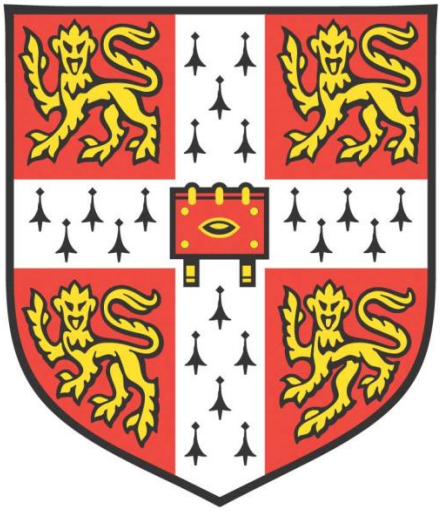
THE ROLE OF ALPHA OXIDATION IN LIPID METABOLISM



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This dissertation is submitted for the degree of Doctor of Philosophy

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DECLARATION

This dissertation is the result of my own work and includes nothing, which is the outcome of work done in collaboration except as declared in the preface and specified in the text.

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ABSTRACT

Recent findings have shown an inverse association between the circulating levels of pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) with the risk of pathological development in type 2 diabetes, cardio vascular disease and neurological disorders. From previously published research, it has been said that both these odd chain fatty acids are biomarkers of their dietary intake and are significantly correlated to dietary ruminant fat intake. However, there are profound studies that show the contrary where they do not display this biomarker correlation. Additionally, several astute studies have suggested or shown odd chain fatty acid endogenous biosynthesis, most often suggested *via* alpha oxidation; the cleavage of a single carbon unit from a fatty acid chain within the peroxisomes. To better understand the correlations and interactions between these two fatty acids with pathological development, the origin of these odd chain fatty acids needed to be determined, along with confirming their association with the disease aetiology.

To minimise animal & human experimentation we made use of existing sample sets made available through institutional collaborations, which produced both animal and human interventional study samples suitable for odd chain fatty acid investigations. These sample collaborations allowed us to comprehensively investigate all plausible contributory sources of these odd chain fatty acids; including from the intestinal microbiota, from dietary contributions, and derived from novel endogenous biosynthesis.

The investigations included two intestinal germ-free studies, two ruminant fat diet studies, two dietary fat studies and an ethanol intake study. Endogenous biosynthesis was assessed through: a stearic acid infusion, phytol supplementation, and an *Hacl1* knockout mouse model. A human dietary intervention study was used to translate the results. Finally, a study comparing circulating baseline C15:0 and C17:0 levels with the development of glucose intolerance.

We found that the circulating C15:0 and C17:0 levels were not significantly influenced by the presence or absence of intestinal microbiota. The circulating C15:0 levels were significantly and linearly increased when the C15:0 dietary composition increased; however, there was no significant correlation in the circulating C17:0 levels with intake. Circulating levels of C15:0 were affected by the dietary composition and factors affecting the dietary intake, e.g. total fat intake and ethanol, whereas circulating C17:0 levels were found to be independent of these variables. In our studies, the circulating C15:0 levels were not significantly affected by any expected variations in alpha oxidation caused by pathway substrate inhibition or gene knockout. However, C17:0 was significantly related, demonstrating it is substantially endogenously biosynthesised. Furthermore, we found that the circulating C15:0 levels, when independent of any dietary variations, did not correlate with the progression of glucose intolerance when induced, but the circulating C17:0 levels did significantly relate and linearly correlated with the development of glucose intolerance.

To summarise, the circulating C15:0 and C17:0 levels were independently derived; the C15:0 levels substantially correlated with its dietary intake, whilst the C17:0 levels proved to be separately derived from its endogenous biosynthesis *via* alpha oxidation of stearic acid. C15:0 was found to be minimally endogenously biosynthesised *via* a single cycle of beta oxidation of C17:0 in the peroxisomes, however, this did not significantly contribute to the circulating levels of C15:0. Additionally, only the baseline levels of C17:0 significantly correlated with the development of glucose intolerance. These findings highlight the considerable differences between both of these odd chain fatty acids that were once thought to be homogeneous and similarly derived. On the contrary, they display profound dietary, metabolic, and pathological differences.

PUBLICATIONS

1. **Jenkins, B**., West, J.A., Koulman, A., 2015. A Review of Odd-Chain Fatty Acid Metabolism and the Role of Pentadecanoic Acid (C15:0) and Heptadecanoic Acid (C17:0) in Health and Disease. Molecules 20, 2425–2444. doi:10.3390/molecules20022425

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7. **Benjamin J Jenkins**; Evelyn de Schryver; Paul P. Van Veldhoven; Albert Koulman. 2-hydroxyacyl-CoA lyase 1; Endogenous Biosynthesis of Heptadecanoic acid. Under co-author review.

8. **Benjamin J. Jenkins**; Edmond Y. Huang; Marianne Molin; Manar Aoun; Charles Coudray; Kari Almendingen; Eugene B. Chang; Martin Ronis; Albert Koulman. “The effect of dietary fat composition and ethanol on the metabolism of pentadecanoic acid and heptadecanoic acid.” In draft.

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butyryl ACP; further additions of acetyl ACP leads to the elongation of this moiety by two carbon units. The cyclic process of condensation followed by reduction, then dehydration, and then reduction continues until a sixteen carbon-acyl ACP is formed. Discontinuation of this biosynthetic process is due to the sixteen carbon-acyl ACP being a preferred substrate for a thioesterase that hydrolyses the sixteen carbon-acyl ACP to yield palmitic acid and ACP. 1) Acetyl ACP and malonyl ACP condense to form acetoacetyl ACP and two by-products: carbon dioxide and ACP, this reaction is catalysed by beta-ketoacyl-acyl-carrier-protein synthase I. 2) acetoacetyl ACP is then reduced to produce D-3-hydroxybutyryl ACP and $NADP^+$, this reaction is catalysed by 3-oxoacyl-(acyl-carrier-protein) reductase. 3) Then D-3-hydroxybutyryl ACP is dehydrated to form crotonyl ACP (a trans-Δ2-enoyl ACP) and water, this reaction is catalysed by 3-Hydroxyacyl ACP dehydrase. 4) Then crotonyl ACP is reduced, producing butyryl ACP and $NADP^+$, this reaction is catalysed by enoyl ACP reductase.

Figure 7: A schematic of the catabolic fatty acid beta oxidation process where an acetyl-CoA molecule is cleaved from the carboxylic acid terminus of a fatty acid. 1) Dehydrogenation of the alpha and beta carbons producing a trans bond. 2) Hydration of the beta carbon producing a β-hydroxyacyl-CoA. 3) Dehydrogenation of the beta hydroxyl of the β-hydroxyacyl-CoA producing a ketone. 4) A thiolytic cleavage; cleavage of β-ketoacyl-CoA cleaving acetyl-CoA and producing a (n-2)-acyl-CoA.

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*Figure 10: This figure shows an example of how the area under the curve can be calculated via the trapezoid rule. The trapezoidal rule uses each data point to construct an individual trapezoid and using the trapezoid area equation to find the individual segment area; area = ((A+B)/2) * H. The total area under the curve can then be calculated by summing up each individual trapezoid.*

Figure 11: Box and whiskers plot showing the influence of intestinal microbiota on plasma odd chain fatty acids in mice, values are percentage of

total fatty acids measured by gas chromatography with mass spectrometry detection. Box represent mean ± standard deviation, whiskers represent the minimum and the maximum values (n = 6-8 per group). Pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0), low fat chow diet (LF) and high fat diet (HF), conventional mice (CV) and germ-free mice (GF).

Figure 12: Comparison between the diet composition (mol %) and the plasma composition (mol %) of pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) between three different isocaloric high fat (37.5 % kcal) diets. The plasma samples were analysed by gas chromatography with mass spectrometry detection. Error bars represent ± standard error of the mean. (n = 3-4 mice per group). C15:0 (●); $R^2 > 0.999$, significance of slope ($p = 0.0134$), slope equation ($Y = 0.1487*X + 0.05577$). C17:0 (▲); $R^2 = 0.914$, significance of slope ($p = 0.19$), slope equation ($Y = 0.08288*X + 0.08343$).

Figure 13: The comparison between the diet composition and the serum composition (Mol %) of pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) in the five-stage ruminant fat diet dose response in rats. The serum samples were analysed by gas chromatography with mass spectrometry detection. Error bars represent ± standard error of the mean. (n = 6-9 per group). C15:0 (●); $R^2 = 0.997$, significance of slope ($p < 0.0001$), slope equation ($Y = 0.4062*X + 0.04530$). C17:0 (▲); $R^2 = 0.912$, significance of slope ($p = 0.0114$), slope equation ($Y = 0.9579*X + 0.06044$).

Figure 14: The effect of changing the proportions of dietary fat (corn oil) from 5% to 35% to 70% of the total energy content (% energy) on the serum composition (Mol %) of pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) whilst maintaining an identical fatty acid composition in between each diet. The serum samples were analysed by gas chromatography with mass spectrometry detection. The significance of the difference between each group is shown by the p-value star system determined from homoscedastic t-tests; where $p \leq 0.05$ was considered significant ($p<0.05$=, $p<0.01$=**,*

*p<0.001=***, p<0.0001=****). (n = 6-7 rats per group). Error bars represent ± standard error of the mean.*

*Figure 15: The effect of changing the proportions of dietary fat from 5% to 30% of the total energy content (% energy) across three different dietary sources of fat (basal fat, lard fat, and fish oil) on the serum composition (Mol %) of pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0). The fatty acid composition for the 5% and 30% fat diets under the same fat source are identical. The serum samples were analysed by gas chromatography with mass spectrometry detection. The significance of the difference between each group is shown by the p-value star system determined from homoscedastic t-tests; where p ≤ 0.05 was considered significant (p < 0.05 = *, p < 0.01 = **, p < 0.001 = ***, p < 0.0001 = ****). (n = 6-7 rats per group). Error bars represent ± standard error of the mean.*

Figure 16: Healthy adult dogs (n=5) were fed a high fat diet (4-8 weeks) where they had their glucose tolerance assessed by an oral glucose tolerance test at the start (x) and end (■) of the dietary intervention. The points represent the average insulin concentration (pmol/L) for each time point (minutes).

Figure 17: Healthy adult dogs (n=5) were fed a high fat diet (4-8 weeks) where they had their glucose tolerance assessed by an oral glucose tolerance test at the start (x) and end (■) of the intervention. The points represent the average glucose concentration (mmol/L) for each time point (minutes).

Figure 18: Oral glucose tolerance test (OGTT) performed at the beginning and at the end of a four to eight-week high fat diet intervention in dogs. Glucose and insulin were measured from 0 to 180 minutes and the area under the curve (AUC) was calculated using the trapezoidal rule. The graph data points represent the difference between the insulin AUC and the glucose

*AUC, from the beginning to the end of the four to eight-week high fat diet in relation to the C15:0 fatty acid levels at baseline (Mol %). Top: INSULIN (Ins.) - C15:0 R^2 = 0·053, Y = 267588*X – 2123, significance of the slope; p = 0.7094. Bottom: GLUCOSE (Glc.) - C15:0 R^2 = 0·048, Y = 4572*X - 36.62, significance of the slope; p = 0.7225.*

*Figure 19: Oral glucose tolerance test (OGTT) performed at the beginning and at the end of a four to eight-week high fat diet intervention in dogs. Glucose and insulin were measured from 0 to 180 minutes and the area under the curve (AUC) was calculated using the trapezoidal rule. The graph data points represent the difference between the insulin AUC and the glucose AUC, from the beginning to the end of the four to eight-week high fat diet in relation to the C17:0 fatty acid levels at baseline (Mol %). Top: INSULIN (Ins.) - C17:0 R^2 = 0·957, Y = -267356*X + 53142, significance of the slope; p = 0.0039. Bottom: GLUCOSE (Glc.) - C17:0 R^2 = 0·755. (n = 5), Y = -4253*X + 854.0, significance of the slope; p = 0.0558.*

LIST OF ABBREVIATIONS

µm	micrometre
µMol/L	micromoles per litre
Da	Dalton
G	gravitational constant
g	grams
Hz	hertz
kcal	kilocalories
mg	milligrams
mg/mL	milligram per millilitre
mL	millilitre
mm	millimetre
mmol/L	milli mol per litre
Mol %	percentage of the total fatty acids measured
nmol / kg / day	nano mol per kilogram per day
^{o}C	degrees Celsius
kcal / day	kilocalories per day
g / day	grams per day
pmol/L	picomol per litre
w / w	weight per weight
14C	radioactive carbon fourteen
Fe^{2+}	ferrous ion
Mg^{2+}	magnesium ion
CO_2	carbon dioxide

U-^{13}C	universally labelled with stable isotope carbon-thirteen
R^2	R squared
EtOH	ethanol
ACP	acyl carrier protein
ATP	adenosine triphosphate
AUC	area under the curve
C1:0	methanoic acid
C2:0	ethanoic acid
C3:0	propanoic acid
C4:0	butanoic acid
C5:0	pentanoic acid
C6:0	hexanoic acid
C7:0	heptanoic acid
C8:0	octanoic acid
C9:0	nonanoic acid
C10:0	decanoic acid
C11:0	undecanoic acid
C12:0	dodecanoic acid
C13:0	tridecanoic acid
C14:0	tetradecanoic acid
C15:0	pentadecanoic acid
C16:0	palmitic acid
C17:0	heptadecanoic acid
C18:0	stearic acid
C19:0	nonadecanoic acid

C20:0	eicosanoic acid
C21:0	heneicosanoic acid
C22:0	docosanoic acid
C23:0	tricosanoic acid
C24:0	tetracosanoic acid
C25:0	pentacosanoic acid
C26:0	hexacosanoic acid
C27:0	heptacosanoic acid
C28:0	octacosanoic acid
C29:0	nonacosanoic acid
C30:0	triacontanoic acid
CoA	co-enzyme A
CV	conventional mice
CVD	cardio vascular disease
Fa2h	fatty Acid 2-Hydroxylase
$FADH_2$	1,5-dihydro-flavin adenine dinucleotide
FAME	fatty acid methyl esters
GC	gas chromatography
GC-MS	gas chromatography with mass spectrometry
GF	germ free mice
Glc.	glucose
G-WAT	gonadal white adipose tissues
Hacl1	2-Hydroxyacyl-CoA Lyase 1 gene
HACL1	2-Hydroxyacyl-CoA Lyase 1 enzyme / protein

Hacl1-/-	2-Hydroxyacyl-CoA Lyase 1 homozygous mutant / mutant knockout
Hacl1+/-	2-Hydroxyacyl-CoA Lyase 1 heterozygous wildtype / mutant
Hacl1+/+	2-Hydroxyacyl-CoA Lyase 1 homozygous wildtype / wildtype
HAO_2	2-hydroxy-acid oxidase
HF	high-fat diet
HPLC	high performance liquid chromatography
I-BAT	interscapular brown adipose tissue
Ins.	insulin
I-WAT	inguinal white adipose tissues
LF	low-fat (chow) diet
m-C10:0	methyl decanoate
m-C11:0	methyl undecanoate
m-C12:0	methyl dodecanoate
m-C13:0	methyl tridecanoate
m-C14:0	methyl tetradecanoate
m-C14:1	methyl tetradecenoate
m-C15:0	methyl pentadecanoate
m-C15:1	methyl pentadecenoate
m-C16:0	methyl hexadecanoate
m-C16:1 cis	methyl hexadecenoate
m-C16:1 trans	methyl trans hexadecanoate
m-C17:0	methyl heptadecanoate
m-C17:1	methyl heptadecenoate

m-C18:0	methyl octadecanoate
m-C18:1 cis	methyl octadecenoate
m-C18:1 trans	methyl trans octadecenoate
m-C18:2 cis	methyl octadecadienoate
m-C18:2 trans	methyl trans octadecadienoate
m-C18:3 cis	methyl octadecatrienoate
m-C20:0	methyl eicosanoate
m-C20:1 cis	methyl icosenoate
m-C20:2 cis	methyl icosadienoate
m-C20:3n3 cis	methyl icosatrienoate
m-C20:3n6 cis	methyl icosatrienoate
m-C20:4n6 cis	methyl icosatetraenoate
m-C21:0 cis	methyl henicosanoate
m-C22:0 cis	methyl docosanoate
m-C22:1n9 cis	methyl docosenoate
m-C22:1n9 cis	methyl docosenoate
MCT	medium chain triglyceride
MS	mass spectrometry
MtBE	methyl tert-butyl ether
NAD^+	nicotinamide adenine dinucleotide
NADH	di-hydro-nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
OC-FA	odd chain fatty acid
OGTT	oral glucose tolerance test
PC	phosphatidylcholine
PE	phosphatidylethanolamine

PI	phosphatidylinositol
PPAR-α	peroxisome proliferator-activated receptor alpha
PS	phosphatidylserine
R-WAT	retroperitoneal white adipose tissue
T2D	type 2 diabetes
TG	triglyceride
TPP	thiamine pyrophosphate
WHO	World Health Organization

DEFINITIONS

Biomarkers	a biological marker that when measured is indicative of a particular characteristic, either a biological process or is representative of its exogenous source.
Conventional mice	mice that have not been adulterated and are representative of 'normal' mice
Endogenous	originated from within the organism / cell
Exogenous	originated from outside the organism / cell
Germ free mice	mice that do not have any microbiota organisms living in or on them
Glucose intolerance	used to describe a metabolic condition which results in a higher than normal blood glucose levels (hyperglycaemia)
Gut / intestinal microbiota	the community of micro-organisms including bacteria and protozoa that reside inside the digestive tract
In vitro	(a Latin term) a biological process and / or cell culture kept in a laboratory vessel rather than inside a living organism
In vivo	(a Latin term) a biological process or cell that resides within a living organism
Isocaloric	diets that possess identical number of calories
Knockout	a genetically modified organism where a gene has been deactivated either by replacing, deleting or disrupting it

Ruminant	a mammal that re-chews its regurgitated cud from its rumen (first stomach containing partially digested food due to microbiota), e.g. cattle and sheep
Western diet	a loosely defined term for a diet that is high in saturated fat, red meats, simple carbohydrate and low in fresh fruit and vegetables; this diet is correlated with the development of metabolic diseases

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The inter- and intra- tissue pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) comparison; where an increase in one odd chain fatty acid correlates with an increase in the other odd chain fatty acid across the four tissue types analysed (plasma, liver, inguinal white adipose tissue, gonadal white adipose tissue and retroperitoneal white adipose tissue, and interscapular brown adipose tissue).

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- Power calculations for the intestinal microbiota – low fat (chow) diet study.
- Power calculations for the intestinal microbiota – high fat (ruminant fat based) diet study.
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- Power calculations for the dietary biomarker – ruminant fat supplementation study.
- Power calculations for the dietary biomarker – dietary fat percentage study.
- Power calculations for the dietary biomarker – dietary fat percentage and fat source study.
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1 INTRODUCTION

This thesis details the investigation of odd chain fatty acids and their role as dietary biomarkers, as well as possibly originating from other non-dietary sources, such as from the intestinal microbiota and/or from novel endogenous biosynthesis.

To preface the results presented within this thesis, a detailed description of the investigated molecules is included as well as their relationship with food, dietary intake, cellular / organism biochemistry and ultimately their relationship with pathology based on the currently available literature. The information gathered is from peer reviewed published articles and esteemed bodies / institutes of knowledge. Included in this introduction is the detailed information and explanations of the analytical methods used.

1.1 Fatty acid biochemistry

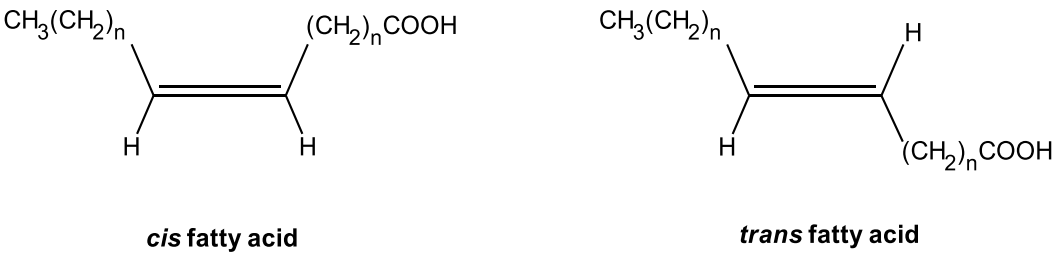
1.1.1 Structure and properties

A fatty acid is an aliphatic structure, typically consisting of a straight chain carbon backbone (either saturated or unsaturated) with at least one carboxylic acid functional group (Rustan and Drevon, 2001). Fatty acid chain lengths vary considerably but the most relevant fatty acids have a chain length ranging from 4-carbons long to 30-carbons and are separated into several groups, which are linked to their physiological relevance These are: short chain fatty acids (≤ 5 carbons), medium chain fatty acids (between 6 to 12 carbons), long chain fatty acids (13 to 21 carbons) and very long chain fatty acids (≥ 22 carbons). In mammalian biochemistry, fatty acids, on the most part have an even number of carbon atoms in the fatty chain; this is because of the *de novo* lipogenesis process (Berg et al., 2002), however, odd numbered fatty acids (also known as odd chain fatty acids) do exist at low concentrations / compositions (University of Alberta et al., 2011). Fatty acids in general do not exist as free carboxylic acids within the circulation (van der Vusse, 2009); this is because of

their high affinity to many types of protein; causing enzymatic inhibition in many of these cases. When free fatty acids are measured this is when the non-esterified fatty acids are bound to albumin in the circulation (Spector, 1975), hence, why free fatty acids are preferably termed non-esterified fatty acids.

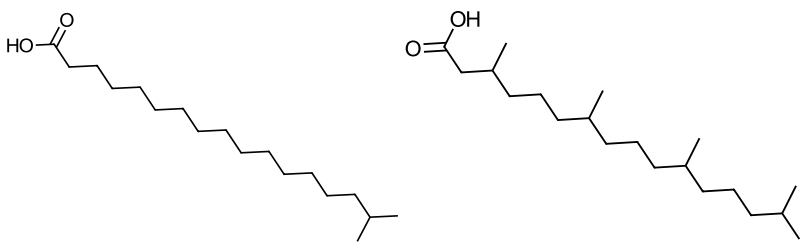
Saturated fatty acids have no carbon-carbon double bonds and therefore, each carbon bond is fully saturated with hydrogen (general molecular formula: $C_nH_{2n+1}COOH$). Due to continuous molecular motion *in vivo* and atomic free rotation of the carbon-carbon bonds, saturated fatty acids are capable of multiple orientations and configurations, however for simplicity; saturated fatty acids are expressed as extended straight chains. An unsaturated fatty acid possesses at least one carbon-carbon double bond within the structure; which can either have a *cis* or a *trans* configuration. An unsaturated fatty acid can have a single carbon-carbon double bond known as a mono-unsaturated fatty acid or they can have two-or-more carbon-carbon double bonds known as a poly-unsaturated fatty acid. An unsaturated fatty acid with a *cis* configuration is where the two present hydrogen atoms are adjacent to each other across the carbon-carbon double bond; the presence of a carbon-carbon double bond restricts the atomic motion around that bond position and also causes a bend in the fatty acid chain structure (Gunstone et al., 2007). By increasing the number of double bonds within the fatty acid chain they increase the contortion of the fatty acid molecule, this malformation increases the longitudinal cross-sectional area of the unsaturated fatty acid molecule and restricts inter-molecular packing & molecular interactions, therefore reduces the melting temperature of that substance. A *trans* configuration is where the two hydrogen atoms are opposite to each other on either side of the carbon-carbon double bond (*see Figure 1*). The *trans* configuration does not cause a significant bend in the acyl chain and has a similar longitudinal cross-sectional area to a saturated fatty acid.

Figure 1: ***Molecular structure of cis fatty acids and trans fatty acids. A cis carbon-carbon double bond configuration is where the two present hydrogen atoms are adjacent to each other across the carbon-carbon double bond. A trans configuration is where the two hydrogen atoms are opposite to each other on either side of the carbon-carbon double bond***.



Another common configuration of fatty acids is known as branched chain fatty acids, these are when there are alkyl chains (either one or more) branching from the authentic parent fatty acid chain. Typical branched chain fatty acids are usually saturated or mono-unsaturated and the alkyl branch is usually a methyl group (*see Figure 2*).

Figure 2: ***Two examples of branched chain fatty acids; on the left is 16-methyl heptadecanoic acid (trivial name is isostearic acid, $C_{18}H_{36}O_2$), on the right is a beta branched chain fatty acid; 3,7,11,15-Tetramethylhexadecanoic acid (trivial name is phytanic acid, $C_{20}H_{40}O_2$)***.



There are theoretically over one thousand different configurations that fatty acids can exhibit varying by differences in the chain length, degree of unsaturation, categorisation of the unsaturated bond(s), along with a range of additional bonded substituents along the chain (LIPID MAPS). However, around forty fatty acids make up the clear majority of measured species, of these, around three fatty acids make up about 80% of all measured / reported species.

Fatty acids are named systematically as carboxylic acid derivatives, numbering the chain from the carboxyl carbon. The names of fatty acids from a one carbon chain length to thirty-two carbon chain length are shown below (*see table 1*) (Chow, 2007). The -anoic ending of the saturated acid is changed to -enoic, -adienoic, -atrienoic, -atetraenoic, -apentaenoic, and -ahexaenoic to indicate the presence of one to six double bonds, respectively (Gunstone et al., 2007). Due to the complexity and length of the systematic names and the trivial names give little structural information; a shorthand notation system is commonly used in the literature where two numbers, prefixed by a capital 'C' are separated by a colon. The first number represents the chain length and the second number represents the quantity of double bonds in that fatty acid. An example of this shorthand notation is as follows; systematic name: octadecenoic acid, trivial name: oleic acid, where it has 18 carbon atoms and 1 carbon-carbon double bond. The shorthand notation for this fatty acid is C18:1.

Table 1: The systematic, trivial, and shorthand names of saturated fatty acids from one carbon in length (methanoic acid; C1:0) to thirty carbons in length (triacontanoic; C30:0).

Systematic name	Trivial name	Shorthand notation
Methanoic	Formic	C1:0
Ethanoic	Acetic	C2:0
Propanoic	Propionic	C3:0
Butanoic	Butyric	C4:0
Pentanoic	Valeric	C5:0
Hexanoic	Caproic	C6:0
Heptanoic	Enanthic	C7:0
Octanoic	Caprylic	C8:0
Nonanoic	Pelargonic	C9:0
Decanoic	Capric	C10:0
Undecanoic	-	C11:0
Dodecanoic	Lauric	C12:0
Tridecanoic	-	C13:0
Tetradecanoic	Myristic	C14:0
Pentadecanoic	-	C15:0
Hexadecanoic	Palmitic	C16:0
Heptadecanoic	Margaric	C17:0
Octadecanoic	Stearic	C18:0
Nonadecanoic	-	C19:0
Eicosanoic	Arachidic	C20:0

Heneicosanoic	-	C21:0
Docosanoic	Behenic	C22:0
Tricosanoic	-	C23:0
Tetracosanoic	Lignoceric	C24:0
Pentacosanoic	-	C25:0
Hexacosanoic	Cerotic	C26:0
Heptacosanoic	Carboceric	C27:0
Octacosanoic	Montanic	C28:0
Nonacosanoic	-	C29:0
Triacontanoic	Melissic	C30:0

NB. '-' indicates a fatty acid that does not have a trivial name.

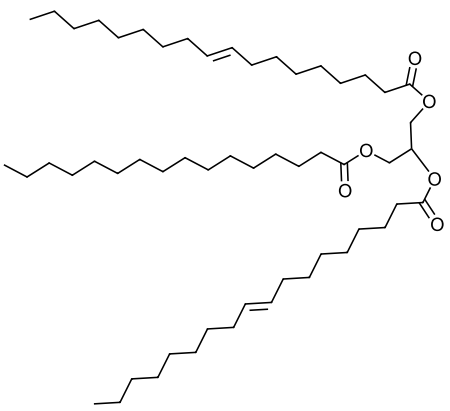
The structure of a fatty acid significantly affects its melting point (the melting point is physiologically relevant in cellular membranes and determining its fluidity); characteristics such as fatty acids with branched chains and *cis* double bonds will produce a lower melting point than their saturated equivalent counterparts. The number of carbons within the fatty acid chain will also affect the melting temperature (by increasing the carbon number the melting point also increases). Additionally, successive fatty acids with an odd number of carbon atoms in the chain have a lower melting point than their predecessor even chain fatty acid (Knothe and Dunn, 2009).

1.1.2 Fatty acid incorporation into whole lipids

The fatty acid structure represents the major lipid building block of many complex lipids and therefore are one of the most fundamental species of whole biological lipids; the term 'whole lipids' is used to describe larger lipid structures with a fatty acid component (Fahy et al., 2011). The most abundant whole lipid classes include: glycerolipids, glycerophospholipids, and sphingolipids (Quehenberger and Dennis, 2011), but many other classes exist.

Glycerolipids are a compound molecule where a glycerol molecule esterifies with up to three fatty acid moieties; mono-, di- and tri- prefix represent the esterification of one, two or three fatty acids to the fundamental glycerol molecule, respectively. The chain lengths of the fatty acids in naturally occurring glycerolipids can be of varying lengths and degrees of saturations but 16, 18 and 20 carbons are the most common. Similarly, with fatty acid species; glycerolipid systematic names are simplified to trivial names and further abbreviated to shorthand notation names (Liebisch et al., 2013). The capitals 'TG' denote the fundamental lipid specie is a triglyceride ('DG' denotes diglycerides and 'MG' denotes monoglycerides) and the individual fatty acid moieties are described similarly to fatty acids (where capital 'C' followed by the number of carbons and the number of unsaturated bonds separated by a colon) with each fatty acid species separated by a forward slash. The typical structure of a triglyceride is shown in the figure below (*see Figure 3*) with the systematic name, trivial name, and the shorthand notation.

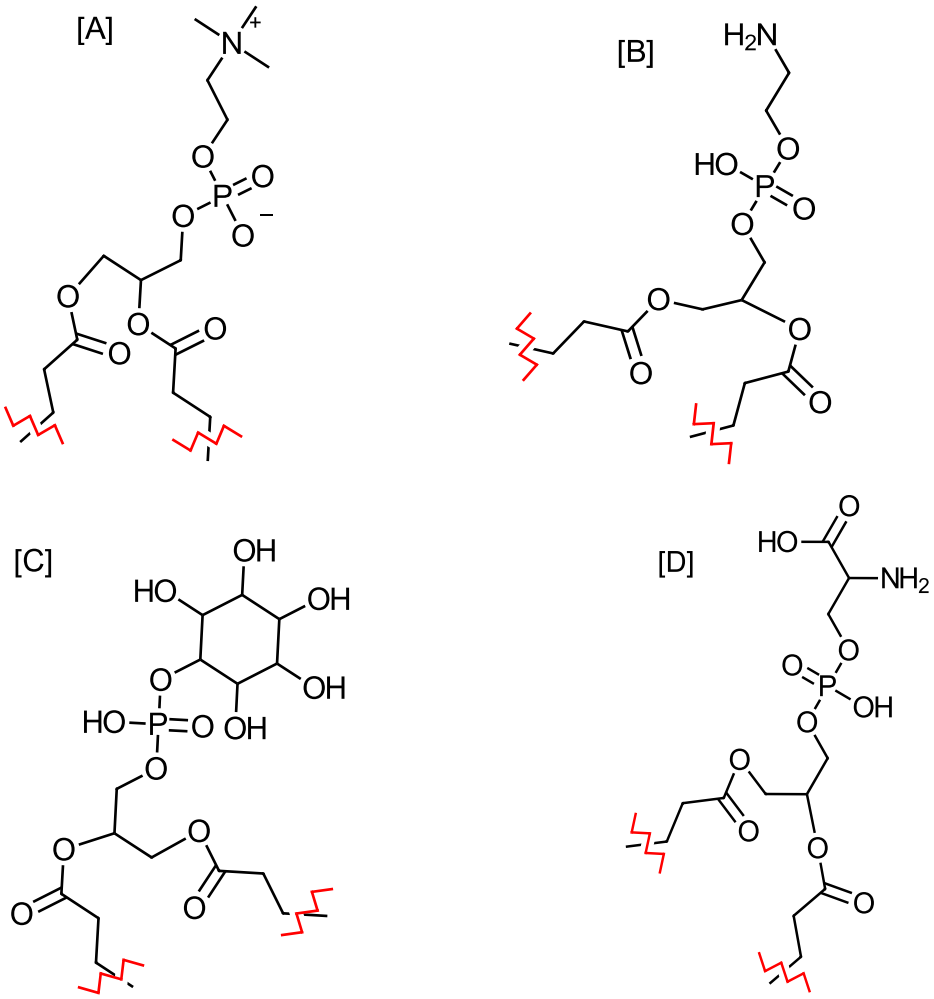
Figure 3: An example of a common triglyceride; systematic name: 1-hexadecanoyl-2,3-di-(9Z-octadecenoyl)-sn-glycerol, trivial name: 1-palmitoyl-2,3-dioleoyl-sn-glycerol, and the shorthand notation: TG(16:0/18:1/18:1), molecular Formula:$C_{55}H_{102}O_6$.



Triglycerides are particularly common within cellular and organism fatty deposits, as well as in circulating lipoproteins & chylomicrons (Feingold and Grunfeld, 2000), where they provide an essential energy store and fatty acid transportation vehicle.

Glycerophospholipids (usually abbreviated to phospholipids) are another category of whole lipids which are further sub-divided into distinct classes based on the nature of the polar head group attached to the glycerol backbone. These distinct classes of glycerophospholipids include (Han, 2016): phosphatidylcholine (abbreviated to PC), phosphatidylethanolamine (abbreviated to PE), phosphatidylinositol (appreciated to PI) and phosphatidylserine (abbreviated to PS). The shorthand notation for glycerophospholipids is similar to glycerolipids where the head group abbreviation is prefixed before the fatty acid moiety description. The head group structure of the mentioned distinct glycerophospholipids classes is shown in the figure below (*see Figure 4*).

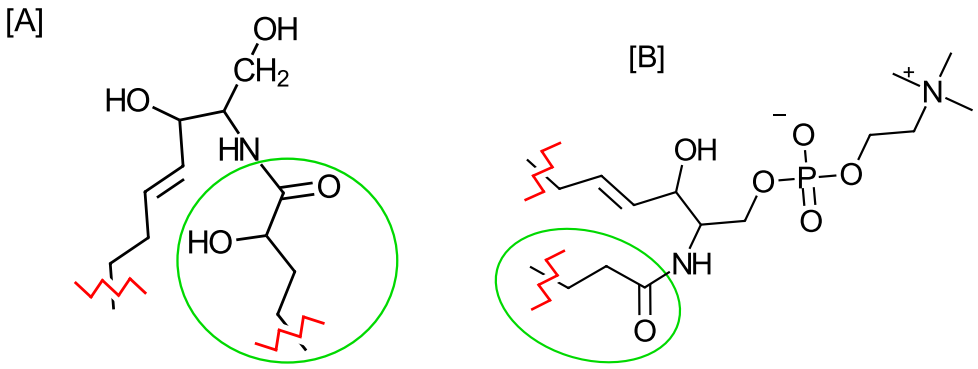
Figure 4: Glycerophospholipids polar head group structures of the four main sub-classes: [A]-phosphatidylcholine; PC, [B]-phosphatidylethanolamine; PE, [C]-phosphatidylinositol; PI, and [D]-phosphatidylserine; PS. The tilde-break (~) identifies an extended carbon chain.



Glycerophospholipids are common in all cells due to their incorporation into the lipid bilayer of cellular membranes (Alberts et al., 2002). Glycerophospholipids are also involved in many metabolic pathways / processes including apoptosis (Chaurio et al., 2009), blood coagulation (Sletnes, 1993) (Nemerson, 1968), and the activation of protein kinase C.

Sphingolipids are another common classification of lipid that possesses a sphingoid base moiety and a long chain fatty acid component. This lipid is readily converted to other lipid species such as ceramides (a sphingoid bases with an amide linked fatty acid), phosphosphingolipids (the head group attached via phosphodiester linkages, sphingomyelins, also known as ceramide phosphocholines), and glycosphingolipids (such as cerebrosides and gangliosides) (Gault et al., 2010). The major subclass of sphingolipids are ceramides (N-acyl-sphingoid bases), where the fatty acid moiety is typically saturated or monounsaturated ranging in carbon chain length from 14 to 26 atoms. The fatty acid component of ceramides commonly has a hydroxyl group on carbon 2 of the chain. Common derivatives of ceramides are sphingomyelins which consist of a ceramide structure usually with either a phosphocholines or a phosphoethanolamine polar head group. The fatty acid moieties of sphingomyelins are typically ≥ long chain fatty acids; either saturated or monounsaturated and often containing odd number of carbons (Furland et al., 2007). The shorthand notation for sphingolipids is similar to glycerolipids and glycerophospholipids where the head group abbreviation is prefixed before the fatty acid moiety description. The ceramide and sphingomyelins structure is shown in the figure below (*see Figure 5*).

Figure 5: The structure of the two-main sphingolipid; [A]-ceramides and [B]- sphingomyelin. The tilde-break (~) identifies an extended carbon chain; the green ring (O) identifies the fatty acid moiety.



Sphingomyelin are a major constituent of animal cellular membranes and ubiquitous across all cell and tissue types, often present as the most abundant lipid overall, e.g. nerve cells, the eye lens and in erythrocytes (Slotte and Ramstedt, 2007).

1.1.3 Saturated fatty acid *de novo* lipogenesis

Fatty acid *de novo* lipogenesis is the biosynthetic pathway in which saturated fatty acids are endogenously produced within the cellular cytosol by the sequential addition of two carbon units derived from acetyl-CoA, thus elongating the base moiety (Berg et al., 2002). The cyclic elongation of the fatty acid by fatty acid synthase terminates when a sixteen carbon-acyl is formed; this is because the sixteen carbon-acyl molecule is the preferred substrate for thioesterase. Thioesterase hydrolyses the sixteen carbon-acyl to yield palmitic acid (Ward, 2015) (Chakravarty et al., 2004).

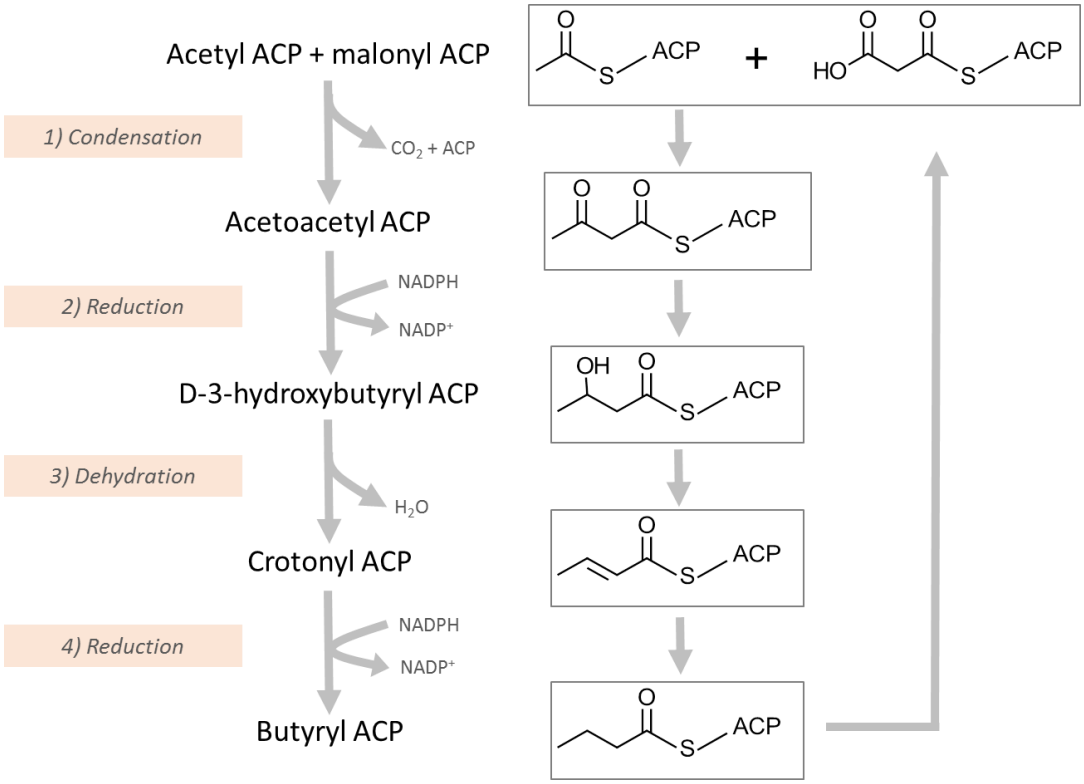
Carboxylation of acetyl-CoA to malonyl-CoA is the initial step in fatty acid biosynthesis (Numa et al., 1965), this reaction irreversibly initiates the cyclic elongation mechanism. However, the essential regulatory step in the fatty acid biosynthesis process lies with the biosynthesis of malonyl-CoA. Malonyl-CoA biosynthesis is catalysed by acetyl-CoA carboxylase containing a biotin

group; with the liberation of an adenosine triphosphate (ATP) molecule an activated carbon-dioxide (CO_2) molecule is produced and transferred to an acetyl-CoA molecule to form malonyl-CoA; *CO2-biotin(enzyme) + acetyl-CoA → malonyl-CoA + biotin(enzyme)* (Berg et al., 2002).

Following the biosynthesis of malonyl-CoA, the fatty acid intermediates as well as the malonyl-CoA are linked to an acyl carrier protein (ACP) (Kresge et al., 2005) at the sulfhydryl terminus, then to the serine residue of the acyl carrier protein by the enzyme malonyl transacylase. In saturated fatty acid *de novo* lipogenesis there are four processes involved, these include (*see Figure 6*); 1) Acetyl ACP and malonyl ACP condense to form acetoacetyl ACP and two by-products: carbon dioxide and ACP, this reaction is catalysed by beta-ketoacyl-acyl-carrier-protein synthase I. 2) acetoacetyl ACP is then reduced to produce D-3-hydroxybutyryl ACP and NADP+, this reaction is catalysed by 3-oxoacyl-(acyl-carrier-protein) reductase. 3) Then D-3-hydroxybutyryl ACP is dehydrated to form crotonyl ACP (a trans-Δ2-enoyl ACP) and water, this reaction is catalysed by 3-Hydroxyacyl ACP dehydrase. 4) Then crotonyl ACP is reduced, producing butyryl ACP and NADP+, this reaction is catalysed by enoyl ACP reductase.

Figure 6: A schematic of the anabolic fatty acid de novo lipogenesis process where acetyl ACP molecule is condensed to malonyl ACP producing butyryl ACP; further additions of acetyl ACP leads to the elongation of this moiety by two carbon units. The cyclic process of condensation followed by reduction, then dehydration, and then reduction continues until a sixteen carbon-acyl ACP is formed. Discontinuation of this biosynthetic process is due to the sixteen carbon-acyl ACP being a preferred substrate for a thioesterase that hydrolyses the sixteen carbon-acyl ACP to yield palmitic acid and ACP. 1) Acetyl ACP and malonyl ACP condense to form acetoacetyl ACP and two by-products: carbon dioxide and ACP, this reaction is catalysed by beta-ketoacyl-acyl-carrier-protein synthase I. 2) acetoacetyl ACP is then reduced to produce D-3-hydroxybutyryl ACP and NADP$^+$, this reaction is catalysed by 3-oxoacyl-(acyl-carrier-protein) reductase. 3) Then D-3-hydroxybutyryl ACP is

dehydrated to form crotonyl ACP (a trans-Δ2-enoyl ACP) and water, this reaction is catalysed by 3-Hydroxyacyl ACP dehydrase. 4) Then crotonyl ACP is reduced, producing butyryl ACP and $NADP^+$, this reaction is catalysed by enoyl ACP reductase.



Fatty acids beyond sixteen carbons (palmitate; C16:0) in length are formed by elongation reactions in the cytosolic face of the endoplasmic reticulum membrane (Berg et al., 2002). These elongation reactions add two carbon units to the carboxyl ends of the sixteen-carbon fatty acyl-CoA substrate producing an eighteen-carbon derivative (stearic acid; C18:0), which is continued until the desired/target fatty acid carbon chain length is obtained (Berg et al., 2002).

By the addition of acetyl-ACP to malonyl-ACP the conventional saturated fatty acid biosynthesis (*see Figure 6*) can produce even chain fatty acids. However, propionyl-ACP incorporation into the fatty acid synthesis instead of acetyl-ACP yields odd chain fatty acids (Berg et al., 2002) (Horning

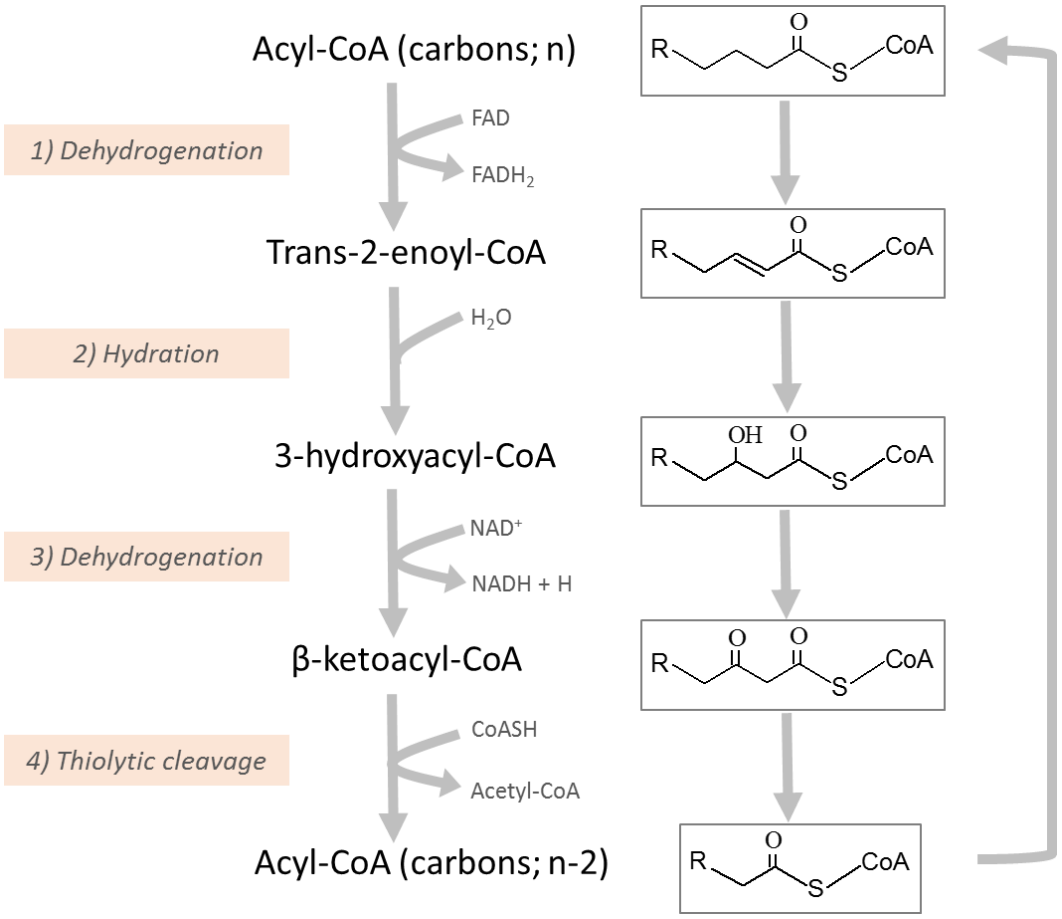
et al., 1960) (Pfeuffer and Jaudszus, 2016); acetyl transacylase produces propionyl-ACP from propionyl-CoA (Ward, 2015). Propionyl-CoA is biosynthesised from valine and isoleucine (branched chain amino acids) catabolism (Crown et al., 2015).

1.1.4 Fatty acid beta oxidation

Fatty acids are primarily metabolised via beta oxidation in the mitochondria or in the peroxisomes of eukaryotic cells (Wanders et al., 2010); this is a catabolic process where fatty acids are broken down to generate acetyl-CoA molecules (which enter the tricarboxylic acid cycle also known as the Krebs cycle) and NADH and $FADH_2$ (which enter the mitochondrial electron transport chain) (Akunekwe, 1993). Beta oxidation is named due to the oxidation occurring on the beta carbon of the fatty acid chain.

There are four processes involved in beta oxidation include (*see Figure 7*) (Houten and Wanders, 2010) (Schulz, 1991); 1) Dehydrogenation of two hydrogen atoms from both the alpha and beta carbon producing a *trans* carbon-carbon double bond and $FADH_2$, this reaction is catalysed by acyl-CoA dehydrogenase. 2) Hydration of the beta carbon producing a β-hydroxyacyl-CoA, this reaction is catalysed enoyl-CoA hydratase. 3) Dehydrogenation of the beta hydroxyl functional group on the β-hydroxyacyl-CoA producing a ketone functional group on the beta carbon and producing NADH, this reaction is catalysed by 3-hydroxyacyl-CoA dehydrogenase. 4) A thiolytic cleavage; cleavage of β-ketoacyl-CoA releasing a two-carbon acetyl-CoA molecule and producing an (n-2)-acyl-CoA, this reaction is catalysed by beta ketothiolase (a.k.a. 3-oxoacyl-CoA thiolase).

Figure 7: A schematic of the catabolic fatty acid beta oxidation process where an acetyl-CoA molecule is cleaved from the carboxylic acid terminus of a fatty acid. 1) Dehydrogenation of the alpha and beta carbons producing a trans bond. 2) Hydration of the beta carbon producing a β-hydroxyacyl-CoA. 3) Dehydrogenation of the beta hydroxyl of the β-hydroxyacyl-CoA producing a ketone. 4) A thiolytic cleavage; cleavage of β-ketoacyl-CoA cleaving acetyl-CoA and producing a (n-2)-acyl-CoA.



The process of acetyl-CoA cleavage from the fatty acid continues until either two units of acetyl-CoA are finally produced in the case of even chain fatty acids (Lodish et al., 2000) or with regards to odd chain fatty acids one acetyl-CoA and one propionyl-CoA are produced (Hama, 2010).

Unsaturated fatty acid beta oxidation requires the conversion of a *cis* conformation carbon-carbon double bond at the gamma position (bond position

3) into an adjacent *trans* carbon-carbon double bond at the beta position (bond position 2) utilising the enzyme enoyl-CoA isomerase (Zhang et al., 2002), this can then be directly beta oxidised. If the unsaturated fatty acid has a *cis* carbon-carbon double bond at the delta position (bond position 4) then 2,4 dienoyl-CoA reductase (Shoukry and Schulz, 1998) (Fillgrove and Anderson, 2001) reduces the unsaturated bond producing a suitable substrate for beta oxidation.

Beta oxidation in the mitochondria and the peroxisomes utilises similar enzymes (Poirier et al., 2006), substrates and initially produces the same products. Peroxisomal beta oxidation differs from mitochondrial beta oxidation in the fact that it is not directly coupled to adenosine triphosphate (ATP) synthesis due to the unconnected tricarboxylic acid cycle and the electron transport chain which are located in the mitochondria organelle (Mannaerts and Van Veldhoven, 1993). Instead, the peroxisomal beta oxidation process produces hydrogen peroxide, which is readily converted to water and oxygen by catalase (Alberts et al., 2002) (Cooper, 2000).

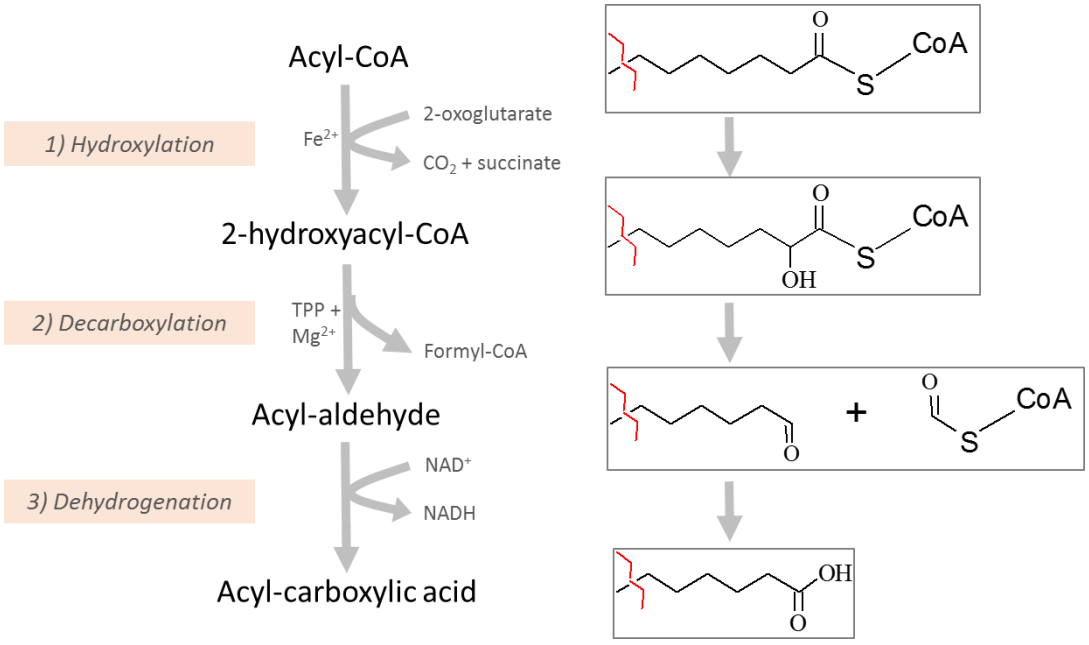
1.1.5 Fatty acid alpha oxidation

Alpha oxidation is a salvage or ancillary metabolic pathway for the catabolism of beta branched chain fatty acids (Jansen and Wanders, 2006); e.g. phytanic acid (*see Figure 2*), that are unable to be broken down via conventional beta oxidation due to the stearic hindrance of the beta branch on the fatty acid carbon chain backbone. The alpha oxidation catabolic process is reported to be located in the peroxisomes of eukaryotic cells; mainly in the peroxisomes of hepatic (liver) cells. The process of alpha oxidation cleaves a formyl-CoA from the parent beta branched chain fatty acids (Casteels, 2006), which is then readily converted to formic acid (by formyl-CoA hydrolase (Sly and Stadtman, 1963)) then carbon dioxide, additionally NAD^+ is reduced to NADH when converting the aldehyde product of alpha oxidation back to a carboxylic acid; a fatty acid. Alpha oxidation is named due to the oxidation occurring at the alpha carbon of the fatty acid chain.

In alpha oxidation of beta branched chain fatty acids there are three steps to this process, these include (*see Figure 8*) (Jansen and Wanders, 2006)

(Casteels, 2006) (Wanders et al., 2003); 1) Hydroxylation of the alpha carbon to form 2-hydroxyacyl-CoA; phytanoyl-CoA hydroxylase catalyses this reaction in the case of phytanoyl-CoA, whereas fatty acid 2-hydroxylase catalyses this reaction for other fatty acid species (Alderson et al., 2004). This reaction requires iron ($Fe^{2+)}$, ascorbate and 2-oxoglutarate. The products of the hydroxylation are carbon dioxide, succinate and the desired 2-hydroxyacyl-CoA. 2) Decarboxylation of the 2-hydroxyacyl-CoA into an (n-1)-acyl-aldehyde, this reaction is catalysed by 2-hydroxyacyl-CoA lyase (formerly known as 2-hydroxyphytanoyl-CoA lyase) (Casteels et al., 2007). The cleavage of formyl-CoA in this decarboxylation reaction requires thiamine pyrophosphate (TPP) and magnesium (Mg^{2+}). The formyl-CoA produced is unstable at the normal physiological pH (neutral; ~ pH 7) and spontaneously breaks down into formic acid and then converted to carbon dioxide. 3) Dehydrogenation of the (n-1)-acyl-aldehyde into an (n-1)-acyl-carboxylic acid (fatty acid shorter by one carbon), this reaction is catalysed by a fatty aldehyde dehydrogenase requiring NAD^+ and producing NADH.

Figure 8: A schematic of the catabolic fatty acid alpha oxidation process where a formyl-CoA molecule is cleaved from the carboxylic acid terminus of a fatty acid. 1) Hydroxylation of the alpha carbon to form 2-hydroxyacyl-CoA; phytanoyl-CoA hydroxylase catalyses this reaction in the case of phytanoyl-CoA, whereas fatty acid 2-hydroxylase catalyses this reaction for other fatty acid species. 2) Decarboxylation of the 2-hydroxyacyl-CoA into an (n-1)-acyl-aldehyde, this reaction is catalysed by 2-hydroxyacyl-CoA lyase (formerly known as 2-hydroxyphytanoyl-CoA lyase). 3) Dehydrogenation of the (n-1)-acyl-aldehyde into an (n-1)-acyl-carboxylic acid (fatty acid shorter by one carbon), this reaction is catalysed by a fatty aldehyde dehydrogenase.



Alpha oxidation in the peroxisomes either leads to the utilisation or translocation of the (n-1)-fatty acid or the (n-1)-fatty acid can go through local peroxisomal beta oxidation; typically, just a single cycle of catabolism (Francoise Le and Jean, 2012), after which, it may be utilised or translocated.

1.2 Odd chain fatty acids as dietary biomarkers

1.2.1 What are biomarkers

The term “biomarker”, a portmanteau of the phrase “biological marker”, which refers to a diverse category of compounds that can be measured and correlated with the rate of biological, pathological, and/or pharmacological processes (Mayeux, 2004). The World Health Organization (WHO) has defined a biomarker as “any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease” (Strimbu and Tavel, 2010).

In relation to nutrition a ‘biomarker’ is further subdivided into three categories, these are: recovery, concentration and predictive biomarkers. A recovery biomarker is a compound that exhibits a direct relationship between the absolute concentrations ingested or administered with the absolute concentration measured in the tissue (Neuhouser et al., 2008) (Strimbu and Tavel, 2010) (Winchester, 2008). A concentration biomarker is a compound that exhibits a statistically significant correlation between the amounts ingested or administered with the amounts measured within tissues. Concentration biomarkers are affected by endogenous biological processes and, therefore are subjected to metabolism, absorption factors and/or personal characteristics (such as compound or drug interactions and other demographical factors). Concentration biomarkers are typically used to correlate between high and low dietary intakes and with the incidence of pathology over time, using high numbers of participants or subjects, where other variables can be normalised across the study population to increase or test the validity of the biomarker relationships. Finally, a predictive biomarker exhibits a dose response relationship similar to concentration biomarkers where the compound is susceptible to endogenous biological processes, however, the relationship with their dietary intake is principal.

1.2.2 Odd chain fatty acids as biomarkers

The two most prominent odd chain fatty acids; pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) are both considered to be biomarkers

of ruminant fat intake (undecidedly, either recovery, concentration and / or predictive). This biomarker status is based on nutritional correlations in human observational studies and from food analysis, where ruminant fat containing food products have the highest content of these odd chain fatty acids (Jenkins et al., 2015) (Brevik et al., 2005) (Albani et al., 2016) (Sofie Biong et al., 2006) (Pfeuffer and Jaudszus, 2016). Ruminant fat is particularly high in C15:0 and C17:0 because these fatty acids are produced in relatively high levels by rumen microbial fermentation and microbial *de novo* lipogenesis (Vlaeminck et al., 2006). Since they are biosynthesised in the rumen microbiota they are also then absorbed and incorporated into the host ruminant animal into their entire lipid metabolic pathways, fat deposits and milk production. Rumen microbiota can produce C15:0 and C17:0 via two pathways, either through the incorporation and elongation of propionic acid into fatty acid *de novo* biosynthesis or via the alpha oxidation breakdown of even chain fatty acids into odd chain fatty acids (Emmanuel, 1978). These two mechanisms are previously thought to be significantly unique to the ruminant microbiota.

All the current evidence for the exclusive dietary source of odd chain fatty acids are based on observational studies that determined the associations between the diet and measured levels in circulating (plasma or serum) and tissues. These presented biomarker correlations in the literature have been statistically significant and exhibited across different studies leading to the assumption that odd chain fatty acids (specifically C15:0 and C17:0) are dietary derived and other non-dietary sources either do not exists or do not significantly contribute to tissue or cellular levels (Brevik et al., 2005) (Albani et al., 2016) (Sofie Biong et al., 2006) (Wolk et al., 1998) (Smedman et al., 1999).

1.2.3 Dietary fatty acid compositions

Dietary fatty acids are derived from both animal and plant sources, where there are different compositions of each fatty acid between each source. Even chain fatty acids such as palmitic acid (C16:0) and stearic acid (C18:0) are apparently ubiquitous across each dietary fat. However, the odd chain fatty acids such as pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) are significantly more abundant in animal fats compared to plant fats and oils; where

these odd chain fatty acids can be completely absent or at least below the limit of detection (*see Table 2*).

Table 2: The fatty acid composition of commonly consumed fats and oils in the typical Western diet. Values are given as number of mg per 100 grams of dietary fat (WFC, 2014).

	C12:0	C14:0	C15:0	C16:0	C17:0	C18:0	C20:0
	mg / 100 grams of edible fat						
Beef tallow	75	2200	300	23000	840	14000	130
Coconut oil	43000	16000	35	8500	-	2600	79
Corn oil	-	-	-	10000	-	1900	410
Lard	140	1600	130	23000	530	13000	200
Olive oil	-	-	-	9800	-	2900	420
Palm oil	420	1100	82	41000	-	4100	350
Rapeseed oil	64	78	-	4000	-	1900	580
Safflower oil	-	68	-	4500	-	1900	400
Butter	2500	8300	830	22000	320	7600	120
Sunflower oil	-	36	-	5700	-	4100	150

NB. '-' identifies undetectable concentrations of that fatty acid.

A typical Western diet generally contains low amounts of beef tallow and lard, however, the consumption of dairy foods such as milk and milk products, yogurts, cheese and butter, as well as food that are produced from these products are a considerable source of dietary dairy fat (NDNS, 2016). For this reason, dairy fat is the main source of dietary odd chain fatty acids, typically containing twice as much C15:0 as C17:0.

1.3 Odd chain fatty acids in pathology

1.3.1 Correlation with all diseases

The study of odd chain fatty acids as biomarkers of dairy fat intake has been based on the association of C15:0 and C17:0 with dietary assessment data yielding varying degrees of reliability when taking the literature as a whole (Wolk et al., 1998) (Sofie Biong et al., 2006). Furthermore, many emerging studies have correlated odd chain fatty acids with the reduced risk of incidence and/or prognosis of developing various pathologies (Forouhi et al., 2014) (Khaw et al., 2012). From the available literature, in most cases odd chain fatty acids have been typically attributed to the ingestion of ruminant fat and are treated as dietary biomarkers rather than diet independent fatty acids. For this reason, the correlations that odd chain fatty acids show with pathology are associated with the dietary ruminant fat rather than with the particular dietary concentration of those fatty acids. However, odd chain fatty acids have been associated with a wide range of different pathologies. Not all the correlations with these pathologies could be simply due to the relative amount of ingested ruminant fat (for example in vegans). It is much more likely that these are a consequence of the odd chain fatty acid directly, either by possessing protective or debilitating properties, or are indirectly related by association with processes that affects the pathological development. The table below outlines different diseases with their correlations with odd chain fatty acids (*see Table 3*).

Table 3: A summary table presenting data from published literature demonstrating the associations between pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) odd chain fatty acids and pathology, either as a biomarker; both risk and identifications, or as a possible application for treatment of pathology. This is not a comprehensive meta-analysis but an illustration showing that C15:0 and C17:0 fatty acids have been associated with several diseases, including metabolic and psychological pathologies.

Pathology	n = (diseased, controls)	Summary	Reference
Disorders of propionate, methyl-malonate and biotin metabolism	n = 24, 12	Odd chain fatty acid concentrations used as a marker for patient response to treatment	(Çoker et al., 1996)
Atherosclerosis	2837 cohort	Plasma phospholipid 15:0 was inversely associated with incident CVD and CHD	(de Oliveira Otto et al., 2013)
Type 2 diabetes	n = 346, 3391	Inverse association between plasma phospholipid pentadecanoic acid and diabetes incidence	(Hodge et al., 2007)
Coronary Heart Disease	n = 1595, 2246	Saturated odd chain fatty acids have an inverse relationship with CHD	(Khaw et al., 2012)
Prediabetes and Type 2 Diabetes	n = 181, 170	Significant negative associations with T2D	(Meikle et al., 2013)

Biotin Deficiency	n = 3, n.d.	Provides evidence for propionyl-CoA carboxylase deficiency in diseased patients	(Mock et al., 1988)
Peroxisomal Disorders	n = 86, 84	Levels of heptadecanoic acid (C17:0) were also increased	(Moser et al., 1981)
Insulin sensitivity	n = 86	associated with insulin sensitivity and inversely with insulin resistance	(Nestel et al., 2014)
Cardiomyopathy and rhabdomyolysis in long chain fat oxidation disorders	n = 107, 50	Improvement in the cardiovascular and muscular status	(Roe et al., 2002)
Anorexia Nervosa	n = 8, 19	Compensatory molecule for polyunsaturated fatty acids with regards to membrane fluidity.	(Rt et al., 1995)

NB. 'n.d.' identifies data not presented in the original study.

As shown in the table above (*see Table 3*) odd chain fatty acids have been correlated with a wide variety of different pathologies; their overall description has varied from being an indicative biomarker of disease risk all the way through to possessing therapeutic properties.

1.4 Gas chromatography and mass spectrometry

1.4.1 Sample preparation for fatty acid analysis

Fatty acids are more commonly found esterified in complex molecules rather than as free unesterified fatty acids (Gunstone et al., 1994), this means that the extraction of the esterified fatty acids needs to be tailored to the fatty acid carbon-carbon chain rather than the whole lipid's functional head group(s).

The hydrophobic carbon-carbon chain of the fatty acids, even in an esterified form make the lipid soluble in organic solvents, such as trichloromethane (chloroform) (Folch et al., 1957) and methyl tert-butyl ether (MtBE) (Matyash et al., 2008). For robustness and increased sensitivity esterified fatty acids once extracted by an organic solvent are generally hydrolysed from the whole lipid, and then methylated to form fatty acid methyl esters (FAMEs). FAMEs are highly hydrophobic making them ideal for gas chromatography analysis, whereas, the methylation of the fatty acid carboxylic acid group producing an ester compounds allows the molecule to be positively ionised which is the favoured mode in mass spectrometry (Hoffmann and Stroobant, 2007).

1.4.2 Gas chromatography

Gas chromatography is the principal analytical method for the separation of relatively low mass (< ~850 Da) hydrophobic compounds that are thermally stable yet volatile. This method requires the vaporisation of the compound(s) which is/are then carried by an inert gas, known as the mobile phase (such as nitrogen or helium) through a polymer coated glass capillary; known as the column. Inside the capillary column there is a material bound to the internal surface area; which is known as the stationary phase. When the compound(s) are injected on the column by the GC instrument, they are immediately evaporated and forced through the column by the flow of the mobile phase gas. As the compound(s) flow through the column they collide with the internal surface and interact with the stationary phase. Depending on the chemistries of the compound(s) and the stationary phase there is either minimal interaction where the compound(s) will pass through the entire column relative to the flow of the mobile phase. However, if the chemistries of the compound(s) and the stationary phase are complimentary then there will be significant interaction, where the compound(s) will be temporarily retained. Gas chromatographic instruments use a temperature ramp to hasten these compound(s) stationary phase interactions so to elute the compounds off the column and depending on the magnitude of these interactions will determine how quickly the compound(s) pass through. By varying the column stationary phase chemistries and dimensions, as well as the temperature ramp of the

instrument on the column then different compounds can be separated so that they can be independently analysed and detected, typically via mass spectrometry.

1.4.3 Mass spectrometry

Mass spectrometry is an analytical method (commonly used in conjunction with gas chromatography) where it relies on the separation of gaseous charged ions according to their mass to charge ratio. The ionisation due to the bombardment of high energy electrons cause substantial molecular fragmentation; since each compound is chromatographically separated then the fragmentation ions produced (at that moment in time) by the ionisation process are products of the original compound and can be used to confirm or deduce the chemical structure. The ion separation process differentiates each specie according to their mass to charge ratio (compound mass < ~850 Da are typically singularly charged) so that they arrive at the detector independently of one another so that the detector quantifies the arriving ions and associates that signal with the relative fragment mass. This data is then used to identify the compound(s) and their relative concentration for further bio-informatic interpretation (Hoffmann and Stroobant, 2007).

1.4.4 Fatty acid reporting

Fatty acid compositions in published literature are predominantly expressed as a fraction of weight percentage (this is performed by normalising the single fatty acid value by the total fatty acid value, then multiplied by 100 to give a percentage; the units are often given as Mol %) rather than a concentration value (usually mol/L) (Schwertner and Mosser, 1993). The reason for expressing fatty acids as a fraction of the weight percentage is because it is understood that endogenous fatty acids are biosynthesised from each other, where an increase in unsaturated moieties would also present a decrease in saturated moieties. Additionally, an increase in elongation products would also result in a decrease in precursor substrates. However, exogenous fatty acids (i.e. biomarkers) would not display the same inter-connectivity. Even though this method of expressing fatty acid variations (Mol %) may lead to inter-dependence of data it is

considered reliable and appropriate when correlating changes in medium to long chain fatty acids and assessing biomarkers relationships.

2 MATERIALS & METHODS

A detailed description of the materials, methods and instrumentation used in the analysis of fatty acids, within plasma / serum, liver and adipose tissue.

2.1 Fatty acid analysis

Both C15:0 and C17:0 fatty acids are incorporated ubiquitously within all the lipid classes, with no apparent preference for an individual class (Jenkins et al., 2015). Therefore, the levels of C15:0 and C17:0 in the whole lipid fraction were determined by analysing the total fatty acid composition via trans-esterification under acidic conditions with an alcohol (e.g. methanol) present to produce fatty acid methyl esters (FAMEs).

2.2 Materials

Chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Dorset, UK). All solvents were of HPLC grade. Tridecanoic-$_{d25}$ acid (a thirteen-carbon fatty acid possessing twenty-five deuterium atoms) used as a stable isotope labelled compound in the gas chromatography with mass spectrometry fatty acid methyl ester analysis was obtained from CDN Isotopes (QMX Laboratories Ltd., Essex, United Kingdom). The gas chromatography mass spectrometry fatty acid methyl ester system suitability solution was purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Dorset, UK). The GCMS column (HP-88) was obtained from Agilent Technologies (Agilent Technologies, California, United States of America).

2.3 Study samples

To minimize experimentation, we aimed to make use of existing sample sets from research institutional collaborations, this resulted in a combination of animal & human models specifically selected for their applicability (i.e. mouse, rat, canine, and human). None of the results seemed to introduce ambiguity

about the interpretation of the presented work, therefore, we are convinced that the consistency of the results across the animal & human models will contribute to the validity of their interpretation. The rodent models were used to investigate the dietary and biochemical interactions and mechanisms since it had been previously published that a rodent is a suitable animal model alternative for these investigations (Panchal and Brown, 2011). The canine models were used due to the sample volume requirements of the study and it had been previously shown that dogs are a suitably comparable model to human glucose intolerance pathology. The animals were maintained under standard laboratory conditions with water *ad libitum* throughout the experiments. Animal body weight, food intake and health were monitored throughout the study.

In each of the studies the appropriate ethical approval was obtained (*see individual sections for the relevant ethical statement*), where necessary signed informed consent was achieved, and all experimental protocols were approved by a named institutional and/or licensing committee. Additionally, all methods were performed in accordance with the relevant guidelines and regulations.

2.4 Sample storage

All plasma and serum samples were stored at sub -20^{o}C. Tissue samples (liver and adipose) were stored at -80^{o}C. Chemicals, standards, compounds, and reagents were all stored as directed by the manufacturer. Tissue (liver and adipose) was dissected on dry ice; -78^{o}C. Surplus solvent extracts and post-analysis samples were stored at sub -20^{o}C for possible future re-analysis.

2.5 Sample preparation

The aim of these extraction procedures was to separate the lipid fraction from other components, such as non-lipid cellular material, proteins, carbohydrates, salts, and other unknown polar compounds. Extraction of the lipids preserved them prior to analysis by terminating all biological and metabolic processes by denaturing enzyme structures, therefore, the measured lipids compositions were representative of the true *in vivo* levels at the moment in time of sample collection.

2.5.1 Serum/plasma lipid extraction

Samples were extracted using the chloroform: methanol: water extraction, adapted from the previously described method by Folch and colleagues (Folch et al., 1957). Briefly, chloroform: methanol solution (2: 1, 1 mL) was added to 100 µL of plasma or serum. The samples were then vortexed and sonicated for 15 minutes. Water (400 µL) was added to each sample, followed by further vortexing then sonication (15 minutes), and an additional vortexing to ensure complete recovery. Samples were centrifuged (~20,000 G, for 5 minutes) and the resulting aqueous (top layer) and organic layers (bottom layer) were separated into amber glass screw cap tubes and dried under a gentle stream of oxygen free nitrogen.

2.5.2 Liver & adipose tissue lipid extraction

Approximately 50 to 100 mg of frozen tissue (dissected on dry ice; -78^{o}C) was added to chloroform: methanol solution (2: 1, 400 µL) with a 5 mm stainless steel ball bearing. The samples were homogenised using a Tissue Lyser (Qiagen, Hilden, Germany) for 10 minutes at 25 Hz. A further 600 µL of chloroform: methanol solution (2: 1) was added to each sample and vortexed thoroughly, followed by sonication (15 minutes) and additional vortexing to ensure optimum recovery. Water (400 µL) was added to each sample. The samples were then centrifuged (at ~20,000 G, for 5 minutes) and the resulting aqueous (top layer) and organic layers (bottom layer) were separated into amber glass screw cap tubes.

To ensure complete lipid extraction from the tissue samples a double extraction was carried out. A further 1 mL of chloroform: methanol solution (2: 1) and water (400 µL) was added to each sample, followed by homogenisation (10 minutes at 25 Hz), vortexing, sonication (15 minutes), and a final vortexing. The samples were then centrifuged (at ~20,000 G, 5 minutes) and the resulting aqueous (top layer) and organic layers (bottom layer) were separated and combined to the corresponding initial extracts.

A 25 mg tissue equivalent portion of the extracts was aliquoted into separate amber glass screw cap tubes for fatty acid derivatisation. This was done

by dividing 25 (mg) by the initial amount of frozen tissue; the resultant value was the proportion of the total organic fraction (2 mL) aliquoted for fatty acid derivatisation. This proportionate 25 mg aliquot was then dried under a gentle stream of oxygen free nitrogen. Surplus extracts were stored at sub -20ºC.

2.6 Total fatty acid derivatisation to fatty acid methyl esters

For the analysis of total fatty acids, the samples were derivatised to fatty acid methyl esters. To the dried organic extracts; boron trifluoride in methanol (14%, 125 µL), chloroform: methanol (1:1, 100 µL) and the internal standard tridecanoic acid-$_{d25}$ in chloroform (100 µL, 200 µMol) were added. The mixture was thoroughly vortexed, sonicated (30 minutes) and vortexed a further time to ensure there was no undissolved material. Samples were heated to 80ºC for 90 minutes to commence the derivatisation process.

After the samples had cooled, water (300 µL) and hexane (600 µL) were added. The samples were vortexed and the organic layer separated into amber glass vials, blown down to dryness under a gentle stream of oxygen free nitrogen and finally reconstituted in 200 µL of hexane, ready for gas chromatography with mass spectrometry detection (GC-MS) analysis.

2.7 Gas chromatography separation

Gas chromatography separation was achieved using a 6890N / 5973 Agilent gas chromatography mass spectrometry system (Agilent Technologies, California, USA) with a HP-88, 30 meter capillary column, 0.25 mm internal diameter and a 0.2 µm film thickness (Agilent 112-8837). The front inlet temperature was 250ºC. The injection volume for plasma, serum and liver samples was 1 µL, for adipose tissue samples the injection volume was reduced to 0.1 µL. Front inlet split ratio for serum and plasma samples was 10:1, liver and adipose tissue samples had a 50:1 split ratio. A total oven gradient was over ~28 minutes, starting from 120ºC to 240ºC (the oven gradient parameters are as follows; initial temperature 120ºC hold of 1 minute, temperature increase of 10ºC per minute to 170ºC followed by a hold of 6 minutes, then temperature increase of 3ºC per minute to 194ºC, then an increase of 43ºC per minute to 240ºC, followed by a hold of 7 minutes).

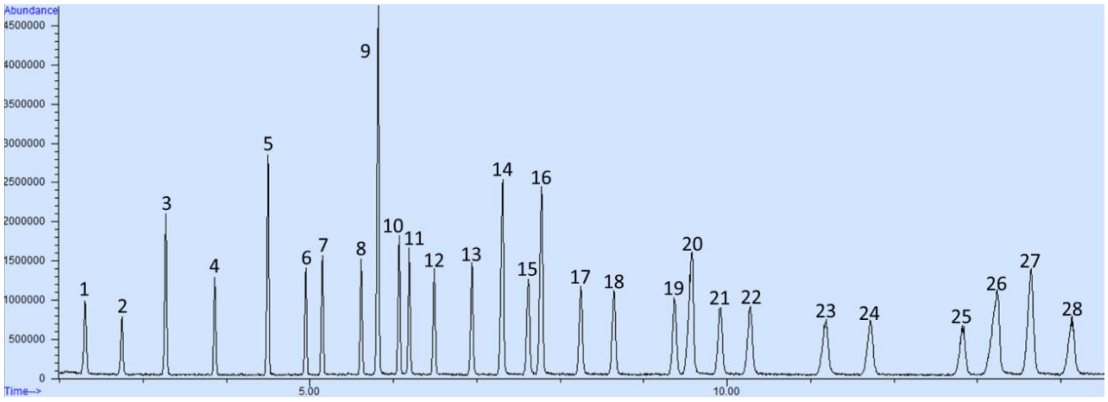
2.8 Mass spectrometry detection

Full scan mass spectrometry detection starting after an initial solvent delay of 2 minutes (positive mode, mass range from 60 to 400 Da, transfer line temperature of 280^{o}C, MS-source temperature of 230^{o}C, MS-quadrupole temperature of 150^{o}C).

2.9 System suitability test

Prior to analysing any test samples on the gas chromatography mass spectrometry instrument (6890N / 5973 Agilent; Agilent Technologies, California, USA) a system suitability test was performed. System suitability tests verify that the resolution and reproducibility of the instrument were sufficient by analysing a quality control standard solution that has the target analytes present at known concentrations. The figure below (*see Figure 9*) shows the total ion chromatogram produced by analysing a system suitability standard solution with twenty-eight fatty acid methyl esters present at a known concentration (2 mg per mL), ranging from m-C10:0 (methyl decanoate) to m-C22:1n9 cis; (methyl docosenoate).

Figure 9: A quality control standard (2 mg per mL) total ion chromatogram to test system suitability and performance prior to analysing each batch of test samples, this ensures the gas chromatography mass spectrometry system was working optimally. Fatty acid methyl ester standards include: 1) m-C10:0; methyl decanoate, 2) m-C11:0; methyl undecanoate, 3) m-C12:0; methyl dodecanoate, 4) m-C13:0; methyl tridecanoate, 5) m-C14:0; methyl tetradecanoate, 6) m-C14:1; methyl tetradecenoate, 7) m-C15:0; methyl pentadecanoate, 8) m-C15:1; methyl pentadecenoate, 9) m-C16:0; methyl hexadecanoate, 10) m-C16:1 trans; methyl trans-hexadecanoate, 11) m-C16:1 cis; methyl hexadecenoate, 12) m-C17:0; methyl heptadecanoate, 13) m-C17:1; methyl heptadecenoate, 14) m-C18:0; methyl octadecanoate, 15) m-C18:1 trans; methyl trans-octadecanoate, 16) m-C18:1 cis; methyl octadecenoate, 17) m-C18:2 trans; methyl trans-octadecadienoate, 18) m-C18:2 cis; methyl octadecadienoate, 19) m-C18:3 cis; methyl octadecatrienoate, 20) m-C20:0; methyl eicosanoate, 21) m-C18:3n3; methyl octadecatrienoate, 22) m-C20:1 cis; methyl icosenoate, 23) m-C21:0 cis; methyl henicosanoate, 24) m-C20:2 cis; methyl icosadienoate, 25) m-C20:3n6 cis; methyl icosatrienoate, 26) m-C22:0 cis; methyl docosanoate, 27) m-C20:4n6 cis; methyl icosatetraenoate and m-C20:3n3 cis; methyl icosatrienoate, 28) m-C22:1n9 cis; methyl docosenoate.



After performing the system suitability test; if the chromatogram produced showed clear resolution of the analytes, as shown in the figure above

(*see Figure 9*) with a comparable intensity (~$1x10^6$ to ~$5x10^6$), then the GC-MS system had been shown to be reproducible and test sample analysis were initiated.

2.10 Data processing

Corresponding fatty acid methyl ester peaks were integrated using GC/MSD ChemStation (Agilent Technologies, California, USA). Integrated fatty acid methyl esters include: methyl dodecanoate; m-C12:0, methyl tetradecanoate; m-C14:0, methyl pentadecanoate; m-C15:0, methyl hexadecanoate; m-C16:0, methyl palmitoleate; m-C16:1 *cis*, methyl heptadecanoate; m-C17:0, methyl octadecanoate; m-C18:0, methyl oleate; m-C18:1 *cis*, methyl linoleate; m-C18:2, and the internal standard; methyl tridecanoate-$_{d25}$. Chromatographic peak picking and integration of the target ion with the associated qualifying ions at the expected retention time determined correct compound identification and quantification. The target and qualifying ions for each fatty acid methyl ester are shown in the table below (*see Table 4*).

Table 4: The fatty acid methyl ester expected chromatographic retention time (minutes) and the mass spectrometry target ions (Da) with three qualifying ions (Da) to confirm fatty acid methyl ester identification. Qualification requires detection of the four ions at the expected retention time. Quantification was via the chromatographic peak area of the target ion. Methyl dodecanoate; m-C12:0, methyl tetradecanoate; m-C14:0, methyl pentadecanoate; m-C15:0, methyl hexadecanoate; m-C16:0, methyl palmitoleate; m-C16:1 cis, methyl heptadecanoate; m-C17:0, methyl octadecanoate; m-C18:0, methyl oleate; m-C18:1 cis, methyl linoleate; m-C18:2, and internal standard; methyl tridecanoate-$_{d25}$.

Fatty acid methyl ester	Retention time (minutes)	Target ion (Da)	First qualifying ion (Da)	Second qualifying ion (Da)	Third qualifying ion (Da)
m-C12:0	3.3	73.9	86.9	142.9	171.0
m-C14:0	4.5	73.9	86.9	198.9	142.9
m-C15:0	5.1	256.0	86.9	212.9	142.9
m-C16:0	5.8	73.9	86.9	227.0	270.1
m-C16:1 *cis*	6.2	68.9	73.9	82.9	237.0
m-C17:0	6.5	284.0	87.0	283.9	241.0
m-C18:0	7.3	73.9	86.9	298.1	255.1
m-C18:1 *cis*	7.8	68.9	264.0	82.9	73.9
m-C18:2 *cis*	8.6	81.1	66.9	78.8	95.1
m-C18:3 *cis*	9.4	79.1	66.9	90.1	67.1
Methyl tridecanoate-$_{d25}$	3.7	77.0	91.0	155.1	203.2

GC/MSD ChemStation integration processed data was then exported to Microsoft Excel™ 2013 (Redmond, Washington, USA) for further processing and statistical analysis.

For the calculation of percentage of the total fatty acids (Mol %), the measured fatty acid quantities was firstly divided by the internal standard (tridecanoic acid-$_{d25}$) response, this normalises the data to correct for extraction, recovery and instrument variations. Then each individual sample fatty acid responses were then divided by the total fatty acid count for that sample (the sum of all the fatty acid responses for that sample), then multiplied by one hundred to give the percentage of the total fatty acids (Mol %).

For the calculation of the absolute concentration (µM), the imported data was divided by the internal standard (tridecanoic acid-$_{d25}$) response for normalisation, then the data was multiplied by two hundred (NB. 200 µM internal standard added in the sample preparation) to give the absolute concentration (µM) relative to the internal standard.

3 STATISTICS

Statistics is the mathematical procedure for collating, organising, analysing, and ultimately the interpretation and presentation of study data. Through statistics, the statistical significance is ultimately determined; this is the likelihood that a measured outcome arouse consequently to an independent variable and is not the product of random chance or change. Statistics basically state if any change measured is real.

Within this study, data was gathered and systematically prepared and presented using Microsoft Excel™ 2013 (Redmond, Washington, USA) and GraphPad Prism (Version 6.03, San Diego, CA, USA.). The interpretation of the study data was achieved through the assessment of the statistical significance, which is discussed in the following sections.

3.1 Paired t-test

A paired t-test is used to assess the comparison between the population means of two data sets, where an observation in one data set is paired with an observation in another data set and whether this comparison is different from zero; typically, this is between an observation from before and after an intervention (Rosner, 1982) (Hsu and Lachenbruch, 2007). The resultant probability value (p-value; obtained from comparing the t-statistic to a t-distribution with (n-1) degrees of freedom) from the paired t-test gives the likelihood that the observation from the two data sets are significantly different and did not occur by chance. Therefore, the lower the p-value means there will be a lower support for the null hypothesis. The null hypothesis is a supposition that there is no significant difference between two population means; where any observable differences are due to experimental errors. The lower level for statistical significance is set at ≤ 0.05, which ensures there is equal to or greater than 95% confidence in the result is statistically significant.

Paired t-test calculated in Microsoft Excel™ 2013 (Redmond, Washington, USA) via this syntax;

=T.TEST(array1,array2,tails,type)

Array1 is the first population data set. Array2 is the second paired data set. A two-tailed t-test is used due to the normal distribution of the data sets with a two-directional consideration. Type 1 t-test identifies it is a paired t-test.

A paired t-test was performed to assess significance in the following study: dietary biomarker investigation - dairy-fat supplementation study (*section 6.2.3.*).

3.2 Homoscedastic t-test

A homoscedastic t-test is used to assess the comparison between the two-independent population means, where an observation in one population cannot be paired with the observations in another population and the two populations must have comparable variances (Kim, 2015) (McCluskey and Lalkhen, 2007) (De Winter, 2013). This homoscedastic t-test assesses whether the difference between each of these population means is different from zero; this is typically between two groups where there is a comparison between a control groups and an experimental group. The p-value produced gives the probability that the observations from the two data sets are significantly different and did not occur by chance. Therefore, the lower the p-value then there will be a lower support for the null hypothesis. A lower level for statistical significance is set at ≤ 0.05, which ensures there is equal to or greater than 95% confidence in the result is statistically significant.

Homoscedastic t-test calculated in Microsoft Excel™ 2013 (Redmond, Washington, USA) via this syntax;

=T.TEST(array1,array2,tails,type)

Array1 is the first indepentent population data set. Array2 is the second indepentent population data set. A two-tailed t-test is used due to the normal

distribution of the data sets with a two directional consideration. Type 2 t-test identifies it is a homoscedastic t-test.

A homoscedastic t-test was performed to assess significance in the following studies: intestinal microbiota investigations – low fat (chow) diet study (*section 5.2.1.*) and high fat (ruminant fat based) diet study (*section 5.2.2.*); dietary biomarker investigations - different dietary fat source comparison study (*section 6.2.1.*), dietary fat percentage study (*section 6.2.4.*), dietary fat percentage and fat source study (*section 6.2.5.*), and ethanol intake study (*section 6.2.6.*); endogenous production investigations – phytol supplementation study (*section 7.2.1.*), stearic acid (C18:0) infusion study (*section 7.2.2.*), and 2-hydroxyacyl-CoA lyase 1 (*Hacl1*) study (*section 7.2.3.*).

3.3 R squared (R^2)

The correlation coefficient (denoted as R^2) is a way to indicate the variance in the dependent measurement that is predictable from the independent measurement (Schneider et al., 2010). The variance in this component is presented as a value between 0 and 1, where 0 represents no predictability of the dependent from the independent measurement and a value closer to 1 indicates that a greater proportion of variance is accounted for by the correlation model.

The R^2 value was calculated within Microsoft Excel™ 2013 (Redmond, Washington, USA) and GraphPad Prism (Version 6.03, San Diego, CA, USA.).

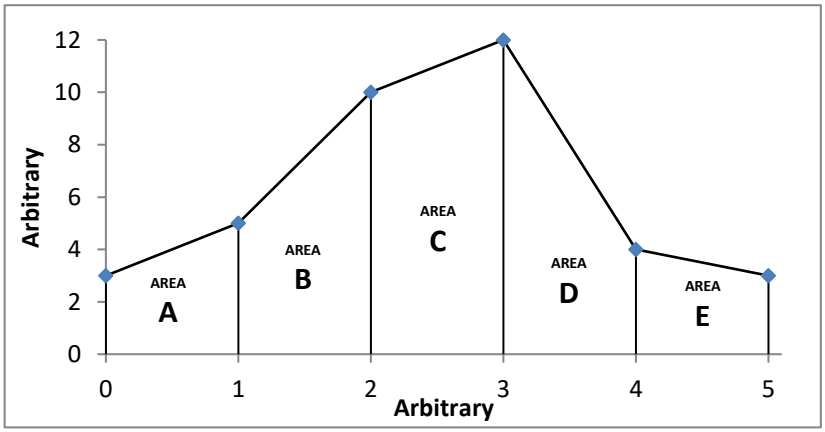
The R^2 statistic was performed to assess the significance in the following studies: dietary biomarker - different dietary fat source comparison study (*section 6.4.1.*), dose response study (*section 6.4.2.*), glucose intolerance (*section 8.2.1.*).

3.4 Trapezoidal rule

The trapezoidal rule is a technique for approximating the region under the graph as a trapezoid to indirectly calculate its area (Yeh, 2002). Typically, this involves dividing the area under the curve into several trapezoids, using each

data point as the determinate of each individual trapezoid boundary as shown in the figure below (*see Figure 10*).

Figure 10: This figure shows an example of how the area under the curve can be calculated via the trapezoid rule. The trapezoidal rule uses each data point to construct an individual trapezoid and using the trapezoid area equation to find the individual segment area; area = ((A+B)/2) * H. The total area under the curve can then be calculated by summing up each individual trapezoid.



The total area under the curve is the sum of all individually calculated trapezoid areas. The individual trapezoid area is calculated in Microsoft Excel™ 2013 (Redmond, Washington, USA) via this formula; (((y value two + y value one) / 2) * (x value two − x value one)).

The trapezoidal rule was used to measure a change in the area under the curve of a graph in the following study: glucose intolerance - glucose intolerance (*section 8.2.1.*).

4 AIM & STUDY DESIGN

The aim of this PhD was to investigate any contributory factors to the circulating C15:0 and C17:0 odd chain fatty acid levels *in vivo* that were suggested in the introductory chapter; from the diet, from non-ruminant gut microbiota and from endogenous biosynthesis. Then to investigate the relationship between the circulating C15:0 and C17:0 fatty acid levels and the prognosis of glucose intolerance, taking into account these contributory factors.

To minimize animal & human experimentation, existing sample sets from research collaborations were used. By making use of existing sample sets we could address each of the main factors that could contribute to circulating levels of pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0), as well as, how these circulating levels correlate with the development of glucose intolerance.

The three contributing factors towards circulating levels of C15:0 and C17:0 include: non-ruminant intestinal microbiota contributions, exogenously derived from the diet, and then endogenously biosynthesised through metabolic pathways.

The contributions from the non-ruminant intestinal microbiota were investigated through the utilisation of a germ-free mouse models. Since germ free mice do not possess any microbiota, then this model will give the greatest difference from conventional mice (smaller differences would result from dietary interventions and/or antibiotic treatment). Therefore, if the intestinal microbiota plays a part in non-ruminant circulating levels of these two odd chain fatty acids then a germ-free mouse model will produce the greatest difference.

Circulating odd chain fatty acids are described as biomarkers of dietary intake, and therefore by definition, the circulating levels of these fatty acids should parallel any changes in the dietary compositions. However, if there is not a homologous relationship between the dietary intake and circulating levels then

this indicates that other factors are significantly interfering. Other possible sources of odd chain fatty acids could be from endogenous biosynthesis via alpha oxidation, however, this suggested metabolic mechanism for the production of odd chain fatty acids has only been minimally investigated *in vitro*. Due to the novelty of the involvement of the alpha oxidation mechanism with *in vivo* odd chain fatty acids, its contributions and influences on circulating levels has only been theorised. By using controlled dietary interventions, supplementations, fatty acid infusions, and gene knockout studies the inter-tangled relationships between the diet, endogenous biosynthesis and circulating levels of C15:0 and C17:0 were investigated.

Each of these factors were fully investigated and reported in the following chapters.

5 INTESTINAL MICROBIOTA

An investigation into the influences the non-ruminant intestinal microbiota has on the host circulating odd chain fatty acid profile across two contrasting diets; a low fat (chow) diet and a high fat (ruminant fat based) diet. The two diets allowed for any non-ruminant intestinal microbiota adaptations to the host dietary intake to be taken into account.

5.1 Introduction

The synthesis of odd chain fatty acids such as C15:0 and C17:0 by ruminant gut microbiota through alpha oxidation of the two most abundant even chain fatty acids; palmitic acid (C16:0) and stearic acid (C18:0) has been well described (Vlaeminck et al., 2006) (Brevik et al., 2005). In the process of *de novo* lipogenesis, C16:0 is formed through the repeating condensation reaction of malonyl-CoA with acetyl-CoA (Kaneda, 1991) (Keeney et al., 1962) (Or-Rashid et al., 2007). This sequential reaction polymerises the substrates and increases the chain by two carbon units per reaction, leading to even chain fatty acids. Termination of this reaction is with C16:0 and through elongation it is readily converted into C18:0. The ratio between these dominant fatty acids is highly dependent on the ratio between protozoa and bacteria within the ruminant's intestinal microbiota. Higher concentrations of C18:0 are dependent on higher levels of bacteria over protozoa in the rumen (Or-Rashid et al., 2007). In the ruminant digestive system, the mix of these two microorganisms generally favours protozoa development and therefore the formation of higher C16:0 concentrations (Vlaeminck et al., 2006). Through alpha oxidation (Emmanuel, 1978) these fatty acids are shortened by one carbon unit and since C16:0 is the dominant even chain fatty acid, then alpha oxidation of this fatty acid makes C15:0 the dominant odd chain fatty acid in the rumen. In addition, it is possible that instead of acetyl-CoA, propionyl-CoA can be used in the synthesis of fatty acid, which would also lead to odd chain fatty acid biosynthesis (Weitkunat et al., 2017). The relative contributions of these two pathways are still unclear.

Non-ruminant gut microbiota also has the ability to produce odd chain fatty acids. A study showed that it is possible to extract odd chain fatty acids from faecal samples of rats fed a complete lipid free diet, which suggests that these minor fatty acid species may be produced by the gut microbiota (Demarne et al., 1979). The importance of understanding the gut microbiota's metabolism is reflected by a surge of recent research linking the gut microbiota to a great range of various conditions and disease states. Furthermore, the gut microbiota has been shown to correlate with some of the findings of odd chain fatty acid and related diseases, for example: cardiovascular disease (Sandek et al., 2007) (Khaw et al., 2012), diabetes (Cani and Delzenne, 2009) (Forouhi et al., 2014), cancer (Guarner and Malagelada, 2003) (Kilner, 2013), inflammation (Tremaroli and Bäckhed, 2012) (Kolak et al., 2007) and even mental illnesses, such as depression (Maes et al., 1996) (Foster and McVey Neufeld, 2013).

Recent research into C15:0 and C17:0 has provided evidence that circulating levels are not exclusively dependent on ingested ruminant fat (Su et al., 2004) (Foulon et al., 2005) (Guo et al., 2010) (Smedman et al., 1999) (Brevik et al., 2005) (Jenkins et al., 2015), which suggests that there are alternative sources contributing to the odd chain fatty acid levels in non-ruminant animals, possibly from their intestinal microbiota.

5.1.1 Study hypothesis

Considering that there has been previous research showing non-ruminant intestinal microbiota produce odd chain fatty acids; which can significantly contribute to the intestinal chyme, then, if the host circulating odd chain fatty acids are only derived from exogenous sources, any variation in the intestinal microbiota composition could influence the host circulating odd chain fatty acid profile. Therefore, we hypothesise that the germ-free mice will have lower circulating odd chain fatty acid compositions compared to conventional mice on the same diet. Additionally, there will also be varying degrees of disparity between the germ-free mice and the conventional mice across different diets, as the diet changes the intestinal microbiota composition which may vary the C15:0 and C17:0 contributions from the intestinal microbiota to the rumen and the host. Mice have been used to study functional and mechanistic

characteristics/interactions between the host and the occupying gut microbiota, mainly due to their extensive similarities in anatomy, physiology and genetics, allowing inferences about human biology to be drawn from murine experimentation (Nguyen et al., 2015). Although there are some differences between the two systems the results collected are not used to directly translate interpretations between each species.

5.2 Intestinal microbiota study design

To test this hypothesis, we measured the circulating odd chain fatty acid composition (Mol %) in germ free and conventional mice across two contrasting diets; a low fat (chow) diet and a high fat (ruminant fat based) diet.

5.2.1 Low-fat (chow) diet study

Two groups of seven to eight-week-old BALB/cByJ female mice (n = 8 per group) were used (Morin et al., 2011); Conventional, specified and opportunistic pathogen free mice were purchased from Charles River Laboratories (Charles River Laboratories, L'Arbresle, France). Germ free female mice were bred in the ANAXEM platform germ free animal facilities (ANAXEM platform, INRA, Jouy-en-Josas, France). All animals were housed in sterile Trexler-type isolators (Getinge-La Calhène, Vendôme, France). Autoclaved tap water and sterile pelleted standard chow (R03, SAFE, Augy, France) were provided *ad libitum*. The absence of gut microbiota in the germ-free mice was monitored by aerobic and anaerobic culture examinations of faecal preparations.

All experiments were performed with permission 91-493 of the French Veterinary Services and in accordance to the European Community rules of animal care. Blood was collected after 32 days and processed into serum, this was then stored at -80^{o}C until analysed.

5.2.2 High-fat (ruminant fat based) diet study

Two groups of C57/BL6 mice (n = 6-7 per group) were used; conventional mice with intestinal microbiota present and germ-free mice (Stepankova et al., 2010). Intestinal colonization of the conventional mice was

achieved through administration of specific pathogen free bacterial cocktail (Central Institute for Laboratory Animal Breeding, Hanover) containing both aerobic microorganisms (*Bacillus species, Lactobacillus lactis, Acinetobacter species and Staphylococcus sciuri) and anaerobic microorganisms (Lactobacillus delbrukii sp., Bulgaricus, L. fermentum, L. catenaforme, Bacteroides distasonis, Bacteroides thetaiotaomicron, Peptostreptococcus micros, P. asaccharolyticus*). The germ-free mice were bred under sterile conditions for the past four generations with faecal samples evaluated weekly for the absence of aerobic and anaerobic bacteria, mould and yeast. The conventional mice were evaluated for the presence of bacteria after 2 days, 7 days and then every week following the intestinal colonisation. Faecal samples were cultivated on peptone bouillon, Sabouraud bouillon, Schadller bouillon (Difco, Detroit, MI) and then on blood agar, Sabourad agar, Schadller agar in anaerobic atmosphere (gas Anaerogen 2.5lt:DIOXO) to detect any presence of anaerobic bacteria. Smears were stained with Gram stain and fluorescence dyes, which stained Gram positive bacteria yellow and Gram negative bacteria green.

After weaning, both groups of mice received sterile water and pelleted 35% high fat (ruminant fat based) feed (C1090-60, Altromin, Lage, Germany) with 13% milk fat and 22% lard fat *ad libitum*. Diet and bedding material were sterilised with gamma radiation (5Mrad, 30 min; Bioster, Czech Republic). Blood was collected after 75 days and processed into plasma, this was then stored at -80^{o}C until analysed.

The study was performed in the department of Immunology and Gnotobiology of the Institute of Microbiology, Czech Academy of Science. The institute is authorised by the Central Committee for Animal Welfare to carry out experiments on laboratory animals and the local ethics guidelines were in compliance with Directive 86/608/EEC on the protection of animals used for scientific purposes and recommendation 2007/526/EC of the European Commission.

5.3 Statistical analysis summary

The statistical approaches utilised were appropriate for the specific experimental design; a homoscedastic t-test was used between two unrelated equal variance groups to identify any significance or non-significance between the two groups. A homoscedastic t-test was used in the following studies: low fat (chow) diet study and the high fat (ruminant fat based) diet study. A value of $p \leq 0.05$ was considered significant.

5.4 Results

To minimize animal experimentation, we aimed to make use of existing sample sets; this resulted in a combination of mouse strains being utilised. Since all mammals perform *de novo* lipogenesis, beta oxidation and alpha oxidation in the production and metabolism of lipids, we therefore expect that these results can be used across the strains. Additionally, published literature and internal studies has shown that the lipid percentage composition is very similar between the strains (Lin et al., 2005). Although this approach is not conventional, we are able for the first time to comprehensively study the influences of the intestinal microbiota on the host circulating odd chain fatty acid profile.

5.4.1 Diet fatty acid composition

The low fat (chow) diet consisted of 5.1% fat, 21.4% protein and 55.7% carbohydrates and the high fat (ruminant fat based) diet consisted of 35% fat, 21.4% protein and 34.4% carbohydrates. The percentage fatty acids of each diet are shown in the table below.

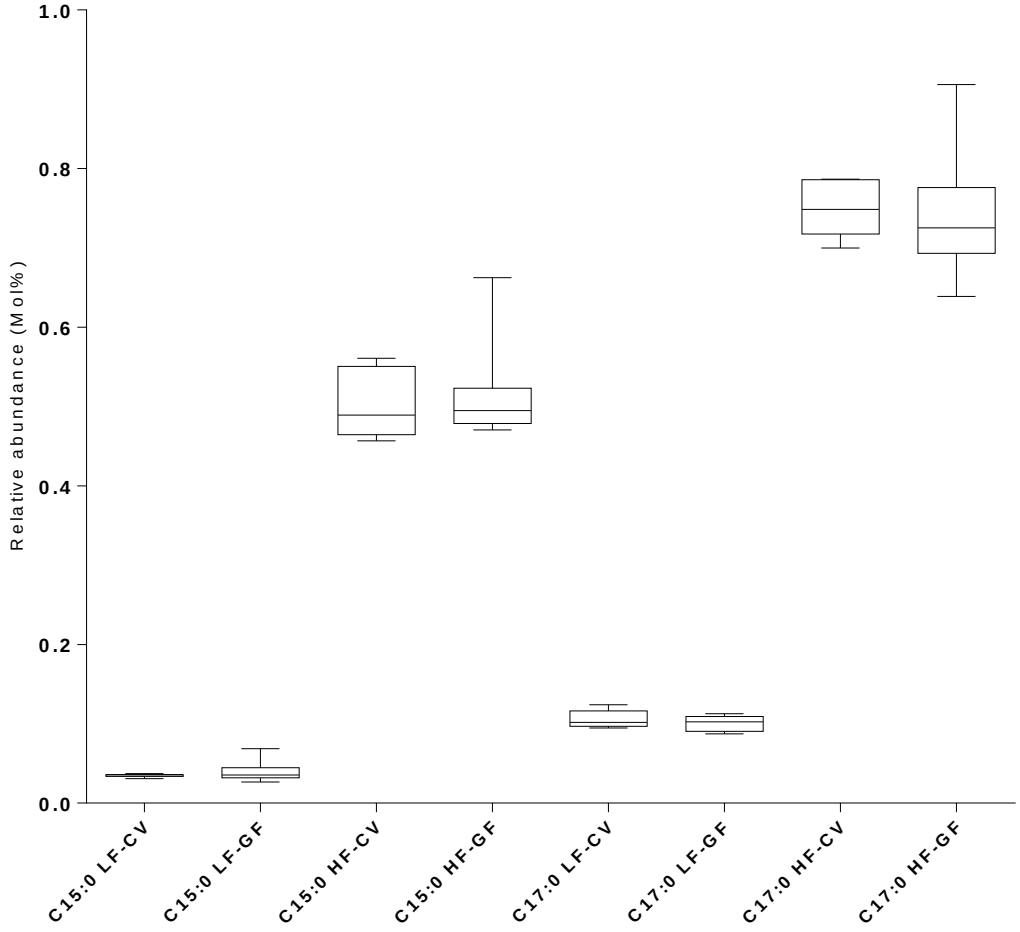
Table 5: The fatty acid composition of the low fat (chow) diet and the high fat (ruminant fat based) diet, as a percentage of the total fatty acids measured (Mol %). The fatty acids were analysed by gas chromatography separation with mass spectrometry detection after boron trifluoride methylation derivatisation.

	Diet	
Fatty acid	Chow (Mol %)	high-fat (Mol %)
C14:0	1.767	9.326
C14:1	0.033	0.467
C15:0	0.035	0.953
C16:0	32.246	36.302
C16:1	0.433	0.935
C17:0	0.089	0.978
C18:0	19.082	28.088
C18:1	9.502	16.818
C18:2	30.602	5.407
C18:3	6.211	0.726

5.4.2 Plasma fatty acid composition

The differences in the odd chain fatty acid levels between the conventional and the germ-free animals were not significant (all p values were greater than 0.05); this observation was independent of the diet (*see Figure 11*). As expected the levels of odd chain fatty acids were greater in the high fat (ruminant fat based) diet animals compared to the low fat (chow) diet animals.

Figure 11: Box and whiskers plot showing the influence of intestinal microbiota on plasma odd chain fatty acids in mice, values are percentage of total fatty acids measured by gas chromatography with mass spectrometry detection. Box represent mean ± standard deviation, whiskers represent the minimum and the maximum values (n = 6-8 per group). Pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0), low fat (chow) diet (LF) and high fat (ruminant fat based) diet (HF), conventional mice (CV) and germ-free mice (GF).



As shown in the figure above (*see Figure 11*) there was no significant differences between the gern free mice and the conventional mice within each dietary intervention (low fat (chow) diet; C15:0 p = 0.370 and C17:0 p = 0.275), high fat (runimant fat based) diet; C15:0 p = 0.626 and C17:0 p = 0.827).

5.5 Discussion & conclusions

The aim of this study was to investigate the relationship between the non-ruminant animals' gut microbiota and the circulation odd chain fatty acids through two different diets; a low fat (chow) diet and a high fat (ruminant fat based) diet, along with comparing mice with conventional intestinal microbiota profiles and germ-free mice (completely absent of any intestinal microbiota).

The low fat (chow) diet was selected because it was completely deficient of ruminant fat sources and would provide information into the absolute baseline measurement of the odd chain fatty acids neglecting both dietary and intestinal microbiota sources. The low fat (chow) diet fat content was only 5.1%, and according to Evrard and colleagues (Evrard et al., 1964) a fat content greater than 6% and the presence of intestinal microbiota reduces the absorption efficiency in the intestines, therefore, the low fat (chow) diet also allowed us to investigate maximum dietary fat absorption. The high fat (ruminant fat based) diet was selected because it aids in the investigation of the influence of the gut microbiota on the host circulating fatty acids. The high fat (ruminant fat based) diet also provided information on any specific interactions between the intestinal microbiota and dietary ruminant fat where it has been previously shown that the intestinal microbiota can be significantly altered when different fat types are consumed over a long duration (Huang et al., 2013). This allowed us to contrast our results with previously published articles, specific to intestinal microbiota and dietary fat (David et al., 2014) (Rueda, 2014) (Alcock and Lin, 2015) (Brown et al., 2012) (Leone et al., 2013) (Huang et al., 2013) (Guzman et al., 2013), leading to a possible cause of the odd chain fatty acid biomarker relationship (i.e. do changes in the intestinal microbiota caused by the dietary fat influence the host circulating odd chain fatty acid profile independent of the dietary odd chain fatty acid content). Previously, the gut microbiota has been shown to increase the host fatty acid oxidation through promotion of PPAR-α (Velagapudi et al., 2010), which in a state of deficient calorific intake could influence circulating odd chain fatty acid levels, meaning it was imperative that the mice received adequate amounts of each pelleted diet during the study. The mice within this study had free access (*ad libitum*) to food and

water representing a natural and healthy environment where there was a normal intestinal microbiota and normal endogenous metabolic pathways can be assumed (Takahashi et al., 1992).

There were no significant differences in the odd chain fatty acid profiles between these different mouse strains (BALB/cByJ and C57/BL6) when fed similar chow diets, therefore the difference in the odd chain fatty acid levels between each of the groups were brought about by the diet rather than the strain.

The germ-free mice were used as this gave the greatest contrast from a normal intestinal microbiota profile and therefore any changes in the microbiota profile of the conventional mice due to the diet would still result in a substantial disparity between the different mouse groups.

It is clear from the box and whiskers plot-figure above (*see Figure 11*) that there were no significant differences caused by the presence or absence of the intestinal microbiota on the host circulating odd chain fatty acid levels, as each p-value was greater than the defined significance score ($p < 0.05$). We can therefore conclude that there were no significant differences brought about by the intestinal microbiota. Interestingly, the results in the low fat (chow) diet fed animals showed that the circulating levels of C17:0 is relatively greater than the dietary source; this indicates that there must be some other factor involved in the homeostasis of this lipid, be it through an alternative endogenous source or through specific lipid retention. Additionally, the odd chain fatty acid ratio between C15:0 and C17:0 in the plasma (C15:0 / C17:0 = 0.67) does not resemble the dietary contributions in the high fat (ruminant fat based) diet (C15:0 / C17:0 = 0.97) where these fatty acids are almost equal. These results agree with previous literature, where there are conflicting relationships between the dietary levels and the circulating levels for these odd chain fatty acids (Jenkins et al., 2015). Since the concentration of circulating odd chain fatty acids do not directly reflect the dietary intake (Smedman et al., 1999) (Jenkins et al., 2015) and the intestinal microbiota has been negated, then endogenous metabolic processes must be responsible for the relationships with diet and disease, which need further investigation.

The limitations of these studies - the results from these studies show that C15:0 and C17:0 are not affected by the presence or absence of gut microbiota in mice this does not exclude the possibility that the human gut microbiota may contain microbiota species that produce either C15:0 or C17:0 and contribute significantly to the human host.

5.6 Summary

To summarise, the presence of odd chain fatty acids within the faecal lipids of previous studies have been attributed to be products of the intestinal microbiota (Demarne et al., 1979), it seems that these odd chain fatty acids are not of a sufficient level to contribute to circulating fatty acid levels as no significant differences were detected. We can therefore conclude that the intestinal microbiota does not influence the host's circulating odd chain fatty acids concentrations. However, this does not exclude the possibility that the human gut microbiota may contain microbiota species that produce either C15:0 or C17:0 but as the *in vivo* levels of C15:0 and C17:0 between rodents and humans are comparable it makes it unlikely that human gut microbiota has a significant impact.

6 DIETARY BIOMARKER

An investigation into the dietary biomarker response of C15:0 and C17:0 fatty acids through different dietary interventions to comprehensively determined if these two fatty acids in circulation correlate with dietary concentrations or compositions (Mol %) in order to ascertain whether they are dietary biomarkers.

6.1 Introduction

Odd chain fatty acids, specifically C15:0 and C17:0 have been described as biomarkers of dairy intake; with varying degrees of reliability for each fatty acid (Jenkins et al., 2015) (Jenkins et al., 2017). Within large human studies both C15:0 and C17:0 fatty acids correlate moderately well with the intake of dairy products, such as milk & butter. However, within strictly controlled animal models, there are significant differences between the correlation of individual odd chain fatty acid intake and their circulating level (Zhukova et al., 2014) (Kim et al., 2011). C15:0 has been shown to be a direct biomarker of intake, and reported to have no notable evidence of endogenous C15:0 biosynthesis. In contrast, there is a convincing amount of evidence showing that C17:0 does not directly relate to dietary intake (Brevik et al., 2005) and that its endogenous biosynthesis has a larger contribution to circulating levels. Dietary or endogenous contributions of these low level fatty acids become less easy to disentangle as the control over the biomarker study decreases as seen in many large epidemiological studies, especially when containing human subjects.

Further confusion around the origin of these two biomarkers in human epidemiology studies is caused by a lack of understanding how dietary factors influence the concentration of odd chain fatty acid in the circulation. We expect that in addition to dietary fat composition, the total amount of dietary fat will impact on the circulating fatty acid levels; presumably by reducing absorption efficiency. We also expect that dietary components that increase *de novo*

lipogenesis, such as ethanol intake, will also impact plasma fatty acid levels. Due to the subjective measurements in dietary assessment mainly due to report bias (Kipnis et al., 2002) (Vitolins et al., 2000), it is extremely difficult to accurately record total dietary fat intake, dietary fat composition and accurate ethanol intake, which reduces the impact of any attempts to normalise these between study groups. Additionally, fatty acid administrations and dietary ethanol intake have been shown to independently influence the composition of *in vivo* fatty acids, regardless of their actual intake. For example, a stearic acid infusion independently increases circulating heptadecanoic acid (Jenkins et al., 2017), increasing dietary fat content reduces linoleic acid; C18:2 (Ronis et al., 2004), and plasma linoleic acid reduces in relation to alcohol intake (Burke et al., 1991).

To investigate how these three dietary factors affect C15:0 and C17:0 as biomarkers, we determined how odd chain fatty acids levels changed in a range of animal studies with very specific dietary interventions. The effect of dietary fat composition was investigated through two animal studies (in mice and rats) where the fat composition was varied between each experimental group and we measured the changes in circulating fatty acid composition. We utilised two separate animal intervention studies (in rats) where different amounts of total dietary fat were given to see if the concentration of these odd chain fatty acids *in vivo* were significantly influenced by the amount of total fat in the diet. The influence of the intake of ethanol on the circulating fatty acid compositions (in rats) were investigated through a controlled animal intervention study utilizing a gastric cannula to directly introduce the animal feed and ethanol.

It is of great importance to understand which factors can change odd chain fatty acid compositions and concentration in the circulation, as many studies have shown that increased odd chain fatty acid levels are associated with a reduced risk for cardio metabolic diseases. Without this detailed understanding the wrong public health message could be formulated, which will decrease the effectiveness of nutritional advice to the public.

6.1.1 Study hypothesis

Several studies have shown that both C15:0 and C17:0 fatty acids show a positive correlation when the dietary composition is compared to the circulating composition. However, these relationships are not consistent between studies in the available literature. There is also published literature showing the contrary to this perceived biomarker response, where there is not any significant biomarker correlation. It is also reasonable to assume that these two fatty acids are involved in metabolic mechanisms, therefore, we hypothesise that these two fatty acids are not true dietary biomarkers since there are other factors that influence the circulating composition (like metabolic catabolism and possible endogenous biosynthesis), besides only the dietary concentration or composition.

6.2 Study design

To test this hypothesis, we measured the circulating C15:0 and C17:0 compositions and compared these to the dietary compositions within both animal and human intervention studies. Then the influences of total fat intake and ethanol intake had on the circulating C15:0 and C17:0 levels independent of the dietary fatty acid composition.

6.2.1 Different dietary fat source comparison study

As described previously (Huang et al., 2013), C57BL/6 mice were kept under standard conditions at the University of Chicago. At 7-10 weeks of age, the mice were separated into four groups (n = 3-4 per group); a control group were maintained on a low fat (chow; 10% fat) control diet (AIN-93M) and three high fat (37.5% fat) experimental diets; a lard fat based diet (TD.110718), a safflower oil based diet (TD.97223) or a milk fat based diet (TD.97222). All diets were manufactured by Harlan-Teklad (Madison, USA). After 4-5 weeks the mice were euthanized by carbon dioxide asphyxiation and blood was collected then processed into plasma. All samples were stored at -80^{o}C until analysed.

All animal protocols and experiments were approved by the Institutional Animal Care and Use Committee at the University of Chicago.

6.2.2 Dose response study

Sprague Dawley rats (Harlan, Indianapolis, USA) were overfed using one of five experimental diets (n = 6-9 per group) at 17% above matched growth as previously described (Ronis et al., 2004) (Ronis et al., 2013). Blood was collected after 21 days and processed into serum.

All experimental procedures were ethically approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Science.

6.2.3 Ruminant fat supplementation study

Healthy human subjects with stable body weight and no family history of diabetes (n = 26) were recruited for an overfeeding intervention study conducted by Alligier and colleagues (Alligier et al., 2013), where samples were analysed and reported in this manuscript. As previously described, the subjects (age 33 ± 1 years, body weight 79.1 ± 1.8 kg and fat mass 19.6 ± 0.8%) followed a 56-day overfeeding intervention, where they ate an additional 760 kcal per day. The additional energy intake was derived from 100 g of cheese, 20 g of butter, and 40 g of almonds. The diet C15:0 and C17:0 compositions as well as the amount of phytanic acid (plus its precursor; phytol) are shown in the table below (*see Table 6*).

Table 6: The concentration of pentadecanoic acid (C15:0), heptadecanoic acid (C17:0), phytol and phytanic acid for each fat source consumed during the human study intervention shown as milligrams (mg) per 100 grams of the fat source. The actual number of milligrams consumed per day is shown in the 'intervention' column.

	Cheese (mg / 100 g)	Butter (mg / 100 g)	Almonds (mg / 100 g)	Intervention (mg)
(C15:0)	350	880	8	529
(C17:0)	200	330	30	278
Phytol	0	2.25	2.3	1.37
Phytanic acid	54	177	0	89.4

During this study, the subjects maintained their usual eating and physical activity behaviours (monitored by questionnaires and accelerometers over three sets of five-day periods). After an overnight fast at day zero and day 56, blood was drawn and processed into plasma for fatty acid analysis.

All the participants gave signed informed consent following the explanation of the experimental protocol. The protocol was approved by the ethics committee of Lyon Sud-Est according to the French 'Huriet-Serusclat' law and the Second Declaration of Helsinki (study registration number NCT00905892; 20th May 2009, www.clinicaltrials.gov).

6.2.4 Dietary fat percentage study

As described previously (Ronis et al., 2004) (Baumgardner et al., 2008), male Sprague Dawley rats (n = 6-7 per group) were housed under standard conditions. Rats had an intragastric cannula surgically inserted seven days before being separated into three groups, each group receiving an experimental diet with either 5%, 35% or 70% fat composition (fat was isocalorically substituted for carbohydrate calories, additionally, protein,

vitamin and mineral contents were identical in all diets). The animals had *ad libitum* access to water throughout the study. Blood was collected and processed into serum after 21 days on the experimental diets. All samples were stored at -80°C until analysed.

Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved animal facility. Animal maintenance and experimental treatments were conducted in accordance with the ethical guidelines for animal research established and were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences.

6.2.5 Dietary fat percentage and fat source study

As previously described (Aoun et al., 2012), six-week-old male Wistar rats (n = 6-8 per group) were housed under standard conditions. The rats were separated into six groups and fed one of six experimental diets made up of three different fatty acid compositions (basal, lard and fish oil based diet) at two different total fat levels (5% and 30%, w/w). The rats had *ad libitum* access to demineralised water and food. After twelve weeks, the animals were euthanized with pentobarbital, blood was collected by puncturing the abdominal vein with a heparinised syringe, and then centrifuged at 1000 G for 10 minutes at 4^{o}C to obtain plasma. All samples were stored at -80^{o}C until analysed.

The INRA institutional guidelines for the care and use of laboratory animals were followed, and all experimental procedures were approved by the local ethics committee in Montpellier, France (reference CEEALR-11 009).

6.2.6 Ethanol intake study

As described previously (Ronis et al., 2004) (Baumgardner et al., 2008), male Sprague Dawley rats (n = 7 per group) were housed under standard conditions. Rats had an intragastric cannula surgically inserted seven days before being separated into two groups, each group receiving an experimental diet with either 35% corn oil or 35% corn oil with 35% ethanol (ethanol was isocalorically substituted for carbohydrate calories, additionally, protein, vitamin and mineral contents were identical in all diets). The animals had *ad*

libitum access to water throughout the study. Blood was collected and processed into serum after 21 days on the experimental diets. All samples were stored at -80^{o}C until analysed.

Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved animal facility. Animal maintenance and experimental treatments were conducted in accordance with the ethical guidelines for animal research established and were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences.

6.3 Statistical analysis summary

The statistical approaches utilised were appropriate for specific experimental designs; a homoscedastic t-test was used between two unrelated equal variance groups to identify any significance or insignificance between them two groups. A homoscedastic t-test was used in these studies. A value of $p \leq 0.05$ was considered statistically significant.

6.4 Results

To minimize animal experimentation, we aimed to make use of existing sample sets; this resulted in a combination of mouse, rat, and human samples being utilised. Since all mammals perform *de novo* lipogenesis, beta oxidation, and alpha oxidation in the production and metabolism of lipids, we therefore expect that these results can be compared and interpreted (but not directly translated) across the species (mice, rats, dogs, and humans). Additionally, the circulating lipid percentage compositions were very similar between these species, where the C15:0 to C17:0 ratio was consistent sub 1.0 Mol %. Although C15:0 and C17:0 fatty acids have a minor composition in plasm or serum of all mammals, their concentration is strikingly consistent between different species (Jenkins et al., 2017). Although this approach is not conventional, we are able for the first time to comprehensively study the influences of dietary odd chain fatty acid compositions of the circulating levels to determine (either disprove or validate) their dietary biomarker classification.

6.4.1 Different dietary fat source comparison study

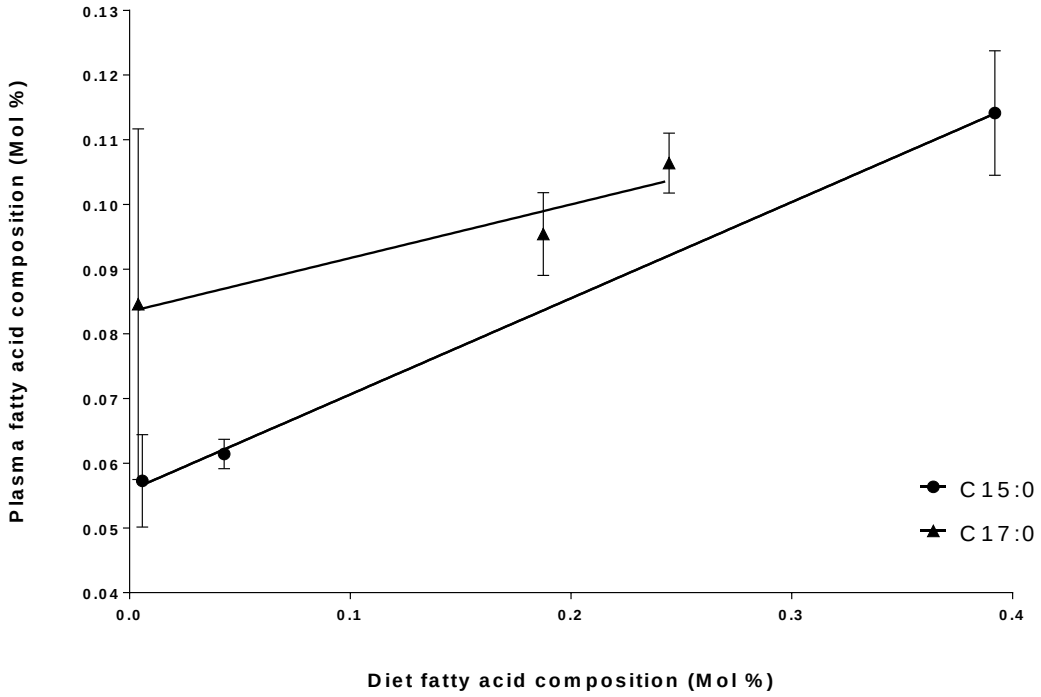
This interventional study allowed a controlled unbiased assessment of the C15:0 and C17:0 fatty acid biomarker response by comparing different isocaloric high fat diets with the resulting circulating odd chain fatty acid compositions. The three experimental diets varied only by the fat source used: either lard, safflower or milk fat. The diet and plasma odd chain fatty acid compositions are shown in the table below (*see Table 7*).

Table 7: Composition of pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) of the three isocaloric high fat (37.5 % kcal) diets. The plasma samples were analysed by gas chromatography with mass spectrometry detection. Values are shown as percentage of total fatty acid composition (mol %). Results are shown with ± standard error of the mean. (n = 3-4 mice per group).

Diet group	Diet (Mol %)		Plasma (Mol %)	
	C15:0	C17:0	C15:0	C17:0
Safflower	0.006	0.004	0.056 ± <0.001	0.085 ± 0.002
Lard	0.043	0.187	0.061 ± <0.001	0.095 ± 0.001
Milk	0.392	0.244	0.116 ± 0.002	0.106 ± <0.001

The relationships between the diet and tissue levels are shown in the figure below (*see Figure 12*).

Figure 12: Comparison between the diet composition (mol %) and the plasma composition (mol %) of pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) between three different isocaloric high fat (37.5 % kcal) diets. The plasma samples were analysed by gas chromatography with mass spectrometry detection. Error bars represent ± standard error of the mean. (n = 3-4 mice per group). C15:0 (●); $R^2 > 0.999$, significance of slope (p = 0.0134), slope equation (Y = 0.1487*X + 0.05577). C17:0 (▲); $R^2 = 0.914$, significance of slope (p = 0.19), slope equation (Y = 0.08288*X + 0.08343).



The C15:0 levels were shown to have a linear relationship between the dietary composition and the circulating composition when comparing the results from three different dietary sources of fat. This result shows that ruminant fat significantly increases the circulating C15:0 composition above both lard fat and safflower oil, which reinforces the conclusion that C15:0 is a reliable biomarker of ruminant fat intake. However, C17:0 did not have a linear correlation with dietary intake. This disparity in the biomarker response between these two odd

chain fatty acids is also shown in the literature (Jenkins et al., 2017) (Albani et al., 2016) (Brevik et al., 2005) (Arab, 2003) (Golley and Hendrie, 2014).

6.4.2 Dose response study

Five groups of rats were fed different isocaloric 70% high fat diets via an intragastric cannula at 17% above growth matched *ad libitum* chow fed rats; this ensured that these experimental rats receive a hypercaloric diet with no deviation in dietary intake between each rat, thus reducing possible animal variations affecting the experimental results. The five experimental diets varied by their composition of ruminant fat (beef tallow) ranging from 0% to 11.7% as this will assess the dose response nature of each odd chain fatty acid. The dietary fat source and fatty acid composition are shown in the table below (*see Table 8*).

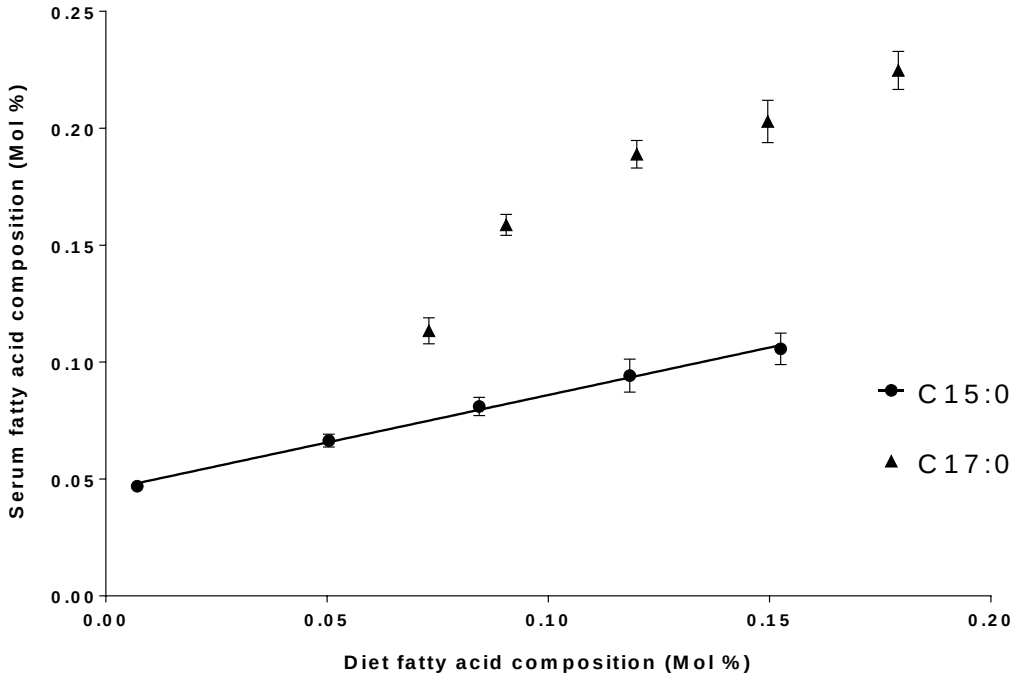
Table 8: Comparison between the diet composition and the serum composition (Mol %) of pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) in a dose response study in rats where five groups were subjected to isocaloric high fat diets with increasing ruminant fat content from 0% to 11·7%.

	Fat source			**Fatty acid composition**	
Diet	Corn oil	MCT oil	Beef tallow	C15:0 (Mol %)	C17:0 (Mol %)
1	70%	0%	0%	0.007	0.073
2	50%	16.4%	3.6%	0.050	0.090
3	35%	28.7%	6.3%	0.084	0.120
4	20%	41%	9%	0.118	0.150
5	5%	53.3%	11.7%	0.152	0.179

To assess these odd chain fatty acids as biomarkers of ruminant fat intake, the dietary C15:0 and C17:0 compositions were compared to the

circulating levels, these comparisons are shown in the figure below (*see Figure 13*).

Figure 13: The comparison between the diet composition and the serum composition (Mol %) of pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) in the five-stage ruminant fat diet dose response in rats. The serum samples were analysed by gas chromatography with mass spectrometry detection. Error bars represent ± standard error of the mean. (n = 6-9 per group). C15:0 (●); R^2 = 0.997, significance of slope (p < 0.0001), slope equation (Y = 0.4062*X + 0.04530). C17:0 (▲); R^2 = 0.912, significance of slope (p = 0.0114), slope equation (Y = 0.9579*X + 0.06044).



The circulating C15:0 compositions significantly linearly correlated (positively) with an increase in the dietary compositions. This shows that not only is C15:0 a biomarker of ruminant fat intake but actually a direct biomarker of the total dietary C15:0 intake (these results agree with the conclusions reached in the previous study; Different dietary fat source comparison study (*section*

6.4.1)). Although the circulating levels of C17:0 increased, there was not the same linear response as seen with C15:0, therefore suggesting other influencing factors which independently effect C17:0.

Interestingly, the largest C17:0 compositional change in the serum is seen between the first and second dietary study groups, this is where there is actually the smallest difference in dietary C17:0 change which further suggests there are other influential factors.

6.4.3 Ruminant fat supplementation study

The ruminant fat supplementation study was designed to increase the participants' daily intake of ruminant fat by an additional 30%, this resulted in an increase of 529 mg and 278 mg of C15:0 and C17:0, respectively, to their daily diet. The supplementation had a duration of fifty-six days which is sufficient for remodelling of tissue lipids (Skeaff et al., 2006) (Sobrecases et al., 2010) (Mensink and Katan, 1992) and should have produced a proportional increase in any biomarkers responses. The table below (*see Table 9*) shows the mean fatty acid compositions of the participants at the start of the study (day zero) and at the end of the study (day fifty-six), along with the percentage change and a measure of statistical significance (paired t-test; p-value) brought on by the diet.

Table 9: The pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) concentrations of the human plasma samples, analysed by gas chromatography with mass spectrometry detection at the start (day = 0) and at the end of the intervention (day = 56). The diet increase of each of the fatty acids due to the intervention is 30% above baseline. Values are given with ± standard error of the mean. Differences between the start of the study (Day = 0) and the end (Day = 56) were determined by a paired t-test (a measure of the significance or insignificance of an observation in one sample set (Day = 0) that is paired with the same observation in the second sample set (Day = 56) within the same study population); a value of $p \leq 0.05$ was considered significant. (n = 26).

	Day = 0 (Mol %)	Day = 56 (Mol %)	Average % change	*p*-value
(C15:0)	0.097 ± 0.003	0.106 ± 0.004	9.98	0.031*
(C17:0)	0.144 ± 0.004	0.145 ± 0.003	0.16	0.951

We aimed to challenge the validity of the results by examining the data reported through the dietary questionnaires to determine if other changes in the habitual diets of the participants lead to an increase in the intake of these two odd chain fatty acids from other sources besides ruminant fat (*see Table 10*).

Table 10: The dietary assessment of the human participants throughout the time course (day 0 to day 56) of the intervention showing that the dairy intake has significantly increased due to the supplementation, 234.1 to 308.1 grams per day. This table also confirms that the participant habitual diet remained compositionally unchanged throughout the intervention. A value of $p \leq 0.05$ was considered significant. (n=26).

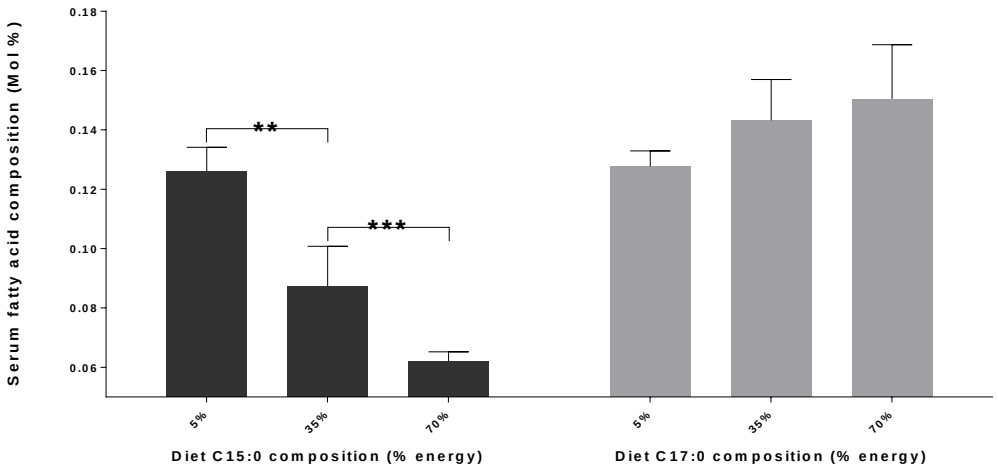
	Day 0	Day 56	*p*-value
Energy intake (kcal / day)	2236 ± 71	3040 ± 76	<0.001
Protein intake (g / day)	92.1 ± 3.1	127.0 ± 3.0	<0.001
Carbohydrate intake (g / day)	254.3 ± 8.4	270.6 ± 10.9	0.054
Lipid intake (g / day)	93.6 ± 4.7	160.3 ± 5.1	<0.001
Saturated fatty acid (g / day)	35.7 ± 2.1	62.9 ± 1.8	<0.001
Mono-unsaturated fatty acid (g / day)	28.5 ± 1.7	52.1 ± 1.9	<0.001
Poly-unsaturated fatty acid (g / day)	11.2 ± 0.8	19.6 ± 1.1	<0.001
Dairy intake (g / day)	234.1 ± 23.7	308.1 ± 23.7	<0.001

From the table above (*see Table 10*) it is clear the participants maintained their habitual diet since the average increase in their energy intake is ~804 kcal / day, this is only ~5% higher than the planned increase of 760 kcal / day by the dietary intervention, which is considered negligible. Additionally, the average participant lipid intake increased by ~66.7 g per day which is representative of the dietary intervention. From these results, we can state that the overfeeding intervention led to a significant increase of dietary ruminant fats and this significantly increased the participant intake of odd chain fatty acids, as intended by the design of the study intervention.

6.4.4 Dietary fat percentage study

Previous publications have shown that C15:0 fatty acid is a consistent biomarker within many intervention studies and correlates with dairy fat intake in human nutrition studies (Lankinen and Schwab, 2015) (Brevik et al., 2005). There has been varying degrees of C15:0 biomarker reliability within the larger observational human studies that do not rigidly control the participant's diet. Within these studies the total dietary fat content appears to influence the tissue C15:0 composition independently of the diet's fatty acid composition but this is difficult to disentangle from the other uncontrolled study factors. With regards to C17:0 the opposite is true, where the perceived biomarker response increases in reliability within larger observational human studies over strictly controlled animal intervention studies. The effects of changing the total dietary fat content (% energy) whilst maintaining the fatty acid composition between diets on the circulating odd chain fatty acid levels are shown in the figure below (*see Figure 14*).

Figure 14: The effect of changing the proportions of dietary fat (corn oil) from 5% to 35% to 70% of the total energy content (% energy) on the serum composition (Mol %) of pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) whilst maintaining an identical fatty acid composition in between each diet. The serum samples were analysed by gas chromatography with mass spectrometry detection. The significance of the difference between each group is shown by the p-value star system determined from homoscedastic t-tests; where $p \leq 0.05$ was considered significant ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***, $p < 0.0001$ = *). (n = 6-7 rats per group). Error bars represent ± standard error of the mean***.



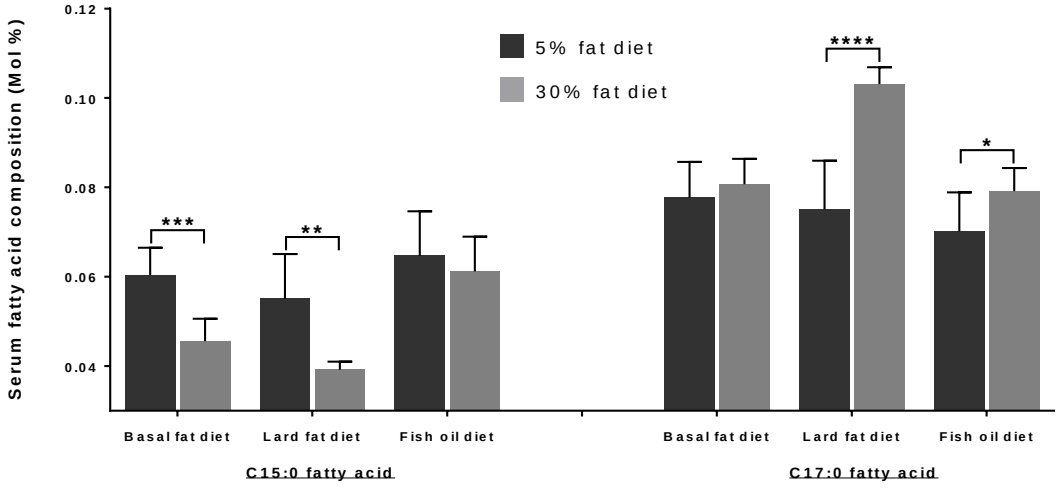
As shown above (*see Figure 14*) the circulating C15:0 composition decreased linearly ($R^2 = 0.9858$, $y = -0.0319x + 0.1556$, $p < 0.01$) as the total fat percentage increased, on the contrary, C17:0 appeared to increase ($R^2 = 0.9566$, $y = 0.0114x + 0.1177$, $p > 0.05$); although statistically non-significantly.

6.4.5 Dietary fat percentage and fat source study

Due to the significantly ($p < 0.01$) decreasing serum composition of C15:0 with the increasing dietary fat (composed of corn oil) composition from 5% to 35% to 70% of the total energy content (% energy); the effects of increasing the dietary total fat content needed to be assessed using different dietary fat sources to understand possible dietary interactions. By utilising three

different dietary fat compositions (Basal diet; composed of 60% sunflower / olive / colza oil, Lard diet; composed of 90% lard fat, and Fish oil diet; composed of 20% fish oil & 45% sunflower / olive / colza oil (Aoun et al., 2012). The correlations between the dietary fatty acid compositions at two different total fat levels (5% and 30%, w/w) were determined (*see Figure 15*), this was to determine if different fat sources were complimentary or deleterious to the circulating odd chain fatty acid compositions independent of the actual dietary fatty acid composition.

Figure 15: The effect of changing the proportions of dietary fat from 5% to 30% of the total energy content (w/w) across three different dietary sources of fat (basal fat, lard fat, and fish oil) on the serum composition (Mol %) of pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0). The fatty acid composition for the 5% and 30% fat diets under the same fat source are identical. The serum samples were analysed by gas chromatography with mass spectrometry detection. The significance of the difference between each group is shown by the p-value star system determined from homoscedastic t-tests; where $p \leq 0.05$ was considered significant ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***, $p < 0.0001$ = *). (n = 6-7 rats per group). Error bars represent ± standard error of the mean***.



As shown in the figure above (*see Figure 15*) the circulating C15:0 composition in the 5%-basal fat diet group was ~32% higher than the 30%-basal fat diet group ($p = < 0.01$). In the lard fat diet groups there was a ~40% higher C15:0 composition (Mol %) in the 5%-lard fat group compared to the 30%-lard fat group ($p < 0.01$). However, in the fish oil diet there was no statistically significant ($p > 0.05$) differences between either dietary total fat composition (5% or 30% total fat content). In the basal groups there was no significant difference seen in the C17:0 composition (Mol %) between the 5% and 30% diets ($p > 0.05$). Both the lard fat diet and the fish oil diets saw a higher composition of C17:0 in the 30% total fat diets when compared to the 5% total fat diets; ~27% higher ($p < 0.0001$), ~11% higher ($p < 0.05$) lard fat and fish oil, respectively.

6.4.6 Ethanol intake study

The true influence that dietary alcohol has on the circulating levels of C15:0 and C17:0 is unclear as human data is complicated by reporting bias and multiple influencing factors. The data is further complicated by the assumption that these fatty acids are only derived through ruminant fat consumption. Therefore, a specifically controlled animal intervention study is needed to see the unadulterated effects of ethanol of circulating fatty acid compositions. In support of this study it has been previously reported that alcohol intake significantly influences other circulating fatty acid levels independent of any dietary contributions (Burke et al., 1991), making it imperative to understand the relationships between odd chain fatty acids in the circulating and the dietary content of ethanol (alcohol).

In the table below (*see Table 11*) the serum C15:0 and C17:0 compositions are shown for the ethanol treated rats and the associated control group along with the statistical *p*-value between them (the p-value was used to assess the statistical significance or non-significance between the ethanol treated study group and the associated control group).

Table 11: The serum pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) composition of the ethanol treated rats (labelled; EtOH) and the associated control group (labelled; Control) measured by gas chromatography with mass spectrometry detection. The significance of the difference between the two groups is shown by the p-value determined from a homoscedastic t-test. A value of $p \leq 0.05$ was considered significant. Results are shown with ± standard error of the mean. (n = 7 per group).

Diet group	Serum C15:0 (Mol %)	Serum C17:0 (Mol %)
Control	0.071 ± 0.003	0.116 ± 0.0
EtOH	0.058 ± 0.003	0.112 ± 0.0
p-value	0.014	0.502

From the table above (*see Table 11*), it is clear that the C15:0 levels in the ethanol treated group displayed a significant difference from the associated control group (~20% reduced in the ethanol group; p = 0.014). When looking at the other fatty acid analysed; C14:0, C14:1 and C16:0 also showed a significant difference in response to the ethanol intervention. The variation in these other fatty acids is supported by published literature and enhances the validity of this study protocol (Burke et al., 1991) (Simon et al., 1996).

6.5 Discussion & conclusion

The aim of this study was to assess the validity of both C15:0 and C17:0 as biomarkers of their dietary intake and if they can be used to assess the dietary content of prominent foods high in odd chain fatty acids such as ruminant fat (i.e. dairy or dairy based products). Their validity was investigated by using

several animal models and a human translational study, involving both dietary supplementation and dietary interventions.

The initial study (*see 6.2.1 Different dietary fat source comparison study*) involved the influence of three difference high fat (37.5%) diets on the circulating odd chain fatty acid compositions (Mol %). The three high fat diets were made up of different fat sources; safflower, lard and milk fat, which offered two main insights into these odd chain fatty acids. Firstly, this dietary approach assessed if C15:0 and C17:0 are valid biomarkers of ruminant fat intake by producing definitive dietary groups rather than having a correlation analysis over an indiscrete range. Secondly, it allowed the consistency of these fatty acids within the circulation to be determined whilst having insignificant contributions from the diet.

As shown in the published literature, C15:0 is a reliable biomarker of its dietary intake (Brevik et al., 2005), which was confirmed in this study (*6.2.1 Different dietary fat source comparison study*) where the circulating composition of C15:0 significantly increased linearly in relation to the dietary composition ($R^2 > 0.999$, significance of the slope; $p = 0.0134$). When looking at the C17:0 compositions there was no significant correlation with the diet ($p = 0.19$), although there was a slight increase in the circulating C17:0 composition in the milk fat group this was not a statistically significantly different from the other two groups. This pattern (i.e. C15:0 is a reliable biomarker and C17:0 is not always) is shown in the published literature where these two fatty acids differ when assessed as biomarkers of dietary intake (Brevik et al., 2005) (Albani et al., 2016). An additional point is that the circulating composition of C17:0 was relatively consistent in all three groups (only 0.021 Mol% variation; which is roughly 20-25%), even though the safflower group had negligible amounts of dietary C17:0 in it; the circulating levels were similar to the lard and milk fat diet groups. To speculate, if C17:0 can only be exogenously derived from the diet then there must be an endogenous mechanism for the retention of this fatty acid so that the negligible dietary amounts could accumulate to the reported levels, however, there has been no reports demonstrating this, suggesting there must be another contributory source of C17:0.

To further investigate the relationship between the dietary odd chain fatty acid compositions and the circulating compositions a dose response study was performed which included a five stage isocaloric high fat diet in rats (*6.4.2 Dose response study*), where the composition of the fat in each diet had increasing amounts of beef tallow from 0% to 11.7% by increments of 2.7% (*see Table 8),* this range represented a normal population range of dairy fat intake in a typical Western human diet. NB. Beef tallow has a fatty acid composition representative of the ruminant animal lipidome, including any fatty acids produced by the rumen microbiome and sourced directly from the diet.

In this study (*see Figure 13*), the circulating composition of C15:0 and C17:0 increased in response to the increase in amount of beef tallow in each diet. According to the literature C15:0 is a more reliable biomarker of ruminant fat intake than C17:0, which is confirmed in this animal study. C15:0 directly correlated with the ruminant fat intake (*see Figure 13*), whilst C17:0 did not show the same linear relationship. An additional point is that the circulating composition of C17:0 was always higher than the dietary composition; since there are no known biological mechanisms specifically for the biological retention of C17:0 *in vivo* then this further confirms that C17:0 is not a reliable biomarker of dietary intake because it actually provides evidence for the endogenous biosynthesis of C17:0 fatty acid. This C17:0 disparity from the dietary content is especially evident between study groups one and study group two in the ruminant fat dose response study, where the smallest difference in dietary C17:0 resulted in the highest change in circulating C17:0 levels, this further suggests the involvement of an endogenous biosynthetic mechanism rather than a direct biomarkers response.

In the human translational study with the ruminant fat supplementation, the diets of twenty-six participants were supplemented for fifty-six days with a 30% increase in ruminant fat sources. An additional 40 grams of almonds was also included to provide a source of unsaturated fat to avoid unhealthy consequences of a high saturated fat supplementation. Across the intervention, the plasma C15:0 compositions increased by ~10% (statistically significant, $p = 0.031$) as a result of a dietary 30% increase. This plasma increase in C15:0

directly concurs with the expected increase according to the ruminant fat dose response study (*see Figure 13*), further reinforcing the validity of the results. However, the plasma C17:0 levels did not change across the supplementation study rebutting the claims that C17:0 is a dietary biomarker.

We have been able to show that C15:0 was a reliable biomarker of its dietary intake across three different studies, include a human supplementation study but have also shown that C17:0 did not have the same biomarker correlation. Although increasing the dietary intake of C17:0 did seemingly increase the circulating levels of C17:0, these are not directly linked as shown in the human supplementation study suggesting the involvement of endogenous mechanisms which dominate over any dietary factors.

Since C15:0 has been shown as a dietary biomarker we set out to investigate factors that would influence dietary processes and interactions to see if these factors affect C15:0 independent to C17:0, these included the total fat and ethanol content in the diet. As shown in the study above (*see 6.2.4 Dietary fat percentage study*), the dietary fatty acid composition was maintained over three diets where total fat content increased from 5% to 35% then to 70%; this produced a three-stage total fat dose response intervention. As shown above (*see Figure 14*) there was a significant ($p < 0.01$) and proportionate decrease in the circulating C15:0 levels with the increase in the dietary total fat content. It has been shown that as the dietary total fat content increases the absorption of essential fatty acids decreases; this may be due to a reduced mixing of the intestinal chyme and/or less complete homogenous lipid emulsification; this may explain the results seen for C15:0 (i.e. higher total fat contents reduce the absorption of C15:0). On the other hand, there was an increase in the circulating C17:0 levels as the dietary total fat content increased (although statistically insignificant). Since there was no notable decrease in the circulating C17:0 with the increased in dietary total fat content then this suggests that it is not significantly obtained from the diet.

To further investigate the relationship between the dietary total fat content and the variations seen in the circulating compositions of C15:0 and C17:0 we analysed samples from a study (*see 6.2.5 Dietary fat percentage and*

fat source study) where there was either 5% or 30% total fat content (w/w) across three different fat sources, these included a basal diet; composed of 60% sunflower / olive / colza oil, a lard fat diet; composed of 90% lard fat, and a fish oil diet; composed of 20% fish oil & 45% sunflower / olive / colza oil. The three different fat sources were intended to show if there would be a complimentary or deleterious effects on the circulating odd chain fatty acid compositions. The same correlation is seen in the previous study (*see 6.2.4 Dietary fat percentage study*) where there was a significant decrease in the circulating C15:0 levels as the dietary total fat content was increased; an increase of 25% to the total dietary fat content caused a ~25% reduction in the circulating C15:0, this directly reflects the expected decrease seen in the previous study (*see Figure 14*). Only the fish oil diet showed no significant decrease in the circulating C15:0 compositions, this may be due to the higher absorption efficiency of unsaturated fatty acids in the diet (Ockner et al., 1972) driving an overall increase in intestinal lumen absorption. Although there was no significant change in the basal fat diet, there was a substantial and statistically significant increase in the circulating C17:0 compositions in the lard fat diet and the fish oil diet with the increase in the dietary total fat content (~27% higher; $p < 0.0001$, ~11% higher; $p < 0.05$, for the lard fat and fish oil diets, respectively). This data further suggests that C15:0 is derived from the diet since intestinal absorption factors are dominant, however, there must be endogenous mechanisms involved in the circulating compositions of C17:0 to maintain these consistent levels.

It has been unclear the true influence that dietary alcohol has on the circulating levels of odd chain fatty acid since within human epidemiology studies the participant intake of alcohol is recorded but the results from these participants are still included in the data processing, even though published literature has shown that alcohol intake can significantly alter the circulating fatty acid profile independently of the dietary contributions (Burke et al., 1991).

In this ethanol study (*see 6.2.6 Ethanol intake study*) the affect that ethanol has on endogenous C15:0 and C17:0 levels were investigated by isocalorically substituting dietary carbohydrates for ethanol (30% kcal). The ethanol experimental group had a ~20% lower C15:0 composition than the

associated control group ($p = 0.014$), but here was no significant difference between the groups for C17:0 ($p = 0.502$). Previous studies have shown that dietary ethanol reduces the intestinal absorption of fatty acids, further suggesting that C15:0 is dietary derived. The stability of C17:0 across each of the interventions suggests that there is either a physiological need for C17:0 at that particular composition or it is the product of a biological mechanism that is separate or independent of other typical fatty acid metabolic and biosynthetic pathways that may be influenced by ethanol.

To summarise, it is clear that each individual odd chain fatty acid is the result of very different processes and that their relations with disease risk cannot be generalised. From our results, we can conclude that C15:0 appears to be a direct, linear biomarker of dietary C15:0 intake. However, this does not mean it can be directly translated into a marker that can quantify ruminant fat intake in all cases due to varying fatty acid compositions of different ruminant fat sources (e.g. milk, butter, beef tallow), as well as other dietary factors that influence C15:0 fatty acid absorbance (e.g. dietary total fat content and ethanol). However, the circulating C15:0 levels can still be used to contrast between different levels of dietary intake (upper and lower quartiles) and also provides further proof of the possible protective role of ruminant fat in the diet against studied pathologies (Forouhi et al., 2014). C17:0 has been misidentified as a ruminant fat biomarker, which leads to the conclusion that it is most likely endogenously biosynthesised. This does not mean that any dietary C17:0 doesn't influence the circulating compositions but the major factor affecting the C17:0 compositions *in vivo* are mainly from endogenous biological processes. These data agree with published literature and explains why the C15:0 biomarker relationship weakens in larger data sets, and why C17:0 rarely correlates with intake; especially within strictly controlled dietary studies.

The limitations of these studies - the results from these studies I collected from a combination of rodent models and human participants, although rodent models have been used to study biochemical and mechanistic characteristics due to their extensive similarities in anatomy, physiology and genetics, the results may not be directly translatable to humans. Each study

offers an additional limitation; where there was the use of high fat diet with the fat percentage higher than the average human diet. The high degree of control within the animal studies do not reflect the diversity in human populations, therefore, the artificial correlations seen with C17:0 may still be useful in epidemiological studies.

6.6 Summary

To summarise, C15:0 fatty acid is a consistent biomarker of dietary intake, its circulating composition (*in vivo*, Mol %) linearly correlates with levels of intake. Additionally, dietary factors such as total fat content and ethanol significantly affect circulating levels independently of the dietary compositions. Due to these diverse range of factors that affect the circulating levels of C15:0 it is difficult to normalise and compare large epidemiological human data to show strong linear correlations between intake and circulating levels, hence explaining why there may be conflicting and contrasting conclusions within the published literature.

C17:0 fatty acids cannot be considered a dietary biomarker since the circulating levels do not reliably correlate; and in some circumstances actually contradict their dietary intake. The consistency of the circulating C17:0 levels, along with the elevated *in vivo* C17:0 levels over C15:0 levels can only suggest that it is endogenously biosynthesised. The published conclusions of C17:0 being a dietary biomarker is most likely attributed to the unavoidable limited control within large epidemiological human studies. Additionally, dietary factors (NB. not reported in this chapter, *see Chapter 7; Endogenous synthesis*) may be independently increasing the endogenous production of C17:0 to a similar extent as the increase in the dietary compositions, manifesting a phantom biomarker response.

7 ENDOGENOUS SYNTHESIS

An investigation into the endogenous production of C17:0 odd chain fatty acids by alpha oxidation, utilising the concept of metabolic substrate competitive inhibition (upregulation and downregulation of alpha oxidation via phytol/phytanic acid and stearic acid administration) as well as utilising an *Hacl1* gene knockout mouse model.

7.1 Introduction

There are two known possible biological mechanisms that could effectuate the endogenous production of odd chain fatty acids, however, these mechanisms have only been strictly investigated within *in vitro* studies and their contribution to *in vivo* circulating levels within whole organisms has only been speculated. The mechanisms proposed, either as an independent source or through a combination are: elongation of propionyl-CoA in *de novo* lipogenesis and/or alpha oxidation of either straight even chain fatty acids or 2-methyl branched chain fatty acids.

Odd chain fatty acid endogenous biosynthesis through elongation of propionyl-CoA has been investigated within *in vitro* studies; prominent manuscripts include the work by Horning and colleagues (Horning et al., 1960) where they showed C15:0 can be produced through adipose tissue enzymatic incubation with radio-labelled 2-13C-malonyl-CoA and propionyl-CoA. Later work by Crown and colleagues (Crown et al., 2015) showed stable isotope labelled branched chain amino acids; [U-13C]-valine and [U-13C]-isoleucine, resulted in labelled propionyl-CoA incorporation into 3T3-L1 adipocyte cellular fatty acids. Although both of these studies showed odd chain fatty acid endogenous biosynthesis by the incorporation of propionyl-CoA into *de novo* lipogenesis, the results do highlight the improbability of this propionyl-CoA mechanism substantially contributing to *in vivo* tissue fatty acid levels due to severely differing compositions. In each of these studies the most abundant fatty acid was C15:0, with C17:0 considerably lower. This configuration is shown in

many other published manuscripts, however, contradicts the widely reported levels of circulating C15:0 and C17:0 *in vivo*, where C17:0 is roughly twice the composition as C15:0 (Jenkins et al., 2015). This disparity between the *in vitro* and *in vivo* compositions shows that propionyl-CoA incorporation into circulating odd chain fatty acids is not a significantly contributing mechanism.

Studies have suggested that differences in circulating odd chain fatty acids may be due to endogenous biosynthesis via alpha oxidation. This work has been done *in vitro* but it remains unclear if alpha oxidation significantly contributes to circulating C15:0 and C17:0 levels *in situ*. A considerable piece of work demonstrating alpha oxidation *in vitro* on straight chain fatty acids was presented by Su and colleagues (Su et al., 2004), where radiolabelled C15:0 was biosynthesised during incubation of differentiating 3T3-L1 Cells with [9,10-3H]-C16:0. This work also demonstrated significant increases in C17:0 fatty acids with progression through differentiation, along with their incorporation into each major lipid class (phospholipids and glycerolipids). Further work published on genes associated with straight chain fatty acid alpha oxidation include: *Fa2h* (Alderson et al., 2004) showed straight chain fatty acid conversion to 2-hydroxy-fatty acids by fatty acid 2-hydroxylase (FA2H) which is encoded by the *Fa2h* gene. This is a key study due to the formation of a straight chain fatty acid substrate that may be incorporated into alpha oxidation. This manuscript also demonstrated the relative affinity of FA2H with fatty acid chain length; where there is a higher affinity between FA2H with fatty acids with eighteen carbon atoms or greater relative to palmitic acid (Alderson et al., 2004). Another key gene involved in alpha oxidation is *Hacl1* which codes for the protein 2-hydroxyacyl-CoA lyase 1 (abbreviated to HACL1). Two key manuscript (Foulon et al., 2005) (Casteels et al., 2007) show cleavage of a carbon-carbon bond at the alpha carbon atom in a straight chain 2-hydroxyacyl-CoA molecule; demonstrating the endogenous biosynthesis of odd chain fatty acids from even chain fatty acids *in vitro*.

A study by Foulon and colleagues (Foulon et al., 2005) showed that there was active competition *in vitro* between 2-hydroxy-straight-chain fatty acids and 2-hydroxy-3-methyl branched chain fatty acids, where there was a

considerable reduction in radiolabelled 14C-formate and 14C-formyl-CoA with the addition of either 2-hydroxyhexadecanoyl-CoA or 2-hydroxyoctadecanoyl-CoA. Since it is well documented that 3-methyl branched chain fatty acids (beta branched chain fatty acids) are the primary/targeted substrate for alpha oxidation, and straight chain fatty acids can be incorporated into this mechanism, although, this has only been demonstrated within *in vitro* studies. Then by increasing the primary/target substrate within an *in vivo* model there would be active competition between these substrates. Due to the substrate competition, there would be a proportionate decrease in the incorporation of straight chain fatty acids into the alpha oxidation pathway. This would result in a decrease in the endogenously biosynthesised odd chain fatty acids (i.e. with an increase in branched chain fatty acids there would be a decrease in odd chain fatty acid production). Hence, a phytol (precursor to phytanic acid; the most abundant branched chain fatty acid) supplementation study should produce a decrease in circulating odd chain fatty acids compared to an appropriate control group.

Due to an independent mechanism for the endogenous production of 2-hydroxy-straight chain fatty acids by fatty acid 2-hydroxylase; which does not influence 3-methyl branched chain fatty acid hydroxylation by phytanoyl-CoA 2-hydroxylase, then an increase in circulating straight chain fatty acids *in vivo* would result in an increase in 2-hydroxy-straight chain fatty acids inhibiting 3-methyl branched chain fatty acids alpha oxidation. This increase in 2-hydroxy-straight chain fatty acid would likely increase odd chain fatty acid biosynthesis (i.e. higher substrate levels typically drive higher product synthesis). This was investigated *in vivo* by the stearic acid infusion and measuring the absolute changes in odd chain fatty acids with no change in dietary intake.

Only one step in the mechanism of alpha oxidation is currently unambiguous (Jenkins et al., 2015), this is the cleavage of formyl-CoA from the parent molecule 2-hydroxy-fatty acid by 2-hydroxyacyl-CoA lyase 1 (Mezzar et al., 2010), since there is only one known enzyme that effectuates this cleavage. Each other step in this mechanism, both anterior and posterior to cleavage of the carbon-carbon bond has many different isoenzymes. Therefore, permutations in 2-hydroxyacyl-CoA lyase 1 would likely give more consistent results when

studying odd chain fatty acid endogenous biosynthesis from straight chain fatty acids. In alpha oxidation, *Hacl1* codes for the principal protein (2-hydroxyacyl-CoA lyase 1; HACL1) and has been shown to cleave formyl-CoA from both branched chain and straight chain fatty acids within *in vitro* studies. Due to the negligible amount of 2-methyl branched chain fatty acids in mammals along with the considerably lower rate of alpha oxidation when compared to beta oxidation, then the contributions of odd chain fatty acids from 2-methyl branched chain fatty acids seems unlikely or insignificant. However, alpha oxidation of straight chain fatty acids may still be a considerable source due to their high abundance in all tissues (C16: and C18:0 usually > 50% total fatty acids). Alpha oxidation has been demonstrated in both adipocytes (Roberts et al., 2009) and hepatocytes (Croes et al., 1996) *in vitro*, however, these tissues have varying amounts of *Hacl1* expression which could correlate with odd chain fatty acid compositions *in vivo*.

7.1.1 Study hypothesis.

Due to the inconsistent results published around both C15:0 and C17:0 fatty acids as biomarkers of dietary intake, along with the results shown in the previous chapters (*see Chapter 5 and 6*; Intestinal microbiota and Dietary biomarker, respectively); we hypothesise that odd chain fatty acids can be endogenously biosynthesised through alpha oxidation of the subsequent neighbouring even chain fatty acids. We further hypothesise that C17:0 is more substantially endogenously biosynthesised than C15:0. Additionally, odd chain species with a shorter carbon chain can be endogenously produced through beta oxidation of longer odd chain species, as seen with even chain fatty acids (i.e. C15:0 may be biosynthesised from C17:0 via beta oxidation).

7.2 Study design

To test this hypothesis, we measured the circulating C15:0 and C17:0 compositions (Mol %; via GC-MS) and compared these compositions between each experimental group within each study to investigate possible endogenous biosynthesis of these odd chain fatty acids. A phytol supplementation study was used to investigate *in vivo* inhibition of 2-hydroxy-fatty acid alpha oxidation by

3-methyl branched chain fatty acids. A stearic acid *in vivo* infusion model was used to investigate the inhibition of 3-methyl branched chain fatty acid alpha oxidation by 2-hydroxy-fatty acids. An *Hacl1* gene knockout mouse model was used to directly investigate this gene, and resulting enzyme's influence on levels of odd chain fatty acids.

7.2.1 Phytol supplementation study

C57BL/6 mice (n = 4-5 per group) were used in a phytol supplemented diet investigation (Selkälä et al., 2015). Mice were fed *ad libitum* a cereal based, pelleted, rodent diet (Harlan Teklad, USA) with *ad libitum* access to water. The phytol supplemented group received an additional 0.5% (w / w) phytol added to the diet, starting at 3 to 4 months of age. After 14 days on the phytol supplemented diet, terminal blood was drawn from inferior vena cava and processed into serum. Serum was separated by centrifugation within 15-30 minutes after blood sampling and stored at -70^{o}C until analysed.

All experiments were executed according to accepted criteria for the humane care and experimental use of laboratory animals. All protocols were approved by the Animal Care and Use Committee of the University of Oulu.

7.2.2 Stearic acid (C18:0) infusion study

As described previously, Sprague Dawley rats (n = 11 per group) were used in a stearic acid (250 or 1000 nmol/kg/day) intraperitoneal infusion procedure lasting five weeks, with control animals receiving saline vehicle treatment only (Pan et al., 2010). The animals were maintained in a 12-hour light–dark cycle, were given food and water *ad libitum* and were acclimatised in the animal facility for at least one week prior to any procedures. Blood was collected after 35 days under pentobarbital aesthesia and processed into serum, all samples were stored at -80^{o}C until analysed.

The animal study was approved by the Animal Care and Use Committee of Taichung Veterans General Hospital, Taiwan.

7.2.3 2-hydroxyacyl-CoA lyase 1 (*Hacl1*) study

Heterozygous (*Hacl1*+/-) mice, from a Swiss Webster background, were mated to obtain *Hacl1* deficient mice (*Hacl1*-/-). Wild type littermates (*Hacl1*+/+) were used as controls (Mezzar et al., 2010) (Mezzar et al., 2017). All animals were kept under conventional conditions in the animal housing facility of the KU Leuven (KU Leuven, Leuven, Belgium). Mice were kept on a regular chow diet and had water *ad libitum*. After 14 weeks, the mice were anesthetized with overdose of Nembutal for tissue collection (n = 15 *Hacl1*-/-; 8 females, 7 males, n = 12 control (*Hacl1*+/+); 7 females, 5 males). Blood was removed and processed into serum. Liver, white adipose tissue (WAT; inguinal, gonadal and retroperitoneal) and interscapular brown adipose tissue (BAT) were collected and frozen. All samples were stored at -80^{o}C until analysed.

All animal experiments were approved by the Institutional Animal Ethical Committee of KU Leuven.

7.3 Statistical analyses

The statistical approaches utilised were appropriate for specific experimental designs; a homoscedastic t-test was used between two unrelated equal variance groups to identify any significance or non-significance between them two groups. A homoscedastic t-test was used in the following studies; Phytol supplementation study, Stearic acid (C18:0) infusion study, and the 2-hydroxyacyl-CoA lyase 1 (*Hacl1*) study. A value of $p \leq 0.05$ was considered significant.

7.4 Results

To minimize animal experimentation, we aimed to make use of existing sample sets; this resulted in a combination of mouse and rat samples being analysed. Since all mammals perform lipogenesis, beta oxidation, and alpha oxidation in the production and metabolism of lipids, we therefore expect that these results can be interpreted across the species (but not directly translated). Additionally, published literature has shown that the circulating lipid percentage composition is very similar between the species, where C15:0 to C17:0 ratio is consistently 1:2 respectively. Although C15:0 and C17:0 fatty acids have a

minor composition in plasm/serum of all mammals, their concentration is strikingly consistent. Although this approach is not conventional, we are able for the first time to comprehensively study the influences of fatty acid alpha oxidation on circulating odd chain fatty acid.

7.4.1 Phytol supplementation study

To investigate inhibition of straight chain fatty acid conversion into odd chain fatty acids by the introduction of the target substrate of alpha oxidation, we conducted a phytol enriched diet study. One group of mice had an additional 0.5% (w / w) phytol supplemented to their chow diet with a control group receiving just the chow diet alone. We then measured the total fatty acids (free and esterified) by gas chromatography with mass spectrometry detection to assess any influences of the phytol supplementation on the levels of serum odd chain fatty acids fatty acid.

The levels of C17:0 significantly decreased in the phytol diet compared with the control (control: 0.130 ± 0.005, phytol: 0.089 ± 0.004; $p < 0.001$). There was no significant difference seen between the phytol supplemented group and the control group for C15:0 fatty acid in the serum (control: 0.049 ± 0.002, phytol: 0.045 ± 0.002; $p = 0.174$).

7.4.2 Stearic acid (C18:0) infusion study

Our previous results suggest that alpha oxidation is important in the endogenous production of C17:0 but not for C15:0. To investigate if C18:0 (the substrate for alpha oxidation to produce C17:0) increases circulating C17:0 we conducted an intraperitoneal infusion in rats with either 250 or 1000 nmol / kg / day of stearic acid or just the dosing vehicle and measured the absolute change in C17:0. The absolute values were used for comparison rather than relative percentages. This was due to an artificial source of C18:0 in the biological system which has several consequences: a reduction in normal processes of fatty acid elongation and increased fatty acid desaturation to try and maintain a natural biological homeostasis, as well as an artificially altered fatty acid percentages due to the introduction of the C18:0.

While there was no statistically significant change in the absolute concentration of C15:0 (control: 3.308 ± 0.310 µmol, 250 nmol/kg/day stearic acid: 4.447 ± 0.466 µmol, $p = 0.055$), there was a statistically significant increase in C17:0 (control: 5.683 ± 0.542 µmol, 250 nmol/kg/day stearic acid: 8.101 ± 0.651 µmol, $p < 0.01$).

A higher concentration (1000 nmol/kg/day) of infused stearic acid did not significantly ($p = 0.810$) increase serum C17:0 above the 250 nmol/kg/day group.

7.4.3 2-hydroxyacyl-CoA lyase 1 (*Hacl1*) study

Total fatty acids (combined free and esterified) were measured in the serum, the liver and four types of adipose tissue; inguinal white adipose tissue, gonadal white adipose tissue, retroperitoneal white adipose tissue and interscapular brown adipose tissue. The relative absolute quantities (µMol) of C15:0 and C17:0 for each of these tissues is shown in the table below (*see Table 12*).

Table 12: The relative quantities of pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) when comparing Hacl1-/- mice and the related control mice in each of the studied tissues; serum, liver, three white adipose tissues (inguinal; I-WAT, gonadal; G-WAT, and retroperitoneal; R-WAT) and interscapular brown adipose tissue (I-BAT). The samples were analysed by gas chromatography with mass spectrometry detection and normalised to an internal standard to account for analytical variation, tissue absolute quantities (µMol) normalised to total lipid content. The significance of the difference between the Hacl1 knockout mice (Hacl1-/-) and the related wildtype mice (control) are shown by the t-test; a value of $p \leq 0.05$ was considered significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Results are shown with ± standard error of the mean. (n = 12-15 per group).

	C15:0 (µMol)		C17:0 (µMol)	
	Control	Hacl1$^{-/-}$	Control	Hacl1$^{-/-}$
Serum	16.4 ± 1.9	16.2 ± 1.1	36.2 ± 3.1	28.7 ± 1.3***
Liver	22.0 ± 1.2	20.6 ± 1.0	53.8 ± 2.4	42.2 ± 1.9***
I-WAT	24.0 ± 2.0	25.0 ± 1.6	24.6 ± 1.8	26.1 ± 1.2
G-WAT	25.9 ± 1.2	23.6 ± 1.0	30.8 ± 1.2	28.7 ± 0.7
R-WAT	25.7 ± 1.1	25.5 ± 1.0	29.9 ± 1.7	30.6 ± 1.0
I-BAT	8.5 ± 0.4	8.0 ± 0.5	18.1 ± 1.0	16.9 ± 0.9

As shown in the table above (*see Table 12*) only the serum C17:0 and liver C17:0 levels displayed a decrease associated with the *Hacl1* gene knockout. There were no statistically significant changes in any tissue C15:0 levels.

It is unlikely to expect a complete C17:0 deficiency in the *Hacl1-/-* group since salvage pathways compensates for a deficiency in HACL1; such as

2-hydroxy-acid oxidase (HAO2) (Jenkins et al., 2017) (Draye et al., 1987), also dietary C17:0 still contributes.

When comparing other fatty acids in the *Hacl1-/-* group, there were no other statistically significant differences (data not shown).

7.5 Discussion & conclusions

The aims of these studies were to investigate the endogenous biosynthesis of odd chain fatty acids by a secondary metabolic pathway, known as alpha oxidation. Through two substrate supplementation studies and a gene knockout study the principal step of alpha oxidation (the cleavage of the carbon-carbon bond by 2-hydroxyacyl-CoA lyase 1 on straight chain fatty acids) was either upregulated, downregulated or knocked out.

Phytol was selected for the assessment of the inhibition of alpha oxidation of straight chain fatty acids because it is the natural precursor of phytanic acid; the target substrate of alpha oxidation. The precursory metabolism of phytol into phytanic acid will still result in a representative inhibition of 2-hydroxy-straight chain fatty acids at that concentration of branched chain fatty acid applied (applied in the diet) to the study model (Mize et al., 1966), i.e. the metabolic break down of the phytol into phytanic acid was inconsequential. A supplementation of 0.5% (*w/w*) phytol was added to the experimental diet, which was then fed to the treatment animal group. This magnitude of supplementation represents the higher level of dietary phytol/phytanic acid content in a typical human Western diet (Brown et al., 1993), however, is far within the metabolic capacity of phytol/phytanic acid metabolism. There was no physiological toxicity brought on by the phytol supplementation since there was no saturation of the alpha oxidation pathway. In a study by Foulon and colleagues (Foulon et al., 2005), they demonstrated that there was metabolic competition in alpha oxidation between 2-hydrox-straight chain fatty acids and 2-hydroxy-3-methyl branched chain fatty acids; this substrate competition is the justification for this phytol supplementation investigation and allows the *in vivo* investigation of odd chain fatty acid endogenous biosynthesis by alpha oxidation.

In this investigation, there was a significant decrease in C17:0 due to the phytol supplementation compared to the matched control group (~32% decreased, $p < 0.001$). Circulating C15:0 fatty acids statistically non-significantly decreased by ~8% ($p = 0.174$), which could be largely attributed to the decrease in C17:0 going though one cycle of chain shortening beta oxidation in the peroxisomes (Wanders et al., 2001). The greater decrease in C17:0 when compared to C15:0 further reinforces the notion of alpha oxidation on straight chain fatty acids. FA2H is known to have a greater affinity for the production of 2-hydroxy-stearoyl-CoA (from C18:0) over the production of any shorter 2-hydroxy-fatty acids from their corresponding fatty acid (Alderson et al., 2004) (Guo et al., 2010). Since 2-hydroxy-stearoyl-CoA is the preferred substrate of FA2H then there would be a greater incorporation of C18:0 into alpha oxidation than any shorter straight chain fatty acid, consequently, C17:0 being predominantly biosynthesised over any other odd chain species.

To compliment the phytol supplementation, a stearic acid infusion study was performed to artificially increase the C18:0 compositions, this was to investigate the alpha oxidation substrate competition theory as shown by Foulon and colleagues (Foulon et al., 2005), where there is 2-hydroxacyl-CoA lyase 1 competition between 2-hydroxy-straight chain fatty acids and 2-hydrox-3-methyl branched chain fatty acids.

In this stearic acid infusion study, three groups of male Sprague–Dawley rats were administered with either 250 nmol/kg/day, 1000 nmol/kg/day, or just the vehicle solution which was used as the control group. This three-tier dose response then allowed the assessment of the change in absolute concentrations of circulating C17:0 fatty acids compared with the varying concentrations of administered stearic acid in order to indirectly investigate the competition between 2-hydroxy-fatty acids and 2-hydroxy-3-methyl branched chain fatty acids in alpha oxidation *in vivo*. Due to the high affinity of fatty acid 2-hydroxylase to stearic acid then increasing the concentrations of stearic acid *in situ* would in turn increase the production of 2-hydroxy-stearoyl-CoA.

The infusion of stearic acid (both 250 and 1000 nmol/kg/day) resulted in a significant increase in circulating C17:0 ($p < 0.01$), whilst there was a

minimal increase in C15:0 it was not statistically significant ($p = 0.055$). The 250 nmol/kg/day infusion group produced a ~43% increase in circulating C17:0, this increase was independent of exogenous sources and therefore can only be accredited to endogenous biosynthesis. The subtle increase in the circulating C15:0 concentration can be attributed to chain shortening by one cycle of beta oxidation within the peroxisomes. It has been previously reported that fatty acid beta oxidation in the peroxisomes does not go through to completion (Mannaerts et al., 2000) since the two-known acyl-CoA oxidases show no activity on butyryl-CoA. Additionally, it has been shown that ~25% of long chain fatty acids go through just one cycle of chain shortening beta oxidation in the peroxisomes; the removal of just one acetyl-CoA molecule (Lee et al., 1998). This approximate amount of 25%, along with the reduced rate of odd chain fatty acid (compared to even chain fatty acid) going through beta oxidation (Gotoh et al., 2008), then there would be an even greater percentage of C17:0 just going through a single cycle of peroxisomal beta oxidation. These two factors combined may account for the increase in the absolute concentration of C15:0 (~34% increase) in the stearic acid infusion groups.

In the 1000 nmol/kg/day stearic acid infusion group there was no further increase in the circulating C18:0 above the levels in the 250 nmol/kg/day group (circulating C18:0 levels in the 250 nmol/kg/day stearic acid group: 656.892 ± 26.304 µmol, and the 1000 nmol/kg/day stearic acid group: 630.487 ± 32.699 µmol, $p = 0.541$). The higher amount of infused saturated fatty acids increased biological mechanisms, such as elongation (C18:0 to C20:0 and C22:0 (Wong et al., 2004) (Kelly et al., 2001)) and desaturation (C18:0 to C18:1) to maintain physiological homeostasis. The higher rates of fatty acid elongation and desaturation in the 1000 nmol/kg/day group explains why there is no difference in the circulating levels of C18:0 between these groups. Since there is no difference in the C18:0 between these groups then it is reasonable to assume that there will be the same amount of C18:0 incorporation into the alpha oxidation pathway for the endogenous production of C17:0, which explains why there is no further increase in C17:0 with higher doses of infused stearic acid.

The *Hacl1* gene knockout mouse model was developed by Paul P. Van Veldhoven and colleagues (Mezzar et al., 2010) where it resulted in a significant decrease in 2-hydroxacyl-CoA lyase 1; there was not a complete cessation of alpha oxidation due to known salvage pathways, such as peroxisomal oxidation utilising 2-hydroxy-acid oxidase (HAO2) (Jones et al., 2000) (Draye et al., 1987), and also bacterial acetolactate synthase-like (HACL2). Even though 2-hydroxy-acid oxidase provides an alternative enzymatic pathway for alpha oxidation of 2-hydroxy-fatty acids, it is still reasonable to expect (based on the data from the phytol supplementation and stearic acid infusion studies (Jenkins et al., 2017)) that a nullification of the *Hacl1* gene would bring about a notable decrease in alpha oxidation, and therefore, a notable decrease in the endogenous production of odd chain fatty acids.

Odd chain fatty acid endogenous production was investigated by utilising an *Hacl1* knockout mouse model and analysing the odd chain fatty acid concentration in the significant tissues associated with alpha oxidation, these tissues include; serum, liver, inguinal white adipose, gonadal white adipose, retroperitoneal white adipose, and interscapular brown adipose. The liver and adipose tissues have the highest *Hacl1* gene expression and via *in vitro* studies (hepatocytes and adipocytes) have been shown to perform alpha oxidation. The fatty acid concentration (μMol) is presented rather than the percentage of total fatty acids (Mol %) because knocking out the *Hacl1* gene upregulates any salvage pathways (for example: HAO2-peroxisomal oxidation and HACL2 decarboxylation) which will affect other fatty acid levels; this artificial mouse model provides further evidence of endogenous odd chain fatty acid biosynthesis but may not be suitable for fatty acid profiling.

In the table above (*see Table 12*) the concentration of C15:0 in each of the analysed tissues was statistically unaffected by the *Hacl1* gene knockout, where there are no significant differences between the two groups (*Hacl1* knockout group and the *Hacl1* control group), this suggests that C15:0 is not significantly produced by 2-hydroxyacyl-CoA lyase 1 enzyme. Although statistically non-significant there is a small decrease in the composition of C15:0 (within these tissues: serum, liver, gonadal white adipose, and interscapular

brown adipose tissue) in the *Hacl1* knockout group compared to the control group. The tissues that manifest a decrease (significant and non-significant) in C17:0 also manifest a decrease in C15:0 but far less attenuated; when comparing the C15:0 concentrations with the C17:0 concentrations for each individual across all the tissues there is a strong correlation ($R^2 = 0.9399$, $p < 0.0001$, see Appendix 1). This further reinforces the notion that C15:0 is not directly biosynthesised from alpha oxidation but is actually the end product of single cycle of C17:0 peroxisomal beta oxidation. This minor contribution of C15:0 via beta oxidation could explain the presence of C15:0 in studies that have an undetectable C15:0 dietary content.

In the table above (*see Table 12*), the serum C17:0 concentrations were ~20% lower in the *Hacl1* knockout group when compared to the *Hacl1* control group. Comparably, in the liver there was a ~22% lower C17:0 concentration in the *Hacl1* knockout group than the *Hacl1* control group. No adipose tissue showed any significant differences in C17:0. This does not exclude the possibility that adipose tissue may produces C17:0 via a different mechanism but the role of HACL1 through alpha oxidation is statistically non-significant. The ratio of C15:0 to C17:0 composition in the serum is ~1:2 respectively, this ratio can be seen in the liver tissue and interscapular brown adipose tissue, but not in any of the white adipose tissues, which further suggests that the liver tissue is the biosynthetic source of circulating C17:0. The interscapular brown adipose tissue also has this ~1:2 ratio but there was no significant difference in C17:0 between the groups, suggesting its fatty acid composition is derived from the serum, unlike the white adipose tissue where *de novo* lipogenesis predominantly affects its fatty acid composition.

The limitations of these studies - the phytol supplementation uses the precursor ‘phytol’ instead of phytanic acid to competitively inhibit alpha oxidation of straight-unbranched-chain fatty acids, this confounds the results. Additionally, the use of the *Hacl1* knockout mice causes salvage pathways to become more active or become initiated, which does not allow us to measure the true influence this gene/protein/enzyme has on circulating levels of these odd chain fatty acids.

7.6 Summary

To summarise, *Hacl1* plays a significant role in the biosynthesis of C17:0 in the liver tissue and on the circulating levels. We can also reinforce previous results showing that C15:0 is not endogenously biosynthesised by HACL1 in alpha oxidation, however, we expect that C15:0 can be biosynthesised through a single cycle of peroxisomal beta oxidation of C17:0. The C15:0 produced from C17:0 peroxisomal beta oxidation does not significantly contribute to the circulating levels under normal dietary conditions.

8 GLUCOSE INTOLERANCE

An investigation into the relationship between the baseline (pre-intervention) circulating odd chain fatty acid levels (Mol %, from the total lipid fraction including free and esterified fatty acids) with the change (assessed through measuring the area under the curve of the insulin and glucose response) in oral glucose tolerance test results across a high fat diet designed to induce glucose intolerance.

8.1 Introduction

It has been recently published that circulating levels of C15:0 and C17:0 fatty acids correlates with a reduced risk of type 2 diabetes (Pfeuffer and Jaudszus, 2016). The most prominent piece of work was produced by Forouhi and colleagues (Forouhi et al., 2014); where they investigated the incidences of type 2 diabetes with their baseline plasma phospholipid fatty acid levels. Their report concludes that saturated even chain fatty acids from the phospholipid fraction correlate with an increased incidence of type 2 diabetes. However, C15:0 and in particular C17:0 fatty acid showed an inverse relationship; with C17:0 showing a greater correlation with reduced incidence over C15:0. There is published work that has shown that there are contradictory results within human observational/epidemiological studies for the correlation between odd chain fatty acids and the incidence of glucose intolerance, within each class of lipids individually, as well as in the total fatty acids composition (both free and esterified fatty acids). For C15:0, as a free fatty acid (un-esterified) has been reported separately as showing both no correlation (Tan et al., 2010) and a positive correlation (circulating levels increased in T2D patients) (Yi et al., 2007) with glucose intolerance. Esterified C15:0 fatty acids have been reported as not correlating with glucose intolerance in several publications (Yi et al., 2007) (Meikle et al., 2013) (Patel et al., 2010). Published manuscripts have demonstrated that esterified C17:0 shows a negative correlation (decreases in T2D patients) (Meikle et al., 2013) (Patel et al., 2010). Additionally, a report by

Yakoob and colleagues shows that both C15:0 and C17:0 fatty acids from the total lipid fraction; both free and esterified fatty acids, decrease significantly in T2D patients (Yakoob et al., 2016). From the literature, it is clear that C15:0 has multi-directional correlations with glucose intolerance, however, C17:0 fatty acid is consistently reported to have a negative correlation with glucose tolerance where participants with lower levels of circulating C17:0 have a greater incidence of developing type 2 diabetes.

Considering both of these fatty acids are present in every lipid class at relatively consistent compositions (Mol %; variation is typically less than half an order of magnitude between each low level fatty acid (< 1 Mol %) within different lipid classes) (Jenkins et al., 2015) then it would be reasonable to assume that the total fatty acid composition would be the best approach for fatty acid comparison with glucose intolerance. Furthermore, it has been shown that labelled saturated fatty acids are primarily incorporated into the glycerolipid fractions (Nestel and Steinberg, 1963), and glycerolipids are the dominant lipid class in plasma/serum (Quehenberger and Dennis, 2011) and are dramatically increased in pathological states such as with increased glucose intolerance (Vergès, 2015), therefore, comparing the fatty acid compositions from the total fatty acid fraction is the first and foremost approach.

It has been shown that odd chain fatty acids increase the fluidity of cellular membranes to a similar degree as polyunsaturated fatty acids (Holman et al., 1989). This increase in fluidity allows for an increased flexibility and movement of integral membrane proteins allowing efficient inter-conversions between inward- and outward-facing conformations facilitating the transport of solutes across the membrane barrier. It has also been suggested that odd chain fatty acids may upregulate glycolysis (*via* propionyl-CoA conversion into succinyl-CoA, increasing substrate drive in the Krebs cycle) essentially increasing glucose utilisation for energy production; aiding in the reduction of extracellular glucose concentrations (Anderson and Bridges, 1984) (Guo et al., 2012). Finally, odd chain fatty acids have been proposed as biomarkers of an overall healthy metabolism (Jenkins et al., 2017); with rapid and efficient multifaceted catabolism of fatty acids and/or whole lipid species. Each of these

three mechanisms would mean that odd chain fatty acids are protective against the development of glucose intolerance and therefore measuring the baseline levels of these fatty acids would be indicative of the risk of pathological development.

With no rigidly controlled studies correlating the circulating odd chain fatty acid compositions with the development of glucose intolerance, from a known starting point under identical dietary intakes and conditions, then these associations are unsubstantiated, thus requiring a strictly controlled animal intervention study to eliminate influential and complicating factors.

8.1.1 Study hypothesis

Published literature from observational and epidemiology studies has shown that the circulating C15:0 and C17:0 fatty acids are protective or predictive of the progression of Type 2 Diabetes. It has been suggested that they may do this by either: increasing the fluidity of cell membranes; aiding in efficient glucose transport across cellular membranes or by upregulating glycolysis essentially increasing glucose utilisation; aiding in the reduction of extracellular glucose concentrations (Anderson and Bridges, 1984) (Guo et al., 2012), or they are biological markers of overall healthy and efficient lipid metabolism. If odd chain fatty acids function in any one of these ways *in vivo*, then we hypothesis that the odd chain fatty acid compositions (C15:0 and C17:0) at baseline will have a strong correlation ($R^2 > 0.75$) with progression of insulin resistance across a glucose intolerance inducing diet in a suitable animal model. Furthermore, since C17:0 has the highest tissue concentration (~1:2, C15:0 to C17:0) and is significantly endogenously biosynthesised (*see Chapter 6 & 7*) then we infer that C17:0 will have the greatest correlation with the development of glucose intolerance.

8.2 Study design

To test this hypothesis, we measured the baseline circulating C15:0 and C17:0 from the total fatty acid composition (via GC-MS) and the glucose tolerance (via an oral glucose tolerance test) in dogs, then again after a high fat glucose intolerance inducing diet. The difference between the baseline glucose

tolerance test results and the post-intervention test results were compared to determine any relationship.

8.2.1 Glucose intolerance

Healthy adult mongrel dogs (~9 months of age, starting weight was 20.5 ± 2.9 kg, n = 5) were acclimated prior to commencing the study intervention on a low fat chow diet with canine suitable laboratory meat (Kal Kan, Franklin, TN, and PMI Nutrition LabDiet, Richmond, IN). The study intervention involved a hypercaloric meat based 52% high fat diet (TestDiet, Indiana, United States of America) administered for four to eight weeks (Coate et al., 2014). The dietary approximate macronutrient values for the acclimation baseline diet were (as % of total energy): protein; 31%, total fat; 26%, saturated fat; 11%, total carbohydrates; 43%, starch; 41%. The approximate dietary macronutrient values for the dietary intervention were (as % of total energy): protein; 22%, total fat; 52%, saturated fat; 23%, total carbohydrates; 26%, starch; 21% (Coate et al., 2014).

Before and after the high fat diet intervention a 180-minute oral glucose tolerance test (OGTT) (0.9 g / kg body weight of glucose polymer administered) was carried out following a 24 hour fast; each dog was fed a can of meat to ensure equivalent energy and macronutrient consumption among groups before beginning the fast. The baseline (collected 10 minutes before glucose bolus administration) plasma samples were used for fatty acid analysis (via gas chromatography with mass spectrometry detection).

The protocol was approved by the Vanderbilt University Institutional Animal Care and Use Committee, and the animals were housed and cared for according to Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

8.3 Statistical analyses summary

The goodness of fit of a trendline expressed as R squared (R^2) that is greater than 0.75 was considered a strong fit, an R^2 between 0.5 and 0.75 was considered moderate. The area under the curve for the glucose tolerance was calculated via the trapezoidal rule, total area under the curve is the sum of all

individually calculated trapezoid areas; individual trapezoid areas are calculated via (((y value two + y value one) / 2) * (x value two - x value one)).

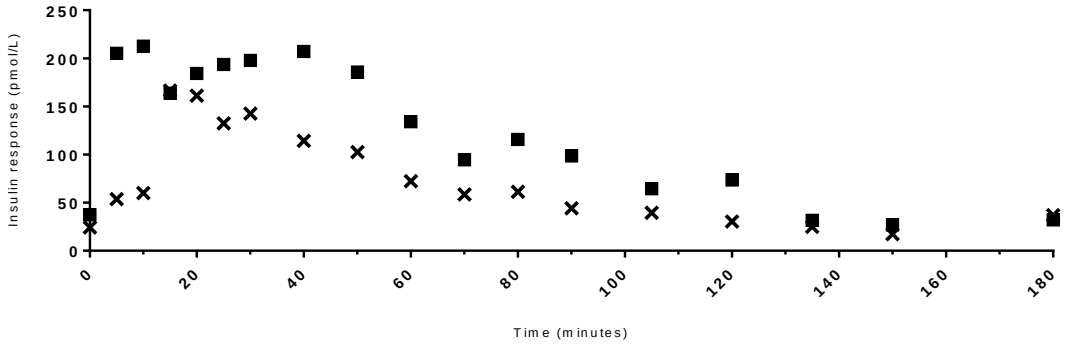
8.4 Results

The relationship between C15:0 and C17:0 with glucose intolerance has been reported through a number of epidemiology studies. Any variation in circulating C15:0 and C17:0 has been attributed to variations in ruminant fat intake with no consideration of endogenous biosynthesis. Therefore, we investigated how these odd chain fatty acids correlate with the development of glucose intolerance during a controlled high fat diet in a canine model; the canine model was selected due to the sample volume requirements of the study and it has been previously shown that dogs are a suitably comparable model to human glucose intolerance pathology (Ionut et al., 2010).

8.4.1 Group average insulin response

The group insulin response was determined by finding the group (n = 5) average for the insulin concentration measured at each time point along the oral glucose tolerance test. This was done for the oral glucose tolerance test results collected at baseline and at the end of the high fat glucose intolerance inducing diet, to produce the group average insulin response (*see Figure 16*).

Figure 16: Healthy adult dogs (n=5) were fed a high fat diet (4-8 weeks) where they had their glucose tolerance assessed by an oral glucose tolerance test at the start (x) and end (■) of the dietary intervention. The points represent the average insulin concentration (pmol/L) for each time point (minutes).

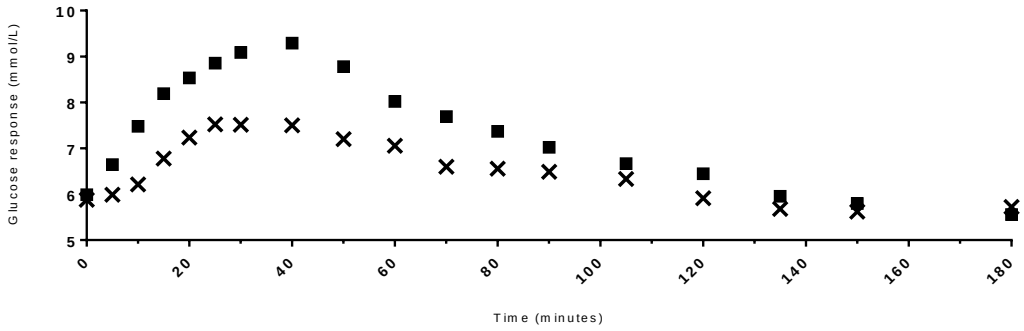


As shown in the figure above (*see Figure 16*) the group average insulin response was significantly increased by ~69% in the post-intervention results (baseline insulin area under the curve = 10997.9, post-intervention insulin area under the curve = 18603.5; $p = 0.045$). The fasting insulin concentration (time = 0 minutes, pmol/L) is not significantly ($p > 0.05$) different between the pre- and post-intervention results, however, each subsequent insulin measurement is higher in the post-intervention results confirming that the high fat glucose intolerance inducing diet did in fact induce glucose intolerance in the animals.

8.4.2 Group average glucose response

The group glucose response was determined by finding the group (n=5) average for the glucose concentration measured at each time point along the oral glucose tolerance test. This was done for the oral glucose tolerance test results collected at baseline and at the end of the high fat glucose intolerance inducing diet, to produce the group average glucose response (*see Figure 17*).

Figure 17: Healthy adult dogs (n=5) were fed a high fat diet (4-8 weeks) where they had their glucose tolerance assessed by an oral glucose tolerance test at the start (x) and end (■) of the intervention. The points represent the average glucose concentration (mmol/L) for each time point (minutes).



As shown in the figure above (*see Figure 17*) the post-intervention group glucose response had increased by ~11% from the baseline response (baseline glucose area under the curve = 1151.1, post-intervention glucose area under the curve = 1280.7; p = 0.005). The fasting glucose concentration (time = 0 minutes, mmol / L) is not statistically significantly different between the baseline and post-intervention results (p = 0.632). The statistically significant increase in the glucose area under the curve signifies that the high fat glucose intolerance inducing diet did indeed induce glucose intolerance in the animals.

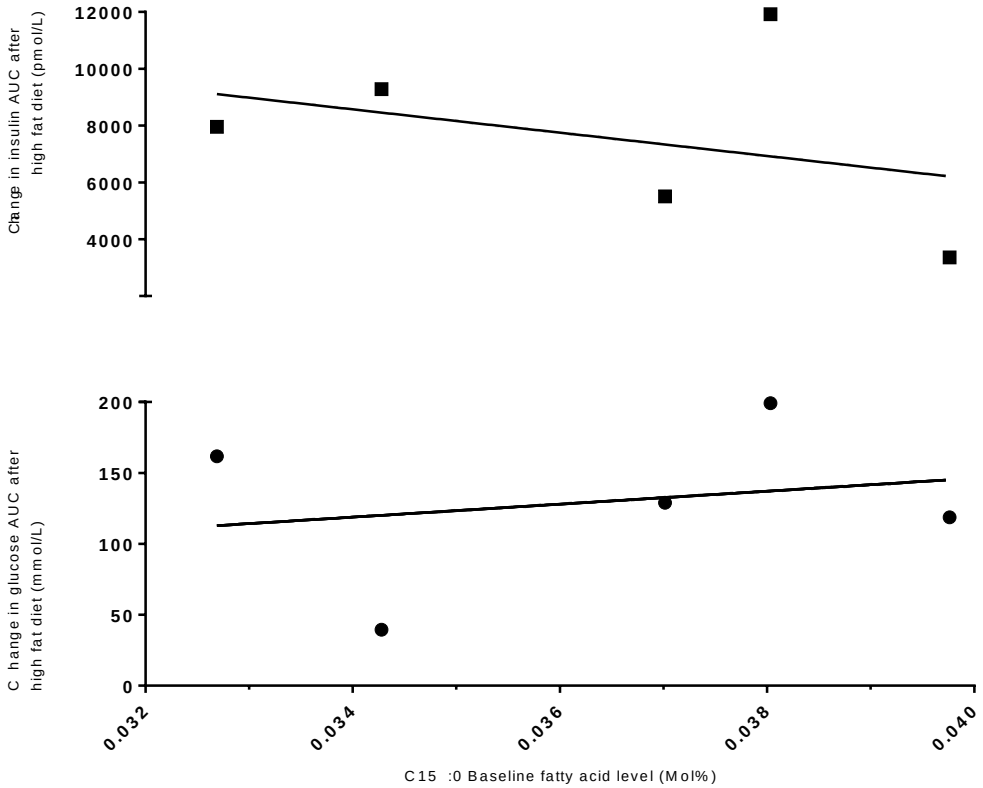
8.4.3 Baseline C15:0 comparison with insulin and glucose response

Each individual had their insulin and glucose measured across an oral glucose tolerance test; the area under the curve for both the insulin and glucose was determined using the trapezoid rule. The process of measuring the insulin and glucose response and calculating the area under the curve for the change in the blood insulin & glucose compositions was repeated for both the baseline and post-intervention blood collections immediately before each of the oral glucose tolerance tests. The development of glucose intolerance was determined by calculating the difference between the baseline area under the curves (both

insulin and glucose) and the post-intervention area under the curves (both insulin and glucose). Each animal in the study developed an increase in area under the curve for both insulin and glucose, which confirms that the animals developed increasing glucose intolerance across the dietary intervention time course (*see Figure 16 & 17*). The baseline results showed that the animals at baseline were healthy and showed no sign of glucose intolerance, and therefore, are a realistic & comparative model for assessing biomarkers of predicting glucose intolerance.

The difference between the individual baseline and the individual post-intervention area under the curves for insulin and glucose was then compared to the associated individual baseline (pre-intervention) C15:0 fatty acid compositions (Mol %) so that any trends can be determined (*see Figure 18*).

Figure 18: Oral glucose tolerance test (OGTT) performed at the beginning and at the end of a four to eight-week high fat diet intervention in dogs. Glucose and insulin were measured from 0 to 180 minutes and the area under the curve (AUC) was calculated using the trapezoidal rule. The graph data points represent the difference between the insulin AUC and the glucose AUC, from the beginning to the end of the four to eight-week high fat diet in relation to the C15:0 fatty acid levels at baseline (Mol %). Top: INSULIN (Ins.) - C15:0 R^2 = 0·053, Y = 267588*X – 2123, significance of the slope; p = 0.7094. Bottom: GLUCOSE (Glc.) - C15:0 R^2 = 0·048, Y = 4572*X - 36.62, significance of the slope; p = 0.7225.



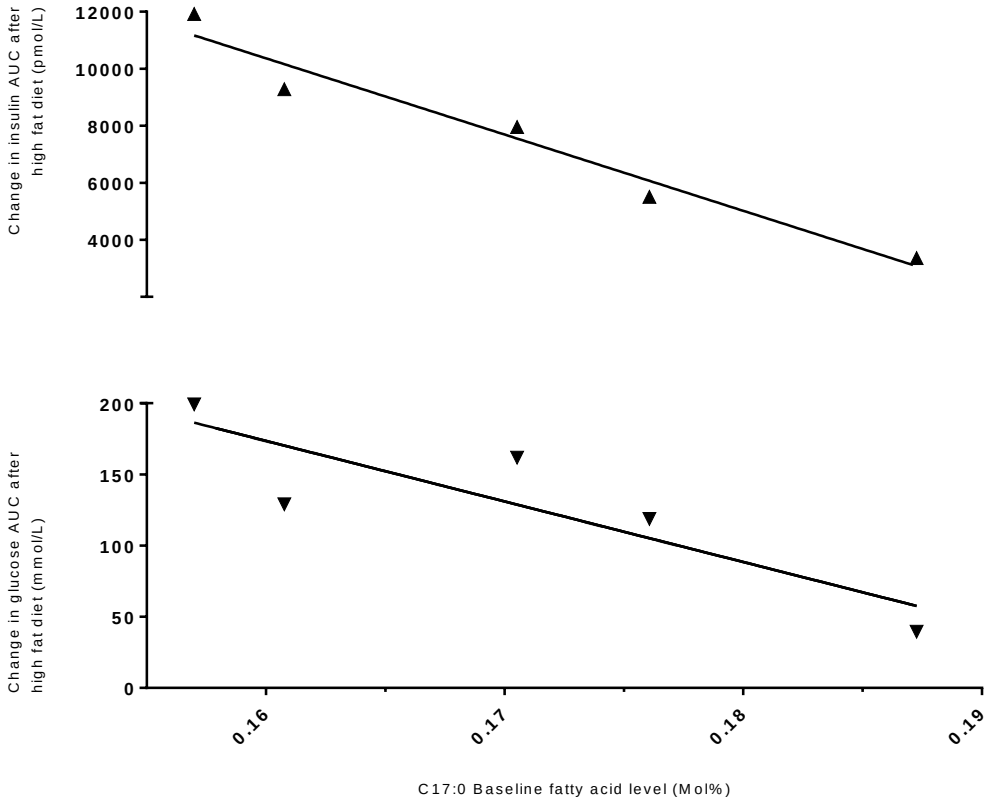
As shown in the figure above (*see Figure 18*) there were no significant correlations (R^2 = 0.053, R^2 = 0.048 for insulin and glucose, respectively) between baseline C15:0 (Mol %) with the development of glucose intolerance

(insulin & glucose area under the curves), across the high fat dietary intervention.

8.4.4 Comparison between the baseline C17:0 level and OGTT results

Similar to the baseline C15:0 (Mol %) comparison with the change in insulin and glucose response across a high fat glucose intolerance inducing diet, the baseline (pre-intervention) C17:0 compositions (Mol %) for each individual was compared to that individual's change in their insulin and glucose response from the oral glucose tolerance tests measured at the start and end of the dietary intervention. This was to see if the baseline C17:0 compositions (Mol %) were predictive of the development in glucose intolerance (*see Figure 19*). Since each animal was kept on identical diets within the same environment then the differences in C15:0 and C17:0 compositions were due to the individual biological mechanisms (differences in absorption or endogenous production) and were independent of any dietary contributions, therefore, the predictive or protective nature of these two odd chain fatty acids could be determined.

Figure 19: Oral glucose tolerance test (OGTT) performed at the beginning and at the end of a four to eight-week high fat diet intervention in dogs. Glucose and insulin were measured from 0 to 180 minutes and the area under the curve (AUC) was calculated using the trapezoidal rule. The graph data points represent the difference between the insulin AUC and the glucose AUC, from the beginning to the end of the four to eight-week high fat diet in relation to the C17:0 fatty acid levels at baseline (Mol %). Top: INSULIN (Ins.) - C17:0 R^2 = 0·957, Y = -267356*X + 53142, significance of the slope; p = 0.0039. Bottom: GLUCOSE (Glc.) - C17:0 R^2 = 0·755. (n = 5), Y = -4253*X + 854.0, significance of the slope; p = 0.0558.



As shown in the figure above (*see Figure 19*) there were significant correlations (R^2 = 0.957, R^2 = 0.755 for insulin and glucose, respectively) between baseline C17:0 (Mol %) with the development of glucose intolerance (insulin & glucose area under the curves) across the high fat dietary intervention.

8.5 Discussion & conclusions

The aim of this study was to investigate the validity of published correlations (pathology biomarker) between C15:0 and C17:0 with the incidence of Type 2 Diabetes (i.e. glucose intolerance) under strictly controlled study conditions where there were no variations in the dietary composition of these fatty acids. The glucose intolerance biomarker validity was assessed through the use of a canine animal model; where the baseline (pre-intervention) circulating odd chain fatty acid composition (C15:0 and C17:0, Mol %) was measured (*via* GC-MS) and compared to the change in the insulin and glucose response (change = final concentration minus initial concentration) to an oral glucose tolerance test conducted at baseline (pre-intervention) and again at the end (post-intervention) of a four to eight week high fat glucose intolerance inducing diet. The correlation between the individual baseline (pre-intervention) circulating fatty acid composition (Mol %) and the change in their insulin and glucose response across the high fat glucose intolerance inducing diet was assessed through coefficient of determination ($R^2 > 0.75$ considered a strong correlation) and regression slope statistics ($p < 0.05$).

The use of a canine model was necessary due to sample volume requirements; the volume of plasma that needed to be collected throughout the intervention included the baseline (pre-intervention) plasma fatty acid composition required ~250 µL of blood to be extracted, then the required volume of blood collected throughout the oral glucose tolerance test excluded smaller species (rodents & lagomorphs) for use in this investigation. Since we aimed to make use of existing sample sets from research institutional collaborations to minimise animal experimentation and were unable to develop collaborations with feline models of glucose intolerance, we then utilised the canine model as a suitable alternative (no ambiguity was introduced to the overall results by using this canine model).

It has been shown that a high fat dietary intervention in a canine animal model was a suitable and translatable representation of prediabetes and mild type 2 diabetes (T2D) in humans (Ionut et al., 2010). All the animals in this study were initially healthy and showed no sign of enhanced glucose intolerance;

however, after the high fat dietary intervention each animal saw an increase in their glucose intolerance (*this data is shown in Figure 8.4.1 & 8.4.2*) both the maximum insulin concentration (pmol / L) and the insulin clearance curve (area under the curve) had increased significantly from the baseline values. This shows that the cellular uptake of glucose was less efficient after the dietary intervention further confirming that the animal models had developed glucose intolerance

When looking at the correlations between the baseline fatty acid compositions and the associated individual change in their insulin and glucose responses there was no significant correlation with their baseline C15:0 levels (*see Figure 18*) but there was a significant correlation seen with the C17:0 levels (*see Figure 19*).

These results further highlight the mechanisms that make odd chain fatty acids beneficial in glucose intolerance pathology since there is only a strong correlation seen with C17:0. If odd chain fatty acids are protective due to their involvement with cellular membranes enhancing the fluidity and facilitating the movement of integral membrane proteins allowing efficient transport of solutes across the membrane barrier then most likely both C15:0 and C17:0 would show a correlation. Furthermore, if this was true then C15:0 would have the greatest protective effect when compared to C17:0 since it has a lower chain length and melting temperature, therefore would produce higher membrane fluidity. Since there is no correlation with the baseline C15:0 then it is reasonable to conclude that the incorporation of odd chain fatty acids into the cellular membrane are not significant in the development of glucose intolerance. If odd chain fatty acids upregulate glucose metabolism aiding in the reduction of extracellular glucose concentrations by increasing succinyl-CoA (converted from propionyl-CoA) in the Krebs cycle then both C15:0 and C17:0 would show a correlation with the development of glucose intolerance. Additionally, if this was the case then C15:0 would have a greater correlation than C17:0 since it can be metabolised via beta oxidation more quickly which would produce greater amounts of propionyl-CoA for conversion into succinyl-CoA. Since there is no correlation between the baseline C15:0 and the development of glucose intolerance then it

is reasonable to conclude that the catabolism of odd chain fatty acids into propionyl-CoA is not significant in the development of glucose intolerance. However, odd chain fatty acids have been suggested as biomarkers of a healthy metabolic state; the rapid & efficient multifaceted catabolism of fatty acids and/or whole lipid species (Foulon et al., 2005) (Galadari et al., 2013) (saturated fatty acids, beta branched chain fatty acids and ceramide-sphingomyelin production). Since it is understood that each odd chain fatty acid is a product of different metabolic mechanisms; C17:0 the product of stearic acid alpha oxidation and C15:0 is the peroxisomal beta oxidation product of C17:0 and dietary biomarker, then each of these fatty acids would allow different correlations with the development of glucose intolerance regardless of their involvement with the cellular membrane and end product metabolism. Since C17:0 is the initial product of alpha oxidation then it would be a better marker of healthy lipid metabolism (being the first stage metabolic product) than C15:0 (because evidentially not all biosynthesised C17:0 will go through a single cycle of peroxisomal beta oxidation). Since the baseline C17:0 levels had a strong correlation with the development of glucose intolerance then it is reasonable to conclude that a healthy lipid metabolism has the greatest influence on the development of glucose intolerance.

Although glucose intolerance / insulin resistance is defined as abnormal glucose metabolism; where pathological symptoms erupt from disruption in these glucose pathways, efforts to understand the pathogenesis are increasingly focused on disorders in lipid metabolism. Whilst the data and interpretations presented suggest that it is mainly C17:0 that is related to the development of glucose intolerance, this is not to say that there are no other mechanism involved, where C15:0 is significantly associated. This glucose intolerance study only investigated the endogenous levels of these odd chain fatty acids at one level, however, external contributions of C15:0 and/or C17:0 may provide further beneficial (or possibly detrimental) associations. The pharmacological associations between C15:0, C17:0 and the development of glucose intolerance needs to be further investigated

The limitations of this study – the use of the canine model may confound the results because dogs quickly develop glucose intolerance in response to a high fat dietary intervention, unlike humans. Therefore, the relationship between the change in the glucose intolerance across the dietary intervention with the baseline C17:0 may be more apparent. The results from this study do not show any correlation between C15:0 and the change in glucose intolerance, however, this does not exclude the possibility that the composition of C15:0 may still be significantly involved in the development of glucose intolerance in human.

8.6 Summary

To summarise, there was no significant correlation between the baseline C15:0 compositions (Mol %) and the development of glucose intolerance. Within large epidemiological human studies there may be a suggestion that since C15:0 is a biomarker of dietary intake then it maybe an identifier for a healthy diet composed of high C15:0 foods, these then may be alleviating or protecting against the development of glucose intolerance; this needs to be further investigated.

With regards to C17:0 there was a strong negative correlation between the baseline compositions and the development of glucose intolerance, independent of dietary variations. We cannot conclude whether C17:0 is protective against glucose intolerance or is a pathology biomarker of initial healthy lipid metabolism which may make the animal / human resistant to developing glucose intolerance; this needs to be further investigated.

9 DISCUSSION & CONCLUSION

The original aim of this PhD was to investigate any contributory factors to the circulating odd chain fatty acid levels *in vivo* that were suggested in the introductory chapter; from the diet, from the intestinal microbiota and from endogenous biosynthesis. Then to investigate the relationship between circulating odd chain fatty acids and the prognosis of glucose intolerance, taking into account, these contributory factors.

Our original hypothesis' included: germ free mice will have lower circulating odd chain fatty acid compositions compared to conventional mice on the same diet, C15:0 and C17:0 are not true dietary biomarkers since there are other factors that influence the circulating composition, and we hypothesised that odd chain fatty acids can be endogenously biosynthesised through alpha oxidation of the subsequent neighbouring even chain fatty acids. These hypothesise are discussed in the following conclusions.

9.1 Gut microbiota

The aim of the first study was to investigate the relationship between the gut microbiota and the circulating odd chain fatty acid compositions in mice. The low fat diet was selected because it was completely deficient of ruminant fat sources and would provide information into the absolute baseline measurement of C15:0 and C17:0 independent of dietary and gut microbiota sources. The high fat diet also provided information on any specific interactions between the gut microbiota and dietary dairy fat which allowed us to put our result into contrast with previously published articles, specifically on interactions between the gut microbiota and dietary fat (Huang et al., 2013). Germ free mice were used as this gave the greatest contrast from a normal gut microbiota and therefore any changes in the microbiota profile of the

conventional mice (due to the diet) would still manifest in a statistically significantly different mouse model from the germ-free mice.

We found that there were no statistically significant differences caused by the presence or absence of gut microbiota on the host circulating C15:0 and C17:0. The results in the low fat diet fed animals showed that the circulating levels of C17:0 are relatively greater than the dietary source, rather suggesting that there must be some other source. We can conclude the gut microbiota did not play a significant role in the endogenous levels of these two fatty acids. However, this does not exclude the possibility that the human gut microbiota may contain microbiota species that produce either C15:0 or C17:0 (Weitkunat et al., 2017) but as the *in vivo* levels of C15:0 and C17:0 between rodents and humans are comparable it makes it unlikely that human gut microbiota has a significant impact.

9.2 Dietary biomarkers & endogenous biosynthesis

To investigate any correlations between the dietary content of odd chain fatty acids and the circulating compositions of C15:0 and C17:0, we conducted a study comparing three different fat source diets, one of which was a milk fat based diet. This was then followed by a ruminant fat dose response study. In both of these dietary interventions, the circulating C15:0 compositions increased linearly with intake, however, there was not a clear correlation between dietary intake and circulating composition for C17:0. According to the literature C15:0 is a reproducible and reliable biomarker of ruminant fat intake, significantly more often than C17:0, which is confirmed in these animal studies.

Other dietary factors like dietary total fat content and ethanol intake significantly decreased circulating levels of C15:0 but showed non-significant or contradicting results for C17:0, which suggests that there are separate biological mechanisms related to each of these fatty acids, completely independent of each other.

It has been suggested previously that C17:0 may be endogenously biosynthesised. This is further reinforced in the presented studies where the circulating composition of C17:0 was far higher than the dietary composition,

however, this pattern was not seen for C15:0, which suggests C15:0 was predominantly obtained from the diet. Several *in vitro* investigations have shown that C17:0 can be produced endogenously both through elongation of propionyl-CoA (Weitkunat et al., 2017) and alternatively, through alpha oxidation of stearic acid (C18:0). However, the results for the C17:0 production through propionyl-CoA do not directly explain tissue C17:0 levels in whole organisms. The comparison between the expected and the actual fatty acid proportions in plasma do not match (i.e. C17:0 is not the dominant fatty acid in the *in vitro* propionyl-CoA studies but it is the highest level odd chain fatty acid *in vivo*). This suggests that propionyl-CoA plays a minor role in the production of odd chain fatty acids *in vivo*. As stated in the literature, C17:0 could be endogenously produced through alpha oxidation of C18:0.

To clarify the results, these studies will be jointly discussed, giving clear evidence of the endogenous production of C17:0.

By combining the results of the phytol supplementation study (where dietary phytol decreased circulating C17:0 levels but unaffected C15:0) and the dose response study results we are able to explain why the circulating C17:0 increased but was not the result of dietary C17:0 but the result of upregulated alpha oxidation on C18:0. Between the five diets in the dose response study the phytanic acid increased linearly, while, the phytol composition drastically dropped between diet one and two (50% reduction), and then followed a linear decrease from diet two to five. The combination of phytanic acid and the phytol content had a direct inverse correlation with the changes in the circulating C17:0 between each diet group (as dietary phytanic acid and phytol increases the plasma C17:0 decreases proportionately). Since phytol and phytanic acid have been shown to inhibit alpha oxidation on stearic acid for the biosynthesis of C17:0, then this explains why the smallest difference between C17:0 in each diet is between groups 1 and 2, where there is actually the largest difference in C17:0 levels in plasma.

Phytanic acid is in very low concentrations (with near zero phytol) in the commonly consumed ruminant fat sources. This, along with the high content of C18:0 demonstrates that a high ruminant fat diet promotes the endogenous

production of C17:0. This suggests that C17:0 is not a direct ruminant fat biomarker but actually a product of endogenous production influenced by dietary substrate intake.

In the human supplementation study, the plasma C15:0 concentration increased by ~10% as a result of a dietary 30% increase, this plasma increase directly concurs with the expected increase according to the ruminant fat dose response study. However, the plasma C17:0 levels did not change across the supplementation study. This can be explained by the target substrate of alpha oxidation: phytanic acid and its precursor phytol. The supplementation included an increase of phytol by ~25% and an increase of phytanic acid by ~100%, which both reduces the endogenous production of C17:0. This reduction in the endogenous production along with the increased dietary consumption of C17:0 accounts for the insignificant change seen in the plasma across the intervention.

Since it is well understood that C17:0 can be endogenously produced through alpha oxidation of C18:0 within *in vitro* studies, so we conducted a controlled C18:0 infusion study in rats to assess the effect of increasing C18:0 *in vivo* on the circulating C17:0 levels. The absolute serum quantities of C17:0 increased significantly by ~46% in rats receiving the C18:0 infusion compared to a control group. Further increasing the infusion of C18:0 above 250 nmol/kg/day *in vivo* did not significantly increase the serum C17:0 levels. We then showed that 2-hydroxyacyl-CoA lyase 1 plays a significant part in the endogenous production of C17:0. Using the *Hacl1* knockout mouse model, there was a ~20% reduction in plasma C17:0 compared to wildtype controls; it has been shown that there are metabolic salvage pathways that compensate for a deficiency in HACL1 which explains why the circulating C17:0 doesn't completely disappear.

9.3 Glucose intolerance

The significance of the different associations of C15:0 and C17:0 with glucose intolerance has been previously reported, however, no consideration for the factors affecting endogenous production of C17:0 has been taken into account. In the glucose intolerance study, we utilised a canine model of glucose

intolerance where we compared the baseline fatty acid levels with the change in glucose and insulin measured during an oral glucose tolerance test after a high fat diet. There was no significant correlation between C15:0 at baseline with the change in either glucose or insulin across the high fat diet, but there was a strong correlation seen with C17:0 at baseline. These results highlight the importance in understanding the origin of these fatty acids and that the endogenous production (not the dietary source) of C17:0 has a strong relationship with the development of glucose intolerance, either as a marker of healthy lipid metabolism or indeed the fatty acid may have protective properties.

9.4 Final conclusion

To summarise, it is clear that individual odd chain fatty acids are the result of very different processes and that their relations with disease risk cannot be generalised. From our results, we can conclude that C15:0 appears to be a linear biomarker of dietary C15:0 intake. This does not mean it can be directly translated into a marker that can quantify ruminant fat intake in all cases due to varying fatty acid compositions of different ruminant fat sources (e.g. milk, butter, beef tallow). However, it can still be used to contrast between different levels (high or low) of dietary intake and may provide further proof of the possible protective role of ruminant fat in the diet against metabolic diseases.

C17:0 has been misidentified as a ruminant fat biomarker due to its relationship with dietary phytol, phytanic acid and stearic acid which all influence the C17:0 endogenous production. Moreover, the disparity between C15:0 and C17:0 agrees with data collected in epidemiological studies showing that C17:0 has a stronger inverse association with diseases but a weak association with diet. This highlights the importance of the need for further investigation into the endogenous production of C17:0.

10 FURTHER WORK

The aim of this research was to comprehensively investigate two odd chain fatty acids (C15:0 and C17:0) and their role in epidemiology as dietary biomarkers. From this research, it has been shown that there are significant differences between these two fatty acids, where C15:0 is primarily obtained exogenously from the diet, whereas, C17:0 is predominantly endogenously biosynthesised via alpha oxidation of stearic acid. This work was presented using data from many different individual studies, including: human & animal dietary intervention studies, dose response studies, fatty acid infusions, and gene knockout investigations, whilst analysing any perturbations in the odd chain fatty acid compositions from the total lipid fraction of serum or plasma; the consistency of the results has further reinforced these conclusions.

From this work, there has arisen future areas of research that would expand the understanding of odd chain fatty acids. An important follow up would be the comprehensive and simultaneous analysis of the composition of odd chain fatty acids within every lipid class/species in the prior mentioned studies, stable isotope labelled fatty acid studies, gene expression analysis within each of the prior mentioned studies, and direct administration of C15:0 and C17:0. Each of these points are further discussed in the following sections.

10.1 Lipid class/species compositional analysis

The work in this investigation looked at the composition of C15:0 and C17:0 within the total lipid fraction, without any acknowledgement of which specific lipid classes these fatty acids were incorporated. This allowed for a thorough and comprehensive analysis of these odd chain fatty acids as biomarkers, their endogenous production, as well as correlations with glucose intolerance. As shown in other studies, the incorporation of C15:0 and C17:0 into the different lipid classes has shown varying & contradictory results within observational/epidemiological studies, since each study had limited control of its participants. The analysis of the C15:0 and C17:0 within each lipid class of

the studies mentioned in this thesis would give dramatic insight into where and how these lipids cause/protect/predict glucose intolerance. By authenticating which lipid class these odd chain fatty acids eventually primarily incorporate would give further evidence for their compartmentalisation and whether it is this compartmentalisation that varies between healthy and pathological subjects.

10.2 Stable isotope labelled fatty acid studies

A specific stable isotope labelled stearic acid infusion (infusion so that a specific/known circulating concentration can be obtained) needs to be carried out. This stable isotope infusion will definitively demonstrate endogenous biosynthesis of C17:0 from stearic acid (if this actually occurs), additionally it will allow the location of this biosynthesis/metabolism to be obtained.

Another study that would be useful would be the administration of stable isotope labelled C17:0 to animal and human participants, this will allow the subsequent metabolism of C17:0 to be followed, possibly giving an idea of how this fatty acid relates to glucose intolerance.

10.3 Gene expression analysis

Regarding odd chain fatty acids little work has been carried out to link biosynthesis/metabolism or odd chain fatty acid specific gene expression to their role in glucose intolerance. We have shown in this thesis that perturbations in the *Hacl1* gene significantly alter C17:0 fatty acid levels in circulation, and these circulating levels then correlating with the development of glucose intolerance. It has been published that there have been no observed/recorded HACL1 deficiency in humans (Casteels et al., 2007), however, *Hacl1* has been shown to significantly vary in different pathologies. Specific correlations between the development of glucose intolerance and the native *Hacl1* gene expression need to be assessed to see if it the initial expression of this gene that correlates with glucose intolerance or does the development of pathology cause a variation in the *Hacl1* gene expression.

10.4 Administration of C15:0 and C17:0

By doing direct administration (adding to the diet at known concentrations) of C15:0 and C17:0 in both animal & human studies we will be able to measure absorbance efficiencies and direct dose responses. A definitive dose response administering of pure C15:0 fatty acid solution would negate other influential factors like alternative fatty acid biosynthesis / metabolism contributing to circulating C15:0 levels, confirming or dismissing its biomarker status. Through a diet administration of C17:0 we should be able to see what dietary levels significantly increase circulating levels. Through both the administrations of C15:0 and C17:0 we will be able to investigate the factors that contribute to their predictive or protective nature in glucose intolerance, and whether they can have a therapeutic action on pathological progression.

10.5 Further work summary

Even though the work presented provides strong evidence for the endogenous biosynthesis of odd chain fatty acids (primarily C17:0 via alpha oxidation with C15:0 secondarily produces possibly via a single cycle of peroxisomal beta oxidation) there has been substantial areas of odd chain fatty acid physiology that has been shown lacking in knowledge and controlled research. There is little to no published literature on how or why odd chain fatty acids relate to glucose intolerance, hence, why the results from this research were necessary to help get the research proverbial ‘ball’ rolling.

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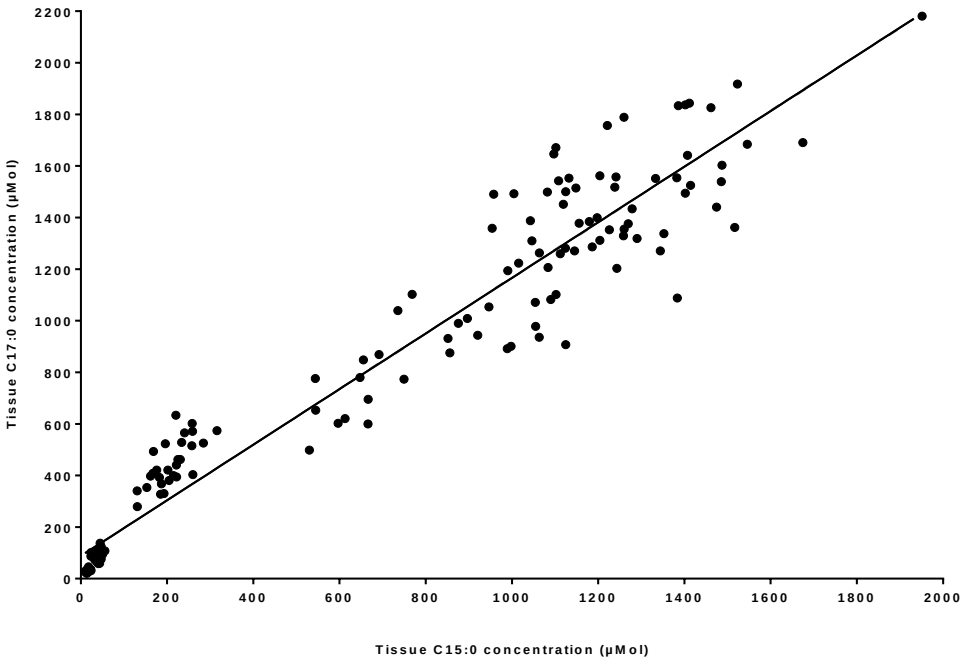
12 APPENDICES

12.1 Appendix 1.

The inter- and intra- tissue pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) comparison; where an increase in one odd chain fatty acid correlates with an increase in the other odd chain fatty acid across the four tissue types analysed (plasma, liver, inguinal white adipose tissue, gonadal white adipose tissue and retroperitoneal white adipose tissue, and interscapular brown adipose tissue.

Appendix figure A: The comparison between the tissue C15:0 composition and the tissue C17:0 composition in mice to show the relationship between these two fatty acids further reinforcing the endogenous biosynthesis of C15:0 from C17:0 via a single cycle of peroxisomal beta oxidation. (R^2 = 0.9399, p < 0.0001, Y = 1.078*X + 88.12).



The tissues that manifest a decrease (significant and insignificant) in C17:0 also manifest a decrease in C15:0 but far less attenuated; when comparing the C15:0 concentrations with the C17:0 concentrations for each individual across all the tissues there is a strong correlation ($R^2 = 0.9399$, $p < 0.0001$, $Y = 1.078*X + 88.12$, *see Appendix figure A*). This minor contribution of C15:0 via beta oxidation of C17:0 may explain the presence of C15:0 in studies that have an undetectable C15:0 dietary content.

12.2 Appendix 2.

Retrospective power calculations were computed to assess the suitability of the sample size used; where statistical significance was seen in both C15:0 and C17:0 then no retrospective power calculations were required. Additionally, if correlations were presented then those studies would also not require power calculations.

Two power calculation approaches were used; if there was statistical significance seen in either fatty acid, then the variation detected was hypothetically applied to the other fatty acid to assess how many biological replicates would be required to see that degree of change (if the two fatty acids are homologous). The second approach was to search the literature and determine what would be the expected change, and hypothetically apply it to each fatty acid, then determine how many biological replicates would be required to see that change reported in the literature.

12.2.1 Power calculations for the intestinal microbiota – low fat (chow) diet study.

To perform a retrospective power calculation to determine if the sample size (n=) within this study was suitable to address the research question (is the gut microbiota the major source of C15:0 and C17:0 in circulation?) an assumption was made; this assumption was that the negation of the gut microbiota (i.e. in the germ free mice) would cause a greater than 50% decrease in the circulating C15:0 and C17:0 composition in the host. The required sample size to see a statistical significant difference of a 50% decrease in the germ free mice was calculated using an online power calculator

(available at: https://www.statisticalsolutions.net/pssZtest_calc.php). A 50% change was considered conservative due to the believed assumption that the gut microbiota are the major source of these two odd chain fatty acids *in vivo*.

Power calculation parameter for C15:0 and C17:0:

	C15:0	**C17:0**
Mu(0):	**0.03484**	**0.10628**
Mu(1):	**0.01742**	**0.05314**
Sigma:	**0.00196**	**0.01084**
Sided test:	**Two**	**Two**
Alpha(α):	**0.05**	**0.05**
Power of the test:	**0.95**	**0.95**
Sample size (n=):	**1**	**1**

To see a statistical significant 50% decrease in the C15:0 and C17:0 composition then more than one biological replicates (n>1) would be required according to this power calculation. However, in the low fat (chow) diet study eight biological replicates (n=8) were used but no statistically significant differences were seen.

12.2.2 Power calculations for the intestinal microbiota – high fat (ruminant fat based) diet study.

To perform a retrospective power calculation to determine if the sample size (n=) within this study was suitable to address the research question (is the gut microbiota the major source of C15:0 and C17:0 in circulation?) an assumption was made; this assumption was that the negation of the gut microbiota (i.e. in the germ free mice) would cause a greater than 50% decrease in the circulating C15:0 and C17:0 composition in the host. The required sample size to see a statistical significant difference of a 50% decrease in the germ free mice was calculated using an online power calculator (available at: https://www.statisticalsolutions.net/pssZtest_calc.php). A 50%

change was considered conservative due to the believed assumption that the gut microbiota are the major source of these two odd chain fatty acids *in vivo*.

Power calculation parameter for C15:0 and C17:0:

	C15:0	C17:0
Mu(0):	**0.50167**	**0.74892**
Mu(1):	**0.25084**	**0.37446**
Sigma:	**0.042935**	**0.03471**
Sided test:	**Two**	**Two**
Alpha(α):	**0.05**	**0.05**
Power of the test:	**0.95**	**0.95**
Sample size (n=):	**1**	**1**

To see a statistical significant 50% decrease in the C15:0 and C17:0 composition then more than one biological replicates (n>1) would be required according to this power calculation. However, in the high fat (ruminant fat based) diet study six-to-seven biological replicates (n=6-7) were used but no statistically significant differences were seen.

12.2.3 Power calculations for the dietary biomarker – different dietary fat source comparison study.

To perform a retrospective power calculation to determine if the sample size (n=) within this study was suitable to address the research question (is there a linear relationship between the plasma composition and the dietary content) an assumption was made; this assumption was that there would likely be a gradient slope of at least 0.1 between diet and plasma compositions (i.e. as the diet composition increases by two-fold the plasma will increase by at least 10%). The required sample size to see a statistical significant difference was calculated using an online power calculator (available at: https://www.statisticalsolutions.net/pssZtest_calc.php). A gradient slope of 0.1

was considered conservative due to the believed assumption that C15:0 and C17:0 are dietary biomarkers with no other notable source *in vivo*.

Power calculation parameter for C15:0:

	Safflower-lard	**Safflower-milk**
Mu(0):	**0.00578**	**0.00578**
Mu(1):	**0.00993**	**0.08895**
Sigma:	**0.00110**	**0.00110**
Sided test:	**Two**	**Two**
Alpha(α):	**0.05**	**0.05**
Power of the test:	**0.95**	**0.95**
Sample size (n=):	**1**	**1**

Power calculation parameter for C17:0:

	Safflower-lard	**Safflower-milk**
Mu(0):	**0.00866**	**0.00866**
Mu(1):	**0.04914**	**0.06148**
Sigma:	**0.00312**	**0.00312**
Sided test:	**Two**	**Two**
Alpha(α):	**0.05**	**0.05**
Power of the test:	**0.95**	**0.95**
Sample size (n=):	**1**	**1**

To see a statistical significant biomarker relationship that produces a 0.1 gradient response then more than a single biological replicate (n>1) would be required according to this power calculation. However, in this dietary biomarker – different dietary fat source comparison study three-to-four

biological replicates (n=3-4) were used but only C15:0 showed statistically significant differences.

12.2.4 Power calculations for the dietary biomarker – ruminant fat supplementation study.

To perform a retrospective power calculation to determine if the sample size (n=) within this study was suitable to address the research question (does supplementation increase *in vivo* C17:0 proportionately to C15:0) an assumption was made; this assumption was that there would a proportionate increase seen for C17:0 as compared to C15:0 *in vivo* in response to the dietary intervention (a 30% increase in the diet results in a ~10% increase in the C15:0 fatty acid *in vivo*). The required sample size to see a statistical significant difference was calculated using an online power calculator (available at: https://www.statisticalsolutions.net/pssZtest_calc.php).

Power calculation parameter for C17:0:

	C17:0
Mu(0):	**0.14441**
Mu(1):	**0.15885**
Sigma:	**0.01794**
Sided test:	**Two**
Alpha(α):	**0.05**
Power of the test:	**0.95**
Sample size (n=):	**21**

To see a statistical significant biomarker relationship we would expect that both C15:0 and C17:0 would have a proportionate increase when a dietary supplementation was applied. The *in vivo* C15:0 increased by ~10% with the 30% dietary increase, therefore we would expect that this increase would be seen in the *in vivo* C17:0. To see a statistical significant increase of ~10% in the C17:0 then twenty-one biological replicates (n>21) would be required

according to this power calculation. However, in this dietary biomarker – ruminant fat supplementation study twenty-six biological replicates (n=26) were used but only C15:0 showed statistically significant differences.

12.2.5 Power calculations for the dietary biomarker – dietary fat percentage study.

To perform a retrospective power calculation to determine if the sample size (n=) within this study was suitable to address the research question (does the amount of dietary fat affect the *in vivo* C15:0 and C17:0 compositions) an assumption was made; this assumption was that there would be a proportionate affect seen in C17:0 as there would be for C15:0 (as dietary fat increase the *in vivo* C15:0 decreases). The required sample size to see a statistical significant difference was calculated using an online power calculator (available at: https://www.statisticalsolutions.net/pssZtest_calc.php). This assumption that C17:0 should be similarly affected as C15:0 (an increase of 25% to the total dietary fat content caused a ~25% reduction in the circulating C15:0) was reasonable because they are thought to be homologous to each other.

Power calculation parameter for C17:0:

	5%-35%	**35%-70%**
Mu(0):	**0.12769**	**0.14323**
Mu(1):	**0.15961**	**017904**
Sigma:	**0.01378**	**0.01371**
Sided test:	**Two**	**Two**
Alpha(α):	**0.05**	**0.05**
Power of the test:	**0.95**	**0.95**
Sample size (n=):	**3**	**2**

To see a statistical significant biomarker relationship we would expect that both C15:0 and C17:0 would see a similar affect when the dietary

intervention was applied. The *in vivo* C15:0 decreased by ~25% with a 25% dietary increase in total fat, therefore we would expect that this increase would be seen in the *in vivo* C17:0. To see a statistical significant decrease of ~25% in the C17:0 between each dietary group then three-and-two biological replicates (n=3-2) would be required according to these power calculations. However, in this dietary biomarker – dietary fat percentage study seven-and-six biological replicates (n=7-6) were used but only C15:0 showed statistically significant decreases with an increase in dietary total fat.

12.2.6 Power calculations for the dietary biomarker – dietary fat percentage and fat source study.

To perform a retrospective power calculation to determine if the sample size (n=) within this study was suitable to address the research question (does the amount of dietary fat affect the *in vivo* C15:0 and C17:0 compositions between different fat sources) an assumption was made; this assumption was that there would be a ~25% reduction in fatty acid where there is a ~25% increase in dietary total fat. The required sample size to see a statistical significant difference was calculated using an online power calculator (available at: https://www.statisticalsolutions.net/pssZtest_calc.php). This assumption that both C15:0 and C17:0 should see a ~25% reduction when a ~25% increase in dietary total fat is applied was reasonable due to the results seen in the previous study (dietary biomarker – dietary fat percentage study).

Power calculation parameter for C15:0:

	BD	**LD**	**FOD**
Mu(0):	**0.06033**	**0.05507**	**0.06469**
Mu(1):	**0.04525**	**0.04130**	**0.04851**
Sigma:	**0.00616**	**0.01002**	**0.00994**
Sided test:	**Two**	**Two**	**Two**
Alpha(α):	**0.05**	**0.05**	**0.05**
Power of the test:	**0.95**	**0.95**	**0.95**
Sample size (n=):	**3**	**7**	**5**

Power calculation parameter for C17:0:

	BD	LD	FOD
Mu(0):	**0.07772**	**0.07015**	**0.07507**
Mu(1):	**0.05829**	**0.05262**	**0.05630**
Sigma:	**0.00795**	**0.01088**	**0.00870**
Sided test:	**Two**	**Two**	**Two**
Alpha(α):	**0.05**	**0.05**	**0.05**
Power of the test:	**0.95**	**0.95**	**0.95**
Sample size (n=):	**3**	**6**	**3**

To see a statistical significant decrease of ~25% in the C15:0 and C17:0 between each dietary group (5% and 30%) then three biological replicates (n=3) for the basal fat (BD) diet group, six-to-seven biological replicates (n=6-7) for the lard fat (LD) diet group and three-to-five biological replicates (n=3-5) for the fish oil diet group would be required according to these power calculations. However, in this dietary biomarker – dietary fat percentage and fat source study six-to-eight biological replicates (n=6-8) were used for the basal fat (BD) diet group, six-to-eight biological replicates (n=6-8) were used for the lard fat diet group, and eight biological replicates (n=8) were used for the fish oil diet (FOD) groups but C15:0-basal fat, C15:0-lard fat, C17:0-lard fat, and C17:0-fish oil diet groups showed statistical significance with an increase in dietary total fat.

12.2.7 Power calculations for the dietary biomarker – ethanol intake study.

To perform a retrospective power calculation to determine if the sample size (n=) within this study was suitable to address the research question (does the ethanol decrease *in vivo* C15:0 and C17:0 fatty acids) an assumption was made; this assumption was that there would be a ~20% reduction in the

C15:0 and C17:0 composition between the control group and the ethanol treated group. The required sample size to see a statistical significant difference was calculated using an online power calculator (available at: https://www.statisticalsolutions.net/pssZtest_calc.php). This assumption that the *in vivo* C15:0 and C17:0 would decrease by ~20% due to the ethanol treatment is based on the belief that ethanol reduces the intestinal absorption of all lipid species (by varying amount but averages to ~30%) (Mansbach, 1983), this value of ~20% was conservative.

Power calculation parameter for C15:0 and C17:0:

	C15:0	**C17:0**
Mu(0):	**0.07066**	**0.11614**
Mu(1):	**0.05652**	**0.09291**
Sigma:	**0.00863**	**0.00928**
Sided test:	**Two**	**Two**
Alpha(α):	**0.05**	**0.05**
Power of the test:	**0.95**	**0.95**
Sample size (n=):	**5**	**3**

To see a statistical significant 20% decrease in the C15:0 and C17:0 composition then five biological replicates (n=5) would be required for C15:0 and three biological replicates (n=3) would be required for C17:0 according to this power calculation. However, in this ethanol intake study seven biological replicates (n=7) were used but only a statistical significant difference was seen for C15:0.

12.2.8 Power calculations for the endogenous synthesis – phytol supplementation study.

To perform a retrospective power calculation to determine if the sample size (n=) within this study was suitable to address the research question (does phytol supplementation affect C15:0 proportionately to C17:0) an

assumption was made; this assumption was that there would a proportionate decrease seen for C15:0 as there would be for C17:0 (i.e. the phytol supplementation caused a ~30% decrease in C17:0). The required sample size to see a statistical significant difference was calculated using an online power calculator (available at: https://www.statisticalsolutions.net/pssZtest_calc.php). It was reasonable to assume that the phytol supplementation would cause a reduction in C17:0 due to the previously investigated alpha oxidation competitive inhibition between straight chain fatty acids (specifically stearic acid) and beta-branched chain fatty acids (e.g. phytanic acid directly from phytol metabolism). If C15:0 and C17:0 were homologous then there would be a proportionate decrease in C15:0 as seen with C17:0.

Power calculation parameter for C15:0:

	C15:0
Mu(0):	**0.04883**
Mu(1):	**0.03418**
Sigma:	**0.00365**
Sided test:	**Two**
Alpha(α):	**0.05**
Power of the test:	**0.95**
Sample size (n=):	**1**

To see a statistical significant proportionate decrease in the *in vivo* C15:0 as was seen with C17:0 (~30% decrease in C17:0 was seen due to the phytol supplementation) if C15:0 was homologous to C17:0 then we would expect there to be a ~30% decrease in its composition in vivo, however, we only saw a ~8% decrease in C15:0 with the phytol supplementation. To see a statistical significant decrease of ~30% in the C15:0 then more than one biological replicates (n>1) would be required according to this power calculation. However, in this endogenous synthesis – phytol supplementation

study four-to-five biological replicates (n=4-5) were used but only C17:0 showed a statistically significant decrease.

12.2.9 Power calculations for the endogenous synthesis – 2-hydroxyacyl-CoA lyase (Hacl1) study.

To perform a retrospective power calculation to determine if the sample size (n=) within this study was suitable to address the research question (does the genetic knockout of *Hacl1* proportionately affect C15:0 and C17:0) an assumption was made; this assumption was that there would a proportionate decrease seen for C15:0 as there would be for C17:0 (i.e. the *Hacl1* knockout resulted in a ~20% decrease in C17:0). The required sample size to see a statistical significant difference was calculated using an online power calculator (available at: https://www.statisticalsolutions.net/pssZtest_calc.php). If C15:0 and C17:0 were homologous then there would be a proportionate decrease in C15:0 as seen with C17:0.

Power calculation parameter for plasma C15:0:

	C15:0
Mu(0):	**16.42**
Mu(1):	**13.136**
Sigma:	**2.20717**
Sided test:	**Two**
Alpha(α):	**0.05**
Power of the test:	**0.95**
Sample size (n=):	**6**

To see a statistical significant proportionate decrease in the *in vivo* C15:0 as was seen with C17:0 (~20% decrease in C17:0 was seen in the *Hacl1* knockout group) if C15:0 was homologous to C17:0 then we would expect there to be a ~20% decrease in its composition *in vivo*, however, we only saw a ~1% decrease in C15:0 with the phytol supplementation. To see a statistical

significant decrease of ~20% in the C15:0 then six biological replicates (n=6) would be required according to this power calculation. However, in this endogenous synthesis – 2-hydroxyacyl-CoA lyase (Hacl1) study twelve-to-fifteen biological replicates (n=12-15) were used but only C17:0 showed a statistically significant decrease.