01^{b}	0.04 ± 0.01^{c}	0.03 ± 0.01^{c}	0.12 ± 0.01^{b}	0.12 ± 0.01^{b}	0.10 ± 0.01^{b}
C22:5n3	0.14 ± 0.01^{d}	0.32 ± 0.01^{c}	0.56 ± 0.04^{b}	1.47 ± 0.12^{a}	0.18 ± 0.02^{d}	0.13 ± 0.01^{d}	0.29 ± 0.01^{c}
C22:6n3	2.11 ± 0.09^{e}	3.36 ± 0.18^{cd}	7.97 ± 0.52^{a}	4.41 ± 0.42^{b}	3.27 ± 0.18^{cd}	2.73 ± 0.22^{de}	3.91 ± 0.14^{bc}
C24:0	0.10 ± 0.01^{ab}	0.09 ± 0.01^{ab}	0.11 ± 0.01^{a}	0.10 ± 0.01^{ab}	0.09 ± 0.01^{ab}	0.08 ± 0.01^{b}	0.11 ± 0.01^{a}
C24:1n9	0.13 ± 0.01^{b}	0.14 ± 0.01^{b}	0.22 ± 0.02^{a}	0.16 ± 0.02^{b}	0.16 ± 0.01^{b}	0.13 ± 0.01^{b}	0.17 ± 0.01^{b}
Total SFA	30.32 ± 0.38	29.27 ± 0.38	29.95 ± 0.65	30.94 ± 1.11	29.78 ± 0.66	30.45 ± 0.90	29.22 ± 0.35
Total MUFA	31.94 ± 0.65^{b}	27.07 ± 0.99^{c}	38.65 ± 1.39^{a}	38.38 ± 1.97^{a}	39.50 ± 1.27^{a}	41.30 ± 1.55^{a}	33.95 ± 0.87^{b}
Total PUFA	37.74 ± 0.42^{b}	43.66 ± 0.71^{a}	31.41 ± 0.88^{c}	30.68 ± 0.96^{c}	30.73 ± 0.78^{c}	28.26 ± 0.78^{d}	36.83 ± 0.57^{b}
Total n6PUFA	35.34 ± 0.35^{b}	38.95 ± 0.63^{a}	21.15 ± 0.57^{f}	21.02 ± 0.57^{f}	27.01 ± 0.64^{d}	25.19 ± 0.61^{e}	31.92 ± 0.49^{c}
Total n3PUFA	2.40 ± 0.09^{d}	4.72 ± 0.15^{b}	10.26 ± 0.63^{a}	9.66 ± 0.82^{a}	3.72 ± 0.18^{c}	3.07 ± 0.21^{cd}	4.91 ± 0.13^{b}
n6:n3	15.25 ± 0.57^{a}	8.34 ± 0.23^{b}	2.14 ± 0.10^{d}	2.31 ± 0.15^{d}	7.40 ± 0.25^{bc}	8.51 ± 0.33^{b}	6.55 ± 0.15^{c}

Note: *The values are % abundance of each fatty acid to total fatty acids. The results are expressed as mean ± SEM (n = 15). The values with different superscript letters in the same row are significantly different from each other ($P < 0.05$). C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil; ND, not detected; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

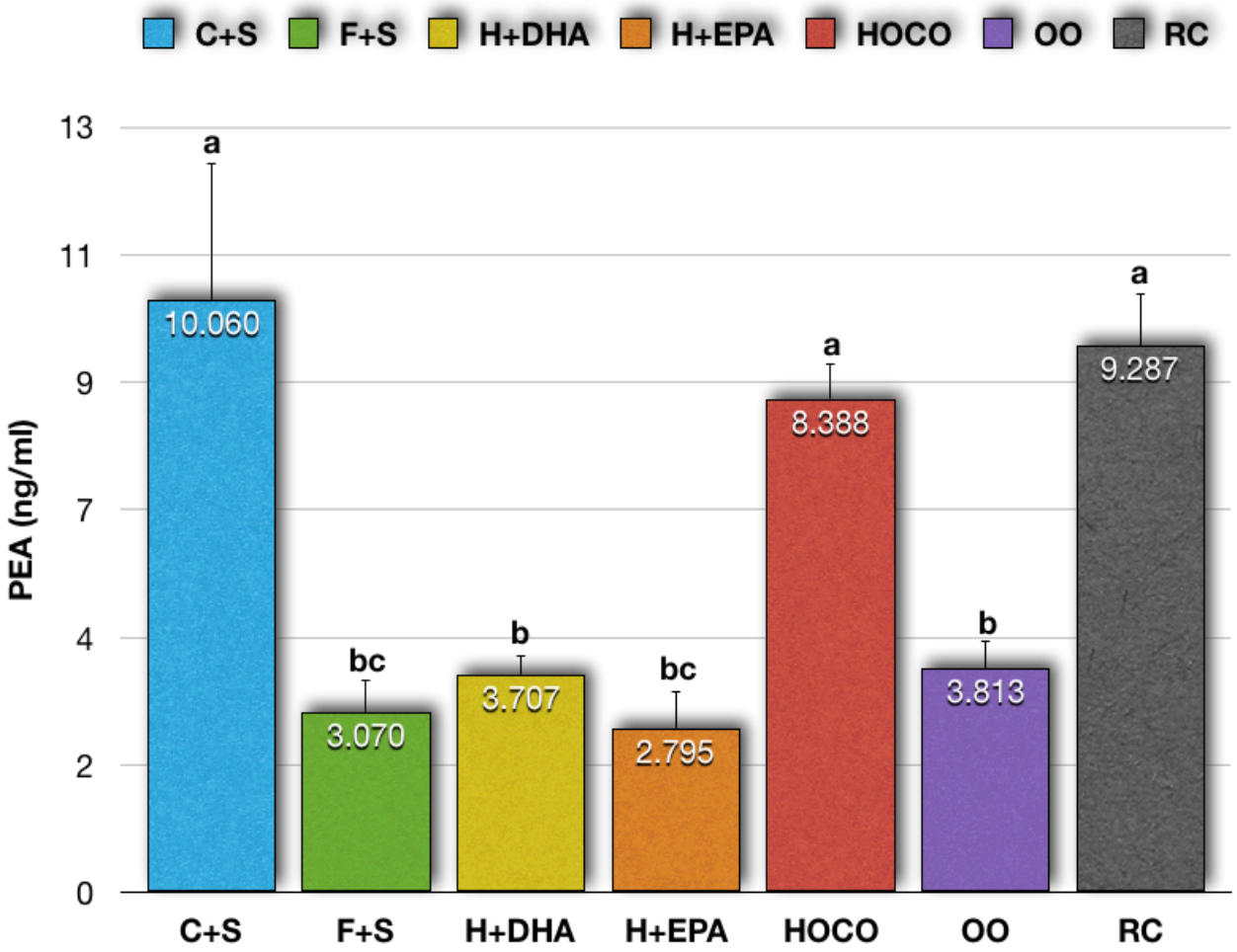
3.4.4 Effect of diet on plasma fatty acid ethanolamide levels

The FAE levels in the plasma are shown in Figures 3.3–3.9. C+S, RC, and HOCO diets reflect higher ($P < 0.05$) levels of PEA (Figure 3.3). The higher percentage of n9 FA in HOCO and OO diets resulted in an increase ($P < 0.05$) in OEA concentrations (Figure 3.4). In addition, higher ($P < 0.05$) levels of LEA were observed in the hamsters fed the C+S, F+S, and OO diets (Figure 3.5). Similarly, significant increases in the plasma ALEA content (Figure 3.6) were consistent with feeding F+S and RC diets. Furthermore, AEA concentrations were higher ($P < 0.05$) in the C+S hamsters in comparison with all other dietary treatments (Figure 3.7), whereas the H+EPA and H+DHA diets showed increased ($P < 0.05$) EPEA and DHEA contents, respectively, when compared to the C+S, F+S, RC, HOCO, and OO diets (Figures 3.8 and 3.9). Moreover, except for H+EPA and H+DHA diets, all other diets failed to generate EPEA (Figure 3.8); on the contrary, all seven dietary treatments did result in the biosynthesis of DHEA (Figure 3.9).

3.4.5 Correlations between dietary fatty acid and fatty acid ethanolamide concentrations

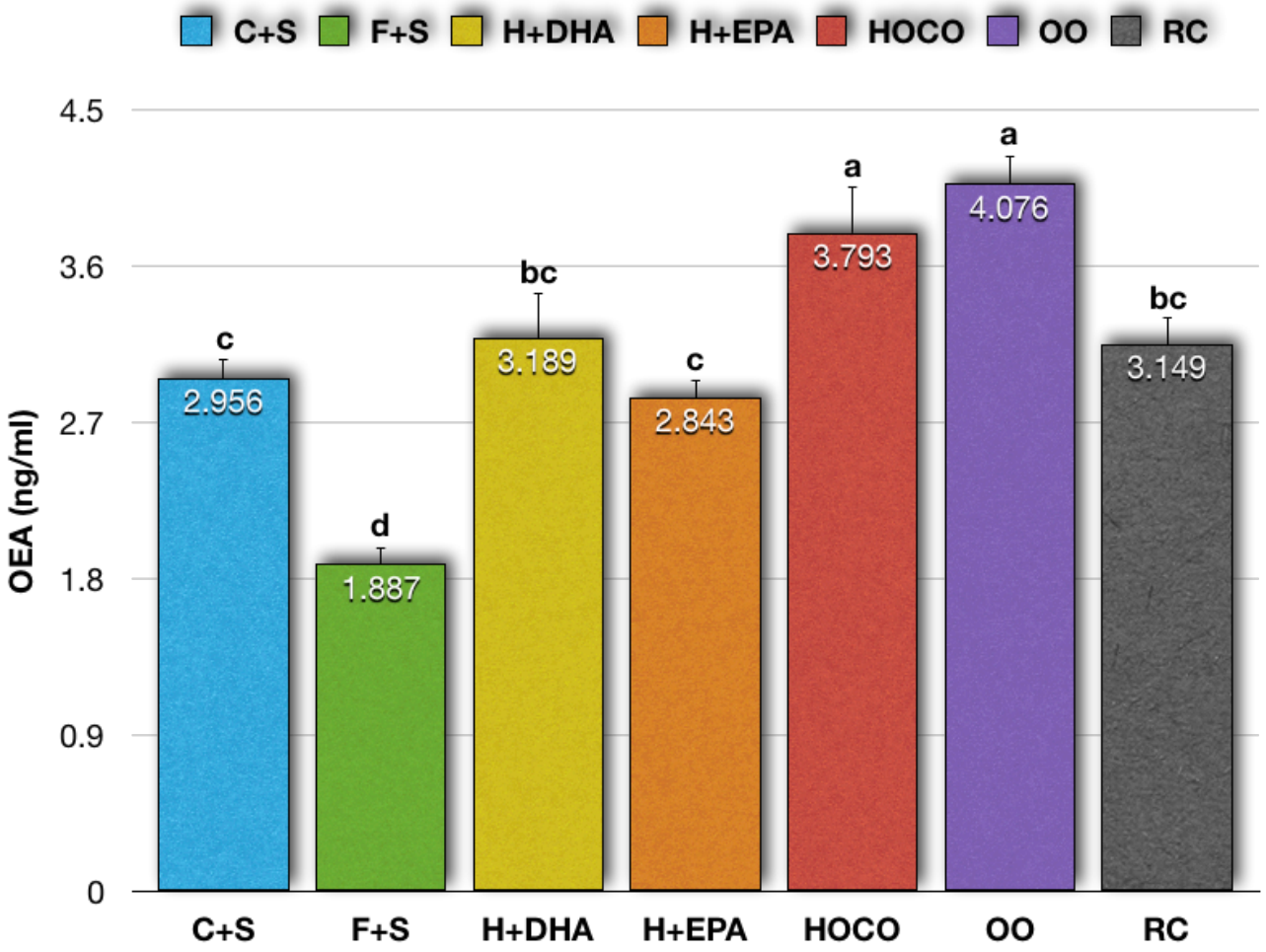
Pearson's correlation coefficients between plasma FAs and their corresponding FAEs are presented in Table 3.7. As expected, correlations ($P < 0.0001$) were observed between endpoint plasma FA, C18:1n9, C18:3n3, C20:4n6, C20:5n3, C22:6n3, and the corresponding FAEs whereas a negative association was found between precursor FA C16:0 and plasma PEA levels ($r = -0.04$; $P < 0.6562$). Moreover, a weak positive

Figure 3.3 Plasma PEA concentrations (ng/ml) in response to various dietary oil blends in hamsters.



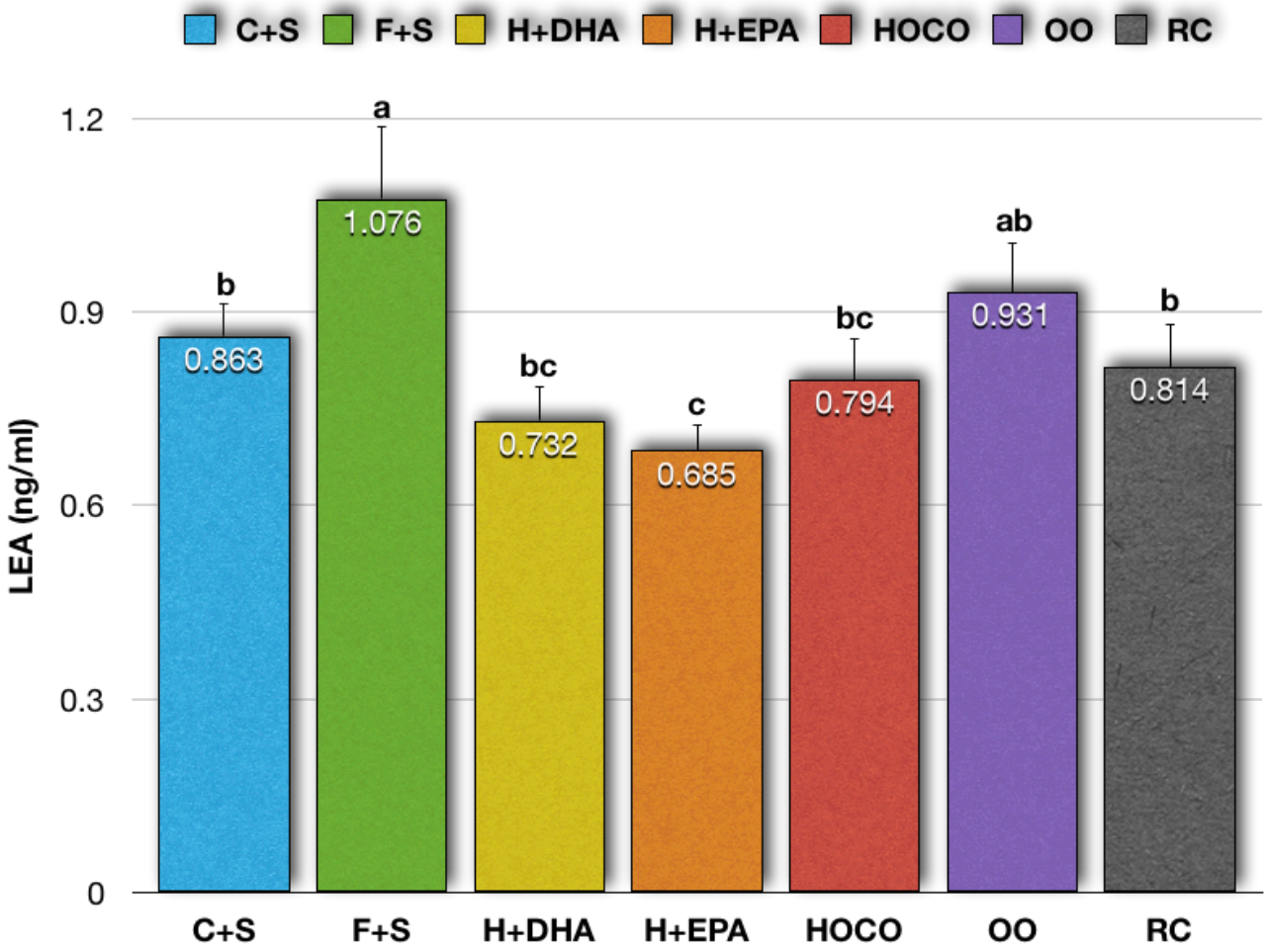
Note: The results are expressed as mean ± SEM (n = 15). The values with different superscript letters are significantly different from each other ($P < 0.05$). PEA, palmitoylethanolamide; C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil.

Figure 3.4 Plasma OEA concentrations (ng/ml) in response to various dietary oil blends in hamsters.



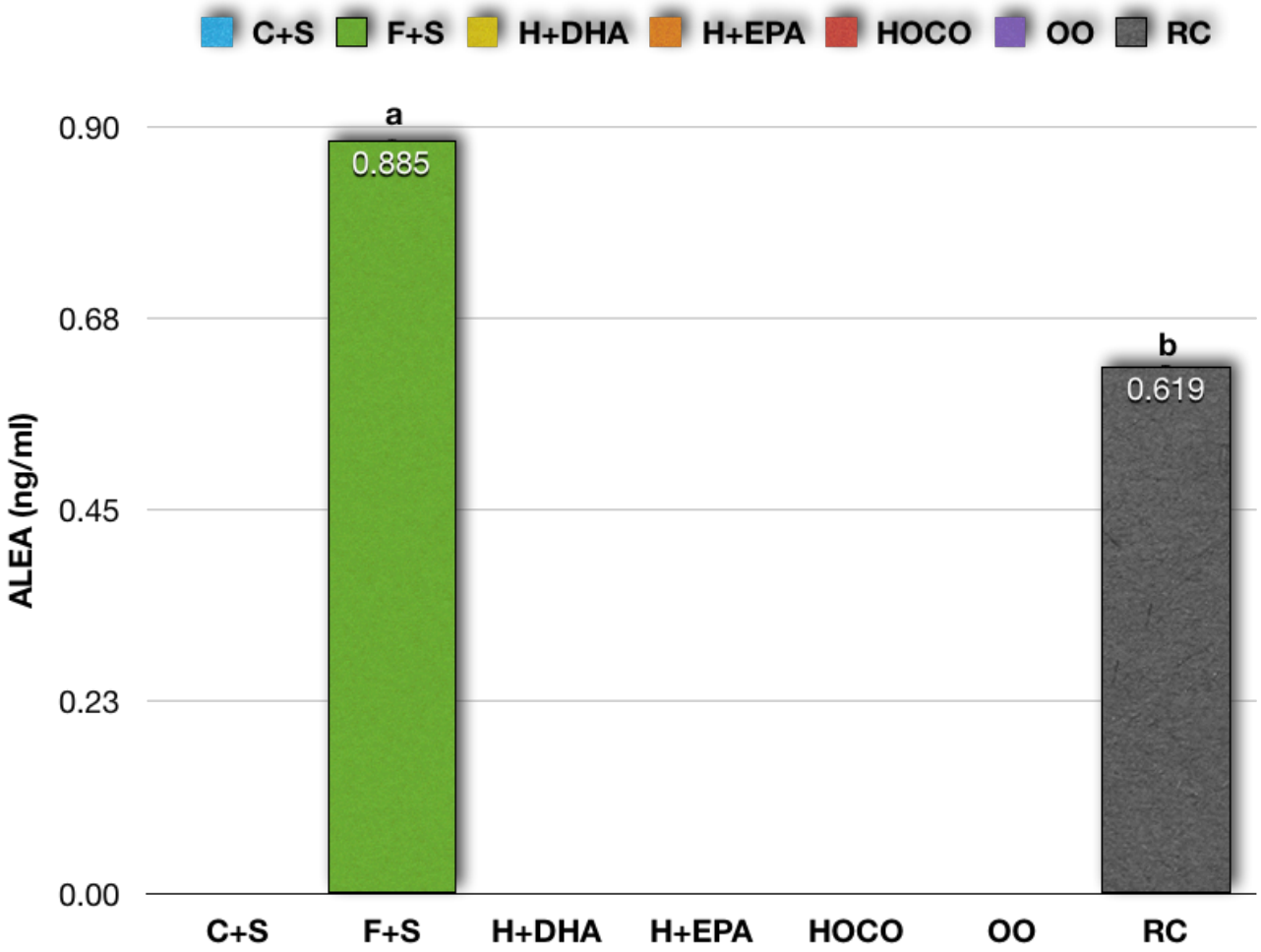
Note: The results are expressed as mean ± SEM (n = 15). The values with different superscript letters are significantly different from each other ($P < 0.05$). OEA, oleoylethanolamide; C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil.

Figure 3.5 Plasma LEA concentrations (ng/ml) in response to various dietary oil blends in hamsters.



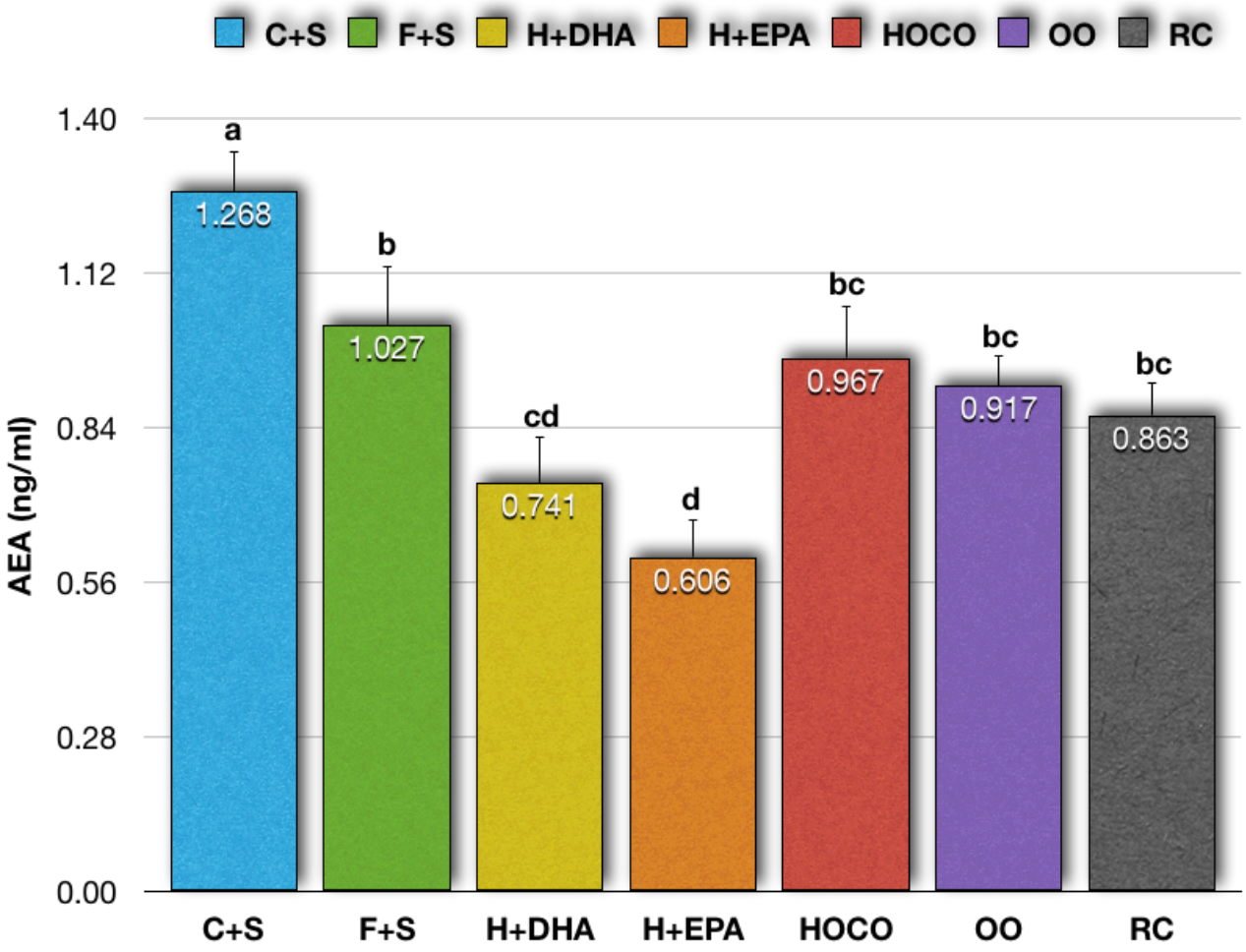
Note: The results are expressed as mean ± SEM (n = 15). The values with different superscript letters are significantly different from each other ($P < 0.05$). LEA, linoleoylethanolamide; C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil.

Figure 3.6 Plasma ALEA concentrations (ng/ml) in response to various dietary oil blends in hamsters.



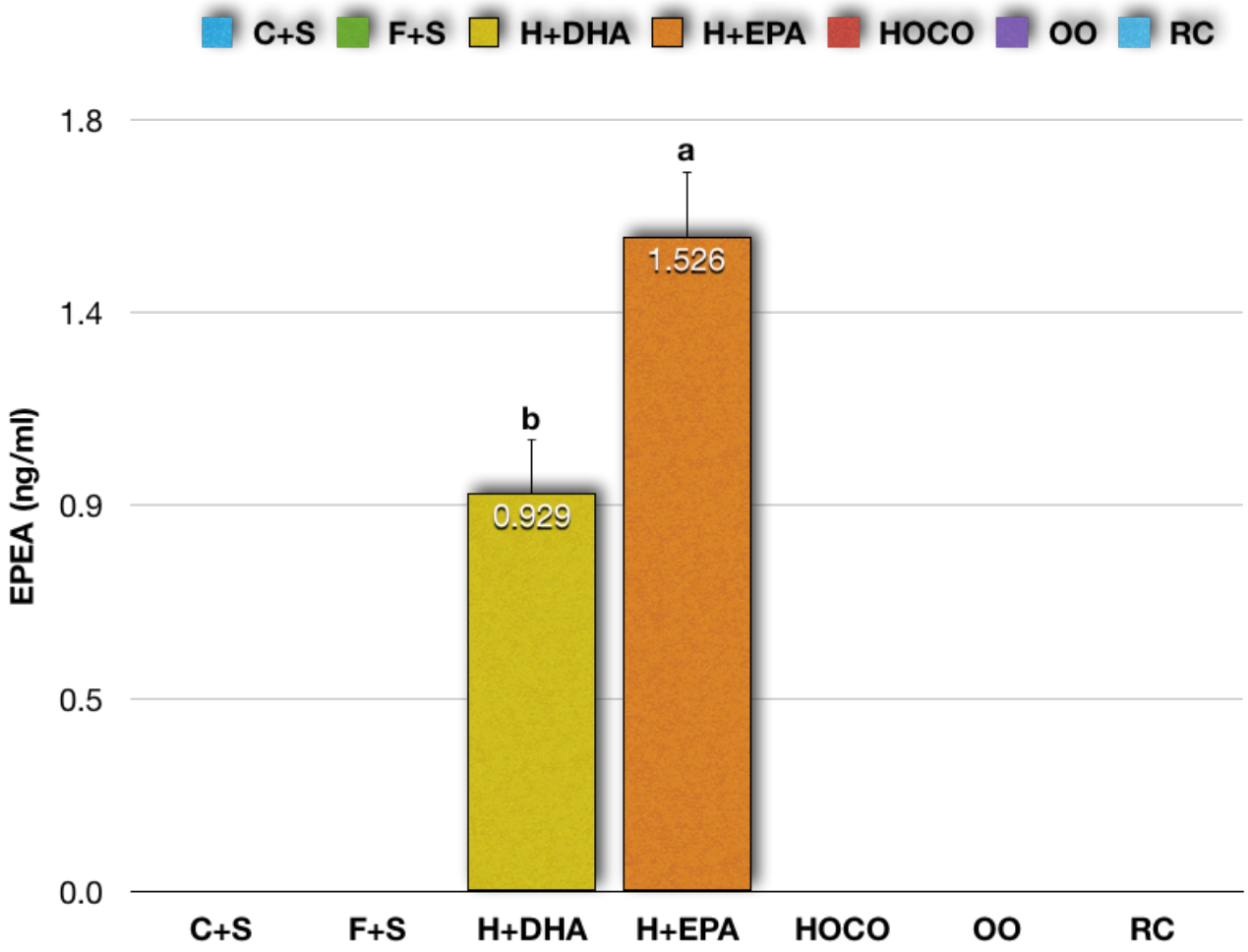
Note: The results are expressed as mean ± SEM (n = 15). The values with different superscript letters are significantly different from each other ($P < 0.05$). ALEA, α-linolenoylethanolamide; C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil.

Figure 3.7 Plasma AEA concentrations (ng/ml) in response to various dietary oil blends in hamsters.



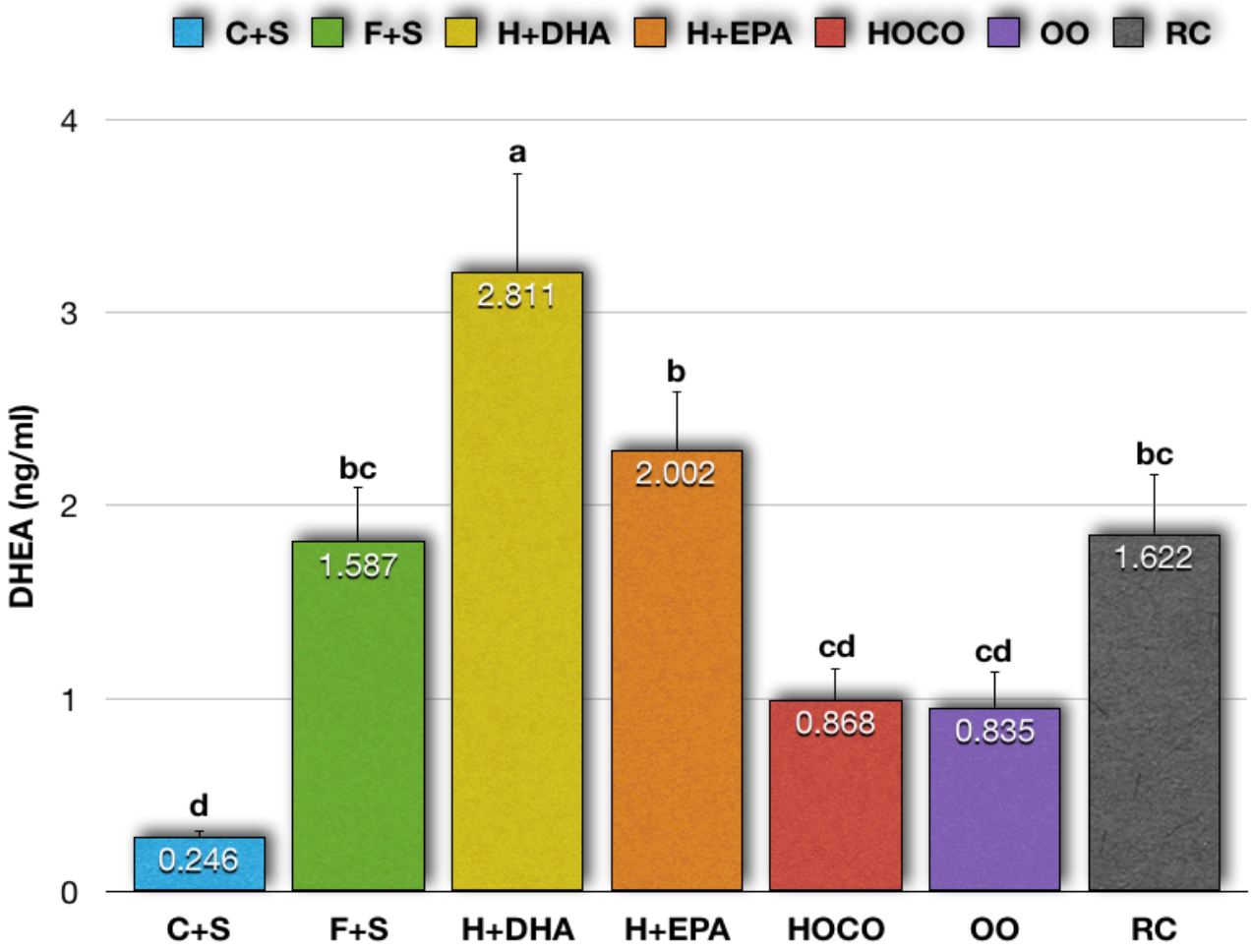
Note: The results are expressed as mean ± SEM (n = 15). The values with different superscript letters are significantly different from each other ($P < 0.05$). AEA, arachidonoylethanolamide; C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil.

Figure 3.8 Plasma EPEA concentrations (ng/ml) in response to various dietary oil blends in hamsters.



Note: The results are expressed as mean ± SEM (n = 15). The values with different superscript letters are significantly different from each other ($P < 0.05$). EPEA, eicosapentaenoylethanolamide; C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil.

Figure 3.9 Plasma DHEA concentrations (ng/ml) in response to various dietary oil blends in hamsters.



Note: The results are expressed as mean ± SEM (n = 15). The values with different superscript letters are significantly different from each other ($P < 0.05$). DHEA, docosahexaenoylethanolamide; C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil.

Table 3.7 Pearson's correlation between dietary fatty acid (precursor fatty acid) and fatty acid ethanolamide levels.

Fatty acids	FAE	*r*	*P*
C16:0	PEA	– 0.04	0.6562
C18:1n9	OEA	0.57	<0.0001
C18:2n6	LEA	0.29	<0.0384
C18:3n3	ALEA	0.93	<0.0001
C20:4n6	AEA	0.44	<0.0001
C20:5n3	EPEA	0.85	<0.0001
C22:6n3	DHEA	0.50	<0.0001

Note: Significant correlation was considered ($P < 0.05$). FAE, fatty acid ethanolamide; *r*, Pearson correlation; PEA, palmitoylethanolamide; OEA, oleoylethanolamide; LEA, linoleoylethanolamide; ALEA, α-linolenoylethanolamide; AEA, arachidonoylethanolamide; EPEA, eicosapentaenoylethanolamide; DHEA, docosahexaenoylethanolamide.

association was also observed between plasma C18:2n6 and LEA ($r = 0.29$; $P < 0.0384$).

3.5 Discussion

The present study demonstrates the anorexic potency of OEA in that both HOCO as well as OO diets attenuated food intake when compared to the H+EPA diet. In addition, no differences were observed in food consumption between HOCO diet and OO treatment. This effect is due to the increased precursor C18:1n9 leading to OEA formation enabling enhanced fat oxidation (23), thereby reducing fat mass and improving lean mass. Furthermore, the elevated fat oxidation observed with OO, H+EPA, H+DHA, and HOCO feeding when compared to C+S was accompanied by weight maintenance and improved overall body composition that includes reduced fat mass and increased lean mass. Moreover, the inconsistencies observed in the treatments such as HOCO and n3-blends as well as RC when compared to other dietary treatments leading to higher lean mass could be the results of higher long-chain FA levels (24). The results of C20:5n3 and C22:6n3 consumption are in alignment with a clinical trial performed with obese children (25), whereas the results of HOCO feeding are in accordance with the trials performed with overweight adults (15, 26). As such, the results of this study are supportive of a role for diets high in C18:1n9 in improving body composition by increasing the lean mass and reducing the fat mass. OO is extensively used in Mediterranean diets and is widely considered to be the best dietary oil with respect to health benefits and satiety (27); however, similar findings were observed with various other n9-enriched oil blends and omega-3 FA blends. Notably, HOCO and OO diets

increased plasma OEA concentrations, which have been shown to stimulate satiety in other animal trials (5, 9, 13, 14) and one human trial (27). The resulting modulation of feeding behaviour has been shown to be altered by the activation of certain lipid sensors, such as CD36, which plays a crucial role in the post-digestive synthesis of OEA (14, 23). The LFHS study diet showed altered plasma FAE levels after a 12 h fasting state. Interestingly, our energy expenditure data demonstrate that higher fat oxidation occurs among OO, HOCO + n3 blends, F+S, and HOCO diets (Table 3.5) and may be due to the fact that n3 FA are highly polyunsaturated and readily undergo oxidation, resulting in peroxisome proliferator-activated receptor alpha (PPAR-α) receptor stimulation (28). However, a latest meta-analysis shows mixed results of fish oil consumption on body composition (29). Furthermore, Sethi and colleagues (28) showed that not only native C20:5n3 and C22:6n3 but also oxidized C20:5n3 and C22:6n3 activate PPAR-α. These investigators also reported that oxidized C20:5n3 and C22:6n3 stimulate this receptor in endothelial cells much more than do native C20:5n3 and C22:6n3. Hence, the present study supports the notion that the oxidation of n3 FAs may transform into stronger PPAR-α agonists. Our results are similar to the previous findings reported by Lin *et al.* (23). Therefore, those data substantiate our findings that H+EPA and H+DHA diets fail to induce obesity despite the increased food consumption, and rather maintain healthy body weights through enhanced fat oxidation.

Our body composition data (Table 3.5) are consistent with data from previous trials (15, 26) that show decreased fat mass with diets enriched in HOCO. In addition to reduced fat mass, the present study shows improved lean mass with n3 FAs-, RC-,

HOCO-, and OO-enriched diets. These findings possibly result from the escalated thermogenesis-associated energy expenditure attributed to transient receptor potential vanilloid 1 (TRPV1) activation in the gut, which aids in body weight maintenance (30–32). The conflicting findings in the present trial supporting that n3 FAs consumption attenuate fat accumulation in hamsters is supported by a detailed review (33). On the contrary, the opposite outcome resulting in increased fat mass in previous human trials (15, 26) is in agreement with the trial performed with obese adults (34). These data suggest that it might be easier to manage dietary compliance when working with laboratory animals compared with humans. This might be a major reason underlying why the anti-obesity effects of C20:5n3 and C22:6n3 supplementations were not obvious in humans as in animals. Moreover, since both FAs, C20:5n3 and C22:6n3, induce PPARα-mediated TRPV1 activation (30) *via* protein kinase C (PKC) (31), any impairment in PKC activity may lead to hyperphagia, impaired satiety, and obesity.

As anticipated, the total plasma FA levels measured at the trial end represented that of dietary treatments. For instance, a difference of 39–44% in C18:1n9 was observed in plasma concentrations when compared to F+S, HOCO, and OO diets. Moreover, plasma C18:3n3 levels increased by 5.8-fold in the F+S hamsters when compared to the C+S counterparts because C18:3n3, being an essential FA, is reflected in higher levels in flaxseed oil-enriched diets (35). Meanwhile, C20:4n6 was the lowest in the H+DHA- and H+EPA-fed animals, indicative of a push–pull mechanism between n3 and n6 synthesis. C22:1n9 was not detected in any of the experimental treatments, except one dietary treatment, HOCO, with very low detection levels to ensure the safe

usage of HOCO oil in human diets (36–38). Also, significant elevations in C20:5n3 and C22:6n3 were observed in H+EPA and H+DHA diets, respectively; however, the conversion of C18:3n3 to C20:5n3 and C22:6n3 followed a distinct pattern. The efficacy of the quantity of dietary C18:3n3 on its conversion and accumulation to longer chain n3 FAs is still a matter of conjecture (39). Therefore, the ratio of C18:2n6 to C18:3n3 is increasingly considered as an influencing factor in this process. Accordingly, our results are consistent with those of previous trials demonstrating a similar ratio between C18:2n6 and C18:3n3 (40, 41), and suggest that the highest plasma C22:6n3 levels are not always observed with diets containing the highest C18:3n3 concentration.

Noticeable alterations in plasma PEA were observed in our study. For instance, the highest amounts of fasting PEA were found with the C+S, RC, and HOCO diets ($P < 0.05$). This is similar to that observed by Izzo *et al.* (42), who reported elevated fasting PEA concentrations in rats. PEA levels have been shown to reflect the inflammatory status, as evidence demonstrates that PEA can act as an on-demand modulator of inflammation or nociception (43, 44). Thus, the observation of higher fasting PEA concentrations in these animals could be explained by differences in vascular hypotension and tissue damage (45). Furthermore, the negative correlation observed between C16:0 and PEA also suggests that the transformation of DFA to FAEs is not necessarily correlative with precursor FA levels, likely since the response of some FAEs is highly dependent on the inflammatory status of the analyzed blood fraction.

As expected, plasma OEA concentrations were the highest in the animals fed

dietary treatments enriched in C18:1n9. A decrease in food consumption was also noted in the same diets, except for the F+S treatment. In the present trial, suppressed food intake was observed with high n9 diets probably due to higher OEA concentrations; is in agreement with the pharmacological studies performed with systemic OEA administration (14, 46–49). However, to conclude that the anorexic effect of OEA enabling attenuated food consumption is due to the systemic OEA administration leading to higher levels of OEA cannot be considered as the only explanation. Food intake homeostasis is governed by a series of bioenergetic, metabolic, and nutritional signaling cascades (50, 51); therefore, the observed changes in plasma OEA concentrations across diets may not be the only reliable indices of reduced food consumption.

Interestingly, we also observed a weak, albeit significant, positive correlation between C18:2n6 and LEA levels, particularly with the OO diet. This result could be explained by the impact of the LFHS diet on the levels or the activity of *N*-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), the key enzyme involved in FAE biosynthesis. Moreover, the alterations in LEA levels have been shown in a previous study demonstrating that LEA movement/upregulation was affected in rats fed a LFHS diet (52). Thus, the study indicates that similar to dietary fat, high dietary sucrose also influences FAE synthesis and homeostasis.

AEA is an endocannabinoid that plays a crucial role in appetite, food modulation, and body weight management. Pharmacological evidence shows that AEA increases food intake (50, 53–55). In our trial, H+EPA diet was found to generate the lowest

concentrations of AEA in the fasting state when compared to the C+S, F+S, RC, HOCO, and OO diets. Furthermore, the H+EPA diet was observed to be the only diet where the animals displayed excess food consumption which might be due to the increased AEA levels during the deglutition; leading these animals to eat more during the eating phase. Moreover, elevated leptin levels effectively decrease AEA concentrations, indicating that mechanistic interactions between AEA and leptin hormone exist, which play a vital role in inducing satiety (56). These viable interactions between AEA and leptin hormone might have resulted in diminished food intake during HOCO and OO dietary feeding. Nevertheless, further experiments are necessary to determine the dietary influence of free feeding on plasma AEA concentrations.

ALEA has been shown to bind cannabinoid receptors (57), but its biological functions remain unclear. In our study, the highest ALEA levels were found with the F+S diet likely due to the presence of C18:3n3-rich flaxseed oil, consistent with previous reports (15). Similarly, elevated EPEA levels were observed with the H+EPA diet and led to increased food consumption; however, further experiments are needed to understand the overall biological significance of EPEA on food intake. In addition, the increased DHEA levels observed with H+DHA diets demonstrate that the dietary incorporation of C22:6n3 shifted the FAE homeostasis to favour DHEA in C22:6n3 enriched diets, and is consistent with the previous findings (58) over n6 and n9 enriched diets. Overall, these data suggest that fasting itself altered plasma FAE concentrations (Figures 3.3–3.9).

The present study has several strengths. In this trial, we successfully demonstrated

the influence of various blends of oils, especially with varieties of n9 oils in combination with different dietary oils generally consumed by human beings; and showed the implication of these dietary oils on FAE levels nutritionally. Furthermore, in this trial, we demonstrated the effects of these diets on long-term plasma levels on seven key FAEs for the first time. As it has been validated that for long-term studies with animal models, a duration of minimum 90 days or nearly two years is recommended, which would translate into minimum four weeks of intervention in humans (59, 60). Conversely, the study has a limitation as well that the trial performed did not address the relationship between food consumption and neurological AEA levels as these interactions would be reflected best in brain concentrations of AEA, which could be considered a limitation.

In conclusion, the present study provides evidence that plasma FAE concentrations are regulated through their precursor FA and demonstrates the potency of FAEs in modulating appetite. The energy expenditure data reveal similar findings with diets containing OO, HOCO, and HOCO with omega-3 FA blends; therefore, we conclude that the incorporation of HOCO into diets could aid in regulating appetite and thereby contribute towards overall wellness. Future experiments on C18:1n9-enriched diets and body composition involving in-depth mechanistic understanding of satiety inducing pathways are warranted to confirm these outcomes that will enable the development of appropriate nutritional and pharmacological approaches to regulating appetite in obesity.

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3.7 Conflict of interest statement

The authors have no conflict of interests to declare in the development of this manuscript.

3.8 Support or funding information

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Bridge to Chapter 4

The findings in Chapter 3 indicated the beneficial impact of the consumption of C18:1n9. The data triggered the investigation of the actions of dietary fatty acids on tissue FAE concentrations in a broad panel of internal organs and tissues such as central and peripheral organs. Therefore, Chapter 4 demonstrates the effect of longer-term feeding of variants of dietary-n9 oils generally consumed by human beings in a feeding trial conducted in hamsters. The findings propose a theoretical model describing the energy homeostasis leading to OEA induced satiety.

Chapter 4

Research Article

Manuscript 3

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Dietary fatty acid profile influences circulating and tissue fatty acid ethanolamide concentrations in a tissue-specific manner in male Syrian hamsters

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4.1 Abstract

Background: The discovery of *N*-acylethanolamines (NAEs) has prompted an increase in research aimed at understanding their biological roles including regulation of appetite and energy metabolism. However, a knowledge gap remains to understand the effect of dietary components on NAE levels, in particular, heterogeneity in dietary fatty acid (DFA) profile, on NAE levels across various organs.

Objective: To identify and elucidate the impact of diet on NAE levels in seven different tissues/organs of male hamsters, with the hypothesis that DFA will act as precursors for NAE synthesis in golden Syrian male hamsters.

Method: A two-month feeding trial was performed, wherein hamsters were fed various dietary oil blends with different composition of 18-C fatty acid (FA).

Results: DFA directly influences tissue FA and NAE levels. After C18:1n9-enriched dietary treatments, marked increases were observed in duodenal C18:1n9 and oleoylethanolamide (OEA) concentrations. Among all tissues; adipose tissue brown, adipose tissue white, brain, heart, intestine-duodenum, intestine-jejunum, and liver, a negative correlation was observed between gut-brain OEA concentrations and body weight.

Conclusion: DFA composition influences FA and NAE levels across all tissues, leading to significant shifts in intestinal-brain OEA concentrations. The endogenously synthesized

increased OEA levels in these tissues enable the gut-brain-interrelationship. Henceforth, we summarize that the brain transmits anorexic properties mediated via neuronal signalling, which may contribute to the maintenance of healthy body weight. Thus, the benefits of OEA can be enhanced by the inclusion of C18:1n9-enriched diets, pointing to the possible nutritional use of this naturally occurring bioactive lipid-amide in the management of obesity.

Keywords: Fatty acid ethanolamides, endocannabinoid, oleoylethanolamide, food intake, satiety.

4.2 Introduction

Ingestion and digestion of dietary fatty acids (DFA) activate the gastrointestinal fatty acid ethanolamide (FAE) system, generating intrinsic signals that mediate many biological functions (1–5). A couple of these signals are imparted by various gut hormones as well as lipid signalling mediators called FAEs which in conjunction with various physiological roles also hold chief importance in regulating appetite and thereby maintaining body weight. Published data (6) support that the dietary fat post metabolism influences the concentrations of endocannabinoid-like compounds also, termed FAEs. In addition, DFA play a crucial role in the physiological function of human tissues (7). However, excess intake of DFA leading to energy disequilibrium may result in overweight and obesity. Therefore, it is essential to understand the impact of DFA consumption on various tissues, especially those that regulate energy homeostasis since DFA act as precursors for FAEs (8, 9). One of these FAEs, called oleoylethanolamide (OEA) is considered to be the most potent appetite suppressing lipid amide among the family of FAEs (1); which include but are not limited to palmitoylethanolamide (PEA), known for its anti-inflammatory, analgesic, and neuroprotective properties (10); linoleoylethanolamide (LEA), which is potentially known for its involvement in regulation of food intake by selective prolongation of feeding latency and post-meal interval (11); and arachidonoylethanolamide (AEA), these are endocannabinoids known for appetite stimulation, fat storage accumulation, and lipogenesis (6). In addition to the above-mentioned *N*-acylethanolamines (NAEs), data on the activity of amine conjugates

from C18:3n3 in animals appear to be scarce, and therefore, full biological significance remains to be established in group of n-3 polyunsaturated fatty acids (PUFA) such as α-linolenoylethanolamide (ALEA), eicosapentaenoylethanolamide (EPEA), and docosahexaenoylethanolamide (DHEA) (12). However, Brown *et al.* (13) have suggested that EPEA and DHEA may possess potential anti-carcinogenic properties. Additionally, Pu *et al.* have demonstrated associations between DHEA and an increase in gynoid fat mass (14) in humans.

Pharmacological data have demonstrated that short-term fat exposure in the stomach induces jejunal AEA mobilization, while duodenal fat exposure leads to OEA synthesis, contributing to the fine-tuning of dietary fat intake (15). Furthermore, pharmacological interventions in animal experiments indicate that OEA regulates body weight and satiety by delaying meal initiation as well as increasing meal-intervals (16). In addition, only limited acute nutritional trials have examined the effects of DFA intake on FAE levels across various tissues (8, 17); so a need exists to understand the impact of long-term feeding, where diets resemble human DFA consumption patterns. Therefore, the present study was designed to investigate tissue compositional changes in ethanolamides and fatty acids (FAs) in response to consumption of different dietary oils varying in their fatty acid (FA) profile, fed for longer-term in a hamster model; including, adipose tissue brown (ATB), adipose tissue white (ATW), brain, heart, intestine-duodenum (I-D), intestine-jejunum (I-J), and liver. An additional objective was to examine the association between OEA levels and endpoint body weight of Golden Syrian male hamsters since detailed review (1) demonstrates that OEA when injected

pharmacologically, induces satiety that might associate with body weight. Henceforth, to address these objectives, a range of n-6, n-9, and n-3 enriched oils were selected and incorporated into the diets. The trial was performed hypothesizing that DFA will act as the precursor for FAEs synthesis.

Moreover, since hyperlipidaemia is a hallmark of human obesity; to achieve the objective, the hamster was used as a model to examine the effects of various proportions of DFA ingestion. Both mammals (18) and hamsters (19) are reported to have the enzymes to synthesize and degrade NAEs. To further expand, hamster as a model was used in the present trial because they have served as models for studying obesity previously (20). Additionally, hamsters, when compared to other rodents, respond to diet modification similarly to humans causing haematological and biochemical changes, identical to what is observed in human obesity (21). Also, Moise et al. (19) demonstrated that endocannabinoids engage cannabinoid receptors, CB1 in hamster brain and undergo enzymatic hydrolysis catalysed by fatty-acid amide hydrolase (FAAH), regulating brain levels of fatty-acid amides in Syrian hamsters. Furthermore, hamsters are more prone to develop hypercholesterolemia (22) characterised by atherosclerotic plaque formation or development more like humans (23). All in all, the features of combined obesity, the endocannabinoid system, predominant enzyme FAAH controlling FAE levels, and hypercholesterolemia, make Syrian hamster as a viable animal model useful for preclinical evaluation of novel anti-obesity agents (24) and obesity research.

4.3 Materials and methods

To investigate our objective, a model diet was prepared to induce dietary obesity presented (Chapter 3, Table 3.1 and 3.2). The diet comprised 36% sucrose and 10% fat of overall ingredient composition of experimental diets (Chapter 3, Table 3.1), which will lead to weight gain over the feeding period. Hamsters were fed two months with various dietary oils incorporated into the diets in the dietary pattern of low-fat high sucrose (LFHS) (22 energy% from fat and 58 energy% from carbohydrate), respectively (Chapter 3, Table 3.2).

4.3.1 Chemicals

Heptadecanoic acid (C17:0) internal standard was obtained from Sigma-Aldrich (St. Louis, MO, USA) and the GLC 461 standards mix was purchased from Nu-Chek Prep Inc. (Elysian, MN, USA). FAE standards—including PEA, OEA, LEA, ALEA, AEA, DHEA, and EPEA—and deuterium-containing internal FAE standards—[$^2H_4$] PEA, [$^2H_4$] OEA, [$^2H_4$] LEA, [$^2H_4$] ALEA, [$^2H_8$] AEA, [$^2H_4$] DHEA, and [$^2H_4$] EPEA—were obtained from Cayman Chemical (Ann Arbor, MI, USA). Liquid chromatography (LC)-grade solvents were purchased from Sigma-Aldrich. All other chemicals and reagents were purchased from Sigma-Aldrich or Fisher Scientific (Waltham, MA, USA).

4.3.2 Animals

All experimental procedures were approved by the Animal Ethics Board, University of Manitoba, Canada. All experiments were conducted according to the Canadian Animal Care guidelines. Four-week-old male Golden Syrian hamsters (n = 105) were purchased

from Charles River Laboratories (Montreal, Quebec, Canada) and allowed to acclimatize for two weeks in the animal facility (temperature, 22 ºC; humidity, 30–60%) with controlled 12-h light/dark cycles (daylight from 6:30 to 18:30 h). During acclimatization, hamsters were provided with access to water and a standard pelleted rodent chow diet (Prolab RMH 3000 Diet) *ad libitum*. After the acclimatization period, hamsters were transferred to individual cages.

4.3.3 Diets

Hamsters were fed a LFHS diet (36 energy% sucrose, 10 energy% fat, 4.10 kcal/g) for 8 weeks. Diets were supplemented with various fats comprising 4% of the total dietary composition as follows: (1) C+S, 25:75 corn oil:n9 safflower oil; (2) F+S, 25:75 flaxseed oil:n6 safflower oil; (3) H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; (4) H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; (5) HOCO, high oleic canola oil; (6) OO, olive oil; and (7) RC, regular canola oil.

4.3.3.1 Intervention

Seven oils with different n-9: n-6: n-3 proportions were selected as the intervention; C+S (62:37:1), F+S (41:48:11), H+DHA (77:10:13), H+EPA (75:11:14), HOCO (86:13:1), OO (91:8:1), and RC (67:26:7). The FA composition varied noticeably among treatment oils (Chapter 3, Table 3.3), allowing comparison of the effects of n-3 FAs, C18:3n3, C20:5n3, and C22:6n3; n-6 FA, C18:2n6; and n-9 FA, C18:1n9; generally incorporated to the diets by humans.

4.3.3.2 Comparison of dietary fat blend

No high saturated fat blend was used in the present trial since the effects of saturated fat have been well established. Moreover, previous reports have examined the impact of HOCO and flaxseed oil in comparison to a diet high in saturated fats (25–27). Therefore, the contrast of oils selected allowed for comparisons of the effects of oils rich in n-9 versus n-6 versus n-3 oil blends.

All dietary treatments were prepared in the animal facility at the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN), University of Manitoba, (Winnipeg, MB, Canada). Dietary ingredients were purchased from Dyets Inc. (Bethlehem, PA, USA). The n9 safflower oil was purchased from Vitahealth (Winnipeg, MB, Canada). HOCO was obtained from Richardson Oilseed (Winnipeg, MB, Canada) and the DHA and EPA rich oils were obtained from Croda Inc. (Edison, NJ, USA). Ingredient composition, nutrient composition, and the FA composition of experimental diets are shown in Chapter 3, Tables 3.1–3.3 and Figure 3.1. Table 4.1 shows the selected FA composition of diets. All animals were healthy throughout the observation period.

4.3.4 Experimental design

4.3.4.1 Study protocol

Following acclimatization, hamsters were systematically randomized into study groups to ensure an even distribution of starting body weights across groups (n = 15 per treatment

Table 4.1 Fatty acid composition of the diets (g/100g)*.

Fatty Acid	C+S	F+S	H+DHA	H+EPA	HOCO	OO	RC
C16:0	14.23	13.80	12.33	12.41	12.35	15.14	13.54
C18:1n9	40.72	26.44	50.25	50.17	58.54	62.36	44.67
C18:2n6	25.75	34.25	6.62	6.85	8.31	6.19	17.06
C18:3n3	0.34	7.59	0.86	1.04	0.97	0.41	4.76
C20:4n6	0.01	0.02	0.40	0.64	0.02	0.02	0.03
C20:5n3	0.24	0.23	0.33	7.20	0.33	0.22	0.30
C22:6n3	ND	ND	7.72	1.80	ND	ND	0.03
SUM rest	18.70	17.68	21.51	19.70	19.49	15.65	19.62
Total SFA	30.57	29.23	30.00	28.28	28.65	28.10	30.09
Total MUFA	42.95	28.52	53.28	52.98	61.36	64.80	47.33
Total PUFA	26.48	42.25	16.72	18.54	9.99	7.1	22.59
Total n6PUFA	25.89	34.42	7.34	8.09	8.64	6.42	17.41
Total n3PUFA	0.59	7.83	9.38	10.45	1.35	0.68	5.18
n6:n3	43.85	4.39	0.78	0.77	6.37	9.49	3.36

Note: *The values are % abundance of each fatty acid to total fatty acids. C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil; ND, not detected; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

group). Blocks of hamsters were initiated into the study protocol every six days. Chapter 3, Figure 3.2 outlines the graphical representation of the protocol for the animal trial.

4.3.4.2 Feeding restriction and sample collection

After feeding on day 59, animals were starved for 12 h (21:00 to 9:00 h) before euthanasia by isoflurane inhalation (2.5%). Tissue samples; namely, ATB, ATW, brain, heart, I-D, I-J, and liver were rapidly collected and snap-frozen in liquid N_2 and immediately stored at – 80 ºC until analysis.

4.3.5 Fatty acid ethanolamide analysis

Tissue FAEs were extracted using a liquid-phase extraction method (28) with minor modifications. Synthetic deuterium-labelled FAEs served as internal standards to frozen tissue samples. The tissue samples were individually subjected to ice-cold acetone and homogenized. The concoction was vortexed for 1 min before centrifuging at 2000 × g for 15 min at 4 ºC. The supernatant was transferred to disposable culture tubes made of borosilicate glass, purchased from Fisher Scientific (Waltham, MA, USA). The collected sample residue was dried under N_2. Lipids were extracted with chloroform:methanol (2:1 vol/vol), by adding 2 ml of the mixture to the N_2 dried sample along with 500 μL of deionized water. The samples were vortexed for 30 secs before centrifuging at 2000 × g for 15 min at 4 ºC. Organic phases were collected, and the chloroform layer was transferred to a fresh borosilicate tube. The eluates obtained were dried under N_2 and reconstituted in acetonitrile. The final sample prepared was transferred to LC-MS/MS

analysis vials. FAE levels were further analyzed using a Waters Acquity UPLC system coupled to a Micromass Quattro *micro* API mass spectrometer (Waters, Milford, MA) equipped with an atmospheric pressure ionization (API) probe and electrospray ionization interface (ESI). FAEs were separated on a Kinetex XB-C18 column (2.1 × 100 mm, 1.7 µm, Phenomenex Inc.). The detailed FAE analysis protocol has been published (Manuscript 2) and has been reported previously (Chapter 3).

4.3.6 Fatty acid analysis

FA profiles in tissues were analyzed via direct transesterification followed by gas chromatography (29). For this, 2 mL of methanol:toluene (4:1 vol/vol) was added to the frozen tissue sample with heptadecanoic acid (17:0) as an internal standard, for homogenization. Acetyl chloride (200 μL) was added while vortexing and heated for 1 hr at 80 °C. The samples were cooled, and then 5 mL of K_2CO_3 was added before centrifuging at 2500 rpm for 5 min at 4 °C. The upper toluene phase containing FA methyl esters was collected and stored at – 80 °C until further analysis.

Methylated FA samples were analyzed by gas chromatography using a fixed capillary column. The detailed FA analysis protocol has been published (Manuscript 2) and has been reported previously (Chapter 3). A known FA mixture was compared with the samples to identify retention peaks using Galaxie software (Varian Inc.). The level of each FA was then calculated according to the corresponding peak area relative to that of all FA of interest to yield the relative percentage of total FA (30).

4.3.7 Statistical analyses

The results are expressed as mean ± SEM. Data were analyzed using SAS 9.4 (IBM Software, Armonk, NJ, USA). Statistical significance was evaluated with one-way analysis of variance (ANOVA) and Tukey's *post-hoc* testing when applicable. Pearson's correlation analyses were performed to examine the associations between tissue FAs and FAEs. Moreover, correlation analyses were performed to examine the associations between OEA concentrations and body weight. Statistical significance was defined as $P < 0.05$.

4.4 Results

4.4.1 Effect of diet treatment on tissue fatty acid ethanolamide levels

Tissue levels of seven FAEs measured after each dietary treatment are shown in Tables 4.2–4.8. Figure 4.1 shows the distribution of FAEs across seven different organs.

4.4.1.1 Adipose tissue brown (ATB) fatty acid ethanolamide levels

Visceral fat depot, ATB isolated from hamsters had significantly higher levels of PEA after H+DHA and H+EPA diets than did those from other dietary treatments (Table 4.2). F+S resulted in the least ($P < 0.05$) concentrations of OEA in ATB when compared to H+EPA, HOCO, and OO. On the contrary, F+S reflected increased ($P < 0.05$) LEA concentrations in comparison to OO diet. ALEA and EPEA were only detectable in the diets enriched with the precursor FAs such as C18:3n3 and C20:5n3, respectively.

Table 4.2 Adipose tissue brown: Fatty acid (g/100g)* and fatty acid ethanolamide (ng/g) levels as a function of diet.

Parameter	C+S	F+S	H+DHA	H+EPA	HOCO	OO	RC
C16:0	15.02 ± 0.59^{abc}	16.54 ± 0.22^{a}	15.53 ± 0.65^{bc}	15.75 ± 0.14^{abc}	14.61 ± 0.23^{c}	16.29 ± 0.29^{ab}	16.11 ± 0.49^{c}
PEA	200.72 ± 19.86^{abc}	203.86 ± 23.75^{abc}	298.26 ± 19.27^{a}	254.80 ± 37.17^{ab}	106.68 ± 14.56^{c}	108.55 ± 28.03^{bc}	200.71 ± 19.62^{abc}
C18:1n9	52.86 ± 0.63^{bc}	43.66 ± 0.60^{d}	56.80 ± 2.23^{abc}	57.12 ± 0.75^{abc}	61.33 ± 0.42^{a}	58.81 ± 1.59^{ab}	51.17 ± 2.51^{c}
OEA	10.74 ± 0.92^{ab}	8.87 ± 0.81^{b}	10.81 ± 0.76^{ab}	12.27 ± 1.21^{a}	12.37 ± 0.72^{a}	12.14 ± 0.71^{a}	10.76 ± 0.86^{ab}
C18:2n6	17.65 ± 0.27^{b}	22.58 ± 0.22^{a}	9.80 ± 0.42^{d}	9.24 ± 0.16^{de}	9.39 ± 0.24^{de}	8.61 ± 0.17^{e}	13.64 ± 0.38^{c}
LEA	6.74 ± 0.49^{ab}	7.58 ± 0.84^{a}	6.09 ± 0.40^{ab}	6.58 ± 1.59^{ab}	6.12 ± 0.30^{ab}	5.42 ± 0.39^{b}	6.96 ± 0.32^{ab}
C18:3n3	ND	0.33 ± 0.15	ND	ND	ND	ND	0.13 ± 0.02
ALEA	ND	1.28 ± 0.01^{a}	ND	ND	ND	ND	1.14 ± 0.01^{b}
C20:4n6	0.47 ± 0.13	0.40 ± 0.02	0.50 ± 0.12	0.45 ± 0.03	0.50 ± 0.05	0.50 ± 0.04	0.65 ± 0.17
AEA	0.91 ± 0.05	0.78 ± 0.09	0.87 ± 0.11	0.90 ± 0.11	0.82 ± 0.05	0.77 ± 0.07	0.78 ± 0.06
C20:5n3	0.02 ± 0.01	0.02 ± 0.01	0.28 ± 0.15	0.50 ± 0.04	0.03 ± 0.01	0.06 ± 0.04	0.31 ± 0.23
EPEA	ND	ND	0.17 ± 0.17^{b}	1.49 ± 0.19^{a}	ND	ND	ND
C22:6n3	0.06 ± 0.01^{b}	0.11 ± 0.03^{b}	0.58 ± 0.12^{a}	0.31 ± 0.06^{ab}	0.08 ± 0.01^{b}	0.16 ± 0.09^{ab}	0.30 ± 0.13^{ab}
DHEA	0.67 ± 0.09^{c}	1.91 ± 0.56^{c}	14.13 ± 1.30^{a}	7.28 ± 1.44^{b}	1.62 ± 0.31^{c}	2.02 ± 0.63^{c}	2.14 ± 0.30^{c}

Note: *The values are % abundance of each fatty acid to total fatty acids. Results are expressed as mean ± SEM (n = 15). Values with different superscript letters in the same row are significantly different from each other ($P < 0.05$). C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil; PEA, palmitoylethanolamide; OEA, oleoylethanolamide; LEA, linoleoylethanolamide; ALEA, α-linolenoylethanolamide; AEA, arachidonoylethanolamide; EPEA, eicosapentaenoylethanolamide; DHEA, docosahexaenoylethanolamide; ND, not detected.

Table 4.3 Adipose tissue white: Fatty acid (g/100g)* and fatty acid ethanolamide (ng/g) levels as a function of diet.

Parameter	C+S	F+S	H+DHA	H+EPA	HOCO	OO	RC
C16:0	18.08 ± 0.26	17.73 ± 0.24	18.47 ± 0.32	17.34 ± 0.76	16.96 ± 0.27	18.36 ± 0.35	17.93 ± 0.43
PEA	287.58 ± 20.56^{a}	97.80 ± 13.12^{bc}	138.11 ± 14.32^{b}	119.64 ± 13.16^{bc}	93.30 ± 10.57^{c}	114.54 ± 12.36^{bc}	128.50 ± 14.10^{bc}
C18:1n9	48.57 ± 1.84^{b}	46.79 ± 0.62^{c}	54.30 ± 1.14^{ab}	55.01 ± 0.66^{ab}	58.35 ± 0.98^{a}	54.58 ± 1.13^{ab}	50.78 ± 1.25^{bc}
OEA	12.42 ± 1.23^{ab}	10.62 ± 1.42^{b}	12.54 ± 1.55^{ab}	12.89 ± 1.01^{ab}	14.75 ± 1.42^{a}	13.75 ± 1.30^{a}	13.16 ± 1.36^{ab}
C18:2n6	17.67 ± 0.44^{b}	20.82 ± 0.31^{a}	12.31 ± 0.49^{d}	12.73 ± 0.34^{cd}	11.78 ± 0.48^{d}	12.11 ± 0.54^{d}	14.81 ± 0.72^{c}
LEA	2.64 ± 0.22^{ab}	2.98 ± 0.32^{a}	1.93 ± 0.13^{bc}	1.93 ± 0.12^{bc}	2.07 ± 0.15^{abc}	1.66 ± 0.07^{c}	2.32 ± 0.18^{abc}
C18:3n3	0.39 ± 0.03^{b}	1.48 ± 0.13^{a}	0.49 ± 0.04^{b}	0.57 ± 0.05^{b}	0.44 ± 0.03^{b}	0.42 ± 0.03^{b}	1.23 ± 0.05^{a}
ALEA	ND	1.30 ± 0.02	ND	ND	ND	ND	1.14 ± 0.01
C20:4n6	0.04 ± 0.01	0.03 ± 0.01	0.27 ± 0.22	0.06 ± 0.03	0.03 ± 0.01	0.12 ± 0.07	0.10 ± 0.07
AEA	0.65 ± 0.42	0.22 ± 0.04	0.18 ± 0.03	0.19 ± 0.03	0.21 ± 0.03	0.16 ± 0.03	0.44 ± 0.16
C20:5n3	0.002 ± 0.01	0.002 ± 0.01	0.03 ± 0.01	0.09 ± 0.02	0.003 ± 0.01	0.006 ± 0.01	0.07 ± 0.06
EPEA	ND	ND	ND	0.75 ± 0.19	ND	ND	ND
C22:6n3	0.005 ± 0.01	0.005 ± 0.01	0.27 ± 0.20	0.04 ± 0.02	0.003 ± 0.01	0.05 ± 0.03	0.04 ± 0.04
DHEA	ND	ND	5.02 ± 1.21	ND	ND	ND	ND

Note: *The values are % abundance of each fatty acid to total fatty acids. Results are expressed as mean ± SEM (n = 15). Values with different superscript letters in the same row are significantly different from each other ($P < 0.05$). C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil; PEA, palmitoylethanolamide; OEA, oleoylethanolamide; LEA, linoleoylethanolamide; ALEA, α-linolenoylethanolamide; AEA, arachidonoylethanolamide; EPEA, eicosapentaenoylethanolamide; DHEA, docosahexaenoylethanolamide; ND, not detected.

Table 4.4 Brain: Fatty acid (g/100g)* and fatty acid ethanolamide (ng/g) levels as a function of diet.

Parameter	C+S	F+S	H+DHA	H+EPA	HOCO	OO	RC
C16:0	22.85 ± 0.75	22.76 ± 0.43	21.10 ± 2.09	22.82 ± 0.95	21.44 ± 2.59	21.50 ± 0.70	21.48 ± 0.60
PEA	215.15 ± 53.03^{b}	350.99 ± 24.92^{ab}	361.94 ± 26.44^{a}	346.76 ± 29.66^{ab}	315.10 ± 39.93^{ab}	381.73 ± 20.02^{a}	322.22 ± 38.14^{ab}
C18:1n9	15.99 ± 0.87^{b}	16.57 ± 0.93^{ab}	16.60 ± 1.49^{ab}	18.46 ± 0.95^{a}	19.40 ± 1.22^{a}	17.93 ± 1.19^{ab}	18.39 ± 0.99^{a}
OEA	65.69 ± 18.11^{b}	65.26 ± 5.67^{b}	63.62 ± 5.61^{b}	66.71 ± 6.08^{b}	100.89 ± 20.25^{a}	92.10 ± 7.71^{a}	96.89 ± 19.62^{a}
C18:2n6	1.19 ± 0.09^{ab}	1.27 ± 0.04^{a}	0.84 ± 0.03^{c}	0.87 ± 0.04^{bc}	1.09 ± 0.11^{abc}	1.01 ± 0.08^{abc}	1.26 ± 0.08^{a}
LEA	1.29 ± 0.11^{ab}	1.56 ± 0.09^{a}	1.09 ± 0.06^{b}	1.14 ± 0.06^{ab}	1.19 ± 0.09^{ab}	1.19 ± 0.15^{ab}	1.32 ± 0.11^{ab}
C18:3n3	ND	0.06 ± 0.03	ND	ND	ND	ND	0.05 ± 0.02
ALEA	ND	1.24 ± 0.01	ND	ND	ND	ND	1.23 ± 0.01
C20:4n6	10.39 ± 0.56^{ab}	10.75 ± 0.43^{a}	7.93 ± 0.47^{c}	8.04 ± 0.39^{bc}	9.29 ± 0.50^{abc}	9.15 ± 0.45^{abc}	8.82 ± 0.48^{abc}
AEA	1.23 ± 0.11	1.61 ± 0.12	1.23 ± 0.10	1.33 ± 0.13	1.28 ± 0.13	1.53 ± 0.11	1.29 ± 0.14
C20:5n3	ND	ND	0.34 ± 0.06	0.48 ± 0.13	ND	ND	ND
EPEA	ND	ND	ND	ND	ND	ND	ND
C22:6n3	13.74 ± 0.99^{ab}	14.98 ± 0.92^{ab}	17.23 ± 1.44^{a}	14.42 ± 1.14^{ab}	12.20 ± 1.16^{b}	13.27 ± 1.06^{ab}	13.36 ± 1.28^{ab}
DHEA	30.74 ± 3.63^{b}	36.48 ± 3.05^{ab}	41.80 ± 2.8^{ab}	33.48 ± 2.45^{ab}	29.74 ± 2.82^{b}	44.63 ± 2.25^{a}	36.17 ± 2.41^{ab}

Note: *The values are % abundance of each fatty acid to total fatty acids. Results are expressed as mean ± SEM (n = 15). Values with different superscript letters in the same row are significantly different from each other ($P < 0.05$). C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil; PEA, palmitoylethanolamide; OEA, oleoylethanolamide; LEA, linoleoylethanolamide; ALEA, α-linolenoylethanolamide; AEA, arachidonoylethanolamide; EPEA, eicosapentaenoylethanolamide; DHEA, docosahexaenoylethanolamide; ND, not detected.

Table 4.5 Heart: Fatty acid (g/100g)* and fatty acid ethanolamide (ng/g) levels as a function of diet.

Parameter	C+S	F+S	H+DHA	H+EPA	HOCO	OO	RC
C16:0	12.59 ± 0.40	13.00 ± 0.57	13.20 ± 0.33	13.63 ± 0.51	12.30 ± 0.35	12.42 ± 0.20	12.79 ± 0.41
PEA	8.60 ± 2.69^{a}	10.47 ± 0.94^{a}	6.81 ± 1.24^{b}	6.65 ± 1.66^{b}	7.86 ± 0.07^{b}	8.19 ± 0.18^{b}	9.50 ± 1.75^{a}
C18:1n9	25.33 ± 2.08^{ab}	24.63 ± 2.43^{b}	33.33 ± 2.62^{a}	32.23 ± 2.02^{a}	32.23 ± 2.43^{a}	33.05 ± 2.34^{a}	28.71 ± 2.59^{a}
OEA	5.97 ± 0.49^{ab}	5.54 ± 0.58^{ab}	4.42 ± 0.44^{b}	4.79 ± 0.70^{b}	7.04 ± 1.08^{a}	7.13 ± 1.84^{a}	6.56 ± 0.44^{ab}
C18:2n6	26.42 ± 0.91^{ab}	29.96 ± 1.31^{a}	17.24 ± 1.01^{d}	17.38 ± 0.74^{d}	21.20 ± 0.99^{cd}	19.98 ± 0.95^{cd}	22.84 ± 1.35^{bc}
LEA	3.35 ± 0.32^{a}	3.47 ± 0.64^{a}	1.35 ± 0.09^{b}	1.63 ± 0.32^{b}	1.52 ± 0.10^{b}	1.48 ± 0.13^{b}	3.09 ± 0.33^{a}
C18:3n3	0.09 ± 0.01^{b}	0.73 ± 0.12^{a}	0.19 ± 0.01^{b}	0.25 ± 0.04^{b}	0.27 ± 0.07^{b}	0.10 ± 0.01^{b}	0.50 ± 0.07^{a}
ALEA	1.08 ± 0.01^{b}	1.25 ± 0.01^{a}	1.08 ± 0.01^{b}	1.09 ± 0.01^{b}	1.08 ± 0.01^{b}	1.08 ± 0.01^{b}	1.25 ± 0.01^{a}
C20:4n6	10.73 ± 0.84^{a}	8.28 ± 0.95^{ab}	4.90 ± 0.42^{c}	5.90 ± 0.40^{bc}	9.83 ± 0.78^{a}	10.02 ± 0.75^{a}	8.66 ± 0.88^{ab}
AEA	0.54 ± 0.04^{a}	0.54 ± 0.08^{a}	0.25 ± 0.02^{b}	0.35 ± 0.05^{ab}	0.40 ± 0.03^{ab}	0.44 ± 0.06^{ab}	0.52 ± 0.05^{a}
C20:5n3	0.02 ± 0.01^{c}	0.06 ± 0.01^{c}	0.79 ± 0.08^{b}	3.29 ± 0.23^{a}	0.03 ± 0.01^{c}	0.07 ± 0.01^{c}	0.07 ± 0.01^{c}
EPEA	ND	ND	ND	0.69 ± 0.22	ND	ND	ND
C22:6n3	3.96 ± 0.38^{c}	4.25 ± 0.59^{c}	12.23 ± 1.24^{a}	7.83 ± 0.65^{b}	4.90 ± 0.42^{c}	5.00 ± 0.45^{bc}	4.75 ± 0.53^{c}
DHEA	3.56 ± 0.74^{b}	4.84 ± 0.98^{b}	12.94 ± 0.98^{a}	11.06 ± 1.40^{a}	3.19 ± 0.80^{b}	4.21 ± 0.67^{b}	4.75 ± 0.64^{b}

Note: *The values are % abundance of each fatty acid to total fatty acids. Results are expressed as mean ± SEM (n = 15). Values with different superscript letters in the same row are significantly different from each other ($P < 0.05$). C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil; PEA, palmitoylethanolamide; OEA, oleoylethanolamide; LEA, linoleoylethanolamide; ALEA, α-linolenoylethanolamide; AEA, arachidonoylethanolamide; EPEA, eicosapentaenoylethanolamide; DHEA, docosahexaenoylethanolamide; ND, not detected.

Table 4.6 Intestine – duodenum: Fatty acid (g/100g)* and fatty acid ethanolamide (ng/g) levels as a function of diet.

Parameter	C+S	F+S	H+DHA	H+EPA	HOCO	OO	RC
C16:0	19.75 ± 0.30	18.95 ± 0.45	20.61 ± 0.37	20.19 ± 0.38	19.43 ± 0.44	20.27 ± 0.42	19.85 ± 0.42
PEA	154.11 ± 16.61^{b}	178.21 ± 17.51^{ab}	306.78 ± 51.21^{a}	296.42 ± 27.47^{a}	291.19 ± 32.56^{a}	306.57 ± 41.38^{a}	291.61 ± 35.25^{a}
C18:1n9	21.55 ± 2.34^{ab}	19.50 ± 2.82^{b}	31.51 ± 2.96^{a}	28.20 ± 4.17^{a}	34.46 ± 4.29^{a}	28.38 ± 3.91^{a}	23.29 ± 2.87^{ab}
OEA	74.60 ± 8.55^{b}	59.56 ± 5.58^{b}	123.54 ± 23.65^{ab}	106.89 ± 8.75^{ab}	182.44 ± 41.01^{a}	160.55 ± 21.95^{a}	117.32 ± 14.89^{ab}
C18:2n6	23.49 ± 0.88^{ab}	27.79 ± 0.96^{a}	14.99 ± 0.96^{c}	15.89 ± 1.18^{c}	15.70 ± 1.27^{c}	17.17 ± 1.33^{c}	22.17 ± 0.86^{ab}
LEA	18.02 ± 1.84	20.04 ± 3.92	11.70 ± 2.72	15.14 ± 1.55	15.56 ± 2.11	14.59 ± 2.07	17.82 ± 2.16
C18:3n3	0.23 ± 0.02^{b}	0.50 ± 0.10^{a}	0.24 ± 0.02^{b}	0.25 ± 0.03^{b}	0.22 ± 0.03^{b}	0.23 ± 0.03^{b}	0.42 ± 0.07^{ab}
ALEA	1.24 ± 0.01	1.54 ± 0.13	1.28 ± 0.01	1.26 ± 0.01	1.27 ± 0.01	1.26 ± 0.01	1.49 ± 0.06
C20:4n6	9.33 ± 0.77^{a}	7.27 ± 0.67^{ab}	4.72 ± 0.56^{b}	5.45 ± 0.75^{ab}	6.94 ± 1.06^{ab}	8.56 ± 1.03^{ab}	7.93 ± 0.73^{ab}
AEA	5.27 ± 0.57^{a}	1.87 ± 0.25^{b}	2.53 ± 0.57^{b}	3.19 ± 0.49^{ab}	3.42 ± 0.86^{ab}	3.96 ± 0.62^{ab}	3.79 ± 0.60^{ab}
C20:5n3	0.03 ± 0.02^{c}	0.14 ± 0.02^{c}	1.06 ± 0.14^{b}	3.18 ± 0.53^{a}	0.05 ± 0.01^{c}	0.07 ± 0.02^{c}	0.16 ± 0.01^{c}
EPEA	ND	ND	2.27 ± 0.44^{b}	5.09 ± 0.55^{a}	ND	ND	0.72 ± 0.12
C22:6n3	1.63 ± 0.17^{c}	2.23 ± 0.25^{bc}	4.67 ± 0.57^{a}	3.12 ± 0.46^{ab}	1.87 ± 0.32^{bc}	2.02 ± 0.24^{bc}	2.67 ± 0.27^{bc}
DHEA	1.38 ± 0.24^{ab}	4.30 ± 0.57^{ab}	33.23 ± 7.74^{a}	15.07 ± 1.80^{ab}	9.66 ± 2.35^{ab}	9.48 ± 2.12^{b}	14.06 ± 2.22^{ab}

Note: *The values are % abundance of each fatty acid to total fatty acids. Results are expressed as mean ± SEM (n = 15). Values with different superscript letters in the same row are significantly different from each other ($P < 0.05$). C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil; PEA, palmitoylethanolamide; OEA, oleoylethanolamide; LEA, linoleoylethanolamide; ALEA, α-linolenoylethanolamide; AEA, arachidonoylethanolamide; EPEA, eicosapentaenoylethanolamide; DHEA, docosahexaenoylethanolamide; ND, not detected.

Table 4.7 Intestine – jejunum: Fatty acid (g/100g)* and fatty acid ethanolamide (ng/g) levels as a function of diet.

Parameter	C+S	F+S	H+DHA	H+EPA	HOCO	OO	RC
C16:0	16.16 ± 0.63^{c}	17.80 ± 0.43^{abc}	19.25 ± 0.55^{a}	18.91 ± 0.47^{ab}	17.74 ± 0.42^{abc}	18.13 ± 0.58^{abc}	17.49 ± 0.37^{bc}
PEA	228.66 ± 26.92	236.15 ± 35.05	295.79 ± 16.59	301.05 ± 42.89	281.93 ± 28.32	276.15 ± 29.41	259.07 ± 32.26
C18:1n9	22.37 ± 2.37^{a}	18.59 ± 1.77^{b}	21.34 ± 0.88^{ab}	23.70 ± 1.31^{a}	23.44 ± 0.86^{a}	24.07 ± 0.88^{a}	22.89 ± 2.21^{a}
OEA	91.03 ± 22.88^{bc}	80.53 ± 10.89^{c}	128.52 ± 3.55^{a}	134.22 ± 25.29^{a}	142.46 ± 21.03^{a}	150.52 ± 24.10^{a}	131.27 ± 21.28^{a}
C18:2n6	19.53 ± 0.90^{bc}	25.23 ± 0.95^{a}	16.19 ± 0.48^{de}	13.80 ± 0.86^{e}	17.77 ± 0.34^{bcd}	17.04 ± 0.44^{cde}	20.21 ± 0.83^{b}
LEA	10.94 ± 1.13^{a}	13.24 ± 1.56^{a}	9.51 ± 1.17^{bc}	8.32 ± 0.64^{c}	11.61 ± 1.71^{a}	11.82 ± 1.76^{a}	12.33 ± 1.00^{a}
C18:3n3	0.10 ± 0.02^{bc}	0.23 ± 0.05^{ab}	0.10 ± 0.04^{abc}	0.07 ± 0.02^{c}	0.08 ± 0.02^{c}	0.09 ± 0.02^{c}	0.24 ± 0.05^{a}
ALEA	1.25 ± 0.01^{c}	1.38 ± 0.03^{a}	1.28 ± 0.01^{bc}	1.27 ± 0.01^{bc}	1.28 ± 0.01^{bc}	1.27 ± 0.01^{bc}	1.33 ± 0.01^{ab}
C20:4n6	6.41 ± 1.39	4.58 ± 1.13	3.77 ± 0.85	3.97 ± 0.90	6.49 ± 1.33	5.87 ± 1.30	4.87 ± 1.17
AEA	5.32 ± 0.92^{a}	1.96 ± 0.25^{c}	2.60 ± 0.43^{abc}	2.71 ± 0.21^{abc}	4.16 ± 0.75^{abc}	4.54 ± 0.82^{a}	2.31 ± 0.33^{bc}
C20:5n3	0.06 ± 0.01^{c}	0.14 ± 0.02^{c}	0.98 ± 0.26^{b}	2.84 ± 0.74^{a}	0.11 ± 0.02^{c}	0.09 ± 0.02^{c}	0.15 ± 0.03^{c}
EPEA	ND	1.17 ± 0.03^{b}	2.95 ± 0.51^{a}	3.97 ± 0.62^{a}	ND	ND	0.13 ± 0.01^{b}
C22:6n3	1.37 ± 0.21^{d}	2.50 ± 0.18^{c}	7.53 ± 0.36^{a}	3.52 ± 0.32^{b}	2.70 ± 0.16^{bc}	2.46 ± 0.14^{cd}	2.93 ± 0.29^{bc}
DHEA	3.30 ± 1.36^{c}	6.07 ± 1.25^{bc}	44.43 ± 7.60^{a}	28.93 ± 13.67^{ab}	9.34 ± 2.58^{bc}	8.08 ± 2.98^{bc}	10.15 ± 3.18^{bc}

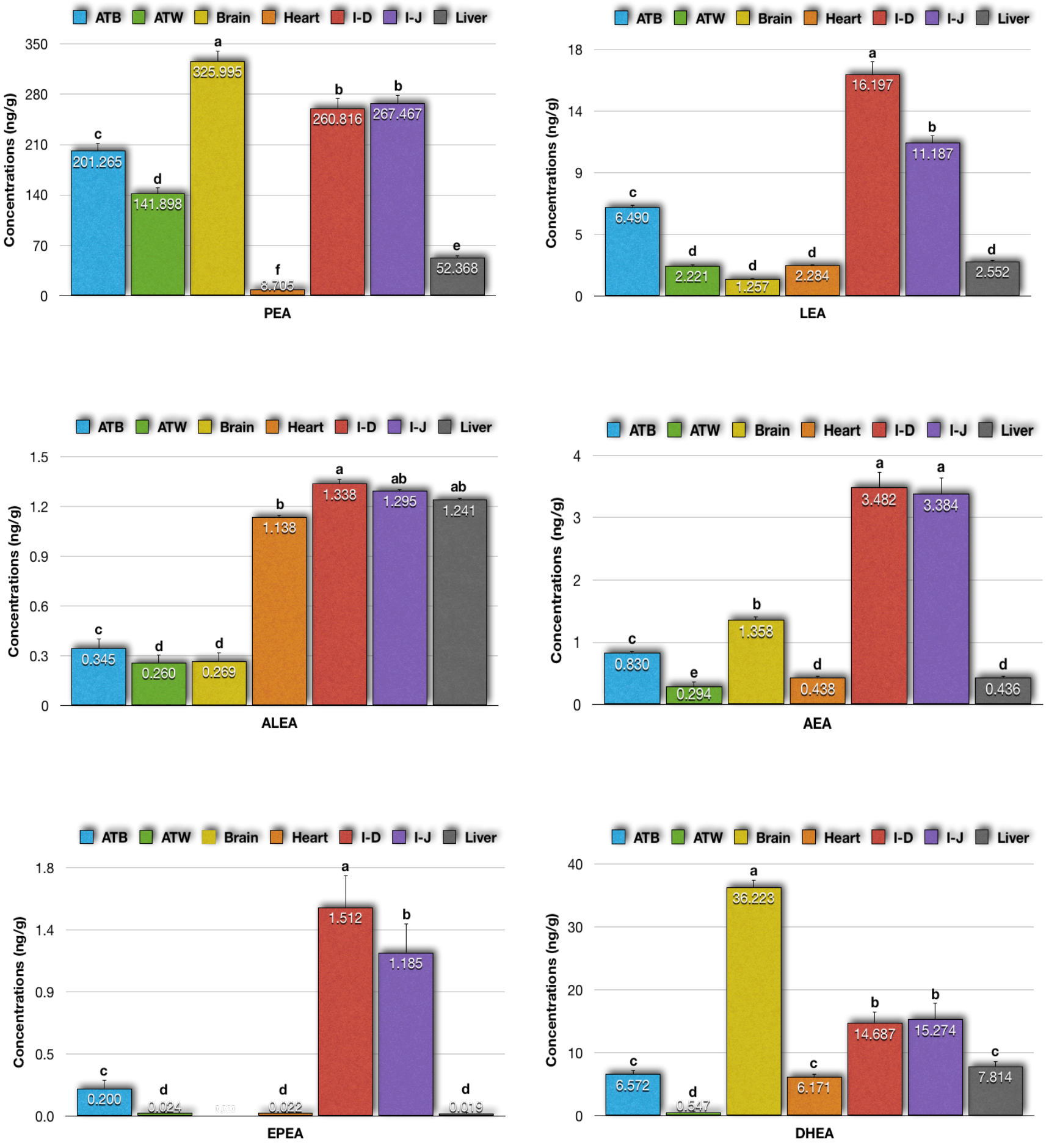
Note: *The values are % abundance of each fatty acid to total fatty acids. Results are expressed as mean ± SEM (n = 15). Values with different superscript letters in the same row are significantly different from each other ($P < 0.05$). C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil; PEA, palmitoylethanolamide; OEA, oleoylethanolamide; LEA, linoleoylethanolamide; ALEA, α-linolenoylethanolamide; AEA, arachidonoylethanolamide; EPEA, eicosapentaenoylethanolamide; DHEA, docosahexaenoylethanolamide; ND, not detected.

Table 4.8 Liver: Fatty acid (g/100g)* and fatty acid ethanolamide (ng/g) levels as a function of diet.

Parameter	C+S	F+S	H+DHA	H+EPA	HOCO	OO	RC
C16:0	9.72 ± 0.27^{c}	9.85 ± 0.27^{c}	11.07 ± 0.22^{a}	10.73 ± 0.16^{ab}	9.17 ± 0.19^{c}	10.03 ± 0.14^{b}	9.83 ± 0.10^{c}
PEA	50.23 ± 6.88	54.65 ± 6.47	51.96 ± 8.74	55.87 ± 7.46	48.85 ± 7.05	56.87 ± 8.20	49.26 ± 7.33
C18:1n9	49.97 ± 0.70^{b}	44.01 ± 1.03^{c}	42.85 ± 1.09^{c}	48.46 ± 0.68^{b}	55.52 ± 0.85^{a}	55.10 ± 0.72^{a}	49.50 ± 0.61^{b}
OEA	38.98 ± 4.95^{b}	37.13 ± 5.36^{b}	35.15 ± 4.09^{b}	35.93 ± 4.49^{b}	44.51 ± 4.16^{a}	48.56 ± 4.12^{a}	38.55 ± 3.91^{b}
C18:2n6	12.78 ± 0.24^{b}	16.68 ± 0.19^{a}	9.48 ± 0.29^{c}	8.73 ± 0.24^{cd}	8.26 ± 0.17^{d}	7.91 ± 0.21^{d}	11.70 ± 0.22^{b}
LEA	3.29 ± 0.31^{ab}	3.78 ± 0.44^{a}	1.83 ± 0.14^{c}	2.05 ± 0.13^{c}	1.97 ± 0.13^{c}	2.13 ± 0.12^{c}	2.76 ± 0.19^{bc}
C18:3n3	0.17 ± 0.07^{c}	1.27 ± 0.10^{a}	0.33 ± 0.16^{c}	0.21 ± 0.01^{c}	0.16 ± 0.01^{c}	0.11 ± 0.01^{c}	0.68 ± 0.02^{b}
ALEA	1.23 ± 0.01^{c}	1.28 ± 0.01^{a}	1.24 ± 0.01^{c}	1.23 ± 0.01^{c}	1.23 ± 0.01^{a}	1.22 ± 0.01^{c}	1.25 ± 0.01^{bc}
C20:4n6	5.37 ± 0.18^{a}	4.52 ± 0.23^{bcd}	3.84 ± 0.20^{cd}	3.41 ± 0.13^{d}	4.75 ± 0.22^{ab}	4.60 ± 0.23^{abc}	4.48 ± 0.19^{abc}
AEA	0.55 ± 0.05^{a}	0.53 ± 0.05^{a}	0.29 ± 0.03^{c}	0.31 ± 0.02^{c}	0.45 ± 0.04^{abc}	0.44 ± 0.05^{abc}	0.45 ± 0.03^{abc}
C20:5n3	0.02 ± 0.01^{c}	0.06 ± 0.01^{c}	1.18 ± 0.10^{b}	2.37 ± 0.14^{a}	0.02 ± 0.01^{c}	0.02 ± 0.01^{c}	0.08 ± 0.01^{c}
EPEA	ND	ND	0.04 ± 0.01^{b}	0.25 ± 0.05^{a}	ND	ND	ND
C22:6n3	2.01 ± 0.07^{d}	3.45 ± 0.20^{c}	11.44 ± 0.43^{a}	5.59 ± 0.18^{b}	3.03 ± 0.13^{cd}	2.58 ± 0.11^{d}	3.89 ± 0.17^{c}
DHEA	3.09 ± 1.05^{b}	6.96 ± 1.50^{b}	17.09 ± 2.70^{a}	11.17 ± 1.75^{ab}	4.70 ± 1.27^{b}	3.69 ± 1.20^{b}	8.30 ± 1.21^{b}

Note: *The values are % abundance of each fatty acid to total fatty acids. Results are expressed as mean ± SEM (n = 15). Values with different superscript letters in the same row are significantly different from each other ($P < 0.05$). C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil; PEA, palmitoylethanolamide; OEA, oleoylethanolamide; LEA, linoleoylethanolamide; ALEA, α-linolenoylethanolamide; AEA, arachidonoylethanolamide; EPEA, eicosapentaenoylethanolamide; DHEA, docosahexaenoylethanolamide; ND, not detected.

Figure 4.1 Tissue fatty acid ethanolamide levels (ng/ml) after two-months feeding of various dietary oil blends[#†].



Note: The results are expressed as mean ± SEM (n = 100). The values with different superscript letters are significantly different from each other ($P < 0.05$). [#]C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil; ATB, adipose tissue brown; ATW, adipose tissue white; I-D, intestine-duodenum; I-J, intestine-jejunum; PEA, palmitoylethanolamide; LEA, linoleoylethanolamide; ALEA, α-linolenoylethanolamide; AEA, arachidonoylethanolamide; EPEA, eicosapentaenoylethanolamide; DHEA, docosahexaenoylethanolamide. [†]Composite of all seven dietary interventions.

Although no significant differences were observed in AEA levels across diets, the H+DHA diet showed a significant elevation in DHEA concentrations when compared to other dietary treatments.

4.4.1.2 Adipose tissue white (ATW) fatty acid ethanolamide levels

Consuming the C+S diet resulted in the highest ($P < 0.05$) levels of PEA in comparison to all other experimental treatments (Table 4.3). The higher percentage of n9 FA in HOCO and OO diets along with n9 enriched diets; namely C+S, H+DHA, H+EPA, and RC diets increased ($P < 0.05$) OEA concentrations. In addition, highest ($P < 0.05$) levels of LEA were observed in hamsters fed the F+S diet, followed by C+S, RC, and HOCO diets. Moreover, ALEA was detectable only in F+S and RC; however, no significant differences were observed across all diet treatments. Similarly, no detectable alterations in AEA concentrations existed across diets. Furthermore, EPEA and DHEA levels were detectable only after consumption of H+EPA- and H+DHA-diets, respectively.

4.4.1.3 Brain fatty acid ethanolamide levels

OO and H+DHA feeding resulted in higher ($P < 0.05$) PEA levels when compared to C+S (Table 4.4). An increase ($P < 0.05$) in brain OEA concentrations post consumption of HOCO, OO, and RC treatments was reflective of C18:1n9 enriched diets. Similarly, F+S being rich in C18:2n6 showed highest ($P < 0.05$) LEA levels. No significant diet related differences in ALEA or AEA concentrations were observed. Furthermore, despite no detectable brain EPEA levels, the OO diet increased ($P < 0.05$) the brain levels of

DHEA in comparison to C+S and HOCO diets but not in comparison to H+DHA diet.

4.4.1.4 Heart fatty acid ethanolamide levels

In the heart, ingestion of F+S, C+S, and RC diets significantly increased PEA levels in comparison to OO and HOCO-enriched diets (Table 4.5). Furthermore, a significant increase in heart OEA, caused by the two highly enriched C18:1n9 diets, HOCO and OO, was reflected by a similar significant change in respective FA incorporated into the heart. Similarly, significant increases in heart LEA and ALEA contents were consistent with feeding F+S, C+S, and RC diets. Moreover, AEA concentrations were higher ($P < 0.05$) in C+S, F+S, and RC hamsters in comparison to H+DHA. Additionally, consumption of the H+DHA diet showed increased ($P < 0.05$) DHEA contents, when compared to the C+S, F+S, RC, HOCO, and OO diets. On the contrary, except for H+EPA diet, all diets failed to generate detectable levels of EPEA. Interestingly, heart showed that higher DHEA levels were associated with blunted ($P < 0.05$) OEA levels resulting in less tissue OEA concentrations in animals fed n3-enriched diets.

4.4.1.5 Intestine-duodenum fatty acid ethanolamide levels

In the duodenum, the total level of PEA was significantly decreased when feeding the C+S diet compared to all other diets (Table 4.6). Overall, the duodenal and jejunal OEA levels were the highest ($P < 0.05$) followed by brain, liver, ATW, ATB, and heart organs when compared with the distribution of OEA concentrations across all the tissues analysed (Figure 4.2A). Figure 4.3A shows the duodenal OEA levels across all dietary

Figure 4.2A Different tissues: OEA levels (ng/g) after two-months feeding of various dietary oil blends[#†].

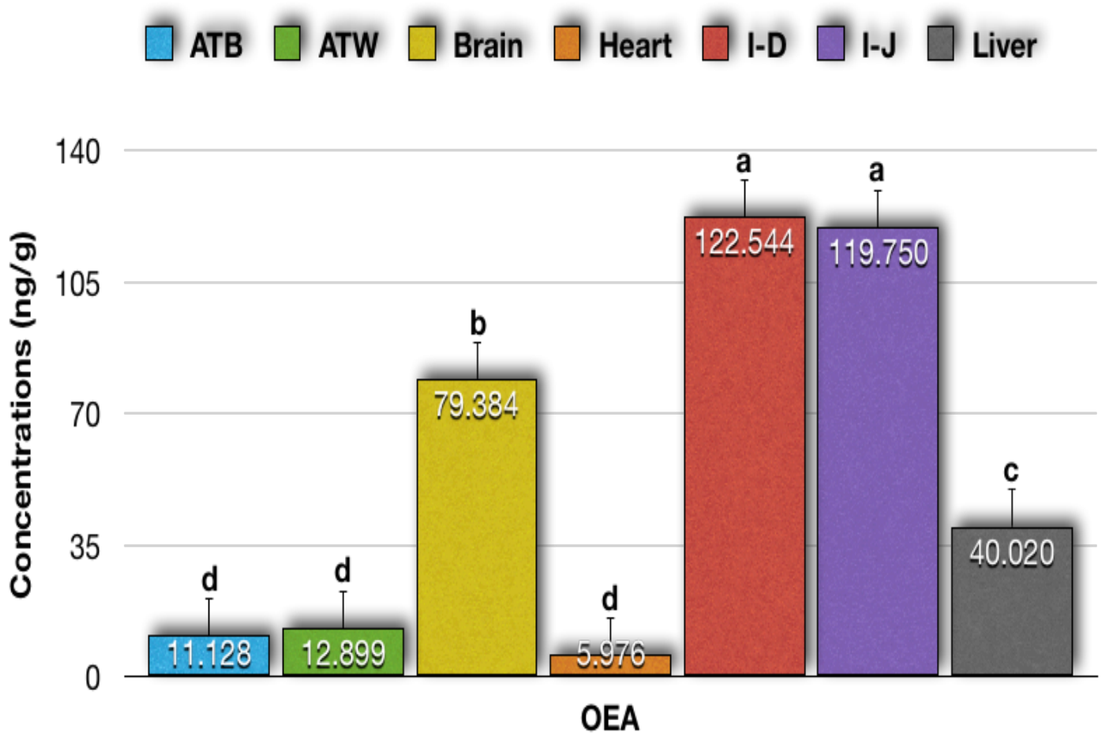
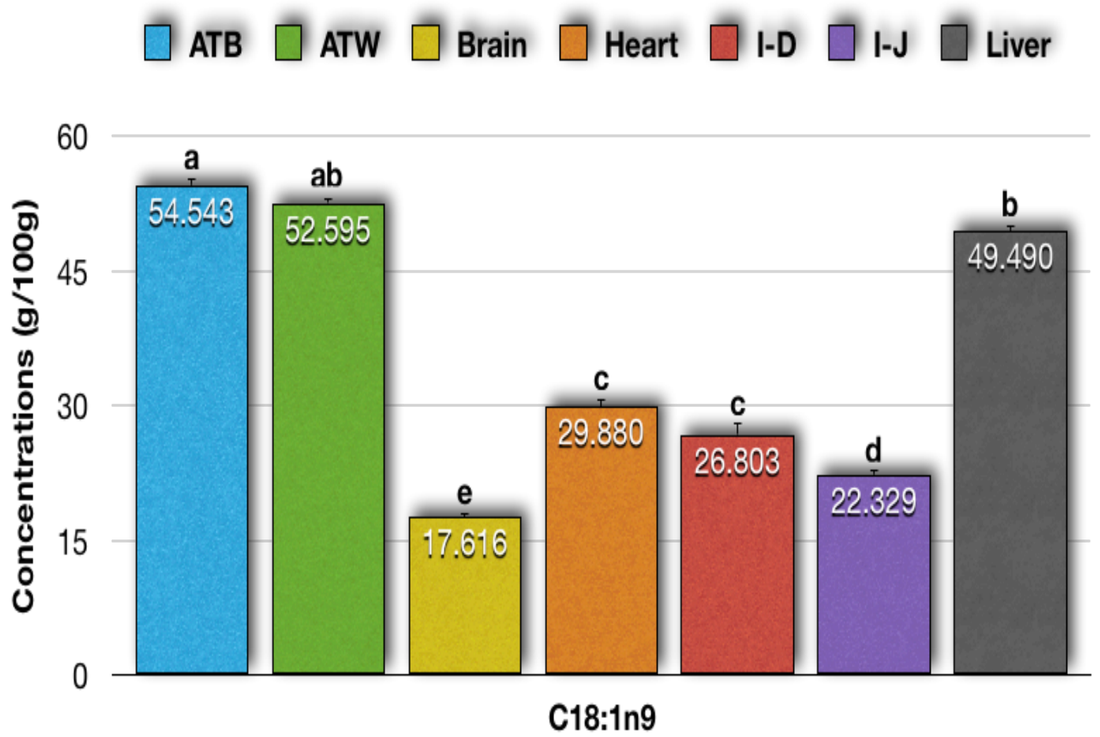


Figure 4.2B Different tissues: C18:1n9 levels (g/100g)* after two-months feeding of various dietary oil blends[#†].



Note: *The values are % abundance of C18:1n9 to total fatty acids. The results are expressed as mean ± SEM (n = 15). The values with different superscript letters are significantly different from each other ($P < 0.05$). OEA, oleoylethanolamide; [#]C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil; ATB, adipose tissue brown; ATW, adipose tissue white; I-D, intestine-duodenum; I-J, intestine-jejunum. [†]Composite of all seven dietary interventions.

Figure 4.3A Intestine – duodenum: OEA levels (ng/g) as a function of diet after two-months feeding of various dietary oil blends[#].

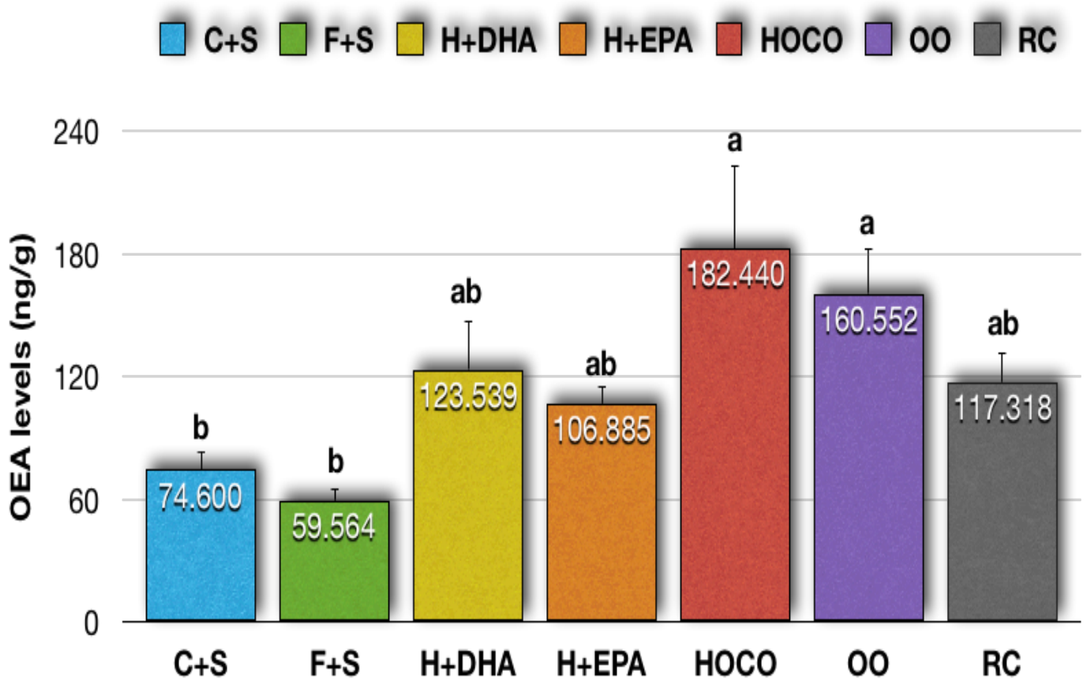
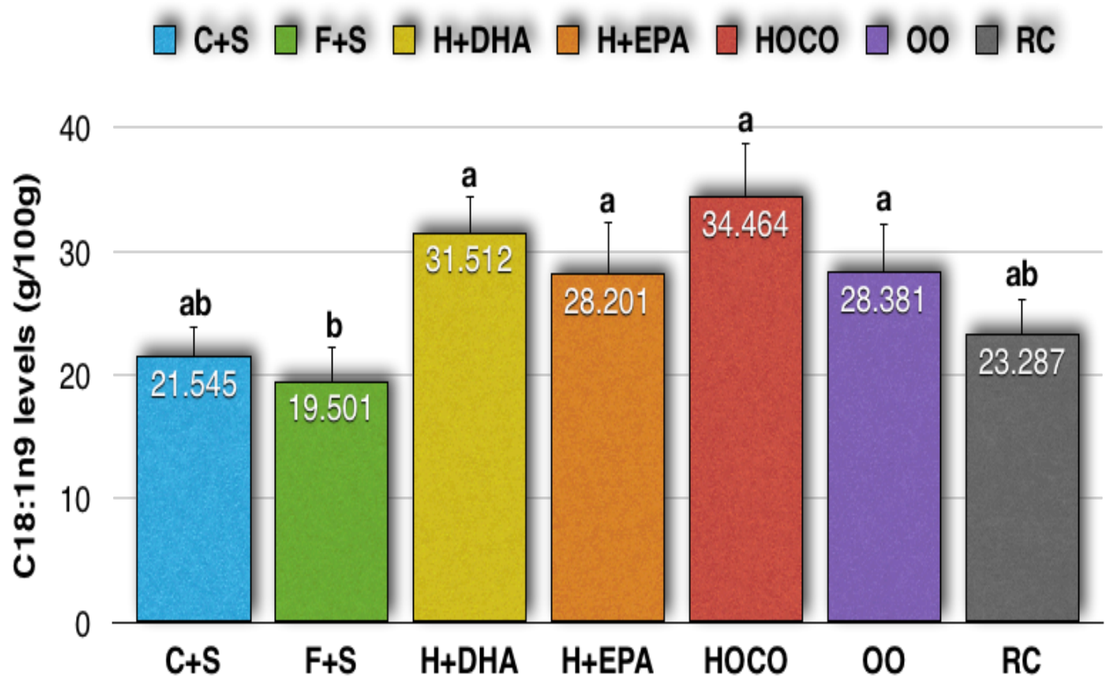


Figure 4.3B Intestine – duodenum: C18:1n9 levels (g/100g)* as a function of diet after two-months feeding of various dietary oil blends[#].



Note: *The values are % abundance of C18:1n9 to total fatty acids. The results are expressed as mean ± SEM (n = 15). The values with different superscript letters are significantly different from each other ($P < 0.05$). OEA, oleoylethanolamide; [#]C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil.

treatments indicating the lowest ($P < 0.05$) concentrations of OEA were present after consumption of F+S and C+S, when compared with HOCO and OO diets. Duodenal LEA and ALEA levels were not changed as a function of diet. Furthermore, duodenal EPEA and DHEA levels tended to follow the same trend as seen for changes in C20:5n3 and C22:6n3 levels, incorporated into the diets, respectively.

4.4.1.6 Intestine-jejunum fatty acid ethanolamide levels

Feeding the experimental diets did not alter the levels of PEA (Table 4.7). In contrast, for the canola-based, HOCO, H+EPA, H+DHA, RC, and OO diets enriched with C18:1n9 levels, significantly increases in OEA levels were observed when compared with the C+S and F+S diets (Figure 4.4A). For jejunal LEA levels, the two n3-rich diets, H+EPA and H+DHA, expressed least ($P < 0.05$) concentrations compared to the other dietary groups. Moreover, in ALEA levels, no shifts were observed across all diets except for the C+S, wherein the concentrations for the same were blunted ($P < 0.05$) compared to F+S and RC. Jejunal AEA levels showed a pattern similar to that of the precursor C20:4n6 present in the tissue, except for OO and F+S diets, where significant differences existed. Furthermore, both H+EPA and H+DHA diets increased ($P < 0.05$) levels of EPEA and DHEA, respectively.

4.4.1.7 Liver fatty acid ethanolamide levels

In the liver, PEA levels remained unchanged regardless of diet (Table 4.8). HOCO and OO diets significantly increased OEA levels compared to other dietary treatments. Also,

Figure 4.4A Intestine – jejunum: OEA levels (ng/g) as a function of diet after two-months feeding of various dietary oil blends[#].

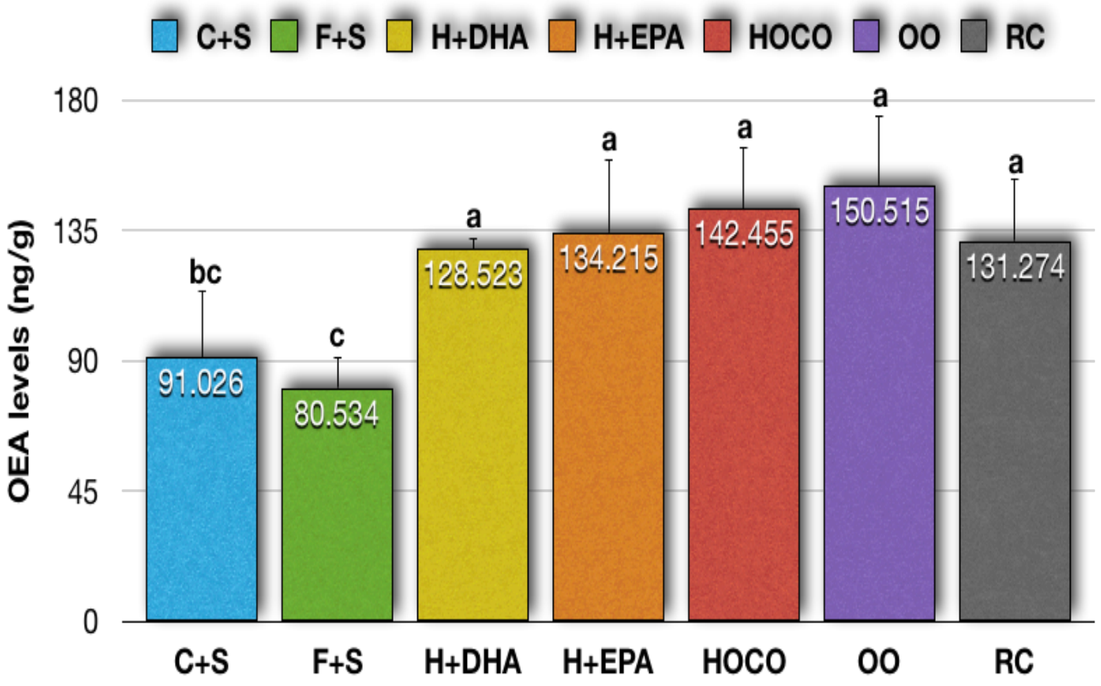
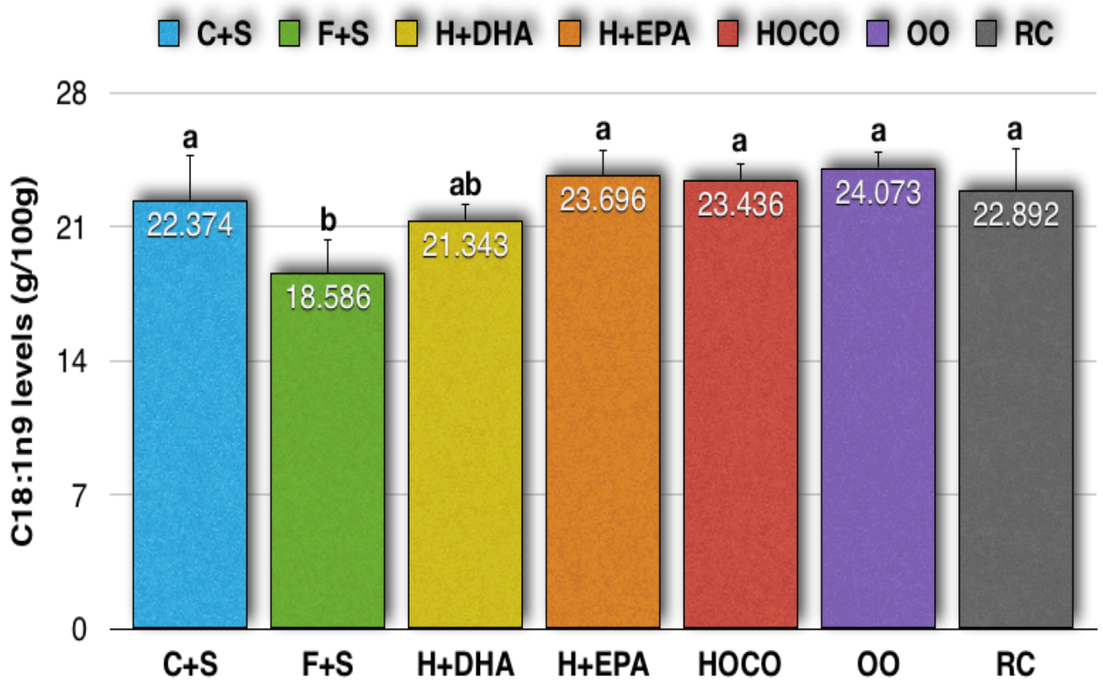


Figure 4.4B Intestine – jejunum: C18:1n9 levels (g/100g)* as a function of diet after two-months feeding of various dietary oil blends[#].



Note: *The values are % abundance of C18:1n9 to total fatty acids. The results are expressed as mean ± SEM (n = 15). The values with different superscript letters are significantly different from each other ($P < 0.05$). OEA, oleoylethanolamide; [#]C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil.

all HOCO and OO based diets reduced ($P < 0.05$) levels of LEA and ALEA, when compared to F+S diet; except for the C+S treatment, which also showed diminished ($P < 0.05$) levels of ALEA, but not LEA concentrations. The H+EPA and H+DHA diets significantly decreased AEA in the liver, while increasing the EPEA and DHEA concentrations compared to all other groups.

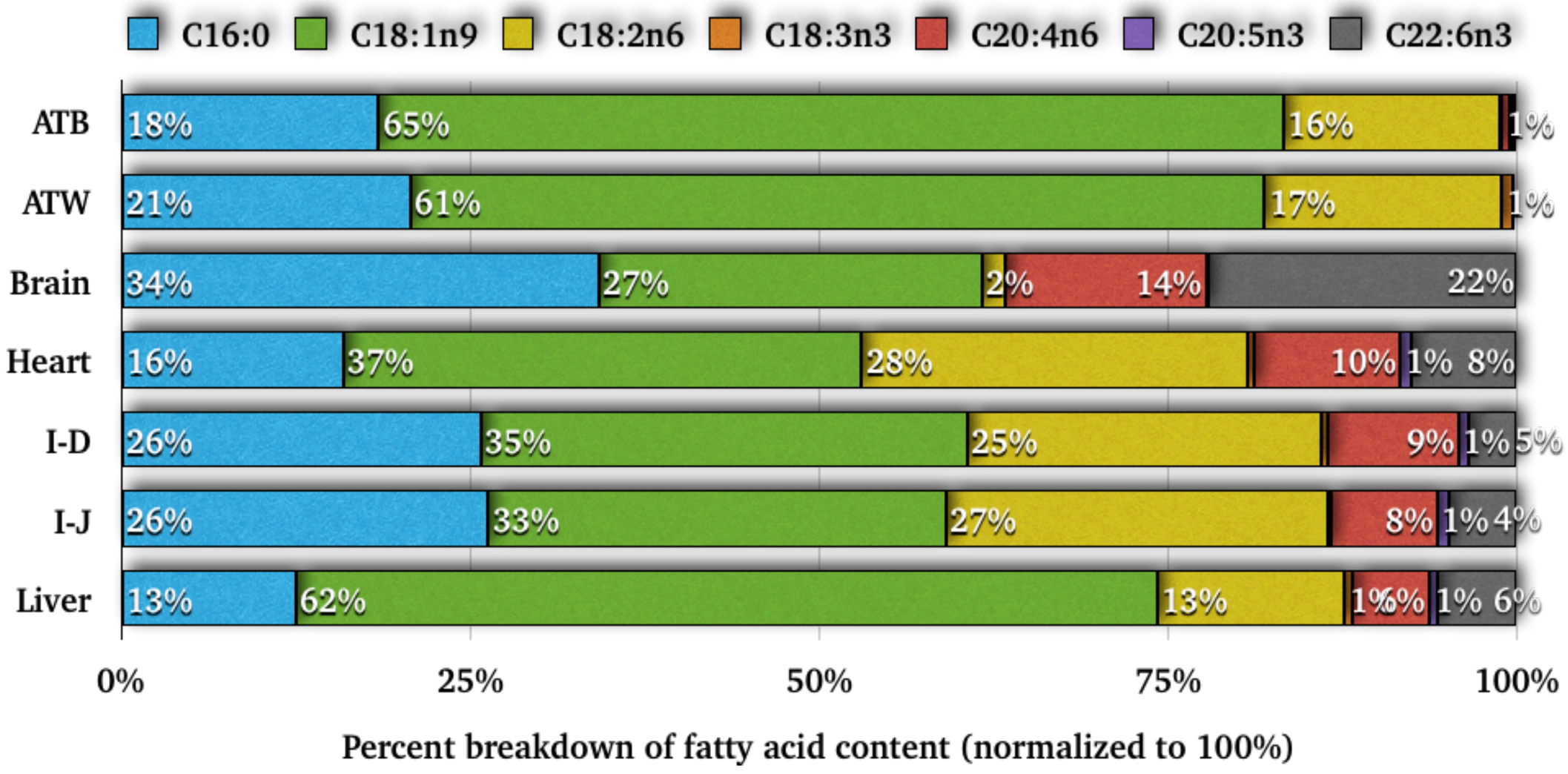
4.4.2 Effect of diet treatment on tissue fatty acid levels

All tissues showed a predominance of C16:0, C18:1n9, and C18:2n6 FAs across all dietary treatments; except brain, wherein chief FAs observed were C16:0, followed by C18:1n9, C22:6n3, and C20:4n6 (Figure 4.5). Tissue weight percentages of C18:1n9 levels after the two-months feeding of various dietary oil blends are shown in Figure 4.2B. The endpoint C18:1n9 levels are shown for each dietary treatment for duodenum (Figure 4.3B) and jejunum (Figure 4.4B).

4.4.2.1 Adipose tissue brown (ATB) fatty acid levels

Comparison of the experimental diets showed no significant differences among C18:3n3, C20:5n3, and C20:4n6 FA levels (Table 4.2). However, increased ($P < 0.05$) levels of C16:0 were observed post feeding F+S, OO, H+EPA, and C+S diets. In addition, feeding F+S diet resulted in the lowest ($P < 0.05$) C18:1n9, but highest ($P < 0.05$) C18:2n6, levels when compared to other dietary treatments. The H+DHA diet followed by H+EPA, RC, and OO dietary treatments significantly increased C22:6n3 levels when compared to F+S, HOCO, and C+S diets.

Figure 4.5 The weight percentage of major fatty acids* in seven different tissues of hamsters after two-months feeding of various dietary oil blends[#†].



Note: *The values are % abundance of each fatty acid to total fatty acids (n = 100). [#]C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil; ATB, adipose tissue brown; ATW, adipose tissue white; I-D, intestine-duodenum; I-J, intestine-jejunum. [†]Composite of all seven dietary interventions.

4.4.2.2 Adipose tissue white (ATW) fatty acid levels

Elevated C18:1n9 levels were observed in hamsters fed HOCO (22% increase; $P < 0.05$), followed by OO, H+EPA, and H+DHA when compared to RC, C+S, and F+S. Similarly, highest ($P < 0.05$) C18:2n6 levels were observed in F+S fed hamsters when compared to all other dietary treatments. Additionally, increased ($P < 0.05$) C18:3n3 levels were only observed with F+S and RC treatments. No differences in levels of C16:0, C20:4n6, C20:5n3, and C22:6n3 were observed across any of the dietary treatments (Table 4.3).

4.4.2.3 Brain fatty acid levels

No detectable differences in C16:0, C18:3n3, and C20:5n3 FA levels were observed across dietary treatments. However, notable alterations were found in three long chain, i.e. C18:1n9, C18:2n6, and C20:4n6; and one very long chain FA, i.e. C22:6n3 levels (Table 4.4). Brain C18:1n9 content was the lowest ($P < 0.05$) with feeding of the C+S diet when compared to HOCO, H+EPA, and RC. Increases ($P < 0.05$) in levels of brain C18:2n6 were observed in animals fed F+S and RC, followed by C+S, HOCO, and OO compared to those given H+EPA and H+DHA. C20:4n6 was the highest ($P < 0.05$) in F+S compared to H+DHA dietary treatment. Moreover, H+DHA-fed animals possessed the highest ($P < 0.05$) content of C22:6n3 when compared to HOCO diet.

4.4.2.4 Heart fatty acid levels

Heart C16:0 levels did not show any statistical differences across diets (Table 4.5). Similarly, comparable contents of C18:1n9 were observed in hearts of hamsters fed n9

enriched dietary treatments; also, the lowest ($P < 0.05$) C18:1n9 levels were only observed in F+S. Furthermore, C18:2n6 content was increased ($P < 0.05$) after feeding the F+S and C+S when compared to other dietary treatments except for RC diets, which was found to have similar C18:2n6 levels as observed in C+S. On the contrary, the animals fed on C18:3n3 FA-enriched diets, i.e. F+S and RC expressed lower ($P < 0.05$) content of C18:3n3, whereas reflected higher ($P < 0.05$) concentrations of C18:2n6. C20:4n6 levels were lowest ($P < 0.05$) in H+DHA and H+EPA dietary treatments. Moreover, H+EPA-fed animals had the highest ($P < 0.05$) content of C20:5n3, while increased ($P < 0.05$) C22:6n3 levels were observed after consumption of H+DHA diet when compared to all other diets.

4.4.2.5 Intestine-duodenum fatty acid levels

Duodenal C16:0 levels did not differ across diets during the course of the study (Table 4.6). However, endpoint duodenum C18:1n9 levels tended to follow the same pattern as seen for dietary C18:1n9 shifts (Table 4.1) and (Figure 4.3B). Here C18:2n6 levels were significantly increased in F+S, C+S, and RC compared to OO and HOCO-enriched groups. Similarly, C18:3n3 rich diets-F+S and -RC, reflected highest ($P < 0.05$) C18:3n3 levels when compared to other dietary groups. Furthermore, consuming the H+DHA diet decreased ($P < 0.05$) C20:4n6 levels compared to the C+S. This decrease ($P < 0.05$) in C20:4n6 in H+DHA was reflected by an increase ($P < 0.05$) in C20:5n3 and C22:6n3 concentrations in the duodenum.

4.4.2.6 Intestine-jejunum fatty acid levels

In jejunum, the percentage of C16:0 was significantly increased when fed the H+DHA diet when compared to RC and C+S experimental diet (Table 4.7). However, comparing all the experimental diets, no significant differences were observed in C20:4n6 levels. Moreover, feeding the C18:1n9 enriched diets reflected the incorporation of that same FA into the tissue. Furthermore, the F+S diet, followed by RC and C+S treatments, resulted in higher ($P < 0.05$), jejunal C18:2n6 levels; indicative of the higher C18:2n6 present in these diets. Feeding both RC and F+S diets significantly increased levels of C18:3n3, while the H+EPA- and H+DHA-diets significantly increased the levels of both C20:5n3 and C22:6n3, respectively, compared to the other dietary treatments.

4.4.2.7 Liver fatty acid levels

In the liver, H+DHA and H+EPA diets significantly increased the levels of C16:0 compared to other dietary treatments (Table 4.8). Moreover, similar C18:1n9 levels were observed post consumption of HOCO and OO diets. The C18:2n6 enriched diets; namely F+S, C+S, and RC, significantly increased liver C18:2n6 levels compared to C18:1n9 enriched-diets. Alterations in the composition of C18:3n3 incorporated into liver similarly followed the same pattern of dietary enrichment of C18:3n3 in F+S and RC diets. On the other hand, n3-enriched diets resulted in decreased ($P < 0.05$) liver C20:4n6 levels, compared to the rest of the dietary fed groups. Also, incorporation of n3-enriched oils to diets significantly increased C20:5n3 and C22:6n3 levels in the liver when feeding the H+EPA and H+DHA diets, respectively, compared to the other dietary treatments.

4.4.3 Correlations between tissue fatty acid and fatty acid ethanolamide concentrations

Pearson's correlation coefficients between tissue FAs and their corresponding FAEs are presented in Table 4.9. Across all tissues, PEA showed no significant association with the root FA. However, positive correlations ($P < 0.01$) were observed between endpoint ATB, I-D, and I-J tissue FA, C18:1n9 and the corresponding OEA level. On the contrary, in ATB, ATW, heart, I-D, I-J, and liver, a positive correlation ($P < 0.02$) was observed between LEA and their corresponding precursor FA, C18:2n6. Furthermore, in all organs/tissues, including ATB, ATW, brain, heart, I-D, I-J, and liver, C18:3n3 FA showed positive associations ($P < 0.02$) with ALEA. In addition, both sections of the intestine-duodenum as well as -jejunum, along with liver, showed positive associations ($P < 0.04$) between AEA and C20:4n6 levels. Also, in ATB, heart, I-D, I-J, and liver, positive associations ($P < 0.02$) were observed between C20:5n3 and EPEA concentrations. Moreover, a moderate positive but highly significant ($P < 0.0001$) association was also observed between C22:6n3 and DHEA in ATB, heart, I-J, and liver. This observation was only noticed for the jejunal section of the intestine and was not seen for the duodenal C22:6n3-DHEA. Overall, except ALEA, none of the FAEs showed associations with corresponding precursor brain FA levels.

4.4.4 Correlations between OEA levels (ng/g) and body weight (g)

A negative significant correlation was observed between overall tissue OEA levels and endpoint body weight ($r = -0.10$; $P = 0.0070$) in hamsters across all diet groups

Table 4.9 Pearson's correlation between tissue fatty acid (g/100g)* and fatty acid ethanolamide (ng/g) concentrations after two-months feeding of various dietary oil blends[#†].

Parameter	ATB	ATW	Brain	Heart	I-D	I-J	Liver
C16:0	15.49 ± 0.17^{d}	17.85 ± 0.16^{c}	21.97 ± 0.26^{a}	12.82 ± 0.16^{e}	19.87 ± 0.16^{b}	17.88 ± 0.21^{c}	10.03 ± 0.10^{f}
PEA	201.27 ± 10.58^{c}	141.90 ± 8.38^{d}	326.00 ± 14.19^{a}	8.71 ± 0.70^{f}	260.82 ± 14.13^{b}	267.47 ± 11.82^{b}	52.37 ± 2.83^{e}
r	0.02	0.17	0.17	– 0.18	0.13	– 0.07	0.14
P	0.8631	0.0921	0.0954	0.2440	0.1994	0.4613	0.1852
C18:1n9	54.54 ± 0.79^{a}	52.60 ± 0.58^{ab}	17.62 ± 0.43^{e}	29.88 ± 0.96^{c}	26.80 ± 1.38^{c}	22.33 ± 0.61^{d}	49.49 ± 0.55^{b}
OEA	11.13 ± 0.34^{d}	12.90 ± 0.52^{d}	79.38 ± 6.27^{b}	5.98 ± 0.37^{d}	122.54 ± 7.94^{a}	119.75 ± 9.11^{a}	40.02 ± 1.74^{c}
r	0.29	0.14	– 0.02	0.03	0.26	0.37	0.16
P	0.0032	0.1637	0.8152	0.7645	0.0100	0.0003	0.1223
C18:2n6	13.06 ± 0.51^{c}	14.62 ± 0.37^{c}	1.08 ± 0.03^{e}	22.26 ± 0.59^{a}	19.56 ± 0.61^{b}	18.64 ± 0.43^{b}	10.81 ± 0.31^{d}
LEA	6.49 ± 0.19^{c}	2.22 ± 0.08^{d}	1.26 ± 0.04^{d}	2.28 ± 0.15^{d}	16.20 ± 0.96^{a}	11.19 ± 0.53^{b}	2.55 ± 0.11^{d}
r	0.31	0.45	0.16	0.42	0.24	0.31	0.61
P	0.0016	<0.0001	0.1183	<0.0001	0.0159	0.0016	<0.0001
C18:3n3	0.07 ± 0.02^{c}	0.71 ± 0.05^{a}	0.01 ± 0.01^{c}	0.31 ± 0.03^{b}	0.30 ± 0.02^{b}	0.14 ± 0.02^{c}	0.42 ± 0.05^{b}
ALEA	0.35 ± 0.06^{c}	0.26 ± 0.05^{c}	0.27 ± 0.05^{c}	1.14 ± 0.01^{b}	1.34 ± 0.03^{a}	1.30 ± 0.01^{ab}	1.24 ± 0.01^{ab}
r	0.44	0.84	0.45	0.57	0.47	0.23	0.52
P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0228	<0.0001
C20:4n6	0.50 ± 0.03^{d}	0.09 ± 0.04^{d}	9.23 ± 0.20^{a}	8.44 ± 0.35^{a}	7.22 ± 0.34^{b}	5.19 ± 0.46^{c}	4.46 ± 0.10^{c}
AEA	0.83 ± 0.03^{c}	0.29 ± 0.07^{e}	1.36 ± 0.05^{b}	0.44 ± 0.02^{d}	3.48 ± 0.25^{a}	3.38 ± 0.26^{a}	0.44 ± 0.02^{d}
r	0.03	– 0.05	0.17	0.16	0.29	0.21	0.28
P	0.7836	0.6501	0.0915	0.1068	0.0041	0.0395	0.0055

Parameter	ATB	ATW	Brain	Heart	I-D	I-J	Liver
C20:5n3	0.17 ± 0.05^{bc}	0.03 ± 0.01^{c}	0.12 ± 0.03^{c}	0.66 ± 0.12^{a}	0.61 ± 0.13^{a}	0.57 ± 0.14^{a}	0.48 ± 0.08^{ab}
EPEA	0.20 ± 0.06^{c}	0.02 ± 0.02^{d}	ND	0.02 ± 0.02^{d}	1.51 ± 0.23^{a}	1.19 ± 0.21^{b}	0.02 ± 0.01^{d}
r	0.24	0.08	–	0.62	0.61	0.45	0.85
P	0.0168	0.4828	–	<0.0001	<0.0001	<0.0001	<0.0001
C22:6n3	0.22 ± 0.04^{e}	0.06 ± 0.03^{e}	14.12 ± 0.46^{a}	6.04 ± 0.37^{b}	2.58 ± 0.16^{d}	3.26 ± 0.21^{d}	4.48 ± 0.31^{c}
DHEA	6.57 ± 0.65^{c}	0.55 ± 0.22^{d}	36.22 ± 1.18^{a}	6.17 ± 0.49^{c}	14.69 ± 1.81^{b}	15.27 ± 2.63^{b}	7.81 ± 0.76^{c}
r	0.53	0.11	0.16	0.59	0.21	0.45	0.53
P	<0.0001	0.3062	0.1146	<0.0001	0.0951	<0.0001	<0.0001

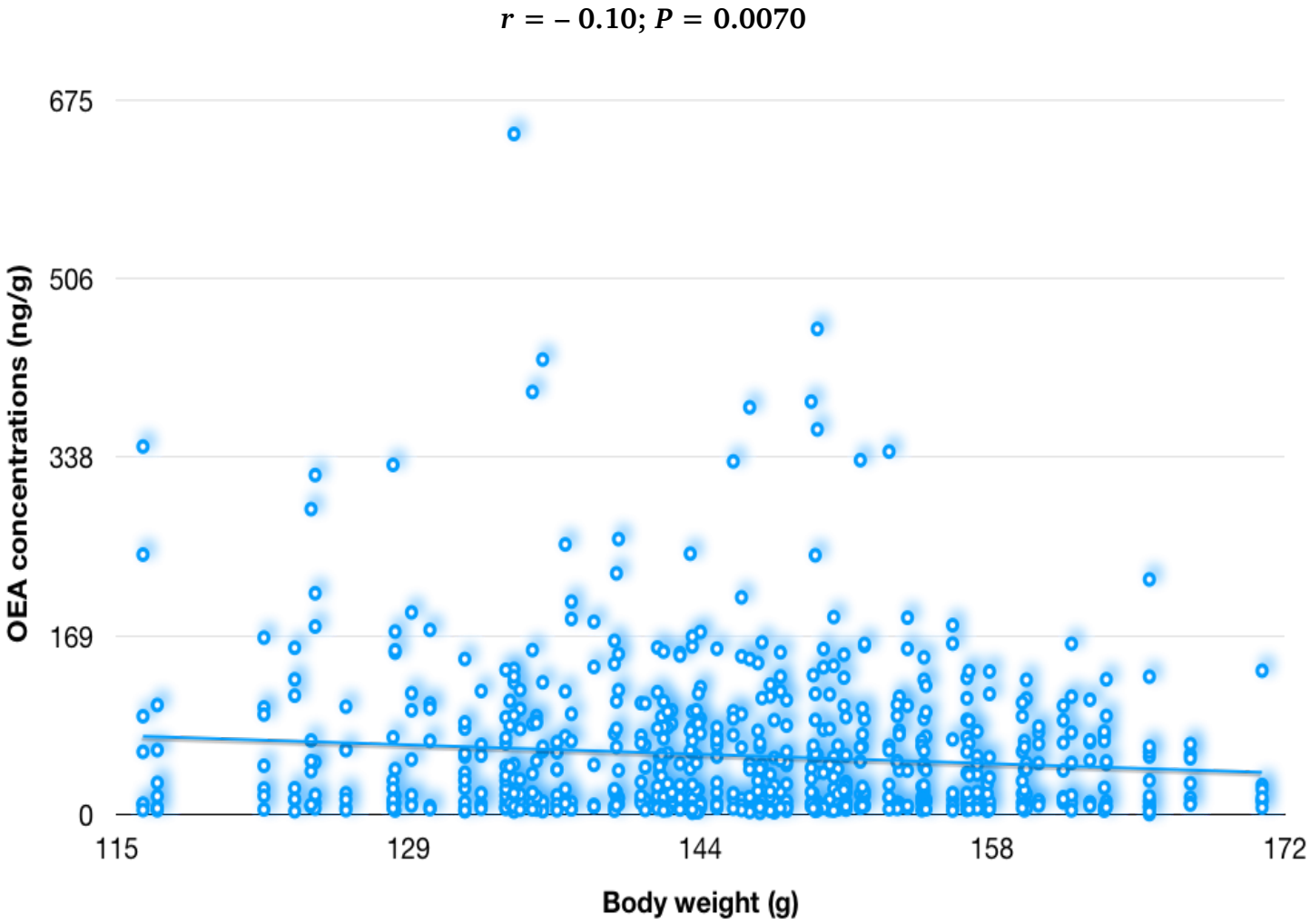
Note: *The values are % abundance of each fatty acid to total fatty acids. Results are expressed as mean ± SEM (n = 99–100); number of diets = 7; number of observations = 696. Values with different superscript letters in the same row are significantly different from each other ($P < 0.05$); r, Pearson correlation coefficients. $^{\#}$C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil; ATB, adipose tissue brown; ATW, adipose tissue white; I-D, intestine-duodenum; I-J, intestine-jejunum; PEA, palmitoylethanolamide; OEA, oleoylethanolamide; LEA, linoleoylethanolamide; ALEA, α-linolenoylethanolamide; AEA, arachidonoylethanolamide; EPEA, eicosapentaenoylethanolamide; DHEA, docosahexaenoylethanolamide; ND, not detected. †Composite of all seven dietary interventions.

(Figure 4.6). Additionally, negative associations were also found between brain OEA concentrations and endpoint body weight ($r = -0.23$; $P = 0.0233$) (Figure 4.7). Similar inverse associations were also observed between gut OEA concentrations and body weight ($r = -0.19$; $P = 0.0074$) (Figure 4.8). Besides, the association between different organs/tissues; namely, ATB, ATW, brain, heart, I-D, I-J, and liver OEA levels and body weight is presented in Table 4.10.

4.5 Discussion

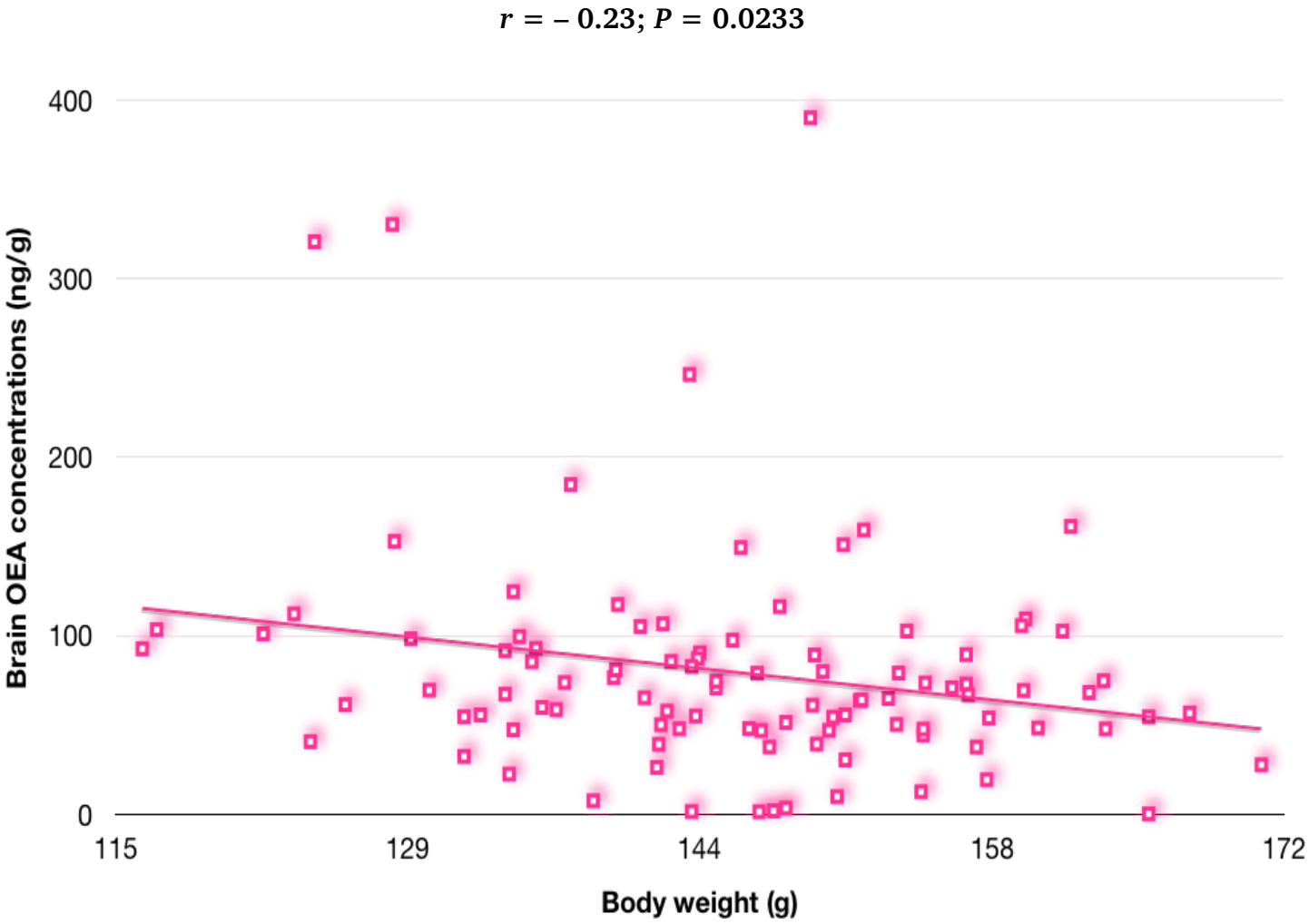
The current study shows a systematic comparison of the effects of diets with different composition of 18-C FA. Moreover, the data show that the long-term feeding of these diets to hamsters influences FAE levels in a broad panel of internal organs and tissues. Furthermore, noticeable differencess in FAE levels across tissues were observed in our study. For instance, the highest ($P < 0.05$) amounts of fasting PEA were found in the brain compared to the remaining six tissues investigated in the trial; namely, I-J, I-D, ATB, ATW, liver, and heart, whereas the levels of PEA remained unaltered in I-D and I-J. Also, consumption of OO- and F+S diets resulted in the highest PEA levels in the brain, when compared with the C+S diet. These findings are similar to those observed by the trial conducted by Izzo *et al.* (31) who reported elevated fasting PEA concentrations in rats. OO and F+S-diets are rich in polyphenols carrying anti-inflammatory properties. Therefore, these diets showed higher PEA values reflecting the anti-inflammatory status (32).

Figure 4.6 The association between OEA levels (ng/g)** and body weight (g) after two-months feeding of various dietary oil blends[#†].



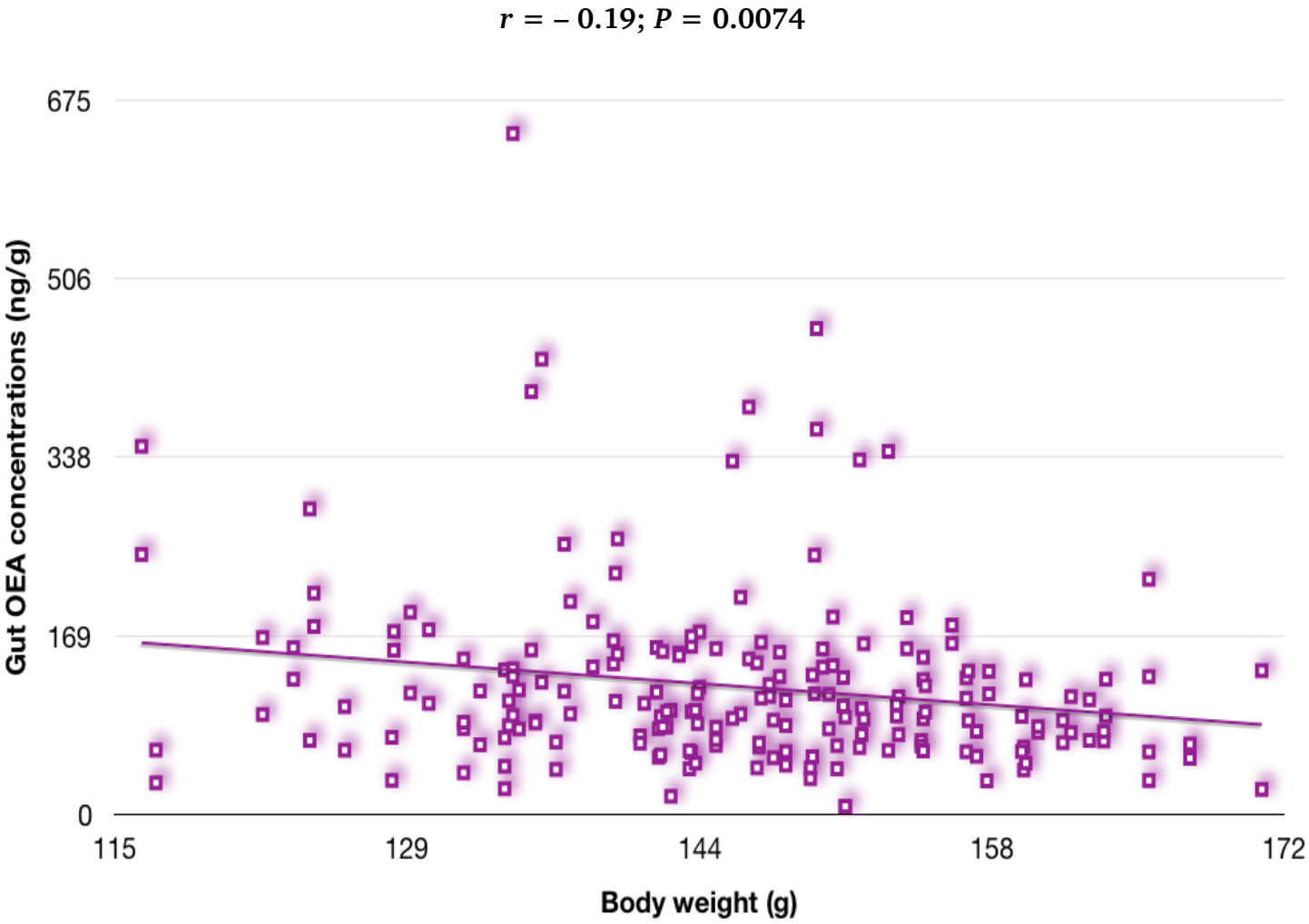
Note: **Across all seven tissues per hamster (n = 100); r, Pearson correlation coefficient; OEA, oleoylethanolamide; [#]C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil. [†]Composite of all seven dietary interventions.

Figure 4.7 The association between brain OEA levels (ng/g) and body weight (g) after two-months feeding of various dietary oil blends[#†].



Note: $n = 100$; r, Pearson correlation coefficient; OEA, oleoylethanolamide; [#]C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil. [†]Composite of all seven dietary interventions.

Figure 4.8 The association between gut[§] OEA levels (ng/g) and body weight (g) after two-months feeding of various dietary oil blends[#†].



Note: $n = 100$; r, Pearson correlation coefficient; OEA, oleoylethanolamide. [§]Gut, combination of intestine-duodenum and intestine-jejunum; [#]C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil. [†]Composite of all seven dietary interventions.

Table 4.10 The association between different organs/tissues oleoylethanolamide levels (ng/g) and body weight (g) after two-months feeding of various dietary oil blends[#].

Organ/Tissue	Parameter	C+S	F+S	H+DHA	H+EPA	HOCO	OO	RC	Composite[†]
ATB	*r*	0.41	– 0.06	0.17	– 0.23	0.16	– 0.04	0.17	0.01
	P	0.1246	0.8321	0.5521	0.4719	0.5709	0.8956	0.5362	0.9065
ATW	*r*	– 0.15	0.08	0.30	– 0.58	– 0.12	0.04	– 0.07	– 0.07
	P	0.5871	0.7789	0.2912	0.0475	0.6729	0.8854	0.7919	0.5792
Brain	*r*	– 0.14	– 0.49	0.04	– 0.66	– 0.26	– 0.27	– 0.29	– 0.23
	P	0.6076	0.0773	0.8908	0.0191	0.3490	0.3366	0.2931	0.0233
Heart	*r*	0.004	– 0.33	0.26	0.55	0.17	0.21	– 0.46	0.06
	P	0.9884	0.2534	0.3657	0.0655	0.5492	0.4437	0.0826	0.5501
I-D	*r*	0.07	– 0.27	– 0.23	– 0.16	0.01	– 0.53	– 0.13	– 0.19
	P	0.8164	0.3489	0.4810	0.6214	0.9705	0.0442	0.6437	0.0568
I-J	*r*	– 0.12	0.16	– 0.18	– 0.53	– 0.15	– 0.36	0.04	– 0.19
	P	0.6796	0.5906	0.5433	0.0765	0.5948	0.1859	0.8929	0.0610
Liver	*r*	– 0.21	– 0.27	– 0.05	0.30	– 0.12	0.05	– 0.33	– 0.13
	P	0.4615	0.3467	0.8694	0.3473	0.6637	0.8696	0.2270	0.2034
Adipose tissue[‡]	*r*	0.09	0.03	0.24	– 0.39	– 0.07	0.01	0.02	– 0.03
	P	0.6453	0.8854	0.2136	0.0592	0.7087	0.9477	0.9137	0.7073
Gut/Intestines[§]	*r*	0.01	– 0.11	– 0.15	– 0.21	– 0.09	– 0.45	– 0.06	– 0.19
	P	0.9429	0.5965	0.4696	0.3144	0.0103	0.0135	0.7540	0.0074

Note: $n = 100$; r, Pearson correlation coefficients; significant correlation was considered ($P < 0.05$). [#]C+S, 25:75 corn oil:n9 safflower oil; F+S, 25:75 flaxseed oil:n6 safflower oil; H+DHA, 85:15 high oleic canola oil:docosahexaenoic acid; H+EPA, 85:15 high oleic canola oil:eicosapentaenoic acid; HOCO, high oleic canola oil; OO, olive oil; RC, regular canola oil; ATB, adipose tissue brown; ATW, adipose tissue white; I-D, intestine-duodenum; I-J, intestine-jejunum. [†]Composite of all seven dietary interventions. [‡]Adipose tissue, combination of adipose tissue brown and adipose tissue white. [§]Gut, combination of intestine-duodenum and intestine-jejunum.

As expected, tissue OEA concentrations were highest in animals fed dietary treatments enriched in C18:1n9 when compared to the C+S and F+S diets in all tissues, the reason might be due to the fact that both the treatments being rich in precursor C18:2n6 were inefficient in endogenously synthesizing high amounts of OEA [33]. The highest OEA were found present in duodenal and jejunal tissues followed by brain, and liver OEA concentrations when compared to ATW, ATB, and heart. Moreover, brain OEA levels were 65% higher when compared to the liver OEA concentrations (Figure 4.2A). The observations confirm that the critical site for FAE synthesis is the intestine which signals the brain via vagal-nigro-striatal pathway (34) increasing c-Fos expression, the marker of neuronal activity involved in appetite regulation, activating oxytocin receptors, thereby enabling dopamine, and histamine release providing a 'reward value' (35), ultimately curbing food intake post consumption of C18:1n9 enriched diets, as shown in our previous experiment (9). Furthermore, CD36 receptors that sense fatty acids are found in abundance in the intestinal sections as well as in villi enterocytes (36), where most lipid absorption occurs, activating the peroxisome proliferator-activated receptor-alpha (PPAR-α) that further potentiates the action of OEA anorexic signalling.

Additionally, differences exist between duodenal (Figure 4.3A) and jejunal (Figure 4.4A) OEA levels at 12 h fasting after long-term feeding of the LFHS diet. Interestingly, the HOCO and OO diets showed increased OEA concentrations when compared to the C+S and F+S dietary treatments, in both sections of the intestine-duodenum and -jejunum. Additionally, when the comparison was made between jejunal

and duodenal OEA levels, both C18:1n9 enriched diets, HOCO and OO expressed diminished OEA levels in the jejunum in contrast to the duodenum. The diminished jejunal OEA concentrations in these diets may exist due to the intestinal fatty acid oxidation at fasting state (37–39), that stimulates various metabolic signals such as PPAR-α and the intestinal vagal afferent activity inducing satiety. The findings substantiate the reduced food consumption observed post HOCO and OO treatments in our previous work (9). Our present results are also consistent with the previous findings (8, 17, 40) demonstrating that feeding of diets resembling human diets affect tissue levels of endocannabinoids and NAEs (8), the critical site for OEA metabolism is the proximal small intestine (17), and high-fat diet decreases the jejunal levels of anorectic NAEs (40). In addition, the concept that fatty acid oxidation controls food intake is explained in detail by Leonhardt and Langhans (41). The findings substantiate that fatty acid oxidation initiates at the jejunal level stimulating satiating signals.

Moreover, in ATB and ATW tissues, the lowest OEA concentrations were observed in F+S diet-fed animals when compared to HOCO- and OO-fed dietary treatments. As such, the outcomes might be due to the presence of higher amounts of C18:3n3 and C18:2n6 present in F+S diet restricting OEA formation. Thereby, the findings suggest that endogenous tissue levels of acylethanolamides seem in some instances to be influenced in a complicated way by the type of dietary fat ingested, a mechanism that perhaps is mediated through changes in expression of enzymes involved in the turnover of endocannabinoids and acylethanolamides (42). Additionally, the OEA levels observed in ATB and ATW may also activate β-adrenergic receptors that further promote ATB β-

oxidation inducing uncoupling protein-1 (UCP1)-mediated thermogenesis (43–45) that burns FA during uncoupled respiration (46); since UCP1 is a mitochondrial molecule involved in diet-induced as well as cold-induced non-shivering thermogenesis. Furthermore, Suárez *et al.* (46) showed that the OEA present in ATB and ATW stimulates lipolysis by activating PPAR-α which further stimulates the β-adrenergic system. The β-adrenergic receptors further in combination with UCP1-mediated thermogenesis help to regulate energy balance by increasing energy expenditure and reducing fat depots; improving overall body composition (9). Notwithstanding the vital role of OEA in white-to-brown adipocyte; in the present study, we failed to see any higher OEA concentrations in either ATB and ATW organs compared to the findings by LoVerme *et al.* (47). The study performed by LoVerme and coworkers demonstrate that cold exposure is a natural stimulus for OEA formation in white fat and suggest a role for the sympathetic nervous system in regulating OEA biosynthesis. The reason behind such a contrast in findings leading to reduced levels of OEA in adipose tissues may be that in the present trial, a stable room temperature was maintained throughout the study protocol. However, the results observed such as reduced food consumption and fat deposits following consumption of high n9 diets (9) suggest that increased OEA concentrations in ATB, as well as ATW, might have stimulated the β-adrenergic activity suggesting the crucial role of β-adrenergic receptors.

Overall, the endpoint FAs in all the full range of tissues including internal organs reflected the FA composition of the DFA intake, and our results are in alignment with previous trials conducted with rodents (8, 17). Furthermore, the highest levels of

C18:2n6 were evident in heart, both sections of the intestine-duodenum and -jejunum; followed by adipose tissues-ATW and -ATB, and liver, which is in agreement with previous studies performed with C18:2n6 containing diets (48–50). Similarly, the significant increase in LEA concentrations post consumption of the n6-rich diets, i.e. F+S followed by C+S and RC diets, reflects the similar enrichment of C18:2n6 in all tissues. Moreover, the intake of C18:2n6 alters the degree of fatty acid *de novo* synthesis in foods containing 20% fat (49, 50), which might influence the synthesis of LEA, thereby explaining the higher levels observed.

The n6 FA C20:4n-6 in phospholipids is the precursor of two of the best-characterized endocannabinoids 2-Arachidonoylglycerol (2-AG) and AEA (51). In our trial, F+S, H+DHA, and H+EPA diets were found to generate lower concentrations of AEA in the fasting state when compared to the C+S, HOCO, OO, and RC diets in heart, intestine-duodenum, intestine-jejunum, and liver. As such the least AEA concentrations reflected after the ingestion of dietary treatments exclusively rich in n3 FAs; H+DHA in heart, F+S and H+DHA in intestine-duodenum, F+S in intestine-jejunum, and H+DHA and H+EPA in the liver, since those diets rich in n3 FAs were inefficient in synthesizing C20:4n6 from C18:2n6 by desaturation-chain elongation (49). Additionally, the brain had the second highest fasting AEA concentrations, after the levels observed in both proximal and distal sections of the intestine. Our findings are consistent with those of previous trials conducted with rodents, showing increased fasting AEA concentrations in intestine followed by the brain (8, 19). Furthermore, presently the period of food deprivation increased the duodenal AEA concentrations by a percent difference of 88%

when compared to brain AEA levels (Figure 4.1), consistent with previous findings in rodents (52). Therefore, the present data indicate that *N*-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) present in the intestinal enterocytes is most likely responsible for the increased AEA levels.

Very little is known about the physiological or biological importance of ALEA, EPEA, and DHEA. Where ALEA has been shown to bind cannabinoid receptors (53), EPEA was observed to promote increased food consumption (9). On the contrary, DHEA has been associated with increased gynoid fat mass (14). Additionally, both long chain, as well as very long chain, FA-derived FAEs, i.e. EPEA and DHEA, have also been shown to activate PPAR-α (8). Further experiments are needed to understand the biological significance of these FAEs in rodent and human physiology. In our study, the highest ALEA levels were found with the F+S diet, likely due to the presence of C18:3n3-rich flaxseed oil, consistent with a previous report (14). Similarly, elevated EPEA levels were observed with the H+EPA diet in all tissues except brain, where the EPEA concentrations remained undetected. This finding is corroborated by previous reports (54–56) which show the notable aspect of brain FA composition being its low content of C20:5n3 precursors. In addition, the increased DHEA levels observed with H+DHA diets demonstrate that dietary incorporation of C22:6n3 shifted FAE homeostasis to favour DHEA in C22:6n3, over n6 and n9, enriched diets, consistent with previous findings (57). Moreover, in heart tissue, increased DHEA concentrations resulted in blunted or diminished OEA levels, which bear a resemblance to the results from previous human (14) as well as rat (58) studies. Overall, these data suggest that precursor DFA in a

fasting state also influences the tissue FAE concentrations (Tables 4.2–4.8) (Figures 4.1–4.4).

Regarding correlations between tissue FA and FAE concentrations, we showed that for the most part changes in FAE coincide with the parallel changes in levels of their precursor FAs (33) (Table 4.9). Since n3 and n6 FAs cannot be synthesised *de novo,* therefore, the tissue FA composition is reflected by the DFA intake (59). However, the data where no such associations or interactions were observed, indicate that mechanisms controlling FAE concentrations are mediated through the regulation of the vital enzyme NAPE-PLD present in each respective tissue (40). For instance, analysis of the FA composition of brain lipids showed a highly preserved FA composition independent of the DFA within the experimental diet (8). Alternatively, these results could be explained by the impact of the LFHS diet on the levels of NAPE-PLD, the key enzyme involved in FAE biosynthesis; since Igarashi *et al.* (60) demonstrated altered upregulation of FAEs in rats fed an LFHS diet. Thus, the present study indicates that similar to dietary fat, high dietary sucrose also influences FAE synthesis and homeostasis. Therefore, the adverse interactions or inverse associations between FAs and FAEs in tissues require a deeper understanding; which can only be answered by further experiments. Henceforth, future nutritional feeding trials should be designed to better understand the influence of DFA intake on *N*-acylphosphatidylethanolamine (NAPE) and *N*-acylethanolamine (NAE) species at the molecular level. Moreover, a better understanding of the underlying mechanism regulating deglutition, digestion, and absorption of DFA leading to the synthesis of FAEs that further activate the fat taste receptors, stimulating satiety signals

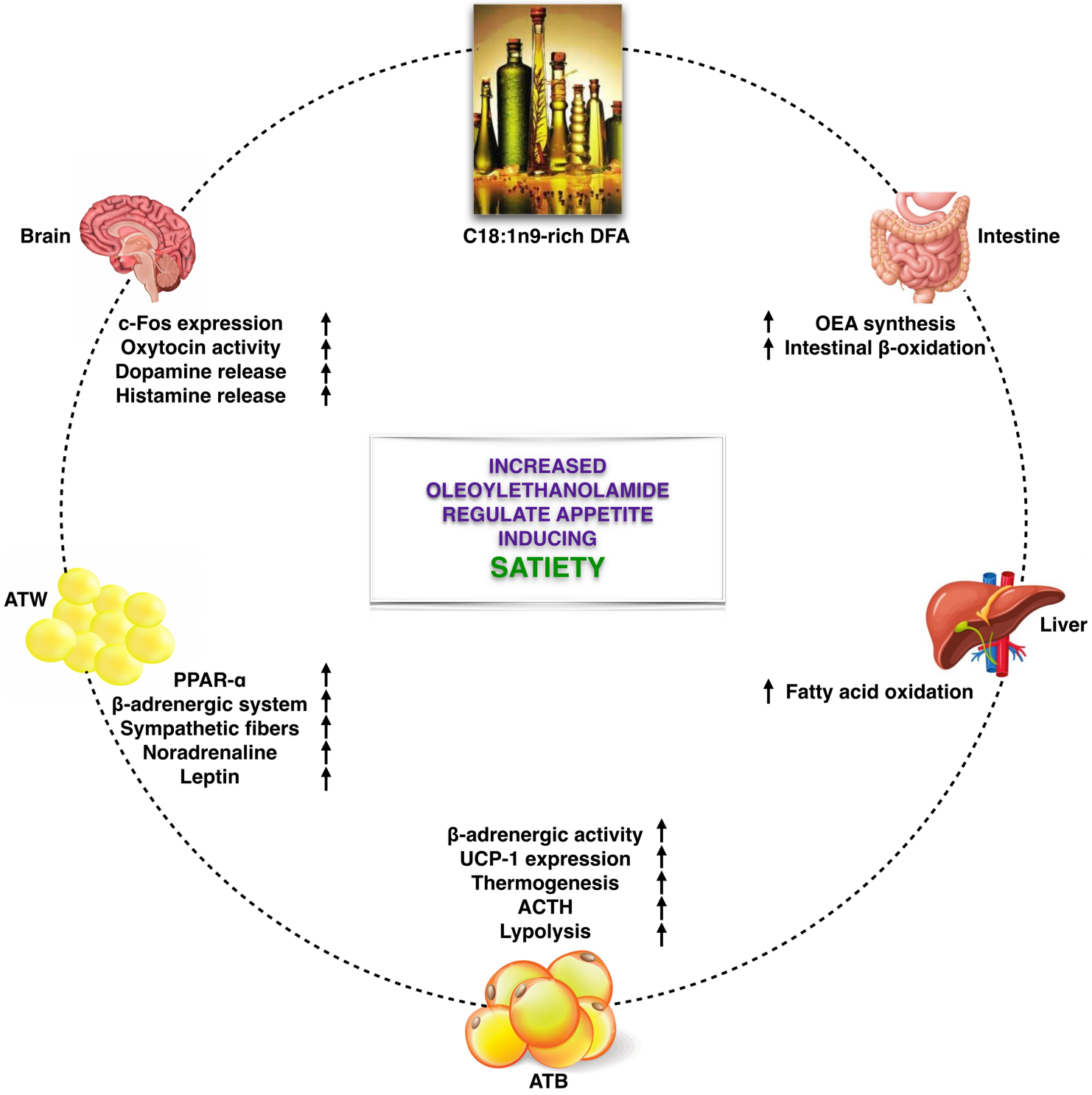
can be highly invasive and cannot be performed on humans. Therefore, animal models such as the hamster can be excellent options in which to conduct future such trials.

The present study has several strengths. To the best of our knowledge, this is the first design that has demonstrated that DFA profile influences tissue FAE concentrations in a broad panel of internal organs and tissues such as central and peripheral organs in male Syrian hamsters. We have in this study shown that longer-term feeding of the LFHS diet influences the fasting FAE concentrations. Furthermore, we propose a theoretical model (Figure 4.9) describing energy homeostasis leading to OEA induced satiety, which is based on previous reports (1, 9, 47) and present findings. Conversely, the study possesses limitations as well in that the trial performed did not measure/analyse adipokines such as leptin and critical neurotransmitters like dopamine, histamine, and noradrenaline which could have explained how well OEA levels helped maintain body weight post consumption of various DFA. An additional yet critical limitation of the present study was that no mechanistic analyses were performed.

Altogether, in the present study, we show the influence of various blends of oils on tissue FA and FAE levels, especially with the variants of n9 oils in combination with different dietary oils generally consumed by human beings. We further show the implication of these dietary oils on FAE concentrations by nutritional feeding for a duration of two months. Furthermore, in this trial, we demonstrated the longer-term feeding effects of LFHS diets on seven different FAEs.

In summary, the present study provides an examination of long-term effects of

Figure 4.9 Oleoylethanolamide inducing satiety model.



Note: DFA, dietary fatty acids; OEA, oleoylethanolamide; ATB, adipose tissue brown; ATW, adipose tissue white; UCP-1, uncoupling protein-1; ACTH, adrenocorticotropic hormone.

various DFA on FAE levels in tissues of golden Syrian hamsters a species commonly used in research on cholesterol metabolism (61), seasonality, circadian rhythms, and social behaviour (19). The findings demonstrate the actions of diet FA composition on FAE patterns in various tissues and organs of hamsters. Additionally, our data establish the feasibility of investigating possible regulatory energy homeostasis mechanisms and modifications in hamsters' circulatory and endocannabinoid system including the cannabinoid receptors that regulate food intake; which could help develop apposite nutritional therapy to control appetite. Furthermore, our study shows the negative associations between OEA levels and endpoint body weight of hamsters; and the brain OEA concentrations and endpoint body weight of hamsters suggesting that OEA exerts anorexic potency that helps maintain healthy body weight. Also, the present trial shows that an inverse relationship exists between gut OEA concentrations and body weight. Moreover, the detailed review (1) presented (Chapter 2, Manuscript 1) demonstrated that the satiety signals are imparted through the gut-brain interrelationship, enhanced by OEA, corroborating the current findings.

In conclusion, the intensity of the OEA satiating component can be amplified/boosted by the inclusion of C18:1n9-enriched diets that could aid in regulating appetite and, thereby help attain overall wellness. Thus, pointing to the possible nutritional use of OEA, a naturally occurring lipid amide in the management of obesity.

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4.7 Conflict of interest statement

The authors have no conflict of interests to declare in the development of this manuscript.

4.8 Support or funding information

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Bridge to Chapter 5

Chapter 3 and 4 dealt with the favourable effect of consumption of MUFA-enriched oils on body composition in male hamsters. These data prompted the design of a full-feeding nutritional intervention trial to explore whether MUFA-enriched diets improve the body composition in human beings since synthesis and breakdown of FAEs partially govern their levels thus efficacy. Moreover, genetic polymorphisms impact the kinetics involving the metabolism of FAEs in enzymes working at both control points suggesting that the alterations in FAE concentrations exhibit inter-individual variability, which may influence body composition either due to FAE involvement, through genetic underpinnings, or by both mechanisms. Therefore, Chapter 5 comprises a manuscript which presents the associations between candidate SNPs in genes related to FAE metabolism and the genes which stimulate the anorexigenic action of FAEs to elucidate the health implications of MUFA-enriched dietary consumption.

Chapter 5

Research Article

Manuscript 4

This manuscript is in preparation and will be submitted for publication in 2019

Effect of high-monounsaturated vs low-monounsaturated diet and genotype pattern on plasma fatty acid ethanolamide concentrations

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5.1 Abstract

Background: Fatty acid ethanolamides (FAEs) are lipid-derived signalling mediators that regulate energy intake and expenditure. Data suggest that FAEs play a role in obesity and that genetic factors impact their rates of synthesis and catabolism. However, how dietary fatty acid (FA) composition and genetics interactively influence FAE levels and their downstream actions on energy balance remain to be fully explained.

Objective: To investigate plasma FAEs concentration in humans in response to consumption of various dietary oils and to elucidate the role of diet × gene interactions on FAEs, and their influence on subcutaneous to android fat mass ratio (SAR), and subcutaneous to visceral fat mass ratio (SVR).

Method: A multi-centre, controlled-feeding, double-blind, randomized, crossover trial was conducted, where participants (n = 115) consumed smoothies containing 20% kcal of total energy from (i) high oleic canola oil (HOCO), (ii) regular canola oil (RCO), or a control oil (CO) (oil blend of butter, safflower, flaxseed and coconut) twice a day for six weeks. Plasma FA and FAE profiles were assessed using GC-FID and UPLC-MS/MS, respectively. DXA was used for body composition analysis. Satiety hormone, leptin levels were measured using ELISA. Genotyping of 9 candidate single nucleotide polymorphisms (SNPs) was performed using qPCR.

Results: Plasma oleoylethanolamide (OEA) concentrations were highest ($P < 0.0001$) after consumption of HOCO (2.49 ± 0.09 ng/ml) followed by RCO (2.34 ± 0.08 ng/ml),

and CO (2.03 ± 0.06 ng/ml). In addition, elevations ($P < 0.0009$) in plasma linoleoylethanolamide (LEA) levels (0.70 ± 0.02 ng/ml) were observed after the CO treatment when compared to the HOCO treatment (0.64 ± 0.01 ng/ml). Furthermore, RCO and CO consumption resulted in the highest ($P < 0.0002$) concentrations of α-linolenoylethanolamide (ALEA) compared to the HOCO treatment. When diet-gene interactions were tested, elevated concentrations of plasma OEA were observed in participants possessing the G protein-coupled receptors (*GRP40*) rs1573611-T ($n = 48$, $P < 0.0037$) allele after the consumption of HOCO ($P < 0.0001$) and RCO ($P < 0.0121$), but not CO. Overall, inverse associations were observed between OEA and SAR ($r = – 0.14$; $P = 0.0098$) as well as OEA and SVR ($r = – 0.16$; $P = 0.0034$).

Conclusion: Plasma OEA concentrations reflect the dietary pattern of C18:1n9 intake and may be influenced by *GRP40* rs1573611 polymorphism. Our data demonstrate that humans possessing *GPR40* rs1573611-T and *LEPR* rs1137101-A/A polymorphisms would benefit more from the ingestion of C18:1n9 enriched dietary oils by elevating energy expenditure and regulating appetite, due to enhanced satiation induced by increased OEA and reduced AEA concentrations, respectively; which in turn influence SVR. This trial was registered at www.clinicaltrials.gov as NCT02029833.

Keywords: Fatty acid ethanolamides, anandamide, oleoylethanolamide, endocannabinoid, diet × gene interactions, subcutaneous to android fat mass ratio, subcutaneous to visceral fat mass ratio.

5.2 Introduction

Obesity has become pandemic, leading to increased metabolic syndrome (MetS) prevalence worldwide (1). Obesity is a heterogeneous condition with substantial inter-individual differences in the pattern of adipose tissue deposition. In addition, different patterns of adipose tissue distribution have different metabolic correlates leading to variations in disease risk (1). For instance, subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) have been shown to play different roles in the pathogenesis of MetS; and these MetS consequently result in ectopic fat storage leading to weight gain (2). However, in making comparisons across weight loss interventions, Merlotti *et al.* (3) have reported that SAT loss is greater than VAT loss in humans. Therefore, in the present study, the ratios such as subcutaneous to android fat mass ratio (SAR) and subcutaneous to visceral fat mass ratio (SVR) were adopted to represent fat distribution. Furthermore, associations or complex interactions between fat intake and the risk of developing obesity have generated interest in elucidating the physiological signals governing satiety. Fatty acid ethanolamides (FAEs) are a class of lipid amides that regulate numerous pathophysiological functions (4), such as enhancing energy expenditure, inducing anorexic signalling, thereby, generating potential anti-obesity effects. Among these FAEs, oleoylethanolamide (OEA) is a lipid amide that is released by enterocytes upon absorption of dietary fat and is involved in eating, energy balance, and feeding behaviours that may engage in the initiation of satiety (5). Conversely, arachidonoylethanolamide (AEA, also known as anandamide) shows a high affinity for

cannabinoid receptor activation, known for inducing appetite. In addition, OEA serves as a naturally occurring lipid mediator that has been clearly implicated in weight regulation in animals due to the enhanced energy expenditure inducing lipolysis (5). However, its role in obesity in humans is still conjectural. Therefore, it is important to elucidate whether OEA exerts any effect on the SAR and SVR.

Furthermore, animal data suggest that OEA acts as the agonist for G protein-coupled receptors (GPCRs) (6), also termed as GPRs. Data suggest that GPR40 is involved in fatty acid (FA) taste perception (7), stimulating leptin release (8). Moreover, the effect of the quality of dietary fat on obesity phenotype and obesity-related genes has been demonstrated (9). For instance, Pu *et al.* (10) showed that A/- allele carriers of fatty acid amide hydrolase (*FAAH*) gene have significantly higher FAE concentrations than individuals with the C/C genotype. However, the scarcity of nutritional trials shedding light on diet × gene interactions to derive maximum benefits out of the potent appetite suppressing lipid amides creates a need to undertake controlled feeding trials to further explore this area. Furthermore, there is considerable scientific interest in identifying genetic variants that help explain inter-individual differences in weight loss success in response to diet interventions (11). Therefore, the objective of the present study was to investigate the dietary interactions with candidate genes and genotypes involved in the synthesis and breakdown of FAEs and genes involved in stimulating the action of FAEs (5, 12). Additionally, the interactions were investigated in genes via which OEA imparts/stimulates anorexic signalling in animals. As such; the objective of the present study was to elucidate the role of diet × gene interactions on FAEs in human

beings; along with to improve our understanding of associations between genotypes, on SAR and SVR, respectively.

5.3 Materials and methods

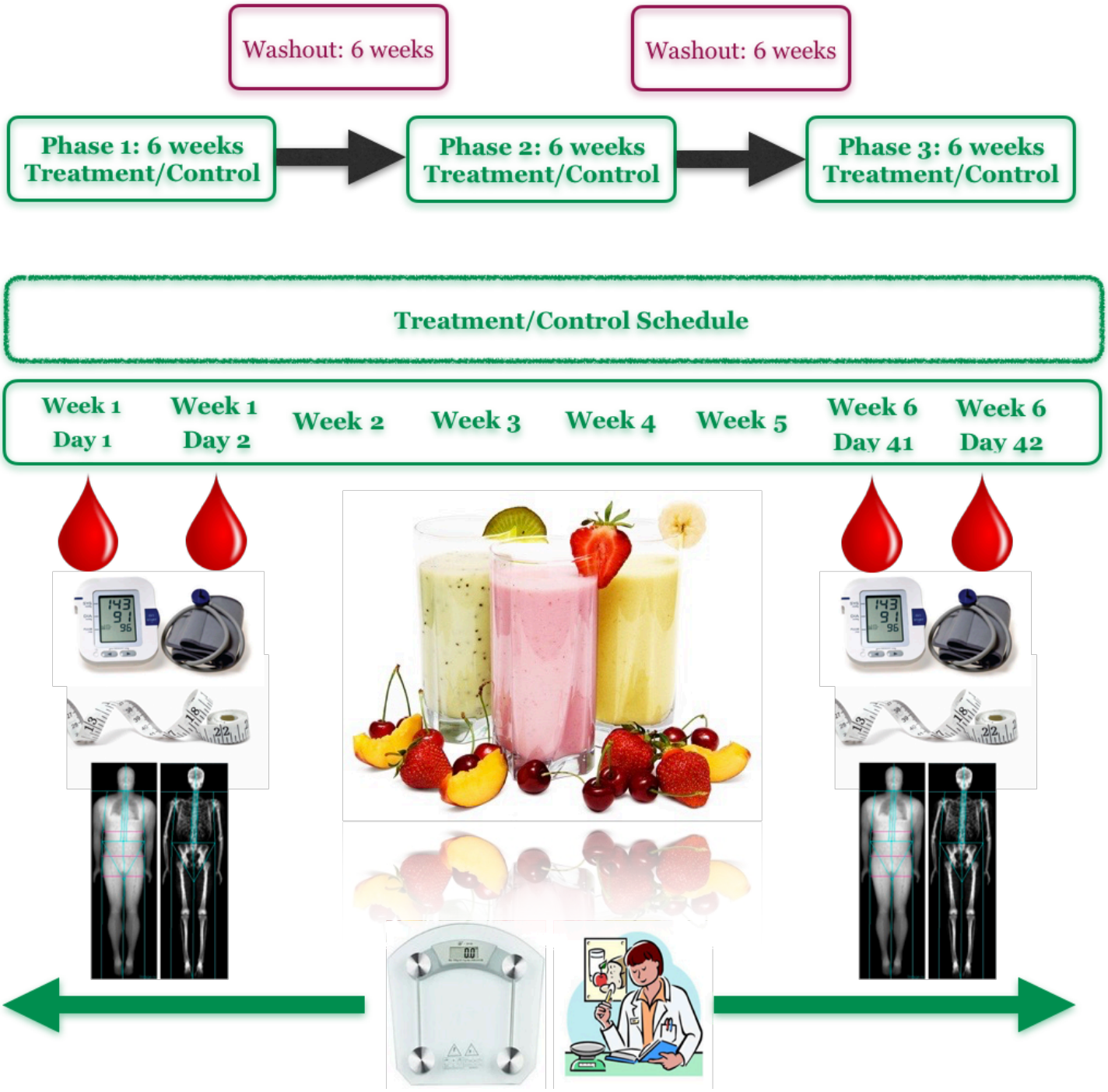
5.3.1 Experimental design

5.3.1.1 Study protocol

A randomized, double-blinded, cross-over, full-feeding, multi-centre trial was conducted from the year 2014 to 2016 at four sites; namely, (i) Institute of Nutrition and Functional Foods (INAF), Laval University, QC, Canada; (ii) The Pennsylvania State University (PSU), PA, USA; (iii) Richardson Centre for Functional Foods and Nutraceuticals (RCFFN), University of Manitoba, Winnipeg, MB, Canada; and (iv) Canadian Center for Agri-Food Research in Health and Medicine (CCARM), St-Boniface Hospital, Winnipeg, MB, Canada. Figure 5.1 outlines the graphical representation of the protocol for the human intervention trial. The study design consisted of 3 treatment phases of 6 weeks each, each separated by 6-week washout periods (ranged from 4–12 weeks for a few participants). All participants were systematically randomized using randomization.com. The trial was conducted according to the principles expressed in the Declaration of Helsinki (13). Trial procedures were approved by the participating sites' Biomedical Research Ethics Board. All participants provided signed and dated written informed consent. This trial was registered at www.clinicaltrials.gov as NCT02029833.

5.3.2 Participants

Figure 5.1 Study design and methods.



5.3.2.1 Inclusion criteria

Before study enrolment, participants underwent a routine physical examination by the study physician. Only participants able to understand and comply with the protocol requirements, instructions, and protocol-stated restrictions were included for the study. Overweight and obese male and female non-smokers (n = 115), aged 20–65 y were recruited for the study. The recruited participants had a fasting blood glucose ≥ 5.6 mmol/L with increased waist circumference, men ≥ 94 cm and women ≥ 80 cm, considered as the primary inclusion criteria. Additionally, the secondary inclusion criteria for the study were that participants meet at least two of the following MetS parameters: serum low density lipoprotein cholesterol (LDL-C) level ≥ 2.6, triglycerides (TG) ≥ 1.7 mmol/L, high density lipoprotein cholesterol (HDL-C) < 1 mmol/L (males) or < 1.3 mmol/L (females), and blood pressure ≥ 130 mmHg (systolic) and/or ≥ 85 mmHg (diastolic). These criteria allowed participants to meet the metabolic syndrome definition of the International Diabetes Federation (IDF) and American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) Consensus group (14). Participants' baseline characteristics are summarized in Table 5.1. Figure 5.2 provides a graphical representation of the participant flow.

5.3.2.2 Exclusion criteria

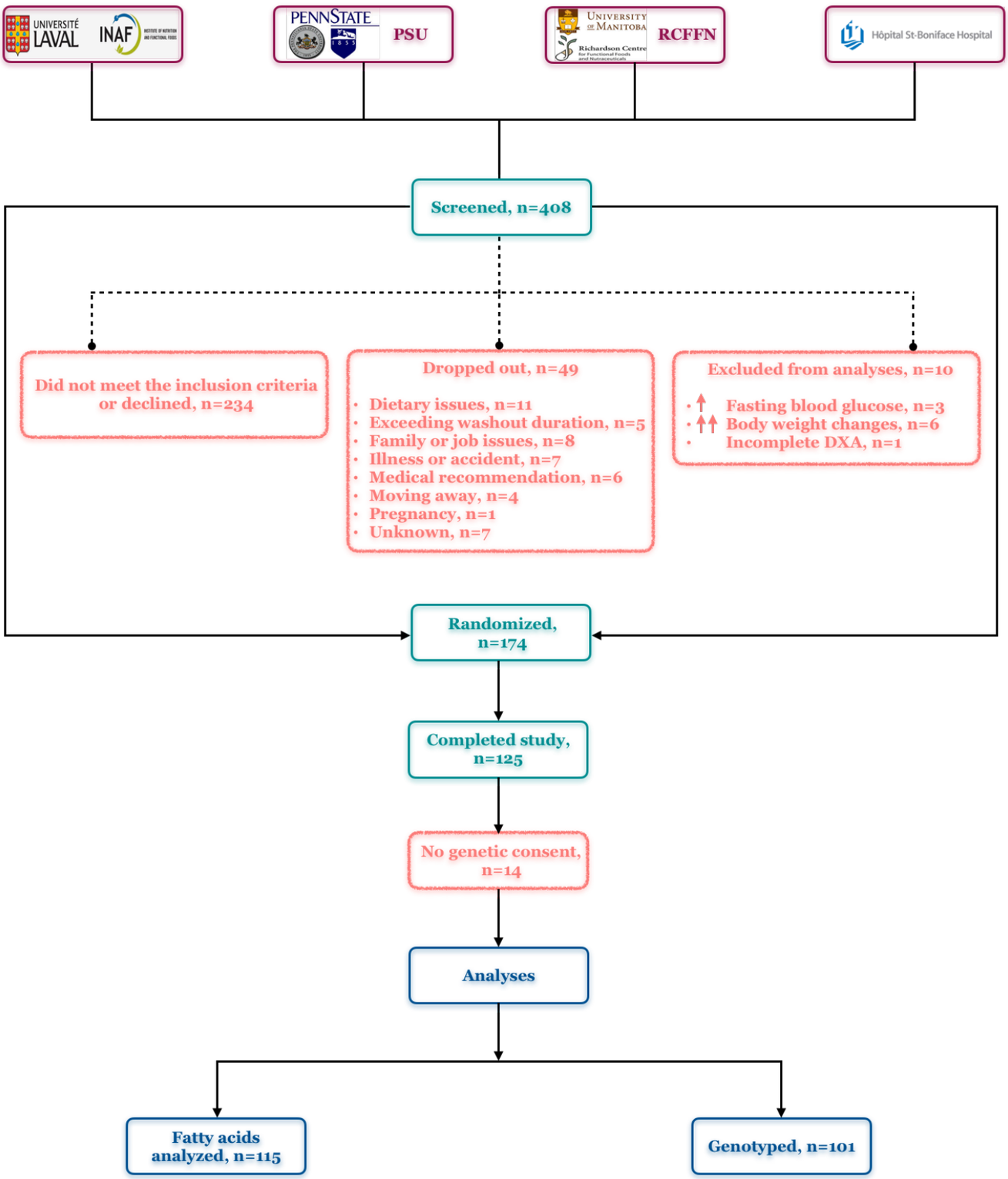
Pregnant or lactating females or those planning to conceive during the study period, or individuals with past or present specific disease states, which as judged by the

Table 5.1 Baseline characteristics of study participants.

Characteristic	Total (n = 99)			Female (n = 59)			Male (n = 40)			$P^{\ddagger}$
	N	Mean	SEM	n	Mean	SEM	n	Mean	SEM	
Age (years)		43.31	1.29		45.65	1.64		39.88	1.98	0.0271
Ethnicity										–
Caucasian	73			45			28			
African	4			3			1			
Asian	8			4			4			
Hispanic	3			1			2			
Others	11			6			5			
Anthropometric measures										
Body weight (kg)		89.76	1.88		83.05	2.21		99.59	2.67	<0.0001
Body Mass Index (kg/m^2)		31.12	0.53		30.99	0.69		31.30	0.84	0.7675
Waist Circumference (cm)		103.80	1.30		100.80	1.60		108.30	1.90	0.0030
Blood pressure (mmHg)										
Systolic BP		118.60	1.30		117.80	1.70		119.70	2.00	0.4647
Diastolic BP		78.10	1.08		77.47	1.41		79.00	1.70	0.4915
Plasma glucose and lipid levels										
Glucose (mmol/L)		5.22	0.04		5.21	0.06		5.23	0.07	0.8271
Total Cholesterol (mmol/L)		5.19	0.09		5.21	0.12		5.15	0.14	0.7369
HDL Cholesterol (mmol/L)		1.35	0.04		1.46	0.04		1.20	0.05	0.0003
LDL Cholesterol (mmol/L)		3.13	0.08		3.09	0.10		3.19	0.12	0.5063
Triglycerides (mmol/L)		1.55	0.07		1.46	0.09		1.67	0.11	0.1523

Note: The results are expressed as mean ± SEM (n = 99). ‡ANOVA was used to analyse between-sex differences in continuous variables. Statistical significance assessed at ($P < 0.05$). BP, blood pressure; HDL, high density lipoprotein; LDL, low density lipoprotein.

Figure 5.2 Participant flow diagram.



Note: INAF, Institute of Nutrition and Functional Foods; PSU, The Pennsylvania State University; RCFFN, Richardson Centre for Functional Foods and Nutraceuticals.

investigator or medical monitor, may affect the outcome of this study or the subject's safety, were excluded from the trial. These diseases included, but were not limited to, cardiovascular disease, hepatic disease, malignancy, gastrointestinal disease, renal disease, hematological disease, neurological disease, or endocrine disease. Participants taking lipid modulating medicines were exempted from the study. Participants with a history of alcohol intake more than the upper limit set by National Institute on Alcohol Abuse and Alcoholism (NIAAA) (15) or drug abuse/dependence within 12 months of the study were excluded from the trial. NIAAA's definition of drinking at high risk is defined as an average weekly intake of >14 drinks/week for men or >7 drinks/week for women. Before the screening, interested participants were advised to stop taking prescription or non-prescription drugs including vitamins and dietary or herbal supplements, for at least 1 month; participants who did not follow the instructions were exempted from the study.

5.3.3 Diets

All dietary treatments and full day meals based on a 7-day rotating menu were prepared in the metabolic kitchen at each site. Participants were offered iso-caloric diets. The overall composition of all experimental diets provided to participants is shown in Table 5.2. Treatment oils were incorporated into smoothies containing 20% kcal of total energy from (1) control oil (CO) (formulated oil blend of butter, safflower, flaxseed and coconut) (Figure 5.3); (2) regular canola oil (RCO); or (3) high oleic canola oil (HOCO), twice a day for six weeks. The FA profile of the dietary oils/fats is presented in Figure 5.4. Remaining energy intake was adjusted in meals as per caloric requirements of

Table 5.2 Overall composition of all experimental diets provided to participants.

Composition	kcal%
Protein	15
Carbohydrate	50
Fat	35
Total	100

Figure 5.3 Composition of control oil blend.

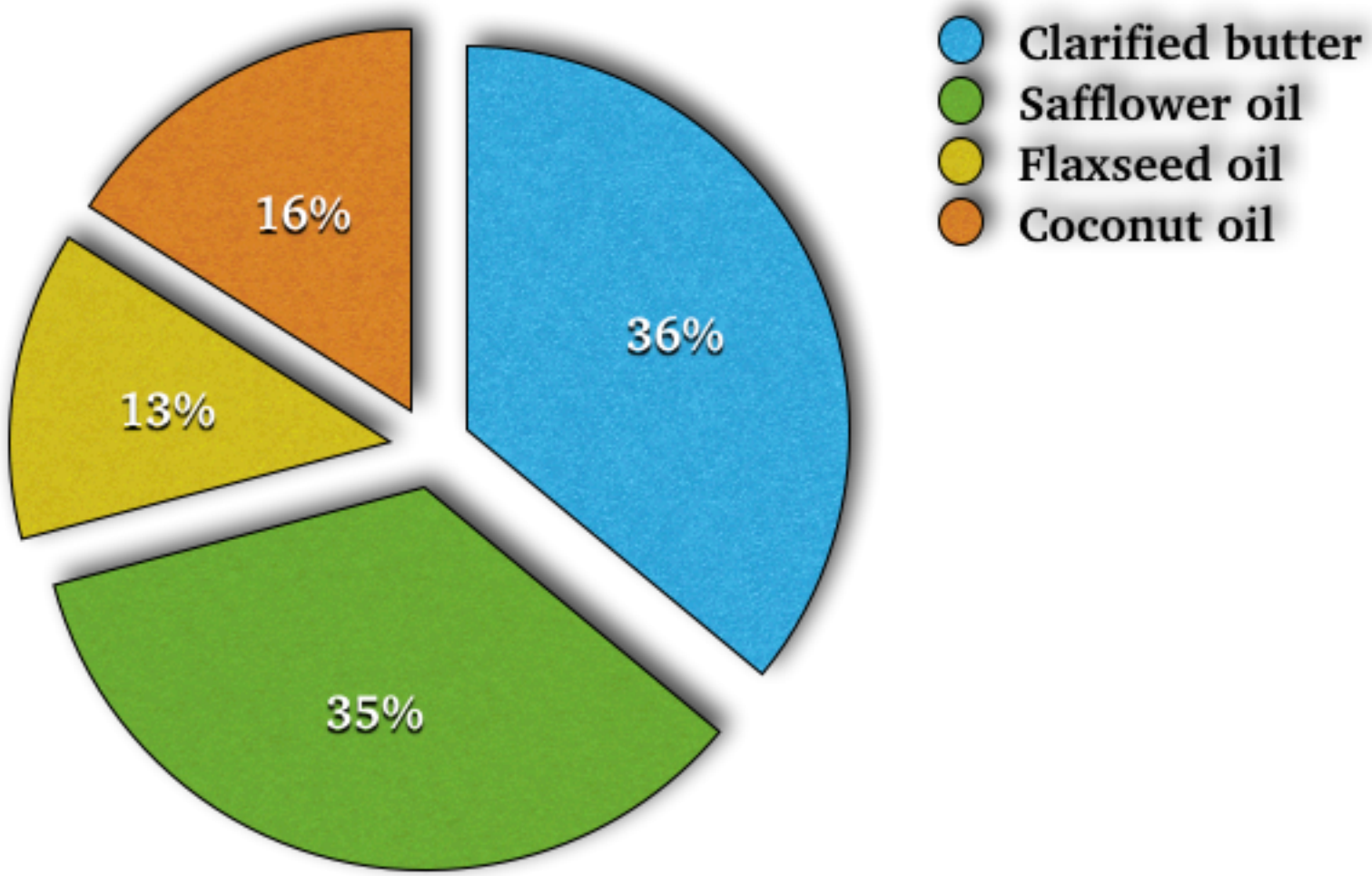
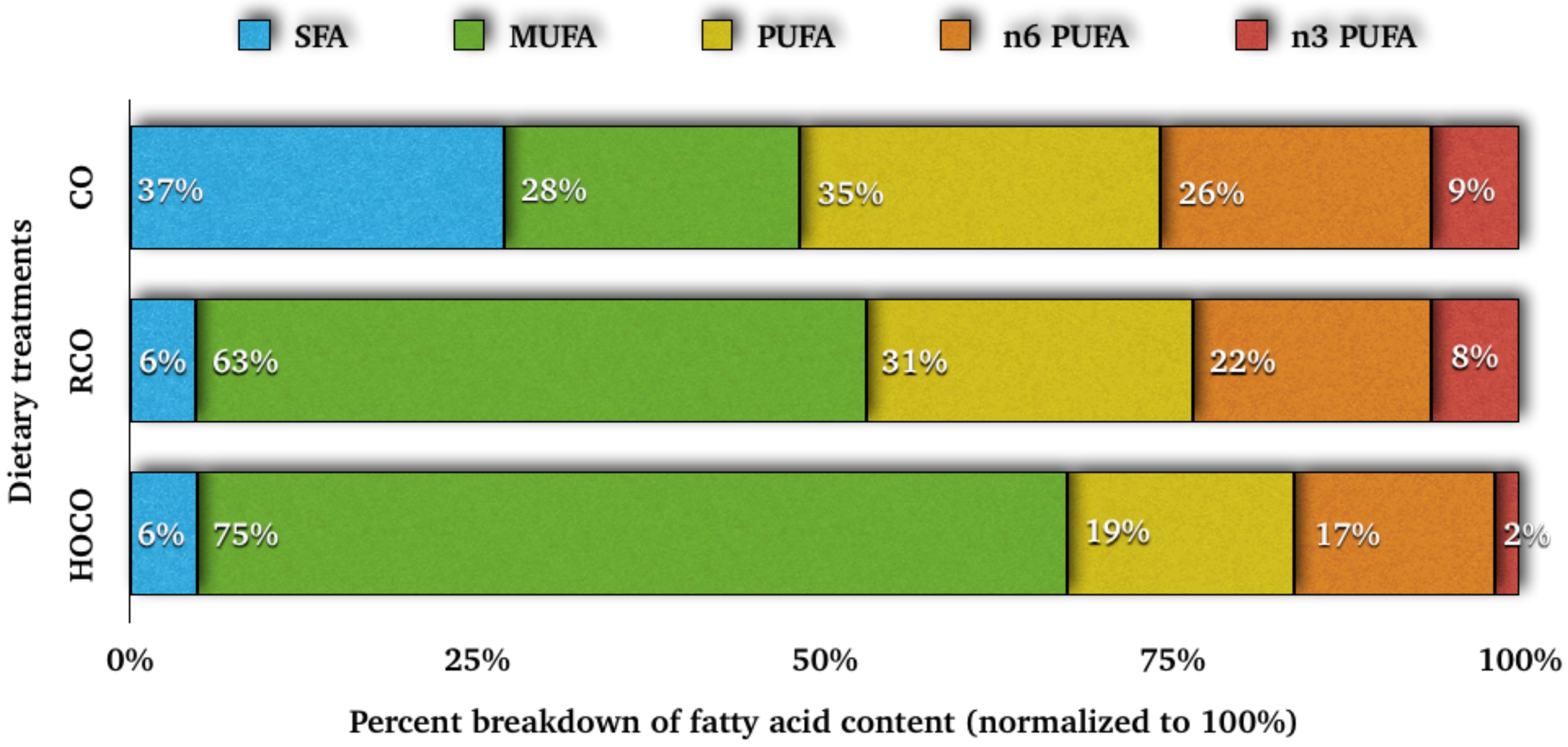


Figure 5.4 Fatty acid composition of the dietary oils.



Note: SFA, total saturated fatty acids; MUFA, total monounsaturated fatty acids; PUFA, total polyunsaturated fatty acids; n6 PUFA, total n6 polyunsaturated fatty acids; n3 PUFA, total n3 polyunsaturated fatty acids; CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil.

participants. Body weights were monitored for the first two weeks on a daily basis to compensate for any distress in food load to avoid overconsumption. If any sudden weight gain or loss was noticed, the caloric intakes were readjusted in the initial two weeks of the intervention phase.

Participants were advised to consume the smoothies twice a day in the morning at breakfast and in the evening as per the randomization sequence/order. Compliance was monitored on a daily basis where one smoothie containing treatment oil along with one meal were consumed in the presence of clinical staff. Participants were advised not to consume food outside the provided meals. All participants were encouraged to maintain the dietary records wherein all the details pertaining to food consumption or medicinal intake if any, were recorded. Participants were strongly recommended to keep consistency in their physical activities throughout the trial. Compliance with the treatment oils was determined by measuring the plasma C18:3n3 levels at the end of each treatment phase. Furthermore, no significant differences in baseline C18:3n3 FA concentrations across the groups indicated no carryover effect and adequate washout periods between the treatment phases (data not shown).

The CO formulation comprised of butter, safflower, flaxseed and coconut oils. The clarified butter was purchased from Verka (New Delhi, India). Flaxseed oil was purchased from Shape Foods (Brandon, MB, Canada). The n6 safflower oil and coconut oil were purchased from eSutras (Chicago, IL, USA). RCO and HOCO were obtained from Richardson Oilseed (Winnipeg, MB, Canada).

5.3.4 Sample collection

5.3.4.1 Anthropometric and clinical data collection

Figure 5.1 outlines the schedule for anthropometric measures that included weight, height, and waist circumference; and clinical procedures included seated blood pressure, dual energy X-ray absorptiometry **(**DXA) scans, and fasting blood draw. At the beginning and end of each phase, blood pressure was monitored at each clinical site by using a digital blood pressure monitor and measured in triplicate after a 5-min rest in a secluded area. Both systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded in the morning before meals while subjects were in a seated position, with the cuff placed at the level of the heart on brachial artery.

5.3.4.2 Dual energy X-ray absorptiometry (DXA)

Figure 5.1 outlines the schedule for DXA scans and weight assessment for the human intervention trial. On day 1 and 42 of each intervention phase, body composition, including subcutaneous adipose tissue mass (SATM); android mass (AM); visceral adipose tissue mass (VATM); body weight (BW); subcutaneous to android fat mass ratio (SAR); subcutaneous to visceral fat mass ratio (SVR) was assessed by DXA (Lunar Prodigy Advance, GE Healthcare, Madison, WI, USA) in fasting state. All DXA scans were performed at the supine position. The data were evaluated with the Encore 2005 software (v. 9.30.044; GE Healthcare, Little Chalfont, UK) by using the same analyst across all scans.

5.3.4.3 Blood sample collection

Figure 5.1 outlines the schedule for fasting blood sample collection for the human intervention trial. 12 h fasting blood samples were collected on day 1, 2, 41, and 42 of each intervention phases. Blood samples were centrifuged at 3000 rpm for 20 mins at 4 °C, aliquoted to yield serum, plasma, red blood cells (RBC), and white blood cells (WBC). Aliquoted samples were immediately stored at – 80 °C until analysis.

5.3.5 Chemicals

Heptadecenoic acid (C17:1) internal standard was obtained from Sigma-Aldrich (St. Louis, MO, USA) and the GLC-463 standards mix was purchased from Nu-Chek Prep Inc. (Elysian, MN, USA). FAE standards—including palmitoylethanolamide (PEA), OEA, linoleoylethanolamide (LEA), α-linolenoylethanolamide (ALEA), AEA, eicosapentaenoylethanolamide (EPEA), and docosahexaenoylethanolamide (DHEA)—and deuterium-containing internal FAE standards—[$^2H_4$] PEA, [$^2H_4$] OEA, [$^2H_4$] LEA, [$^2H_4$] ALEA, [$^2H_8$] AEA, [$^2H_4$] DHEA, and [$^2H_4$] EPEA—were obtained from Cayman Chemical (Ann Arbor, MI, USA). Liquid chromatography (LC)-grade solvents were purchased from Sigma-Aldrich. All other chemicals and reagents were purchased from Sigma-Aldrich or Fisher Scientific (Waltham, MA, USA).

5.3.6 Fatty acid analysis

FA profiles in plasma were analyzed after direct transesterification followed by gas chromatography (16). For this, 2 mL of methanol:toluene (4:1 vol/vol) was added to the

plasma sample with heptadecenoic acid (17:1) as an internal standard. Acetyl chloride (200 μL) was added while vortexing and heated for 1 hr at 80 °C. The samples were cooled, and then 5 mL of K_2CO_3 was added before centrifuging at 2500 rpm for 5 min at 4 °C. The upper toluene phase containing FA methyl esters was collected and stored at – 80 °C until further analysis.

Methylated FA samples were analyzed by gas chromatography using a FUSED SILICA Capillary Column (100 m × 0.25 mm; film thickness, 0.20 μm, SP™-2560; SUPELCO Analytical, Bellefonte, PA, USA) on a Varian 430 gas chromatograph equipped with a flame ionization detector. The injector and detector ports were set at 250 °C and 290 °C, respectively. Oven temperature was set to 130 °C for 2 min and then increased to 175 °C (25 °C/min), held for 20 min. The temperature was then subsequently increased to 240 °C (3 °C/min), where it remained constant for 5 min, and the same temperature was maintained throughout, for a total run time of 50.47 min. A split ratio of 20:1 and an injection volume of 1 μL were used. A known FA mixture was compared with the samples to identify retention peaks using Galaxie software (Varian Inc.). The level of each FA was then calculated according to the corresponding peak area relative to that of all FA of interest to yield the relative percentage of total FA (17).

5.3.7 Fatty acid ethanolamide analysis

Plasma FAEs were extracted using a solid-phase extraction method (18, 19) with minor modifications. FAE levels were analyzed using a Waters Acquity UPLC system coupled to a Micromass Quattro *micro* API mass spectrometer (Waters, Milford, MA) equipped with

an atmospheric pressure ionization (API) probe and electrospray ionization interface (ESI). The detailed FAE analysis protocol has been published (Manuscript 2) and has been reported previously (Chapter 3).

5.3.8 Plasma leptin analysis

Plasma leptin levels were measured using high-sensitivity enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's guidelines (R&D Systems, Minneapolis, MN, USA). The intra-assay and inter-assay CV values were 3.17% and 4.37%, respectively.

5.3.9 DNA extraction and genotyping

Genomic DNA was extracted from WBC by using a column-based DNA extraction kit (DNeasy Blood and Tissue Kit, QIAGEN Sciences Inc., Toronto, ON, Canada) according to the manufacturer's instructions. The concentration and purity of the genomic DNA were assessed by microvolume spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific Inc, Waltham, MA, USA). Genotyping of the candidate genes involved in the synthesis and breakdown of FAEs and the genes involved in stimulating the action of FAEs was accomplished by using the TaqMan GTXpress Master Mix (Applied Biosystems, Life Technologies, Inc., Burlington, ON, Canada). DNA samples were further analysed on a StepOnePlus Real-Time PCR System (Applied Biosystems; Life Technologies Inc., Burlington, ON, Canada). For quality control, the genotyping was performed in duplicate. Overall, 9 single nucleotide polymorphisms (SNPs) in 9 genes were

investigated; out of which 2 essential candidate genes and genotypes involved in the synthesis and breakdown of FAEs; namely, *N*-acyl phosphatidylethanolamine phospholipase D (*NAPE-PLD*) rs12540583 and *FAAH* rs324420 were investigated. Furthermore, cluster of differentiation 36 (*CD36*) rs1761667, G protein-coupled receptors 40 (*GPR40*) rs1573611, Leptin receptors (*LEPR*) rs1137101, cannabinoid receptors (*CNR1*) rs1049353, melanocortin-4 receptor (*MC4R*) rs17782313, uncoupling protein-1 (*UCP1*) rs1800592, and dopamine receptor D2 (*DRD2*) rs1800497, were also investigated; via which OEA imparts/stimulates anorexic signalling in animals.

5.3.10 Data analyses and interpretation

The mechanism of OEA's anorexic signalling to induce satiety remains the same in every individual (5). However, mounting *in vivo* evidence from animal and human trials (5) demonstrate an immense variability in FA intake perception by individuals (20). The variability exists due to the distinct activity of receptors in each individual's gut, which plays a critical role in food consumption and obesity (5, 20). Additionally, Stewart et al. (20) demonstrated similar FA detection threshold in both male and female participants, suggesting that both sexes have similar gustatory and gastrointestinal sensitivity to C18:1n9 (21). Therefore, the FA and FAE analyses data for both sexes, male and female are presented together. Furthermore, previous studies (22–25) have reported that the effect modification claims observed in randomized clinical trials are often spurious when subgroup analyses are conducted. Besides, these results are more frequent when small sample sizes and *post-hoc* analyses are involved; failing to produce rare validations of

such claims. To substantiate it further, Wallach *et al*. (23) performed Cochrane meta-analyses and reported that sex-treatment interactions typically had limited biological plausibility or clinical significance. Thus, no subgroup analyses based on the sex-treatment effects are indicated in the present trial.

5.3.11 Statistical analyses

The results are expressed as means ± SEM. Data were analysed using SAS 9.4 (IBM Software, Armonk, NJ, USA). Abnormally distributed variables were natural log-transformed before statistical analysis. Statistical significance for the effects of treatment was analyzed by the SAS MIXED procedure and Tukey's *post-hoc* testing when applicable. Treatment, sex and age were included in the model as fixed factors, and sequence of treatments, clinical site and participant were included as a random factor, with participant repeated by phase. ANOVA with sex included as a fixed factor was used to investigate the effect of dietary treatment × gene interactions, followed by Tukey-Kramer adjustments for multiple comparisons. Gene and dietary treatment × gene were included as fixed factors to examine the effect of genotype. The SAS SLICE function investigated statistically significant dietary treatment × gene effects. Pearson's correlation analyses were performed to examine the associations between plasma FA and FAEs. Moreover, correlation analyses were conducted to investigate the associations between OEA concentrations and ratios of SAR and SVR, respectively. Statistical significance was defined as $P < 0.05$. The primary outcome of the trial was to investigate the implications of monounsaturated fatty acids (MUFA) on body composition.

Therefore, the power calculation was performed based on the previous clinical trial (26). The sample size of 140 was determined to offset for a 20% dropout rate. The power calculation was performed to detect a 55 g change in android fat mass using the variance parameter in android fat mass (27, 28). For analysis of the secondary outcomes, the sample size indicated the power of 100% ($\alpha = 0.05$) allowing us to detect significant differences in post-treatment plasma OEA concentrations among three dietary interventions (10).

5.4 Results

5.4.1 Participant characteristics

Figure 5.2 outlines the graphical representation of the participant flow. A total of 174 individuals were randomly assigned to the study, with the dropout rate of 28%. Three individuals were excluded due to increased blood glucose levels, 6 individuals were exempted from the analyses due to drastic body weight changes, and 1 individual was excluded from the study due to incomplete DXA measurement. In sum, the study had a dropout rate of ~28%. In total, 125 participants completed the intervention, out of which FA and FAE analyses were performed on 115 individuals. Participants' baseline characteristics are summarized in Table 5.1. Genotyping was performed on 101 participants who provided consent for genetic analyses. Characteristics of the selected polymorphisms are shown in Table 5.3. No significant differences were observed in body weight after any of the dietary treatments, and the body composition results in each dietary phase are tabulated in Table 5.4.

Table 5.3 Characteristics of the selected genetic polymorphisms.

Gene	SNP	Region	Allele	Genotype (n)			MAF%
			Major/Minor	MM	Mm	mm	
CD36	rs1761667	Intron	A/G	25	48	24	49.5
GPR40	rs1573611	Exon	C/T	46	41	7	29.3
LEPR	rs1137101	Missense	A/G	25	50	22	48.5
NAPE-PLD	rs12540583	Missense	A/C	75	20	4	14.2
FAAH	rs324420	Missense	C/A	58	33	8	24.7
CNR1	rs1049353	Coding	C/T	59	32	6	22.7
MC4R	rs17782313	Coding	T/C	55	36	6	24.7
UCP1	rs1800592	Promoter	T/C	43	41	13	34.5
DRD2	rs1800497	Missense	G/A	55	35	7	25.3

Note: SNP, single nucleotide polymorphism; MM, major allele homozygous; Mm, heterozygous; mm, minor allele homozygous; MAF, minor allele frequency; *CD36*, cluster of differentiation 36; *GPR40*, G protein-coupled receptors 40; *LEPR*, Leptin receptors; *NAPE-PLD*, *N*-acyl phosphatidylethanolamine phospholipase D; *FAAH*, fatty acid amide hydrolase; *CNR1*, cannabinoid receptors; *MC4R*, melanocortin-4 receptor; *UCP-1*, uncoupling protein-1; dopamine receptor D2.

Table 5.4 Body composition at each dietary phase[‡].

BC Variables	Parameter	CO		RCO		HOCO		*P*
		Mean	SEM	Mean	SEM	Mean	SEM	
Baseline SATM (kg)		2.22	0.13	2.21	0.13	2.20	0.13	0.8639
Endpoint SATM (kg)		2.16	0.13	2.14	0.13	2.15	0.13	0.7141
	SATM Δ	– 0.05	0.02	– 0.07	0.02	– 0.06	0.02	0.8705
	SATM Δ (%)	– 2.44	0.95	– 3.13	0.95	– 2.54	0.95	0.7368
Baseline VATM (kg)		1.50	0.13	1.49	0.13	1.48	0.13	0.7738
Endpoint VATM (kg)		1.31	0.09	1.31	0.09	1.33	0.09	0.7614
	VATM Δ	– 0.06	0.02	– 0.06	0.02	– 0.05	0.02	0.9277
	VATM Δ (%)	– 4.96	2.15	– 1.98	2.14	– 2.52	2.15	0.4534
Baseline AM (kg)		3.70	0.23	3.68	0.23	3.67	0.23	0.6765
Endpoint AM (kg)		3.54	0.20	3.51	0.20	3.54	0.20	0.7742
	AM Δ	– 0.16	0.03	– 0.16	0.03	– 0.14	0.03	0.7591
	AM Δ (%)	– 3.69	0.87	– 3.11	0.87	– 2.86	0.87	0.6459
Baseline BW (kg)		91.06	2.61	90.81	2.61	91.14	2.61	0.2942
Endpoint BW (kg)		89.85	2.47	89.89	2.47	89.99	2.47	0.8084
	BW Δ	– 1.21	0.31	– 0.92	0.31	– 1.16	0.31	0.1923
	BW Δ (%)	– 1.32	0.85	– 1.02	0.85	– 1.27	0.85	0.9010

Note: [‡]SAS MIXED model with Tukey-Kramer adjustment. The results are expressed as mean ± SEM ($n = 115$). Statistical significance assessed at ($P < 0.05$). BC, body composition; CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil. SATM, subcutaneous adipose tissue mass; VATM, visceral adipose tissue mass; AM, android mass; BW, body weight.

5.4.2 Dietary compliance

Returned empty food bags were used as one of the measures to assess compliance. Besides, participants consumed the treatment incorporated as a smoothie, as well as one meal, under the supervision of clinical staff. To affirm compliance with intake of the smoothie and meal, participants also signed a daily checklist in the presence of clinical staff. Additionally, the observation of increased C18:3n3 levels post-CO and -RCO treatments as compared to HOCO treatment substantiated the high level of compliance towards the experimental diets. All participants showed good tolerance to the experimental diets with no reported side effects or discomfort.

5.4.3 Effect of diet treatment on plasma fatty acid levels

Plasma showed a predominance of C16:0, and C18:2n6 fatty acids (FAs) across all dietary treatments, followed by C18:1n9, and C20:4n6 (Table 5.5). Also, C18:1n9 levels at the end of dietary intervention are shown in Figure 5.5A. Elevated C18:1n9 levels were observed in participants after HOCO consumption (23% increase; $P < 0.0001$), when compared to CO. Similarly, the highest ($P < 0.0001$) C18:2n6 levels were observed after the consumption of the CO treatment when compared to RCO and HOCO, respectively. Additionally, C18:3n3 rich treatments-CO and -RC reflected highest ($P < 0.0001$) C18:3n3 levels when compared to HOCO group. C20:4n6 levels were lowest ($P < 0.0168$) in CO dietary treatments. Moreover, post consumption, RCO showed the highest ($P < 0.0001$) content of C20:5n3, while no changes in levels of C22:6n3 were

Table 5.5: Plasma fatty acid levels[‡] of each dietary phase (g/100g)*.

Fatty acid	CO		RCO		HOCO		*P*
	Mean	SEM	Mean	SEM	Mean	SEM	
C16:0	26.39[a]	0.20	25.88[b]	0.20	25.69[b]	0.20	<0.0001
C18:1n9	11.33[c]	0.14	13.63[b]	0.18	14.28[a]	0.16	<0.0001
C18:2n6	22.65[a]	0.23	21.35[b]	0.21	20.69[c]	0.20	<0.0001
C18:3n3	0.59[a]	0.01	0.56[a]	0.01	0.45[b]	0.01	<0.0001
C20:4n6	8.64[b]	0.29	8.72[ab]	0.31	9.06[a]	0.32	0.0168
C20:5n3	1.01[a]	0.04	1.07[a]	0.04	0.89[b]	0.03	<0.0001
C22:6n3	2.91	0.06	2.91	0.06	2.94	0.05	0.7192

Note: [‡]The values are endpoint plasma fatty acids. *The values are % abundance of each fatty acid to total fatty acids. The results are expressed as mean ± SEM (n = 115). The values with different superscript letters in the same row are statistically different from each other. Statistical significance assessed at ($P < 0.05$) by ANOVA with Tukey-Kramer adjustment. CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil.

Figure 5.5A Plasma C18:1n9 levels (g/100g)* at the end of dietary interventions.

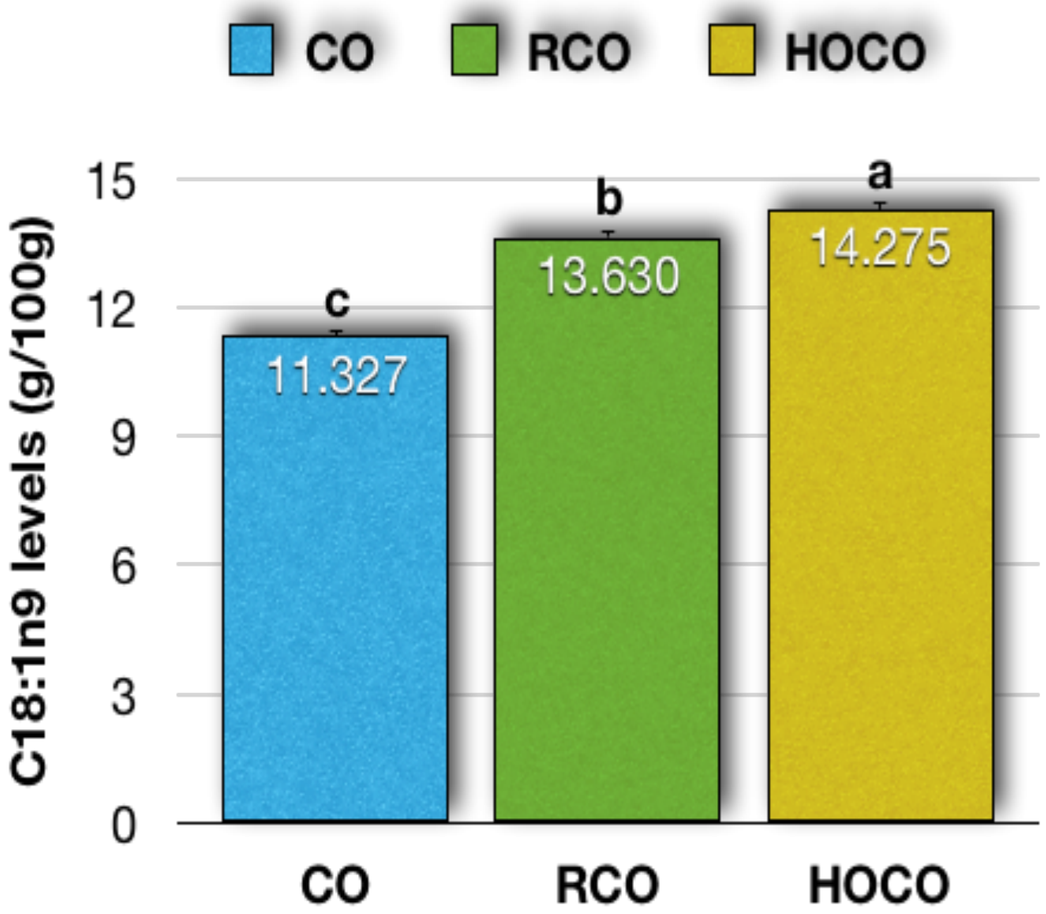
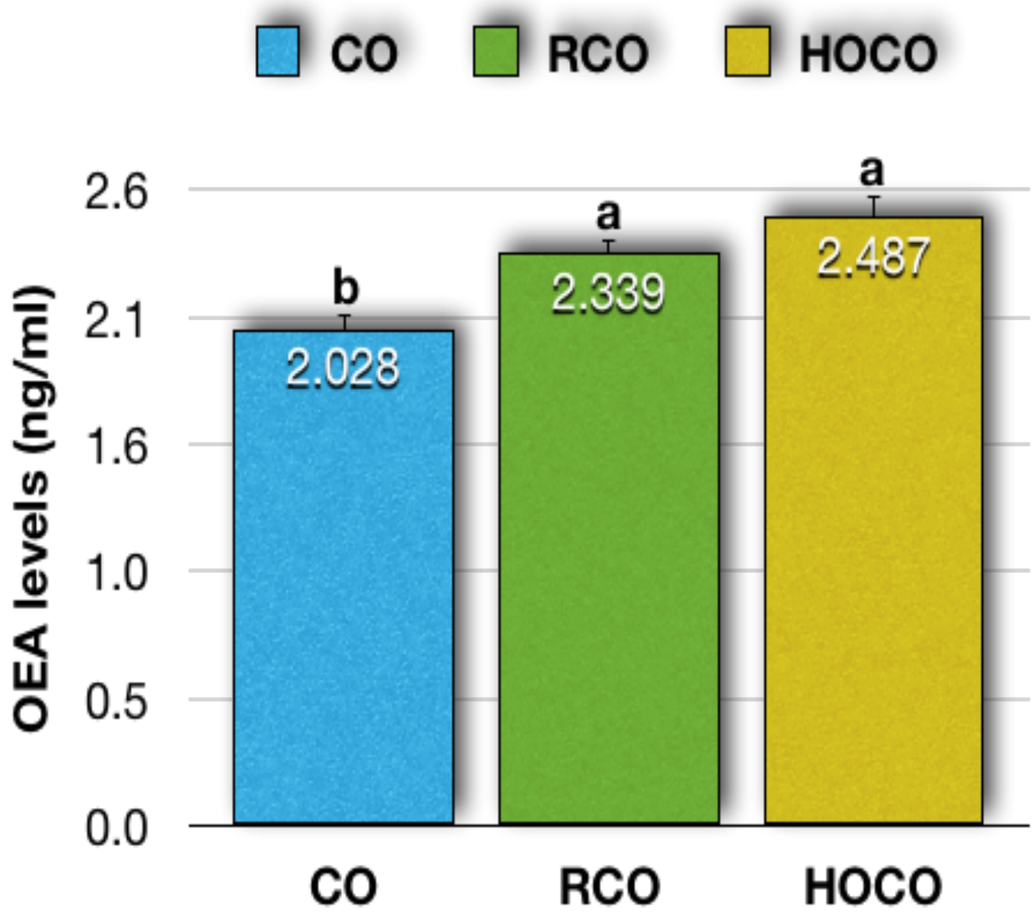


Figure 5.5B Plasma OEA levels (ng/ml) at the end of dietary interventions.



Note: *The values are % abundance of C18:1n9 to total fatty acids. The results are expressed as mean ± SEM (n = 115). The values with different superscript letters are significantly different from each other ($P < 0.05$). OEA, oleoylethanolamide; CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil.

observed across any of the dietary treatments.

5.4.4 Effect of diet treatment on plasma fatty acid ethanolamide levels

Plasma levels of seven FAEs measured after each dietary treatment are shown in Table 5.6. Also, OEA levels at the end of dietary intervention are shown in Figure 5.5B. The most predominant FAE observed was PEA, followed by OEA. Feeding the experimental diets did not alter the fasting levels of PEA, AEA, EPEA, and DHEA. In contrast, for the canola-based, RC and HOCO diets enriched with C18:1n9 levels, significant increases in OEA levels were observed when compared with the CO diet ($P < 0.0001$). For fasting plasma LEA levels, HOCO treatment resulted in the lowest ($P = 0.0013$) concentrations compared to the other dietary groups. Moreover, plasma ALEA concentrations showed similar changes to that of the precursor C18:3n3 levels present in the human plasma.

5.4.5 Correlations between plasma fatty acid and fatty acid ethanolamide concentrations

Pearson's correlation coefficients between plasma FAs and their corresponding FAEs are presented in Table 5.7. Among all FAEs, fasting human plasma PEA, LEA, and EPEA showed no significant association with their root FAs. However, positive significant ($P < 0.0001$) correlations were observed between endpoint plasma FA, C18:1n9 and the corresponding OEA levels. Similarly, in fasting state, C18:3n3 FA showed positive associations ($P < 0.0192$) with ALEA levels. Furthermore, a negative association ($P < 0.0047$) was observed between AEA and C20:4n6. Moreover, a positive ($P < 0.0048$)

Table 5.6 Plasma fatty acid ethanolamides[‡] of each dietary phase (ng/ml).

FAE	CO		RCO		HOCO		*P*
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	
PEA	3.24	0.38	2.92	0.21	2.70	0.17	0.1932
OEA	2.03[b]	0.06	2.34[a]	0.08	2.49[a]	0.09	<0.0001
LEA	0.70[a]	0.02	0.67[ab]	0.02	0.64[b]	0.01	0.0013
ALEA	0.21[a]	0.01	0.21[a]	0.00	0.19[b]	0.00	0.0002
AEA	1.12	0.07	1.04	0.06	1.00	0.06	0.1430
EPEA	0.48	0.05	0.41	0.04	0.35	0.03	0.0685
DHEA	1.12	0.13	1.07	0.13	0.92	0.08	0.5407

Note: [‡]The values are endpoint plasma fatty acid ethanolamides. The results are expressed as mean ± SEM (n = 115). The values with different superscript letters in the same row are statistically different from each other. Statistical significance assessed at ($P < 0.05$) by ANOVA with Tukey-Kramer adjustment. CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil; FAE, fatty acid ethanolamide; PEA, palmitoylethanolamide; OEA, oleoylethanolamide; LEA, linoleoylethanolamide; ALEA, α-linolenoylethanolamide; AEA, arachidonoylethanolamide; EPEA, eicosapentaenoylethanolamide; DHEA, docosahexaenoylethanolamide.

Table 5.7 Pearson's correlation between plasma fatty acid and fatty acid ethanolamide levels after six-weeks consumption of dietary oils[#†].

Fatty acids	Mean	SEM	FAE	Mean	SEM	$r^{‡}$	*P*
C16:0	25.98	0.20	**PEA**	2.95	0.27	– 0.00	0.9666
C18:1n9	14.28	0.20	**OEA**	2.49	0.08	0.39	<0.0001
C18:2n6	20.69	0.23	**LEA**	0.64	0.02	– 0.10	0.0692
C18:3n3	0.45	0.01	**ALEA**	0.19	0.00	0.13	0.0192
C20:4n6	9.06	0.31	**AEA**	1.00	0.06	– 0.15	0.0047
C20:5n3	0.89	0.04	**EPEA**	0.35	0.04	– 0.02	0.7020
C22:6n3	2.91	0.06	**DHEA**	0.92	0.12	0.18	0.0048

Note: Significant correlation was considered ($P < 0.05$). [‡]Pearson correlation coefficients between means of fatty acids and FAE. *r,* Pearson correlation coefficients; FAE, fatty acid ethanolamide; PEA, palmitoylethanolamide; OEA, oleoylethanolamide; LEA, linoleoylethanolamide; ALEA, α-linolenoylethanolamide; AEA, arachidonoylethanolamide; EPEA, eicosapentaenoylethanolamide; DHEA, docosahexaenoylethanolamide; [#]CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil. [†]Composite of all three dietary interventions.

association was observed between C22:6n3 and DHEA concentration.

5.4.6 Correlations between AEA (ng/ml) and leptin (ng/ml) levels

A positive albeit significant correlation was observed between plasma AEA levels and leptin concentrations ($r = 0.17$; $P < 0.0019$) in human plasma regardless of diet (Table 5.8). Additionally, a positive association was also found between AEA concentrations and leptin levels post HOCO treatment ($r = 0.33$; $P < 0.0003$).

5.4.7 Interaction testing between FAE levels and genetic variants

Interaction testing was performed with 3 out of the 7 FAEs tested; namely OEA, LEA, and AEA (Tables 5.9–5.11). The reason for investigating the interactions for the three mentioned FAEs was that OEA is considered to be the most potent appetite suppressing lipid amide among the family of FAEs (5), followed by LEA. In contrast, AEA along with 2-arachidonoylglycerol (2-AG) is considered to be appetite inducing lipid amide among the family of FAEs (29). When diet × gene interactions were tested, elevated concentrations of plasma OEA were observed in participants possessing the *GPR40* rs1573611-T ($n = 48$, $P < 0.0037$) allele after consumption of HOCO ($P < 0.0001$) and RCO ($P < 0.0121$), when compared to CO. Conversely, the test for the diet × gene interactions resulted in higher concentrations of fasting plasma AEA in participants possessing *LEPR* rs1137101-AA ($n = 25$, $P < 0.0058$) genotype after consumption of CO ($P < 0.0031$) and RCO ($P < 0.05$), in comparison to HOCO. Additionally, with *CNR1* rs1049353, a statistical trend ($P = 0.0587$) was observed in diet × gene interactions

Table 5.8 Pearson's correlation between AEA (ng/ml) and leptin (ng/ml) levels after six-weeks consumption of dietary oils.

Diet	*r*	*P*
CO	0.05	0.6135
RCO	0.15	0.1015
HOCO	0.33	0.0003
Composite[†]	0.17	0.0019

Note: Significant correlation was considered ($P < 0.05$). ($n = 115$). r, Pearson correlation coefficients; AEA, arachidonoylethanolamide; CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil. [†]Composite of all three dietary interventions.

Table 5.9 Effect of genetic polymorphisms on plasma oleoylethanolamide levels (ng/ml) after six-weeks consumption of dietary oils.

Gene	SNP	Genotype	Gene effect[†]	Diet × Gene effect[§]			Gene	Diet	Diet × Gene
				CO	RCO	HOCO	P_{gene}	P_{diet}	$P_{interaction}$
CD36	rs1761667						0.7968	<0.0001	0.2607
		A/- (n = 72)	2.19 ± 0.07	1.92 ± 0.09	2.22 ± 0.09	2.41 ± 0.09			
		G/G (n = 25)	2.15 ± 0.13	1.89 ± 0.16	2.35 ± 0.16	2.21 ± 0.16			
GPR40	rs1573611						0.0258	<0.0001	0.0037
		C/C (n = 46)[‡]	2.07 ± 0.27[b]	1.91 ± 0.28	2.16 ± 0.28	2.03 ± 0.28			
		T/- (n = 48)[‡]	2.39 ± 0.27[a]	1.96 ± 0.10[b]	2.41 ± 0.14[a]	2.70 ± 0.16[a]			
LEPR	rs1137101						0.9053	<0.0001	0.7836
		A/A (n = 25)	2.20 ± 0.12	1.88 ± 0.16	2.27 ± 0.16	2.43 ± 0.16			
		G/- (n = 72)	2.18 ± 0.07	1.93 ± 0.09	2.26 ± 0.09	2.34 ± 0.09			
NAPE-PLD	rs12540583						0.0328	<0.0001	0.8026
		A/A (n = 75)	2.24 ± 0.08[a]	1.98 ± 0.10	2.32 ± 0.10	2.42 ± 0.10			
		C/- (n = 24)	1.99 ± 0.13[b]	1.76 ± 0.16	2.04 ± 0.16	2.17 ± 0.16			
FAAH	rs324420						<0.0001	<0.0001	0.2116
		A/- (n = 41)	2.49 ± 0.08[a]	2.15 ± 0.11	2.57 ± 0.11	2.76 ± 0.11			
		C/C (n = 58)	1.97 ± 0.07[b]	1.79 ± 0.09	2.04 ± 0.09	2.10 ± 0.09			
CNR1	rs1049353						0.5105	<0.0001	0.2477
		C/C (n = 59)	2.24 ± 0.08	1.94 ± 0.10	2.29 ± 0.10	2.47 ± 0.10			
		T/- (n = 38)	2.10 ± 0.10	1.89 ± 0.12	2.21 ± 0.12	2.21 ± 0.12			

Gene	SNP	Genotype	Gene effect[†]	Diet × Gene effect[§]			Gene	Diet	Diet × Gene
				CO	RCO	HOCO	P_{gene}	P_{diet}	$P_{interaction}$
MC4R	rs17782313						0.6459	<0.0001	0.4255
		C/- (n = 42)	2.20 ± 0.09	1.96 ± 0.11	2.23 ± 0.11	2.40 ± 0.11			
		T/T (n = 55)	2.25 ± 0.08	1.89 ± 0.11	2.38 ± 0.11	2.48 ± 0.11			
UCP1	rs1800592						0.2408	<0.0001	0.5403
		C/- (n = 54)	2.10 ± 0.09	1.81 ± 0.11	2.20 ± 0.11	2.30 ± 0.11			
		T/T (n = 43)	2.26 ± 0.09	2.03 ± 0.11	2.32 ± 0.11	2.43 ± 0.11			
DRD2	rs1800497						0.1927	<0.0001	0.5321
		A/- (n = 42)	2.09 ± 0.09	1.85 ± 0.12	2.22 ± 0.12	2.22 ± 0.12			
		G/G (n = 55)	2.25 ± 0.08	1.98 ± 0.10	2.29 ± 0.10	2.48 ± 0.10			

Note: The results are expressed as mean ± SEM (n = 97). P values are from SAS MIXED model. [†]Values with different superscript letters in the same column are significantly different from each other (P < 0.05). [§]Values with different superscript letters in the same row are significantly different from each other (P < 0.05). [‡]Mixed-model simple effects of treatment sliced by genotype by using SAS SLICE function when diet and diet by gene were statistically significant, CC (P = 0.1293) and T/- (P < 0.0001). SNP, single nucleotide polymorphism; CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil; *CD36*, cluster of differentiation 36; *GPR40*, G protein-coupled receptors 40; *LEPR*, Leptin receptors; *NAPE-PLD*, *N*-acyl phosphatidylethanolamine phospholipase D; *FAAH*, fatty acid amide hydrolase; *CNR1*, cannabinoid receptors; *MC4R*, melanocortin-4 receptor; *UCP-1*, uncoupling protein-1; dopamine receptor D2.

Table 5.10 Effect of genetic polymorphisms on plasma linoleoylethanolamide levels (ng/ml) after six-weeks consumption of dietary oils.

Fatty acid	SNP	Genotype	Gene effect[†]	Diet × Gene effect[§]			Gene	Diet	Diet × Gene
				CO	RCO	HOCO	P_{gene}	P_{diet}	$P_{interaction}$
CD36	rs1761667						0.4095	0.0097	0.2363
		A/- (n = 72)	0.65 ± 0.02	0.68 ± 0.03	0.64 ± 0.03	0.62 ± 0.03			
		G/G (n = 25)	0.65 ± 0.01	0.67 ± 0.02	0.65 ± 0.03	0.63 ± 0.02			
GPR40	rs1573611						0.3684	0.0431	0.3157
		C/C (n = 46)	0.63 ± 0.02	0.66 ± 0.02	0.62 ± 0.02	0.62 ± 0.02			
		T/- (n = 48)	0.65 ± 0.02	0.67 ± 0.02	0.67 ± 0.02	0.63 ± 0.02			
LEPR	rs1137101						0.8890	0.0319	0.6062
		A/A (n = 25)	0.65 ± 0.02	0.67 ± 0.03	0.67 ± 0.03	0.62 ± 0.03			
		G/- (n = 72)	0.65 ± 0.01	0.67 ± 0.02	0.65 ± 0.02	0.63 ± 0.02			
NAPE-PLD	rs12540583						0.1174	0.0417	0.8848
		A/A (n = 75)	0.66 ± 0.02	0.68 ± 0.02	0.66 ± 0.02	0.64 ± 0.02			
		C/- (n = 24)	0.62 ± 0.03	0.64 ± 0.03	0.63 ± 0.03	0.59 ± 0.03			
FAAH	rs324420						<0.0001	0.0353	0.7071
		A/- (n = 41)	0.70 ± 0.02[a]	0.72 ± 0.02	0.70 ± 0.02	0.67 ± 0.02			
		C/C (n = 58)	0.62 ± 0.01[b]	0.64 ± 0.02	0.62 ± 0.02	0.59 ± 0.02			
CNR1	rs1049353						0.7535	0.0569	0.8834
		C/C (n = 59)	0.66 ± 0.02	0.68 ± 0.02	0.66 ± 0.02	0.63 ± 0.02			
		T/- (n = 38)	0.64 ± 0.02	0.66 ± 0.02	0.65 ± 0.02	0.62 ± 0.02			

Fatty acid	SNP	Genotype	Gene effect[†]	Diet × Gene effect[§]			Gene	Diet	Diet × Gene
				CO	RCO	HOCO	P_{gene}	P_{diet}	$P_{interaction}$
MC4R	rs17782313						0.0932	0.0212	0.4553
		C/- (n = 42)	0.67 ± 0.02	0.69 ± 0.02	0.69 ± 0.02	0.64 ± 0.02			
		T/T (n = 55)	0.63 ± 0.02	0.66 ± 0.02	0.62 ± 0.02	0.61 ± 0.02			
UCP1	rs1800592						0.3138	0.0296	0.1305
		C/- (n = 54)	0.64 ± 0.02	0.68 ± 0.02	0.63 ± 0.02	0.60 ± 0.02			
		T/T (n = 43)	0.66 ± 0.02	0.66 ± 0.02	0.67 ± 0.02	0.65 ± 0.02			
DRD2	rs1800497						0.4693	0.0219	0.5616
		A/- (n = 42)	0.64 ± 0.02	0.66 ± 0.02	0.65 ± 0.02	0.61 ± 0.02			
		G/G (n = 55)	0.66 ± 0.02	0.68 ± 0.02	0.65 ± 0.02	0.64 ± 0.02			

Note: The results are expressed as mean ± SEM (n = 97). P values are from SAS MIXED model. [†]Values with different superscript letters in the same column are significantly different from each other (P < 0.05). [§]Values with different superscript letters in the same row are significantly different from each other (P < 0.05). SNP, single nucleotide polymorphism; CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil; *CD36*, cluster of differentiation 36; *GPR40*, G protein-coupled receptors 40; *LEPR*, Leptin receptors; *NAPE-PLD*, *N*-acyl phosphatidylethanolamine phospholipase D; *FAAH*, fatty acid amide hydrolase; *CNR1*, cannabinoid receptors; *MC4R*, melanocortin-4 receptor; *UCP-1*, uncoupling protein-1; dopamine receptor D2.

Table 5.11 Effect of genetic polymorphisms on plasma arachidonoylethanolamide levels (ng/ml) after six-weeks consumption of dietary oils.

Fatty acid	SNP	Genotype	Gene effect[†]	Diet × Gene effect[§]			Gene	Diet	Diet × Gene
				CO	RCO	HOCO	P_{gene}	P_{diet}	$P_{interaction}$
CD36	rs1761667						0.2919	0.0890	0.4654
		A/- (n = 72)	1.04 ± 0.05	1.09 ± 0.07	1.04 ± 0.07	0.97 ± 0.07			
		G/G (n = 25)	1.15 ± 0.08	1.30 ± 0.12	1.11 ± 0.12	1.06 ± 0.13			
GPR40	rs1573611						0.0097	0.2623	0.7334
		C/C (n = 46)	0.94 ± 0.06[b]	0.98 ± 0.09	0.94 ± 0.09	0.90 ± 0.09			
		T/- (n = 48)	1.14 ± 0.05[a]	1.25 ± 0.08	1.13 ± 0.09	1.06 ± 0.09			
LEPR	rs1137101						0.5492	0.0023	0.0058
		A/A (n = 25)[‡]	1.10 ± 0.08	1.35 ± 0.12[a]	1.11 ± 0.12[a]	0.86 ± 0.12[b]			
		G/- (n = 72)[‡]	1.05 ± 0.05	1.07 ± 0.07	1.04 ± 0.07	1.04 ± 0.07			
NAPE-PLD	rs12540583						0.2617	0.4325	0.8316
		A/A (n = 75)	1.09 ± 0.05	1.18 ± 0.07	1.06 ± 0.07	1.02 ± 0.07			
		C/- (n = 24)	0.98 ± 0.08	1.01 ± 0.13	1.00 ± 0.13	0.92 ± 0.13			
FAAH	rs324420						0.0333	0.1919	0.5643
		A/- (n = 41)	1.16 ± 0.06[a]	1.28 ± 0.10	1.10 ± 0.10	1.10 ± 0.10			
		C/C (n = 58)	1.00 ± 0.05[b]	1.04 ± 0.08	1.02 ± 0.08	0.93 ± 0.08			
CNR1	rs1049353						0.4587	0.0349	0.0587
		C/C (n = 59)	1.08 ± 0.06	1.09 ± 0.09	1.07 ± 0.09	1.07 ± 0.09			
		T/- (n = 38)	1.05 ± 0.07	1.23 ± 0.10	1.04 ± 0.10	0.88 ± 0.10			

Fatty acid	SNP	Genotype	Gene effect[†]	Diet × Gene effect[§]			Gene	Diet	Diet × Gene
				CO	RCO	HOCO	P_{gene}	P_{diet}	$P_{interaction}$
MC4R	rs17782313						0.6299	0.2499	0.7616
		C/- (n = 42)	1.07 ± 0.06	1.11 ± 0.09	1.10 ± 0.09	1.01 ± 0.09			
		T/T (n = 55)	1.11 ± 0.06	1.22 ± 0.09	1.09 ± 0.09	1.03 ± 0.09			
UCP1	rs1800592						0.0316	0.1102	0.4604
		C/- (n = 54)	0.97 ± 0.06[b]	1.05 ± 0.09	0.95 ± 0.09	0.92 ± 0.09			
		T/T (n = 43)	1.15 ± 0.06[a]	1.23 ± 0.09	1.17 ± 0.09	1.06 ± 0.09			
DRD2	rs1800497						0.7646	0.1233	0.3833
		A/- (n = 42)	1.05 ± 0.06	1.07 ± 0.10	0.99 ± 0.10	1.08 ± 0.10			
		G/G (n = 55)	1.06 ± 0.06	1.18 ± 0.09	1.10 ± 0.09	0.91 ± 0.09			

Note: The results are expressed as mean ± SEM (n = 97). P values are from SAS MIXED model. [†]Values with different superscript letters in the same column are significantly different from each other (P < 0.05). [§]Values with different superscript letters in the same row are significantly different from each other (P < 0.05). [‡]Mixed-model simple effects of treatment sliced by genotype by using SAS SLICE function when diet and diet by gene were statistically significant, AA (P = 0.0006) and G/- (P = 0.9302). SNP, single nucleotide polymorphism; CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil; *CD36*, cluster of differentiation 36; *GPR40*, G protein-coupled receptors 40; *LEPR*, Leptin receptors; *NAPE-PLD*, *N*-acyl phosphatidylethanolamine phospholipase D; *FAAH*, fatty acid amide hydrolase; *CNR1*, cannabinoid receptors; *MC4R*, melanocortin-4 receptor; *UCP-1*, uncoupling protein-1; dopamine receptor D2.

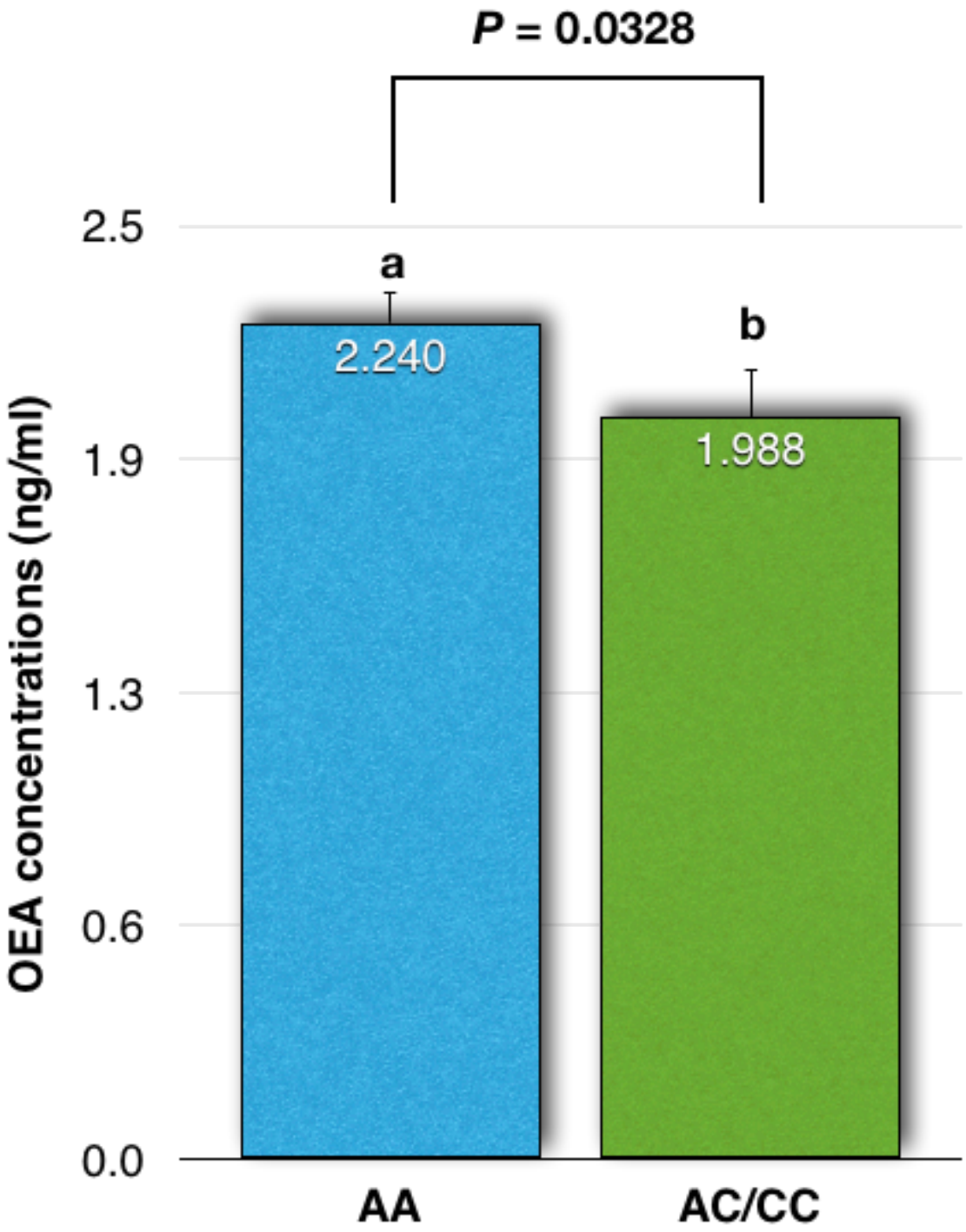
with AEA. Apart from the above 3 genes, the remaining 6 genes investigated failed to show any diet × gene interaction. However, the only gene effect was observed in OEA concentrations by *GPR40*, *NAPE-PLD* ($P = 0.0328$) (Figure 5.6), and *FAAH* ($P = 0.0001$) (Figure 5.7) genes, respectively. In addition to OEA, *FAAH* genotype expressed the effect ($P < 0.0001$) on LEA concentrations. On the contrary, rs1800592 in the *UCP1* gene was observed to influence AEA concentrations only with TT allele carriers showing increased ($P < 0.0316$) AEA concentrations when compared to C/- allele carriers. Furthermore, the effect of *LEPR* rs1137101 was investigated on leptin concentrations (Figure 5.8). Here, individuals possessing the *LEPR* rs1137101-G allele was observed to possess higher ($P = 0.0334$) leptin levels when compared to AA-allele carriers.

5.4.8 Correlations with body composition

5.4.8.1 Correlations between plasma fatty acid and body composition

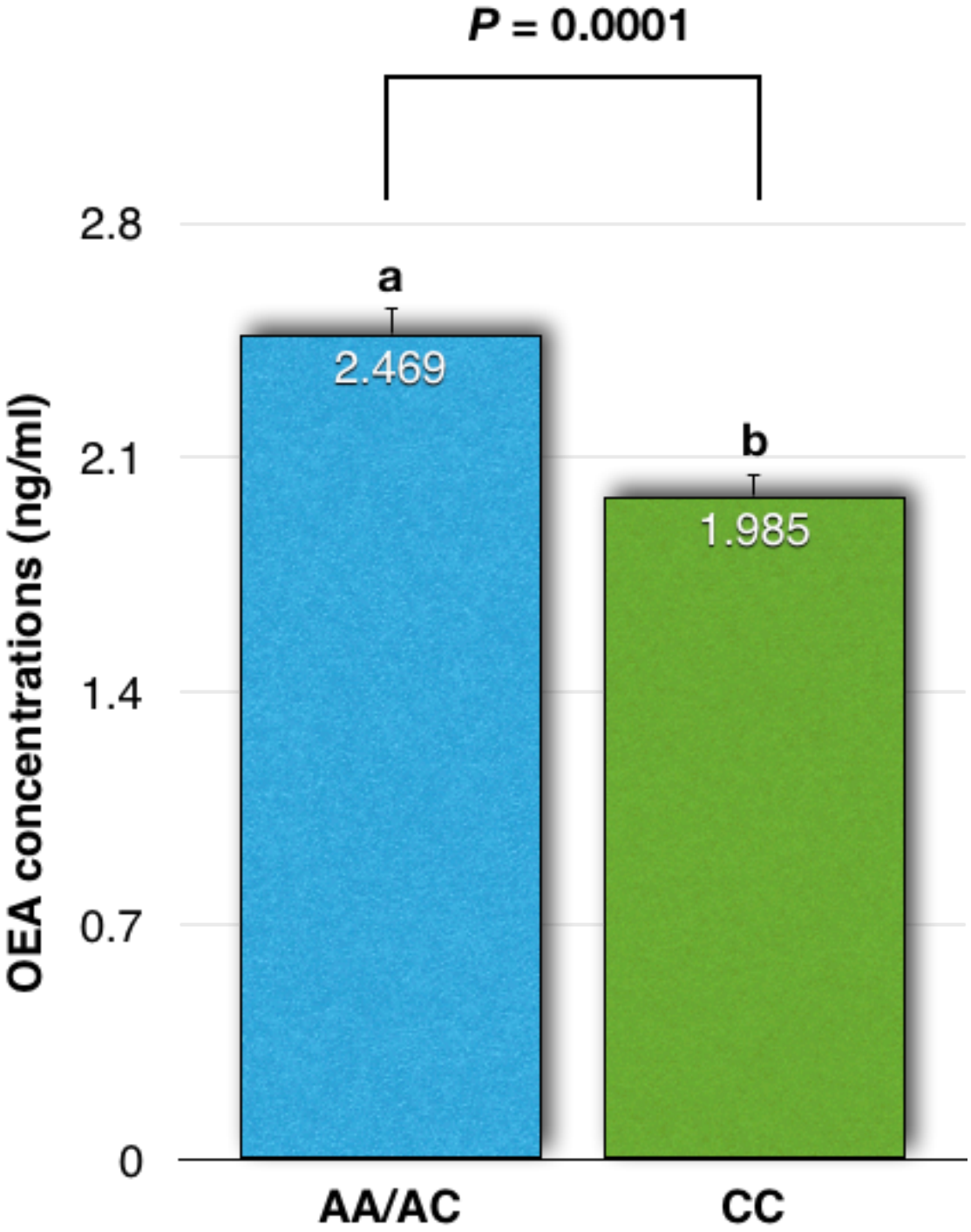
When correlations were investigated between circulating FA levels and SAR, significant positive associations were observed with C20:4n6 and C22:6n3 in the overall group (Table 5.12). Furthermore, correlational analyses for all subjects across all dietary treatments failed to reveal any statistically significant relationship between plasma C18:2n6, C20:4n6, and C20:5n3 levels and SVR. On the contrary, for the composite of all three dietary interventions, plasma C18:1n9 and C18:3n3 levels were negatively correlated with SVR ($r = – 0.13$; $P < 0.0195$) and ($r = – 0.14$; $P < 0.0132$), respectively. Additionally, plasma C16:0 showed a positive association with SVR ($r = 0.13$; $P < 0.0202$). Also, C22:6n3 expressed a weak positive but significant relationship

Figure 5.6 Effect of *NAPE-PLD* rs12540583 polymorphism on OEA concentrations (ng/ml) after six-weeks consumption of dietary oils[#†].



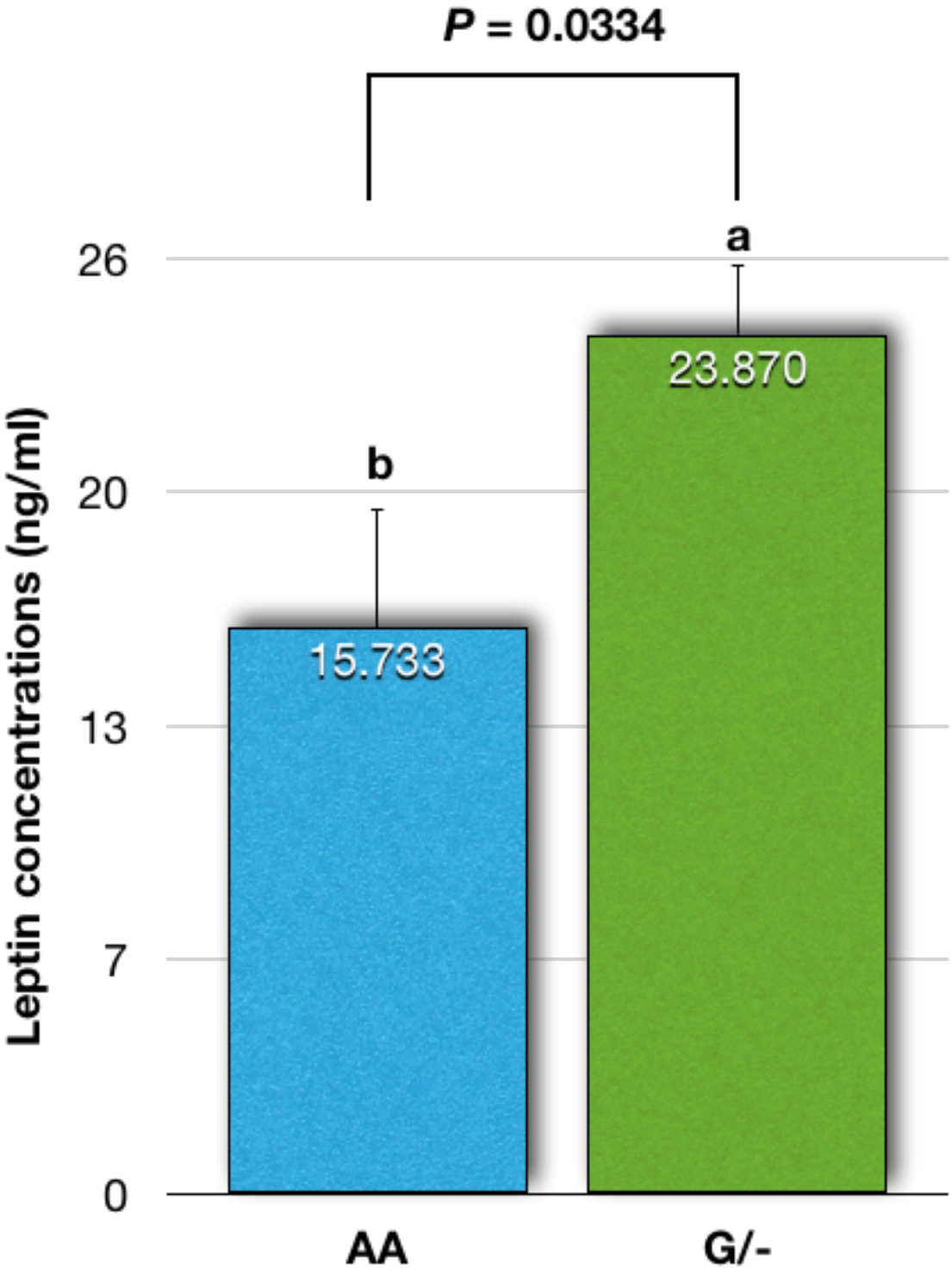
Note: The results are expressed as mean ± SEM (n = 101). The values with different superscript letters are significantly different from each other ($P < 0.05$). *NAPE-PLD*, *N*-acyl ethanolamide phospholipase D; OEA, oleoylethanolamide; [#]CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil. [†]Composite of all three dietary interventions.

Figure 5.7 Effect of *FAAH* rs324420 polymorphism on OEA concentrations (ng/ml) after six-weeks consumption of dietary oils[#†].



Note: The results are expressed as mean ± SEM (n = 101). The values with different superscript letters are significantly different from each other ($P < 0.05$). *FAAH*, Fatty acid amide hydrolase; OEA, oleoylethanolamide; [#]CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil.
[†]Composite of all three dietary interventions.

Figure 5.8: Effect of leptin receptor, *LEPR* rs1137101 genotype on plasma leptin levels after six-weeks consumption of dietary oils[#†].



Note: The results are expressed as mean ± SEM (n = 97). The values with different superscript letters are significantly different from each other ($P < 0.05$). *LEPR*, leptin receptors; [#]CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil. [†]Composite of all three dietary interventions.

Table 5.12 Pearson's correlation between plasma fatty acid[‡] (g/100g)* and body composition variables by dietary treatments after six-weeks consumption of dietary oils.

Fatty acid	Parameter	Dietary treatments							
		CO		RCO		HOCO		Composite[†]	
		Body Composition Variables							
		SAR	SVR	SAR	SVR	SAR	SVR	SAR	SVR
C16:0	***r***	– 0.04	0.19	0.01	0.06	– 0.03	0.06	– 0.02	0.13
	P	0.7116	0.0430	0.9245	0.5039	0.7594	0.5520	0.7572	0.0202
C18:1n9	***r***	– 0.08	– 0.14	– 0.15	– 0.10	– 0.12	– 0.21	– 0.10	– 0.13
	P	0.4222	0.1354	0.1237	0.2986	0.1973	0.0305	0.0642	0.0195
C18:2n6	***r***	0.07	0.05	0.03	0.05	0.07	0.16	0.06	0.07
	P	0.4836	0.6303	0.7196	0.5935	0.4300	0.0938	0.2794	0.1957
C18:3n3	***r***	– 0.26	– 0.25	– 0.10	– 0.13	– 0.18	– 0.18	– 0.16	– 0.14
	P	0.0057	0.0071	0.2809	0.1575	0.0574	0.0570	0.0024	0.0132
C20:4n6	***r***	0.13	0.10	0.16	0.17	0.11	0.10	0.13	0.09
	P	0.1688	0.2822	0.0885	0.0709	0.2261	0.3005	0.0133	0.1147
C20:5n3	***r***	– 0.19	– 0.08	– 0.28	– 0.24	– 0.25	– 0.20	– 0.23	– 0.10
	P	0.0437	0.3833	0.0028	0.0107	0.0077	0.0359	<0.0001	0.0811
C22:6n3	***r***	0.16	0.17	0.13	0.16	0.18	0.22	0.15	0.13
	P	0.1011	0.0755	0.1625	0.0948	0.0575	0.0206	0.0046	0.0137

Note: [‡]The values are endpoint plasma fatty acids (n = 111–113). *The values are % abundance of each fatty acid to total fatty acids. Statistical significance assessed at ($P < 0.05$). r, Pearson correlation coefficients. CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil; SAR, subcutaneous to android fat mass ratio; SVR, subcutaneous to visceral fat mass ratio. [†]Composite of all three dietary interventions.

with both SAR ($r = 0.15$; $P < 0.0046$) and SVR ($r = 0.13$; $P < 0.0137$) in the group overall.

Moreover, in response to HOCO consumption, similar inverse associations were detected between plasma C18:1n9 and SVR ($r = -0.21$; $P < 0.0305$) as well as between plasma C20:5n3 levels and SVR ($r = -0.20$; $P < 0.0359$). Furthermore, HOCO ingestion also led to a weak negative association between C18:3n3 levels and SVR ($r = -0.18$; $P < 0.0570$) with a statistical trend; whereas C22:6n3 levels showed a weak positive relationship with SVR ($r = 0.22$; $P < 0.0206$) which is similar to the findings observed with the composite group. In addition, C20:5n3 resulted in a weak negative association with both SAR ($r = -0.28$; $P < 0.0028$) and SVR ($r = -0.24$; $P < 0.0107$) post RCO consumption. In addition, post-CO intake led to a negative correlation with SAR as well as SVR with C18:3n3 levels. Furthermore, CO intake resulted in inverse associations between C20:5n3 and SAR ($r = -0.19$; $P < 0.0437$); whereas no significant associations were observed with SVR. Moreover, CO treatment showed positive associations with C16:0 and SVR.

5.4.8.2 Correlations between plasma fatty acid ethanolamide and body composition

Table 5.13 outlines correlations observed between FAE concentrations and body composition measurements for the composite as well as individual intervention groups. Correlational analysis for the composite group showed an inverse relationship between

Table 5.13 Pearson's correlation between plasma fatty acid ethanolamides (ng/ml)[‡] and body composition variables by dietary treatments after six-weeks consumption of dietary oils.

FAE	Parameter	Dietary treatments							
		CO		RCO		HOCO		Composite[†]	
		Body Composition Variables							
		SAR	SVR	SAR	SVR	SAR	SVR	SAR	SVR
PEA	***r***	– 0.01	– 0.04	0.03	– 0.02	– 0.04	– 0.03	– 0.01	– 0.03
	P	0.8956	0.6705	0.7864	0.8381	0.7131	0.7889	0.9084	0.5973
OEA	***r***	– 0.21	– 0.23	– 0.12	– 0.20	– 0.13	– 0.18	– 0.14	– 0.16
	P	0.0236	0.0152	0.2151	0.0351	0.1574	0.0362	0.0098	0.0034
LEA	***r***	– 0.10	– 0.09	– 0.04	– 0.19	– 0.06	– 0.16	– 0.07	– 0.09
	P	0.2729	0.3262	0.6521	0.0499	0.5376	0.0877	0.2150	0.1154
ALEA	***r***	0.13	0.03	– 0.11	– 0.13	0.11	0.13	0.06	0.03
	P	0.1821	0.7497	0.2554	0.1866	0.2571	0.1810	0.2764	0.5638
AEA	***r***	– 0.08	– 0.11	– 0.15	– 0.20	– 0.04	– 0.09	– 0.09	– 0.10
	P	0.4150	0.2538	0.1119	0.0320	0.6893	0.3717	0.1101	0.0784
EPEA	***r***	0.07	0.01	0.14	0.08	0.09	0.16	0.10	0.03
	P	0.5171	0.9346	0.2077	0.4731	0.4559	0.1730	0.1497	0.5900
DHEA	***r***	0.10	– 0.00	– 0.17	– 0.18	– 0.11	– 0.03	– 0.04	– 0.01
	P	0.3516	0.9951	0.1261	0.1065	0.3239	0.7685	0.5587	0.8723

Note: [‡]The values are endpoint plasma fatty acid ethanolamides (n = 111–113). Statistical significance assessed at ($P < 0.05$). r, Pearson correlation coefficients. CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil; SAR, subcutaneous to android fat mass ratio; SVR, subcutaneous to visceral fat mass ratio; FAE, fatty acid ethanolamide; PEA, palmitoylethanolamide; OEA, oleoylethanolamide; LEA, linoleoylethanolamide; ALEA, α-linolenoylethanolamide; AEA, arachidonoylethanolamide; EPEA, eicosapentaenoylethanolamide; DHEA, docosahexaenoylethanolamide.
[†]Composite of all three dietary interventions.

OEA concentrations and both ratios of SAR and SVR, ($r = – 0.14$; $P < 0.0098$) and ($r = – 0.16$; $P < 0.0034$), respectively. Similarly, the inverse association between plasma OEA levels and SVR observed in the composite group were also replicated in the HOCO ($r = – 0.18$; $P < 0.0362$), RCO ($r = – 0.20$; $P < 0.0351$), and CO ($r = – 0.23$; $P < 0.0152$) groups when analyzed separately. Additionally, a negative association was also detected between OEA and SAR in the CO group ($r = – 0.21$; $P < 0.0236$). Furthermore, fasting plasma LEA ($r = – 0.19$; $P < 0.0499$) and AEA ($r = – 0.20$; $P < 0.0320$), levels were inversely associated with SVR in response to RCO consumption, but this association was not observed in the composite group.

5.4.8.3 Correlations between plasma fatty acid ethanolamide and body composition by dietary treatments in GPR40 rs1573611

When correlational analyses were performed based on genotype, plasma OEA expressed a weak negative association with SVR ($r = – 0.19$; $P < 0.0292$) in overall/composite group by rs1573611-C/C ($n = 46$) (Table 5.14). Similarly, rs1573611-C/T ($n = 41$) showed a negative association between OEA and SVR ($r = – 0.20$; $P < 0.0275$). Furthermore, in the same group, weak negative associations were detected in plasma LEA and SAR; and similar negative associations were seen between plasma AEA and SAR. However, no such associations were observed with any of the FAEs and SAR or SVR in rs1573611-T/T ($n = 7$).

5.4.8.4 Correlations between plasma fatty acid ethanolamide and body

Table 5.14 Pearson's correlation between plasma fatty acid ethanolamides (ng/ml)[‡] and body composition variables by dietary treatments in *GPR40* rs1573611 genotypes after six-weeks consumption of dietary oils.

Genotype	FAE	Parameter	Dietary treatments							
			CO		RCO		HOCO		Composite[†]	
			Body Composition Variables							
			SAR	SVR	SAR	SVR	SAR	SVR	SAR	SVR
C/C (n = 46)										
	OEA	***r***	– 0.22	– 0.25	– 0.03	– 0.20	0.10	– 0.11	– 0.05	– 0.19
		P	0.1393	0.1022	0.8482	0.1863	0.4890	0.4612	0.5773	0.0292
	LEA	***r***	0.08	0.11	0.13	– 0.14	– 0.00	– 0.13	0.07	– 0.05
		P	0.5864	0.4854	0.3988	0.3678	0.9741	0.3961	0.4021	0.5864
	AEA	***r***	0.08	0.06	– 0.04	– 0.15	0.20	0.21	0.09	0.00
		P	0.5802	0.7027	0.7952	0.3240	0.1777	0.1641	0.3218	0.9848
C/T (n = 41)										
	OEA	***r***	– 0.13	– 0.34	– 0.25	– 0.27	– 0.03	– 0.12	– 0.13	– 0.20
		P	0.4298	0.0314	0.1214	0.0917	0.8585	0.4563	0.1578	0.0275
	LEA	***r***	– 0.24	– 0.15	– 0.24	– 0.29	– 0.16	– 0.25	– 0.21	– 0.13
		P	0.1382	0.3529	0.1375	0.0697	0.3099	0.1170	0.0210	0.1677
	AEA	***r***	– 0.20	– 0.23	– 0.34	– 0.30	– 0.10	– 0.20	– 0.21	– 0.16
		P	0.2058	0.1556	0.0324	0.0560	0.5514	0.2185	0.0208	0.0714
T/T (n = 7)										
	OEA	***r***	– 0.24	– 0.31	– 0.13	– 0.08	– 0.66	– 0.55	– 0.34	– 0.28
		P	0.6003	0.5014	0.7769	0.8595	0.1042	0.1992	0.1304	0.2160

Genotype	FAE	Parameter	Dietary treatments							
			CO		RCO		HOCO		Composite[†]	
			Body Composition Variables							
			SAR	SVR	SAR	SVR	SAR	SVR	SAR	SVR
	LEA	r	– 0.07	– 0.13	0.15	0.07	0.36	0.28	0.13	0.07
		P	0.8880	0.7745	0.7467	0.8792	0.4212	0.5475	0.5623	0.7778
	AEA	***r***	0.47	0.47	0.28	0.32	0.01	0.14	0.26	0.26
		P	0.2831	0.2918	0.5362	0.4883	0.9916	0.7944	0.2601	0.2618

Note: [‡]The values are endpoint plasma fatty acid ethanolamides. Statistical significance assessed at ($P < 0.05$). r, Pearson correlation coefficients. *GPR40*, G protein-coupled receptors 40; CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil; SAR, subcutaneous to android fat mass ratio; SVR, subcutaneous to visceral fat mass ratio; FAE, fatty acid ethanolamide; OEA, oleoylethanolamide; LEA, linoleoylethanolamide; AEA, arachidonoylethanolamide. [†]Composite of all three dietary interventions.

composition by dietary treatments in LEPR rs1137101

Table 5.15 depicts correlations observed between FAE concentrations and body composition measurements for the composite as well as intervention groups by *LEPR*. Among, all three genotypes, rs1137101-AA, AG, and GG; significant inverse associations were observed only with participants possessing rs1137101-AG allele ($n = 50$) across all diets. In both composite as well as the intervention groups, plasma OEA levels were negatively associated with SVR ($r = -0.22$, $P = 0.0064$), ($r = -0.32$, $P = 0.0249$), ($r = -0.28$, $P = 0.0473$), and ($r = -0.31$, $P = 0.0276$), in composite, HOCO, RCO, and CO, respectively. In comparison, when treatment-specific FAE levels were tested against SAR and SVR parameters, plasma AEA levels exhibited an inverse association with SAR post-RCO ($r = -0.33$, $P = 0.0213$) consumption. Similar negative relationship was also observed between composite group and SAR ($r = -0.17$, $P = 0.0368$). However, the inverse association between plasma LEA levels and SAR observed in the composite group was not replicated when tested within each treatment ($r = -0.23$, $P = 0.0058$).

5.5 Discussion

The current study shows that plasma OEA concentrations reflect the dietary pattern of C18:1n9 intake and are influenced by *GRP40* rs1573611 and *LEPR* rs1137101 polymorphisms. Our data demonstrate that humans possessing *GPR40* rs1573611-T and *LEPR* rs1137101-A/A polymorphisms would benefit more from the ingestion of C18:1n9 enriched dietary oils by regulating appetite, due to enhanced satiation induced by

Table 5.15 Pearson's correlation between plasma fatty acid ethanolamides (ng/ml)[‡] and body composition variables by dietary treatments in *LEPR* rs1137101 genotypes after six-weeks consumption of dietary oils.

Genotype	FAE	Parameter	Dietary treatments							
			CO		RCO		HOCO		Composite[†]	
			Body Composition Variables							
			SAR	SVR	SAR	SVR	SAR	SVR	SAR	SVR
A/A (n = 25)										
	OEA	***r***	– 0.16	– 0.27	0.23	0.05	0.11	– 0.10	0.06	– 0.13
		P	0.4403	0.1953	0.2734	0.8159	0.6108	0.6412	0.6206	0.2491
	LEA	***r***	0.04	– 0.13	0.19	– 0.03	– 0.06	– 0.30	0.06	– 0.13
		P	0.8466	0.5485	0.3751	0.8985	0.7644	0.1438	0.6114	0.2805
	AEA	***r***	0.07	– 0.06	0.01	– 0.16	– 0.23	– 0.28	– 0.03	– 0.10
		P	0.7247	0.7880	0.9537	0.4339	0.2715	0.1745	0.7667	0.3738
A/G (n = 50)										
	OEA	***r***	– 0.33	– 0.31	– 0.35	– 0.28	– 0.17	– 0.32	– 0.27	– 0.22
		P	0.0175	0.0276	0.0145	0.0473	0.2348	0.0249	0.0008	0.0064
	LEA	***r***	– 0.28	– 0.11	– 0.21	– 0.21	– 0.17	– 0.27	– 0.23	– 0.09
		P	0.0492	0.4365	0.1395	0.1500	0.2274	0.0611	0.0058	0.2547
	AEA	***r***	– 0.19	– 0.16	– 0.33	– 0.25	0.01	– 0.12	– 0.17	– 0.13
		P	0.1779	0.2751	0.0213	0.0792	0.9477	0.4139	0.0368	0.1273
G/G (n = 22)										
	OEA	***r***	– 0.09	0.14	– 0.23	– 0.27	– 0.14	0.03	– 0.15	– 0.09
		P	0.6879	0.5340	0.3092	0.2240	0.5322	0.8966	0.2325	0.4999

Genotype	FAE	Parameter	Dietary treatments							
			CO		RCO		HOCO		Composite[†]	
			Body Composition Variables							
			SAR	SVR	SAR	SVR	SAR	SVR	SAR	SVR
	LEA	r	0.04	0.24	– 0.06	– 0.32	– 0.01	0.16	– 0.01	– 0.12
		P	0.8494	0.2905	0.7836	0.1498	0.9704	0.4859	0.9435	0.3619
	AEA	***r***	– 0.06	– 0.00	– 0.19	– 0.21	0.17	0.36	– 0.04	– 0.03
		P	0.7977	0.9866	0.3939	0.3549	0.4610	0.1170	0.7642	0.8457

Note: [‡]The values are endpoint plasma fatty acid ethanolamides. Statistical significance assessed at ($P < 0.05$). r, Pearson correlation coefficients. *LEPR*, leptin receptors; CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil; SAR, subcutaneous to android fat mass ratio; SVR, subcutaneous to visceral fat mass ratio; FAE, fatty acid ethanolamide; OEA, oleoylethanolamide; LEA, linoleoylethanolamide; AEA, arachidonoylethanolamide. [†]Composite of all three dietary interventions.

increased OEA and reduced AEA concentrations, respectively; which in turn influence SVR. Furthermore, the present trial demonstrates that the longer-term consumption of different types of fat influences FAE levels in overweight humans in fasting state. Specifically, consumption of dietary treatments enriched in C18:1n9 from HOCO as well as RCO, C18:2n6 from CO, and C18:3n3 from RCO, resulted in elevated concentrations of plasma OEA, LEA, and ALEA, respectively. The findings are similar to those observed by previous human trials (10, 30). Moreover, plasma C18:1n9 and OEA levels showed inverse associations with SVR suggesting that OEA initiates lipolysis and helps reduce body fat.

Furthermore, two key diet × gene interactions were observed with *GPR40* rs1573611 and *LEPR* rs1137101. The *GPR40* rs1573611-T ($n = 48$) interactions with OEA levels and inverse association with SVR suggest that *GPR40* rs1573611 influences body composition (31) and, hence, might play a role in regulation of fat storage (32). The receptor is present in the omental adipose tissue (31, 33), which works in synergy with *GPR119* (31, 34), improving insulin sensitivity and thereby reducing the excess fat mass. Therefore, the diet × gene interactions observed between HOCO ingestion and OEA levels in the *GPR40* gene, suggest that the C18:1n9 enriched diet results in increased OEA concentrations that may increase energy expenditure and help control appetite. Such actions can be thought to induce fat loss that may check weight gain.

In addition, endocannabinoids are a family of polyunsaturated fatty acid derivatives that function as lipid signaling molecules by acting as endogenous ligands at

two known cannabinoid receptors; namely, CB1 and CB2 (35). Activation of the endocannabinoid system, especially the CB1 receptors, induces hyperphagia and weight gain (36, 37). On the contrary, LEPR is known to regulate appetite (38, 39). Although both metabolites, endocannabinoids and leptin, are released from the hypothalamus, leptin hormone and the endocannabinoid system act differently in opposing directions on whole-body energy metabolism (40). The n6 FA C20:4n6 in phospholipids serves as the precursor of the two best-characterized endocannabinoids; namely, 2-AG and AEA (29). Furthermore, AEA can be obtained by conjugation of ethanolamine and C20:4n6 from the reverse reaction of FAAH (41) suggesting the associations between AEA and FAAH exist.

Moreover, the central nervous system regulating all networks of energy homeostasis is made up of a convoluted matrix, generating interest and curiosity to investigate in depth associations among AEA, leptin, and FAAH. Therefore, in the present study we looked at these associations; and we found diet × gene interactions between *LEPR* rs1137101-A/A ($n = 25$) and AEA. Furthermore, the *FAAH* gene effect was also detected in plasma AEA concentrations ($P < 0.0333$). Since the FAAH enzyme is a key component of the endocannabinoid system responsible for endocannabinoid degradation, chiefly AEA (42), present data suggest that the associations exist among AEA, LEPR, and FAAH enzyme. The findings from the present trial suggest that overweight participants, carriers of *FAAH* rs324420-A allele express significantly increased levels of FAEs including OEA and AEA. Therefore, our results agree with a recent animal trial conducted by Balsevich *et al.* (40) and previous human trials (35,

43); wherein Sipe and colleagues (35) demonstrated that *FAAH* rs324420-A mutant alleles directly influence plasma levels of anandamide and related *N*-acylethanolamines (NAEs) in humans and therefore, result in higher concentrations of the same. Similarly, Vazquez-Roque and coworkers (43) illustrated that endocannabinoids are metabolized less in the *FAAH* rs324420-A allele carriers, leading to elevated plasma NAE levels. However, we failed to see significant differences in leptin concentrations across *FAAH* genotypes, rs324420-A and rs324420-C/C, respectively as reported by animal (40) and human trials (44). A reason explaining the contrast in findings may be that in the present trial all participants were not morbidly obese (body mass index, BMI > 40 kg/m^2). Therefore, the findings from the present trial suggest that participants carrying the *FAAH* rs324420-A-allele show normal catalytic properties, however, the increased sensitivity to proteolytic degradation (45) renders the FAAH enzyme less effective in these carriers, thereby leading to increased plasma FAE concentrations, due to inefficient metabolism of endocannabinoids (43). By contrast, although participants with the rs324420-C/C genotype demonstrated reduced FAE concentrations, these carriers may have manifest blunted appetite and thereby, increased satiation because of more efficient cannabinoid degradation.

Furthermore, in the present study we failed to see significant diet × gene interactions with most of the genotypes except 2; namely *GPR40* rs1573611 and *LEPR* rs1137101. Only one additional diet × gene interaction trend ($P = 0.0587$) was observed between *CNR1* rs1049353 and AEA concentrations. The reason behind such null interactions might be due to the poor/impaired anorexic signalling induced by OEA

in humans because of lower EC_{50} of OEA concentrations (6, 46–48). Additionally, although we missed the diet × gene interactions, the gene effect of *NAPE-PLD* and *FAAH* was detected on OEA concentrations. However, since we observed the diet × gene interactions with two genes specifically, we also checked the influence of these genotypes on body composition; and inverse associations were detected between plasma OEA and SVR by *GPR40* rs1573611-C/T allele (Figure 5.9A). Similar associations were observed post-CO consumption (Figure 5.9B). In addition, *LEPR* rs1137101-A/A genotype showed negative correlations with changes in SATM in the composite group (Figure 5.10A) and HOCO intervention group (Figure 5.10B). These negative associations might be due to the influence of effective anorexic signalling mechanisms induced by OEA in participants possessing the *LEPR* rs1137101-A/A allele, because carriers of rs1137101-A/A genotype respond well to diet with regulated levels of leptin concentrations (49) and a more effective endocannabinoid system. Henceforth, individuals with this genetic characteristic demonstrate a lower risk of obesity.

Additionally, the benefits associated with the inverse correlation between plasma OEA and SVR might be the result of activated β-adrenergic receptors present in the *UCP-1* gene (50); which were activated by increased OEA concentrations regardless of diet, especially in participants carrying T/T-allele. In constrast, C/- carriers of *UCP-1* gene reportedly demonstrate higher SATM and BMI due to reduced mRNA expression at the functional promoter region (51). The UCP-1 further promotes adipose tissue β-oxidation inducing UCP1-mediated thermogenesis (52–54) that burns FA during uncoupled respiration (45); since UCP-1 is a mitochondrial molecule involved in diet-induced as

Figure 5.9A The association between OEA levels (ng/ml) and subcutaneous to visceral fat mass ratio by *GPR40* rs1573611, genotype C/T after six-weeks consumption of dietary oils[#†].

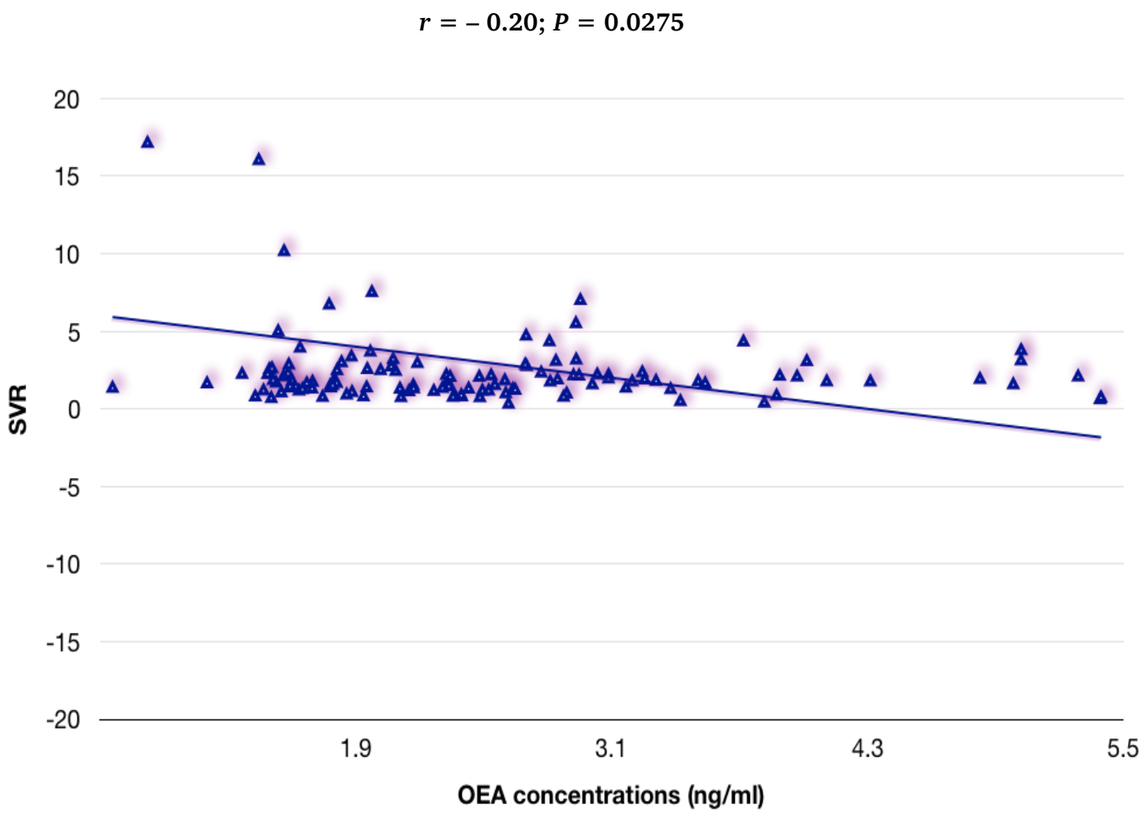
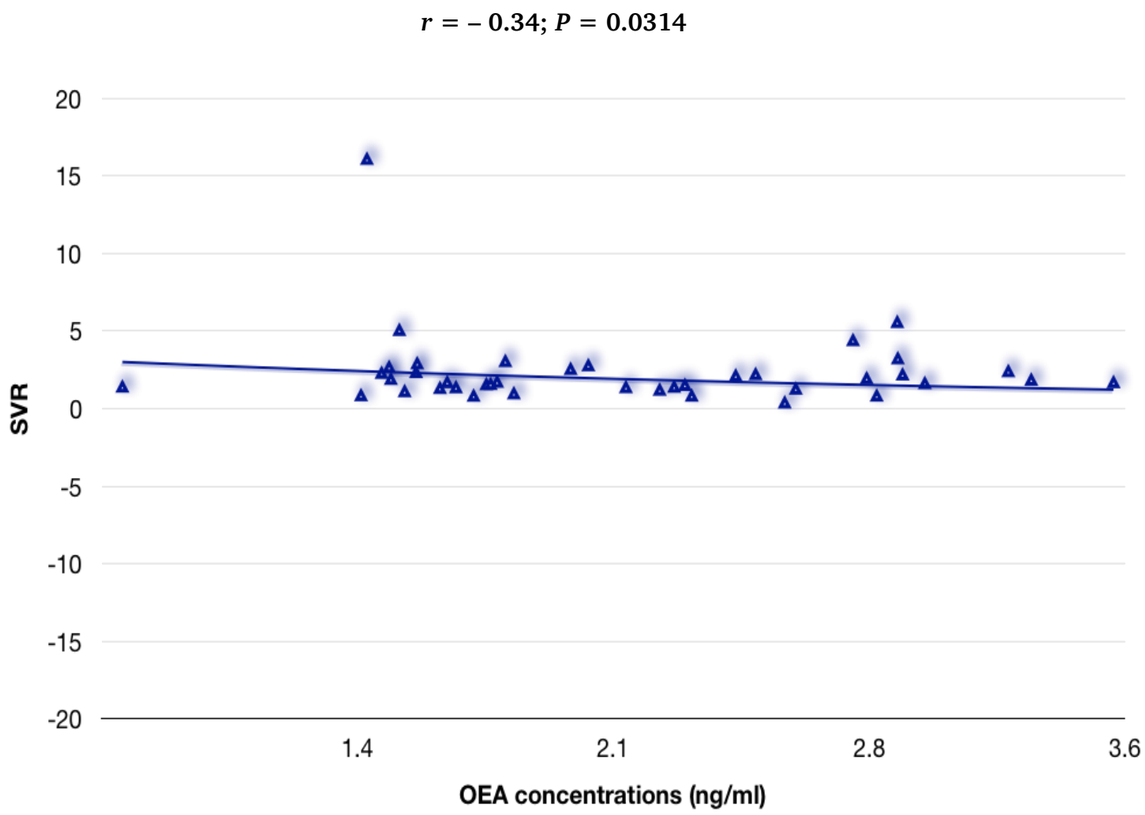


Figure 5.9B The association between OEA levels (ng/ml) and subcutaneous to visceral fat mass ratio by *GPR40* rs1573611, genotype C/T after six weeks consumption of CO.



Note: $n = 41$; r, Pearson correlation coefficient; OEA, oleoylethanolamide; SVR, subcutaneous to visceral fat mass ratio; *GPR40*, G protein-coupled receptors 40; [#]CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil. [†]Composite of all three dietary interventions.

Figure 5.10A The association between OEA levels (ng/ml) and subcutaneous adipose tissue mass changes (g) by *LEPR* rs1137101, genotype A/A after six-weeks consumption of dietary oils[#†].

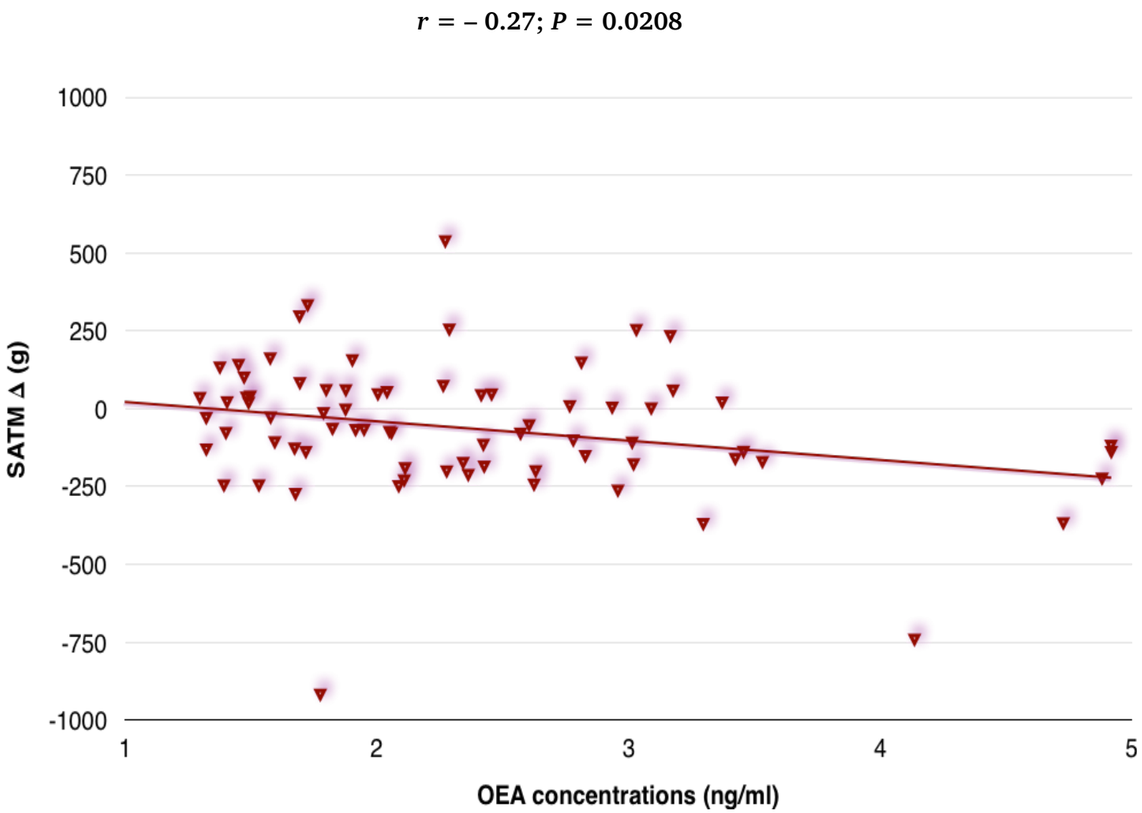
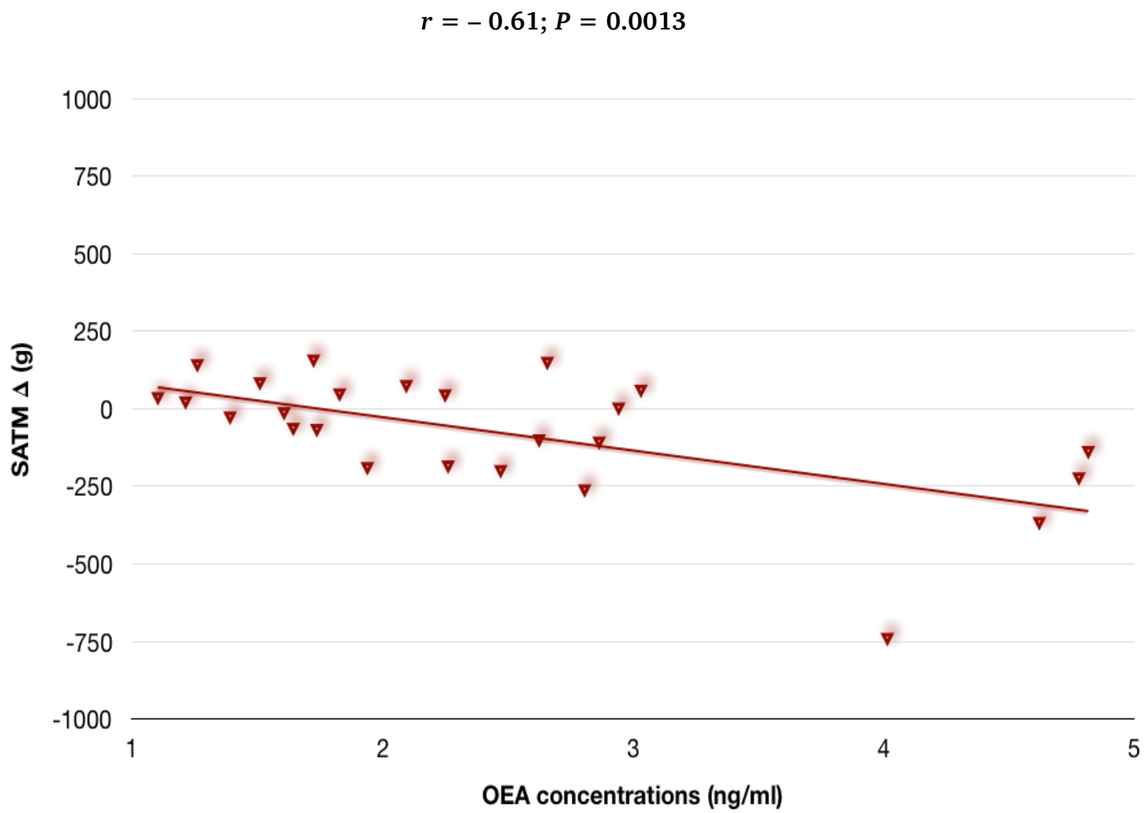


Figure 5.10B The association between OEA levels (ng/ml) and subcutaneous adipose tissue mass changes (g) by *LEPR* rs1137101, genotype A/A after six weeks consumption of HOCO.



Note: $n = 25$; r, Pearson correlation coefficient; OEA, oleoylethanolamide; SATM, subcutaneous adipose tissue mass; *LEPR*, leptin receptors; [#]CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil. [†]Composite of all three dietary interventions.

well as cold-induced non-shivering thermogenesis. Furthermore, Suárez *et al.* (55) showed that the OEA present in adipose tissue stimulates lipolysis by activating PPAR-α which further stimulates the β-adrenergic system. The β-adrenergic receptors in combination with UCP1-mediated thermogenesis increase energy expenditure and reduce fat depots; improving overall body composition (4).

The present study has several strengths. To the best of our knowledge, this is the first study that demonstrates novel diet × gene interactions in 9 genes, with FAEs. Moreover, different adipose tissue compartments have levels of disease risk associated with them, therefore, another strength of the study was the usage of the DXA system to assess adipose tissue compartmentalization; which prevented the misclassification of individuals in terms of MetS risk. Furthermore, the study showed the positive associations between AEA and leptin concentrations for the first time. Additionally, this is the first human intervention study investigating associations between plasma FAEs and SAR as well as SVR. The study design strength includes the focus on dietary quality and compliance control, repeated measure design that provides for repeated major-time points of data collection, and the extensive range of types of data collected. Robustness of the study conduct included meeting the sample size target of 125 participants. Furthermore, in this trial, we measured seven FAEs altogether and demonstrated the influence of long-term consumption of dietary oils on these seven variants of FAEs.

Conversely, the study possesses limitations as well in that the trial performed did not measure/analyse energy expenditure and critical neurotransmitters such as

dopamine, histamine, and noradrenaline, which could have explained how OEA imparted anorexic signalling induced satiation. Also, the leptin system is known to change with the menstrual cycle; therefore, after the menopause, the present findings should be investigated in more controlled parameters, such as examining the parameters in the present trial exclusively on post-menopausal women. Additionally, in the present trial FAEs were measured during the fasting state only; future 'acute trials' should be conducted to assess the influence of post-feeding/non-fasting FAEs on body composition since fasting and non-fasting state modulates the FAEs differentially (56). Furthermore, the finding of no significant difference in weight loss by dietary interventions highlights the importance of conducting large 'acute trials' based on (diet × genotype) pattern. Future 'free-living trials' should also be conducted to investigate the effect of C18:1n9 perception threshold and synthesis of OEA, to examine its implications on overall body composition. To explore these parameters is crucial since Stewart and colleagues (20, 21, 57) demonstrated that humans with less detection threshold for fatty acids (hypo-sensitive) or individuals with impaired responses to fatty acids both in the oral cavity and the gastrointestinal tract, consumed more energy with the inclusion of greater amounts of dietary fat and had higher BMI (58).

In conclusion, the present study demonstrates that dietary fat consumption influence body composition in genetic subgroups through OEA mediated mechanisms. Moreover, the trial shows the actions of diet on FA and FAE levels, and the role of genetic factors on these systems, in humans. Furthermore, our study shows negative associations between OEA levels and SVR, suggesting that OEA exerts anorexic potency

that helps maintain healthy body weight; that might be due to the increased energy expenditure and satiety signals imparted through the gut-brain interrelationship (5, 59), enhanced by OEA. Therefore, the intensity of the OEA satiating component might be amplified/boosted by the inclusion of C18:1n9-enriched diets that could aid in regulating appetite and thereby help maintain body weight, attaining overall wellness. Additionally, we show that the plasma OEA concentrations reflected the dietary pattern of C18:1n9 intake and may be influenced by *GPR40* rs1573611 polymorphism. Our data demonstrate that humans possessing *GPR40* rs1573611-T and *LEPR* rs1137101-A/A polymorphisms would benefit more from the ingestion of C18:1n9 enriched dietary oils by regulating the appetite, due to enhanced satiation induced by increased OEA and reduced AEA concentrations, respectively. These data advance our knowledge in the expanding field of 'nutrigenetics;' suggesting that the interactions among dietary fatty acids, FAE and candidate genes in energy homeostasis exist. Therefore, taking these new insights and translating them into appropriate paradigms for functional research in humans may lead to a deeper understanding of the human physiology and disease, thereby facilitating the development of apposite nutritional strategies to check appetite in obesity.

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5.7 Authors' contributions

The authors' responsibilities were as follows - PJHJ, CT, BL, and PMK-E: principal investigators at RCFFN, St-Boniface Hospital, INAF, and PSU, respectively; PJHJ, DJAJ, PMK-E, SGW, CT, BL, and PWC: study concept and design; PE: contributed expertise for genotyping studies; TCR: contributed expertise for gene expression work; PJHJ, DJAJ, PMK-E, SGW, BL, PC, CT, and PWC: administrative, technical, or material support; PJHJ, DJAJ, PMK-E, PWC, SGW, BL, PC, and CT: study supervision; JS, SSH, XC, KJB, PZ, and PC: clinical trial conduction at four different sites designated for performing Multi-Centre Clinical Trial and acquisition of data; JS: project lead on the human trial at RCFFN, involving clinical trial coordination, meals preparation, laboratory work, analysis and interpretation of the data from all four sites, and drafting of the manuscript; PJHJ: contributed to the preparation of the manuscript.

5.8 Conflict of interest statement

JS, SSH, XC, KJB, PE, and PC have no conflicts of interest to declare in the development of this manuscript.

BL is Chair of Nutrition at Université Laval, which is supported by private endowments from Pfizer, La Banque Royale du Canada and Provigo-Loblaws. BL has received funding in the last 5 years from the Canadian Institutes for Health Research, the Natural Sciences and Engineering Research Council of Canada, Agriculture and Agri-Food Canada [Growing Forward program supported by the Dairy Farmers of Canada

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CT has received funding in the past 5 years from CIHR, NSERC, Agriculture and Agri-Food Canada (Growing Forward and the Canola/Flax Agri-Science Cluster, Pulse Agri-Science Cluster, Canada-Manitoba Agri-Food Research Development Initiative), Research Manitoba, Manitoba Energy Science and Technology, Manitoba Agri-Health Research Network, Alberta Innovates, Alberta Crop Industry Development Fund, Alberta Canola Producers Commission, Alberta Pulse, Saskatchewan Pulse Growers, Canadians Diabetes Association, and MITACS.

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DJAJ has received research grants from Saskatchewan Pulse Growers, the Agricultural Bioproducts Innovation Program through the Pulse Research Network, the Advanced Foods and Material Network, Loblaw Companies Ltd., Unilever, Barilla, the Almond Board of California, Agriculture and Agri-food Canada, Pulse Canada, Kellogg's

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Bridge to Chapter 6

Chapter 5 showed associations among SNPs, FAE, and ratios of body compartments in response to DFA consumption. These data prompted the examination of associations between FA and alterations in body composition at major fat storage sites to understand the impact on overall body weight in the human trial conducted in Chapter 5 since FA influenced the circulating FAE concentrations (Chapter 5) thereby modulating the body composition. Henceforth, Chapter 6 comprises a manuscript which presents the associations between candidate SNPs in genes related to fatty acid receptors since many factors are involved in the intertwined effect of consumption of dietary fat and initiation of satiety influencing overall body composition, many of which may be due to genetic variations.

Chapter 6

Research Article

Manuscript 5

This manuscript is in preparation and will be submitted for publication in 2019

Effect of high-monounsaturated vs low-monounsaturated dietary oils on plasma fatty acids and body composition in overweight adults and the association with single nucleotide polymorphisms in candidate fatty acid receptors

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6.1 Abstract

Background: To date, data suggest that alterations in fatty acids (FAs) composition are found in patients with metabolic syndrome (MetS), which might be partly influenced by genetics. Furthermore, numerous studies have evaluated the effects of consumption of fats high in monounsaturated fatty acids (MUFA) vs low MUFA or polyunsaturated fatty acids (PUFA) on whole body energy expenditure. However, limited data exist to demonstrate the unique relationships between plasma FAs (synthesized post consumption of dietary fats) and body composition based on genetic architecture.

Objective: To examine the associations among plasma FAs and changes in body composition at primary fat storage sites to understand the impact on overall body weight in subjects with obesity and dyslipidemia when fed isocaloric diets varying in fatty acid (FA) composition, and to elucidate the action of diet × gene interactions on plasma FA composition.

Method: In a multi-centre, controlled-feeding, double-blind, randomized, cross-over experiment, subjects (n = 115) consumed smoothies (20% kcal of total energy) twice per day for six weeks containing either (1) high oleic canola oil (HOCO), (2) regular canola oil (RCO), or (3) control oil (CO; a formulated oil blend of butter, safflower, flaxseed, and coconut to match the FA profile of a Western diet). Plasma FA profile was assessed using GC-FID. DXA was used for body composition analysis and genotyping of single nucleotide polymorphisms (SNPs); namely, *CD36*, *GPR40*, and *LEPR*, within genes

coding for enzymes in pathways for energy metabolism was performed using qPCR.

Results: Body composition did not differ across any of the dietary treatments examined from endpoint to endpoint. Independent of diet, a negative correlation was observed between plasma C18:1n9 and changes in android mass (AM) as well as body weight (BW) for the HOCO treatment ($r = -0.26$, $P = 0.0058$) and ($r = -0.38$, $P < 0.0001$), respectively. Similar inverse associations were observed between plasma C18:1n9 and changes in AM as well as BW post-RC ingestion ($r = -0.27$, $P = 0.0038$) and ($r = -0.31$, $P < 0.0008$), respectively. In contrast, there were no correlations between C18:1n9 and changes in AM as well as BW post-CO-consumption. Furthermore, a weak inverse relationship was detected between the intake of C18:1n9 and alterations in BW by the leptin receptors gene (*LEPR*) ($r = -0.21$, $P < 0.0004$). A similar inverse correlation was replicated when the associations were investigated by rs1137101-AA ($r = -0.36$, $P < 0.0014$), whereas GG-genotype showed no such changes.

Conclusion: Our results suggest that dietary HOCO and RCO consumption may have beneficial effects on changes in BW by preventing ectopic fat storage. Furthermore, data indicate that participants possessing *LEPR*-AA polymorphisms would benefit more from the intake of C18:1n9 enriched oils by regulating body weight gain. This trial was registered at www.clinicaltrials.gov as NCT02029833.

Keywords: Dietary fat, fatty acids, leptin receptors, obesity.

6.2 Introduction

To date, data suggest that alterations in fatty acid (FA) composition are found in patients with metabolic syndrome (MetS) (1) which might be partly influenced by genetics. Furthermore, over the past three decades, the global prevalence of overweight and obesity has doubled, leading to 4 million deaths worldwide in the year 2015 (2). Multiple reasons have been advanced for a climb in numbers of obese; as such, accumulation of body fat is also one of the predominant causes of obesity occurring due to the excessive calorie ingestion, leading to retention of excess fat in adipose tissues. Ectopic fat deposition (3) and altered FA (1, 4) composition can synchronously enhance the pathogenesis of MetS. Additionally, the obesity epidemic is chiefly driven by a chronic positive energy balance, which is sustained over the years with a difference of $< 0.1\%$ between daily intake and expenditure (5). Over-consumption of energy-dense foods, which is typically associated with a high-fat content in foods, is a crucial contributor to positive energy balance (6). A detailed review (7) and a human trial (8) have suggested that the dietary fatty acid (DFA) composition associated with a high-fat diet may act differentially on energy utilization and storage, influencing weight gain and loss, leading to obesity. As such, the studies (7, 8) demonstrate that the unsaturated fats appear to be more metabolically beneficial, specifically, monounsaturated fatty acids (MUFA) $\geq$ polyunsaturated fatty acids (PUFA) $>$ saturated fatty acids (SFA), as evidenced by the higher diet-induced thermogenesis (DIT) and fat oxidation (FO); wherein SFAs are likely more obesigenic than MUFA and PUFA. Therefore, suggesting

that DFA composition may influence metabolism, perhaps affecting weight management. Thus, dietary fats are implicated in the development of several metabolic diseases, including obesity (9, 10). For instance, Belury *et al.* (11) showed that human erythrocyte C18:2n6 levels were associated with decreased trunk fat mass. Conversely, Liu *et al.* (12) demonstrated that MUFA reduced central obesity in humans, improving the MetS risk factors. On the other hand, a recent study indicates that both MUFA, as well as PUFA, have a similar metabolic influence on weight management (13); since fat oxidation rates were similar between the diets.

Furthermore, in recent years, C18:1n9 and C18:2n6 fatty acids (FAs) have gathered attention due to their role in modulating the endocannabinoid system by synthesis of lipid signalling amides, oleoylethanolamide (OEA) and linoleoylethanolamide (LEA), respectively. An animal trial (14) has demonstrated that a C18:2n6 enriched diet led to obesity in mice in part by raising peripheral endocannabinoid levels. In addition, C20:4n6 derived from C18:2n6 is the key precursor of two appetite stimulating compounds; namely, 2-Arachidonoylglycerol (2-AG), and arachidonoylethanolamide (AEA, also known as anandamide) that leads to the excessive endocannabinoid signaling which by dysregulating the cannabinoid system results in weight gain and larger adipocytes. Therefore, it is important to understand the influence of the plasma FA profile on the compartmentalization of body fat, since the data are scant. Hence, the objective of the present trial was to investigate the impact of modulating dietary FAs on body composition where the calorie intake was balanced based on the Acceptable Macronutrient Distribution Range (AMDR) (15). An additional

objective of the trial was to investigate the relationship between FA composition and changes in three key fat storage sites; namely, subcutaneous adipose tissue mass (SATM), visceral adipose tissue mass (VATM), and android mass (AM) to understand the impact on overall body weight (BW). In addition, the lack of research on longer-term ingestion of varying DFA and impact on body composition requires a longer-term nutritional intervention trial. Therefore, the present trial also examined the associations between FAs and body composition with MUFA as well as PUFA, in a setup of longer-term dietary intervention trial. Besides, one comprehensive review (16) and a recent human trial (13) compared the effect of short-term feeding of 5-d meal-challenges rich in MUFA and PUFA with mixed findings on diet-induced thermogenesis (DIT) and energy expenditure; a better understanding of which can be achieved by performing longer-term dietary intervention trials. Also, since factors that influence obesity are multifaceted and complex, we investigated the relationship of genotype pattern of two FA receptors, cluster of differentiation 36 (*CD36*) rs1761667 and G protein-coupled receptors 40 (*GPR40*) rs1573611; and a hypothalamic regulatory marker, leptin receptors (*LEPR*) rs1137101, on body composition in humans.

6.3 Materials and methods

6.3.1 Experimental design

6.3.1.1 Study protocol

A randomized, double-blinded, cross-over, full-feeding, multi-centre trial was conducted

from the year 2014 to 2016 at four sites; namely, (i) Institute of Nutrition and Functional Foods (INAF), Laval University, QC, Canada; (ii) The Pennsylvania State University (PSU), PA, USA; (iii) Richardson Centre for Functional Foods and Nutraceuticals (RCFFN), University of Manitoba, Winnipeg, MB, Canada; and (iv) Canadian Center for Agri-Food Research in Health and Medicine (CCARM), St-Boniface Hospital, Winnipeg, MB, Canada. Chapter 5, Figure 5.1 outlines the graphical representation of the protocol for the human intervention trial. The study design consisted of 3 treatment phases of 6 weeks each, separated by 6-week washout periods (ranged from 4–12 weeks for a few participants). All participants were systematically randomized using randomization.com. The trial was conducted according to the principles expressed in the Declaration of Helsinki (17). Trial procedures were approved by the participating sites' Biomedical Research Ethics Board. All participants provided signed and dated written informed consent. This trial was registered at www.clinicaltrials.gov as NCT02029833.

6.3.2 Participants

6.3.2.1 Inclusion criteria

Overweight and obese male and female non-smokers (n = 115), aged 20–65 y were recruited for the study as has been reported previously (Chapter 5). The recruited participants were non-diabetic (fasting blood glucose ≥ 5.6 mmol/L) with increased waist circumference, men ≥ 94 cm and women ≥ 80 cm, considered as the primary inclusion criteria. Additionally, the secondary inclusion criteria for the study were that

participants meet at least two of the MetS parameters, such as higher serum low density lipoprotein cholesterol (LDL-C) level ≥ 2.6, triglycerides (TG) ≥ 1.7 mmol/L, high density lipoprotein cholesterol (HDL-C) < 1 mmol/L (males) or < 1.3 mmol/L (females), and blood pressure ≥ 130 mmHg (systolic) and/or ≥ 85 mmHg (diastolic). These criteria allowed participants to meet the metabolic syndrome definition of the International Diabetes Federation (IDF) and American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) Consensus (18). Participants' baseline characteristics are summarized (Chapter 5, Table 5.1). Chapter 5, Figure 5.2 outlines the graphical representation of the participant flow.

6.3.2.2 Exclusion criteria

Pregnant or lactating females or those planning to conceive during the study period, or individuals with past or present specific disease states were excluded from the trial. These diseases included, but were not limited to, cardiovascular disease, hepatic disease, malignancy, gastrointestinal disease, renal disease, hematological disease, neurological disease, or endocrine disease. Participants taking lipid modulating medicines were exempted from the study. Participants with a history of alcohol intake more than the upper limit set by National Institute on Alcohol Abuse and Alcoholism (NIAAA) (19) or drug abuse/dependence within 12 months of the study were excluded from the trial. NIAAA's definition of drinking at high risk is defined as an average weekly intake of >14 drinks/week for men or >7 drinks/week for women. Participants were advised to stop taking prescription or non-prescription drugs including vitamins and dietary or herbal

supplements 1 month before screening. Participants who did not follow the instructions were exempted from the study.

6.3.3 Diets

All dietary treatments and full day meals based on a 7-day rotating menu were prepared in the metabolic kitchen at each site. Participants were offered iso-caloric diets. The overall composition of all experimental diets provided to participants is shown (Chapter 5, Table 5.2). Treatment oils were incorporated into the smoothies containing 20% kcal of total energy from (1) control oil (CO) (formulated oil blend of butter, safflower, flaxseed and coconut) (Chapter 5, Figure 5.3); (2) regular canola oil (RCO); or (3) high oleic canola oil (HOCO), twice a day for six weeks. The FA profile of the dietary oils/fats is presented (Chapter 5, Figure 5.4). Remaining energy intake was adjusted in meals as per the caloric requirements of the participants. Body weights were monitored for the first two weeks on a daily basis to compensate for any distress in food load to avoid overconsumption. If any sudden weight gain or loss was noticed, the caloric intakes were readjusted in the initial two weeks of the intervention phase.

Participants were advised to consume the smoothies twice a day in the morning at breakfast and in the evening as per the randomization sequence/order. Compliance was monitored on a daily basis where one smoothie containing treatment oil along with one meal was consumed in the presence of clinical staff. Participants were advised not to consume food outside the provided meals. All participants were encouraged to maintain dietary records wherein all the details pertaining to food consumption or medicinal

intake if any, were recorded. Participants were strongly recommended to keep consistency in their physical activities throughout the trial. Compliance with the treatment oils was determined by measuring the plasma C18:3n3 levels at the end of each treatment phase. Furthermore, no significant differences in baseline C18:3n3 FA concentrations across the groups indicated no carryover effect and adequate washout periods between the treatment phases (data not shown).

The CO blend was formulated using various dietary oils (butter, safflower, flaxseed and coconut). The clarified butter was purchased from Verka (New Delhi, India). Flaxseed oil was purchased from Shape Foods (Brandon, MB, Canada). The n6 safflower oil and coconut oil were purchased from eSutras (Chicago, IL, USA). RCO and HOCO were obtained from Richardson Oilseed (Winnipeg, MB, Canada).

6.3.4 Sample collection

6.3.4.1 Anthropometric and clinical data collection

Chapter 5, Figure 5.1 outlines the schedule for anthropometric measures that included weight, height, and waist circumference; and clinical procedures included seated blood pressure, dual energy X-ray absorptiometry **(**DXA) scans, and fasting blood draw. At the beginning and end of each phase, blood pressure was monitored at each clinical site by using a digital blood pressure monitor and measured in triplicate after a 5-min rest in a secluded area. Both systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded in the morning before meals while subjects were in a seated position, with

the cuff placed at the level of the heart on brachial artery.

6.3.4.2 Dual energy X-ray absorptiometry (DXA)

Chapter 5, Figure 5.1 outlines the schedule for DXA scans and weight assessment for the human intervention trial. On day 1 and 42 of each intervention phase, body composition, including SATM, VATM, AM, and BW were assessed by DXA (Lunar Prodigy Advance, GE Healthcare, Madison, WI, USA) in fasting state. All DXA scans were performed at the supine position. The data were evaluated with the Encore 2005 software (v. 9.30.044; GE Healthcare, Little Chalfont, UK).

6.3.4.3 Blood sample collection

Chapter 5, Figure 5.1 outlines the schedule for fasting blood sample collection for the human intervention trial. 12 h fasting blood samples were collected on day 1, 2, 41, and 42 of each intervention phases. Blood samples were centrifuged at 3000 rpm for 20 min at 4 °C, aliquoted to yield serum, plasma, red blood cells (RBC), and white blood cells (WBC). Aliquoted samples were immediately stored at – 80 °C until analysis.

6.3.5 Chemicals

Heptadecenoic acid (C17:1) internal standard was obtained from Sigma-Aldrich (St. Louis, MO, USA) and the GLC-463 standards mix was purchased from Nu-Chek Prep Inc. (Elysian, MN, USA). Liquid chromatography (LC)-grade solvents were purchased from Sigma-Aldrich. All other chemicals and reagents were purchased from Sigma-Aldrich or

Fisher Scientific (Waltham, MA, USA).

6.3.6 Fatty acid analysis

FA profiles in blood plasma were analyzed after direct transesterification followed by gas chromatography (20). The detailed FA analysis protocol has been reported previously (Chapter 5). A known FA mixture was compared with the samples to identify retention peaks using Galaxie software (Varian Inc.). The level of each FA was then calculated according to the corresponding peak area relative to that of all FA of interest to yield the relative percentage of total FA (21).

6.3.7 DNA extraction and genotyping

Genomic DNA was extracted from WBC by using a column-based DNA extraction kit (DNeasy Blood and Tissue Kit, QIAGEN Sciences Inc., Toronto, ON, Canada) according to the manufacturer's instructions. The concentration and purity of the genomic DNA were assessed by microvolume spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific Inc, Waltham, MA, USA). Genotyping of the candidate genes involved in the fat taste receptors and energy homeostasis was accomplished by using the TaqMan GTXpress Master Mix (Applied Biosystems, Life Technologies, Inc., Burlington, ON, Canada). DNA samples were further analysed on a StepOnePlus Real-Time PCR System (Applied Biosystems; Life Technologies Inc., Burlington, ON, Canada). For quality control, the genotyping was performed in duplicate. Overall, genetic variants in 3 genes were investigated; namely, *CD36* rs1761667, *GPR40* rs1573611, and *LEPR* rs1137101.

6.3.8 Plasma inflammatory biomarker analyses

High-sensitivity enzyme-linked immunosorbent assay (ELISA) was used to measure plasma interleukin-6 (IL-6), interleukin-8 (IL-8), leptin, and adiponectin levels, according to the manufacturer's guidelines (R&D Systems, Minneapolis, MN, USA) and (Human Adiponectin ELISA Kit; B-Bridge International Inc., San Jose, CA), respectively. The intra-assay and inter-assay CV values were 7.80 and 7.20% for IL-6; 5.50 and 8.50% for IL-8; 3.17 and 4.37% for leptin; and 3.45 and 5.20% for adiponectin, respectively. High-sensitivity C-reactive protein (hs-CRP) levels were measured using ***Cardio***Phase® ***hs***CRP (Siemens, Newark, DE, USA).

6.3.9 Data analyses and interpretation

Mounting in vivo evidence from animal and human trials (16) demonstrate an immense variability in FA intake perception by individuals (22). The variability exists due to the distinct activity of receptors in each individual's gut, which plays a critical role in food consumption and obesity (16, 22). Additionally, Stewart et al. (22) demonstrated similar FA detection threshold in both male and female participants, suggesting that both sexes have similar gustatory and gastrointestinal sensitivity to C18:1n9 (23). Therefore, the FA analyses data for both sexes, male and female are presented together. Furthermore, previous studies (24–27) have reported that the effect modification claims observed in randomized clinical trials are often spurious when subgroup analyses are conducted. Besides, these results are more frequent when small sample sizes and *post-hoc* analyses are involved; producing rare validations of such claims. To substantiate it further,

Wallach *et al*. (25) performed Cochrane meta-analyses and reported that sex-treatment interactions typically had limited biological plausibility or clinical significance. Thus, no subgroup analyses based on the sex-treatment effects are indicated in the present trial.

6.3.10 Statistical analyses

The results are expressed as mean ± SEM. Data were analysed using SAS 9.4 (IBM Software, Armonk, NJ, USA). Abnormally distributed variables were natural log-transformed before statistical analysis. Statistical significance for the effects of treatment was analyzed by the SAS MIXED procedure and Tukey's *post-hoc* testing when applicable. Treatment, sex and age were included in the model as fixed factors, and sequence of treatments, clinical site and participant were included as a random factor, with participant repeated by phase. Gene and dietary treatment × gene interactions were included as fixed factors to examine the effect of genotype. The SAS SLICE function investigated the data for dietary treatment × gene effects. Pearson's correlation analyses were performed to examine associations between plasma FA levels and body composition variables/parameters assessed by DXA. Moreover, correlation analyses were conducted to investigate the associations between C18:1n9 levels and changes in BW by *LEPR*. Statistical significance was defined as $P < 0.05$. The primary outcome of the trial was to investigate the implications of MUFA on body composition. Therefore, the power calculation was performed based on the previous clinical trial (12). The sample size of 140 was determined to offset for a 20% dropout rate. The power calculation was performed to detect a 55 g change in android fat mass using the variance parameter in

android fat mass (28, 29). For analysis of the secondary outcomes, the sample size indicated the power of 100% ($\alpha = 0.05$) allowing us to detect significant differences in post-treatment plasma C18:1n9 concentrations among three dietary interventions (30).

6.4 Results

6.4.1 Participant characteristics

Chapter 5, Figure 5.2 outlines the graphical representation of the participant flow. A total of 174 individuals were randomly assigned to the study, with the dropout rate of 28%. Three individuals were excluded due to increased blood glucose levels, 6 individuals were exempted from the analyses due to drastic BW changes, and 1 individual was excluded from the study due to incomplete DXA measurements. In total, 125 participants completed the intervention, out of which FA analyses were performed on 115 individuals. Participants' baseline characteristics are summarized (Chapter 5, Table 5.1). Genotyping was performed on 101 participants who provided consent for genetic analyses. Characteristics of the selected polymorphisms are shown in Table 6.1. No significant differences were observed in BW or composition after any of the dietary treatments, and the body composition results in each dietary phase are tabulated (Chapter 5, Table 5.4).

6.4.2 Dietary compliance

Returned empty food bags were used to assess compliance. Participants consumed the treatment smoothies as well as one meal under the supervision of clinical staff. To affirm

Table 6.1 Characteristics of the selected genetic polymorphisms.

Gene	SNP	Region	Allele	Genotype (n)			MAF%
			Major/Minor	MM	Mm	mm	
CD36	rs1761667	Intron	A/G	25	48	24	49.5
GPR40	rs1573611	Exon	C/T	46	41	7	29.3
LEPR	rs1137101	Missense	A/G	25	50	22	48.5

Note: SNP, single nucleotide polymorphism; MM, major allele homozygous; Mm, heterozygous; mm, minor allele homozygous; MAF, minor allele frequency; *CD36*, cluster of differentiation 36; *GPR40*, G protein-coupled receptors 40; *LEPR*, Leptin receptors.

the smoothie and meal intake, participants also signed the daily checklist in the presence of clinical staff. Additionally, increased plasma C18:3n3 levels post-CO and -RCO treatments, as compared to HOCO treatment substantiated the compliance towards the experimental diets. All of the participants showed good tolerance to experimental diets with no reported side effects or discomfort.

6.4.3 Effect of diet treatment on plasma fatty acid levels

Plasma FA profiles at the end of each dietary phase are shown in Table 6.2. Higher plasma FA levels of C12:0, C14:0, and C15:0 ($P < 0.0001$), ($P < 0.0001$), and ($P < 0.0228$), respectively were observed in post consumption of CO group in comparison to RCO and HOCO groups. On the contrary, in comparison to *t*-C16:1n7 to *c*-C16:1n7, CO showed lower ($P < 0.0001$) *t*-C16:1n7 levels but higher ($P < 0.0001$) *c*-C16:1n7; whereas, both RCO and HOCO showed inverse results. Increased ($P < 0.0001$) plasma levels of C17:0, C18:0, *t*-18:1n9, C19:0, and *t*-C18:1n7 ($P = 0.0004$) were observed in the CO group when compared to other dietary treatments. All *cis*-isomers of C18:1 were detected in higher ($P < 0.0001$) levels post ingestion of RCO and HOCO treatments when compared to CO group. Moreover, plasma showed a predominance of C16:0, and C18:2n6 concentrations across all dietary treatments, followed by C18:1n9, and C20:4n6 (Table 6.2). Also, C18:1n9 levels at the end of dietary interventions are shown (Chapter 5, Figure 5.5A). Additionally, by *CD36* gene, C18:1n9 levels are shown in Figure 6.1. Elevated C18:1n9 levels were observed in participants after HOCO consumption (23% increase; $P < 0.0001$), when compared to CO. Similarly, highest ($P < 0.0001$) C18:2n6

Table 6.2 Plasma fatty acids[‡] of each dietary phase (g/100g)*.

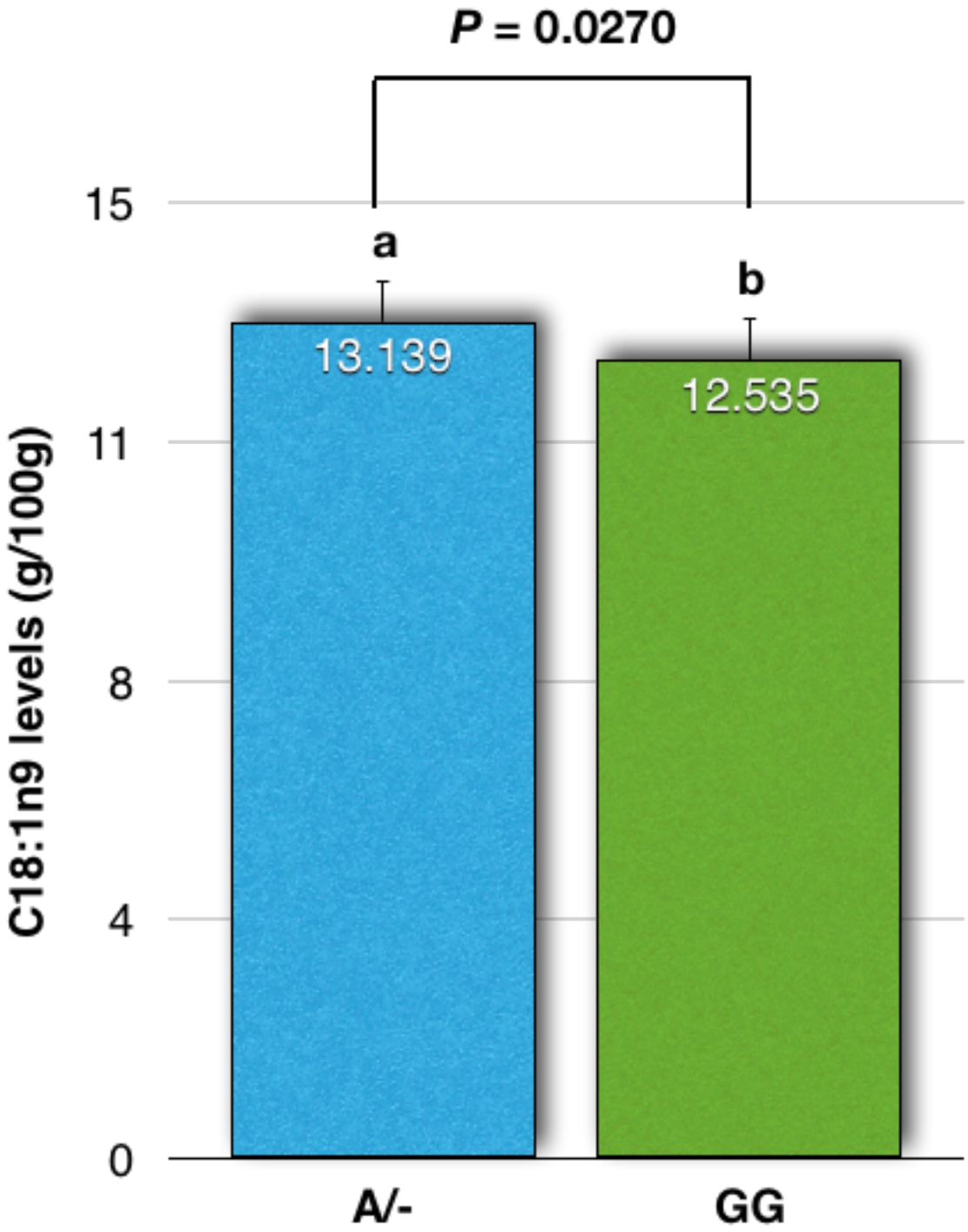
Fatty acid	CO		RCO		HOCO		*P*
	Mean	SEM	Mean	SEM	Mean	SEM	
C7:0	0.02	0.00	0.32	0.07	0.14	0.02	0.9207
C8:0	0.20	0.04	0.15	0.03	0.12	0.02	0.7263
C9:0	0.10	0.01	0.12	0.01	0.10	0.01	0.1079
C10:0	0.20	0.01	0.22	0.02	0.21	0.01	0.1476
C11:0	0.18	0.01	0.18	0.01	0.18	0.01	0.1057
C11:1n9	0.01	0.00	0.01	0.00	0.02	0.00	0.0763
C12:0	0.06[a]	0.00	0.05[b]	0.00	0.05[b]	0.00	<0.0001
C12:1n9	0.01	0.00	0.01	0.00	0.02	0.00	0.4830
C13:0	0.40	0.23	0.04	0.01	0.04	0.01	0.2308
C13:1n9	0.01	0.01	0.02	0.00	0.02	0.00	0.2525
C14:0	0.55[a]	0.01	0.49[b]	0.01	0.50[b]	0.01	<0.0001
C14:1n9	0.02	0.00	0.02	0.00	0.03	0.00	0.2809
C15:0	0.35[a]	0.02	0.33[b]	0.02	0.33[b]	0.02	0.0228
C15:1n9	0.10	0.02	0.09	0.02	0.09	0.02	0.6806
C16:0	26.39[a]	0.20	25.88[b]	0.20	25.69[b]	0.20	<0.0001
***t*-C16:1n7**	0.16[b]	0.01	0.17[a]	0.01	0.19[a]	0.01	<0.0001
***c*-C16:1n7**	0.94[a]	0.02	0.87[b]	0.03	0.88[b]	0.03	<0.0001
C17:0	0.44[a]	0.01	0.41[b]	0.01	0.41[b]	0.01	<0.0001
C18:0	12.59[a]	0.11	12.22[b]	0.12	12.11[b]	0.12	<0.0001
***t*-C18:1n11**	0.08	0.01	0.09	0.01	0.10	0.02	0.6213
***t*-C18:1n9**	0.24[a]	0.01	0.16[b]	0.01	0.15[b]	0.01	<0.0001
***t*-C18:1n7**	0.11[a]	0.01	0.07[b]	0.01	0.07[b]	0.01	0.0004

Fatty acid	CO		RCO		HOCO		*P*
	Mean	SEM	Mean	SEM	Mean	SEM	
***c*-C18:1n9**	11.33[c]	0.14	13.63[b]	0.18	14.28[a]	0.16	<0.0001
***c*-C18:1n7**	1.38[b]	0.02	1.72[a]	0.04	1.72[a]	0.03	<0.0001
***t, t*-C18:2n6**	0.03	0.00	0.03	0.00	0.03	0.00	0.1717
C19:0	0.06[a]	0.01	0.04[b]	0.00	0.05[b]	0.00	<0.0001
C18:2n6	22.65[a]	0.23	21.35[b]	0.21	20.69[c]	0.20	<0.0001
C19:1n9	0.05	0.01	0.05	0.01	0.05	0.01	0.6738
C18:3n6	0.12	0.01	0.12	0.01	0.13	0.01	0.1683
C18:3n3	0.59[a]	0.01	0.56[a]	0.01	0.45[b]	0.01	<0.0001
C20:0	0.21[b]	0.01	0.23[ab]	0.01	0.24[a]	0.01	0.0281
***c*-9, *t*-11-C18:2n6, CLA**	0.14[a]	0.01	0.11[b]	0.01	0.11[b]	0.01	<0.0001
C20:1n15	0.03	0.00	0.03	0.00	0.03	0.00	0.4435
C20:1n12	0.03	0.00	0.02	0.00	0.02	0.00	0.6778
C20:1n9	0.17[b]	0.01	0.26[a]	0.01	0.27[a]	0.01	<0.0001
C20:2n6	0.40	0.03	0.37	0.02	0.36	0.01	0.0899
C20:3n6	1.99	0.13	1.92	0.12	2.05	0.13	0.4332
C20:4n6	8.64[b]	0.29	8.72[ab]	0.31	9.06[a]	0.32	0.0168
C20:3n3	0.06[b]	0.01	0.11[ab]	0.03	0.15[a]	0.04	0.0163
C22:0	2.75	0.38	2.88	0.40	2.84	0.39	0.3700
C22:1n9	0.25	0.03	0.22	0.03	0.22	0.03	0.2048
C20:5n3	1.01[a]	0.04	1.07[a]	0.04	0.89[b]	0.03	<0.0001
C22:2n6	0.17[a]	0.03	0.11[b]	0.01	0.15[ab]	0.02	0.0204
C22:3n3	0.28	0.02	0.24	0.02	0.27	0.02	0.2039
C22:4n6	0.20	0.02	0.20	0.02	0.21	0.02	0.8732

Fatty acid	CO		RCO		HOCO		*P*
	Mean	SEM	Mean	SEM	Mean	SEM	
C24:0	0.44[a]	0.03	0.39[b]	0.02	0.40[ab]	0.02	0.0476
C22:5n6	0.77	0.06	0.70	0.06	0.82	0.06	0.0525
C24:1n9	0.23	0.03	0.25	0.03	0.20	0.03	0.0730
C22:5n3	1.15[a]	0.03	1.03[b]	0.03	0.97[c]	0.02	<0.0001
C22:6n3	2.91	0.06	2.91	0.06	2.94	0.05	0.7192
Total MCFA	0.45	0.03	0.47	0.04	0.43	0.02	0.2972
Total LCFA	99.39	0.14	99.49	0.04	99.55	0.04	0.6192
Total SFA	44.04[a]	0.32	43.05[b]	0.38	42.72[b]	0.35	<0.0001
Total MUFA	14.99[c]	0.17	17.52[b]	0.22	18.18[a]	0.20	<0.0001
Total PUFA	40.96[a]	0.37	39.43[b]	0.37	39.11[b]	0.36	<0.0001
Total n6 PUFA	35.02[a]	0.39	33.55[b]	0.39	33.52[b]	0.38	<0.0001
Total n3 PUFA	5.94[a]	0.09	5.88[a]	0.11	5.59[b]	0.08	<0.0001
n6:n3	6.08[ab]	0.13	5.93[b]	0.13	6.17[a]	0.12	0.0307
SFA:MUFA	2.98[a]	0.04	2.51[b]	0.04	2.39[c]	0.04	<0.0001
SFA:PUFA	1.10[b]	0.02	1.11[a]	0.02	1.11[a]	0.02	0.0101
MUFA:PUFA	0.37[c]	0.01	0.45[b]	0.01	0.47[a]	0.01	<0.0001
EPA:AA	0.16[ab]	0.02	0.17[a]	0.02	0.13[b]	0.01	0.0479
DHA:AA	0.46	0.04	0.45	0.04	0.43	0.04	0.6869
(EPA+DHA):AA	0.62	0.05	0.63	0.07	0.57	0.05	0.3818

Note: [#]The values are endpoint plasma fatty acids. *The values are % abundance of each fatty acid to total fatty acids. The results are expressed as mean ± SEM (n = 115). The values with different superscript letters in the same row are statistically different from each other. Statistical significance assessed at (P < 0.05) by ANOVA with Tukey-Kramer adjustment. CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil; *t*, trans; *c*, cis; CLA, conjugated linoleic acid; ND, not detected; MCFA, medium chain fatty acids; LCFA, long chain fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; AA; arachidonic acid; DHA, docosahexaenoic acid.

Figure 6.1 Effect of *CD36* receptor rs1761667 polymorphism on C18:1n9 levels (g/100g)* after six-weeks consumption of dietary oils[#†].



Note: *The values are % abundance of C18:1n9 to total fatty acids. The results are expressed as mean ± SEM (n = 97). The values with different superscript letters are significantly different from each other ($P < 0.05$). *CD36*, cluster of differentiation 36; [#]CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil. [†]Composite of all three dietary interventions.

levels were observed after the consumption of CO treatment when compared to RCO and HOCO, respectively. Additionally, C18:3n3 rich treatments-CO and -RC, reflected the highest ($P < 0.0001$) C18:3n3 levels when compared to the HOCO group. In addition, conjugated linoleic acid (CLA, *c*-9, *t*-11-C18:2n6) was observed to be the highest ($P < 0.0001$) in the CO group when compared to the RCO and HOCO groups. C20:4n6 levels were lowest ($P < 0.0168$) in CO dietary treatments. Moreover, post consumption, RCO feeding showed the highest ($P < 0.0001$) content of C20:5n3, while no differences in levels of C22:6n3 were observed across any of the dietary treatments. In addition, total medium chain fatty acids (MCFA) and long chain fatty acids (LCFA) failed to show significant differences across all dietary treatments. Overall, both RCO and HOCO intervention groups showed significantly lower levels of total SFA ($P < 0.0001$). By contrast, total MUFA were detected in the highest ($P < 0.0001$) concentrations in HOCO treatment when compared to RCO and CO treatments. Moreover, the CO group expressed highest ($P < 0.0001$) levels of total PUFA, followed by RCO and HOCO. A similar pattern of n6 PUFA profile was detected in the CO group. Additionally, HOCO treatment was observed to show the lowest ($P < 0.0001$) total n3 PUFA profile in comparison to other two dietary treatments. Also, on the one arm, highest ($P < 0.0001$) SFA:MUFA ratios were observed in the CO group, on the other, lowest ($P < 0.0101$) SFA:PUFA ratios were also observed, indicative of higher PUFA levels. In addition, the highest ($P < 0.0001$) MUFA:PUFA ratios were observed in the HOCO group, followed by RCO and CO groups, expressing the richest content of MUFA present in the treatment oil.

6.4.4 Effect of diet treatment on plasma inflammatory biomarkers

Plasma levels of inflammatory biomarkers measured after each dietary treatment are shown in Table 6.3. Overall, no significant differences were observed in endpoint concentrations of IL-6, IL-8, leptin, adiponectin, and CRP levels across dietary treatments. However, genotype differences in leptin concentrations were detected by *LEPR*; wherein rs1137101-A/A showed decreased ($P = 0.0334$) leptin concentrations when compared to the participants carrying the G/- allele (Chapter 5, Figure 5.8).

6.4.5 Interaction testing between diet and genotyping patterns on fatty acid levels

Results of the test for the interaction between diet and genotype pattern in the six-week intervention period were not statistically significant (Tables 6.4–6.6). However, a gene association was observed for *CD36* rs1761667 ($P = 0.0270$) polymorphism on C18:1n9 levels (Figure 6.1). A similar gene association was observed on MUFA concentrations, wherein participants possessing the *CD36* rs1761667-A allele showed increased ($P = 0.0431$) MUFA content when compared to participants carrying G-allele carriers (Table 6.4). Moreover, gene associations were also observed for *LEPR* rs1137101 on C18:2n6 concentrations (Table 6.6); wherein individuals with the rs1137101-A/A allele expressed increased ($P = 0.0194$) C18:2n6 levels when compared to participants carrying the G-allele.

6.4.6 Correlations between plasma fatty acid and body composition variables by dietary treatments

Table 6.3 Plasma inflammatory biomarkers in study participants[‡].

Parameters	CO		RCO		HOCO		*P*
	Mean	SEM	Mean	SEM	Mean	SEM	
IL-6 (pg/ml)	1.43	0.28	1.25	0.28	1.17	0.28	0.1710
IL-8 (pg/ml)	4.04	0.40	3.69	0.40	3.87	0.40	0.4929
Leptin (ng/ml)	22.19	2.14	21.99	2.14	21.83	2.14	0.9812
Adiponectin (μg/ml)	8.09	0.34	8.23	0.24	7.96	0.34	0.2227
CRP (mg/l)	4.31	0.62	3.51	0.62	3.17	0.62	0.1793

Note: [‡]The values are endpoint plasma inflammatory biomarkers. The results are expressed as mean ± SEM (n = 115). Statistical significance assessed at ($P < 0.05$). CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil; IL-6, interleukin-6; IL-8, interleukin-8; CRP, C-reactive protein.

Table 6.4 Effect of *CD36* rs1761667 and diet × *CD36* rs1761667 on fatty acid levels (g/100g)* after six-weeks consumption of dietary oils.

Fatty acid	*CD36* Genotype	Gene effect[†]	Diet × Gene effect[§]			Gene	Diet	Diet × Gene
			CO	RCO	HOCO	P_{gene}	P_{diet}	$P_{interaction}$
C16:0						0.4815	0.0001	0.2465
	A/- (n = 72)	26.06 ± 0.32	26.53 ± 0.33	25.92 ± 0.33	25.73 ± 0.33			
	G/G (n = 25)	25.71 ± 0.49	25.95 ± 0.52	25.89 ± 0.52	25.29 ± 0.52			
C18:1n9						0.0270	<0.0001	0.7575
	A/- (n = 72)	13.14 ± 0.65[a]	11.24 ± 0.65	13.72 ± 0.65	14.46 ± 0.65			
	G/G (n = 25)	12.54 ± 0.68[b]	10.78 ± 0.70	12.98 ± 0.70	13.85 ± 0.70			
C18:2n6						0.3181	<0.0001	0.2478
	A/- (n = 72)	21.81 ± 0.50	23.03 ± 0.51	21.44 ± 0.51	20.95 ± 0.51			
	G/G (n = 25)	21.32 ± 0.62	22.44 ± 0.65	21.34 ± 0.65	20.17 ± 0.65			
C18:3n3						0.1315	<0.0001	0.1173
	A/- (n = 72)	0.53 ± 0.01	0.59 ± 0.02	0.56 ± 0.02	0.44 ± 0.02			
	G/G (n = 25)	0.49 ± 0.02	0.56 ± 0.03	0.54 ± 0.03	0.36 ± 0.03			
C20:4n6						0.1405	0.0497	0.2349
	A/- (n = 72)	9.40 ± 0.27	9.13 ± 0.29	9.41 ± 0.29	9.64 ± 0.29			
	G/G (n = 25)	10.21 ± 0.50	10.24 ± 0.54	9.82 ± 0.54	10.56 ± 0.54			
C20:5n3						0.3784	<0.0001	0.2697
	A/- (n = 72)	0.95 ± 0.04	0.96 ± 0.05	1.07 ± 0.05	0.84 ± 0.04			
	G/G (n = 25)	1.01 ± 0.07	1.11 ± 0.08	1.08 ± 0.08	0.88 ± 0.08			
C22:6n3						0.8525	0.3274	0.5610
	A/- (n = 72)	2.95 ± 0.07	2.93 ± 0.08	2.96 ± 0.08	2.94 ± 0.08			
	G/G (n = 25)	2.92 ± 0.13	2.84 ± 0.14	2.96 ± 0.14	2.95 ± 0.14			

Fatty acid	*CD36* Genotype	Gene effect[†]	Diet × Gene effect[§]			Gene	Diet	Diet × Gene
			CO	RCO	HOCO	P_{gene}	P_{diet}	$P_{interaction}$
MCFA						0.1337	0.8583	0.6554
	A/- (n = 72)	0.51 ± 0.03	0.52 ± 0.04	0.54 ± 0.44	0.47 ± 0.04			
	G/G (n = 25)	0.41 ± 0.06	0.40 ± 0.08	0.40 ± 0.08	0.42 ± 0.08			
LCFA						0.4418	0.1320	0.0786
	A/- (n = 72)	99.32 ± 0.09	99.34 ± 0.13	99.26 ± 0.13	99.37 ± 0.13			
	G/G (n = 25)	99.21 ± 0.14	98.81 ± 0.21	99.41 ± 0.21	99.41 ± 0.21			
SFA						0.7716	<0.0001	0.5785
	A/- (n = 72)	42.48 ± 0.30	43.36 ± 0.33	42.28 ± 0.33	41.80 ± 0.33			
	G/G (n = 25)	42.66 ± 0.56	43.28 ± 0.61	42.52 ± 0.61	42.18 ± 0.61			
MUFA						0.0431	<0.0001	0.7653
	A/- (n = 72)	19.08 ± 0.20[a]	16.75 ± 0.25	19.76 ± 0.25	20.73 ± 0.25			
	G/G (n = 25)	18.26 ± 0.37[b]	16.08 ± 0.45	19.00 ± 0.45	19.70 ± 0.45			
PUFA						0.4782	<0.0001	0.5739
	A/- (n = 72)	42.89 ± 0.32	44.59 ± 0.39	42.21 ± 0.39	41.87 ± 0.39			
	G/G (n = 25)	43.35 ± 0.59	44.72 ± 0.71	43.15 ± 0.71	42.19 ± 0.71			

Note: *The values are % abundance of each fatty acid to total fatty acids. The results are expressed as mean ± SEM (n = 97). P values are from SAS MIXED model. [†]Values with different superscript letters in the same column are significantly different from each other ($P < 0.05$). [§]Values with different superscript letters in the same row are significantly different from each other ($P < 0.05$). *CD36*, cluster of differentiation 36; CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil; MCFA, medium chain fatty acids; LCFA, long chain fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 6.5 Effect of *GPR40* rs1573611 and diet × *GPR40* rs1573611 on fatty acid levels (g/100g)* after six-weeks consumption of dietary oils.

Fatty acid	*GPR40* Genotype	Gene effect†	Diet × Gene effect§			Gene	Diet	Diet × Gene
			CO	RCO	HOCO	P_{gene}	P_{diet}	$P_{interaction}$
C16:0						0.4173	<0.0001	0.4435
	C/C (n = 46)	25.74 ± 0.32	26.27 ± 0.34	25.58 ± 0.34	25.37 ± 0.34			
	T/- (n = 48)	26.09 ± 0.30	26.44 ± 0.32	26.11 ± 0.32	25.72 ± 0.32			
C18:1n9						0.8503	<0.0001	0.6412
	C/C (n = 46)	13.08 ± 0.18	11.09 ± 0.22	13.67 ± 0.22	14.48 ± 0.22			
	T/- (n = 48)	13.03 ± 0.17	11.21 ± 0.21	13.55 ± 0.21	14.35 ± 0.21			
C18:2n6						0.0761	<0.0001	0.4179
	C/C (n = 46)	22.13 ± 0.32	23.47 ± 0.35	21.84 ± 0.35	21.09 ± 0.35			
	T/- (n = 48)	21.36 ± 0.30	22.45 ± 0.33	21.12 ± 0.33	20.52 ± 0.33			
C18:3n3						0.0661	<0.0001	0.0759
	C/C (n = 46)	0.54 ± 0.02	0.63 ± 0.02	0.58 ± 0.02	0.43 ± 0.02			
	T/- (n = 48)	0.50 ± 0.02	0.55 ± 0.02	0.54 ± 0.02	0.42 ± 0.02			
C20:4n6						0.3014	0.0169	0.4442
	C/C (n = 46)	9.29 ± 0.36	9.17 ± 0.39	9.04 ± 0.39	9.67 ± 0.39			
	T/- (n = 48)	9.80 ± 0.34	9.53 ± 0.37	9.82 ± 0.37	10.04 ± 0.37			
C20:5n3						0.8937	<0.0001	0.1561
	C/C (n = 46)	0.96 ± 0.05	0.95 ± 0.06	1.11 ± 0.06	0.83 ± 0.06			
	T/- (n = 48)	0.97 ± 0.05	1.02 ± 0.06	1.04 ± 0.06	0.86 ± 0.06			
C22:6n3						0.4317	0.3960	0.2221
	C/C (n = 46)	2.88 ± 0.09	2.81 ± 0.10	2.94 ± 0.10	2.89 ± 0.10			
	T/- (n = 48)	2.98 ± 0.09	2.98 ± 0.09	2.97 ± 0.09	2.98 ± 0.09			

Fatty acid	*GPR40* Genotype	Gene effect[†]	Diet × Gene effect[§]			Gene	Diet	Diet × Gene
			CO	RCO	HOCO	P_{gene}	P_{diet}	$P_{interaction}$
MCFA						0.2595	0.3052	0.4900
	C/C (n = 46)	0.46 ± 0.05	0.43 ± 0.06	0.51 ± 0.06	0.43 ± 0.06			
	T/- (n = 48)	0.53 ± 0.04	0.56 ± 0.06	0.54 ± 0.06	0.48 ± 0.06			
LCFA						0.6455	0.3732	0.7083
	C/C (n = 46)	99.41 ± 0.10	99.23 ± 0.16	99.45 ± 0.16	99.55 ± 0.16			
	T/- (n = 48)	99.47 ± 0.09	99.43 ± 0.15	99.44 ± 0.15	99.54 ± 0.15			
SFA						0.3615	<0.0001	0.4051
	C/C (n = 46)	42.22 ± 0.41	43.14 ± 0.44	42.15 ± 0.44	41.36 ± 0.44			
	T/- (n = 48)	42.72 ± 0.38	43.55 ± 0.41	42.45 ± 0.41	42.16 ± 0.41			
MUFA						0.5244	<0.0001	0.5642
	C/C (n = 46)	18.82 ± 0.27	16.55 ± 0.33	19.62 ± 0.33	20.30 ± 0.33			
	T/- (n = 48)	19.06 ± 0.26	16.70 ± 0.31	19.66 ± 0.31	20.81 ± 0.31			
PUFA						0.6643	<0.0001	0.7820
	C/C (n = 46)	43.11 ± 0.43	44.59 ± 0.52	42.53 ± 0.52	42.21 ± 0.52			
	T/- (n = 48)	42.86 ± 0.40	44.55 ± 0.49	42.33 ± 0.49	41.69 ± 0.49			

Note: *The values are % abundance of each fatty acid to total fatty acids. The results are expressed as mean ± SEM (n = 97). P values are from SAS MIXED model. [†]Values with different superscript letters in the same column are significantly different from each other ($P < 0.05$). [§]Values with different superscript letters in the same row are significantly different from each other ($P < 0.05$). *GPR40*, G protein-coupled receptors 40; CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil; MCFA, medium chain fatty acids; LCFA, long chain fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 6.6 Effect of *LEPR* rs1137101 and diet × *LEPR* rs1137101 on fatty acid levels (g/100g)* after six-weeks consumption of dietary oils.

Fatty acid	*LEPR* Genotype	Gene effect†	Diet × Gene effect§			Gene	Diet	Diet × Gene
			CO	RCO	HOCO	P_{gene}	P_{diet}	$P_{interaction}$
C16:0						0.1284	<0.0001	0.0860
	A/A (n = 25)	25.46 ± 0.41	25.81 ± 0.44	25.68 ± 0.44	24.90 ± 0.44			
	G/- (n = 72)	26.19 ± 0.25	26.62 ± 0.27	26.02 ± 0.27	25.92 ± 0.27			
C18:1n9						0.6984	<0.0001	0.4547
	A/A (n = 25)	12.96 ± 0.24	10.89 ± 0.29	13.62 ± 0.29	14.35 ± 0.29			
	G/- (n = 72)	13.06 ± 0.14	11.25 ± 0.18	13.58 ± 0.18	14.36 ± 0.18			
C18:2n6						0.0194	<0.0001	0.9487
	A/A (n = 25)	22.60 ± 0.41[a]	23.82 ± 0.46	22.38 ± 0.46	21.61 ± 0.46			
	G/- (n = 72)	21.51 ± 0.24[b]	22.71 ± 0.27	21.22 ± 0.27	20.59 ± 0.27			
C18:3n3						0.3558	<0.0001	0.4879
	A/A (n = 25)	0.50 ± 0.02	0.56 ± 0.03	0.53 ± 0.03	0.42 ± 0.03			
	G/- (n = 72)	0.53 ± 0.01	0.59 ± 0.02	0.57 ± 0.02	0.43 ± 0.02			
C20:4n6						0.2826	0.0382	0.2255
	A/A (n = 25)	9.07 ± 0.47	9.12 ± 0.52	8.69 ± 0.52	9.41 ± 0.52			
	G/- (n = 72)	9.66 ± 0.28	9.37 ± 0.30	9.66 ± 0.30	9.93 ± 0.30			
C20:5n3						0.5470	<0.0001	0.2238
	A/A (n = 25)	0.92 ± 0.07	0.96 ± 0.08	0.97 ± 0.08	0.85 ± 0.08			
	G/- (n = 72)	0.97 ± 0.04	0.99 ± 0.05	1.09 ± 0.05	0.83 ± 0.05			
C22:6n3						0.6523	0.4556	0.4988
	A/A (n = 25)	2.89 ± 0.12	2.86 ± 0.13	2.95 ± 0.13	2.85 ± 0.13			
	G/- (n = 72)	2.95 ± 0.07	2.92 ± 0.08	2.96 ± 0.08	2.97 ± 0.08			

Fatty acid	*LEPR* Genotype	Gene effect[†]	Diet × Gene effect[§]			Gene	Diet	Diet × Gene
			CO	RCO	HOCO	P_{gene}	P_{diet}	$P_{interaction}$
MCFA						0.3652	0.4238	0.3778
	A/A (n = 25)	0.53 ± 0.06	0.60 ± 0.08	0.51 ± 0.08	0.48 ± 0.08			
	G/- (n = 72)	0.47 ± 0.04	0.46 ± 0.05	0.51 ± 0.05	0.446 ± 0.045			
LCFA						0.7717	0.7129	0.6839
	A/A (n = 25)	99.49 ± 0.13	99.51 ± 0.21	99.43 ± 0.21	99.52 ± 0.21			
	G/- (n = 72)	99.44 ± 0.08	99.30 ± 0.13	99.47 ± 0.13	99.56 ± 0.13			
SFA						0.8567	<0.0001	0.1820
	A/A (n = 25)	42.56 ± 0.54	43.41 ± 0.58	42.67 ± 0.58	41.60 ± 0.58			
	G/- (n = 72)	42.45 ± 0.32	43.29 ± 0.34	42.15 ± 0.34	41.90 ± 0.34			
MUFA						0.7523	<0.0001	0.2846
	A/A (n = 25)	18.91 ± 0.36	16.25 ± 0.44	19.82 ± 0.44	20.66 ± 0.44			
	G/- (n = 72)	18.96 ± 0.21	16.77 ± 0.26	19.61 ± 0.26	20.51 ± 0.26			
PUFA						0.9555	<0.0001	0.0137
	A/A[‡] (n = 25)	43.00 ± 0.57	45.48 ± 0.69[a]	41.59 ± 0.69[b]	41.94 ± 0.69[b]			
	G/-[‡] (n = 72)	42.97 ± 0.33	44.31 ± 0.40[a]	42.68 ± 0.40[b]	41.92 ± 0.40[b]			

Note: *The values are % abundance of each fatty acid to total fatty acids. The results are expressed as mean ± SEM (n = 97). P values are from SAS MIXED model. [†]Values with different superscript letters in the same column are significantly different from each other ($P < 0.05$). [§]Values with different superscript letters in the same row are significantly different from each other ($P < 0.05$). [‡]Mixed-model simple effects of treatment sliced by genotype by using SAS SLICE function when diet and diet by gene were statistically significant, AA ($P < 0.0001$) and G/- ($P < 0.0001$). *LEPR*, Leptin receptors; CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil; MCFA, medium chain fatty acids; LCFA, long chain fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Pearson's correlation between plasma FAs and body composition variables are presented in Table 6.7–6.8. Significant inverse associations were observed between C18:1n9 and changes in VATM, AM, and BW in the overall/composite group as well as HOCO group. Additionally, intake of RCO treatment led to negative associations ($r = -0.19$; $P < 0.0480$) between MUFA and changes in AM. Also, inverse associations were observed between MUFA and changes in BW post-HOCO intake ($r = -0.27$; $P < 0.0032$) and composite group ($r = -0.13$; $P < 0.0149$), respectively. In comparison, the CO group failed to express significant negative associations with all of the body composition variables investigated.

6.4.7 Correlations with body composition by dietary treatments in genetic variants

6.4.7.1 Correlations between plasma fatty acid and body composition variables by dietary treatments in CD36 rs1761667

When correlations were investigated between changes in AM and plasma PUFA, post-RCO consumption in rs1761667-A/G genotype carriers, moderate positive associations were observed ($r = 0.31$; $P < 0.0305$) (Table 6.9). Moreover, in response to RCO and HOCO consumption, an inverse association ($r = -0.36$; $P < 0.0108$) and ($r = -0.50$; $P < 0.0003$) was detected, respectively, between plasma C18:1n9 and changes in BW. Furthermore, correlational analyses for all subjects across all dietary treatments (overall group) also revealed similar statistically significant negative relationships between plasma C18:1n9 and alterations in BW as well as changes in AM in AG-genotype. On the

Table 6.7 Pearson's correlation between plasma fatty acid (g/100g)* and body composition variable changes[‡] by dietary treatments.

Fatty acid	Parameter	Dietary treatments															
		CO				RCO				HOCO				Composite[†]			
		Body Composition Variables															
		VATM Δ	SATM Δ	AM Δ	BW Δ	VATM Δ	SATM Δ	AM Δ	BW Δ	VATM Δ	SATM Δ	AM Δ	BW Δ	VATM Δ	SATM Δ	AM Δ	BW Δ
C18:1n9	***r***	– 0.06	– 0.04	– 0.06	– 0.06	– 0.15	– 0.21	– 0.27	– 0.31	– 0.25	– 0.06	– 0.26	– 0.38	– 0.11	– 0.08	– 0.14	– 0.18
	P	0.5445	0.6565	0.5152	0.5150	0.1094	0.0284	0.0038	0.0008	0.0069	0.5171	0.0058	<0.0001	0.0418	0.1571	0.0075	0.0006
C18:2n6	***r***	0.02	0.03	0.04	0.07	– 0.05	0.07	0.01	– 0.03	0.04	– 0.07	– 0.03	– 0.03	– 0.01	0.00	– 0.01	0.00
	P	0.7925	0.7492	0.6689	0.4361	0.5771	0.4694	0.9474	0.7610	0.6943	0.4335	0.7110	0.7709	0.8440	0.9790	0.9081	0.9841
MUFA	***r***	– 0.06	– 0.00	– 0.03	– 0.10	– 0.09	– 0.15	– 0.19	– 0.15	– 0.12	– 0.08	– 0.17	– 0.27	– 0.06	– 0.06	– 0.10	– 0.13
	P	0.5551	0.9643	0.7658	0.2671	0.3154	0.1071	0.0480	0.1065	0.1997	0.3744	0.0710	0.0032	0.2398	0.2474	0.0739	0.0149
PUFA	***r***	– 0.07	– 0.11	– 0.14	0.02	– 0.01	0.03	0.01	– 0.05	0.10	0.01	0.08	– 0.16	– 0.00	– 0.03	– 0.03	– 0.07
	P	0.4463	0.2632	0.1409	0.8508	0.8777	0.7746	0.9360	0.6063	0.3035	0.9517	0.3678	0.0873	0.9469	0.6043	0.6358	0.2258

Note: *The values are % abundance of each fatty acid to total fatty acids. [‡]Changes measured as difference between endpoint and baseline values. $n = 115$; r, Pearson correlation coefficients; statistical significance assessed at ($P < 0.05$). CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil; VATM, visceral adipose tissue mass; SATM, subcutaneous adipose tissue mass; AM, android mass; BW, body weight; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. [†]Composite of all three dietary interventions.

Table 6.8 Pearson's correlation between plasma fatty acid (g/100g)* and multiple variable changes‡ by dietary treatments.

Fatty acid	Parameter	Dietary treatments							
		CO		RCO		HOCO		Composite†	
		Body Composition Variables							
		AM Δ (%)	BW Δ (%)	AM Δ (%)	BW Δ (%)	AM Δ (%)	BW Δ (%)	AM Δ (%)	BW Δ (%)
C18:1n9	***r***	– 0.02	– 0.03	– 0.30	– 0.30	– 0.25	– 0.36	– 0.13	– 0.17
	P	0.7987	0.7669	0.0012	0.0010	0.0068	<0.0001	0.0164	0.0017
C18:2n6	***r***	– 0.07	0.03	– 0.08	– 0.05	– 0.11	– 0.06	– 0.10	– 0.03
	P	0.4694	0.7792	0.3785	0.6240	0.2490	0.5288	0.0611	0.5806
MUFA	***r***	0.03	– 0.07	– 0.21	– 0.15	– 0.15	– 0.24	– 0.07	– 0.11
	P	0.7508	0.4693	0.0252	0.1092	0.1165	0.0088	0.1817	0.0338
PUFA	***r***	– 0.18	– 0.02	– 0.08	– 0.07	0.06	– 0.18	– 0.08	– 0.09
	P	0.0570	0.8376	0.3978	0.4818	0.5100	0.0602	0.1509	0.0983

Note: *The values are % abundance of each fatty acid to total fatty acids. ‡Changes measured as percentage change between endpoint and baseline values. n = 112–115; r, Pearson correlation coefficients; statistical significance assessed at ($P < 0.05$). CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil; AM, android fat mass; BW, body weight; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. †Composite of all three dietary interventions.

Table 6.9 Pearson's correlation between plasma fatty acid (g/100g)* and body composition variable changes[‡] by dietary treatments in *CD36* rs1761667 genotypes.

Genotype	Fatty acid	Parameter	Dietary treatments															
			CO				RCO				HOCO				Composite[†]			
			Body Composition Variables															
			VATM Δ	SATM Δ	AM Δ	BW Δ	VATM Δ	SATM Δ	AM Δ	BW Δ	VATM Δ	SATM Δ	AM Δ	BW Δ	VATM Δ	SATM Δ	AM Δ	BW Δ
A/A (n = 24)																		
	C18:1n9	***r***	– 0.18	– 0.04	– 0.18	0.31	0.02	– 0.22	– 0.15	– 0.27	– 0.33	0.11	– 0.23	– 0.27	– 0.14	0.08	– 0.06	– 0.03
		P	0.3886	0.8614	0.3991	0.1421	0.9388	0.3045	0.4726	0.1955	0.1185	0.6243	0.2772	0.2071	0.2402	0.5217	0.6396	0.7807
	C18:2n6	***r***	– 0.06	0.21	0.12	0.25	0.01	0.09	0.08	0.04	– 0.01	– 0.20	– 0.21	– 0.26	– 0.01	– 0.04	– 0.04	0.00
		P	0.7960	0.3296	0.5654	0.2448	0.9631	0.6626	0.7113	0.8552	0.9582	0.3409	0.3308	0.2163	0.9302	0.7490	0.7276	0.9768
	MUFA	***r***	– 0.12	0.00	– 0.09	0.18	0.08	– 0.15	– 0.05	– 0.21	– 0.17	0.15	– 0.02	– 0.17	– 0.05	0.10	0.04	– 0.03
		P	0.5867	0.9965	0.6615	0.4088	0.7016	0.4749	0.8112	0.3281	0.4316	0.4696	0.9198	0.4252	0.6710	0.3979	0.7263	0.7938
	PUFA	***r***	– 0.02	– 0.15	– 0.14	0.03	– 0.31	0.04	– 0.22	– 0.21	– 0.04	– 0.04	– 0.08	– 0.33	– 0.13	– 0.08	– 0.18	– 0.19
		P	0.9432	0.4801	0.5283	0.8906	0.1448	0.8628	0.3123	0.3219	0.8525	0.8553	0.7149	0.1098	0.2734	0.4881	0.1218	0.1091
A/G (n = 48)																		
	C18:1n9	***r***	– 0.12	– 0.04	– 0.10	– 0.22	– 0.24	– 0.17	– 0.33	– 0.36	– 0.15	– 0.13	– 0.27	– 0.50	– 0.13	-0.14	– 0.21	– 0.29
		P	0.4272	0.7956	0.4916	0.1403	0.1068	0.2356	0.0211	0.0108	0.3148	0.3712	0.0630	0.0003	0.1134	0.1048	0.0118	0.0003
	C18:2n6	***r***	0.02	– 0.08	– 0.04	– 0.02	0.08	0.12	0.16	– 0.07	– 0.06	– 0.10	– 0.16	– 0.06	0.02	0.01	0.02	– 0.04
		P	0.8871	0.5749	0.7974	0.9150	0.5678	0.4131	0.2774	0.6414	0.4944	0.2919	0.7032	0.0870	0.8510	0.9407	0.8346	0.6619
	MUFA	***r***	– 0.13	– 0.02	– 0.10	– 0.21	– 0.14	– 0.13	– 0.22	– 0.22	– 0.04	– 0.14	– 0.17	– 0.42	– 0.08	– 0.12	– 0.16	– 0.25
		P	0.3760	0.8763	0.4948	0.1604	0.3348	0.3604	0.1308	0.1282	0.7896	0.3582	0.2430	0.0027	0.3275	0.1438	0.0580	0.0027
	PUFA	***r***	– 0.17	0.00	– 0.11	– 0.09	0.21	0.18	0.31	0.08	0.18	– 0.11	0.06	0.10	0.09	0.05	0.11	0.03
		P	0.2534	0.9809	0.4606	0.5528	0.1520	0.2226	0.0305	0.5794	0.2309	0.4728	0.6838	0.4793	0.2978	0.5776	0.2051	0.6875

Genotype	Fatty acid	Parameter	Dietary treatments															
			CO				RCO				HOCO				Composite[†]			
			Body Composition Variables															
			VATM Δ	SATM Δ	AM Δ	BW Δ	VATM Δ	SATM Δ	AM Δ	BW Δ	VATM Δ	SATM Δ	AM Δ	BW Δ	VATM Δ	SATM Δ	AM Δ	BW Δ
G/G (n = 25)																		
	C18:1n9	***r***	0.07	– 0.05	– 0.01	– 0.16	0.17	– 0.23	– 0.03	– 0.55	– 0.27	0.26	0.08	– 0.23	0.17	0.09	0.19	– 0.21
		P	0.7558	0.8072	0.9520	0.4324	0.4288	0.2675	0.8717	0.0045	0.1940	0.2111	0.7099	0.2714	0.1398	0.4256	0.0957	0.0720
	C18:2n6	***r***	– 0.08	0.09	0.04	0.16	– 0.29	– 0.11	– 0.27	– 0.27	0.28	– 0.09	0.09	– 0.02	– 0.14	– 0.06	– 0.14	– 0.01
		P	0.6972	0.6691	0.8392	0.4569	0.1538	0.6054	0.1878	0.1928	0.1676	0.6540	0.6556	0.9353	0.2435	0.5846	0.2149	0.9447
	MUFA	***r***	0.05	– 0.05	– 0.02	– 0.26	0.01	– 0.10	– 0.06	– 0.25	– 0.15	0.08	– 0.02	– 0.23	0.12	0.07	0.14	– 0.17
		P	0.8288	0.8117	0.9095	0.2143	0.9528	0.6266	0.7861	0.2222	0.4615	0.6882	0.9316	0.2699	0.2926	0.5444	0.2230	0.1352
	PUFA	***r***	– 0.17	0.05	– 0.05	0.33	0.21	– 0.15	0.04	0.17	0.05	– 0.10	– 0.07	– 0.36	– 0.02	– 0.08	– 0.08	0.08
		P	0.4296	0.8108	0.8077	0.1098	0.3245	0.4658	0.8370	0.4280	0.8025	0.6292	0.7572	0.0750	0.8679	0.5057	0.4888	0.4749

Note: *The values are % abundance of each fatty acid to total fatty acids. [‡]Changes measured as difference between endpoint and baseline values. *r*, Pearson correlation coefficients; statistical significance assessed at ($P < 0.05$). *CD36*, cluster of differentiation 36; CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil; VATM, visceral adipose tissue mass; SATM, subcutaneous adipose tissue mass; AM, android mass; BW, body weight; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. [†]Composite of all three dietary interventions.

contrary, for the AA-genotype, no such associations were detected. However, GG-genotype showed a moderate negative correlation ($r = -0.55$; $P < 0.0045$) post-RCO consumption between C18:1n9 and overall changes in BW.

6.4.7.2 Correlations between plasma fatty acid and body composition variables by dietary treatments in GPR40 rs1573611

When correlational analyses were performed based on the genotype, plasma C18:1n9 expressed a negative association with changes in BW ($r = -0.17$; $P < 0.0436$) in the overall group by rs1573611-C/C ($n = 46$) (Table 6.10). However, both RCO and HOCO consumption showed moderate negative associations between C18:1n9 and changes in BW ($r = -0.38$; $P < 0.0087$) and ($r = -0.32$; $P < 0.0309$), respectively. Similarly, rs1573611-C/T ($n = 41$) showed negative associations between C18:1n9 and changes in BW in RCO as well as HOCO groups. Furthermore, in the participants carrying rs1573611-T/T genotype ($n = 7$), a strong positive association was detected between plasma PUFA and changes in BW ($r = 0.92$; $P < 0.0037$) post-CO intake. On the contrary, post-RCO consumption showed a strong inverse relationship ($r = -0.89$; $P < 0.0080$) between C18:1n9 and changes in SATM in carriers of TT genotype. Similarly, HOCO consumption expressed very strong negative associations ($r = -0.85$; $P < 0.0156$), between C18:1n9 and changes in BW in participants carrying TT genotype.

6.4.7.3 Correlations between plasma fatty acid and body composition variables by dietary treatments in LEPR rs1137101

Table 6.10 Pearson's correlation between plasma fatty acid (g/100g)* and body composition variable changes[‡] by dietary treatments in *GPR40* rs1573611 genotypes.

Genotype	Fatty acid	Parameter	Dietary treatments															
			CO				RCO				HOCO				Composite[†]			
			Body Composition Variables															
			VATM Δ	SATM Δ	AM Δ	BW Δ	VATM Δ	SATM Δ	AM Δ	BW Δ	VATM Δ	SATM Δ	AM Δ	BW Δ	VATM Δ	SATM Δ	AM Δ	BW Δ
C/C (n = 46)																		
	C18:1n9	***r***	0.21	– 0.15	0.06	– 0.10	– 0.28	– 0.20	– 0.37	– 0.38	– 0.20	0.13	– 0.03	– 0.32	– 0.10	0.10	– 0.01	– 0.17
		P	0.1671	0.3116	0.7112	0.4972	0.0635	0.1814	0.0112	0.0087	0.1818	0.3804	0.8357	0.0309	0.2405	0.2545	0.9465	0.0436
	C18:2n6	***r***	– 0.15	0.12	– 0.04	0.18	0.09	– 0.02	0.06	– 0.04	0.13	– 0.23	– 0.10	0.04	0.02	– 0.14	– 0.08	0.04
		P	0.3066	0.4323	0.7999	0.2299	0.5469	0.8733	0.6909	0.7796	0.3801	0.1306	0.5277	0.8129	0.8162	0.1132	0.3298	0.6008
	MUFA	***r***	0.13	– 0.15	– 0.00	– 0.23	– 0.28	– 0.10	– 0.31	– 0.25	– 0.10	0.22	0.12	– 0.17	– 0.11	0.13	0.01	– 0.15
		P	0.4004	0.3151	0.9867	0.1286	0.0579	0.5075	0.0383	0.0898	0.5186	0.1344	0.4329	0.2595	0.2104	0.1295	0.8845	0.0818
	PUFA	***r***	– 0.07	– 0.00	– 0.06	0.11	0.12	– 0.07	0.05	0.14	0.00	– 0.17	– 0.14	– 0.25	0.04	– 0.14	– 0.07	– 0.01
		P	0.6280	0.9814	0.7091	0.4711	0.4285	0.6219	0.7440	0.3575	0.9821	0.2641	0.3584	0.1001	0.6759	0.1012	0.3836	0.8770
C/T (n = 41)																		
	C18:1n9	***r***	– 0.28	– 0.09	– 0.25	– 0.14	– 0.10	– 0.14	– 0.16	– 0.41	– 0.15	– 0.09	– 0.25	– 0.37	– 0.06	– 0.11	– 0.13	– 0.31
		P	0.0785	0.5703	0.1078	0.3779	0.5543	0.3796	0.3298	0.0077	0.3634	0.5547	0.1164	0.0177	0.5424	0.2425	0.1567	0.0005
	C18:2n6	***r***	0.09	0.12	0.16	– 0.15	– 0.02	0.07	0.03	– 0.16	– 0.15	– 0.05	– 0.21	– 0.28	– 0.06	0.06	– 0.00	– 0.14
		P	0.5779	0.4553	0.3165	0.3619	0.9106	0.6511	0.8561	0.3151	0.3350	0.7695	0.1953	0.0721	0.4830	0.5373	0.9614	0.1265
	MUFA	***r***	– 0.22	– 0.07	– 0.20	– 0.09	0.04	– 0.05	0.00	– 0.13	0.02	– 0.14	– 0.13	– 0.33	0.04	– 0.09	– 0.04	– 0.21
		P	0.1604	0.6489	0.1991	0.5706	0.7931	0.7647	0.9812	0.4247	0.8978	0.3931	0.4310	0.0359	0.6616	0.3164	0.6421	0.0211
	PUFA	***r***	– 0.19	0.07	– 0.06	0.04	0.10	0.28	0.24	0.04	0.19	– 0.17	0.01	– 0.07	0.02	0.06	0.07	0.04
		P	0.2377	0.6765	0.7037	0.8187	0.5361	0.0758	0.1275	0.8059	0.2258	0.2792	0.9558	0.6792	0.8305	0.4913	0.4715	0.6691

Genotype	Fatty acid	Parameter	Dietary treatments															
			CO				RCO				HOCO				Composite[†]			
			Body Composition Variables															
			VATM Δ	SATM Δ	AM Δ	BW Δ	VATM Δ	SATM Δ	AM Δ	BW Δ	VATM Δ	SATM Δ	AM Δ	BW Δ	VATM Δ	SATM Δ	AM Δ	BW Δ
T/T (n = 7)																		
	C18:1n9	*r*	– 0.58	0.51	– 0.10	0.01	0.71	– 0.89	– 0.42	– 0.42	– 0.52	– 0.63	– 0.63	– 0.85	– 0.01	– 0.21	– 0.25	– 0.05
		P	0.1715	0.2461	0.8312	0.9910	0.0712	0.0080	0.3436	0.3434	0.2349	0.1323	0.1318	0.0156	0.9489	0.3614	0.2740	0.8225
	C18:2n6	*r*	– 0.26	– 0.34	– 0.47	0.71	– 0.02	0.09	0.14	0.00	0.16	– 0.34	– 0.05	– 0.10	– 0.08	– 0.07	– 0.17	0.02
		P	0.5669	0.4535	0.2904	0.0763	0.9737	0.8398	0.7635	0.9938	0.7340	0.4523	0.9124	0.8286	0.7380	0.7513	0.4666	0.9406
	MUFA	*r*	– 0.41	0.47	0.01	– 0.13	0.58	– 0.87	– 0.60	– 0.58	– 0.77	– 0.72	– 0.83	– 0.94	– 0.04	– 0.28	– 0.36	– 0.21
		P	0.3611	0.2896	0.9812	0.7738	0.1723	0.0117	0.1537	0.1704	0.0441	0.0687	0.0196	0.0016	0.8560	0.2205	0.1114	0.3651
	PUFA	*r*	– 0.27	– 0.06	– 0.27	0.92	– 0.07	– 0.19	– 0.45	– 0.63	0.47	– 0.03	0.30	0.18	0.01	– 0.13	– 0.13	– 0.13
		P	0.5554	0.8960	0.5629	0.0037	0.8766	0.6785	0.3117	0.1324	0.2860	0.9552	0.5153	0.7053	0.9765	0.5833	0.5628	0.5672

Note: *The values are % abundance of each fatty acid to total fatty acids. [‡]Changes measured as difference between endpoint and baseline values. *r*, Pearson correlation coefficients; statistical significance assessed at ($P < 0.05$). *GPR40*, G protein-coupled receptors 40; CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil; VATM, visceral adipose tissue mass; SATM, subcutaneous adipose tissue mass; AM, android mass; BW, body weight; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. [†]Composite of all three dietary interventions.

Table 6.11 outlines the correlations observed between FA levels and body composition measurements for the composite as well as intervention groups by *LEPR*. In participants with rs1137101-AA ($n = 25$), significant inverse associations were observed between C18:1n9 and changes in BW post-RCO and -HOCO consumption. Similar weak negative albeit significant ($r = -0.36$, $P = 0.0013$) associations were also detected in the composite group. Furthermore, in AA genotype, where all three treatments failed to show the significant associations between C18:2n6 and changes in BW, a negative correlation was revealed in composite group ($r = -0.25$, $P = 0.0305$). On the other hand, individuals with the rs1137101-AG ($n = 50$) allele were found to have a moderate inverse relationship ($r = -0.41$, $P = 0.0033$) between C18:1n9 and BW post-HOCO ingestion, where all other FAs failed to show significant associations across all dietary treatments. Additionally, the rs1137101-GG ($n = 50$) allele expressed significant inverse associations ($r = -0.43$, $P = 0.0444$) between C18:1n9 and changes in VATM, in response to HOCO consumption. Furthermore, the GG-allele showed a strong inverse relationship ($r = -0.66$, $P = 0.0008$) between plasma total PUFA level and changes in SATM post-HOCO intake. Moreover, in GG genotype, the comparison between the overall group (composite of all three dietary interventions) and individual intervention groups, PUFA was positively associated with changes in VATM ($r = 0.26$, $P = 0.0387$), whereas a negative relationship ($r = -0.24$, $P = 0.0498$) was observed with changes in SATM.

6.5 Discussion

Table 6.11 Pearson's correlation between plasma fatty acid (g/100g)* and body composition variable changes[‡] by dietary treatments in *LEPR* rs1137101 genotypes.

Genotype	Fatty acid	Parameter	Dietary treatments															
			CO				RCO				HOCO				Composite[†]			
			Body Composition Variables															
			VATM Δ	SATM Δ	AM Δ	BW Δ	VATM Δ	SATM Δ	AM Δ	BW Δ	VATM Δ	SATM Δ	AM Δ	BW Δ	VATM Δ	SATM Δ	AM Δ	BW Δ
A/A (n = 25)																		
	C18:1n9	***r***	0.03	– 0.13	– 0.10	– 0.29	– 0.13	– 0.37	– 0.31	– 0.59	– 0.18	– 0.13	– 0.23	– 0.44	0.02	– 0.17	– 0.12	– 0.36
		P	0.8867	0.5357	0.6477	0.1596	0.5512	0.0685	0.1260	0.0018	0.3892	0.5443	0.1792	0.0294	0.8879	0.1392	0.3090	0.0013
	C18:2n6	***r***	0.14	0.14	0.21	– 0.32	– 0.31	0.34	– 0.04	– 0.13	– 0.24	– 0.03	– 0.26	– 0.35	– 0.20	0.14	– 0.05	– 0.25
		P	0.5168	0.5014	0.3233	0.1143	0.1255	0.0985	0.8422	0.5306	0.2386	0.8866	0.2103	0.0901	0.0786	0.2268	0.6710	0.0305
	MUFA	***r***	0.04	– 0.05	– 0.02	– 0.13	– 0.05	– 0.35	– 0.24	– 0.43	– 0.01	– 0.26	– 0.22	– 0.36	0.09	– 0.20	– 0.08	– 0.28
		P	0.8662	0.8213	0.9229	0.5260	0.8308	0.0816	0.2401	0.0317	0.9639	0.2079	0.2823	0.0803	0.4459	0.0895	0.4844	0.0166
	PUFA	***r***	– 0.18	– 0.14	– 0.23	– 0.03	– 0.21	0.13	– 0.09	0.01	– 0.11	0.16	0.03	– 0.10	– 0.21	0.04	– 0.13	– 0.04
		P	0.3905	0.5014	0.2641	0.8741	0.3073	0.5319	0.6837	0.9707	0.6000	0.4340	0.8908	0.6328	0.0645	0.7272	0.2502	0.7554
A/G (n = 50)																		
	C18:1n9	***r***	– 0.15	0.10	– 0.03	– 0.04	– 0.11	– 0.18	– 0.24	– 0.22	– 0.16	– 0.05	– 0.20	– 0.41	– 0.10	– 0.01	– 0.09	– 0.16
		P	0.3138	0.4965	0.8503	0.8019	0.4523	0.2191	0.0974	0.1264	0.2650	0.7445	0.1652	0.0033	0.2247	0.8890	0.2682	0.0537
	C18:2n6	***r***	– 0.10	0.02	– 0.06	0.12	0.04	0.00	0.04	– 0.14	– 0.04	– 0.03	– 0.07	– 0.02	– 0.03	– 0.02	– 0.04	0.01
		P	0.4805	0.8964	0.7022	0.4245	0.7578	0.9815	0.7707	0.3188	0.8063	0.8249	0.6387	0.8912	0.7127	0.7833	0.5959	0.9201
	MUFA	***r***	– 0.13	0.05	– 0.05	– 0.09	0.00	– 0.08	– 0.06	0.02	0.03	– 0.01	0.02	– 0.27	– 0.02	0.01	– 0.01	– 0.09
		P	0.3688	0.7074	0.7376	0.5299	0.9780	0.5837	0.6807	0.8995	0.8370	0.9467	0.9091	0.0535	0.7928	0.9129	0.9021	0.2728
	PUFA	***r***	– 0.20	0.05	– 0.10	0.07	0.22	0.08	0.26	– 0.01	0.02	– 0.01	0.01	– 0.17	0.03	0.02	0.04	– 0.03
		P	0.1726	0.7531	0.4912	0.6147	0.1337	0.5604	0.0710	0.9231	0.9002	0.9557	0.9576	0.2325	0.7596	0.7762	0.6261	0.7462

Genotype	Fatty acid	Parameter	Dietary treatments															
			CO				RCO				HOCO				Composite†			
			Body Composition Variables															
			VATM Δ	SATM Δ	AM Δ	BW Δ	VATM Δ	SATM Δ	AM Δ	BW Δ	VATM Δ	SATM Δ	AM Δ	BW Δ	VATM Δ	SATM Δ	AM Δ	BW Δ
G/G (n = 22)																		
	C18:1n9	***r***	0.01	– 0.44	– 0.30	– 0.09	– 0.16	– 0.11	– 0.16	– 0.34	– 0.43	0.35	– 0.03	– 0.36	– 0.17	0.13	– 0.03	– 0.15
		P	0.9740	0.0421	0.1678	0.7056	0.4863	0.6261	0.4859	0.1247	0.0444	0.1112	0.8806	0.0954	0.1751	0.2858	0.8386	0.2302
	C18:2n6	***r***	0.02	0.12	0.10	0.32	0.24	– 0.06	0.13	– 0.01	0.40	– 0.42	– 0.05	0.01	0.22	– 0.22	– 0.00	0.08
		P	0.9146	0.6046	0.6568	0.1420	0.2827	0.7863	0.5753	0.9645	0.0616	0.0531	0.8258	0.9486	0.0786	0.0729	0.9795	0.5415
	MUFA	***r***	0.07	– 0.48	– 0.29	– 0.24	– 0.22	– 0.00	– 0.14	– 0.25	– 0.52	0.41	– 0.05	– 0.41	– 0.22	0.17	– 0.03	– 0.19
		P	0.7632	0.0236	0.1868	0.2815	0.3266	0.9898	0.5201	0.2634	0.0138	0.0575	0.8383	0.0588	0.0747	0.1643	0.7848	0.1203
	PUFA	***r***	0.14	0.11	0.14	0.21	0.26	– 0.01	0.17	0.07	0.51	– 0.66	– 0.19	0.04	0.26	– 0.24	0.01	0.06
		P	0.5470	0.6254	0.5266	0.3391	0.2394	0.9639	0.4603	0.7419	0.0164	0.0008	0.4016	0.8693	0.0387	0.0498	0.9435	0.6347

Note: *The values are % abundance of each fatty acid to total fatty acids. ‡Changes measured as difference between endpoint and baseline values. *r*, Pearson correlation coefficients; statistical significance assessed at ($P < 0.05$). *LEPR*, leptin receptors; CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil; VATM, visceral adipose tissue mass; SATM, subcutaneous adipose tissue mass; AM, android mass; BW, body weight; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. †Composite of all three dietary interventions.

Our results suggest that dietary HOCO and RCO consumption may have beneficial effects on BW and obesity risk by preventing ectopic fat storage, since inverse associations were observed between C18:1n9 and BW changes post-HOCO and RCO ingestion. On the contrary, post-CO consumption failed to express such relationships. Furthermore, data suggest that participants possessing *LEPR*-AA polymorphisms would benefit more from the intake of C18:1n9 enriched oils by regulating appetite, due to enhanced satiation induced by DIT increasing energy expenditure (7, 8). In the present study, correlations between FAs and changes in variables of DXA by diet were investigated. Although no diet × gene interactions were observed, we successfully demonstrated that based on genotype pattern, increases in C18:1n9 composition in regular diets may bring favourable alterations in body composition in a manner that depends on genetic architecture; and to the best of our knowledge, this is the first study showing such associations.

A diet enriched in C18:1n9 MUFA has been found to be more highly oxidized for energy than one rich in C18:2n6 PUFA (16, 31). This is likely because C18:1n9 synthesizes OEA (16) stimulating the β-adrenergic signalling by enhancing cyclic adenosine monophosphate (cAMP) concentration and protein kinase A (PKA) activity, which subsequently induces peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) activation and fatty acid oxidation (32). In addition, FAs stimulate the interaction between PGC-1α and peroxisome proliferator-activated receptors (PPARs) activating the thermogenic genes such as uncoupling protein-1

(*UCP1*) (33); which further activate the β-adrenergic receptors, demonstrating the synergistic effect of DFA and β-adrenergic signalling on thermogenesis. Furthermore, β-adrenergic activation and signalling, inducing DIT, is a known mechanism of thermogenic stimulation in brown adipose tissue (BAT) which activates the oxidative genes in multiple tissues that alters the metabolism as well as oxidation of DFA (34); subsequently, enhancing glucose uptake in skeletal muscle and adipose tissue inducing lipolysis (35). Therefore, the overall synthesis of OEA inducing satiety, and further activation, as well as stimulation of β-adrenergic receptors, could potentially result in improved FA clearance leading to reduced obesogenic fat deposition with monounsaturated fat consumption.

Furthermore, previous reports (16, 22, 36, 37) suggest that FAs stimulate fat taste receptors inducing satiating pathways in the oral/buccal cavity and in the alimentary canal. However, studies evaluating the relationship between fat taste receptors and body composition are sparse. Therefore, we investigated the effect of the fat taste receptor genes on various fat depots in humans. The *CD36* and *GPR40* have been studied extensively for their role in facilitating LCFA uptake and oxidation, positioning both of these genes as critical players in FA metabolism (38). When the *CD36* rs1761667-AG carriers were compared with the rs1761667-AA, the negative relationship between C18:1n9 and changes in BW were observed post-HOCO and -RCO ingestion; this might be due to the enhanced CD36 protein expression in AG-carriers stimulating the anorexic signalling by upregulating the FA oxidation. A similar effect detected with GG-genotype might be due to the highly expressed *CD36* receptor which has a significant role in the

regulation of FA entry into the cell (39).

Moreover, the major agonists of GPRs are FAs with a chain length greater than 6 carbons, namely MCFA and LCFA (40). Also, the C-allele and TT-genotype in *GPR40* rs1573611 are observed to be associated with increased body fat (41). Regardless, in our study, consumption of RCO, as well as HOCO, showed inverse associations between C18:1n9 and changes in BW. This beneficial negative relationship might be due to the improved insulin sensitivity in these participants because of the integrated two-receptor signal transduction cascade (42); also, because stimulation of GPR40 activates the PPAR-**γ** dependent transcription through the downstream effects on PGC-1α, enhancing FA oxidation. Moreover, inactivation of PPAR-**γ** in adipose tissue decreases the fat mass expansion since PPAR-**γ** plays a critical role in cell differentiation converting preadipocytes to adipocytes, which alters the hyperplasia as well as the hypertrophy of the adipocytes (43). Additionally, the attenuated PPAR-**γ** expression in BAT leads to the increase in the hepatic PPAR-**γ** and CD36 gene expressions (44) thereby influencing SATM. Therefore, the impact on overall phenotype may occur due to the fact that SATM contains beige precursor cells/adipocytes in comparison to VATM (45). Henceforth, the cumulative actions may counteract the ectopic lipid accumulation in non-adipocyte cells preventing insulin resistance and subsequently preventing lipotoxicity (46).

Additionally, LEPR plays a vital role in the modulation of body energy homeostasis and fat storage (47). Genetic variants in the human *LEPR* may profoundly influence BW and insulin resistance. For instance, the human trial conducted by Hart

Sailors *et al.* (48) showed that Caucasian participants with A-allele carriers express lower circulating leptin levels, consistent with the findings from our present trial. On the contrary, G-allele is primarily associated with increased adiposity and escalated percent fat mass (47). Moreover, in the present trial, a weak positive relationship was detected between overall PUFA intake and changes in VATM in G-allele carriers, which might be the effect of insulin resistance as well as dysregulation of Janus kinase 2-signal transducers and activators of transcription 3 (JAK2/STAT3) cascades (49) in G-allele carriers leading to leptin insensitivity, hyperphagia, metabolic and endocrine abnormalities such as obesity. These effects may occur since leptin binds to the long form of LEPR of multiple neuronal populations activating JAK2/STAT3 cascades which play a key role in the modulation of appetite. Present results are consistent with the previous trial conducted by Phillips *et al.* (50) who showed the insulin resistance was prominent in G-allele carriers of *LEPR* post-n6 PUFA consumption. In contrast, C18:1n9 consumption and plasma MUFA status did not adversely affect the phenotype, indicative of leptin sensitivity (51).

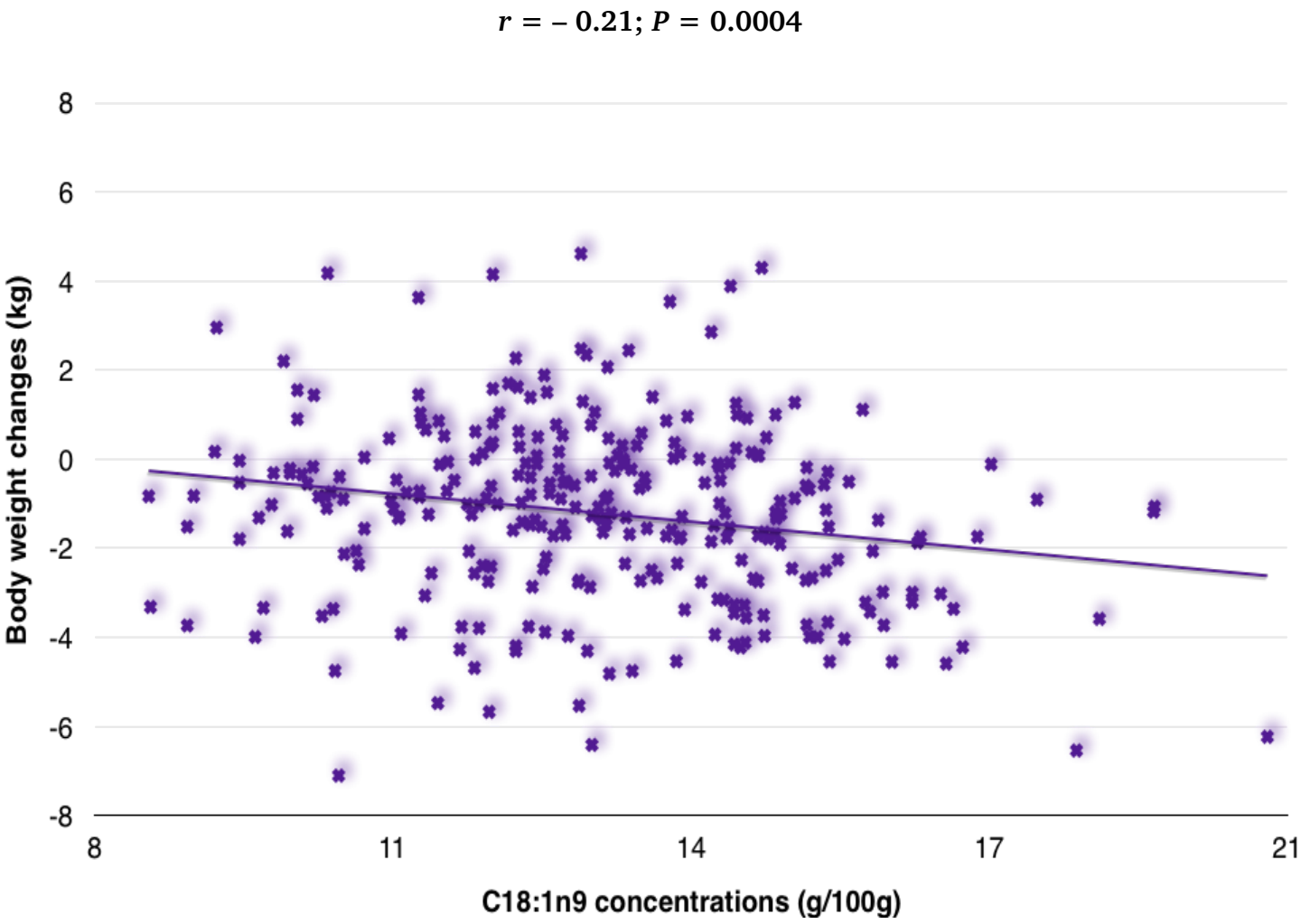
In addition, data suggest that the two FA receptors CD36 (52) and GPR40 (53, 54) are involved in nutrient sensing (55) and may play a crucial role in signalling pathways that mediate FA detection in the mouth as well as the gut (16, 56). On the contrary, LEPR are involved in energy homeostasis by the release of leptin hormone (57), which diminishes food intake by modulating the endocannabinoid system in a tissue- or cell-specific manner (58). Moreover, leptin stimulates FA oxidation in skeletal muscle (59). Therefore, in the present trial, we showed that an inverse relationship

exists between the intake of C18:1n9 and changes in BW, likely mediated at least in part by *LEPR* (Figure 6.2). A similar inverse correlation was replicated when the associations were investigated by *LEPR* rs1137101-AA allele (Figure 6.3). The negative associations observed between ingestion of C18:1n9 and changes in BW in carriers of rs1137101-AA genotype is likely due to greater leptin sensitivity, maintaining resting energy expenditure at lower leptin concentrations, thereby, promoting decreased caloric intake, and improving overall body composition through maintenance of lower BW (60).

The present study has several strengths. To the best of our knowledge, this is the first study that has demonstrated the relationships between plasma FAs and body composition based on genotypes under controlled DFA feeding states with low vs high MUFA DFA. Moreover, the present trial explored the differential effects of altering DFA composition across different adipose tissues accurately by the use of DXA system. We successfully demonstrated that genetic influences modulated the plasma FA composition, with beneficial effects more evident against a background of high MUFA intake. The study design strength includes the focus on dietary quality, repeated measure design that provides for repeated major-time points of data collection, and the extensive range of types of data collected. Robustness of the study conduct includes large sample size. Furthermore, in this trial, we used a 100m GC column wherein we successfully identified and reported fifty different FAs in human plasma post consumption of dietary oils.

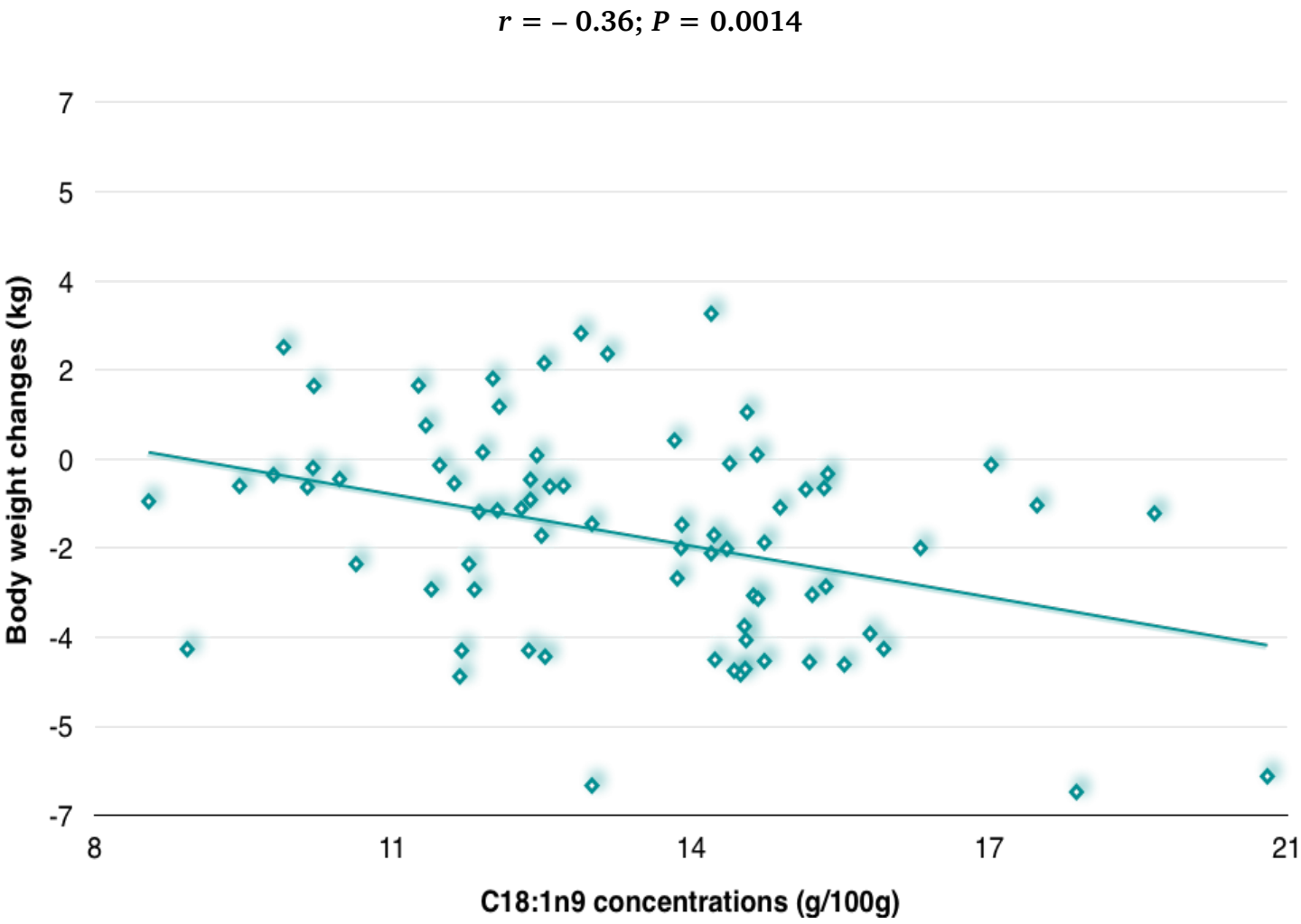
At the same time, limitations of the study exist. To report a few, it is important to mention that plasma and RBC FA patterns both correlate with that of dietary fat

Figure 6.2 The association between C18:1n9 levels (g/100g)* and body weight changes[‡] (kg) by *LEPR* rs1137101 after six-weeks consumption of dietary oils[#†].



Note: *The values are % abundance of C18:1n9 to total fatty acids. [‡]Changes measured as percentage change between endpoint and baseline values. $n = 97$; r, Pearson correlation coefficient; *LEPR*, leptin receptors; [#]CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil. [†]Composite of all three dietary interventions.

Figure 6.3 The association between C18:1n9 levels (g/100g)* and body weight changes[‡] (kg) by *LEPR* rs1137101-A/A after six-weeks consumption of dietary oils[#†].



Note: *The values are % abundance of C18:1n9 to total fatty acids. [‡]Changes measured as percentage change between endpoint and baseline values. $n = 25$; r, Pearson correlation coefficient; *LEPR*, leptin receptors; [#]CO, control oil; RCO, regular canola oil; HOCO, high oleic canola oil. [†]Composite of all three dietary interventions.

consumption (61). However, the RBC FA has been suggested to reflect the long-term DFA intake, and act as a better biomarker or indices of DFA ingestion. Furthermore, RBC is proposed to be a better reflector of FA metabolism (62). Additionally, in the present trial, the associations were conducted based on total FAs analysed; however, it would be interesting to see the associations with FAs investigated based on each lipid fraction such as phospholipid or cholesterol esters. Henceforth, additional controlled longer-term feeding trials should be conducted to understand the influence of DFA consumption on body composition in more depth by elucidating the relationships among DFA, subfractions of plasma FA pool, and variables of body composition. A further limitation of the present trial was that the satiety and energy expenditure were not measured.

In conclusion, our study shows a negative association between C18:1n9 levels and changes in variables of DXA influencing overall alterations in BW suggesting that C18:1n9 exerts anorectic signals via gut-brain interrelationship (16, 63) that helps maintain healthy BW (12) by enhancing energy expenditure (31). Furthermore, the relationship of plasma C18:1n9 with improved body composition in this overweight population, as a function of genetic architecture, suggests that obesity is a preventable disease that can be treated through personalized nutrition under rapidly emerging multidisciplinary science of nutrigenetics.

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6.7 Authors' contributions

The authors' responsibilities were as follows - PJHJ, CT, BL, and PMK-E: principal investigators at RCFFN, St-Boniface Hospital, INAF, and PSU, respectively; PJHJ, DJAJ, PMK-E, SGW, CT, BL, and PWC: study concept and design; PE: contributed expertise for genotyping studies; TCR: contributed expertise for gene expression work; PJHJ, DJAJ, PMK-E, SGW, BL, PC, CT, and PWC: administrative, technical, or material support; PJHJ, DJAJ, PMK-E, PWC, SGW, BL, PC, and CT: study supervision; JS, SSH, XC, KJB, and PC: clinical trial conduction at four different sites designated for performing Multi-Centre Clinical Trial and acquisition of data; JS: project lead on the human trial at RCFFN, involving clinical trial coordination, meals preparation, laboratory work, analysis and interpretation of the data from all four sites, and drafting of the manuscript; PJHJ: contributed to the preparation of the manuscript.

6.8 Conflict of interest statement

JS, SSH, XC, KJB, PE, and PC have no conflicts of interest to declare in the development of this manuscript.

BL is Chair of Nutrition at Université Laval, which is supported by private endowments from Pfizer, La Banque Royale du Canada and Provigo-Loblaws. BL has received funding in the last 5 years from the Canadian Institutes for Health Research, the

Natural Sciences and Engineering Research Council of Canada, Agriculture and Agri-Food Canada [Growing Forward program supported by the Dairy Farmers of Canada (DFC), Canola Council of Canada, Flax Council of Canada, Dow Agrosciences], Dairy Research Institute, Dairy Australia, Danone Institute, Merck Frosst, Pfizer and Atrium Innovations for which Douglas Laboratories manufacture and market omega-3 supplements. BL served as the Chair of the peer-review Expert Scientific Advisory Council of DFC. BL is also an Advisory Board member of the Canadian Nutrition Society; the Conseil pour les initiatives de progrès en alimentation and has served as Advisory Expert for the Saturated Fat panel of Heart and Stroke Foundation of Canada. BL has also received honoraria from the International Chair on Cardiometabolic risk, DFC and the World Dairy Platform as an invited speaker in various conferences.

CT has received funding in the past 5 years from CIHR, NSERC, Agriculture and Agri-Food Canada (Growing Forward and the Canola/Flax Agri-Science Cluster, Pulse Agri-Science Cluster, Canada-Manitoba Agri-Food Research Development Initiative), Research Manitoba, Manitoba Energy Science and Technology, Manitoba Agri-Health Research Network, Alberta Innovates, Alberta Crop Industry Development Fund, Alberta Canola Producers Commission, Alberta Pulse, Saskatchewan Pulse Growers, Canadian Diabetes Association, and MITACS.

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PMK-E has received research funding from California Walnut Commission, Ag Canada and Canola Oil Council, California Strawberry Commission, Ocean Spray Cranberries, National Cattlemen's Beef Association, McCormick Science Institute, International Nut & Dried Fruit Council, Hass Avocado Board. PMK-E has served on the following advisory boards: California Walnut Commission, HumanN, Avocado Nutrition Science Advisors, Seafood Nutrition Partnership. SGW has received consulting funds, travel funds, and research funding from the Canola Council of Canada and the McCormick Science Institute.

DJAJ has received research grants from Saskatchewan Pulse Growers, the Agricultural Bioproducts Innovation Program through the Pulse Research Network, the Advanced Foods and Material Network, Loblaw Companies Ltd., Unilever, Barilla, the Almond Board of California, Agriculture and Agri-food Canada, Pulse Canada, Kellogg's Company, Canada, Quaker Oats, Canada, Procter & Gamble Technical Center Ltd., Bayer Consumer Care, Springfield, NJ, Pepsi/Quaker, International Nut & Dried Fruit (INC), Soy Foods Association of North America, the Coca-Cola Company (investigator initiated, unrestricted grant), Solae, Haine Celestial, the Sanitarium Company, Orafti, the International Tree Nut Council Nutrition Research and Education Foundation, the

Peanut Institute, the Canola and Flax Councils of Canada, the Calorie Control Council (CCC), the CIHR, the Canada Foundation for Innovation and the Ontario Research Fund. DJAJ has received in-kind supplies for trial as research support from the Almond Board of California, Walnut Council of California, American Peanut Council, Barilla, Unilever, Unico, Primo, Loblaw Companies, Quaker (Pepsico), Pristine Gourmet, Bunge Limited, Kellogg Canada, WhiteWave Foods. DJAJ has been on the speaker's panel, served on the scientific advisory board and/or received travel support and/or honoraria from the Almond Board of California, Canadian Agriculture Policy Institute, Loblaw Companies Ltd, the Griffin Hospital (for the development of the NuVal scoring system, the Coca-Cola Company, EPICURE, Danone, Diet Quality Photo Navigation (DQPN), FareWell, Verywell, True Health Initiative, Saskatchewan Pulse Growers, Sanitarium Company, Orafti, the Almond Board of California, the American Peanut Council, the International Tree Nut Council Nutrition Research and Education Foundation, the Peanut Institute, Herbalife International, Pacific Health Laboratories, Nutritional Fundamental for Health, Barilla, Metagenics, Bayer Consumer Care, Unilever Canada and Netherlands, Solae, Kellogg, Quaker Oats, Procter & Gamble, the Coca-Cola Company, the Griffin Hospital, Abbott Laboratories, the Canola Council of Canada, Dean Foods, the California Strawberry Commission, Haine Celestial, PepsiCo, the Alpro Foundation, Pioneer Hi-Bred International, DuPont Nutrition and Health, Spherix Consulting and WhiteWave Foods, the Advanced Foods and Material Network, the Canola and Flax Councils of Canada, the Nutritional Fundamentals for Health, Agri-Culture and Agri-Food Canada, the Canadian Agri-Food Policy Institute, Pulse Canada, the Saskatchewan Pulse Growers, the Soy

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PJHJ's research related to a variety of oils and fats has been supported by grants and contracts from both industry and non-industry sources, including the Canola Council of Canada, Dairy Farmers of Canada, Canadian Institutes for Health Research, Natural Sciences and Engineering Research Council of Canada, Heart and Stroke Foundation of Canada, and National Institutes of Health Rare Diseases Network. PJHJ also serves as a committee member for the Soy Nutrition Institute, and the North American International Life Sciences Institute's Lipids Committee. PJHJ is President of Nutritional Fundamentals for Health Inc., which markets functional foods and nutraceuticals.

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Chapter 7

Overall Conclusion

7.1 Summary and implications

Environmental factors along with genetics appear to play a significant role in the regulation of body weight (1). In addition, controversies regarding interactions between fat intake and obesity exist (2–4), leading to the argument whether monounsaturated fatty acids (MUFA) or polyunsaturated fatty acids (PUFA) from dietary fats provide better health effects in terms of maintaining body weight to check obesity. Therefore, the present research was conducted to gain insight into this debate; and the results of the current investigation have implications for the ingestion of MUFA, especially C18:1n9-enriched dietary oils for the prevention of obesity.

Furthermore, evidence reveals that oleoylethanolamide (OEA) may engage in the initiation of satiety along with regulating numerous other pathophysiological functions (5, 6). The data from the present research show that ingestion of C18:1n9 oils, leads to low feed consumption, which might be the result induced by the anorexic potency of OEA due to the increased precursor C18:1n9, enabling enhanced fat oxidation (5, 7),

thereby reducing fat mass and improving lean mass. Therefore, the conclusion of enhanced fat oxidation in hamsters due to the diet effects is in the context of 8 weeks of intake of C18:1n9-enriched oils when incorporated as 14 kcal%. Importantly, we conclude that oral ingestion of C18:1n9 @ 1.8 $g.kg^{-1}$ effectively induced fat oxidation in the hamster. Moreover, the present research for the first time reveals that dietary fatty acid (DFA) profile influences tissue fatty acid ethanolamide (FAE) concentrations in a broad panel of internal organs and tissues such as central and peripheral organs of male Syrian hamsters, which in humans would have been a challenge to investigate. Additionally, the present research proposed a hypothetical model (Chapter 4, Figure 4.8) describing the energy homeostasis leading to OEA induced satiety, based on previous reports (5, 7, 8) and present findings.

Additionally, the present research enhances our understanding of dietary and genetic factors that regulate FAE metabolism. Specifically, advancing our knowledge in fatty acid amide hydrolase (*FAAH*) gene following previous studies (9, 10). Furthermore, data demonstrate that humans possessing *GPR40* rs1573611-T and *LEPR* rs1137101-A/A polymorphisms would benefit more from the intake of C18:1n9 enriched dietary oils by regulating the appetite, due to enhanced satiation induced by increased OEA and reduced AEA concentrations, respectively. Overall, the findings from the present work promote the notion of precision nutrition to deliver more preventive and practical dietary advice, concluding that ‘one size fits all' theory is no longer applicable nutritional advice.

The findings of the present research comply with previous reports and evidence

(9, 11–13) suggesting that MUFA consumption improves body composition. Moreover, the work conducted by Hammad *et al.* (11) shows that SNPs within lipoprotein lipase (*LPL*), fat mass and obesity-associated gene (*FTO*), peroxisome proliferator activated receptor-alpha (*PPARA*), adiponectin (*ADIPOQ*), apolipoprotein E (APOE), hepatic lipase (*LIPC*), and adrenoceptor beta-2 (*ADRB2*) genes modulate body fat mass distribution in response to dietary MUFA consumption. In addition to the previous work, the associations and interactions presented in the present research advance our understanding that polymorphisms in *LEPR* and *GPR40* genes help improve the body composition via the bioactive lipid amides, fatty acid ethanolamides (FAEs).

On the whole, the findings from the present study substantiate all four hypotheses proposed in the thesis; demonstrating that various dietary oils impact the plasma and tissue FAE concentrations (Hypotheses 1 and 2) covered in (Chapters 3 and 4 conducting an animal trial; Chapter 5 performing a human intervention trial). Endogenously synthesized OEA inversely associates with body weight (Hypothesis 2) covered in (Chapter 4, using an animal model). Genetic polymorphisms in humans related to FAE metabolism affect FAE concentrations (Hypothesis 3), covered in (Chapter 5, in the human trial). Single nucleotide polymorphisms (SNPs) involved in fatty acid receptors as well as a hypothalamic regulatiory marker influence body composition in humans (Hypothesis 4) covered in (Chapters 5 and 6, accomplished in the human clinical trial).

By comparing the animal- and human-derived data from the trials conducted in the present research, it is noted that the consumption of C18:1n9-enriched dietary oils

increased the circulating OEA concentrations. The enhanced OEA levels helped improve body composition, pointing to the possible use of these nutritional vegetable oils and natural OEA compound to control appetite, which may contribute to the maintenance of healthy body weight.

Nevertheless, the convergence exists between both animal and human trials; divergence cannot be excluded in terms of the food intake/satiety and energy expenditure not measured in humans. Similarly, the genetic variations explored or investigated in humans were mechanistically not examined in the animal trial, which could have aided in understanding the molecular details of OEA-mediated signalling, deficits in which might be implicated in human obesity.

In sum, current theories (14) emphasize three non-exclusive mechanisms as potential causes of hyperphagia, disrupting the satiating pathway regulated by the central nervous system (CNS). Disruption in any of these cascades may negatively affect the body composition: (i) deficits in the ability to generate satiety signals in response to feeding; (ii) dysfunctions of hypothalamic centers that control energy homeostasis; and (iii) abnormally high activation of 'reward pathways' in the brain by food-related stimuli. However, the present research performed in addition to the mounting evidence (5) demonstrates that in a different way and with a particular specialization such as increasing the feeding latency coupled with reduced meal size, OEA can modulate physiological mechanisms underpinning appetite as well as food intake, through gut-brain interrelationship mediated via neuronal signalling to maintain energy homeostasis

thus, improving body composition. Finally, along with human physiology, anatomy, and genetics, the sociocultural implication would also play an essential role in maintaining overall wellness and as such, the significance of the Mediterranean diet cannot be ruled out, and therefore, the benefits of the same can partly be attained by the inclusion of C18:1n9-dense dietary oils.

Altogether, the observations of a health-modulating genetic heterogeneity from the present research are essential in establishing population-wide recommendations for dietary oils over the long run in that they may help identify individuals who could benefit the most from consuming C18:1n9-enriched dietary oils. Hence, results from the present trial support the promising concept of personalised nutrition and health assessment.

7.2 Strengths

The trials conducted in the present research have several strengths. The studies performed used dietary oils generally consumed by human beings; and their implications on FAE levels by achievable nutritional interventions.

In the present research, recruitment and retention strategies were effective in achieving a sufficient number of participants who completed the study protocol to enable adequate statistical power to identify small differences in outcome measures. The robustness of the study conduct includes meeting the sample size target of 125 participants, under full feeding and randomized crossover design; since randomized,

controlled trials are considered to be the ‘gold standards’ in clinical nutrition research (15, 16).

7.3 Limitations

Although the research study possesses strengths, the limitations cannot be ruled out. As such, it is important to mention that erythrocyte fatty acids were not analyzed, as erythrocyte fatty acid concentrations are proposed to be a better reflector of the long-term DFA intake and fatty acid metabolism (17). Furthermore, the crucial parameters such as appetite and energy expenditure were not measured in the human intervention trial. Additionally, in the present trial, FAEs were measured in the fasting state only; future ‘acute trials’ should be conducted to assess the influence of post-feeding/non-fasting FAEs on body composition since fasting and non-fasting states modulate FAE levels differentially.

An additional limitation of the human trial was the population size of genotyped participants. While the population was large enough to see significant genetic associations in dietary intervention trials, it is still considered small when compared to the size of populations used in genome wide association studies (GWAS). Therefore, replication of genetic associations is critical, especially when gene by nutrient interactions are being investigated and study populations are small. The novel associations reported in this thesis will require replication in subsequent clinical trials.

7.4 Future directions

The oils are not only fatty acids (18). Therefore, the biological effects of additional chemical compounds present in fats/oils including antioxidants, such as polyphenols and flavonoids; fat-soluble vitamins, such as retinoids and tocopherols; polar and nonpolar compounds; namely, triglycerides, diglycerides, monoglycerides, phospholipids, and sterols should be investigated. As such, the future trials will shed light on the influence of these bioactive compounds (if any) on the key anabolic and catabolic enzymes (19, 20) involved in the synthesis and degradation of *N*-acylethanolamines (NAEs) that may modulate the biological activities thereof.

Future experiments examining the impact of C18:1n9-enriched diets on body composition involving an in-depth mechanistic understanding of satiety inducing pathways are warranted in both sexes, male and female, to confirm the outcomes of the research conducted. Sex is a critical biological variable (21), and the reduction in the rodent gender gap will improve the understanding of the metabolic activity of NAEs. These will enable the development of appropriate nutritional and pharmacological approaches to regulating appetite in obesity.

Furthermore, the findings from the present trial are based on a candidate SNP selection approach which may cause bias due to the restriction of all possible variations not selected as a candidate. Thus, the present research provides substantial ground/evidence highlighting the importance of conducting large (diet × genotype) pattern based 'acute trials' using the next-generation sequencing approach (22). Future 'free-living trials' should also be undertaken to investigate the effect of C18:1n9

perception threshold yielding OEA and its implications on overall body composition.

Future trials investigating the genetic heterogeneity of responsiveness to FAE signalling should seek to recruit for individuals with particular genotypes that have been associated with response to fatty acids and FAEs. Henceforth, GWAS are warranted to develop appropriate paradigms for functional research in humans by looking at diet × genotype interactions or associations that may lead to a deeper understanding of the human physiology and disease, thereby facilitating the development of apposite nutritional strategies to check appetite in obesity.

7.5 Final conclusion

Obesity has become pandemic, leading to increased metabolic syndrome prevalence worldwide. Data from the present research demonstrate that physiological signals such as FAEs, in particular, OEA may govern satiety and energy expenditure. Overall, the present research performed indicates that among a variety of nutritional signalling cascades, OEA induces anorexic signalling generating potential anti-obesity roles via one of the mechanisms of action by enhancing energy expenditure. Henceforth, our findings provide evidence for inclusion of C18:1n9-enriched dietary oils/fats, predominately found in canola and olive oils, as well as high-C18:1n9 vegetable oils such as sunflower oil, which may enhance OEA levels by increasing precursor substrate availability for endogenous OEA synthesis that could aid in regulating appetite and thereby help attain overall wellness. Furthermore, the data from the present research advance our knowledge in the booming scientific field of ‘nutrigenetics;’ suggesting that the

interactions among dietary fatty acids, FAE and candidate genes in energy homeostasis exist. Hence, the findings provide a fundamental step towards an era of personalised nutrition to control appetite in obesity.

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Appendices

Appendix A

Co-authored manuscripts on the human trial

A.1

Co-Authored Research Article

Manuscript 6

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Diets enriched with conventional or high-oleic acid canola oils lower atherogenic lipids and lipoproteins compared to a diet with a western fatty acid profile in adults with central adiposity

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Nutrition and Disease

Diets Enriched with Conventional or High-Oleic Acid Canola Oils Lower Atherogenic Lipids and Lipoproteins Compared to a Diet with a Western Fatty Acid Profile in Adults with Central Adiposity

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ABSTRACT

Background: Novel oils high in monounsaturated fatty acids (MUFAs) and low in saturated fatty acids (SFAs) are an alternative to partially hydrogenated oils high in *trans*-unsaturated fatty acids. There is widespread use of high-MUFA oils across the food industry; however, limited knowledge of their cardiovascular impact exists.

Objective: We investigated the effects of diets containing canola oil, high-oleic acid canola oil (HOCO), and a control oil blend (diet formulated to emulate a Western fat profile) on lipids, lipoproteins, and apolipoproteins (apos), as secondary outcomes of the trial.

Methods: In a multi-center, double-blind, randomized, 3-period crossover, controlled feeding trial, men ($n = 44$) and women ($n = 75$) with a mean age of 44 y, mean body mass index (BMI; in kg/m^2) of 31.7, and an increased waist circumference plus ≥1 metabolic syndrome criteria consumed prepared, weight-maintenance diets containing canola oil [17.5% MUFAs, 9.2% polyunsaturated fatty acids (PUFAs), 6.6% SFAs], HOCO (19.1% MUFAs, 7.0% PUFAs, 6.4% SFAs), or control oil (10.5% MUFAs, 10.0% PUFAs, 12.3% SFAs) for 6 wk with ≥4-wk washouts. Fasting serum lipids were assessed at baseline and 6 wk. Diet effects were examined using a repeated measures mixed model.

Results: Compared with the control, canola and HOCO diets resulted in lower endpoint total cholesterol (TC; −4.2% and −3.4%; $P < 0.0001$), LDL cholesterol (−6.6% and −5.6%; $P < 0.0001$), apoB (−3.7% and −3.4%; $P = 0.002$), and non-HDL cholesterol (−4.5% and −4.0%; $P = 0.001$), with no differences between canola diets. The TC:HDL cholesterol and apoB:apoA1 ratios were lower after the HOCO diet than after the control diet (−3.7% and −3.4%, respectively). There were no diet effects on triglyceride, HDL cholesterol, or apoA1 concentrations.

Conclusions: HOCO, with increased MUFAs at the expense of decreased PUFAs, elicited beneficial effects on lipids and lipoproteins comparable to conventional canola oil and consistent with reduced cardiovascular disease risk in adults with central adiposity. This trial was registered at www.clinicaltrials.gov as NCT02029833. *J Nutr* 2019;149:471–478.

Keywords: apolipoproteins, canola oil, cardiovascular disease risk, dietary fatty acids, dietary intervention, high-oleic acid canola oil, lipids, lipoproteins, metabolic syndrome, Western diet

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Introduction

Reduction of dietary SFAs and replacement with unsaturated fatty acids in the context of a healthy diet represents a cornerstone of nutritional recommendations for the prevention and treatment of cardiovascular disease (CVD) (1–4). Canola oil is a commonly consumed vegetable oil that is low in SFAs, moderate in PUFAs, and rich in MUFAs (62% oleic acid) (5), with numerous cardioprotective benefits (6, 7). Canola oil is also available in a high-oleic acid variety (HOCO; 71% oleic acid) that is equivalent in SFAs and proportionally lower in PUFAs (8).

The development of HOCO and incorporation into the food supply was spurred, in part, by the recognition of the adverse cardiovascular health effects of industrially produced *trans*-unsaturated fatty acids (TFAs) from partially hydrogenated vegetable oils (PHVOs) (9). High-MUFA oils are a reasonable substitute for TFA-containing fats and oils given their favorable fatty acid profiles that are consistent with dietary guidance and their ability to achieve or exceed the functional characteristics of PHVOs (i.e., oxidative stability, shelf life, fry life, neutral flavor) (10–12). Food applications for HOCO include replacing margarine and shortening in commercial baked goods and frying oil for restaurant deep-frying and commercial frying of packaged snacks and chips (10, 12). These foods are primary energy sources among US adults (13). Given the FDA's required removal of added TFAs (14), high-MUFA oils are becoming the

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BL has received funding in the last 5 years from the Canadian Institutes for Health Research, the Natural Sciences and Engineering Research Council of Canada, Agriculture and Agri-Food Canada (Growing Forward program supported by the Dairy Farmers of Canada (DFC), Canola Council of Canada, Flax Council of Canada, Dow Agrosciences), Dairy Research Institute, Dairy Australia, Danone Institute, Merck Frosst, Pfizer, and Atrium Innovations for which Douglas Laboratories manufacture and market omega-3 supplements. BL served as the Chair of the peer-review Expert Scientific Advisory Council of DFC. BL is also an Advisory Board member of the Canadian Nutrition Society, the Conseil pour les initiatives de progrès en alimentation and has served as an Advisory Expert for the Saturated Fat panel of the Heart and Stroke Foundation of Canada. BL has also received honoraria from the International Chair on Cardiometabolic risk, DFC, and the World Dairy Platform as an invited speaker in various conferences. 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He received an honorarium from the United States Department of Agriculture to present the 2013 W.O. Atwater Memorial Lecture. DJAJ received the 2013 Award for Excellence in Research from the International Nut and Dried Fruit Council. DJAJ received funding and travel support from the Canadian Society of Endocrinology and Metabolism to produce mini cases for the Canadian Diabetes Association (CDA). DJAJ is a member of the International Carbohydrate Quality Consortium (ICQC). His wife, ALJ, is a director and partner of Glycemic Index Laboratories, Inc., and his sister, CB, received funding through a grant from the St. Michael's Hospital Foundation to develop a cookbook for one of his studies. CGT has received funding in the past 5 years from CIHR, NSERC, Agriculture and Agri-Food Canada (Growing Forward and the Canola/Flax Agri-Science Cluster, Pulse Agri-Science Cluster, Canada-Manitoba Agri-Food Research Development Initiative), Research Manitoba, Manitoba Energy Science and Technology, Manitoba Agri-Health Research Network, Alberta Innovates, Alberta Crop Industry Development Fund, Alberta Canola Producers Commission, Alberta Pulse, Saskatchewan Pulse Growers, Canadian Diabetes Association, and MITACS. 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PJHJ also serves as a committee member for the Soy Nutrition Institute, and the North American International Life Sciences Institute's Lipids Committee. PJHJ is President of Nutritional Fundamentals for Health Inc., which markets functional foods and nutraceuticals.

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Abbreviations used: COMIT II, canola oil multi-center intervention trial II; HOCO, high-oleic acid canola oil; INAF, Institute of Nutrition and Functional Foods; MetS, metabolic syndrome; PHVO, partially hydrogenated vegetable oils; PSU, The Pennsylvania State University; RCFFN, Richardson Center for Functional Foods and Nutraceuticals; SBRC, St. Boniface Hospital Albrechtsen Research Center; TC, total cholesterol; TFA, *trans*-unsaturated fatty acids.

"new standard" of oil across the food industry and, thus, intake is likely to become pervasive.

Research involving the cardiovascular health impact of HOCO on atherogenic biomarkers is scarce (15, 16). Of particular concern is that widespread consumption of higher-MUFA, lower-PUFA oils will decrease the intake of total PUFAs, the preferred class of unsaturated fatty acids to replace SFAs in the context of a healthy diet for cardioprotection (17). Although conventional canola oil has beneficial effects on CVD lipid and lipoprotein biomarkers (6), we cannot assume that increased MUFAs at the expense of decreased PUFAs in HOCO will elicit identical impacts. We previously investigated the effects of consuming oils with differing unsaturated fat profiles, including HOCO and regular canola oil, in individuals with metabolic syndrome (MetS) criteria in the Canola Oil Multi-center Intervention Trial I (COMIT I), the trial preceding the project herein. The canola and HOCO treatments did not differ in endpoint lipids, lipoproteins, or apos following 4 wk of feeding (16). COMIT II was conducted to address additional knowledge gaps of the effects of HOCO on novel and established CVD risk markers.

The objective of the present study was to examine the effects of diets containing conventional canola oil and HOCO on lipids, lipoproteins, and apos compared to a control diet with a fatty acid composition characteristic of a Western diet in individuals with MetS risk factors. We hypothesized the lipid, lipoprotein, and apo response would be similar between the two canola diets, with greater benefit relative to the Western diet. This article presents the first systematic assessment of the shift in fatty acids in reformulated canola oil compared with conventional canola oil, as well as a Western diet fat profile.

Methods

Participants

Males and females (aged 20–65 y) with MetS risk factors were eligible for the study. Risk for MetS was defined as an increased waist circumference (International Diabetes Federation cut points: men ≥94 cm, women ≥80 cm) plus at least one of the following secondary inclusion criteria: elevated fasting blood glucose (≥5.6 mmol/L), TG (≥1.7 mmol/L), systolic blood pressure (≥130 mmHg), diastolic blood pressure (≥85 mmHg); and/or decreased high-density lipoprotein cholesterol (HDL cholesterol; men <1 mmol/L, women <1.3 mmol/L). Exclusion criteria included: smokers; consumption of >14 alcoholic beverages per week; use of prescription lipid-modifying medications in the last 3 mo or chronic anti-inflammatory medications; kidney disease, liver disease, diabetes, or uncontrolled thyroid disease; and pregnant or lactating women.

Study design

COMIT II was a double-blind, randomized, controlled feeding, crossover, clinical trial that consisted of three, 6-wk feeding periods separated by ≥4-wk washout periods. The trial was conducted from 2014–2016 at 4 research centers in North America [Richardson Center for Functional Foods and Nutraceuticals, University of Manitoba (RCFFN); Institute of Nutrition and Functional Foods, Laval University (INAF); Canadian Center for Agri-Food Research in Health and Medicine, St. Boniface Hospital Albrechtsen Research Center (SBRC); Departments of Nutritional Sciences and Biobehavioral Health, The Pennsylvania State University]. The respective centers' ethics review boards approved the COMIT II protocol and related documents, and the procedures followed were in accordance with the Declaration of Helsinki as revised in 1983. All participants provided written informed consent at screening prior to enrollment. Randomization.com was used to generate the random allocation sequence, with 6 possible sequences and an allocation ratio of 1:1:1:1:1:1. The sequences were assigned to each participant in the prespecified order as he or she was enrolled in the trial by the study coordinators. This trial was registered at www.clinicaltrials.gov as NCT02029833.

TABLE 1 Macronutrient composition of the 3 experimental diets containing the oils[1]

	Canola oil diet	HOCO diet	Control oil diet
Protein	15.87	15.87	15.71
Carbohydrate	50.79	50.79	50.75
Fat	35.26	35.26	35.21
MUFA	17.45	19.11	10.50
Oleic acid	15.55	17.86	5.92
PUFA	9.21	7.02	9.96
α-Linolenic acid	2.10	0.76	1.73
Linoleic acid	6.42	5.56	7.28
SFA	6.56	6.43	12.26

[1]The average macronutrient composition from the 7-d rotating menu, estimated at 3000 kcal, using Food Processor Nutrition Analysis Software (ESHA Research). Nutrients are presented as percentage of total energy. HOCO, high-oleic acid canola oil.

In COMIT I, the feeding periods were 4 wk in length (18), however, a 6-wk feeding period was chosen for COMIT II to allow assessment of the effects of prolonged intervention on CVD risk markers. Although lipids are responsive to dietary intervention by 14 d, 6 wk allowed the participants to reach a steady state of lipid concentrations (19) and also accommodated assessment of other outcomes that require longer duration for measurable change (i.e., body composition, vascular measures). A break of a minimum of 4 wk between diet periods was selected for compliance purposes and to reduce participant scheduling burden; this also ensured sufficient washout of the prior diet effects.

Controlled diets and oil interventions

During the feeding periods, participants were provided with an isocaloric, healthy, weight-maintenance base diet with one of the following oils: canola oil (Canola Harvest 100% Canola Oil, Richardson International), HOCO (Canola Harvest High Oleic Low Linolenic Canola Oil, Richardson International, Canada), or control oil [blend of ghee (Verka), safflower oil (eSutras), coconut oil (eSutras), and flaxseed oil (Shape Foods)]. The conventional canola oil and HOCO contained approximately 60% and 70% oleic acid, respectively. HOCO is a specialty canola cultivar that was developed through traditional plant breeding (11) to selectively reduce the total PUFA content, namely linoleic and α-linolenic acids, resulting in a higher oleic acid and proportionately lower PUFA content compared with conventional canola. The oil blend in the control diet was approximately 49% ghee, 29% safflower oil, 14% flaxseed oil, and 8% coconut oil, and was designed so that when it was added to the base diet the overall fat profile approximated the average fatty acid profile of a contemporary Western-style diet. The most recent estimate of average intake among US adults (NHANES 2015–2016) for SFAs, MUFAs, and PUFAs is 12%, 12%, and 8% of total energy, respectively (20).

The 3 experimental diets were identical in percentage of energy from macronutrients, but differed in fatty acid composition due to the presence of the intervention oils (Table 1). The kitchen staff at each site prepared breakfast, lunch, dinner, and snacks for the participants, adhering to a 7-d rotating menu. The diets were calorie controlled for weight maintenance, calculated using the Harris Benedict Formula, and monitored by daily weighing at each participating center prior to food pick-up. If a participant exhibited weight change during the first 2 wk of diet period 1, the caloric content was adjusted appropriately by switching to a higher or a lower calorie menu (menus were available in 300 kcal increments). The canola experimental diets were higher in MUFAs and lower in SFAs compared with the control diet.

The oils were incorporated into a smoothie containing frozen strawberries, orange sherbet, and skim milk, which was divided into 2 portions and consumed daily in the morning and evening to avoid

gastrointestinal distress from the fat load. The total volume of the smoothie and relative proportion of the ingredients (non-oil ingredients 1:1:1) was adjusted to participants' caloric needs. For example, for a 3,000 kcal/d diet, the smoothie contained 60 g oil, 200 g skim milk, 200 g strawberries, and 200 g orange sherbet. The intervention oils provided approximately 18% of total energy for all levels of caloric intake.

Participants were instructed to consume all foods provided and to avoid consumption of extraneous food items and calorie-containing beverages. Measures to optimize compliance have been described previously (16). All study personnel and participants were blinded to the diets, with the exception of the kitchen staff responsible for smoothie preparation.

Sample collection and analyses

Participants underwent various clinical tests on 2 consecutive days at baseline (days 1 and 2) and endpoint (days 41 and 42) of each diet period, and the mean values were calculated for all parameters. Anthropometric measures included weight, height, and waist circumference, and clinical procedures included seated blood pressure, DXA scans, and fasting blood draw. All blood draws followed 12 h without food or drink except water and 48 h without alcohol. Blood was allowed to clot, separated by centrifugation, aliquoted into microtubes, and stored at −80°C. Frozen serum samples were shipped on dry ice to St. Michael's Hospital (Toronto, ON, Canada), the central laboratory for multi-site analyses of lipids, lipoproteins, and apos.

The endpoints of interest were total cholesterol (TC), TG, LDL cholesterol, HDL cholesterol, non-HDL cholesterol, apolipoprotein A1 (apoA1), apolipoprotein B (apoB), and the TC:HDL cholesterol and apoB:apoA1 ratios. TC, TG, and HDL cholesterol were quantitatively determined by an enzymatic, colorimetric method on a Roche/Hitachi cobas c 501 analyzer (Roche Diagnostics). LDL cholesterol was estimated according to the Friedewald equation (21). However, for 4 time point samples, due to high serum TG concentrations (>4.52 mmol/L), LDL cholesterol was not calculated and recorded as a missing value. Non-HDL cholesterol was calculated as TC − HDL cholesterol. ApoA1 and apoB were quantitatively determined by endpoint nephelometry on a BN ProSpec nephelometer (Siemens). The TC: HDL cholesterol and apoB: apoA1 ratios were calculated from original values.

Statistical methods

The primary outcome of COMIT II was body composition with supplementary measurement of visceral adiposity measured by DXA (www.clinicaltrials.gov NCT02029833). Outcomes reported herein are secondary outcomes. Statistical analyses were performed using SAS 9.4 (SAS Institute Inc.). The primary analysis was endpoint-to-endpoint comparison (mean of days 41 and 42) of lipids, lipoproteins, and apos across the 3 diets. A secondary analysis was performed to assess absolute change from baseline within each diet. Data were analyzed per protocol to assess the efficacy of the diet response and missing data were not imputed. Participants with a weight change of >5% during any diet were removed from the analyses to eliminate the confounding effects of substantial weight change on the outcomes. All values for the primary and secondary analyses are presented as least squares mean ± SEMs and $P \leq 0.05$ was considered significant.

The effects of the diets on the lipid, lipoprotein, and apo outcomes were assessed using a repeated measures mixed model (proc mixed), with subject, diet sequence, and study center as random effects and time as the repeated effect. Factors assessed in the model include diet (canola oil diet, HOCO diet, control oil diet), time (diet period 1, 2, 3), sex (male, female), center (RCFFN, INAF, SBRC, The Pennsylvania State University), and diet sequence, and the following interactions: diet-by-time, diet-by-sex, diet-by-center, and diet-by-sequence. The diet-specific baseline value of the dependent variable was used as a covariate. Final models included diet and only significant terms. Tukey–Kramer adjusted P values were used for multiple pairwise comparisons between diets, only when there was a significant main effect of diet. Normality of the residuals from the final models was assessed and nonnormal dependent variables were log transformed. Within-diet changes from baseline were assessed by the least squares means P values from the final mixed model output. The effect of the diets on DXA-measured weight (both endpoint and change from baseline) was assessed as described above.

The COMIT II sample size was calculated according to the primary outcome, body composition, and a sample size of 140 was determined to detect a 55 g change in android fat mass using the variance parameter in android fat mass from the COMIT I trial (22), and assuming a 20% dropout rate. For analysis of the secondary outcomes, a sample size of 119 offered 97% power to detect a difference of 10% in LDL cholesterol between diets, with $\alpha = 0.017$.

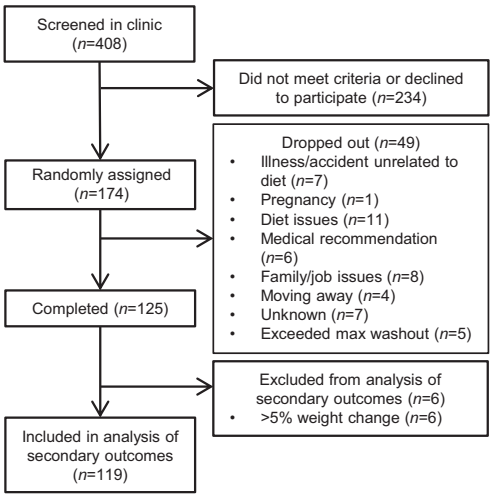

FIGURE 1 Flow diagram of the COMIT II participants for inclusion in the lipid and lipoprotein outcome analyses. COMIT, canola oil multicenter intervention trial; max, maximum.

Results

Baseline characteristics

The flow of participants through COMIT II and inclusion for the lipid and lipoprotein analyses are depicted in **Figure 1**. One hundred and twenty-five participants completed the study, with a dropout rate of ~28%. Participants who had a weight change of >5% during any diet period were removed ($n = 6$). **Table 2** presents the baseline characteristics (diet period 1, days 1 and 2) of the remaining COMIT II participants included in the analyses ($n = 119$). Participants were predominately female and middle-aged with class I obesity (BMI 30.0–34.9 kg/m^2). Approximately 38% of the participants met the clinical criteria for a MetS diagnosis at baseline (i.e., at least 3 risk factors). The individual MetS criteria of TG, HDL cholesterol, glucose, and blood pressure were on average within healthy ranges at baseline, suggesting one individual criterion did not drive study enrollment. Among the 119 participants enrolled who finished the trial, the percentage with elevated blood glucose, hypertriglyceridemia, reduced HDL cholesterol, or hypertension was 23%, 35%, 41%, and 33%, respectively.

Weight stability

Table 3 shows mean DXA-measured body weight at baseline (days 1 or 2) and endpoint (days 41 or 42), and the absolute

TABLE 2 Baseline characteristics of the COMIT II participants[1]

Variable	Value[2]
Sex	
Female	75 (63%)
Male	44 (37%)
Anthropometric measures	
Age, y	44 ± 13 (22–65)
Weight, kg[3]	91.3 ± 18.7 (60.4–146.4)
BMI, kg/m^2	31.7 ± 5.3 (22.6–52.6)
MetS criteria	
Waist circumference, cm	105 ± 13 (80–151)
Female	103 ± 12 (80–131)
Male	109 ± 13 (94–151)
TGs, mmol/L[4]	1.60 ± 0.73 (0.33–3.67)
HDL-C, mmol/L[4]	1.33 ± 0.35 (0.67–2.49)
Female (n = 75)	1.41 ± 0.35 (0.87–2.49)
Male (n = 43)	1.20 ± 0.31 (0.67–1.97)
Glucose, mmol/L[4]	5.30 ± 0.59 (4.16–8.00)
Blood pressure, mmHg	
Systolic blood pressure	120 ± 14 (88–164)
Diastolic blood pressure[4]	79 ± 11 (54–100)
Number of MetS criteria[5]	
0	1, 0.85%
1	29, 24.79%
2	43, 36.75%
3	27, 23.08%
4	12, 10.26%
5	5, 4.27%
Additional cardiovascular disease risk biomarkers	
Total cholesterol, mmol/L[4]	5.17 ± 0.90 (3.38–7.36)
LDL-C, mmol/L[4]	3.11 ± 0.75 (1.04–5.33)

[1] Values are means ± SDs (minimum–maximum) or frequency (%), n = 119. HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; MetS, metabolic syndrome.
[2] Data collected on days 1 and 2 of diet period 1. Fasting lipids, lipoproteins, and glucose were assessed in serum.
[3] Weight was measured using a scale at each participating center (i.e., not DXA weight).
[4] n = 118 due to missing values.
[5] n = 117 due to missing values. Enrolled participants met the requirements of an increased waist circumference plus one additional factor at the screening visit; values present here are from the baseline visits of diet period 1.

weight change for the COMIT II participants used in the lipid and lipoprotein analyses (n = 119). All diets modestly reduced body weight from baseline (<1 kg; P < 0.0001 for all). No differences between the 3 diets in endpoint weight or weight change were observed (P = 0.19).

Endpoint-to-endpoint mean comparisons

The primary analysis of endpoint-to-endpoint comparisons (mean of days 41 and 42) between the 3 diets is presented in **Table 4**. Compared with the control oil diet, consumption of both regular canola oil and HOCO diets resulted in lower endpoint means for TC (canola compared with control: P = < 0.0001; HOCO compared with control: P = 0.002), LDL cholesterol (canola compared with control: P = < 0.0001; HOCO compared with control: P = 0.0002), apoB (canola compared with control: P = 0.005; HOCO compared with control: P = 0.01), and non-HDL cholesterol (canola compared with control: P = 0.002; HOCO compared with control: P = 0.008). There were no significant differences between canola oil and HOCO diets for these parameters. The TC: HDL cholesterol ratio was lower following the HOCO diet compared with the control (HOCO compared with the control: P = 0.01), as well as the apoB: apoA1 ratio (HOCO compared with the control: P = 0.02; canola compared with the control: P = 0.06). There was a trend toward a diet effect on HDL cholesterol (P = 0.09); no diet effects on TG or apoA1 were observed. An effect of time was observed on TC, HDL cholesterol, LDL cholesterol, apoA1, and apoB, with no significant diet-by-time interaction for any parameters (data not shown). There was a significant diet-by-center interaction for apoB, with a higher endpoint value after HOCO at RCFFN compared with SBRC (data not shown; differences of LSM estimate = 0.09 g/L; P for interaction = 0.04).

Absolute change from baseline

The secondary analysis of change from baseline for all lipid and lipoprotein parameters within each diet is shown in **Figure 2**. All diets reduced TC, LDL cholesterol, non-HDL cholesterol, HDL cholesterol, apoB, and apoA1 from baseline (P < 0.0001 for all). TG (canola: P = 0.0182, HOCO: P = 0.0053, control: P = 0.0002), the TC: HDL cholesterol ratio (canola and HOCO: P < 0.0001, control: P = 0.0002), and the apoB: apoA1 ratio (canola and HOCO: P < 0.0001, control: P = 0.006) were also reduced from baseline. Differences between diets in change from baseline were similar to the endpoint comparisons, with the exception of apoB.

Discussion

COMIT II is the first double-blind, randomized, controlled feeding, crossover study to compare the effects of diets containing conventional canola oil and HOCO against a control diet with a fatty acid composition consistent with Western intakes. Herein, we report the lipid, lipoprotein, and apo response, secondary outcomes of the COMIT II study, in participants with MetS risk factors. The principal finding is that 42 d of canola oil and HOCO consumption similarly lowered endpoint TC, LDL cholesterol, apoB, and non-HDL cholesterol, and to a greater magnitude than the Western diet control oil. Further, the TC: HDL cholesterol and apoB: apoA1 ratios were reduced after HOCO compared with the control. These data indicate that HOCO, with increased MUFAs at the expense of decreased PUFAs, elicited beneficial effects on atherogenic lipids and lipoproteins comparable to canola oil and consistent with CVD risk reduction.

High-oleic oils are being increasingly incorporated into the food supply to replace PHVOs high in TFAs (10, 12), although the health effects of the widespread consumption of high-oleic oils remain unclear. Investigation into the clinical cardiovascular impact of HOCO is necessary to identify any unfavorable effects of this novel oil on cardiovascular biomarkers. Only 2 clinical trials to date have assessed the effects of HOCO on lipid and lipoprotein endpoints (15, 16), the primary biomarker targets for atherosclerotic CVD risk reduction (23). A previous study from our group, COMIT I, assessed the effects of controlled feeding of 5 dietary oils that varied in unsaturated fatty acid compositions, including canola oil and HOCO, on lipids and lipoproteins in individuals at risk of or with MetS (n = 130) (16). Endpoint values following 28 d of canola oil and HOCO in COMIT I were 4.81 ± 0.14 and 4.77 ± 0.14 mmol/L for TC, and 2.91 ± 0.08 and 2.86 ± 0.08 mmol/L for LDL cholesterol, respectively. Herein, we report numerically lower TC and LDL cholesterol endpoint values after 42 d of canola oil and HOCO. Analogous to the current report, the 2 COMIT I canola diets

TABLE 3 DXA-measured weights at baseline, endpoint, and the changes from baseline after consumption of diets containing canola oil, HOCO, or control oil for 6 wk in adults with central adiposity plus at least one additional MetS factor[1]

Diet	Baseline (kg)	Endpoint (kg)	Change (kg/6 wk)
Canola Oil	90.03 ± 1.71	89.12 ± 1.68	−0.65 ± 0.16 *
HOCO	90.37 ± 1.69	89.46 ± 1.68	−0.92 ± 0.15 *
Control Oil	90.28 ± 1.69	89.41 ± 1.69	−0.87 ± 0.16 *

[1]Values are means ± SEMs, $n = 119$. *Different from 0, $P \leq 0.05$. HOCO, high-oleic acid canola oil; MetS, metabolic syndrome.

did not differ in endpoint values for any parameters. Gillingham et al. was the first to investigate the effects of a high-oleic acid rapeseed oil diet compared with a Western control diet on lipids and lipoproteins (15). Following 28 d of controlled feeding in hypercholesterolemic participants ($n = 36$), endpoint TC, LDL cholesterol, and non-HDL cholesterol were lower after the high-oleic phase (5.27 ± 0.14, 3.10 ± 0.12, and 3.94 ± 0.14 mmol/L) compared with the Western diet control phase (5.65 ± 0.16, 3.53 ± 0.14, and 4.28 ± 0.17 mmol/L). These findings are consistent with the COMIT II study results, with differences in endpoint values likely due to the variation in populations studied. While these trials provide important insights into the effects of HOCO on CVD biomarkers compared with canola oil (16) and a Western diet (15), COMIT II is the first study to simultaneously examine diets containing conventional canola oil or HOCO and compared to a diet with a contemporary Western fatty acid profile.

We were not surprised to report no differences in the 2 diets containing the canola oil and HOCO on lipid outcomes. We utilized the Katan Calculator for a post hoc predicted differences in blood lipids and lipoproteins following replacement of the COMIT II control diet with the canola oil and HOCO diets, and found that the predicted changes were very similar (data not shown). Although HOCO and canola oil have unique fatty acid profiles when analyzed as independent oils, the COMIT II study design diluted assessment of the proportional fatty acid difference. The intervention oils provided approximately 50% of the total daily fat; thus, the remaining 50% was provided by other fat sources (i.e., mayonnaise, salad dressing, dairy fat) in equivalent amounts across diets, resulting in very modest fatty acid differences between the canola oil diet and HOCO diet. Therefore, the conclusion of a lipid and lipoprotein benefit of HOCO similar to canola oil and relative to control is in the context of 6 wk of intake when incorporated as approximately 18% of total energy (60 g per 3,000 kcal). Diet effects on lipids and lipoproteins following higher intakes of HOCO and canola oil (i.e., >18% of total energy) are unknown. A higher oil dosage is not recommended in the Dietary Guidelines for Americans healthy US-style eating pattern (i.e., 2,000 kcal, 27 g oil; 3,000 kcal, 44 g oil) (2) and modeling exercises suggest risk of essential fatty acid deficiency following elevated intake of high-oleic acid oils (24). Thus, we cannot conclude the longer-term implications of high-oleic oil consumption or the effects of higher dosages, and future research should consider the potential adverse effects of overconsumption for pertinent dietary recommendations.

MetS is defined as a cluster of 3 or more co-occurring inter-related conditions, including abdominal obesity, dysglycemia, dyslipidemia, and/or hypertension, and is associated with increased risk of cardiometabolic disease (25). The COMIT II study participants were required to have at least 2 MetS criteria at the screening visit, one of which was required to be an elevated waist circumference. In contrast to the NCEP ATP III waist circumference criteria (men ≥102 cm, women ≥88 cm), the International Diabetes Federation cut points were used (men ≥94 cm, women ≥80 cm) to identify individuals who may benefit from dietary intervention in the earlier stages of cardiometabolic disease risk. These inclusion criteria were also consistent with those of COMIT I (18). Further, the inclusion criteria of 2 rather than the syndrome definition of 3 factors (25) were selected to increase the generalizability of our findings to a sample that is highly representative of the North American population. An analysis of 2003–2012 NHANES data reported MetS prevalence among adults as 33%, with higher rates among women and Hispanics (26); the COMIT II sample had slightly higher rates at baseline (38%), likely due to the predominance of women (63%). MetS prevalence increases markedly with age [approximately 18% among 18–39 y and 50% among 60+ y in the US (26)], underscoring the relevance of this syndrome as the

TABLE 4 Endpoint-to-endpoint comparisons of fasting serum lipids, lipoproteins, and apos following the consumption of diets containing canola oil, HOCO, or control oil for 6 wk in adults with central adiposity plus at least one additional MetS factor[1]

	Canola oil diet	HOCO diet	Control oil diet	*P* for diet effect	*P* for time effect
TC, mmol/L	4.54 ± 0.04 [a]	4.58 ± 0.04 [a]	4.74 ± 0.04 [b]	<0.0001	0.01
TGs, mmol/L	1.45 ± 0.04	1.44 ± 0.04	1.40 ± 0.04	NS	NS
HDL-C, mmol/L	1.25 ± 0.01	1.28 ± 0.01	1.26 ± 0.01	NS	0.005
LDL-C, mmol/L	2.64 ± 0.04 [a]	2.67 ± 0.04 [a]	2.83 ± 0.04 [b]	<0.0001	0.02
TC:HDL-C ratio	3.82 ± 0.04 [a,b]	3.77 ± 0.04 [a]	3.92 ± 0.04 [b]	0.02	NS
apoA1, g/L	1.44 ± 0.01	1.46 ± 0.01	1.45 ± 0.01	NS	0.003
apoB, g/L	0.87 ± 0.01 [a]	0.88 ± 0.01 [a]	0.91 ± 0.01 [b]	0.002	0.04
apoB:apoA1 ratio	0.619 ± 0.01 [a,b]	0.616 ± 0.01 [a]	0.64 ± 0.01 [b]	0.01	NS
Non-HDL-C, mmol/L	3.30 ± 0.05 [a]	3.31 ± 0.05 [a]	3.45 ± 0.05 [b]	0.001	NS

[1]Values are least squares means ± SEMs, $n = 119$. Labeled means in a row without a common superscript letter differ, $P \leq 0.05$. A repeated measures mixed model was used to assess the effects of diet, time, sex, center, and sequence, and the interactions diet-by-time, diet-by-sex, diet-by-center, and diet-by-sequence. The diet-specific baseline value was used as a covariate. Final models included diet and only significant terms. Pairwise comparisons were assessed using the Tukey–Kramer method when there was a significant effect of diet. HOCO, high-oleic acid canola oil; MetS, metabolic syndrome; NS, $P > 0.05$; TC, total cholesterol.

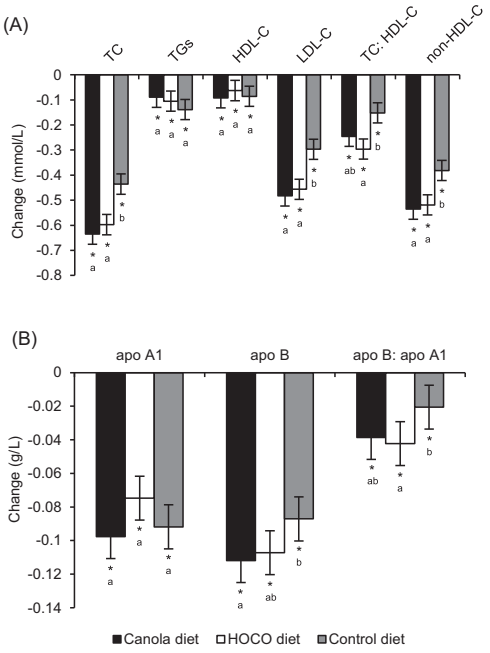

FIGURE 2 Absolute change (endpoint–baseline) in (A) lipids and lipoproteins and (B) apos following the consumption of diets containing canola oil, HOCO, and control oil for 6 wk in adults with central adiposity plus at least one additional MetS factor. Values are least squares mean ± SEM, $n = 119$. *Different from 0, $P \leq 0.05$. Labeled means in a group without a common letter differ, $P \leq 0.05$. HOCO, high-oleic acid canola oil; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; MetS, metabolic syndrome; TC, total cholesterol.

proportion of the older population rapidly grows. Further, MetS prevalence is ~21% among Canadian adults, with substantially higher estimates of individuals having components of the syndrome (i.e., 67% have ≥1 and 44% have ≥2 criteria) (27). These rates are concerning since MetS is associated with a 5-fold greater risk of incident diabetes (28) and a 2-fold greater risk of incident CVD events and mortality (29). Thus, selecting a sample of metabolically compromised adults is relevant to a considerable portion of the population and is appropriate for lifestyle intervention trials that aim to identify dietary strategies for chronic disease prevention and risk reduction among North Americans, with important implications for dietary counseling and nutrition policy recommendations. Previous investigations of the effects of canola oil on lipid and lipoprotein parameters in individuals at risk for or with MetS have been limited to 3 trials, including COMIT I, all of which reported lipid-lowering benefits of canola oil (16, 30, 31).

Although there is a substantial evidence base to support the cardioprotective benefits of canola oil, very few trials have directly compared a canola oil-based diet to a control diet with the fatty acid composition of the average, contemporary Western diet (6). This was a noted limitation of COMIT I (16) and prevents the determination of how diets enriched in canola oil fare as a replacement for a diet with the fat profile typical of Western intakes, as well as confirmation of the absence of adverse lipid effects. According to the latest NHANES food consumption data (2015–2016), the average intake of SFAs, MUFAs, and PUFAs among US adults is 12%, 12%, and 8% of total energy, respectively (20), percentages that the COMIT II control diet aimed to emulate (i.e., 12% SFAs, 11% MUFAs, and 10% PUFAs). The SFA content of the control diet was roughly 2-fold that of the canola oil and HOCO diets, and the MUFA content was appreciably lower than the 2 canola diets, although still aligned with average intakes. Ghee, coconut oil, safflower oil, and flaxseed oil were included in the control blend to generate the targeted fatty acid profile, which was based on an exhaustive evaluation of oil combinations during the COMIT II study design. Some of the individual fatty acids in the control diet were not directly congruent with Western intake. However, approximately 50% of the control oil blend was butter based (i.e., butter oil/ghee), a major source of animal fat in the Western diet, and only 8% was from coconut oil. Future research should incorporate fats and oils more representative of Western sources (i.e., corn oil, animal fats) when designing a control arm with a Western diet fatty acid profile, or provide a single fat source for the control for a comparative test of culinary oils.

COMIT II had numerous strengths, including a tightly regulated, controlled feeding, double-blind, multi-center, randomized, crossover design with a large sample size and inclusion of a commonly consumed oil. The crossover design allowed subjects to act as their own controls during each diet period, and the controlled feeding aspect reduced confounding variables characteristic of free-living designs. Moreover, blood was sampled on 2 consecutive days at the baseline and 2 consecutive days at the endpoint of each experimental period, allowing calculation of mean values and possible attenuation of intra-individual variability of lipid parameters. Further, collection of diet-specific blood samples on days 1 and 2 ensured attainment of precise baseline values (data not shown), in contrast to assumed return to initial baseline value post-washout, for inclusion as a covariate in the primary analysis. A limitation of COMIT II is small reductions in body weight (<1 kg) that were observed across all diets; however, it is not uncommon to see some degree of weight loss in controlled feeding trials, and is likely attributable to shifts from the habitual diet to a generally healthier controlled feeding eating pattern (i.e., lower sodium, lower SFAs, higher fiber, among others). This may explain the differences in all measured outcomes compared with baseline, particularly in the control condition. Importantly, the magnitude of weight reduction did not differ across diets and, thus, it is unlikely that the weight loss meaningfully mediated the lipid and lipoprotein diet response. Future assessments of individuals with MetS criteria should utilize the NCEP ATP III abdominal obesity cut points with a higher waist circumference threshold for inclusion (25).

In summary, canola oil and HOCO improved the lipid and lipoprotein profile compared to a control oil with a fatty acid composition characteristic of Western intakes in individuals with at least 2 MetS symptoms. Incorporating high-oleic acid and/or conventional canola oils into the diet by replacing dietary sources higher in SFA is an effective strategy to improve lipid and lipoprotein parameters and thus, reduce atherosclerotic CVD risk.

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KJB: analyzed data; KJB: wrote the paper; PJHJ, PMK-E, SGW, PWC, BL, DJAJ, and CGT: had primary responsibility for the final content; and all authors: read and approved the final manuscript.

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A.2

Co-Authored Research Article

Manuscript 7

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Common variants in lipid metabolism-related genes associate with fat mass changes in response to dietary monounsaturated fatty acids in adults with abdominal obesity

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Common Variants in Lipid Metabolism–Related Genes Associate with Fat Mass Changes in Response to Dietary Monounsaturated Fatty Acids in Adults with Abdominal Obesity

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ABSTRACT

Background: Different fatty acids (FAs) can vary in their obesogenic effect, and genetic makeup can contribute to fat deposition in response to dietary FA composition. However, the antiobesogenic effects of the interactions between dietary MUFAs and genetics have scarcely been tested in intervention studies.

Objective: We evaluated the overall (primary outcome) and genetically modulated (secondary outcome) response in body weight and fat mass to different levels of MUFA consumption.

Methods: In the Canola Oil Multicenter Intervention Trial II, a randomized, crossover, isocaloric, controlled-feeding multicenter trial, 44 men and 71 women with a mean age of 44 y and an increased waist circumference (men ∼108 cm and women ∼102 cm) consumed each of 3 oils for 6 wk, separated by four 12-wk washout periods. Oils included 2 high-MUFA oils—conventional canola and high-oleic canola (<7% SFAs, >65% MUFAs)—and 1 low-MUFA/high-SFA oil blend (40.2% SFAs, 22.0% MUFAs). Body fat was measured using DXA. Five candidate single-nucleotide polymorphisms (SNPs) were genotyped using qualitative PCR. Data were analyzed using a repeated measures mixed model.

Results: No significant differences were observed in adiposity measures following the consumption of either high-MUFA diet compared with the low-MUFA/high-SFA treatment. However, when stratified by genotype, 3 SNPs within lipoprotein lipase (*LPL*), adiponectin, and apoE genes influenced, separately, fat mass changes in response to treatment ($n = 101$). Mainly, the *LPL* rs13702-CC genotype was associated with lower visceral fat (high-MUFA: −216.2 ± 58.6 g; low-MUFA: 17.2 ± 81.1 g; $P = 0.017$) and android fat mass (high-MUFA: −267.3 ± 76.4 g; low-MUFA: −21.7 ± 102.2 g; $P = 0.037$) following average consumption of the 2 high-MUFA diets.

Conclusions: Common variants in *LPL*, adiponectin, and apoE genes modulated body fat mass response to dietary MUFAs in an isocaloric diet in adults with abdominal obesity. These findings might eventually help in developing personalized dietary recommendations for weight control. The trial was registered at clinicaltrials.gov as NCT02029833 (https://www.clinicaltrials.gov/ct2/show/NCT02029833?cond=NCT02029833&rank=1). *J Nutr* 2019;149:1749–1756.

Keywords: dietary fatty acids, fat quality, genotype, gene–nutrient interaction, fatness, adiposity

Introduction

The composition of dietary fatty acids (FAs) has been recognized as a determinant of fat deposition and distribution (1–5). FAs can vary in their obesity-inducing effects by influencing energy expenditure, fat oxidation, and thermogenesis, and/or modulating appetite sensation (6–8). Increasing evidence has demonstrated that dietary MUFAs increase fat oxidation, diet-induced thermogenesis (8–10), and resting energy expenditure (11), and promote weight loss (12, 13) compared with SFAs. Our recent controlled feeding study showed that 2 test diets high in MUFAs—canola oil and a high-oleic

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canola oil—significantly reduced android fat mass compared with a high-PUFA flaxseed/safflower oil in participants with abdominal obesity (1). These favorable effects might be attributed partly to interactions between FAs and genetic polymorphisms.

The genetic contribution to obesity is well recognized, and heritability of obesity is estimated to be 40–70% (14, 15). The responses of individuals with obesity to weight-gain prevention and reduction strategies can also vary broadly based on their genetic makeup (16). Therefore, examining gene–nutrient interactions assists in estimating the role of qualitative intake of FAs on the onset/progression of obesity in a genotype-specific manner.

Evidence from controlled trials and observational studies suggests a contribution of the interactions between genetic polymorphisms and dietary FAs in modulating adiposity via several mechanisms. For instance, the consumption of a higher proportion of MUFAs (17–19) and PUFAs (20, 21) relative to SFAs was found to be associated with lower body weight in peroxisome proliferator activated receptor γ (*PPARG*) rs1801282-G allele carriers in different ethnic populations. Additionally, a meta-analysis of 14 studies in US and European Caucasians revealed a direct association between SFA consumption and BMI, as well as waist-to-hip ratio, in the rs2306692-TT genotype carriers of low-density lipoprotein receptor-related protein 1 (22).

Although current research provides emerging evidence for gene–MUFA interactions (17–19, 23–25), numerous polymorphisms in lipid metabolism–related genes have yet to be investigated. Moreover, to investigate fat deposition and distribution, surrogate biomarkers have been often used, potentially masking outcomes. This study aimed to assess associations of common genetic variants and changes of total and regional fat mass following 6-wk controlled isocaloric dietary interventions with different concentrations of dietary MUFAs. To meet our objective, we used whole-body DXA scanning, which provides a reliable identification of fat distribution and discrimination between different fat depots.

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Supplemental Tables 1–4 and Supplemental Figure 1 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/.

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Abbreviations used: ADIPOQ, adiponectin; COMIT, Canola Oil Multicenter Intervention Trial; FA, fatty acid; HOCO, high-oleic canola oil; LPL, lipoprotein lipase; RCFFN, Richardson Centre for Functional Foods and Nutraceuticals; RCO, regular canola oil; ROI, region of interest; SCAT, subcutaneous adipose tissue; SNP, single nucleotide polymorphism; TG, triglyceride; UTR, untranslated region; VAT, visceral adipose tissue.

Methods

Study design and population

This study of gene–nutrient interactions was conducted within the framework of the Canola Oil Multicenter Intervention Trial (COMIT) II. COMIT II was a randomized, controlled, double-blind, crossover study designed to evaluate the response of body composition to 3 oils with different MUFA concentrations, including regular canola oil (RCO), high-oleic acid canola oil (HOCO), and a low-MUFA/high-SFA oil blend. This trial was conducted from 2014 to 2016 at 3 sites in Canada and 1 site in the United States: the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN) at the University of Manitoba in Winnipeg; the Canadian Centre for Agri-Food Research in Health and Medicine at St Boniface Hospital Albrechtsen Research Centre in Winnipeg; the Institute of Nutrition and Functional Foods at Laval University in Quebec City; and the Departments of Nutritional Sciences and Biobehavioral Health at The Pennsylvania State University in University Park. The protocol was reviewed and approved by institutional ethics boards across the participating clinical sites. The trial was registered at clinicaltrials.gov as NCT02029833.

Participants aged 20–65 y were included if they had abdominal obesity according to the International Diabetes Federation cutoff point for waist circumference (94 cm in men and 80 cm in women) in addition to at least 1 of the following metabolic syndrome criteria: fasting concentrations of blood glucose ≥5.6 mmol/L, triglycerides (TGs) ≥1.7 mmol/L, and HDL-cholesterol <1 mmol/L (men) or <1.3 mmol/L (women); and blood pressure ≥130 mmHg (systolic) and/or ≥85 mmHg (diastolic). Individuals were excluded if they had unstable thyroid disease, kidney disease, diabetes mellitus, or liver disease. Current smokers, individuals consuming more than 14 alcoholic beverages per week, individuals taking medication known to affect lipid metabolism for at least the last 3 mo, or individuals who were unwilling to stop taking any supplement at least 2 wk before the study were not permitted to participate. Written informed consent was obtained from all participants upon enrollment. Participants were randomly assigned to 1 of 6 treatment sequences using a random number generator at randomization.com.

Study diets

This study consisted of 3 treatment periods during which the participants consumed a controlled isocaloric, full-feeding diet with a fixed macronutrient composition of 35% fat, 50% carbohydrate, and 15% protein of total energy, as well as ∼208 mg/3000 kcal/d cholesterol and ∼38 g/3000 kcal/d fiber. The macronutrient composition of the 3 experimental diets has been previously reported (26). Menus for the 3 phases were identical except for the type of treatment oil provided. Treatment phases extended for 6 wk and were separated by 6-wk washout periods (ranged from 4 to 12 wk for a few participants). During the washout periods, participants were instructed to consume their habitual diets. Participants were asked to maintain their usual level of physical activity throughout the entire study. Physical activity changes were monitored by a weekly checklist.

The treatment oils, which comprised 20% of total energy, were incorporated into a smoothie beverage and were divided equally into 2 portions consumed at breakfast and supper. Treatment oils included: *1*) RCO (Canola Harvest Canola Oil; Richardson International), which provided 6.6% SFAs, 65.3% MUFAs, 19.6% n–6 PUFAs, 8.5% n–3 PUFAs α-linolenic acid; *2*) HOCO (Canola Harvest Canola Oil; Richardson International), which provided 6.7% SFAs, 75.9% MUFAs, 14.8% n–6 PUFAs, 2.6% n–3 PUFAs α-linolenic acid; and *3*) a low-MUFA/high-SFA oil blend that provided 40.2% SFAs, 22.0% MUFAs, 29.6% n–6 PUFAs, 8.2% n–3 PUFAs α-linolenic acid. The low-MUFA/high-SFA oil blend was prepared using commercially available ghee/butter oil (36.0%, Verka), safflower oil (34.9%, eSutras), coconut oil (16.0%, eSutras), and flaxseed oil (13.1%, Shape Foods). Study food and treatment shakes were prepared based on a 7-d rotating menu cycle in the metabolic kitchen of the participating sites. Compliance was assessed by smoothie consumption where the participants were required to consume ≥90% of the smoothies provided at each phase. Participants signed a daily checklist to verify consumption of smoothies. To maximize compliance rate, participants were required to consume 1 smoothie at breakfast under the supervision of a clinical coordinator for 5 d/wk. During weekdays, participants were provided the rest of their meals and a second smoothie in a food cooler bag for consumption off-site. Weekend meals and treatment shakes were delivered to the participants' residences or handed out to them, upon their request, at the clinical site on Fridays.

Measurement of fat mass

DXA scans were performed by a trained operator using Lunar Prodigy Advance DXA (GE Healthcare) with the default configurations. A DXA scan was performed for all participants at the initiation and termination of each dietary phase. Participants were asked to remove any metal items and heavy clothes before scanning. Regions of interests (ROIs) were manually adjusted using enCORE 2012 software (version 14.10.022) according to the manufacturer's instructions. Fat mass was analyzed as total fat mass, as well as 4 different districts including trunk, legs, android, and gynoid fat masses. The android and gynoid ROIs were identified as per the manufacturer's instructions. The android region has been defined as a portion of the abdomen that starts at the pelvis cut line and extends upward to include 20% of the distance between the pelvis and neck cut lines, with the outer arms' cuts as the lateral boundaries. The gynoid region has been defined as a portion of the legs with upper boundary below the pelvis cut line by 1.5 times the height of the android region, extending downward to twice the height of the android ROI, with the outer legs' cuts as the lateral boundaries. Further, visceral adipose tissue (VAT) was assessed by the CoreScan feature in enCORE 2012 software (version 14.10.022), and used to calculate the subcutaneous adipose tissue (SCAT) by subtracting VAT mass from android fat mass (27). VAT measurement using the CoreScan has been validated using computed tomography scanning (28). Criteria used to identify the anatomical ROIs were identical across all sites.

Genotyping

Twelve-hour fasting blood samples were collected and processed at the beginning of the trial, then stored at −80°C until being shipped to the RCFFN for analysis. Genomic DNA was extracted from the buffy coat samples of the first day of the first phase using a Qiagen DNeasy Blood and Tissue Kit according to the manufacturer's instructions (Qiagen Sciences, Inc.). A Thermo Scientific NanoDrop 2000 microvolume spectrophotometer was used to assess the concentration and purity of the extracted DNA (Thermo-Fisher Scientific, Inc.). TaqMan GTXpress Master Mix with allele-specific probes (Applied Biosystems, Life Technologies Inc) was used for genotyping of the single-nucleotide polymorphisms (SNPs) of interest. Amplification and detection of DNA were conducted with the 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies Inc). Data were acquired by software StepOne 2.1 (Applied Biosystems, Life Technologies Inc). Polymorphisms located in lipogenesis/adipogenesis-related genes were selected for their various roles in obesity development, where each SNP chosen was either a functional SNP, had a minor allele frequency ≥5, and/or had been previously reported for gene–nutrient interactions. This study assessed possible gene–diet interactions in a total of 5 candidate variants (**Supplemental Table 1**), within 3 genes, namely adiponectin (*ADIPOQ*), apoE (*APOE*), and lipoprotein lipase (*LPL*). The role of LPL as the rate-limiting enzyme that catalyzes the hydrolysis of TGs underscores *LPL* as a candidate gene for obesity. The functional *LPL* rs13702 and rs3200218 are located in the 3′ untranslated region (UTR) region and they are involved in translational regulation (29, 30). *LPL* rs13702-C allele is suggested to disrupt the microRNA recognition elements seed site and abolish the microRNA-410–mediated repression of mRNA at the *LPL* 3′ UTR, therefore increasing the activity of LPL (31). Despite the master role of LPL in regulating the supply of FAs to adipose tissue, the effects of possible interactions between *LPL* polymorphism and dietary FA interactions on obesity have been scarcely studied. *ADIPOQ* rs266729 (−11,377 C/G) is located in the promoter region and has

been shown to alter the circulating adiponectin concentration as well as the risk of obesity and insulin resistance (24, 32). Lastly, *APOE4* isoform has been associated with abnormal lipid metabolism and increased risk for several health problems including obesity (33–35).

Statistical analyses and sample size

The primary aim of the COMIT II trial was to evaluate the effect of MUFA consumption on body composition, mainly VAT. Therefore, the sample size was calculated to detect a 55-g change in android fat mass using the variance parameter in android fat mass from our previous controlled trial (1). A total sample size of 140 was required to account for a dropout rate of 20%.

Statistical analyses were performed using SAS 9.4 (SAS Institute, Inc.) based on a per protocol approach. Normality was assessed using the Shapiro–Wilk test and the skewness values. Nonnormally distributed variables were log-transformed before analysis. The results are expressed as least-squares means ± SEMs unless otherwise specified, and statistical significance was set at $P < 0.05$. Multiple comparison was assessed using the Tukey–Kramer test. Changes in fat mass and body weight represent the difference over 6 wk between end point and baseline of each dietary phase. PROC MIXED (SAS Institute, Inc.) with repeated-measures procedure was used to assess the effect of the 3 dietary treatments on changes in body fat and body weight. Treatment, sex, age, and genotype were used as fixed effects, with participants as a repeated factor. Random effects were treatment sequence, clinical site, and participants. Prespecified potential confounders such as ethnicity, baseline body composition, baseline fasting concentrations of glucose, homeostatic model of insulin resistance, and cholesterol were investigated in all models. The Hardy–Weinberg equilibrium was assessed with a chi-square test.

For diet–gene interaction analysis, due to the considerable not exactly similar comparable concentrations of MUFAs in the 2 canola treatments compared with the low-MUFA/high-SFA treatment, the statistical analysis of diet-by-SNP interaction was conducted to compare the combined effect of the 2 high-MUFA diets (HOCO + RCO, averaged) with the low-MUFA/high-SFA diet on changes in body fat and body weight. This decision was also based on our inability to detect statistical differences between HOCO and RCO in body composition in the overall population in our previous trial (COMIT I) (1), and based on the predefined hypothesis that the small variation in the concentration of MUFAs between the 2 high-MUFA treatments will not significantly influence the effect of genes on obesity. Although our sample size was lower than the longitudinal, survey-based diet–gene interaction studies, the controlled, full-feeding, crossover design of this study reduced the need for a larger sample size because it eliminated a wide range of confounders associated with the former designs. However, we consider this analysis an exploratory study to identify SNPs that might influence the body fat response to dietary fat type.

Each individual SNP was assessed separately using the aforementioned statistical model. All SNPs were analyzed in the additive model. Dominant and recessive models were analyzed only when the simple effect of heterozygous-by-MUFAs (in addition, to 1 homozygotes-by-MUFAs) showed a significant interaction. Only 4 APOE isoforms (encoded by rs429358 and rs7412) were obtained, and were analyzed and presented as non-*E4* (ε2/ε3 and ε3/ε3 genotypes) and *E4* (ε3/ε4 and ε4/ε4 genotypes).

Results

A total of 124 participants completed the counterbalanced trial and all of the required DXA scans. Three participants were excluded due to a high fasting blood glucose concentration (>7 mmol/L) and 6 participants were excluded due to large changes in body weight (weight change from baseline to endpoint >5%) at any dietary period (**Supplemental Figure 1**). Therefore, 115 participants (71 women and 44 men) were included in the analysis of the effect of dietary MUFAs on changes in body composition, as the primary outcome of the COMIT II trial. No significant differences were observed in changes in body weight or fat mass following the consumption of any of the 3 treatments (**Supplemental Table 2**).

The assessment of gene-by-diet interactions, the secondary outcome of the COMIT II trial, included 101 participants, because 14 participants did not consent for genetic analyses. Baseline characteristics are displayed in **Table 1**. Hardy–Weinberg equilibrium was not achieved for the *ADIPOQ* rs266729 and *APOE*. The effects of gene–diet interactions on changes in body weight, total fat mass, and selected regional fat mass were tested (**Supplemental Tables 3** and **4**). Diet was found to interact with common variants in *LPL*, *ADIPOQ*, and *APOE* to modify changes in body fatness in an isocaloric diet (**Figures 1–3**), as detailed below.

The *LPL* rs13702-CC genotype (Figure 1) was found to be associated with lower VAT and android fat mass following high-MUFA consumption compared with the low-MUFA diet. Likewise, carriers of the *LPL* rs13702-CC genotype showed trends toward less total fat mass and body weight following high-MUFA consumption compared with low-MUFA intake.

The consumption of high-MUFA diets protected the *ADIPOQ* rs266729-GG homozygotes from the increase in SCAT that was observed following consumption of the low-MUFA diet (Figure 2A). Further, in response to the low-MUFA diet, the carriers of *ADIPOQ* rs266729-GG homozygotes showed higher SCAT and android fat mass compared with C allele carriers (Figure 2). Lastly, *E4* carriers had greater reductions in total fat mass following consumption of the low-MUFA diet compared with high-MUFA diets (Figure 3).

Discussion

Results of the current study indicate that changes in total and compartmental fat mass and body weight in response to dietary fat substitutions are modified by common variants within lipid metabolism–related genes. Minor allele homozygotes of either *LPL* or *ADIPOQ* had lower body fat indices following consumption of high-MUFA diets compared with a low-MUFA diet, whereas *APOE4* carriers had lower body fat indices upon the consumption of a low-MUFA diet compared with a high-MUFA intake. These results highlight the genetic contribution to the responsiveness of body fatness to dietary MUFAs and could explain our inability to detect significant effects of MUFA consumption on body weight and fat mass compared with the low-MUFA/high-SFA diet in spite of the existing evidence (12, 13). Identifying the contribution of genetic architecture to the body's response to dietary modifications can direct the pathway toward the era of personalized nutrition. From our dietary intervention trial, we cannot conclude on the exact mechanisms of the observed phenomena and we need to refer to future biochemical studies. However, some existing knowledge might help to illuminate the biochemistry underlying the present findings.

LPL is the rate-limiting enzyme that catalyzes the hydrolysis of TGs in the core of TG-rich lipoprotein constituents as well as facilitating the uptake of FAs by adipocytes (36, 37). These functions highlight *LPL* as a candidate gene for obesity. Ma et al. (38) reported no influence of the SNP *LPL* rs13702 under different dietary FA interventions on BMI or waist

TABLE 1 Characteristics of participants at the baseline of dietary intervention[1]

Characteristic[2]	Total ($n = 101$)	Female ($n = 60$)	Male ($n = 41$)
Age, y	43.3 ± 1.29	45.7 ± 1.64[a]	39.9 ± 1.98[b]
Ethnicity, *n*			
Caucasian	74	45	29
African	4	3	1
Asian	8	4	4
Hispanic	3	1	2
Others	12	7	5
Waist circumference, cm	104 ± 1.30	101 ± 1.60[a]	108 ± 1.90[b]
Systolic BP, mmHg	119 ± 1.30	118 ± 1.70	120 ± 2.00
Diastolic BP, mmHg	78.1 ± 1.08	77.5 ± 1.41	79.0 ± 1.70
Total cholesterol, mmol/L	5.19 ± 0.09	5.21 ± 0.12	5.15 ± 0.14
TGs, mmol/L	1.55 ± 0.07	1.46 ± 0.09	1.67 ± 0.11
HDL-cholesterol, mmol/L	1.35 ± 0.04	1.46 ± 0.04[a]	1.20 ± 0.05[b]
LDL-cholesterol, mmol/L	3.13 ± 0.08	3.09 ± 0.10	3.19 ± 0.12
Glucose, mmol/L	5.22 ± 0.04	5.21 ± 0.06	5.23 ± 0.07
Insulin, pmol/L	98.7 ± 6.10	94.3 ± 7.92	105.0 ± 9.58
VAT mass, g	1334 ± 84.0	1056 ± 99.0[a]	1741 ± 120[b]
SCAT mass, g	2213 ± 90.0	2293 ± 117	2097 ± 141
Legs fat mass, g	12,645 ± 430	13,790 ± 531[a]	10,969 ± 642[b]
Trunk fat mass, g	19,472 ± 721	18,584 ± 930	20,772 ± 1124
Android fat mass, g	3548 ± 145	3349 ± 187	3838 ± 226
Gynoid fat mass, g	6002 ± 206	6334 ± 264	5516 ± 319
Total fat mass, g	36,282 ± 1119	36,565 ± 1459	35,869 ± 1765
Body weight, kg	89.8 ± 1.88	83.0 ± 2.21[a]	99.6 ± 2.67[b]
BMI, kg/m^2	31.1 ± 0.53	31.0 ± 0.69	31.3 ± 0.84

[1] Values are means ± SEMs unless otherwise specified. PROC MIXED (SAS Institute, Inc.) procedure was used to assess sex differences, $P < 0.05$ was considered significant. PROC MEANS (SAS Institute, Inc.) was used to determine the mean characteristics of the overall population. Labeled means within the same row without a common letter indicate sex-based statistical difference. BP, blood pressure; SCAT, subcutaneous adipose tissue; TG, triglyceride; VAT, visceral adipose tissue.

[2] Lipid profiles and glucose concentrations were determined using Cobas enzymatic reagents on Roche/Hitachi c 501e automated clinical chemistry analyzers using serum samples. Serum insulin concentrations were measured with the Roche/Hitachi Cobas e immunoassay analyzer and electrochemiluminescence immunoassay kits.

circumference in 2 independent populations. The C allele in the functional *LPL* rs13702 is suggested to increase the hydrolytic activity of *LPL* (30, 31); however, to our knowledge, no interactions between *LPL* polymorphisms and dietary FAs to modulate regional fat masses have been reported. The consistent decrease of 4 distinct regions of fat mass in *LPL* rs13702-CC homozygotes following the high-MUFA diets (Δ high-MUFA compared with low-MUFA greater than −200 g per site) in an isocaloric condition provides validity to this interaction. Given the previously proposed *LPL* rs13702-C allele–induced elevation in *LPL* activity, MUFA-rich diets might, therefore, protect the *LPL* rs13702-CC carriers from an increment in fat mass by the activation of obesity-opposing pathways, such as increasing the activity of hormone-sensitive lipase (39) or elevating the ratio of skeletal muscle to adipose tissue LPL activity (40), which would reduce the propensity for fat deposition.

The *ADIPOQ* gene encodes the peptide hormone adiponectin, which modulates a number of metabolic processes including lipid oxidation in muscle and liver (41). The *ADIPOQ* rs266729-G allele has been identified as a risk factor for obesity in several studies (32, 42), and has been associated with a lower risk for obesity following the consumption of a higher percentage of energy derived from fat (43). The present study shows, despite the controlled isocaloric diet, that compared with the low-MUFA diet a higher MUFA intake significantly reduced SCAT in the android region (Δ high-MUFA compared with low-MUFA approximately −160 g) among the *ADIPOQ* rs266729-GG homozygotes. However, the C-allele carriers had greater benefits following low-MUFA consumption (Δ low-MUFA compared with high-MUFA approximately −160 g and −190 g in SCAT and android fat mass, respectively) compared with high-MUFA intake. This finding constitutes further refinement of existing obesity associations, specifically because a previous study found no effect of dietary MUFAs on the association between the *ADIPOQ* rs266729 and obesity (24). However, we did not assess depot-specific concentrations of adiponectin or its receptors, leaving the validation of the underlying mechanism to future studies.

The *APOE* gene encodes apoE, which mediates the catabolism of the TG-rich lipoprotein particles in an isoform-dependent manner (44, 45). The *E4/E4* genotype has been associated with abnormal lipid metabolism and increased risk for several health problems including obesity (33–35, 46–49) and has previously shown responsiveness to dietary interventions (45, 46, 50). Mice carrying the human non-*E4* allele were heavier when fed low- and high-fat diets compared with *E4/E4* mice (51). The same study found that FA mobilization was lower in non-*E4* than in *E4/E4* mice, whereas *E4* mice overexpressed proteins involved in FA oxidation in skeletal muscle. Our results add another dimension to the evidence that *APOE* isoforms differentially influence fat mass, through demonstrating that the concentration of dietary MUFAs modulated fat loss in *E4* carriers (Δ low-

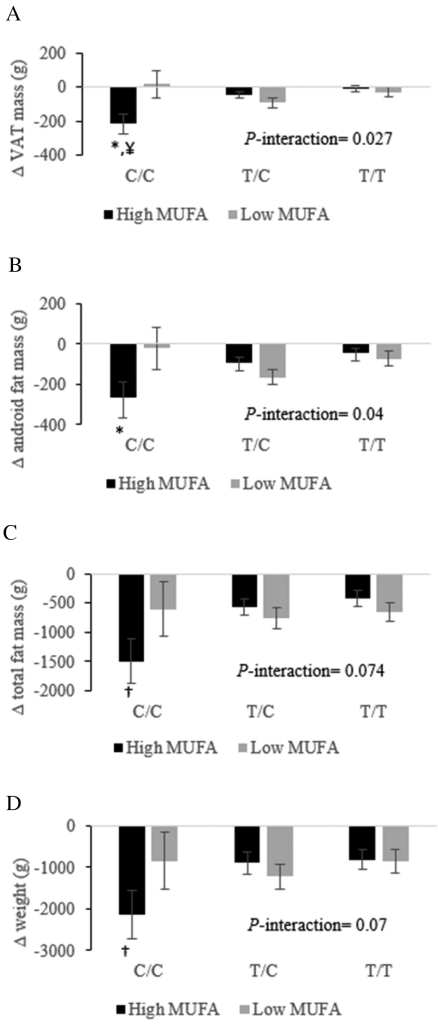

FIGURE 1 *LPL* rs13702 genotypes determine the effect of high- compared with low-MUFA consumption on 6-wk changes in VAT mass (A), android fat (B), total fat mass (C), and body weight (D) in adults with abdominal obesity. Changes were calculated by subtracting the baseline value of the selected fat mass from its corresponding 6-wk end-point value. Total participants = 101: $n = 50$ *LPL* rs13702-TT, $n = 45$ *LPL* rs13702-CT, and $n = 6$ *LPL* rs13702-CC. Values are least-squares means ± SEMs. PROC MIXED (SAS Institute, Inc.) with repeated-measures procedure was used to assess the effect of gene–MUFA interactions on fat mass changes, using participants' identification code as a repeated factor. $P < 0.05$ was considered significant. *Statistical significance in the response of a specific fat mass to different concentrations of dietary MUFAs within the same genotype. †Trend toward statistical significance ($0.06 > P > 0.05$) in the response of a specific fat mass to different concentrations of dietary MUFAs within the same genotype. ¥Statistically significant ($P = 0.017$) greater reduction in VAT mass following high-MUFA consumption in CC carriers compared with TT carriers of *LPL* rs13702. *LPL*, lipoprotein lipase gene; VAT, visceral adipose tissue.

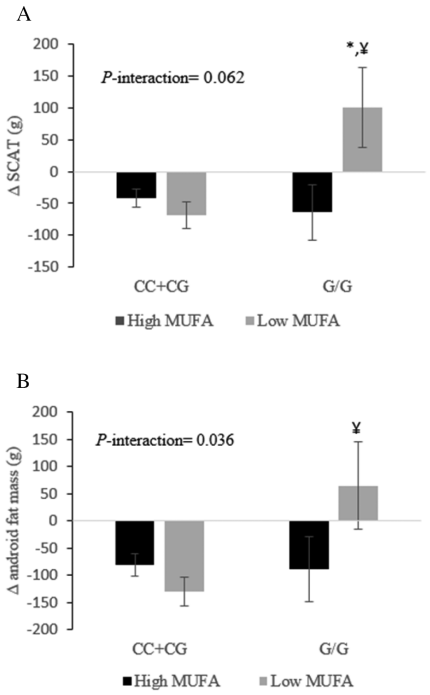

FIGURE 2 *ADIPOQ* rs266729 genotypes determine the effect of high- compared with low-MUFA consumption on 6-wk changes in SCAT (A) and android fat mass (B) in adults with abdominal obesity. Changes were calculated by subtracting the baseline value of the selected fat mass from its corresponding 6-wk end-point value. Total participants = 101: $n = 91$ *ADIPOQ* rs266729-CC + CG and $n = 10$ *ADIPOQ* rs266729-GG. Values are least-squares means ± SEMs. PROC MIXED (SAS Institute, Inc.) with repeated-measures procedure was used to assess the effect of gene–MUFA interactions on fat mass changes, using participants' identification code as a repeated factor. $P < 0.05$ was considered significant. *Statistical significance in the response of a specific fat mass to different concentrations of dietary MUFAs within the same genotype. ¥Statistical significance of the greater reduction in SCAT mass and android fat mass following low-MUFA consumption in C carriers ($P = 0.012$ and 0.022, respectively) compared with GG carriers of *ADIPOQ* rs266729. Recessive model (CC + CG compared with GG) was analyzed because the simple effect of heterozygous-by-MUFA showed a significant interaction on ≥1 compartmental fat masses. *ADIPOQ*, adiponectin gene; SCAT, subcutaneous adipose tissue.

MUFA compared with high-MUFA approximately −360 g) in an isocaloric condition. The *E4/E4* genotype was found to be associated with increased basal mitochondrial uncoupling and FA oxidation in mice (52), and this mechanism might be modulated by the quantity of dietary MUFAs, especially given that dietary MUFAs could increase fat oxidation and thermogenesis (8).

Assessing adiposity using DXA scanning provided a comprehensive assessment of the effect of these SNPs on total and regional adiposity. Another strength of this study was the crossover design with a controlled, isocaloric dietary intervention, which eliminated a range of confounders that might be

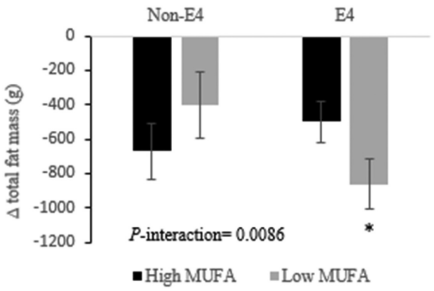

FIGURE 3 *APOE* genotypes determine the effect of high- compared with low-MUFA consumption on 6-wk changes in total fat mass in adults with abdominal obesity. Changes were calculated by subtracting the baseline value of the total fat mass from its corresponding 6-wk end-point value. Total participants = 101: $n = 35$ non-*E4* and $n = 66$ *E4*. Values are least-squares means ± SEMs. PROC MIXED (SAS Institute, Inc.) with repeated-measures procedure was used to assess the effect of gene–MUFA interactions on fat mass changes, using participants' identification code as a repeated factor. $P < 0.05$ was considered significant. *Statistical significance in the response of a specific fat mass to different concentrations of dietary MUFAs within the same genotype. *APOE*, apoE gene.

inherent with free-living and/or parallel study designs. However, an important limitation of the current study was that we did not apply stringent control for multiple testing, which could lead to a potential overstatement of our findings. Thus, large-scale studies are highly encouraged to evaluate these associations between the quality of dietary fat and polymorphisms within lipid metabolism–related genes. Additionally, Kien et al. (11) reported an attractive effect of dietary MUFA in which a high-MUFA consumption elevated physical activity levels compared with a high-SFA intake. The fact that participants of this study were instructed to maintain the same level of physical activity throughout the trial, and the lack of objective evaluation of physical activity, could have hindered the effect of different dietary FAs on physical activity; and consequently adiposity. The mixed ethnicity of this study population could also be perceived as a limitation, but might also provide generalizability of the current findings.

In summary, we report the contribution of common variants in *LPL*, *ADIPOQ*, and *APOE* genes to changes in body fatness in response to dietary MUFAs. These changes in body fat were observed regardless of the controlled isocaloric scenario. Although the observed changes in total and compartmental fat mass in response to gene–diet interactions were small over 6 wk, their statistical significance might indicate a potentially substantial clinical effect in weight reduction/maintenance regimens over prolonged periods.

Acknowledgments

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Appendix B

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This manuscript has been published in the Obesity Reviews: An official journal of the International Association for the study of obesity 2018; **19**: 178–197.

Oleoylethanolamide: The role of bioactive lipid amide in modulating eating behaviour

Jyoti Sihag and Peter J. H. Jones

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Dietary fatty acid composition impacts plasma fatty acid ethanolamide levels and body composition in golden Syrian hamsters

Jyoti Sihag and Peter J. H. Jones

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Dietary fatty acid profile influences circulating and tissue fatty acid ethanolamide concentrations in a tissue-specific manner in male Syrian hamsters

Jyoti Sihag and Peter J. H. Jones

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Author: Jyoti Sihag,Peter J.H. Jones

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B.4 Copyright for manuscript 6: A.1

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Diets enriched with conventional or high-oleic acid canola oils lower atherogenic lipids and lipoproteins compared to a diet with a western fatty acid profile in adults with central adiposity

Kate J. Bowen, Penny M. Kris-Etherton, Sheila G. West, Jennifer A. Fleming, Philip W. Connelly, Benoît Lamarche, Patrick Couture, David J.A. Jenkins, Carla G. Taylor, Peter Zahradka, Shatha S. Hammad, Jyoti Sihag, Xiang Chen, Valérie Guay, Julie Maltais-Giguère, Danielle Perera, Angela Wilson, Sandra Castillo San Juan, Julia Rempel, and Peter JH Jones

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Common variants in lipid metabolism-related genes associate with fat mass changes in response to dietary monounsaturated fatty acids in adults with abdominal obesity

Shatha S. Hammad, Peter Eck, Jyoti Sihag, Xiang Chen, Philip W. Connelly, Benoît Lamarche, Patrick Couture, Valérie Guay, Julie Maltais-Giguère, Sheila G. West, Penny M. Kris-Etherton, Kate J. Bowen, David J.A. Jenkins, Carla G. Taylor, Danielle Perera, Angela Wilson, Sandra Castillo, Peter Zahradka, and Peter J.H. Jones

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Appendix C

Ethics approval for studies in Chapters 3, 4, 5, and 6

C.1 Animal trial: Chapters 3 and 4

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Animal Care & Veterinary Services
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6 March 2013

TO: Dr. P. Jones, RCFFN, 196 Innovation Drive

FROM: Dr. H. Aukema, Chair, Fort Garry Campus Animal Care Committee

RE: Your protocol entitled "**The impact of fatty acid ethanolamines on cardiovascular health, energy expenditure and weight change in a Syrian hamster model**"

Please be advised that your Animal Use Protocol form was reviewed by the Fort Garry Campus Animal Care Committee (FG ACC) at its meeting of January 24 2013. The committee recommended **APPROVAL** of your protocol.

Protocol Reference Number: **F13-004**
Animals approved for use: **118 Syrian Hamsters**
Protocol approval is valid from: **March 6, 2013 to March 5, 2014**
Category of Invasiveness: **B**

The protocol reference number must be used when ordering animals. It is understood that these animals will be used only as described in your protocol. Failure to follow this protocol will result in the termination of your ability to use animals.

The protocol must be kept current. Minor modifications to the protocol must be submitted in the form of an amendment. Major changes would necessitate preparation and submission of a new protocol. Failure to renew this protocol prior to the expiry date will result in the termination of your ability to continue ordering animals.

On behalf of the Fort Garry Campus Animal Care Committee, I would like to extend our best wishes for the successful completion of your research.

HA/tvo

copy: Veterinary Services
Amanda Stevenson, Animal Technician, RCFFN

C.2 Human trial: Chapters 5 and 6

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PROTOCOL NUMBER:	PROJECT OR PROTOCOL TITLE:
	Effects of Canola Oil on Body Composition and Lipid Metabolism in Participants with Metabolic Syndrome/Canola Oil Multi-Center Intervention Trial 2 (COMIT II) (Linked to B2010:052 and B2007:071)
SPONSORING AGENCIES AND/OR COORDINATING GROUPS:	
Canola Council of Canada, DOW Agrosciences and Agriculture and Agri-Food Canada	

Submission Date(s) of Investigator Documents:	REB Receipt Date(s) of Documents:
September 3 and October 1, 2013	September 3 and October 7, 2013

THE FOLLOWING ARE APPROVED FOR USE:

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Protocol		September 3, 2013
Consent and Assent Form(s):		
Research Subject Information and Consent Form	V. 2	October 1, 2013
RCFFN Preliminary Trial Screening Consent		September 3, 2013
Additonal Research Subject Information and Consent Form for Genetic Analysis	V. 1	September 3, 2013
Other:		
Poster 1 and 2	V. 1	September 3, 2013
Participant Screening Form		
General Information Sheet	V. 1	September 3, 2013

CERTIFICATION
The University of Manitoba (UM) Biomedical Research Board (BREB) has reviewed the research study/project named on this ***Certificate of Final Approval*** at the ***full board meeting*** date noted above and was found to be acceptable on ethical grounds for research involving human participants. The study/project and documents listed above was granted final approval by the Chair or Acting Chair, UM BREB.

BREB ATTESTATION
The University of Manitoba (UM) Biomedical Research Board (BREB) is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement 2, and the applicable laws and regulations of Manitoba. In respect to clinical trials, the BREB complies with the membership requirements for Research Ethics Boards defined in

Division 5 of the Food and Drug Regulations of Canada and carries out its functions in a manner consistent with Good Clinical Practices.

QUALITY ASSURANCE
The University of Manitoba Research Quality Management Office may request to review research documentation from this research study/project to demonstrate compliance with this approved protocol and the University of Manitoba Policy on the Ethics of Research Involving Humans.

CONDITIONS OF APPROVAL:

1. The study is acceptable on scientific and ethical grounds for the ethics of human use only. ***For logistics of performing the study, approval must be sought from the relevant institution(s).***
2. This research study/project is to be conducted by the local principal investigator listed on this certificate of approval.
3. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to the research study/project, and for ensuring that the authorized research is carried out according to governing law.
4. **This approval is valid until the expiry date noted on this certificate of approval. A Bannatyne Campus Annual Study Status Report** must be submitted to the REB within 15-30 days of this expiry date.
5. Any changes of the protocol (including recruitment procedures, etc.), informed consent form(s) or documents must be reported to the BREB for consideration in advance of implementation of such changes on the **Bannatyne Campus Research Amendment Form.**
6. Adverse events and unanticipated problems must be reported to the REB as per Bannatyne Campus Research Boards Standard Operating procedures.
7. The UM BREB must be notified regarding discontinuation or study/project closure on the **Bannatyne Campus Final Study Status Report**.

Sincerely,

[redacted]

Lindsay Nicolle, MD, FRCPC
Chair, Biomedical Research Ethics Board
Bannatyne Campus

Please quote the above Human Ethics Number on all correspondence.
Inquiries should be directed to the REB Secretary **Telephone:** (204) 789-3255/ **Fax:** (204) 789-3414

Appendix D

Study material and forms

D.1 Poster: Study advertisement

UNIVERSITY OF MANITOBA

Richardson Centre for Functional Foods and Nutraceuticals

Do you have extra belly fat?

The Richardson Centre for Functional Foods and Nutraceuticals is conducting a nutrition study to investigate how dietary oils can improve your health while potentially reducing android (belly) fat.

The study is open to men and women who meet the following criteria:

- Aged 20-65
- Elevated waist circumference
- Not taking medication to lower blood lipids/cholesterol
- Non-smoker

Volunteers will be compensated for their participation.

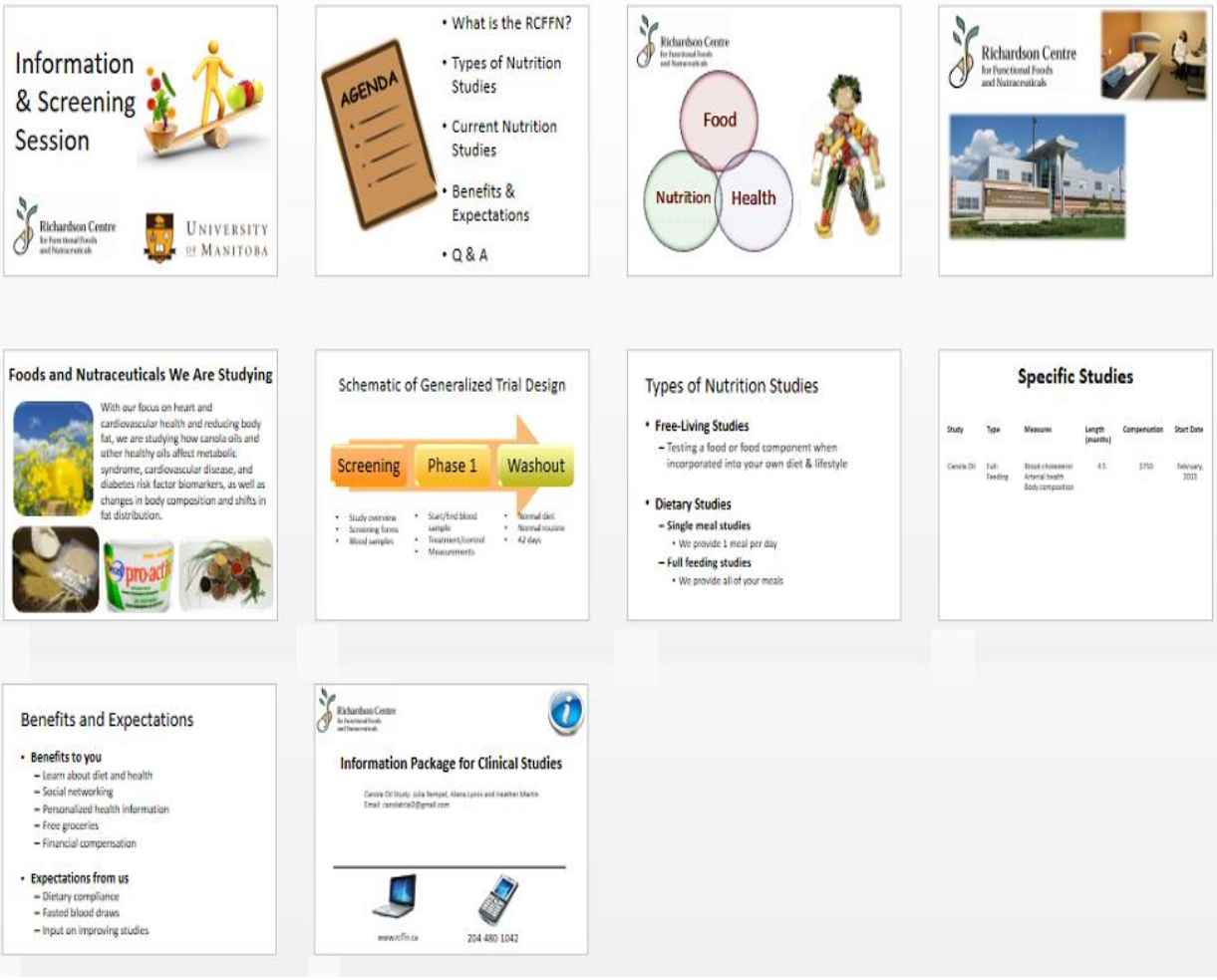
Phone: (204) 480-1042

Email: canola.trial@umanitoba.ca

Website: www.rcffn.ca

Dr. Peter Jones, Principal Investigator

D.2 Presentation: Study information session



D.3 Medical examination form

Phase Pre-Study	Study Physician Dr._____	
Date of Visit ____/____/____ MM/DD/YR	Investigator Dr. Peter Jones	Subject Code

COMPLETE PHYSICAL EXAMINATION

A. Vital Signs

Body Weight _____ lbs _____ kg

Height _____cm

Respiration _____

Blood Pressure (seated) _____/_____ mmHg systolic diastolic

Heart Rate _____bpm

Race/Ethnic Origin:
☐ **Caucasian** ☐ **African-American/Canadian** ☐ **Asian** ☐ **Other** _____

B. Body Systems (Check the appropriate box if organ system was examined. If not done, write N/D in the box)

	Normal	Abnormal	*Details of abnormal finding
1) Ears, Nose, Throat			
2) Eyes			
3) Dermatological			
4) Musculoskeletal			
5) Lymph Nodes			
6) Neurological			
7) Cardiovascular			
8) Respiratory			

9) Endrocrine			
10) Urogenital			
11) Gastrointestinal (complete section C)			

C. Gastrointestinal Cont…

Bowel Habits:
Frequency _____ /Day

Consistency _____

Urination:
Frequency _____/Day

Nocturia _____/Night

Medications:

Hospitalization:

Family History:

D. Medical History

	YES	NO
Have you taken a glucose lowering medication or a medication affecting lipid metabolism (cholestyramine, colestipol, niacin, colfibrate, gemfibrozil, probucol, HMG-CoA reductase inhibitors, and high-dose dietary supplements, plant sterols or fish oil capsules) within the past 3 months?		
Do you take systemic aspirin, NSAIDS. antibodies, corticosteroids, androgens or phenytoin within the past 3 months?		
Are you on anticoagulant therapy?		
Do you smoke?		
Do you consume large amounts of alcohol? (more than 2 drinks per day or 12 drinks per week)		
Do you follow a specific diet?		
Do you have a major food allergy?		
Do you have lactose intolerance?		
Have you had major surgery in the last 6 months?		
Do you have diabetes mellitus?		

Do you have kidney disease?		
Do you have liver disease?		
Do you have heart disease?		
Do you have gastrointestinal, pancreatitis or biliary disease (onset within past three months)?		
Have you had cancer? If yes, occurrence of therapy within past 1 year?		
Do you have anemia, bleeding disorder or significant blood loss/donation?		
Do you have uncontrolled thyroid disease or hypertension? (Subject will be accepted if she is on a stable dose of a thyroid or blood pressure medication that has no known effects on blood lipid metabolism).		
Do you have a history of eating disorders?		

E. Additional Physician Notes

Based on the medical examination and medical history, is the subject eligible to participate in the study protocol (circle one):

YES **NO**

Physician's Signature _____

Date _____

D.4 Participant screening consent form

Richardson Centre for Functional Foods and Nutraceuticals

Room 106
196 Innovation Drive
Winnipeg, Manitoba
Canada R3T 2N2
Telephone (204) 474-8883
Fax (204) 474-7552
peter_jones@umanitoba.ca

RCFFN preliminary trial screening consent

You have expressed an interest in participating in a study at the Richardson Center for Functional Foods and Nutraceuticals. You have been invited to have your health assessed to determine if you meet the requirements of the study.

The clinical coordinator team will assess your cholesterol level, medical history, body measurements, and your availability over the next 2 years. Depending on your results you will be offered the opportunity to participate in a study.

To allow the necessary information to be obtained, you agree to provide fasting blood samples (approximately 10 ml or 2 teaspoons) for the measurement of blood cholesterol, blood count, and iron levels.

Prior to taking part in any study, you will be given the specific study consent form to read and sign if you are still interested in participating.

The blood is taken from a vein in the forearm, as is usually done during a blood test. Some known risks, although rare, are associated with placing a needle into a vein. These include the possibility of infection, perforation or penetration of the needle through the vein, and bleeding, pain, or bruising at the site.

I understand I can withdraw from this process at any time at my discretion.

Participant's Signature	Participant's Name (please print)	Date
Investigator's Signature **(or Clinical Coordinator)**	Investigator's Name (please print) **(position)**	Date

D.5 Participant informed consent form

Richardson Centre for Functional Foods and Nutraceuticals

RESEARCH SUBJECT INFORMATION AND CONSENT FORM

Title of Study:

Canola Oil Multi-center Intervention Trial II (COMIT II): Effects of Oleic Acid Enriched and Regular Canola Oil on Body Composition and Lipid Metabolism in Participants with Metabolic Syndrome Risk Factors

Investigator: Peter J.H. Jones, PhD
Richardson Centre for Functional Foods and Nutraceuticals
University of Manitoba
196 Innovation Drive
Winnipeg, Manitoba R3T 6C5
Phone: 204 474 9787

Sponsor: Canola Council of Canada, 400-167 Lombard Avenue, Winnipeg, Manitoba, R3B OT6

You are being asked to participate in a research study. Please take your time to review this Information and Consent Form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family. This consent form may contain words that you do not understand. Please ask the study staff to explain any words or information that you do not clearly understand. The study doctor and institution are receiving professional fees and financial support to conduct this study.

Purpose of study
Obesity is a contributing factor to most chronic diseases and a burden on human health throughout the world. The ability to limit or even reduce excess weight gain through modification of fatty acid intake would be beneficial in combating the obesity epidemic. The primary objectives of the study are to examine the health benefits of dietary canola oils on body composition and cardiovascular disease risk factors. It is anticipated that consuming these healthy oils will favorably alter body composition, specifically through

the reduction of android (stomach) fat. Furthermore, we are also examining the effects of dietary canola oils on many other cardiovascular disease risk factors.

Study procedures

Pre-screening procedures

If you agree to take part in this study, you will be asked to give a fasting (nothing to eat or drink 12 hours before the test) blood sample (approximately two teaspoons) to measure your blood lipid levels and additional biochemistry parameters. In addition, we will measure your blood pressure and waist circumference. Prior to beginning the study, you will undergo a physical examination by a physician to ensure that you are in good health. During the physical examination, the physician will measure your vital signs, examine the normality of body systems and ask you some questions regarding your medical history. The study physician and or study staff will review medical history and ask questions to determine whether you are eligible to participate. Any change in your health status at any point during the study needs to be reported to the study investigators.

Study procedures

The study will consist of 3 phases of 42 days (6 weeks) each during which you will consume a fixed composition of a precisely controlled weight-maintaining diet. Each study phase will be followed by a washout period of 6 weeks where you can consume your habitual diets. During each study phase, you will be provided with a balanced, precisely controlled weight-maintaining diet (35% energy from fat, 50% carbohydrate, and 15% protein). Each phase will differ only in the dietary oils provided as part of the controlled diet as follows:

1) Control phase: Dietary fat consumed will provide 35% of total energy and will be comprised of "Western diet" fats in the form of a mixture of common dietary oils.

2) Canola oil phase: Dietary fat consumed will provide 35% of total energy and will be comprised of up to 60% of total energy from fat from *canola oil*.

3) High oleic acid canola oil phase: Dietary fat consumed will provide 35% of total energy and will be comprised of up to 60% of total energy from fat from *high oleic acid canola oil*. Oleic acid is a specific dietary fatty acid of interest with health benefits.

This study is a double-blind design, which means that neither you nor the study staff will know which oil variety you are receiving in each phase. In the unlikely event of an emergency, this information will be made available.

Study diets will be prepared in the metabolic kitchen of the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN). You will consume at least 1 of 3 daily meals at the RCFFN under supervision. The other meals will be prepared and packed to be taken out. The treatment oils will be provided as a part of the meals, as appropriate for each phase. You will be asked to consume only the food provided by our metabolic kitchen during each study phase. Additionally, we ask that you do limit caffeinated

beverages consumption to one to two beverages a day, alcoholic beverages consumption to 2 beverages a week. No alcoholic beverages are to be consumed within 48 hours prior to blood draws during the study periods. No caffeinated beverages consumption within 12 hours prior to blood draws during the study periods

We will measure the amount of fat in your body using a procedure called dual energy x-ray absorptiometry (DXA). These analyses will be performed 6 times in total during the study, once at the beginning of each phase and once at the end of each phase. For this procedure, you will need to lie in a horizontal position for about 5-10 minutes while the scan arm passes from your head to your feet. The radiation from this test is very low dosage (equivalent to approximately 1 day of natural background radiation). The dosage is 1000 times less than the limit for trivial exposure. You will be asked not to wear anything metal (metal may affect bone density values which will affect body composition calculations). In addition, you will need to ensure that you will not undergo barium tests/exams, or a nuclear medicine scan or injection with an x-ray dye within two weeks prior to your DXA scan. If you are female and are not post-menopausal you will be asked to take a pregnancy test prior to beginning the study and subsequently before each DXA scan. To measure abdominal subcutaneous and visceral fat, the DXA scan you will undergo will be used to measure this.

From the 28th to 35th day of each dietary period participants will be asked to wear activity monitors around the waist. This activity monitoring period is optional and you may choose not to participate. If you do choose to participate these small devices are about the size of a wrist watch and can be worn on a belt or with special belts that are made for the monitors. These devices measure movement and ambient light and this data will be used to measure 24 hour physical activity, energy expenditures, and sleep/wake measurements. On the 35th day of each period the data stored on the devices will be downloaded and saved under code and the data on the device will be deleted.

During days 1, 2, 41 and 42 of each six-week test diet phase, fasting blood samples will be obtained for assessment of blood fat, fatty acid profile and other cardiovascular diseases (CVD) biomarkers including insulin glucose concentrations and inflammatory markers, oxidative stress markers and markers of adiposity.

Each blood draw will require taking approximately 10 teaspoons of blood and will take approximately 5 minutes. The total amount of blood drawn during each phase of the study will be approximately 10 tablespoons. The total blood volume required for this trial will be approximately 2 cups.

On day 41, you will be required to consume a small amount of deuterated water (about 2-3 tablespoons). The movement of these tagged materials will permit assessment of the change in fatty acid metabolism of your body in response to your diet. All of the above tagged materials are non-radioactive, non-toxic, and do not pose any health risk to you.

During the last week of each phase, you will also be required to undergo a non-invasive ultrasound test called flow mediated dilatation (FMD), which will help us measure how efficiently your arteries are regulating blood flow. In addition, your arterial health will be further measured through another non-invasive procedure using a Mobil-O-Graph which will only require you to wear a blood pressure cuff which measures blood pressure at the same time as determining your blood vessel elasticity.

Risks and discomforts
As with any clinical trial, there may be as yet unknown or unforeseen risks of taking part.

The dietary oils contained within the meals at the proposed level has been shown to have no known direct negative side effects on health in several dozen existing animal and human experiments. Some known risks, although rare, are associated with placing a needle into a vein. These include the possibility of infection, perforation or penetration of the needle through the vein, and bleeding, pain, or bruising at the site.

Vascular ultrasound test discomfort and risks
There are no known risks associated with ultrasound. Measuring FMD requires the forearm blood pressure cuff to be inflated tightly. This may cause participants' hands and arms below the blood pressure cuff to go numb or feel a tingling or pricking sensation while the cuff is inflated and after it is released; it is similar to the sensation of your hand or arm "falling asleep." During the 5 minutes that the blood pressure cuff is inflated on your forearm, your arm could become numb and we will ask you not to move it. This might be moderately painful. However, any discomfort or numbness should go away within minutes of cuff deflation and there are no known long-term risks associated with this test. There is a possibility for red blotching or mild bruising (petechiae) appearing on the skin above and below the location of the blood pressure cuff. Studies in adults indicate that petechiae is rare (occurring in less than 0.5% of patients), is typically not uncomfortable, and does not require treatment. There are no risks associated with measurement of blood pressure, heart rate, or EKG as long as the participant is not allergic to adhesive tape. Paper and/or foam EKG electrodes are available for individuals with adhesive tape sensitivities. You will be given the opportunity to inspect these electrodes prior to participating so you can determine if you will have a reaction to them. Temporary redness at the site of the electrode placement is possible.

In case you feel any discomfort during the experimental trial a physician, Dr. Erica Luong, will be available to contact at any time. Dr. Erica Luong can be reached at 204 890 3441.

Benefits
You may not benefit from participation in this research; however, the study should contribute to a better understanding of the effects of dietary oils on body composition and blood fat levels as well as CVD biomarkers. You will also receive access to your test results when they become available.

Costs
All clinic and professional fees, diagnostic and laboratory tests that will be performed as part of this study are provided at no cost to you. There will be no cost for the study treatment that you will receive.

Remuneration for participation
You will receive up to a maximum of $750.00 for your time and inconvenience of the study schedule. This amount will be provided as $175.00 remunerated at the end of each of the first two phases $400.00 will be provided upon completion of the 3rd and final phase. If you withdraw early from the study, you will receive an appropriate pro-rated fraction of this amount.

Alternatives
You are not obligated to participate in this study. The study coordinators, physician and principal investigator will answer any questions you have about the experimental group of this study.

Confidentiality
Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. The RCFFN staff involved with your care may review/copy medical information that may reveal your identity. With your permission, the study doctor may also write to your Family Doctor to tell him/her that you are taking part in a study or to obtain further medical information. The Biomedical Research Ethics Board at the University of Manitoba and National Research Council Research Ethics Board may also review your research-related records for quality assurance purposes. If you are a research participant from the St. Boniface General Hospital, your research related records may be reviewed by the St. Boniface General Hospital for quality assurance purposes. Other agencies that may review your research related records for quality assurance and data analysis include; St. Boniface Research Centre, Manitoba; University of Toronto, Ontario; University of Laval, Quebec; Pennsylvania State University, Pennsylvania, United States; Canola Council of Canada; and Agriculture and Agri-Food Canada. But these agencies will not be able to link your research related data with your personal information. If the results of the trial are published, your identity will remain confidential. Personal information such as your name, address, telephone number and/or any other identifying information will not leave the RCFFN.

Study samples will be stored in a locked freezer at the RCFFN. Only the study coordinators and the principal investigator will have access to the samples. Your samples will not be used for any additional analyses, nor stored for any longer than 5 years, nor shared with any other group, other than is indicated in the protocol, without your prior specific consent.

Voluntary participation/withdrawal from the study
Your decision to take part in this study is voluntary. You may refuse to participate or you may withdraw from the study at any time. Your decision to not participate or to withdraw from the study will not affect your other medical care.

Your participation in this study may be terminated without your consent by the study coordinators, physician or principal investigator. The study staff will withdraw you if he/she feels that participation is no longer in your best interest, or if you fail to follow the directions of the study staff.

If you decide to participate, you will agree to cooperate fully with the study visit schedule, and will follow the study staff's instructions.

We will tell you about new information that may affect your health, welfare, or willingness to stay in this study.

Should you wish to withdraw your participation from the study, you must inform the study coordinators so that your file can be officially close.

Medical care for injury related to study
In the event of an injury that occurs to you as a direct result of participating in this study, or undergoing study procedures you should immediately notify the study physician, Dr. Erica Luong at 204 890 3441 or go to your nearest emergency room to receive necessary medical treatment. You are not waiving any of your legal rights by signing this consent form nor releasing the investigator or the sponsor from their legal and professional responsibilities. If any health abnormalities are identified in the clinical tests conducted during this experiment, Dr. Erica Luong will be contacted, who will inform you of the results.

Questions
You are free to ask any questions that you may have about your treatment and your rights as a research subject. If any questions come up during or after the study or if you have a research-related injury, contact the study doctor and the study staff.

Investigator:	Dr. Peter Jones	Tel No.	204 474 9787
Coordinator:	Julia Rempel	Tel No.	204 480 1042
Study Physician:	Dr. Erica Luong	Tel No.	204 890 3441

For questions about your rights as a research subject, you may contact:

The Biomedical Research Ethics Board, University of Manitoba at 204 789 3389

Do not sign this consent form unless you have a chance to ask questions and have received satisfactory answers to all of your questions.

This study is registered on a publicly available Registry Databank at Clinicaltrials.gov. ClinicalTrials.gov is a website that provides information about federally and privately

supported clinical trials. A description of this clinical trial will be available on http://ClinicalTrials.gov. This website will not include information that can identify you. At most, the website will include a summary of the results. You can search this website at any time.

Consent

I agree to allow the study doctor to inform my family doctor that I am participating in this study or to obtain information regarding my medical history.

Yes ☐ No ☐

1. I have read and understood this Information and Consent Form, and I freely and voluntarily agree to take part in the clinical trial (research study) described above.

2. I understand that I will be given a copy of the signed and dated Information and Consent Form. I have received an explanation of the purpose and duration of the trial, and the potential risks and benefits that I might expect. I was given sufficient time and opportunity to ask questions and to reflect back my understanding of the study to study personnel. My questions were answered to my satisfaction.

3. I agree to cooperate fully with the study doctor and will tell him if I experience any side effects, symptoms or changes in my health.

4. I am free to withdraw from the study at any time, for any reason, and without prejudice to my future medical treatment.

5. I have been assured that my name, address and telephone number will be kept confidential to the extent permitted by applicable laws and/or regulations.

6. By signing and dating this document, I am aware that none of my legal rights are being waived.

Signature:_____ Date: _____

Printed name of above:_____

I confirm that I have explained the purpose, duration etc of this clinical trial, as well as any potential risks and benefits, to the subject whose name and signature appears above. I confirm that I believe that the subject has understood and has knowingly given their consent to participate by his/her personally dated signature.

Signature:_____ Date: _____

Printed name of above:_____ Study role:_____

ALL SIGNATORIES MUST DATE THEIR OWN SIGNATURE

D.6 Genetic consent form

Richardson Centre for Functional Foods and Nutraceuticals

ADDITIONAL RESEARCH SUBJECT INFORMATION AND CONSENT FORM FOR GENETICS ANALYSIS

Title of Study: **Canola Oil Multi-center Intervention Trial II (COMIT II): Effects of Oleic Acid Enriched and Regular Canola Oil on Body Composition and Lipid Metabolism in Participants with Metabolic Syndrome Risk Factors**

Investigator: Peter Jones, PhD
Richardson Centre Functional Foods and Nutraceuticals
University of Manitoba
196 Innovation Drive, Smartpark
Winnipeg, Manitoba R3T 6C5
Phone: (204) 474-9787

Sponsor: Canola Council of Canada, 400-167 Lombard Avenue, Winnipeg, Manitoba, R3B OT6

You are being asked to participate in a research study. Please take your time to review this Information and Consent Form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

The study doctor and institution are receiving professional fees and financial support to conduct this study.

Nature and duration of procedure
From the blood drawn during the clinical study as outlined in the Research Subject Information and Consent Form, we would like to extract genetic information from your cells and perform analyses using laboratory techniques that augment and recognize specific genes to determine

how your genetic makeup influences the efficiency of your body in converting dietary oil-derived fatty acids into longer chain fatty acids that are known to have health benefits, and how the intake of these dietary oil-derived fatty acids change your gene expression profile. Genetic information which includes DNA and RNA are molecules found in the cells of your body and are organized into genes that contain all of the information needed to make the proteins that perform specific biological functions in your body.

Confidentiality and safekeeping of biological samples containing genetic information

All of the information obtained about you and the results of the research will be treated confidentially. We will protect your confidentiality by assigning your samples containing genetic information a specific code. This code will link you to your samples containing genetic information and can only be decoded by the principal researcher or an individual authorized by the latter. Samples containing your genetic information will be kept at the Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, under the supervision of Dr. Peter Jones for a 5-year period following the end of the research project. After this time, all samples will be destroyed. Your samples containing genetic information will only be used for the purpose of this research project.

Your participation and the results of the research will not appear in your medical record. Although the results of this study may be published or communicated in other ways, it will be impossible to identify you. Unless you have provided specific authorization or where the law permits or a court order has been obtained, your personal results will not be made available to third parties such as employers, government organizations, insurance companies, or educational institutions. This also applies to your spouse, other members of your family and your physician. However, for the purposes of ensuring the proper management of research, it is possible that a member of an ethics committee, a Health Canada representative, or a representative from the Richardson Centre for Functional Foods and Nutraceuticals may consult your research data and record. You can communicate with the research team to obtain information on the general progress or the results of the research project. Project updates will be mailed at the end of the project. However, we will not communicate any individual results to you.

Potential risks and/or benefits

Some known risks, although rare, are associated with placing a needle into a vein. These include the possibility of infection, perforation or penetration of the needle through the vein, and bleeding, pain, or bruising at the site. In case you feel any discomfort during the experimental trial a physician, Dr. Erica Luong, will be available to contact at any time. Dr. Erica Luong can be reached at 204 890 3441.

While there may be no direct benefits to you for taking part in these additional analyses, we hope that these results will provide us with the information on genetic characteristics of people in response to the intake of the treatment oils.

ADDITIONAL RESEARCH SUBJECT INFORMATION AND CONSENT FORM FOR GENETIC ANALYSIS

Signature of participant

The content of the procedure and procedures associated with it have been fully explained to me, and all experimental procedures have been identified. I have had the opportunity to ask questions concerning any and all aspects of the project and procedures involved, and may continue in the future to ask further questions at any time, as it is my right to do so. I am aware that I may refuse to participate as well as withdraw my consent at any time. I acknowledge that no guarantee or assurance has been given by anyone as to the results to be obtained and that my participation in this study is completely voluntary. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. Samples will not be utilized for any additional analyses, nor stored for any prolonged period, nor shared with any other group, other than is indicated in the protocol, without my specific consent.

I, _____, have read the above description. I have been made aware of all the procedures, advantages and disadvantages of the study, which have been explained to me.

_____ _____

Signature of Subject Date

I confirm that I have explained the purpose, duration etc of this clinical trial, as well as any potential risks and benefits, to the subject whose name and signature appears above. I confirm that I believe that the subject has understood and has knowingly given their consent to participate by his/her personally dated signature.

Signature:_____ Date/Time: _____

Printed name of above:_____ Study role:_____

D.7 Participant information sheet

Canola Oil Multi-Centre Intervention Trial (COMIT II) Trial Instructions

Controlled Feeding Helpful Hints

We recognize that participating in a controlled feeding study is a significant commitment, so we have put together some helpful hints to guide you along the way. But please, if you have any problems, concerns or questions please ask any of the staff – we will try to help you as best we can.

Study Contact Info

Please let us know as soon as possible if you are not coming at your "regular" time, are having any problems or need to fill out a "pack out request" (to have meals packed out for a trip or meeting).

Allowed Beverages

1) Caffeine-free diet or unsweetened beverages may be consumed in any amount desired. These include:

    a. Water
    b. Calorie-free mineral water
    c. Diet caffeine-free soda
    d. Crystal Light or sugar-free KoolAid (the Crystal Light cannot be the fortified kind)
    e. Decaf Coffee and Tea

2) Caffeinated no-calorie diet soda beverages and caffeinated coffee and tea are limited to:

    a. **2 servings per day**
    b. Diet Soda - 1 serving is one 12 oz can
    c. Coffee and tea - 1 serving is 8 oz

Beverages Not Allowed

1) Alcoholic beverages are limited to 2/week for this study. (1 alcoholic drink is considered to be a 12 oz beer, 5 oz of wine, or 1.5 oz of hard liquor and if you drink hard liquor the mixer must be non-caloric, i.e., diet soda, water, etc.)

2) Regular soda or beverages with calories are not allowed, this includes all regular sodas, fruit juices, vegetable juices, milk etc.

Allowed Seasonings & Sweeteners

1) You may use the following seasonings as desired:
    a. Lemon Pepper
    b. Pepper
    c. Salt-free seasonings
    d. Mrs. Dash
    e. Lemon Juice
    f. Tabasco or hot pepper sauce

2) The following condiments are allowed in limited amounts (you may have up to 5 units/day). One unit is listed for each.
    a. Ketchup - 1 packet
    b. Mustard - 2 packets
    c. Horseradish - 1 tbsp

3) Sweeteners:
   Any non-caloric sweetener, e.g., Sweet-n-Low, Equal

Sweeteners Not Allowed

The following sweeteners are **not** allowed:

Any sweetener with calories, e.g., sugar of any kind (brown, raw, white etc), honey

Allowed Medications

During the study, you will be asked on a daily basis if you have been ill and if so, have you taken any medication. If necessary, and on an occasional basis, it is OK to take over-the-counter and prescription medication as listed below. For any medication not listed below, please ask.

Headache/Pain Medications	Tylenol – check before taking any other pain medication (such as Advil, Ibuprofen, etc)
Sleep/Sedative Medications	OTC Preparations – check with study staff
Cold/Allergy Medications	Check with study staff
Laxatives	Senna - only for occasional use

Antidiarrheal	Lomotil, Kaopectate - only for occasional use
Cough	Check with study staff

Do not take Aspirin, or vitamin/herb supplements. If you need to take an antibiotic, please check with the study staff before taking it.

Gum

Sugar-free chewing gum is allowed.

Fasting Blood Draws

Please be sure to fast for 12 hrs prior to each blood draw. You may only drink water during these fasting periods (we advise you to drink plenty of water 2 days prior to your visit as this will facilitate blood sampling). **Also, please do not drink alcohol for 48 hrs prior to each blood draw, and caffeinated beverages 12 hrs prior to each blood draw**. We will remind you when the blood draws are approaching so that you will remember about the fasting and alcohol restriction.

Exercise

Please do not alter your level of physical activity during the study. Ideally, we would like you to maintain a consistent level of activity, with very few changes to your normal routine. It also is important not to engage in very strenuous activity (i.e. aerobics class, jogging, etc) on the day before a blood draw.

HINTS FOR THE STUDY:

1) If you are a coffee or tea drinker, you may use some of the milk from your breakfast in your beverage. You may NOT use additional milk.
2) You may save some breakfast butter for use in that day's dinner. There is the entrée, and usually a vegetable and dinner roll to divide it up between if you feel there is too much for breakfast.

Thanks for your participation!!!! Questions??

Please ask study staff:
Phone: 204-480-1042 (available Monday-Friday 8:30-4:30)
Email: canolatrial2@gmail.com

D.8 Participants' weight monitoring form

Participant Code: _____

Phase: _____

Date	Weight (lb)	Weight (kg)

D.9 Shake consumption checklist

Participant Code: _____

Phase: _____

Date	Shake taken?		Comments
	Participant Initial	Coordinator Initial	

D.10 Data collection forms

Participant Code:_____ Phase: ____

Participant Code _____

Study Phase _____ Treatment (i.e., A, B, C) _____

Start Date _____ End Date _____

SECTION 1: START AND END WEIGHT

Instructions: *Ask participants to remove heavy footwear and/or heavy articles of clothing (i.e., jackets, outerwear), before each weight measurement.*

Day 1: Weight (lbs) _____ Staff Initials _____

Day 2: Weight (lbs) _____ Staff Initials _____

Day 41: Weight (lbs) _____ Staff Initials _____

Day 42: Weight (lbs) _____ Staff Initials _____

SECTION 2: WAIST CIRCUMFERENCE

Instructions: *Waist circumference is measured at either the natural waist, i.e., smallest circumference of the abdomen, or midway between the lowest rib and iliac crest. The measuring tape should be snug around the body, but not pulled so tight that it is constricting.*

Day 1 or 2:

a. First measurement (cm): _____

b. Second measurement (cm): _____

c. Average (1st and 2nd) waist circumference measurement: _____

Staff Initials _____

Day 41 or 42:

a. First measurement (cm): _____

b. Second measurement (cm): _____

c. Average (1st and 2nd) waist circumference measurement: _____

Staff Initials _____

SECTION 3: SEATED BLOOD PRESSURE

Has the participant consumed caffeine, used any medication or eaten any food in the last 12 hours, and/or exercised in the past 2 hours? □ Yes* □ No

*If yes, participant needs to be rescheduled for a blood pressure measurement.

Instructions: *The participant should be instructed to relax as much as possible; ideally, at least 5 minutes should elapse before the first reading is taken. Apply cuff to non-dominant arm. After applying the cuff, the participant must be quiet and remain continuously seated without legs crossed for 5 minutes. Instruct the participant not to talk during the reading. Wait 3 minutes after each reading before taking the next reading.*

Day 1 or 2:

a. First blood pressure measurement (mmHg): ___ ___ / ___ ___ (SBP/DBP)
b. Second blood pressure measurement (mmHg): ___ ___ / ___ ___ (SBP/DBP)
c. Third blood pressure measurement (mmHg): ___ ___ / ___ ___ (SBP/DBP)
d. Average (2nd and 3rd) blood pressure measurement: ___ ___ / ___ ___ (SBP/DBP)

Staff Initials _____

Day 41 or 42:

a. First blood pressure measurement (mmHg): ___ ___ / ___ ___ (SBP/DBP)
b. Second blood pressure measurement (mmHg): ___ ___ / ___ ___ (SBP/DBP)
c. Third blood pressure measurement (mmHg): ___ ___ / ___ ___ (SBP/DBP)
d. Average (2nd and 3rd) blood pressure measurement: ___ ___ / ___ ___ (SBP/DBP)

Staff Initials _____

SECTION 4: BLOOD COLLECTION

DAY 1

Date (Month Day, Year) _____ Time (HH:MM) _____

Fasted over last 12 hours? Yes ___ / No ___

Caffeine in last 12 hours? Yes ___ / No ___ If Yes, when _____

Alcohol in last 48 hours? Yes ___ / No ___ If Yes, when _____

Arm Right ___ / Left___

Collect:
2 X 7.5 ml serum (red/grey SST tube) ____ (day 1, 2, 41, 42)
2 X 4 ml plasma heparin (green top) ____ (day 1, 2, 41, 42)
2 X 4 ml plasma EDTA (purple top) ____ (day 1, 2, 41, 42)

Have all blood samples been obtained? Yes ___ / No** ___

*** If No, please state reasons why:* _____

_____

_____

_____

Ease of Blood Draw: Excellent ___ Good ___ Difficult ____ Very Difficult ____

Comments (Anything which could impact blood draw results? i.e., Are you feeling stressed? How did you sleep last night? Has anything out of the ordinary happened over the past 48 hours?):_____

_____

_____

_____

Name of Phlebotomist:_____

SECTION 4: BLOOD COLLECTION (continued)

DAY 2

Date (Month Day, Year) _____ Time (HH:MM) _____

Fasted over last 12 hours? Yes ___ / No ___

Caffeine in last 12 hours? Yes ___ / No ___ If Yes, when _____

Alcohol in last 48 hours? Yes ___ / No ___ If Yes, when _____

Arm Right ___ / Left___

Collect:
2 X 7.5 ml serum (red/grey SST tube) ____ (day 1, 2, 41, 42)
2 X 4 ml plasma heparin (green top) ____ (day 1, 2, 41, 42)
2 X 4 ml plasma EDTA (purple top) ____ (day 1, 2, 41, 42)

Have all blood samples been obtained? Yes ___ / No** ___

*** If No, please state reasons why:* _____

_____

_____

_____

Ease of Blood Draw: Excellent ___ Good ___ Difficult ____ Very Difficult ____

Comments (Anything which could impact blood draw results? i.e., Are you feeling stressed? How did you sleep last night? Has anything out of the ordinary happened over the past 48 hours?)**:**_____

_____

_____

_____

Name of Phlebotomist:_____

SECTION 4: BLOOD COLLECTION (continued)

DAY 41

Date (Month Day, Year) _____ Time (HH:MM) _____

Fasted over last 12 hours? Yes ___ / No __

Caffeine in last 12 hours? Yes ___ / No ___. If Yes, when _____

Alcohol in last 48 hours? Yes ___ / No ___ If Yes, when _____

Deuterium water provided?* Yes ___ / No ___ ***Only required on day 41 of each phase***

Arm Right ___ / Left___

Collect:
2 X 7.5 ml serum (red/grey SST tube) ____ (day 1, 2, 41, 42)
2 X 4 ml plasma heparin (green top) ____ (day 1, 2, 41, 42)
2 X 4 ml plasma EDTA (purple top) ____ (day 1, 2, 41, 42)

Have all blood samples been obtained? Yes ___ / No** ___

*** If No, please state reasons why:* _____

_____

_____

_____

Ease of Blood Draw: Excellent ___ Good ___ Difficult ____ Very Difficult ____

Comments (Anything which could impact blood draw results? i.e., Are you feeling stressed? How did you sleep last night? Has anything out of the ordinary happened over the past 48 hours?):_____

_____

_____

_____

Name of Phlebotomist:_____

SECTION 4: BLOOD COLLECTION (continued)

DAY 42

Date (Month Day, Year) _____ Time (HH:MM) _____

Fasted over last 12 hours? Yes ___ / No __

Caffeine in last 12 hours? Yes ___ / No ___ If Yes, when _____

Alcohol in last 48 hours? Yes ___ / No ___ If Yes, when _____

Arm Right ___ / Left___

Collect:

2 X 7.5 ml serum (red/grey SST tube)	____ (day 1, 2, 41, 42)
2 X 4 ml plasma heparin (green top)	____ (day 1, 2, 41, 42)
2 X 4 ml plasma EDTA (purple top)	____ (day 1, 2, 41, 42)
1 X 2.5 ml whole blood (PAXgene tube)	**____ (day 42)**

Have all blood samples been obtained? Yes ___ / No** ___

*** If No, please state reasons why:*

☐ No PAXgene tube collected since genetic analysis consent not given

☐ Other (please describe): _____

_____

_____

_____

Ease of Blood Draw: Excellent ___ Good ___ Difficult ____ Very Difficult ____

Comments (Anything which could impact blood draw results? i.e., Are you feeling stressed? How did you sleep last night? Has anything out of the ordinary happened over the past 48 hours?)

:_____

_____

_____

_____

Name of Phlebotomist:_____

SECTION 5: DXA

To be completed by study staff:

BASELINE DXA, DAY: 1 2 3 (please circle one)

Date (Month Day, Year) _____ Time of scan (HH:MM): _____

Since screening has the volunteer had any radiation exposure (e.g. x-rays, radiotherapy, CT scan)? Yes ___ / No ___

Date & details: _____

_____

_____

FEMALES OF CHILDBEARING AGE ONLY:

Possibility of being pregnant Yes ___ / No ___

Has a pregnancy test been offered? Yes ___ / No ___

Has a test been taken? Yes ___ / No*** ___ Test Results (circle): **Negative / Positive**

Result Confirmation:

Participant signature:_____ Study staff signature:_____

***If no pregnancy test taken please explain why:

_____

_____

Check:

Jewelry removed (including glasses): Yes ___ / No ___

Spine straight: Yes ___ / No ___

Comments:_____

_____

_____

Staff Initials:_____

SECTION 5: DXA (continued)

To be completed by study staff:

ENDPOINT DXA, DAY: 40 41 42 (please circle one)

Date (Month Day, Year): _____ Time of scan (HH:MM): _____

Since screening has the volunteer had any radiation exposure (e.g. x-rays, radiotherapy, CT scan)? Yes ___ / No ___

Date & details: _____

_____

_____

FEMALES OF CHILDBEARING AGE ONLY:

Possibility of being pregnant Yes ___ / No ___

Has a pregnancy test been offered? Yes ___ / No ___

Has a test been taken? Yes ___ / No*** ___ Test Results (circle): **Negative / Positive**

Result Confirmation:

Participant signature:_____ Study staff signature:_____

***If no pregnancy test taken please explain why:

_____

_____

Check:

Jewelry removed (including glasses): Yes ___ / No ___

Spine straight: Yes ___ / No ___

Comments:_____

_____

_____

Staff Initials:_____

D.11 Weekly monitoring forms

Week _____

1. In the past week has your exercise level changed?

   ☐ Yes ☐No

   If Yes, was it: ☐ More Active ☐ Less Active ☐ No Exercise

   *__Please remember to keep your exercise level constant throughout the study.__

2. Have you taken any prescription or non-prescription drugs in the past week?

   ☐ Yes ☐No

   If Yes, specify:

Name	Dosage	Frequency	Reason	Start Date	End Date

3. Have you taken any vitamins, minerals or other supplements in the past week?

   ☐ Yes ☐No

   If Yes, specify:

Name	Dosage	Frequency	Reason	Start Date	End Date

4. Have you had any changes in a medical condition, new illness or injury in the past week?

   ☐ Yes ☐No

   If Yes, describe: _____

5. If you were ill in the past week, did your eating change as a result?

☐ Yes ☐No

If Yes, describe: _____

6. Have you eaten any foods outside of the study diet?

☐ Yes ☐No

If Yes, describe: _____

7. Any specific comments regarding study food:

_____

_____

_____

_____

D.12 Adverse event report form

Description	Start Date	Intensity*	Outcome	Date Ended	Diet Related?

Note: Mild*: An event that is easily tolerated by the volunteer, causing minimal discomfort and not interfering with everyday activities; **Moderate*:** An event that is sufficiently discomforting to interfere with normal everyday activities; **Severe*:** An event which is incapacitating and prevents normal everyday activities

D.13 Study comments and protocol deviation form (if any)

Date (Month Day, Year)	Comments	Study Personnel Initials

D.14 Participant status summary

☐ Participant completed the study

☐ Participant withdrew from the study Date of withdrawal: _____

REASON FOR WITHDRAWAL. Tick appropriate box(es):

☐ Informed consent withdrawn by the participant

☐ Failure to comply with study requirements

☐ Investigator decision to withdraw participant

Provide rationale for withdrawal (if applicable):

_____

_____

_____

_____

☐ Participant's final results sent

☐ Statement supplier form completed

Investigator Initials _____

D.15 Instructions: Blood sample processing

Sample	Blood Collection Tube	Tube Volume	Processing Instructions	Aliquoting Instructions	Study Days
Serum	**Red**/**Grey** SST tube	2 × 7.5 mL	• Invert 5 times • Room temp for 30 min • Spin for 10 min @ 1000 × *g*	• Aliquot serum into cryovials[1] with **brown**[2] caps (0.5mL/tube) • Store at -80°C	1, 2, 41, 42
Plasma heparin	**Green** top (lithium heparin)	2 × 4.0 mL	• Invert 8 times • Spin immediately for 10 min @ 1300 × *g*	• Aliquot plasma into cryovials with **green**[3] caps (0.5mL/tube) • Aliquot WBC (buffy coat) into 1 (one) Cryo.s™ (RNase and DNase free vials)[4] • Aliquot RBC into cryovials with **red**[5] caps (0.5mL/tube) • Store all fractions at -80°C	1, 2, 41, 42
Plasma EDTA	**Purple** top (K2 EDTA)	2 × 4.0 mL	• Invert 8 times • Spin immediately for 10 min @ 1300 × *g*	• Aliquot plasma into cryovials with **purple**[6] caps (0.5mL/tube) • Aliquot WBC (buffy coat) into 1 (one) Cryo.s™ (RNase and DNase free vials)[4] • Aliquot RBC into cryovials with **red**[5] caps (0.5mL/tube) • Store all fractions at -80°C	1, 2, 41, 42
Whole blood	PAXgene tube[7]	1 × 2.5 mL	• Invert 8 times • Store tube upright at room temperature for 2 hrs	• Store at -20°C for 24hrs, then transfer to -80°C	42

Note: [1]Cryovials, microtubes, 0.5ml with skirted base, without screw cap, bag of 500, Sarstedt #72.730.007; [2]**Brown** cap, screw cap for microtubes, color coded, **brown**, bag of 500, Sarstedt #65.716.009; [3]**Green** cap screw cap for microtubes, color coded, **green**, bag of 500, Sarstedt #65.716.005; [4]RNase and DNase free vials Cryo.s™ Cryogenic Storage Vials, Polypropylene, Sterile, Greiner Bio-One, 2ml, case of 500, VWR #82050-206; [5]**Red** cap, Screw cap for microtubes, color coded, **red**, bag of 500, Sarstedt #65.716.003; [6] **Violet** cap: Screw cap for microtubes, color coded, **violet**, bag of 500, Sarstedt #65.716.008; [7] PAXgene Blood RNA tube, 2.5 mL, Qiagen Product #762165

Appendix E

Sample menu: Background diet provided to participants

Based on 3000 kcal: 35% fat, 50% carbohydrate, and 15% protein of total energy

Breakfast	**gm**
English muffin, whole grain	90
Egg substitute	75
Canadian bacon	38
American cheese	28
Shake	330
Lunch	**gm**
Chicken salad:	
Chicken (cooked and diced)	75
Scallions (chopped)	6
Grapes (halved)	45
Light mayonnaise	16
Non-fat sour cream	16
Lemon juice	6
Lettuce (shredded)	35
Tomatoes	35
Pita bread - whole wheat	105
Melon balls (frozen)	285
Crackers – whole wheat (low-fat)	42
Dinner	**gm**
Jambalaya	220
Chicken breast (cooked)	120
Turkey sausage	40

Lettuce (romaine)	85
Tomatoes	62
Carrots (grated)	55
Reduced fat Italian dressing	30
Dinner roll	55
Margarine	8
Applesauce (1 container)	112
Snack	**gm**
Banana muffin (1 muffin = 43 g)	86
Shake	330