# ANGPTL4 and omega-3 polyunsaturated fatty acids as mediators of lipid homeostasis in different metabolic states

by

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# ABSTRACT

ANGPTL4 AND OMEGA-3 POLYUNSATURATED FATTY ACIDS AS MEDIATORS OF LIPID HOMEOSTASIS IN DIFFERENT METABOLIC STATES

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This thesis investigated the relationship between omega-3 polyunsaturated fatty acids (n-3 PUFA), white adipose tissue (WAT) angiopoietin-like 4 (ANGPTL4) expression, and metabolic state-dependent lipid homeostasis. C57BL/6J mice fed low-fat diets containing n-3 PUFA had reduced plasma NEFA and liver TAG accumulation in the fasted state, while in WAT, markers of lipogenesis were reduced, and markers of lipolysis were increased. Additionally, n-3 PUFA increased WAT *Angptl4* gene expression. In contrast, n-3 PUFA reduced plasma TAG in the fed state but had no effect on *Angptl4* gene expression. Collectively, this thesis suggests that n-3 PUFA regulate lipid metabolism in a metabolic-state dependent manner, and that these important fatty acids may work via differential regulation of *Angptl4* in WAT.

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| | |
|---|---|
| ACS | Acyl-CoA synthase |
| AGPAT | 1-acylglycerol-3-phosphate acyltransferase |
| AIN-93G | American Institute of Nutrition diet 93g |
| ALA | Alpha-linolenic acid |
| AMPK | AMP activated protein kinase |
| ANGPTL4, *Angptl4* | Angiopoietin-like 4 |
| ANOVA | Analysis of variance |
| ATGL, *Atgl* | Adipose triglyceride lipase |
| ATP | Adenosine triphosphate |
| AUC | Area under the curve |
| cAMP | Cyclic adenosine monophosphate |
| CCD | Coiled-coil domain |
| cGMP | Cyclic guanosine monophosphate |
| CoA | Co-enzyme A |
| CPT | Carnitine palmitoyltransferase |
| CVD | Cardiovascular disease |
| DAG | Diacylglycerol |
| DGAT, *Dgat* | Diacylglycerol acyltransferase |
| DHA | Docosahexaenoic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| EPA | Eicosapentaenoic acid |
| eWAT | Epididymal WAT |
| F | Flaxseed oil diet |
| FABP-4 | Fatty acid binding protein 4 |
| FADS2 | Fatty acid desaturase 2 |
| FAS | Fatty acid synthase |
| FATP4 | Fatty acid transport protein 4 |
| FIAF | Fasting induced adipose factor |
| FLD | Fibrinogen-like domain |
| G3P | Glycerol-3-phosphate |
| GPAT | G3P acyltransferase |
| GPIHBP1 | glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1 |
| GR | Glucocorticoid receptor |
| GRE | GR response element |
| HBA1C | Hemoglobin A1C |
| HFD | High-fat diet |
| HOMA-IR | Homeostatic model assessment of insulin resistance |
| HSL, *Hsl* | Hormone-sensitive lipase |
| IDL | Intermediate-density lipoprotein |
| IGTT | Intraperitoneal glucose tolerance test |
| kDa | Kilo Dalton |
| L | Lard diet |
| LA | Linoleic acid |

| | |
|---|---|
| LDL | Low-density lipoprotein |
| LMF1 | Lipase maturation factor 1 |
| LPL, *Lpl* | Lipoprotein lipase |
| M | Menhaden diet |
| MAG | Monoacylglycerol |
| n-3 PUFA | Omega-3 polyunsaturated fatty acids |
| n-6 PUFA | Omega-6 polyunsaturated fatty acids |
| NAFLD | Non-alcoholic fatty liver disease |
| NASH | Non-alcoholic steatohepatitis |
| NEFA | Non-esterified fatty acids |
| NP | Natriuretic peptide |
| NSERC | Natural Sciences and Engineering Research Council |
| PCK1, *Pck1* | Phosphoenolpyruvate carboxykinase 1 |
| PCSK | Proprotein convertase subtilisin/kexin |
| PGAR | PPARγ angiopoietin related |
| PKA | Protein kinase A |
| PKG | Protein kinase G |
| PLIN1 | Perilipin-1 |
| PPAR | Peroxisome proliferator-activated receptors |
| PPRE | PPAR response element |
| qPCR | Quantitative polymerase chain reaction |
| SEM | Standard error of the mean |
| T2D | Type-2 diabetes |
| TAG | Triacylglycerol |
| TBST | Tris-buffered saline-0.1% Tween 20 |
| USRA | Undergraduate Student Research Award |
| VLDL | Very low-density lipoprotein |
| WAT | White adipose tissue |

# 1 ANGPTL4 and omega-3 polyunsaturated fatty acids as mediators of lipid homeostasis

## 1.1 Lipids and disease

Lipids are important molecules that are either consumed in the diet or synthesized through various biosynthesis pathways. Many classes and subclasses of lipids exist. In general, lipids are energy dense molecules comprised of a hydrogenated carbon backbone that serve many essential functions in the body, including formation of plasma membranes, serving as signaling molecules, and regulating gene expression. From a physiological perspective, the primary function of lipid is to act as a storage form of energy to be used in times of need.

The primary source of lipid in the body is from the diet. Additionally, when energy demands are low, carbohydrates (the primary source of energy in the body) are converted into lipid in a process called lipogenesis, further increasing the lipid storage pool. Ultimately, a chronic positive caloric balance results in whole body dyslipidemia and ectopic lipid storage. Excessive lipid storage in tissues like skeletal muscle, vasculature, and the liver are risk factors for the development of common chronic diseases such as obesity, type-2 diabetes (T2D), cardiovascular disease (CVD), and non-alcoholic fatty liver disease (NAFLD). Collectively, dyslipidemia place significant financial and logistical burdens on healthcare systems and individuals globally [1–4]. As such, treatment modalities targeting the underlying mechanisms regulating dyslipidemia are imperative.

## 1.2 Lipid metabolism

### 1.2.1 Digestion and absorption of dietary lipids

Dietary lipid is a major determinant of lipid levels in the body. Consumed lipid enters the intestine as an emulsion of large lipid droplets. Lipid droplets are primarily comprised of triacylglycerol (TAG); three fatty acids esterified to a glycerol backbone [131]. Bile acids secreted from the liver emulsify the lipid droplets in the proximal region of the small intestine [130]. The hydrophobic region of bile acids associate with the lipid droplet, and the polar side chains interact with the aqueous environment, acting as a detergent that reduces lipid droplet size [130]. Along with the protein cofactor colipase, bile acids grant lipases access to TAG. Intestinal lipases hydrolyze TAG to monoacylglycerols and fatty acids, which diffuse across the apical membrane of the enterocyte or are transported by either fatty acid transporter CD36 or fatty acid transport protein 4 (FATP4) [5,130]. TAG are resynthesized within the endoplasmic reticulum of the enterocyte and packaged into large lipoproteins (35 to >250 nm) called chylomicrons [131]. In general, lipoproteins contain a hydrophobic core of lipids surrounded by a hydrophilic monolayer of phospholipids, cholesterol, and apolipoproteins [5]. The size of the chylomicron is directly related to the amount of TAG in the intestinal lumen, and thus fluctuates with metabolic state (i.e., fed vs. fasted) [131]. Mature chylomicrons are secreted from enterocytes into lymphatic circulation and enter the bloodstream via the thoracic duct, delivering TAG to tissues throughout the body [5]. When the chylomicron TAG pool is diminished, the remnant chylomicron is taken up and catabolized in the liver [5].

### 1.2.2 Endogenous lipid production and transport

Liver-derived lipoproteins are secreted into circulation from hepatocytes via the hepatic veins to transport lipids, namely TAG and cholesterol esters [131]. Following a meal, approximately 50% of TAG within liver-derived lipoproteins are from endogenous stores and

15-17% are from dietary sources [6,7]. Multiple classes of liver-derived lipoproteins exist in circulation and can be distinguished based on their relative buoyant densities. Very-low density lipoproteins (VLDL) are initially secreted from hepatocytes containing a large amount of TAG, hence the very-low buoyant density, in addition to a single molecule of full-length ApoB100 and 10-20 molecules of ApoC2 [5]. As VLDL circulates, the buoyant density gradually increases due to hydrolysis of TAG via interaction between ApoC2 and lipoprotein lipase (LPL) [5,131].

### 1.2.3 LPL mediated uptake of circulating lipid

As lipoproteins circulate to metabolically active tissues, they interact with LPL that is bound to the vascular endothelial surface through glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1 (GPIHBP1) [8,131]. Human LPL is a 448 amino acid secreted protein that is expressed primarily by adipocytes and myocytes. The amino-terminal α/β-hydrolase domain contains a serine hydrolase active site triad, and the carboxyl-terminal β-barrel domain contains a lipoprotein and GPIHBP1 binding site [9,131]. Before LPL is secreted, lipase maturation factor 1 (LMF1)-mediated folding and post-translation modifications occur. It is traditionally thought that LPL functions as a dimer; however, recent evidence suggests it is active as a monomer [9,10]. Once LPL is secreted into the interstitial space, GPIHBP1 binds and transports LPL across endothelial cells to the capillary lumen [9].

LPL hydrolyzes the 1(3)-ester linkages of TAG within circulating lipoproteins that express ApoC2, generating non-esterified fatty acids (NEFA) and monoacylglycerols (MAG) [5]. LPL can further process MAG into glycerol and NEFA. Lipolysis rates are maintained until ~80% of TAG are hydrolyzed, and ApoC2 is recycled to other circulating lipoproteins [131]. GPIHBP1 stabilizes the catalytic active site during hydrolysis [8], preventing LPL unfolding and inactivation by endogenous inhibitor proteins (see 1.4.2.1) [10]. Both NEFA and MAG are transported into

adipocytes and myocytes via CD36 and FATP4 [5]. A small portion of NEFA may also passively diffuse into adipocytes at the site of hydrolysis; however, the majority remain in circulation bound to albumin for uptake later [131]. As the primary lipid storage site, the capacity of LPL-mediated lipoprotein TAG hydrolysis in adipose is proportional to TAG availability, whereas muscle tissue LPL activity is saturated quickly even in low TAG environments [131].

### 1.2.4 Lipid fates in different metabolic states

The metabolic state of an organism profoundly affects how lipid is handled in the body. In general, metabolic state refers to the balance between nutrient availability (i.e., fed state) and energy demand (i.e., fasted state). Insulin and glucagon are peptide hormones produced by the beta and alpha cells of the pancreas, respectively [130]. These hormones are regulated by metabolic state and have contrasting properties regarding lipid metabolism.

#### 1.2.4.1 Storage of dietary lipid in WAT in the fed state

Following a meal (i.e., fed state), there is an abundance of macronutrients in the bloodstream. Circulating TAG (in chylomicrons primarily) peak at approximately 3 hours after consuming a meal [11]. Insulin is secreted in response to the presence of carbohydrates, which stimulates LPL activity and inhibits WAT lipolysis [11]. Correspondingly, plasma TAG and NEFA levels drop.

The uptake and storage of fatty acids in adipose tissue is a coordinated process. Following a meal, LPL mediates the hydrolysis and import of dietary TAG from circulating chylomicrons into WAT for up to 5 hours after a meal, and throughout the day during a typical 3-meal dietary pattern [11]. Several fatty acid transporters are responsible for the uptake of NEFA into adipocytes. Acyl-CoA synthase (ACS) esterifies NEFA to coenzyme A (CoA), forming fatty-acyl CoA [12,13]. To a lesser extent, fatty-acyl CoA is also formed within the adipocyte *de novo*

from carbohydrate sources through a series of carboxylation reactions [12,14]. Regardless of origin, in the fed state three fatty-acyl CoAs are sequentially esterified to glycerol-3-phosphate (G3P) via G3P-acetyltransferase (GPAT), 1-acylglycerol-3-phosphate acyltransferase (AGPAT), and diacylglycerol acyltransferase (DGAT) isoforms 1 and 2 to form TAG [12,15,16]. The primary storage form of lipid in adipocytes is TAG, accounting for ~90% of the lipid in these cells [17,18].

#### 1.2.4.2 Lipoprotein circulation and TAG hydrolysis in the fasted state

In the fasted state, dietary macronutrients are not in circulation, insulin levels are low and glucagon levels are high. Consequently, plasma TAG are low and NEFA concentrations are high. From a whole-body perspective, hydrolysis of TAG-containing lipoproteins in the fasted state is low due to reduced TAG substrate and lower LPL activity. The latter is due to lower circulating levels of ApoC2-containing lipoproteins and insulin, in addition to changes in LPL expression and secretion [5,131]. Fasting reduces LPL expression in WAT, while muscle LPL expression is upregulated [131]. The tissue specificity for LPL expression is likely due to energy demand, as muscle requires fatty acids for oxidation in the fasted state. In adipocytes, LPL secretion is further reduced due to reduced rates of post-translational modification, resulting in LPL retention and reduced LPL activity [131].

The source of TAG within circulating lipoproteins change with fasting. Approximately 77% of VLDL TAG is from adipose derived NEFA due to elevated rates of lipolysis [6].

#### 1.2.4.3 WAT lipolysis and lipid mobilization in the fasted state

In the fasted state, the bulk of stored TAG is sent to other high energy demand tissues as NEFA. NEFA are liberated from stored TAG in WAT via lipolysis, which involves sequential hydrolysis reactions, ultimately resulting in three NEFA and G3P [12]. Approximately 50% of plasma NEFA is WAT-derived in the postprandial state and increases with prolonged fasting [11].

WAT lipolysis is stimulated by the activation of several cell surface receptors. β-adrenergic receptors, glucagon receptors, and natriuretic peptide (NP) receptors initiate protein kinase A (PKA) and G (PKG) activity through adenylate cyclase mediated cAMP/cGMP formation, respectively [12,19]. PKA and PKG facilitate the transfer of high energy phosphate groups from donor molecules to target proteins [12,19,20], including perilipin-1 (PLIN1) and hormone sensitive lipase (HSL). The phosphorylation of PLIN1 does two things to prime the lipid droplet for lipolysis: 1) it induces the release of CGI-58, and 2) displaces itself from the surface of the lipid droplet. Free CGI-58 promotes the full activation of adipose triglyceride lipase (ATGL), which hydrolyzes TAG, producing a NEFA and diacylglycerol (DAG). ATGL has a greater specificity for TAG than DAG [19]. Displaced PLIN1 from the lipid droplet allows active HSL bound to fatty acid binding protein-4 (FABP-4) to hydrolyze DAG, yielding a second NEFA and monoacylglycerol (MAG) [12,18,20–22]. MAG lipase hydrolyzes the final fatty acid from MAG, resulting in a third NEFA and glycerol [18,20]. *In vitro* studies have shown that ATGL and HSL are responsible for upwards of 95% of TAG hydrolysis [12].

The activation of HSL is dependent on phosphorylation at specific serine residues of the protein. Phosphorylation at serine 563 by cAMP activates HSL [23]; however, phosphorylation at serine 565 by AMPK prevents serine 563 phosphorylation and therefore blunts HSL activity [24]. Additionally, serines 659 and 660 are phosphorylated by PKA and may have the greatest effect on HSL activation *in vivo* [25].

Insulin inhibits lipolysis through phosphodiesterase-3B mediated degradation of cAMP (thus preventing PKA activation), whereas glucagon promotes cAMP formation [12]. In the fasted state, lipolysis is activated due to a high glucagon:insulin ratio, thereby liberating WAT TAG stores and enabling NEFA to be available for energy production.

#### 1.2.4.4 Fatty acid oxidation in the fasted state

Under fasted conditions, fatty acids can be oxidized within WAT. However, WAT oxidative activity is relatively low compared to that of liver, kidney, brain or muscle [26]. WAT derived NEFA are delivered to more highly oxidative tissues for use in times of fasting. In general, fatty acids are transported across the plasma membrane destined for the mitochondria, endoplasmic reticulum, or peroxisome, where they become activated by ATP-dependent ACS to form acyl-CoA [131]. Carnitine palmitoyltransferase (CPT) I and II found on the outer and inner membranes of the mitochondria, respectively, facilitate the uptake of the fatty acids into the matrix via carnitine substitution. Once in the matrix, acyl-CoA are reformed by CPT II [131]. From here, acyl-CoA undergoes a series of chemical reactions that result in the formation of acetyl-CoA (destined for the citric acid cycle) and a shortened acyl-CoA by 2 carbon atoms from the carboxyl end [130]. It is important to note that oxidation of unsaturated fatty acids requires additional enzymes due to the presence of carbon double bonds within the backbone [131].

In conclusion, lipid handling is a coordinated process that is dependent on metabolic state. In the fed state, TAG are carried by lipoproteins to WAT for storage. The uptake of TAG into WAT is regulated by LPL activity. Fasting reduces LPL activity and increases mobilization of stored TAG as NEFA via lipolysis. Circulating NEFA in the fasted state are subsequently used for energy by the body.

## 1.3 Effects of omega-3 fatty acids on lipid handling

Dietary omega-3 polyunsaturated fatty acids (n-3 PUFA) are associated with reduced risk of developing various chronic metabolic diseases including NAFLD, T2D, and CVD. The protective effects are, in part, attributed to the ability of n-3 PUFA to regulate aspects of lipid cycling.

### 1.3.1 Omega-3 source, structure, and classification

n-3 PUFA are long-chain fatty acids (≥18 carbons) that contain a double bond on the third carbon from the omega end. The shortest n-3 PUFA, alpha-linolenic acid (ALA; 18:3 n3), is an essential dietary n-3 PUFA that is predominantly found in plant-based sources [27]. Longer-chain n-3 PUFA are either consumed in the diet from marine sources or made endogenously from ALA. In hepatocytes and adipocytes, ALA undergoes a series of desaturation and elongation reactions leading to the formation of EPA (20:5 n3) and DHA (22:6 n3). In humans, the conversion efficiency of ALA to EPA and DHA has been estimated as 0.2-6% and 0.1%, respectively [27,28]. The low conversion efficiency of ALA to downstream EPA and DHA, coupled with the fact that ~80% of the world population does not consume the recommended amounts of EPA and DHA [29], emphasizes the importance of obtaining EPA and DHA in the diet.

### 1.3.2 N-3 PUFA regulation of NAFLD

The effect n-3 PUFA have on dyslipidemia is perhaps best exemplified in relation to NAFLD. NAFLD is a progressive metabolic disorder in which the liver accumulates large amounts of TAG, leading to non-alcoholic steatohepatitis (NASH), and eventual fibrosis or cirrhosis [30]. NAFLD has become the main predictive factor in the development of chronic liver disease worldwide [31]. A recent meta-analysis found that clinically diagnosed patients with NAFLD or NASH had a liver-specific mortality rate of 0.77/1000 person years and 11.77/1000 person years, respectively [32]. In 2016, the global prevalence estimate of NAFLD was ~25% [32], which may increase given that NAFLD is positively associated with the obesity pandemic [32–34]. N-3 PUFA have been investigated as a treatment modality for NAFLD.

Dyslipidemia is a key determinant of NAFLD, as hypertriglyceridemia and elevated circulating NEFA are significant predictors of NAFLD [35,36]. In humans with NAFLD, WAT lipolysis

rates and liver VLDL secretion were elevated, as determined using stable isotope tracers[37]. Multiple experimental models have shown that treatment of NAFLD with long-chain n-3 PUFA reduce liver TAG levels [38]. A meta-analysis found that 13 of 17 human clinical studies investigating EPA and DHA treatment of NAFLD patients saw reductions in liver TAG concentrations and inflammation [39]. The following section will discuss some of the proposed mechanisms by which n-3 PUFA regulate lipid homeostasis.

#### 1.3.2.1 TAG lowering properties of n-3 PUFA

##### 1.3.2.1.1 Lipoprotein secretion

Perhaps the most well-established effect of n-3 PUFA is their ability to lower postprandial and fasting circulating TAG levels [40]. Lower circulating postprandial and fasting TAG may be attributed to a reduction in hepatic VLDL production [40].

*In vitro* treatment of hepatocytes with chylomicron remnants isolated from rats orally administered fish oil led to a reduction in VLDL secretion in comparison to corn oil [41,42], which suggests that liver n-3 PUFA from dietary sources is responsible for lower VLDL secretion. When rat hepatocytes were treated directly with either EPA or DHA, VLDL maturation and secretion was reduced in comparison to oleic acid [43–45]. This aligns with *in vivo* findings that rodents fed fish oil exhibit reduced plasma TAG and VLDL formation in comparison to diets high in monounsaturated and n-6 PUFA [46–48].

However, reduced VLDL secretion may be due to reduced TAG substrate [49]. In rodents, reductions in VLDL secretion occur concomitantly with reduced hepatic TAG [46–48]. N-3 PUFA mediated reduction in hepatic TAG was associated with suppressed hepatic fatty acid synthase (FAS) and DGAT expression [47], suggesting that n-3 PUFA may suppress hepatic lipogenesis. Hepatic TAG accumulation may also be reduced by n-3 PUFA through enhanced fatty acid

oxidation. In study using strategic tracers, rats fed n-3 PUFA in comparison to lard showed greater partitioning of acyl-CoA towards oxidation rather than esterification, ultimately resulting in reduced TAG secretion as VLDL in the postprandial state [50]. This is in line with the finding that rats fed fish oil exhibited increased whole-body fat and reduced carbohydrate metabolism [51]. Finally, reduced hepatic TAG (and consequently VLDL secretion) may also be a byproduct of reduced circulating lipid returning to the liver. This may be due to enhanced LPL hydrolysis of TAG within lipoproteins, or suppressed WAT lipolysis of stored TAG.

#### 1.3.2.1.2 WAT lipolysis

Dysregulation of WAT lipid homeostasis contributes to the progression of dyslipidemia. Tracer studies show that ~60% of liver TAG originate from WAT-derived NEFA [52,53]. It is estimated that a reduction in WAT-derived NEFA release would impart the greatest reduction in VLDL TAG [54]. However, physical evidence of n-3 PUFA mediated reduction in WAT-liver NEFA flux is limited [54]. In animal models, evidence suggests that n-3 PUFA reduce NEFA flux to the liver in diseased states. Alcohol consumption increased hepatic TAG accumulation due to increased WAT lipolysis [55]. Flaxseed oil rich in ALA suppressed ethanol induced WAT lipolysis and TAG accumulation in liver [55]. Similarly, oral administration of EPA prevented cancer cachexia-induced NEFA release and WAT mass loss in mice [56]. However, a recent clinical trial showed that supplementation with EPA and DHA did not alter markers of adipose lipolytic rate in insulin resistant adults [57].

In non-diseased states, dietary supplementation with EPA and DHA reduced post-prandial plasma NEFA concentrations and adipocyte volume in the postprandial state in comparison to lard-fed rats [50,51,58,59]. Fatty acid suppression of WAT lipolysis may be specific to n-3 PUFA, as no differences were observed with saturated and monounsaturated fatty acid

consumption in rats [59,60]. Additionally, *ex vivo* adipocytes from n-3 PUFA fed rats had blunted basal and isoproterenol-stimulated lipolytic activity [59]. Taken together, these studies suggest that n-3 PUFA suppress WAT lipolysis in diseased and non-diseased states, thereby reducing NEFA flux to the liver.

#### 1.3.2.1.3 LPL mediated uptake

Reduced supply of lipid to the liver may also be attributed to enhanced LPL hydrolysis of TAG in circulating lipoproteins. Fractional clearance rate of VLDL TAG increased with fish oil supplementation in normal individuals, but not in hypertriglyceridemic individuals [61]. In hypertriglyceridemic individuals, n-3 PUFA increased the conversion of VLDL into intermediate-density lipoproteins (IDL), and IDL into low-density lipoproteins (LDL); however, this may be due to a change in the ratio of lipoprotein to LPL, rather than changes in LPL activity *per se* [40,62].

In the fed state, n-3 PUFA increase clearance of chylomicrons [40]. 33 healthy individuals were supplemented with either EPA or DHA ethyl esters for 4 weeks [63]. The authors found that both EPA and DHA increased hydrolysis of TAG in chylomicrons, LPL activity, and chylomicron diameter in the fed, but not fasted, state [63]. This aligns with mouse studies where menhaden oil-fed mice had reduced plasma TAG in the absorptive (2-3 hours), but not post absorptive state (6-8 hours) [50]. A follow-up analysis determined that the effect was attributed to either increased abundance of LPL or increased LPL affinity for lipoproteins. This is supported by the fact that in dyslipidemic men, n-3 PUFA supplementation was associated with greater increases in postprandial WAT LPL gene expression [64], and pre-heparin LPL activity is increased with n-3 PUFA treatment [40].

Taken together, there is evidence to suggest that dietary n-3 PUFA reduce hepatic TAG by reducing the hepatic lipid supply. This may occur through upregulation of postprandial LPL

hydrolysis and/or reduced NEFA flux from WAT lipolysis. The mechanisms by which n-3 PUFA regulate lipid homeostasis are not fully defined. A potential mechanism of action may involve angiopoietin-like 4 (ANGPTL4).

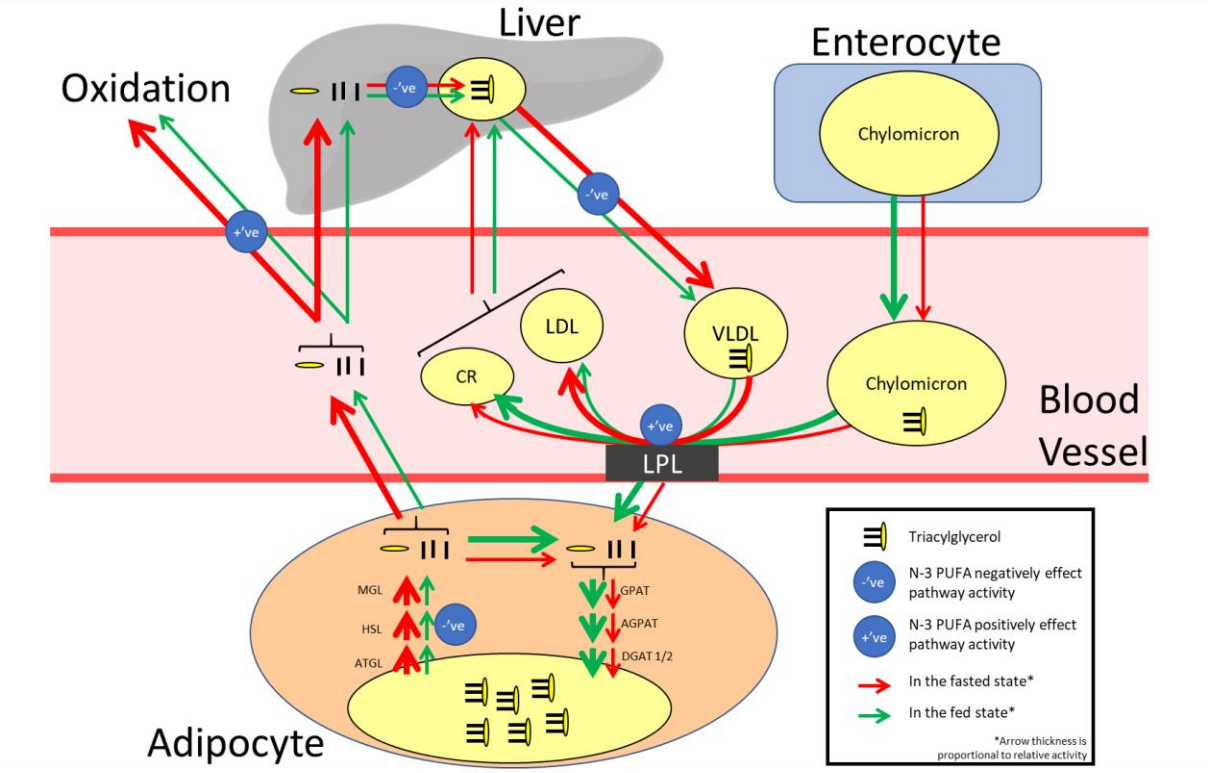


**Figure 1. Summary of n-3 PUFA and lipid metabolism in different metabolic states.** Dietary TAG in the fed state and endogenously synthesized TAG in the fasted state circulate in the bloodstream within chylomicrons and VLDL, respectively. Circulating TAG within lipoproteins are hydrolyzed by LPL, liberating fatty acids from the glycerol backbone. The activity of LPL is dependent on substrate availability and is upregulated by n-3 PUFA, thereby reducing circulating TAG. NEFA and glycerol are eventually transported into adipocytes to resynthesize TAG for storage. Within WAT, the synthesis and breakdown of TAG are coordinated by key enzymes that are upregulated in the fed and fasted states, respectively. WAT lipolysis results in the release of NEFA and glycerol into circulation for oxidation in other tissues. N-3 PUFA reduce circulating NEFA concentrations by suppressing WAT lipolysis and stimulating oxidation. Remaining TAG containing lipoproteins and NEFA in circulation are transported into the liver for temporary storage and repackaging for VLDL secretion in times of energy demand. N-3 PUFA reduce liver TAG accumulation and secretion of VLDL particles. Ultimately, n-3 PUFA and metabolic state regulate the life cycle of lipids.

## 1.4 Adipose tissue-derived ANGPTL4

### 1.4.1 Structure, source, and circulating form

ANGPTL4 is a secreted protein containing an N-terminal signal sequence, a coiled-coil domain (CCD), and a C-terminus fibrinogen-like domain (FLD) [65,66]. In mice, ANGPTL4 gene is encoded on chromosome 17 and the protein is 410 amino acids in length. In contrast, the *ANGPTL4* gene in humans is located on chromosome 19 and the protein is 405 amino acids long. Mice and humans share 75% nucleotide identity and 77% amino acid identity between ANGPTL4 orthologs [65].

ANGPTL4 is primarily expressed in adipose tissue and liver [65,67]. In circulation, ANGPTL4 is present as the native full-length form, truncated forms, and as an oligomer bound by intermolecular disulfide bonds [66,68]. Once secreted, native full-length ANGPTL4 (~50 kDa) circulates as a monomer or oligomer, and monomers are proteolytically cleaved between Arg-161 and Arg-164 into two distinct truncated fragments; the CCD (~15kDa) and the FLD (~37kDa) [66,68,69]. The CCD circulates as an oligomer, whereas the FLD circulates as a monomer [66]. Both the full-length form and the FLD are glycosylated [66,68,69].

### 1.4.2 Discovery

In 2000, subtractive hybridization strategies were employed to identify target genes of PPARγ [65] and PPARα [67]. Both studies identified a target gene whose protein product belonged to the angiopoietin-like family, and its expression was dependent on both PPAR isoforms. Collective findings suggested that this protein was most highly expressed in WAT, was detected in plasma as a secreted protein, was upregulated in genetic mouse models of obesity and diabetes, and its expression increased with fasting. Each study provided a unique name for the protein. Yoon et al., named it PGAR, short for PPARγ angiopoietin related, while Kersten et al.,

chose FIAF for fasting-induced adipose factor. Since these original characterizations, the function, processing, and regulation of PGAR/FIAF (now globally referred to as angiopoietin-like 4, or ANGPTL4) has been investigated. Several other angiopoietins have been identified and shown to possess unique functions[70]; however, this thesis will focus strictly on ANGPTL4.

### 1.4.3 Function

It is generally accepted that ANGPTL4 transiently induces hyperlipidemia through two distinct mechanisms: 1) inhibition of LPL-mediated TAG uptake into peripheral tissues, and 2) by stimulating WAT lipolysis to mobilize TAG stores.

#### 1.4.3.1 LPL inhibition

Initial transgenic ANGPTL4 overexpression studies in mice showed elevated plasma TAG [71,72], in addition to hepatic lipid accumulation [73]. These effects were amplified when mice were challenged with a high-fat diet (HFD) [72,74]. ANGPTL4-induced hypertriglyceridemia was explained by impaired TAG clearance [71,72,75] and not by changes in VLDL production [72]. In ANGPTL4 knock-out mice, plasma TAG is reduced due to amplified TAG uptake [71,76,77]. This effect was replicated in wild-type mice and monkeys treated with ANGPTL4 neutralizing antibodies [76,78]. Further, hypertriglyceridemia induced by an LDL receptor knockout is blunted when ANGPTL4 is also knocked out [79]. Mice lacking ANGPTL4 expression specifically in WAT had enhanced LPL activity and circulating TAG clearance, demonstrating that WAT-derived ANGPTL4 is required for LPL inhibition [80]. Collectively, this suggests that WAT-derived ANGPTL4 plays a key role in regulating TAG clearance. The following provides a brief overview of the mechanism by which ANGPTL4 regulates LPL activity.

A highly conserved amino acid sequence (~40-53aa) within the N-terminal CCD domain [70,81] of ANGPTL4 transiently binds the lid domain of LPL complexed with GPIHBP1 on the

surface of endothelial cells, thereby preventing TAG hydrolysis at the LPL active site [82,83]. Additionally, the protein interaction between ANGPTL4 and LPL results in the cleavage of catalytically active dimers of LPL into inactive monomers [81,84], which subsequently reduces LPL affinity for GPIHBP1 [82].

Mutating ANGPTL4 by replacing 1-3 amino acids within the binding domain impairs its inhibitory action on LPL *in vitro* [70,81]. Further, treating mice with this mutant ANGPTL4 failed to elevate plasma TAG when compared to the native protein [81]. This is supported by evidence in humans possessing the p.E40K inactivation variant of the *ANGPTL4* gene caused by a missense single nucleotide polymorphism (G>A; rs116843064) of the 40th amino acid, resulting in a glutamine (E) substitution for lysine (K). Carriers of the *ANGPTL4* inactivation p.E40K variant have 13-35% reduced circulating TAG [78,85–89], and homozygotes are further reduced [78].

It is traditionally thought that the inhibitory action of ANGPTL4 on LPL occurs within the cell, which is supported by the fact that siRNA knockdown of ANGPTL4 reduced extracellular LPL activity [90]. However, more recent evidence suggests that ANGPTL4 may also promote cleavage of LPL intracellularly through a PCSK-mediated mechanism[91].

#### 1.4.3.2 Lipolysis stimulation

ANGPTL4 overexpression also results in increased plasma NEFA and glycerol [72,92], suggesting that ANGPTL4 upregulates WAT lipolysis. In ANGPTL4-/- mice, fasting and glucocorticoid treatment-induced lipolysis was blunted, indicating that ANGPTL4 plays a role in the release of NEFA [93,94]. Since lipolysis, and therefore plasma NEFA, were not upregulated in mice lacking ANGPTL4, there was no accumulation of TAG in the liver upon glucocorticoid treatment [93]. It is likely that ANGPTL4 induced lipolysis is mediated by cAMP, as ANGPTL4-deficient mice prevented fasting and glucocorticoid-induced expression of cAMP and its

downstream lipolytic targets [94]. This suggests that the native full-length ANGPTL4 protein can induce WAT lipolysis.

It was postulated by Ge et al. that, given the cleavage of ANGPTL4 in circulation into the N-terminal coiled coil domain (CCD) and the C-terminal fibrinogen-like domain (FLD), ANGPTL4 may possess two unique domain-specific functions. While convincing evidence exists for the function of the CCD (see 1.4.3.1 LPL inhibition), a function of the FLD remained elusive until 2017. McQueen et al. investigated whether the FLD of ANGPTL4 alone was responsible for the lipolytic properties of ANGPTL4. In comparison to vehicle control mice, both mice overexpressing the native full-length ANGPTL4 protein and mice over-expressing just the FLD had elevated plasma NEFA. In primary adipocytes, NEFA release was also greater when treated with purified native full length ANGPTL4 or the FLD. Importantly, plasma TAG in mice overexpressing FLD was unchanged. Interestingly, mice over-expressing FLD were resistant to diet-induced obesity, as shown by lower body weight, WAT weight, and fat mass in comparison to vehicle control animals. Surprisingly, the FLD-induced increase in plasma NEFA translated to reduced liver and muscle TAG, which the authors attributed to WAT browning and increased energy expenditure at 23°C.

### 1.4.4 ANGPTL4 and metabolic states

The expression and secretion of ANGPTL4 is regulated by metabolic state. In general, environments which simulate energy demand upregulate ANGPTL4 expression. Perhaps the best documented type of stimuli is with fasting.

In humans, circulating ANGPTL4 is upregulated with fasting [95,96]. This is likely due to fasting-induced increases in gene and protein expression of ANGPTL4 in mouse liver and WAT [65,67,77,97,98]. Human studies have also shown that fasting upregulates ANGPTL4 gene expression

in adipose tissue [95,96,99]. This is supported by the fact that ANGPTL4 is suppressed by insulin and stimulated by glucagon in humans [100,101], key hormones involved in the metabolic adaptions to nutrient availability. Fasting-induced changes in ANGPTL4 expression are mediated by changes in basal rates of transcription.

### 1.4.5 Transcriptional regulation of ANGPTL4

The transcriptional regulation of ANGPTL4 involves several enhancer response elements. As mentioned previously, ANGPTL4 was independently discovered as both a PPARγ [65] and a PPARα [67] target gene. A conserved PPAR response element (PPRE) was identified within intron 3 of human and mouse ANGPTL4 gene [68]. PPARγ is a master regulator of adipogenesis, while PPARα is master regulator of fatty acid oxidation in the liver. It seems that PPARα is responsible for regulating transcription of *Angptl4* in liver [68], whereas PPARδ and PPARγ regulate *Angptl4* expression in muscle [102,103] and adipocytes [68] respectively. In human HepG2 liver cells, PPARα and PPARδ agonists increased *Angptl4* transcription [68]. Fasting or treatment with a PPARα agonist increased PPARα binding to the PPRE in mouse liver as determined by chromatin immunoprecipitation, which was absent in PPARα-/- mice [68]. Meanwhile in differentiated 3T3-L1 adipocytes, PPARγ was found bound to the PPRE, but not in undifferentiated 3T3-L1 adipocytes [68], suggesting that ANGPTL4 may be involved in PPARγ-mediated adipogenesis.

In addition to the PPRE, the ANGPTL4 gene contains a glucocorticoid receptor response element (GRE) and a forkhead box transcription factor response element (FRE) [93,104]. Glucocorticoid treatment and fasting increases ANGPTL4 expression via stimulation of glucocorticoid receptor (GR) and Fox01 recruitment to their respective response elements *in vitro* and *in vivo*, which is suppressed with insulin treatment or refeeding [93,94,104]. *In vitro*

experiments also showed that the classical insulin signaling molecules PI3k and Akt were required for insulin-mediated suppression of glucocorticoid induced ANGPTL4 expression [104]. Collectively, this suggests transcriptional control of ANPGLT4 expression is mediated by GR, Fox01, and PPAR recruitment to their respective enhancers.

### 1.4.6 Lifestyle regulation of ANGPTL4

Human studies have documented that ANGPTL4 expression occurs concomitant with elevated plasma NEFA [95,99,100,105,106]. In a series of human trials, Kersten et al. sought to determine if elevated NEFA levels is the cause of increased circulating ANGPTL4. Fasting, caloric restriction and endurance exercise all independently increased plasma ANGPTL4 and NEFA concentrations. In participants undergoing a hyperinsulemic euglycemic clamp, infusion of fatty acids or a lipolysis inducing β-adrenergic agonist raised plasma ANGPTL4. The authors concluded that plasma NEFA induce ANGPTL4 expression. The findings of Kersten et al. highlight that high energy demand upregulates ANGPTL4 expression. Several others have found that exercise induces ANGPTL4 expression and may be involved in cAMP/AMPK mediated oxidation [95,101,107]. Through a similar mechanism, ANGPTL4 may also be required for oxidation in BAT for non-shivering thermogenesis [108,109]. Low calorie diets and obesity-associated weight loss in humans have also been shown to increase ANGPTL4 expression and activity [99,110]; however, Van der Kolk et al. did not find a weight loss effect.

### 1.4.7 Regulation of ANGPTL4 by lipids

Circulating plasma NEFA may induce ANGPTL4 expression [95]. *In vitro* work showing that treatment of rat hepatoma, human primary myocytes, and mouse intestinal cells with oleic acid, linoleic acid (LA), and known PPAR agonists increased *Angptl4* gene expression [95]. Follow-up studies by the same group investigated the effect of single fatty acids on global gene

expression in the heart of gavaged mice [102,103]. Both microarray studies identified *Angptl4* as one of the top 10 most significantly upregulated genes following treatment with oleic, linoleic, and ALA, but not DHA. Interestingly, ALA treatment induced expression of *Angptl4* was abolished in PPARδ, but not PPARα-/- mice [102,103]. Ultimately, this suggests that acute delivery of dietary fatty acids to the heart regulate the transcription of *Angptl4*. In a feeding model of genetically obese rats, diets rich in LA increased *Angptl4* gene expression in skeletal muscle comparatively to ALA [111]. Treatment of intestinal cells with short chain fatty acids *in vitro* has been shown to increase ANGPTL4 transcription and secretion in a PPARδ-dependent manner [112,113]. Collectively, this work suggests that *Angptl4* expression is regulated by specific fatty acids.

Little research has investigated long-chain n-3 PUFA regulation of ANGPTL4 in liver and WAT. In cultured adipocytes, short-chain fatty acids failed to elevate *Angptl4* expression [112]. In rat hepatoma cells, DHA greatly increased *Angptl4* gene expression [114]. It was also shown that humans undergoing a hyperinsulinemic-euglycemic clamp, intravenous infusion of n-3 PUFA prevented the insulin-mediated suppression of circulating ANGPTL4 expression [114].

### 1.4.8 Epidemiological relevance

Given the role ANGPTL4 plays in regulating lipid homeostasis, it has also been investigated as a determinant of dyslipidemic disease in humans.

In 490 patients undergoing voluntary angiography for coronary artery disease evaluation, elevated plasma ANGPTL4 was associated with cardiovascular events after 4.5 years of follow up [115]. Circulating ANGPTL4 has also been associated with large artery stroke and hypertension [116,117]. Hypertensive subjects also had increased *ANGPTL4* gene expression in subcutaneous WAT [117]. In addition, several recent large-scale human studies suggest that the

hypotriglyceridemic phenotype of the p.E40K inactivation variant were protected from coronary artery disease [78,87,118,119]. Collectively, this is strong evidence that ANGPTL4 is associated with the development of cardiovascular disease.

ANGPTL4 has also been investigated in relation to T2D. Plasma ANGPTL4 levels have been positively associated with T2D [105], HOMA-IR and HBA1C [110], and metabolic syndrome [115]. Interestingly, recent large-scale studies have found that carriers of the p.E40K variant have reduced risk of developing T2D [118–120]. Gusarova et al. also found p.E40K carriers had better glucose tolerance and were associated with reduced fasting glucose levels in non-diabetics. Taken together, these findings implicate functional ANGPTL4 in the development of insulin resistance and T2D.

Little research has been done to determine the effect of ANGPTL4 or ANGPTL4 variants on the development of NAFLD in humans. Rodent experiments would suggest that ANGPTL4-mediated hyperlipidemia translates to excessive accumulation of hepatic TAG [73,93,121]; however, more research is warranted.

## 1.5 Conclusion

Dyslipidemic diseases are becoming increasingly prevalent and place a significant burden on health care systems worldwide. Therefore, understanding how dietary factors can regulate and potentially alleviate dyslipidemia are of paramount importance. Alterations in WAT lipid handling contributes to the development of dyslipidemic disease, such as NAFLD. N-3 PUFA protect against dyslipidemic disease through regulation of WAT lipid handling processes, including LPL-mediated uptake and lipolysis. It is unknown whether ANGPTL4, a secreted protein from WAT known to regulate lipid handling, is implicated in the n-3 PUFA induced changes to WAT lipid handling.

# 2 Rationale

During my NSERC USRA research position and the first few months of my MSc degree, I completed a study begun by a former PhD student in the lab that compared the differential effects of dietary linoleic acid (LA) and ALA on the skeletal muscle secretome of genetically obese rats [111]. Microarray analysis identified *Angptl4* gene expression to be differentially regulated by LA (n-6) and ALA (n-3). qPCR confirmed that rats fed ALA-rich diets blunted the obesity-induced increase in *Angptl4* gene expression, which was not seen in LA fed rats. Subsequent *in vitro* studies showed that treating L6 myotubes with LA also increased *Angptl4* gene expression in comparison to ALA. However, ANGPTL4 protein expression was reduced in the serum of ALA fed rats and the cell culture media of LA and ALA treated myotubes in comparison to respective controls. This discrepancy between skeletal muscle *Angptl4* gene expression and serum ANGPTL4 protein expression may be attributed to the fact that muscle is not the primary source of ANGPTL4. Therefore, we hypothesized that the differential effect of LA and ALA on ANGPTL4 may be more strongly related to events occurring in WAT. In a follow up *in vitro* study (unpublished data), the effect of individual n-3 PUFA on *Angptl4* gene expression was investigated in primary rat adipocytes. EPA and DHA treatment both increased *Angptl4* gene expression in comparison to palmitic acid and ALA. Given the aforementioned evidence, a feeding study in mice using specific n-3 PUFA diets was conceived to explore the effects of these fatty acids on ANGPTL4 in different metabolic states (i.e., fed versus fasted), the results of which serve as the basis of this thesis.

# 3 Research Objectives and Hypotheses

## 3.1 Research Objective

The objective of this thesis was to determine if different dietary n-3 PUFA regulate whole-body lipid homeostasis and WAT ANGPTL4 expression in a metabolic state-dependent manner. To address this objective, C57BL/6J mice were fed custom-formulated diets containing either no n-3 PUFA (lard control), just the precursor ALA, or EPA/DHA. Plasma and liver lipid levels were investigated, along with molecular markers of WAT lipogenesis and lipolysis. WAT gene and protein expression of ANGPTL4 was measured by qPCR and western blot, respectively.

## 3.2 Hypotheses

The ability of n-3 PUFA to regulate ANGPTL4 in WAT is unexplored. Based on preliminary *in vitro* data, it was hypothesized that mice fed the EPA/DHA-rich diet would show higher WAT ANGPTL4 expression level compared to the other dietary groups. Given the known functions of ANGPTL4, it was further hypothesized that greater ANGPTL4 expression would be concomitant with increased plasma TAG, plasma NEFA, and liver TAG concentrations.

# 4 Omega-3 dietary fat regulation of adipose ANGPTL4 expression is dependent on metabolic state

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## 4.1 Abstract

White adipose tissue (WAT) is the primary site for lipid storage and plays an important role in maintaining whole-body lipid homeostasis. Dietary omega-3 polyunsaturated fatty acids (n-3 PUFA) are known to regulate WAT lipolytic rates and triglyceridemia in a metabolic state-dependent manner. Fasting-induced angiopoietin-like 4 (ANGPTL4) is a secreted protein from WAT known to influence lipemia through inhibition of lipoprotein lipase (LPL) and stimulation of WAT lipolysis. However, the relationship between dietary n-3 PUFA and WAT ANGPTL4 expression is not known. Here we show that dietary n-3 PUFA upregulate epididymal WAT (eWAT) ANGPTL4 expression following a 24h fast in C57BL/6J mice. Concomitantly, n-3 PUFA reduced markers of WAT lipolysis and liver triacylglycerol (TAG) accumulation, consistent with previous n-3 PUFA feeding studies in rodents. These results implicate ANGPTL4 as a potential mediator of the beneficial effects of n-3 PUFA on whole-body lipid homeostasis. We expect this work will prompt deeper investigation into the n-3 PUFA-ANGPTL4 relationship using time-course experiments and genetic animal models.

## 4.2 Introduction

WAT plays a central role in regulating whole-body lipid homeostasis in different metabolic states. In the fed state, when macronutrient availability is greater than energy demand, circulating TAG within chylomicrons are hydrolyzed by LPL to be stored in WAT. When energy demand exceeds macronutrient availability (i.e. fasted state), stored WAT TAG is hydrolyzed to non-esterified fatty acids (NEFA) via lipolysis to be distributed throughout the body for oxidation. Dysregulation of WAT lipid handling contributes to the development of dyslipidemic diseases including non-alcoholic fatty liver disease (NAFLD), type-2 diabetes (T2D), and cardiovascular disease (CVD).

ANGPTL4 is a WAT-derived protein secreted into circulation with fasting [65,67,77]. ANGPTL4 functions to transiently induce hyperlipidemia through simultaneous inhibition of LPL [70,80,81] and stimulation of WAT lipolysis [92–94]. As such, ANGPTL4 has been implicated in the development of dyslipidemic disease. Humans possessing inactivation variants in *ANGPTL4* have hypolipidemic phenotypes [78,85–87,89,118] that are associated with protection from coronary artery disease [78,87,88,118,119] and T2D [88,118–120].

It is well documented that dietary n-3 PUFA, particularly long-chain eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), reduce circulating TAG through a variety of mechanisms, including enhanced LPL activity [40] and reduced WAT-derived NEFA flux to the liver [54]. Animal models have shown that consumption of n-3 PUFA in a low-fat diet suppresses WAT lipolysis and reduces plasma TAG concentrations in comparison to lard-fed controls [51,58,59].

Given the potential mechanistic overlap between n-3 PUFA and ANGPTL4-mediated regulation of WAT lipid handling, surprisingly little research has investigated the relationship between dietary n-3 PUFA and WAT ANGPTL4 expression. In mouse cardiac and skeletal

muscle, n-3 PUFA have been shown to regulate ANGPTL4 gene expression [102,103,111]. *In vitro* work in rodent cardiomyocytes and hepatoma cells suggest that long-chain n-3 PUFA induce ANGPTL4 gene expression greater than shorter-chain PUFAs [102,114], which was evident in cultured primary adipocytes in our preliminary experiments (unpublished data). Since ANGPTL4 is highly expressed in WAT [67], the purpose of this study was to determine if different dietary n-3 PUFA regulate the expression of WAT ANGPTL4 in fed and fasted states.

## 4.3 Materials and Methods

### 4.3.1 Animals

#### 4.3.1.1 Care and Feeding

Animals were individually housed in shoebox cages in a temperature and humidity-controlled room (22 ± 2˚C, 60% humidity) on a 12:12-h light-dark cycle. At three weeks of age, 60 male wild type C57Bl/6J mice were weaned onto one of three modified AIN93G diets for eight weeks (n=20/diet). Food consumption and body weight was measured biweekly and weekly, respectively. Prior to euthanization, ten mice on each diet were deprived of food for 24 hours (fasted) beginning at the start of the light cycle. Blood was collected by cardiac puncture immediately following anesthetization with isoflurane. Mice were euthanized by decapitation. Animal protocols were approved by the University of Guelph Animal Care Committee in accordance to the requirements of the Canadian Council of Animal Care.

#### 4.3.1.2 Experimental Diets

Mice were given *ad libitum* access to one of three isocaloric (4.0 kcal/g) low-fat (16% kcal from fat) AIN93G-modified diets (Research Diets, New Brunswick, NJ, USA) for 8 weeks containing either 6.625% lard w/w (L diet; 0% ALA, 0% EPA, 0% DHA), 6.625% flaxseed oil w/w (F diet; ~37% ALA, 0% EPA, 0% DHA), or 7% menhaden oil w/w (M diet; 1.1% ALA, ~10% EPA, ~7% DHA) (Table 1). The L and F diets were supplemented with 0.375% ARASCO oil w/w to match the amount of arachidonic acid present in the M diet.

#### 4.3.1.3 Intraperitoneal glucose tolerance tests

Mice underwent intraperitoneal glucose tolerance tests (IGTT) six weeks-post weaning. Following a six hour fast, mice received an intraperitoneal weight-adjusted (2g/Kg) bolus injection of D-glucose. Blood glucose concentrations were obtained from tail vein blood at

baseline and 15, 30, 45, 60, 90, 120 minutes post-injection using a handheld glucometer (Abbott Freestyle Lite).

#### 4.3.1.4 Blood plasma collection

Needles were flushed with EDTA immediately prior to whole blood collection by cardiac puncture. Blood was inverted four times in EDTA coated microcentrifuge tubes (Sarstedt, Montreal, QC, Canada) and incubated at room temperature for 30 minutes. Following 15 minutes of centrifugation at 2000 RCF at 4˚C, upper-layer plasma was collected and immediately stored at -80˚C.

### 4.3.2 Gene expression

#### 4.3.2.1 RNA extraction

Total RNA was isolated from eWAT using TRIzol pre-treatment and the RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada), which included a DNase digestion step. The concentration of RNA was determined using a Nanodrop 2.0 (Fisher Scientific, Waltham, MA, USA). Only samples with sufficient RNA concentration for cDNA synthesis and having purity ratios (260/230 and 260/280) between 1.8-2.2 were used.

#### 4.3.2.2 qPCR

Primers were designed using the Roche online Universal Probe Library Assay Design Centre and sequence specificity was confirmed using NCBI Primer-BLAST. Total RNA (0.5 µg) extracted from eWAT was used to synthesize cDNA with the High-Capacity Reverse Transcription kit (Life Technologies, Burlington, ON, Canada). qPCR was performed on a Bio-Rad CFX-96 system using 0.025µg cDNA and SsoFast Evagreen Supermix (Bio-Rad Laboratories, Mississauga, ON, Canada), as previously described [111]. *Nono* was used as a

housekeeping gene and had Ct values with a coefficient of variance <3% across experimental groups.

### 4.3.3 Protein extraction and expression

Adipose tissue samples were homogenized in a 3-fold dilution of NP-40 cell lysis buffer (Fisher Scientific, Waltham, MA, USA) supplemented with phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Millipore Sigma, Etobicoke, ON, Canada). Homogenates were centrifuged at 4°C for 15 minutes at 1500 RCF and protein concentrations of collected infranatant was determined using a bicinchonic acid assay. Equal amounts of protein were heat denatured for 5 min at 95°C and separated by polyacrylamide gel electrophoresis on 10% or 15% resolving gels. Proteins were transferred to nitrocellulose membranes at 100V and subsequently blocked in Tris buffered saline-0.1% Tween 20 (TBST) supplemented with 5% nonfat dry milk with gentle agitation for 1 h at room temperature. Membranes were incubated in primary antibody overnight at 4°C with gentle agitation. All primary antibodies were diluted in TBST supplemented with 5-20% BSA or 5% nonfat dry milk. Primary antibodies ATGL (no. 2439), phospho Ser-660 HSL (no. 4126), phospho Ser-563 HSL (no. 4139), phospho Ser-565 HSL (no. 4137), and total-HSL (no. 4107) were purchased from Cell Signaling Technology (Danvers, MA, USA) and used at a dilution of 1:1000. Primary antibodies α-tubulin (ab7291; 1:10,000 dilution) and ANGPTL4 (sc-373762; 1:100 dilution) were purchased from Abcam (Toronto, ON, Canada) and Santa Cruz Biotechnology (Mississauga, ON, Canada), respectively. Membranes were incubated in TBST 5% nonfat dry milk supplemented with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories, Mississauga, ON, Canada) at a dilution of 1:3000 for 2 h at room temperature. Protein bands were detected with Western Lightning Plus-ECL (Perkin Elmer) and imaged using a FluorChem HD2 Imaging System (ProteinSimple, San Jose, CA, USA). Relative band intensities were normalized to

respective internal loading control (α-tubulin or total-HSL) and subsequently quantified using AlphaView (Alpha Innotech Software (Alpha Innotech Software, San Leandro, CA, USA).

### 4.3.4 Biochemical analyses

#### 4.3.4.1 Liver TAG

Quantification of TAG in liver was performed as described [122]. Briefly, frozen tissue was homogenized in 2:1 chloroform:methanol and gently agitated overnight at 4˚C. Following the addition of 1mL of 4mM $MgCl_2$, samples were centrifuged for 1 h at 1000 RCF and 4˚C. The aqueous organic phase was removed, evaporated overnight, and resuspended in a 3:2 butanol:triton-x114 solution. A commercially available colorimetric kit (Millipore Sigma, Etobicoke, ON, Canada) was used to determine TAG concentrations.

#### 4.3.4.2 Plasma analyses

All plasma analyses were conducted using commercially available kits according to manufacturer guidelines. Plasma TAG concentrations were determined using a Triglyceride Colorimetric Assay Kit (Cayman Chemical, Ann Arbour, MI, USA). Plasma glucose concentrations were determined using a Glucose Assay Kit (Abcam, Cambridge, MA, USA). Plasma NEFA concentrations were measured using the HR Series NEFA-HR (2) kit (Wako Diagnostics, Mountain View, CA, USA). Plasma glycerol was determined using the Free Glycerol Determination Kit (Millipore Sigma, Etobicoke, ON, Canada).

### 4.3.5 Statistical analyses

Body weight and food intake were analyzed by repeated measures two-way ANOVA. Glucose tolerance test area under the curve (AUC) was analyzed by one-way ANOVA between the three diet groups. All other measures were analyzed by two-way ANOVA between the six experimental groups. Post-hoc Tukey's multiple comparison test assessed pairwise

comparisons for all measures. Statistical significance was determined at $p<0.05$ using GraphPad Prism 7 (GraphPad Software Incorporated, La Jolla, CA, USA.).

## 4.4 Results

### 4.4.1 Anthropometric characteristics

Mice fed the F and M diets gained more weight than mice fed the L diet over the 8 weeks of feeding (Figure 2A) and tended to consume more food than mice fed the L diet (Figure 2B). Despite differences in body weight, feed efficiency (Figure 2C) and glucose tolerance (Figure 2D) were not different between diet groups.

### 4.4.2 Fasting-induced metabolic changes

A 24h fast reduced body weight (Figure 3A), liver weight (Figure 3B), plasma glucose concentrations (Figure 3D), and plasma TAG (Figure 3E) regardless of diet. Plasma NEFA (Figure 3F) and liver TAG (Figure 3C) were increased in the fasted state with all diets.

### 4.4.3 State-dependent regulation of lipid homeostasis

In the fasted state, there was no effect of diet on liver weight (Figure 3B) or plasma glucose concentrations (Figure 3D). In comparison to the L diet, mice fed the F diet did not lose as much weight following the fast, while there was a trend for weight retention in mice fed the M diet ($p=0.15$) (Figure 3A). Concentrations of plasma NEFA trended to be lower with the F diet compared to the L diet ($p=0.085$) and were significantly lower with the M diet (Figure 3F). Concentrations of liver TAG were significantly lower in fasted mice on the F and M diets (Figure 3C).

In the fed state, mice on the M diet had lower liver weight (Figure 3B) than the L diet, and lower plasma TAG (Figure 3E) than both the L and F diets.

### 4.4.4 N-3 PUFA alter markers of WAT lipid handling

Some markers of lipid handling in eWAT appear to be regulated by n-3 PUFA in different metabolic states. For genes related to the synthesis of TAG, fasting generally reduced the expression of *Dgat*, and significantly so in mice on the M and L (p=0.07) diets in comparison to mice on the same diets in the fed state (p=0.07) (Figure 4A). Similarly, fasting significantly reduced *Dgat2* expression for all diets (Figure 4B). Conversely, fasting generally increased the expression of *Pck1* (Figure 4C), a marker of re-esterification. Fasting significantly increased *Pck1* expression in mice on the F diet in comparison to fed mice on the F diet, but this was not the case for mice on the L or M diets. Expression of *Lpl*, a marker of TAG uptake into WAT, was significantly higher in mice on the F and M diets than the L diet in the fasted state (Figure 4D).

For markers of WAT lipolysis, fasting increased *Atgl* gene expression in mice on the F and M diets, but not the L diet in comparison to fed counterparts (Figure 4E); however, no changes in ATGL protein expression with diet or metabolic state was observed (Figure 5A). Fasting reduced *Hsl* expression in mice on the L diet, but not the F and M diets (Figure 4F). Additionally, mice on the M diet had significantly less *Hsl* gene expression than mice on the L diet in the fed state (Figure 4F). Fasting generally decreased protein expression of inactivation site p565 (Figure 5B) and increased expression of HSL activation sites p563 (Figure 5C) and p660 (Figure 5D). However, no significant differences between diets on HSL protein activation were observed (Figure 5B-D).

### 4.4.5 N-3 PUFA regulation of ANGPTL4

*Angptl4* eWAT expression was not different between diets in the fed state (Figure 6A). In contrast, fasted mice on the M diet exhibited ~2.5 fold greater *Angptl4* expression compared to those on the L diet (Figure 6A). Fasted mice on the F diet had intermediate *Angptl4* expression between M ($p=0.21$) and L ($p=0.11$) diets (Figure 6A). Fasting generally increased full length (~50kDa) ANGPTL4 protein expression; however, no significant diet effects were detected (Figure 6B). With the same ANGPTL4 primary antibody, a second band at ~25kDa was detected. There was a general effect of diet on the ~25kDa band; however, no differences were found between the diets (Figure 6C).

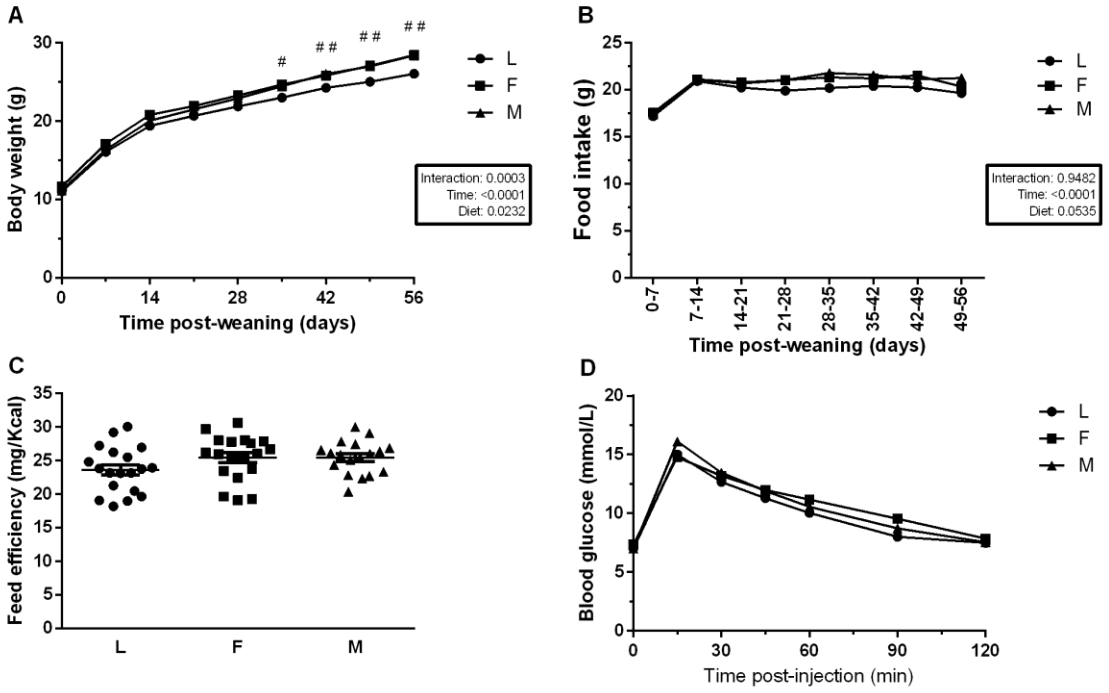


**Figure 2. Anthropometric characteristics.** Total body weight (A; n=19) and food intake (B; n=19) measured weekly and biweekly respectively throughout the eight-week feeding period. Total feed efficiency calculated as a ratio of body weight gain to calories consumed (C; n=19). Time course of blood glucose concentrations of tail vein blood following IGTT (D, n=14-16). Data presented as means, and error bars represent SEM (C). # indicates statistical difference compared to the L diet ($p<0.05$).

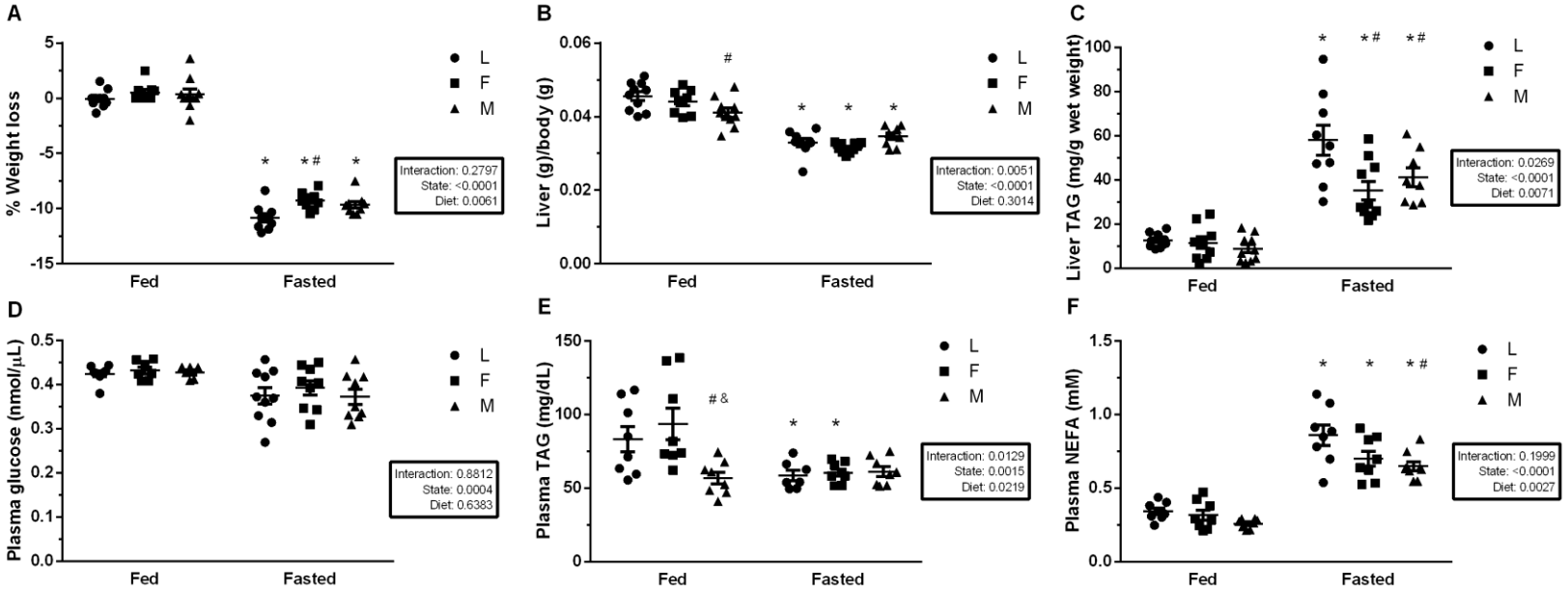


**Figure 3. Metabolic state-dependent changes in metabolism and lipid homeostasis.** Change in body weight in the 24 hours before sacrifice calculated as the difference between initial and final weight divided by the initial weight (A; n=9-10). Total liver weight normalized to final body weight (B; n=9-10). Concentrations of liver TAG (C; n=8-10), plasma glucose (D; n=7-10), plasma TAG (E; n=7-8), and plasma NEFA (F; n=7-8). Data presented as means ±SEM. * indicates a difference for a specific diet between the two metabolic states. Within a metabolic state, # indicates a difference from L diet and & indicates a difference from the F diet ($p<0.05$).

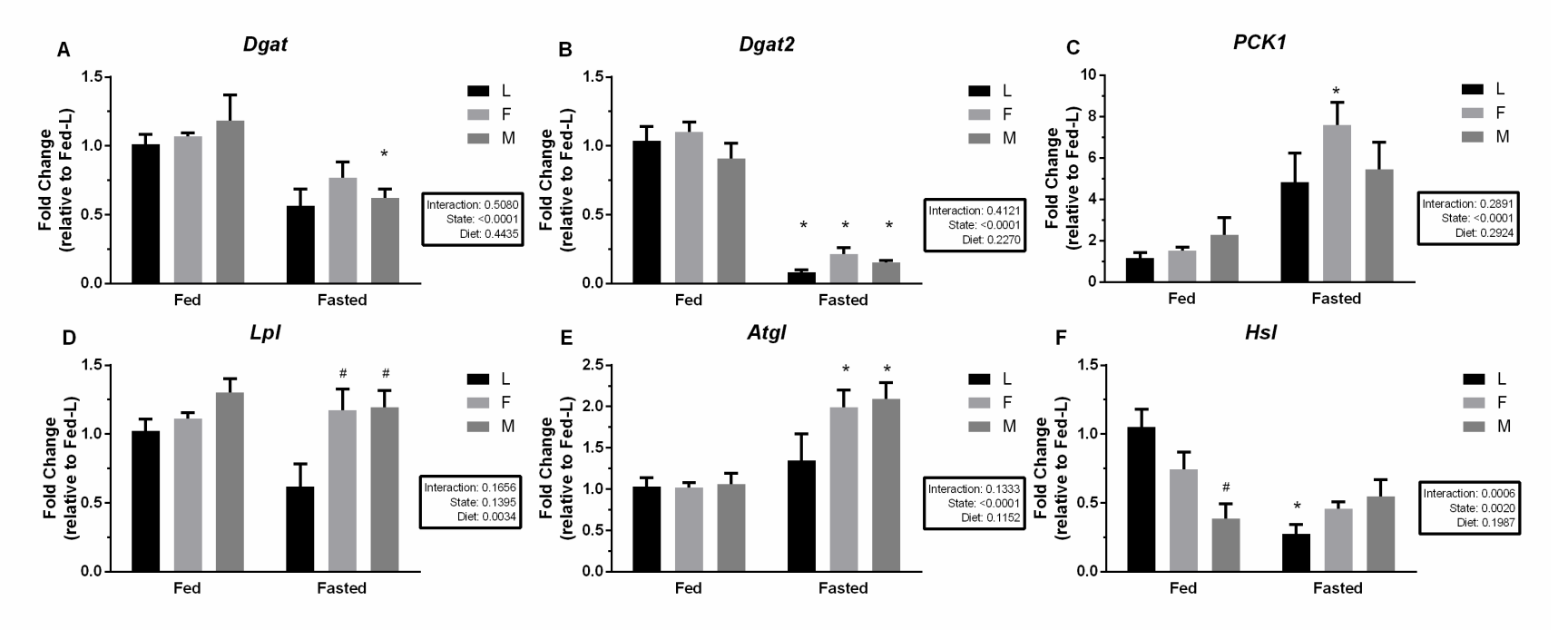


**Figure 4. N-3 PUFA regulation of WAT gene expression.** eWAT gene expression of *Dgat* (A), *Dgat2* (B), *Pck1* (C), *Lpl* (D), *Atgl* (E), and *Hsl* (F). Data are presented as mean fold changes ±SEM relative to the Fed-L group (n=6-7/experimental group). * indicates a difference for a specific diet between the two metabolic states, and # indicates a difference from L diet within a metabolic state ($p<0.05$).

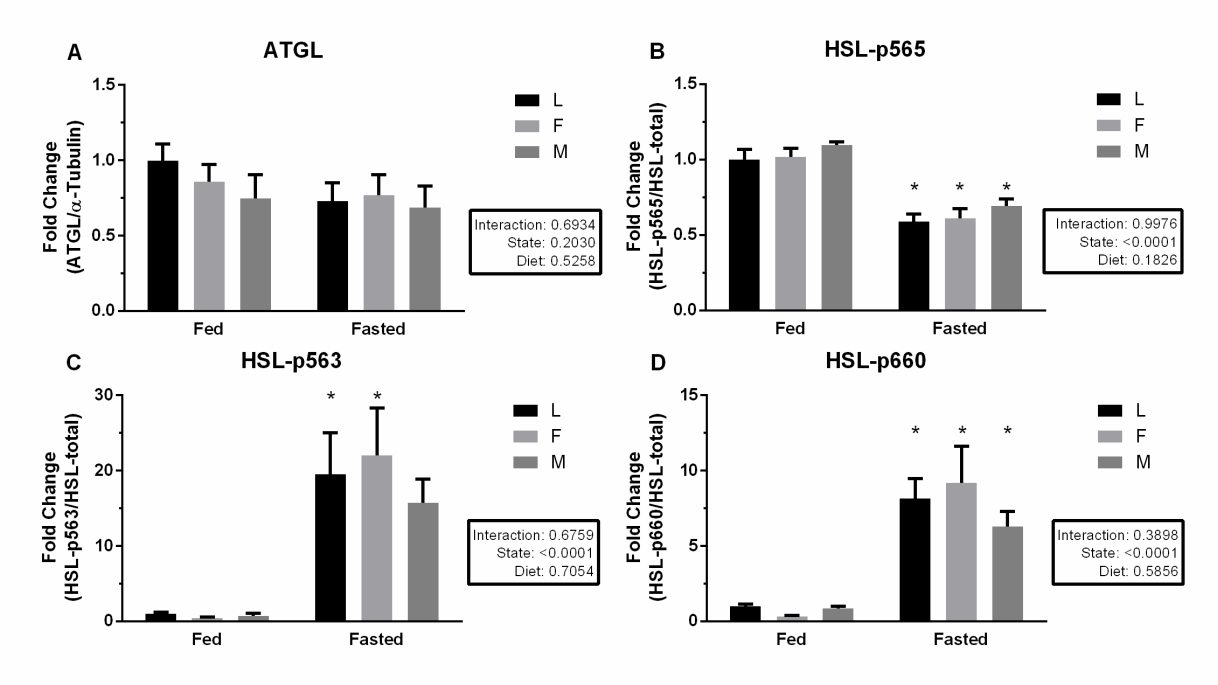


**Figure 5. N-3 PUFA regulation of WAT protein expression.** eWAT protein expression of ATGL normalized to α-Tubulin (A). Protein expression of HSL inhibitory p565 (B), and activation sites p563 (C) p660 (D) normalized to HSL-total expression. Data are represented as mean fold changes ±SEM relative to the Fed-L group (n=7/group). * indicates a difference for a specific diet between the two metabolic states ($p<0.05$).

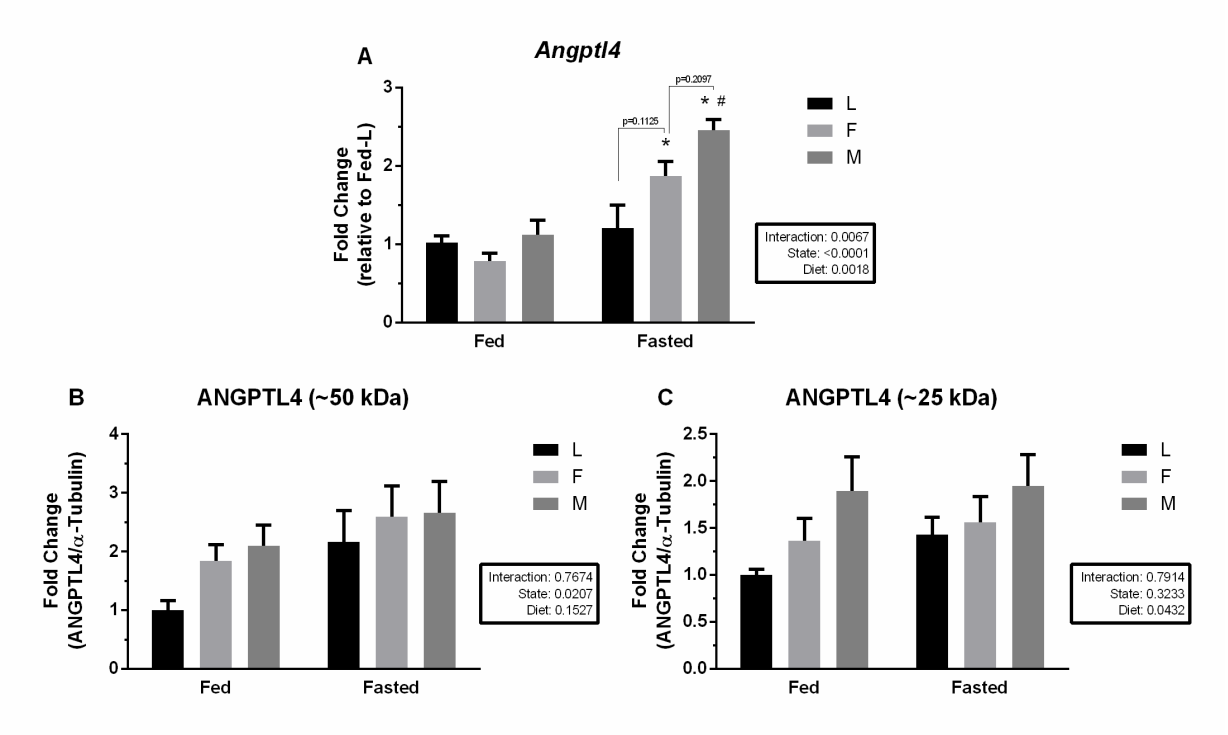


**Figure 6. N-3 PUFA regulation of ANGPTL4.** eWAT gene expression (A) and protein expression of the ~50kDa (B) and ~25kDa (C) bands of ANGPTL4 normalized to α-Tubulin. Data a represented as mean fold changes ±SEM relative to the Fed-L group * indicates a difference for a specific diet between the two metabolic states, and # indicates a difference from L diet within a metabolic state ($p<0.05$).

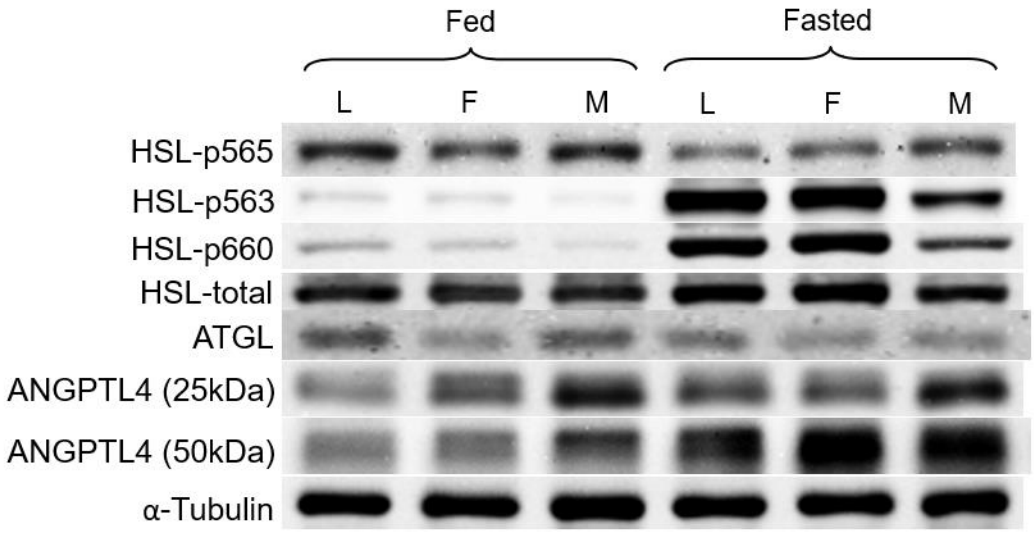


**Figure 7. Representative Western blots.** Equal amounts of protein were run on 10-15% resolving gels and transferred onto nitrocellulose membranes. One of eight representative blots is shown for each primary antibody. ATGL and ANGPTL4 were normalized to α-Tubulin from the same gel. HSL phosphorylation sites were normalized to HSL-total from the same gel.

## 4.5 Discussion

The present study investigated the influence of different dietary n-3 PUFA on WAT ANGPTL4 expression and lipid handling. Here we show that a diet containing EPA and DHA upregulated *Angptl4* gene expression in the fasted state, concomitant with reduced plasma NEFA and liver TAG concentrations, which might be attributed to reduced lipolytic activity. These findings suggest that ANGPTL4 may be implicated in the lipid regulatory properties of n-3 PUFA.

A well-established effect of long-chain n-3 PUFA is a reduction in circulating TAG levels [40]. However, most studies are conducted in a post-prandial state or following an overnight fast [123]. In the current study, we found that n-3 PUFA reduced plasma TAG only in the fed state. This aligns with findings in humans that n-3 PUFA consumption reduces circulating TAG in the fed state [63,123], in part due to enhanced chylomicron clearance [63,124]. N-3 PUFA mediated reduction of plasma TAG in the fed state may be of significance as non-fasting TAG has been inversely associated with all-cause mortality [125]. The current study also showed that in comparison to lard-fed mice, n-3 PUFA reduced plasma NEFA concentrations in the 24h fasted state, but not the fed state. Studies in rats have shown n-3 PUFA mediated reduction in plasma NEFA in comparison to lard-fed controls; however, the effect was observed in the first 3 hours following a meal but not following a 6-12 hour fast [50,51,58].

Elevated circulating TAG-rich lipoproteins and NEFAs result in the accumulation of lipid in the liver [37,126]. N-3 PUFA were previously reported to reduce liver TAG [38], an effect attributed in part to reduced lipid supply via elevated LPL hydrolysis and reduced WAT lipolysis [54]. The present study found that n-3 PUFA fed mice had reduced liver TAG concentrations in the fasted state, which might be explained by reduced plasma NEFA in the fasted state and reduced

plasma TAG in the fed state. Reductions in plasma NEFA may have had a dominant effect on reduction of liver TAG, as ~60% of their composition are WAT-derived NEFA [37].

ANGPTL4 is transcriptionally activated by ligand-bound PPAR isoforms binding to the PPAR response element [68]. N-3 PUFA and their derivatives are natural ligands of PPAR and together have been shown to upregulate ANGPTL4 transcription in muscle, liver, and intestinal cells [95,102,103,114]. However, little research has studied the effects of long-chain n-3 PUFA on adipocytes [112]. Administration of ALA by gavage increased *Angptl4* gene expression in cardiac muscle, which was abolished in PPARδ/β-/- mice [102,103]. Similarly, in liver cells and myocytes, treatment with oleic acid, linoleic acid, and ALA have been shown to increase *Angptl4* gene expression [95,102]. Interesting, treatment of liver cells with DHA increased *Angptl4* gene expression greater than oleic and linoleic acids [114], suggesting that the length and/or degree of saturation influences *Angptl4* transcription. We show that diets containing EPA and DHA upregulated eWAT *Angptl4* expression to a greater extent than mice fed lard. Of particular interest, we showed that the F diet rich in ALA, the precursor of EPA and DHA, had intermediate effects on *Angptl4* expression to that of the EPA/DHA rich diet. This suggests that: 1) longer chain n-3 PUFA have a greater effect on *Angptl4* expression, and/or 2) the intermediate effects of ALA may reflect the poor conversion efficiency of ALA to EPA/DHA [127].

Based on existing evidence of ANGPTL4 functionality, increased abundance of ANGPTL4 in circulation would lead to reduced LPL activity and increased plasma TAG, as ANGPTL4 overexpression [71,72,75] and deficiency [70,71,76–81] studies *in vivo* demonstrate ANGPTL4 inhibits LPL mediated TAG hydrolysis. The current study did not find that the n-3 PUFA-mediated increase in eWAT ANGPTL4 gene expression in the fasted state occurred concomitantly with increased plasma TAG. Additionally, based on whole-body ANGPTL4

overexpression [72,92] and deficiency [93,94] studies, an increase in ANGPTL4 expression would be expected to increase WAT lipolysis and NEFA release. However, a recent study investigating WAT-specific deletion of ANGPTL4 showed an enhancement of WAT lipolysis *in vivo* and *ex vivo* [80], suggesting that WAT-derived ANGPTL4 does not stimulate lipolysis. In the current study, the n-3 PUFA increase in eWAT *Angptl4* gene expression occurred concomitant with reduced plasma NEFA and minor, but statistically insignificant, reductions in HSL activation; suggesting that n-3 PUFA lead to an inhibition of lipolysis.

The discrepancy between eWAT *Angptl4* gene expression and markers of ANGPTL4 function in the present study might be explained by the lack of statistically significant differences in eWAT ANGPTL4 protein concentrations between the different diet groups. The primary ANGPTL4 antibody used in the current study was raised against the N-terminal domain. This antibody detected two distinct bands; a ~50kDa band and a ~25kDa band. Previous work has shown that the ~50kDa band represents the full-length form of ANGPTL4, and the ~25kDa band represents the oligomerized dimer of the N-terminal coiled-coil domain (~12kDa) following post-translational cleavage of the full-length protein [66,68,69,95]. We found that fasting generally increased the expression of full-length ANGPTL4, which is expected based on previous findings [67], and that there was a general diet effect on the expression of the ~25kDa band. While it appeared that the expression of both bands was highest in mice fed the M diet, no statistically significant differences were detected. This is notable since the protein concentration of ANGPTL4 may be more reflective of ANGPTL4 activity than gene expression. It may also be attributed to the length of fast, as any appreciable diet effect on ANGPTL4-mediated changes in plasma TAG may be washed out after 24 hours of food restriction.

The length of fast may also explain why fasting did not result in an increase in *Angptl4* gene expression in mice fed the lard diet. WAT *Angptl4* gene expression time course experiments in response to fasting have not previously exceeded 12 hours in length [77,98]. Since *Angptl4* mRNA turns over rapidly [84,98], it is possible that transcriptional rates begin to fall with extended fasting, which has been shown after 12 hours of fasting in eWAT [77]. Therefore, the current results would suggest n-3 PUFA sustain/preserve *Angptl4* expression better than a lard diet. This may align with findings in humans showing that fish oil infusion following an overnight fast prevented the insulin-mediated reduction in circulating ANGPTL4 during a six-hour hyperinsulinemic euglycemic clamp [114]. Alternatively, the lack of fasting-induced changes in lard-fed animals may also be due to their lower body weight compared to n-3 PUFA-fed mice, as ANGPTL4 expression has been positively associated with obesity in mice [65]. However, this seems unlikely as mice used in the current study were not obese, and it would not provide an explanation for the difference in ANGPTL4 expression between mice fed the F and M diet because they had similar weights. Another possible explanation may be that fed mice had *ad libitum* access to food until sacrifice, making it difficult to determine when food was last consumed. This implies that at the time of sacrifice, some “fed” mice may have just consumed food, while others may be in a post-prandial state. Since eWAT *Angptl4* transcription is induced as early as two hours into a fast [77], mice in the fed groups may have had inadvertent *Angptl4* expression similar to that of the fasted group. Future studies should consider this in their experimental design.

Collectively, our results provide new insights into the metabolic state-dependent mechanisms by which n-3 PUFA function to regulate lipid handling and the expression of WAT ANGPTL4. We expect this will prompt further research investigating the relationship between

WAT ANGPTL4 expression and n-3 PUFA in time course experiments using genetic animal models.

## 4.6 Acknowledgments

We thank Dr. Sander Kersten for his ANGPTL4 expertise and supply of ANGPTL4-/- plasma. Additionally, we thank Logan Townsend for his technical expertise with liver TAG analyses.

# 5 Integrative Discussion and Conclusions

## 5.1 Integrative Discussion

This thesis investigated how dietary n-3 PUFA regulate ANGPTL4 expression in WAT. Following a 24-hour fast, mice fed diets containing EPA and DHA had higher eWAT *Angptl4* expression than lard-fed mice, while mice fed flaxseed oil (rich in ALA) had intermediate expression levels. The n-3 PUFA induced increase in *Angptl4* might contribute to the concomitant reductions in plasma NEFA and liver TAG concentrations. However, there are several potential limitations associated with the study presented in Chapter 4. The subsequent discussion outlines additional research that will help to fully characterize the relationship between n-3 PUFA, ANGPTL4 expression, and lipid homeostasis.

### 5.1.1 Investigating the essentiality of ANGPTL4 in the n-3 PUFA regulation of lipid homeostasis

This thesis proposes that ANGPTL4 may play a role in the n-3 PUFA-mediated reduction in liver TAG and plasma NEFA in the fasted state, or reductions in plasma TAG in the fed state. Since the findings in Chapter 4 are not causative, further research is required to determine if changes in ANGPTL4 expression are required for the lipid regulatory benefits of n-3 PUFA. To accomplish this, several ANGPTL4 deficiency models could be used. For example, mice deficient in ANGPTL4 could be fed diets similar to those used in Chapter 4 (Table 1) to determine if the lipid-related outcomes seen with n-3 PUFA in wild-type mice are lost. Further, mice with a deletion of ANGPTL4 specifically in WAT [80] could be used to determine if WAT-derived ANGPTL4 is required for any n-3 PUFA mediated changes in lipid homeostasis. Additionally, the current study suggests that increased ANGPTL4 expression may be implicated in n-3 PUFA-mediated reduction in WAT lipolysis. *Ex vivo* explants of eWAT from mice lacking

ANGPTL4 expression in WAT treated with n-3 PUFA could mechanistically determine if n-3 PUFA suppression of WAT lipolytic activity is dependent on ANGPTL4 expression.

N-3 PUFA dependency on ANGPTL4 could also be investigated in humans. Participants prescreened for the single nucleotide polymorphism rs116843064 in the *ANGPTL4* gene could be randomized in a clinical trial to receive either n-3 PUFA supplements or a control. If n-3 PUFA supplementation fails to reduce circulating NEFA or TAG levels in those possessing the inactivation variant p.E40K in comparison to control individuals, it may suggest that functional ANGPTL4 is required for n-3 PUFA mediated changes in lipid homeostasis. Collectively, these studies would generate further evidence to position ANGPTL4 as a mediator of n-3 PUFA induced changes in WAT lipid homeostasis, thereby representing a mechanism by which n-3 PUFA prevents development of dyslipidemic disease.

### 5.1.2 The effects of individual n-3 PUFA on ANGPTL4 regulation

Based on previous literature and the results of the study presented in Chapter 4, it is likely that individual n-3 PUFA have different potencies for *Angptl4* induction. In the diets used in Chapter 4, ALA comprised the majority of total n-3 PUFA content in the F diet (Table 1). While limited, endogenous desaturation and elongation of ALA into EPA/DHA occurs in the liver and adipose. Therefore, it is difficult to determine if the intermediate effects of the F diet on *Angptl4* gene expression are attributed to ALA or downstream products (EPA and/or DHA). On the other hand, the majority of the n-3 PUFA content in the M diet corresponds to equivalent amounts of EPA and DHA. It is therefore not possible to comment on the individual effects of EPA or DHA on eWAT *Angptl4* expression based on this mouse feeding study. This is a commonly cited limitation in the literature when using menhaden oil (or fish oil) in a study, as it prevents investigations into the comparative functions of EPA and DHA. Our preliminary data in primary

rat adipocytes treated with 100μM individual fatty acids showed that EPA increased *Angptl4* expression greater than DHA (Figure 8). To confirm the differential regulation of *Angptl4* gene expression by EPA and DHA, one approach would be to block endogenous FADS2-mediated interconversion between these fatty acids. Specifically, cultured adipocytes could be pretreated with a pharmacological FADS2 inhibitor before treatment with ALA, EPA, or DHA. *In vivo*, mice deficient in FADS2 could be fed diets supplemented with EPA or DHA. These proposed experiments would serve to isolate the individual effect EPA and DHA have on *Angptl4* gene expression.

### 5.1.3 Exploring the metabolic state effects of n-3 PUFA-ANGPTL4 regulation

The study presented in Chapter 4 used wild-type mice fed low-fat diets to examine the effect of dietary n-3 PUFA on eWAT ANGPTL4 expression in a healthy state. We observed an upregulation of ANGPTL4 expression concomitant with reductions in plasma NEFA and liver TAG concentrations. It would be valuable to study the relationship between n-3 PUFA and eWAT ANGPTL4 expression in a dyslipidemic state for several reasons: 1) eWAT ANGPTL4 has been shown to be upregulated in obese models [65], 2) elevated plasma NEFA due to hypertrophic adipocytes significantly contributes to the accumulation of TAG in the liver [37], and 3) n-3 PUFA prevent TAG accumulation in adipocytes with a HFD [129]. HFD induces whole-body dyslipidemia characterized by excessive accumulation of TAG in adipocytes and elevated rates of lipolysis. If n-3 PUFA similarly upregulate ANGPTL4 expression under a HFD context as it did in wild-type mice (Chapter 4), it is plausible that ANGPTL4 plays a role in the n-3 PUFA mediated maintenance of adipocyte lipid homeostasis. This is supported by the fact that whole-body overexpression of ANGPTL4 in rodents fed a HFD prevents lipid accumulation in adipocytes by inhibiting LPL-mediated uptake and stimulation of WAT lipolysis [72,74]. This suggests the upregulation of WAT ANGPTL4 expression with a HFD is a protective mechanism

by the adipocyte to prevent hypertrophy. While this may be beneficial for the adipocyte, the resultant increase in circulating NEFA and TAG may be detrimental for the body over time, as evidenced by increased deposition of lipid in the liver [72,74]. As n-3 PUFA are known to reduce liver lipid levels in humans with NAFLD [38], it seems unlikely that n-3 PUFA induced ANGPTL4 expression would directly lead to accumulation of lipid in the liver. This suggests that either: 1) ANGPTL4 does not mediate the effects of n-3 PUFA, or 2) transient n-3 PUFA induced ANGPTL4 expression is not equivalent to whole body ANGPTL4 over-expression models. Given that we show n-3 PUFA only increased ANGPTL4 expression in the fasted state, the latter explanation seems likely. This is because transgenic overexpression of ANGPTL4 would be constant, whereas n-3 PUFA induced expression may depend on the metabolic state.

Since the current study looked at only the fed or a 24-hour fasted state, the time course of n-3 PUFA induced ANGPTL4 expression in the fasted state should be investigated. In a similar study design to that presented in Chapter 4, mice from each diet group sacrificed at several time intervals (e.g. every 2-hours) throughout the 24-hour fast would help to resolve: 1) when in a 24-hour fast n-3 PUFA begin to increase ANGPTL4 expression in comparison to lard fed mice, and 2) when in a 24-hour fast n-3 PUFA induced ANGPTL4 expression peaks. This would provide mechanistic insight into how n-3 PUFA and ANGPTL4 interact to regulate lipid homeostasis.

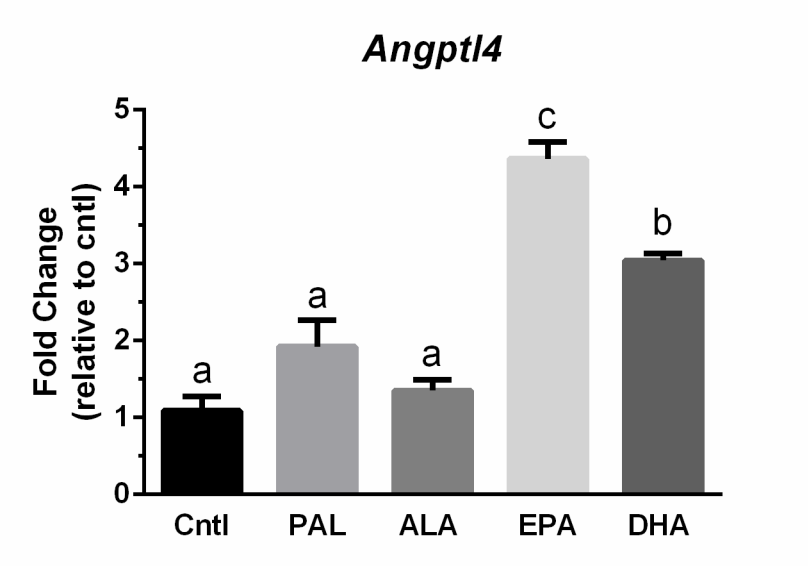


**Figure 8. Differential regulation of *Angptl4* by long chain n-3 PUFA.** The gene expression of *Angptl4* in primary rat adipocytes treated with 100µM of control, palmitic acid, ALA, EPA, or DHA for 48 hours. Pre-adipocytes were isolated from eWAT of Wistar rats by collagenase digestion and size filtration. Adipocytes were plated (~70,000 cells/cm$^2$) and allowed to grow to confluence while progressively being weaned off serum prior to differentiation. Differentiation was done using standard protocols in serum-free conditions. Fatty acid stock solutions were suspended in ethanol and then complexed with 2% BSA. The "Cntl" condition corresponded to ethanol + 2% BSA. Data is presented as mean ±SEM (n=6/treatment). Treatments sharing letters are not statistically different from each other, as determined by one-way ANOVA followed by post-hoc Tukey's multiple comparison test.

## 5.2 Conclusion

This thesis presented evidence that dietary n-3 PUFA, specifically EPA and DHA, upregulate ANGPTL4 expression in the fasted state and not the fed state. Therefore, ANGPTL4 may be implicated in the concomitant reductions in plasma NEFA and liver TAG concentrations. However, studies investigating the essentiality of ANGPTL4 in the lipid regulatory properties of dietary n-3 PUFA are required. Additionally, time course experiments of n-3 PUFA induced ANGPTL4 expression are needed to better understand this relationship and advance our understanding of how n-3 PUFA exert their beneficial effects.

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# APPENDIX

**Table 1. Chemical and nutrient composition of experimental diets**. Reported diet composition is the ingredient analysis information provided by Research Diets.

| Diet | Lard Diet (L) (D16090607) | | Flaxseed Oil Diet (F) (D12041404) | | Menhaden Oil Diet (M) (D12041407) | |
|---|---|---|---|---|---|---|
| | gm% | kcal% | gm% | kcal% | gm% | kcal% |
| Protein | 20 | 20 | 20 | 20 | 20 | 20 |
| Carbohydrate | 64 | 64 | 64 | 64 | 64 | 64 |
| Fat | 7 | 16 | 7 | 16 | 7 | 16 |
| Total | | 100 | | 100 | | 100 |
| kcal/g | 4.0 | | 4.0 | | 4.0 | |
| | | | | | | |
| Ingredient | | | | | | |
| Casein | 200 | 800 | 200 | 800 | 200 | 800 |
| L-Cystine | 3 | 12 | 3 | 12 | 3 | 12 |
| Corn Starch | 397.486 | 1590 | 397.486 | 1590 | 397.486 | 1590 |
| Maltodextrin 10 | 132 | 528 | 132 | 528 | 132 | 528 |
| Sucrose | 100 | 400 | 100 | 400 | 100 | 400 |
| Cellulose, BW200 | 50 | 0 | 50 | 0 | 50 | 0 |
| Lard | 66.25 | 596 | 0 | 0 | 0 | 0 |
| Flaxseed Oil | 0 | 0 | 66.25 | 596 | 0 | 0 |
| Menhaden Oil | 0 | 0 | 0 | 0 | 70 | 630 |
| ARASCO (40%AA) | 3.75 | 34 | 3.75 | 34 | 0 | 0 |
| t-Butylhydroquinone | 0.014 | 0 | 0.014 | 0 | 0.014 | 0 |
| Mineral Mix S10022G | 35 | 0 | 35 | 0 | 35 | 0 |
| Vitamin Mix V10037 | 10 | 40 | 10 | 40 | 10 | 40 |
| Choline Bitartrate | 2.5 | 0 | 2.5 | 0 | 2.5 | 0 |
| FD&C Yellow Dye #5 | 0.025 | 0 | 0.025 | 0 | 0 | 0 |
| FD&C Red Dye #40 | 0 | 0 | 0.025 | 0 | 0 | 0 |
| FD&C Blue Dye #1 | 0.025 | 0 | 0 | 0 | 0.05 | 0 |
| Total | 1000.05 | 4000 | 1000.05 | 4000 | 1000.05 | 4000 |
| Total, gm | 70 | | 70 | | 70 | |
| Fatty Acid (gm/4000 kcal) | | | | | | |
| C12:0 | 0.1 | | 0 | | 0 | |
| C14:0 | 0.8 | | 0 | | 4.8 | |
| C15:0 | 0.1 | | 0 | | 0.3 | |
| C16:0 | 13.3 | | 3.2 | | 10.4 | |
| C16:1, n-7 | 0.9 | | 0 | | 6.8 | |
| C17:0 | 0.2 | | 0 | | 0.3 | |
| C18:0 | 7.3 | | 2.4 | | 1.8 | |
| C18:1, n-9 | 22.2 | | 12.4 | | 6.7 | |
| C18:2, n-6 | 16.4 | | 10.9 | | 1.3 | |
| C18:3, n-3 | 1.0 | | 36.6 | | 1.1 | |
| C18:4, n-3 | 0 | | 0 | | 2.2 | |
| C20:0 | 0.1 | | 0 | | 0.1 | |
| C20:1, n-11 | 0.4 | | 0 | | 1.1 | |
| C20:2, n-6 | 0.5 | | 0 | | 0.1 | |
| C20:3, n-6 | 0.1 | | 0 | | 0.3 | |
| C20:4, n-6 | 1.7 | | 1.5 | | 1.5 | |
| C20:5, n-3 | 0 | | 0 | | 9.9 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| C22:0 | 0 | | 0 | | 0.1 | |
| C22:1, n-9 | 0.1 | | 0.1 | | 0.2 | |
| C22:4, n-6 | 0 | | 0 | | 0.1 | |
| C22:5, n-3 | 0.1 | | 0 | | 2.0 | |
| C22:6, n-3 | 0 | | 0 | | 7.2 | |
| C24:0 | 0 | | 0 | | 0.4 | |
| C24:1, n-9 | 0 | | 0 | | 0.1 | |
| Total | 65.4 | | 67.0 | | 62.6 | |
| Saturated (g) | 22.0 | | 5.6 | | 18.2 | |
| Saturated (%) | 33.6 | | 8.4 | | 29.1 | |
| Monounsaturated (g) | 23.6 | | 12.4 | | 14.9 | |
| Monounsaturated (%) | 36.1 | | 18.6 | | 23.9 | |
| Polyunsaturated (g) | 19.8 | | 48.9 | | 29.5 | |
| Polyunsaturated (%) | 30.3 | | 73.1 | | 47.0 | |
| n-6 (gm) | 18.2 | | 12.4 | | 3.2 | |
| n-3 (gm) | 1.1 | | 36.6 | | 22.3 | |
| n-6/n-3 ratio | 17.1 | | 0.3 | | 0.1 | |