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Allometric scaling of dietary linoleic acid on changes in tissue arachidonic acid using human equivalent diets in mice

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Abstract

The ability to extrapolate nutritional intervention data from experimental rodent models to humans requires standardization of dietary design. The inability to translate the level of nutrients from animal models to humans has contributed to contradictory findings between species. It is hypothesized that dietary linoleic acid (LA) promotes chronic and acute diseases by enriching tissues with arachidonic acid (AA), its downstream metabolite. However, levels of LA in rodent diets are notoriously erratic making interspecies comparisons unreliable. Therefore, the ability to extrapolate the biological effects of dietary LA from experimental rodents to humans necessitates an allometric scaling model that is rooted within a human equivalent context. To determine the physiological effect of dietary LA on tissue AA, a mathematical model for extrapolating nutrients based on energy was designed to mimic human equivalent doses. *C57BL/6J* mice were divided into 9 groups fed a background diet equivalent to that of the US diet (including LA, ALA, AA, EPA, DHA) with supplemental doses of LA (up to 2.3x) or AA (up to 5x). Changes in the phospholipid fatty acid compositions were monitored in plasma and erythrocytes and compared to data from humans supplemented with equivalent doses of LA or AA. Increasing dietary LA had little effect on tissue AA, while supplementing diets with AA significantly increased tissue AA levels, recapitulating results from human trials. Thus, interspecies comparisons for dietary LA between rodents and humans can be achieved when rodents are provided human equivalent doses based on differences in metabolic activity as defined by energy consumption.

List of abbreviations:

ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid

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Introduction

The consumption of dietary fat and the incidence of cardiovascular disease, inflammation, and cancer have been evolving concepts of nutrition since Burr and Burr's 1929 discovery of fat as a essential component of the diet [1]. Various animal studies have examined the role of polyunsaturated fatty acids (PUFAs), particularly n-6 fatty acids, for their functional role in tissues and for their implication in disease prevention. However, the ability to extrapolate nutritional intervention data from experimental rodent models to humans remains a multifaceted problem. The inability to translate the level of nutrients from animals to humans along with the lack of standardization in dietary design, have contributed to contradictory results often observed between the two. Specifically, inappropriate background doses of linoleic acid (LA, 18:2n-6) have been proposed to benefit cancer, inflammation, and atherosclerosis [2-5]. Current research suggests a growing concern that overconsumption of LA, the precursor to arachidonic acid (AA, 20:4 n-6), is responsible for the increased predisposition to disease [6-7]. This study intends to verify a mathematical model for formulating rodent diets that will more closely mimic the typical human diet and human responses. The following questions will be addressed:

1. What is the biological impact of n-6 fatty acid, LA, as a metabolic precursor for AA?
2. What methodological improvements can be made in relevant dosing of n-6 fatty acids in rodent studies that will allow for standardization of dosing between animal models and human data across different research designs?

Part I

Overview

Abstract

In the absence of other n-6 PUFAs (including dietary AA), dietary LA is the sole contributor to tissue AA. Following consumption, LA is desaturated and elongated to AA. It is hypothesized that robust downstream metabolism of LA to AA benefits cancer, inflammation, and atherosclerosis; however dietary composition and dosing of n-6 PUFAs among animal research is highly variable. As such, rodent dietary design is of immense importance in the field of nutrition research, not only in determining this relationship, but as a way to assess the translational ability of individual dietary constituents, through appropriate dosing of nutrients, to physiological effects observed in humans consuming similar levels of nutrients. Currently, there have been only a few attempts to standardize the levels of nutrients in a rodent diet to have human relevance. A random search of the literature supports this lack of standardization in dietary design and dosing of LA. The concern governing the heterogeneity of rodent dietary designs ultimately makes it challenging to extrapolate the findings to humans with a level of confidence. Therefore, investigating appropriate scaling measures for interspecies comparisons is critical. Establishing a model that provides standardization and a hierarchical approach to designing diets seems pressing. If the ultimate goal is to translate findings to humans, identifying allometric scaling models is fundamental for all disciplines.

REVIEW OF THE LITERATURE

1.0 Overview of Omega-6 Fatty Acids

Description

Fatty acids are denoted by straight chain hydrocarbons possessing a carboxyl (COOH) group at one end and a methyl (CH_3) group at the other. N-6 fatty acids are polyunsaturated lipid compounds that consist of a hydrocarbon skeleton and classified by the location of the first double bond on the sixth carbon from the methyl end [8]. Each double bond is separated by a methyl group, a process termed methylene-interrupted, which provides structural stability but the potential for oxidation. LA and AA are the two predominant n-6 PUFAs. Since humans lack the ability to synthesize fatty acids with double bonds greater than the ninth carbon from the carboxyl end; these PUFAs are considered essential, indispensible, and must be included in the diet [8-9]. Additionally, LA is an essential component of ceramides involved in maintenance of the transdermal water barrier of the epidermis and both LA and AA are required as precursors for eicosanoid production [10-11].

Molecular Structure

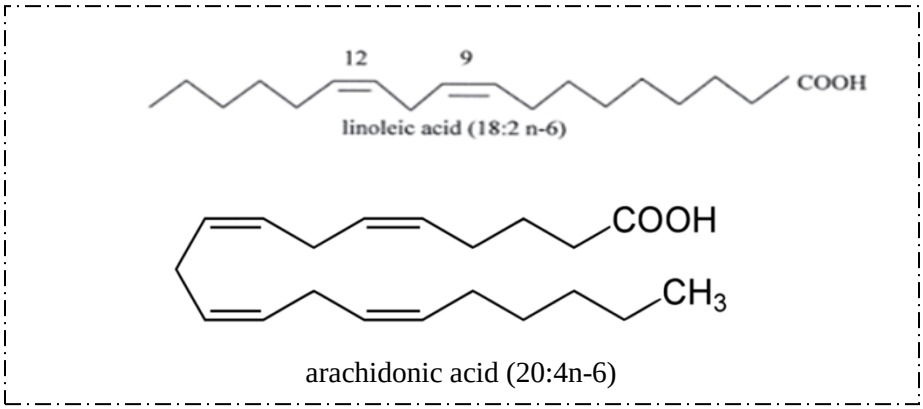


Figure 1. The molecular structure of LA and AA, derived from Laura Jones's Thesis, 2005.

Dietary Sources

Foods rich in LA include most vegetable oils (i.e., sunflower, safflower, evening primrose, sesame, corn and soy) [8, 12], animal products (i.e., chicken, pork, steak) and dairy products (i.e., eggs, cheese) [13]. AA is found exclusively in animal products (i.e., beef ribeye, chicken, eggs, pork loin, turkey, fish) [14]. A comparison of food sources high in LA and AA is depicted in **(Table 1)**.

Western Consumption of LA

Dietary LA is the most abundant PUFA in Western consumption [13-15]. Median intakes of LA are held respectively between 11–17 g/day for women and men (contributing to ~4-10% energy based on a 2,000 kcal/d diet) [15-17]. Notably, LA recommendations are 10 to 100 times higher than alpha-linolenic acid (ALA) and other essential long-chain fatty acids [15], **(Table 2).** Hence, LA represents 90% of n-6 fatty acids in the Western diet [12]. The rise in LA-containing oils (i.e., corn, sunflower, safflower, soybean) and the shift in the n-6 to n-3 ratio mirrors the growth of the innovative vegetable industry [18].

Table 1. Dietary sources of linoleic acid and arachidonic acid [14]

Food Item	Linoleic acid	Arachidonic acid
	mg fatty acid/100g cooked food	
Chicken thigh	838 ± 27	121 ± 6
Chicken breast	272 ± 17	83 ± 8
Egg (whole)	1884 ± 183	239 ± 21
Beef rib eye	320 ± 51	77 ± 14
Canned tuna	25 ± 2	33 ± 4

LA Deficiency

The severity of dermatitis, skin barrier dysfunction, and cutaneous inflammation are associated with the depletion of essential n-6 PUFAs at levels required in the diet; observed in both rodent and human studies [1, 19]. Dietary consumption of LA at 1-3% of energy is sufficient for AA synthesis and turnover [12, 20]. Biochemical and dysfunctional consequences of n-6 PUFA deprivation in rodents under normal n-3 PUFA concentrations, further explain the essentiality in a tissue specific manner (i.e. reductions in brain, heart, liver, etc) [21]. Interestingly, n-6 PUFA deprivation is associated with increased concentrations of n-3 PUFAs and monounsaturated fatty acids (MUFAs) for maintenance of adequate membrane fluidity and body function [21]. Membrane saturation requirements of both n-3 and n-6 PUFAs are therefore essential for establishing an appropriate dietary design in both humans and rodents.

Availability to Human Tissues

Dietary consumption of LA above 2-3% of energy in humans is not reportedly accompanied by an increase in AA plasma/serum and erythrocytes lipid levels [12, 22-25]. In human peripheral tissues, increasing dietary LA from 4% to 18% of energy resulted in lower levels of AA in the cholesterol esters and phospholipid fractions of hyperlipemic subjects [26]. Adam and colleagues found significant increases in LA plasma cholesterol esters and HDL-phosphatidylcholine when LA was supplemented up to 20% of energy compared to a fat-free diet. Tissue levels of AA significantly decreased in the high dietary LA groups (4% and 20% of energy) while increasing in the fat-free group [20]. These results are indicative of the influential power of dietary LA to increase tissue LA, but not AA in this human study. Interestingly, neither group given differential levels of dietary LA (4% and 20% of energy) increased prostaglandin

(PG) synthesis [20]. These findings are in agreement with the concept that desaturation of fatty acids regulates cell fluidity and does not favor increased PG formation via the AA cascade when fed adequate amounts of LA (at least 1-3% of energy) [12]. Nonetheless, the relationship between LA and AA in rodents has yet to mimic the responses observed in humans. A perspective look at LA in the diet of both rodent and human studies may provide more of a consensus on the biological effects of LA-derived foods and the relationship to acute and chronic diseases.

Table 2. Suggested adequate intake and recommended intakes of n-6 polyunsaturated fatty acids versus current estimated consumption in the United States [27]

	Linoleic Acid (LA) 18:2n-6	Linolenic Acid (ALA) 18:3n-3
National Academy of Sciences (AI)		
(DRI, 2002)	6.4% energy	0.6% energy
Men	17 g/d @ 2400 kcal	1.6 g/d @ 2400 kcal
Women	12 g/d @ 1700 kcal	1.1 g/d @ 2400 kcal
Recommendations [27]		
All adults	5-8% energy	0.75% energy
	~15 g/d @ 2000 kcal	~1.7 g/d @ 2000 kcal
Current Mean Estimated Intakes	6.3% energy	0.75% energy
[28]	14 g/d @ 2000 kcal	1.7 g/d @ 2000 kcal

Western Consumption of AA

AA is arguably the most important fatty acid associated with membrane phospholipids and is involved in eicosanoid production, and cellular proliferation and differentiation [14-15]. While dietary LA is a contributor to tissue AA, variations in LA consumption, specifically LA intakes above 2% of calories in humans, were reported to have only minimal alterations on changes in the content of AA in phospholipids of human neutrophils [24]. Additional human clinical trials report dietary variations of LA from (3%-20% of energy) did not significantly increase tissue AA concentration [22, 25]. A human tracer study that examined median fractional turnover rate of LA, found as little as 0.3% to 0.6% of LA was converted to AA [29]. In rodents, the in vivo conversion of supplemented C18 fatty acids (i.e., LA, ALA) to downstream end-products (i.e., AA, DHA), measured by stable isotopes, was found less efficient when compared to C20 fatty acids, possibly indicating the control of Δ-6 desaturase in regulating metabolic activity when dietary PUFA intake is high [30].

The consumption of AA-derived foods, however, significantly impacts tissue AA concentrations in both animal studies [31-33] and human trials [34]. Estimated median intake of AA in humans is 100-500 mg/day (~0.9 % energy), respectively, notably lower than LA. Due to the unreliability of food composition databases for highly unsaturated fatty acids (HUFAs), the accuracy of daily AA intake is still unclear [13].

Digestion, Absorption, and Transport

The composition of cellular membranes are regulated by both genetic and environmental factors, in particular, dietary fat profiles [35]. Dietary n-6 fatty acids are consumed in the form of triglycerides and phospholipids found in food. The majority of dietary lipids ~98% are found as triacylglycerols (TAG) which contain three fatty acids esterified to a glycerol backbone [36]. N-6 fatty acids, LA and AA, are located in the sn-2 position of the triglyceride molecule [11]. While these fatty acids have a specialized location on the TAG they still follow the same digestion, absorption, and transport as all other lipid components.

Digestion of dietary lipids begins in the stomach where short and medium chain fatty acids are hydrolyzed by gastric lipase, an enzyme secreted by the gastric mucosa [36-37]. Gastric lipase works in the stomach under acidic conditions to cleave medium chain fatty acids into diacylglycerol (DAG) and free fatty acids (FFAs) [37]. Some short and medium chain fatty acids can be readily absorbed through the stomach, while longer fatty acids must be broken down in the small intestine. Fat digestion continues through the action of pancreatic lipase and colipase at the interface of the oil and aqueous phase [37]. Working mainly on the sn-1 and sn-3 position of the TAG, pancreatic lipase releases 2-monoacylglycerol (MAG) and FFAs, important for further emulsion by the action of bile salts [37]. The presence of bile in the lumen of the intestine increases the solubility of lipolytic products in the aqueous intestine to emulsify the intestinal contents for formation of micelles [38]. The components of the micelle are transported into the enterocyte for reesterification into TAGs, phospholipids, and cholesterol [36]. Translocation across the membrane occurs by both passive diffusion and carrier-mediated transfer [38]. After the reassembly within the enterocyte, triglycerides and phospholipids are combined with apolipoproteins to form chylomicrons which can then enter the lymphatic

circulation as TAG-rich lipoproteins. Once in the circulation, lipoprotein lipase works similarly to pancreatic lipase, in that it hydrolyzes the TAG-rich lipoproteins into one monoacylglycerol and two FFAs for tissue utilization [36]. Glycerol and chylomicron remnants are subsequently cleaved by the liver.

Biomarkers of Dietary Fat

Lipid metabolism is highly altered by variations in dietary intake that work to maintain a stable membrane composition. Daily fluctuations in fatty acids reflect the competitive nature of n-3 and n-6 PUFAs, thus providing useful information about average tissue maintenance [39]. The assessment for fatty acid composition in various compartments such as adipose tissue (long-term), erythrocytes (medium-term), and plasma or serum (short-term) enable stable readings over a given period of time [40]. A common fatty acid biomarker for human and rodent studies is the erythrocyte (RBC) membrane. RBC membranes vary with environmental factors, oxidative stress, and diet [41]. It is held that erythrocyte fatty acid measurements more accurately correlate with dietary intake than do measurements of serum FA's [41]. While RBC turnover (120 day lifespan) provides a better marker of long-term fat intake compared to plasma, phospholipids, TAGs, this view is not consistently held across all research [42]. Alternatively, plasma lipid fractions provide the most accurate dietary reflection of short-term fatty acids, particularly those fatty acids after a meal [42]. It has been observed that maximal saturation of RBCs and plasma TAGs was achieved within 14 days [42]. This is important in determining the length of a human or animal dietary intervention study to ensure that fatty acids have reached their maximal

threshold in tissues. Additionally, choosing the most appropriate biomarker accessible for interspecies comparisons is an essential component of experimental design.

1.1 Conversion of LA to Downstream Metabolites

It is well established that mammals lack the ability to desaturate fatty acyl chains beyond the Δ 9 carbon, therefore making consumption of precursor long-chain fatty acids, such as LA, an essential component of the diet [43]. Following consumption, LA can participate in oxidization, incorporation into membrane phospholipids, storage in TAGs, or convert to long-chain metabolites [13]. PUFAs, both n-3 and n-6 are synthesized by membrane-bound desaturase families; Δ-6 and Δ-5 desaturases [43]. The conversion of LA to its downstream metabolites is first catalyzed by Δ-6 desaturase, also known as the rate-limiting step for enzymatic PUFA biosynthesis [35]. All desaturases require molecular oxygen for addition of intermittent double bonds along the substrate in a way that maintains the methylene-interrupted distribution [44]. Elongases which are enzymes responsible for adding two carbons to the carboxylic end of the fatty acid, help regulate metabolic fate and degree of unsaturation. The Δ-5 desaturase which places a double bond at the Δ-5 position of the 20-carbon fatty acid is responsible for the final conversion of LA to its preferred form as AA in tissues [43]. In humans, further desaturation of AA is limited by the body's preference to maintain a precise quantity of AA in cells and tissues [10] **(Figure 1).**

The majority of LA conversion to long-chain metabolites, with the help of elongases and desaturases, is presumed to take place in the endoplasmic reticulum [45]. However, biosynthesis of PUFAs is indeed a coupled process between the endoplasmic reticulum and β-oxidation in

peroxisomes during final conversion steps [46-47]. Intermediates can either be incorporated into phospholipids or become the substrates for further downstream conversion [16]. LA may additionally participate in low-density lipoprotein (LDL) clearance and decreased LDL production important in maintaining adequate cholesterol levels [27]. Importantly, LA appears to hold a membrane "threshold" requirement affected by various environmental (i.e. diet) and genetic factors (as reviewed by [27]). Furthermore, exaggeration of plasma and tissue fatty acid composition of specific fatty acids (i.e., AA, EPA, DHA) may result if LA levels fall below the threshold of essentiality (as reviewed by [27]). Additionally, LA and AA are important for eicosanoid signaling, pinocytosis, ion channel modulation, and gene expression regulation [43].

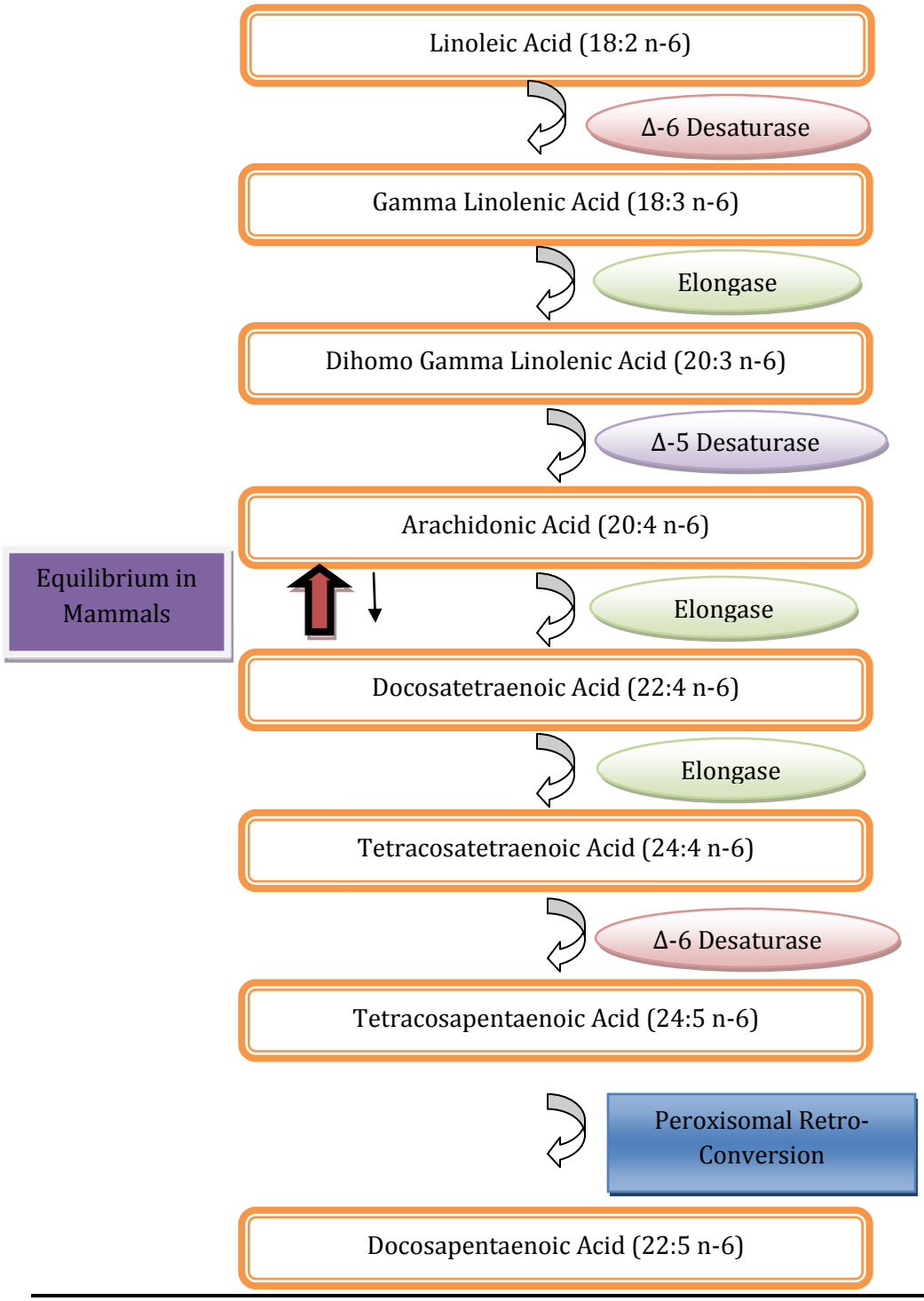


Figure 2. Omega-6 fatty acid metabolism

Metabolism of Arachidonic Acid

AA is considered one of the most abundant PUFAs involved in membrane phospholipids and production of eicosanoids [33]. The initial step in eicosanoid synthesis is translocation of phospholipase A_2 (PLA_2) to the cell membrane by a receptor-mediated influx of Ca^{2+} ions [48]. Hydrolysis by phospholipase A_2 (PLA_2) cleaves the ester bond of AA at the sn-2 position, generating a FFA and a lysophospholipid [49]. Free AA is a precursor for synthesis of PGs in the conversion through cyclooxgensase (COX) and lipoxygenase (LOX) pathways to generate eicosanoids (i.e. PGs and leukotrienes) involved in the inflammatory process [8, 48-49] **(Figure 2).** An imbalance between high intakes of LA and eicosanoid production through AA metabolism has been proposed to promote atherosclerosis, tumor growth, and immune-related pathologies [50]. Therefore, targeting tissue AA content for regulation of eicosanoid expression is a growing topic of interest [10, 33]. Like LA, there appears to be a membrane "threshold" requirement of AA in tissues [15]. However, dietary alterations of LA to control AA metabolism, as proposed by many authors, would require drastic changes in the Western diet (i.e. elimination of nearly all animal and plant products) [15]. Furthermore, dietary LA within the context of a typical human diet has not been found to increase the risk of acute and chronic disease states through AA metabolism [15, 51-52].

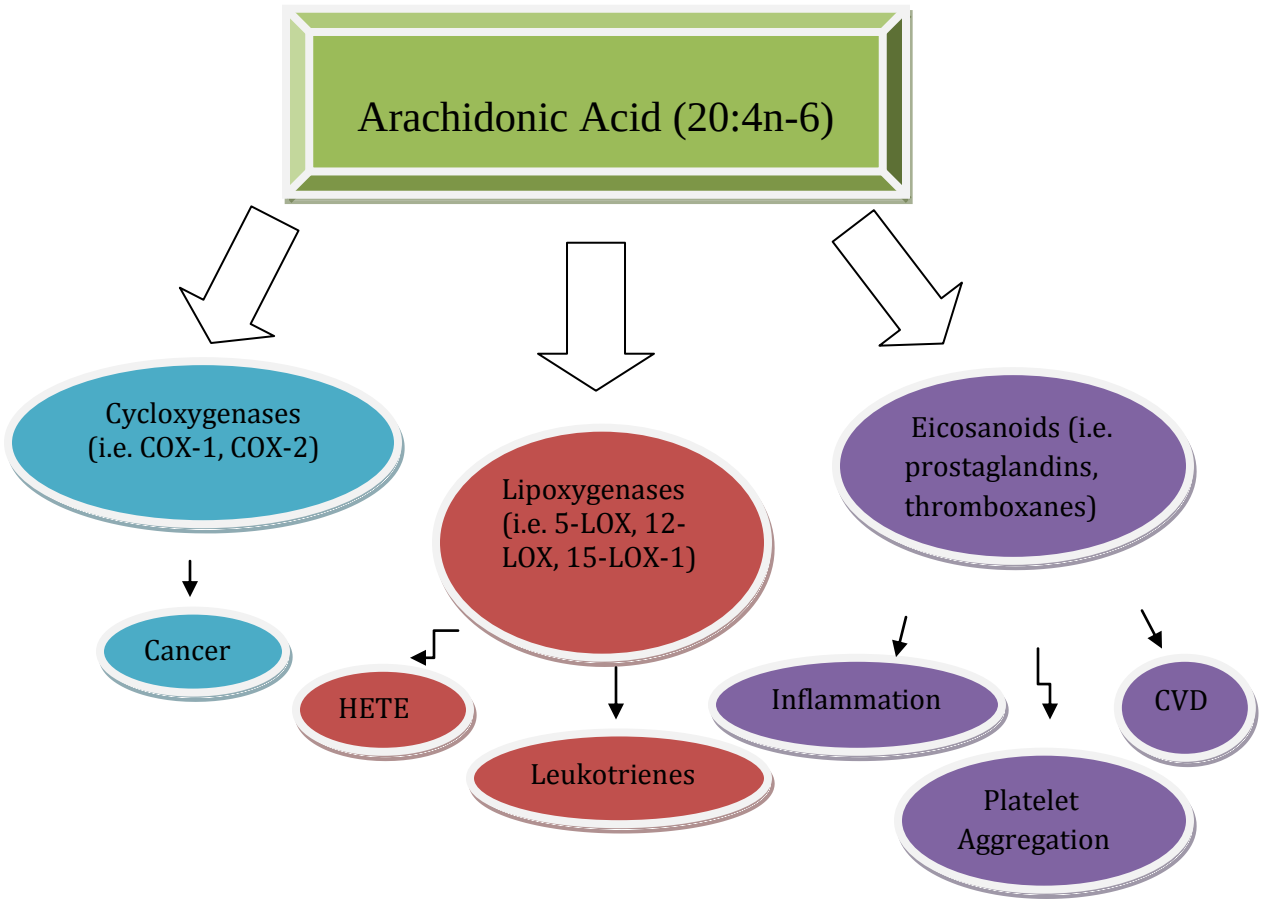


Figure 3. Downstream metabolism of AA

1.2 Overconsumption Concerns Governing LA

The role of n-6 PUFAs in disease prevention is an unrelenting topic of interest and controversy. Relative changes in the course of evolution have been shaped by a variety of genetic and environmental changes impacting the modern-day diet. Paleolithic dietary habits (45,000 years ago) were comprised mainly of fish, wild plants, and nuts and seeds that were much lower in total fat than the current Western diet; however, there is no available data on the health status of these predecessors [16, 28]. Moreover, the ratio of n-6 and n-3 fatty acids was once thought to be 1:1 [28, 53-54]. The increase in technological advancements and the food supply has shifted this ratio toward an increased consumption of n-6 fatty acids at the expense of n-3; proposed to be 15:1 and 20:1 [6, 11, 16, 54]. This shift, however, mirrors the growth of the innovative vegetable oil industry [28]. In all actuality, the mean ratio of n-6 to n-3 fatty acids in the United States is held respectively at ~9.8:1 [28]. Since it has been proposed that dietary fatty acids are a reflection of membrane fatty acid composition, considerable speculation of the n-6 to n-3 ratio have arisen for implication in various diseases [53]. The concern of increased dietary n-6 PUFAs lies in the production of AA, a central pathway for eicosanoid synthesis. Simopoulos and colleagues propose *"a diet rich in omega-6 fatty acids shifts the physiological state to one that is prothrombotic and proaggregatory, with increases in blood viscosity, vasospasm, and vasoconstriction and decreases in bleeding time* [54]." The common term for n-6 PUFAs, as consistently noted by many authors, is seamlessly interchanged with dietary LA. This generic term, while correct, negates the differential tissue specific effects of individual n-6 PUFA metabolites.

Higher dietary n-6 PUFAs is suggestive of greater antagonist effect on n-3 PUFA metabolism of α-linolenic acid (ALA) and reduced cardioprotection [54-55]. Additional

arguments propose lower LA intakes will produce a higher n-3 index for protection against coronary heart disease (CHD) [56] by reducing the exposure of eicosanoid metabolism and inflammation [55]. However, data strongly suggests a direct inverse association of decreased LA and increased CHD risk [57]. Interestingly, a study by Riediger and colleagues found a diet high in n-6 PUFAs, relative to n-3, actually reduced plasma TG levels comparable to baseline values in mice [58]. Nonetheless, membrane fatty acid composition is influenced by exogenous fatty acids and the balance of substrates in the diet [55, 59].

While the n-6 to n-3 ratio has indeed increased, targeting the consumption of LA may not be the answer. Kris-Etherton and colleagues argue that a variation in LA intake does not substantially affect tissue AA levels in humans, with only ~0.2% dietary LA actually converted to AA in tissues [60-61]. Additional support from rodents, found dietary LA to have little effect on altering AA hepatic phospholipids compared to the supplementation of dietary AA which significantly enriched AA tissue levels [62]. One possible explanation of dietary AA accumulation in mice may be a result of reduced oxidation to CO_2 ,with only 14% compared to 42% from dietary LA [62] or that the accumulation of dietary AA in mice may be subject to a dietary experimental design that lacks one or more essential fatty acids. Nonetheless, the debate on the overconsumption of LA in both rodent and human studies remains inconclusive.

1.3 Health Implications

Cardiovascular Health

Dietary profiles may provide one way for assessing the link of PUFA consumption and optimal heath. The American Heart Association supports an n-6 PUFA intake of at least 5-10% of energy for heart health (as reviewed by [60]). The 'consume less' n-6 theory (as reviewed by [6]) lies in the formation of atherosclerotic plaque initiated by the accumulation of LDL and pro-inflammatory cytokines that is believed to be driven by the competition of AA and EPA for metabolic conversion [8]. It is assumed that the link between PUFA consumption and cardiovascular disease (CVD) lies in eicosanoid regulation [8] and protection from oxidative damage [63]. While the n-3 index is an important marker for CHD there are other factors that contribute to cardioprotection [60]. Therefore, the antagonistic role of LA or AA with EPA does not appear to substantially lower the n-3 index [60]. Clearly this paradigm is complex and often misinterpreted.

A review of the literature by Harris [57] found most data in favor of increasing dietary LA to decrease the risk of CHD. In humans, a meta-analysis found that n-6 PUFA intake of 10-21% energy reduced the risk of CHD compared to individuals with lower intakes [57]. In fact, LA is found to be the most potent dietary fatty acid in reducing total cholesterol and LDL levels [64]. In a human study, feeding 25% of energy from LA over a 5 year span, there was found to be reduced atherosclerosis with no adverse side effects [65-66]. Salmeron and colleagues report that women with a mean intake of 7% of energy from LA had a significantly lower risk of type 2 diabetes compared to those consuming less than 4% of energy from LA [67]. There is evidence of higher LA intakes improving insulin resistance [68] and effectively lowering blood pressure [69]. While many human studies demonstrate the ineffectiveness of various dietary LA doses to

significantly increase cardiovascular risk [27, 70], the majority of animal research and several human researchers continue to advocate the relationship of n-6 PUFAs and increased susceptibility to disease [6, 50].

Inflammation

Similar to CVD, LA is proposed to have pro-inflammatory activities independent of its role as a precursor for AA synthesis [71-72]. The lack of consensus on an appropriate biomarker for inflammation has left many researchers at a loss for concise comparison [17]. Since, LA is a substrate for two metabolically active oxidation products (i.e. leukotoxin (LT), leukotoxin diol (LTD)), in vitro treatment of endothelial cells with LA, LT, or LTD was found to promote oxidative stress responses [73]. This study would imply that LA metabolism, in part, possesses pro-inflammatory capabilities. The interference of n-6 PUFAs on n-3 PUFA anti-inflammation is a well explored topic. In a prospective human cohort, it was found that consumption of n-6 fatty acids did not inhibit the beneficial effects of n-3 PUFAs, and taken together, higher intakes of both n-6 and n-3 PUFAs were associated with the lowest inflammation [74]. Similarly, a study by Liou and colleagues found no significant changes in inflammatory markers when comparing 4% to 12% of energy from LA [22]. In fact, Whelan and colleagues report that LA levels were significantly lower in tissues when AA was included in the diet [33]. Even when LA was increased by 55%, AA concentrations did not significantly change or significantly impact eicosanoid production [33].

In addition to assessing LA, dietary AA has been a central focus of the inflammatory process. Two human studies report no significant effect on inflammatory biomarkers with the addition of 1200 mg of AA/day maintaining a constant n-3 intake [75] or with the addition of

700 mg of AA/day [76]. In contrast, it was found that AA at ~2.4% of energy or, 11 grams respectively, significantly enhanced the production of PGE_2 in peritoneal macrophages of hamsters, similar to the results found in human studies [33]. Therefore, it is perceivable that AA also holds a specific threshold in tissues that is affected by other fatty acids present in the diet. Nonetheless, AA consumed at average human intakes of 180 mg/day, respectively, seems unlikely to create robust inflammatory stress that could potentiate chronic diseases.

Cancer

There is growing concern over the possible link between dietary fat, particularly animal fat, and cancer (i.e. breast, colon, intestinal, prostate, etc) [77-81]. Epidemiological studies have observed a linear relationship with increasing dietary fat and incidence of breast and colon cancer [79]. Like CVD and inflammation, much speculation has been placed on dietary LA consumption. Rogers and colleagues observed fewer colon tumors in animals fed 28% beef fat than the controls fed 15% corn oil (~9% energy LA), while Carroll and colleagues similarly found a lowered incidence of mammary tumors in animals fed beef fat versus corn oil [79, 82]. This would imply the pro-tumorigenic capability of dietary LA in the form of corn oil-based diets. Additional rodent studies have found the feeding of LA up to 14% of energy promoted the growth and progression of mammary tumors in mice [5]. However, in a follow-up study, no significant differences in tumor progression were found between mice fed the 23% corn oil diet (~14% of energy from LA) and mice fed the 5% corn oil diet (~3% energy of from LA) [83]. Buckman and colleagues demonstrated the direct stimulatory effect of LA (12% of energy) and oleate on the growth of mammary tumor cells; suggestive of an independent role of unsaturated

fatty acids and prostaglandin synthesis [2]. Conversely, Hubbard and colleagues found mammary tumorigenesis was enhanced by LA and was not dependent of oleic acid (18:1n-9) levels [4]. Two opposing roles of LA on mammary tumorigenesis were observed by Lane and colleagues. In one experiment, 20% corn oil (~12% of energy from LA) induced metastasis, however the second experiment found that 20% corn oil did not significantly affect mammary tumor incidence [2]. In examining lipid profiles of rodents fed corn oil, soybean oil, or palm oil, no consistent changes were observed in mammary tumor carcinogenesis [84]. Overall, the consensus on LA's role in mammary cancer remains inconclusive, most notably due to differences in experimental design.

The diets of rodents fed 20% menhaden oil (primarily n-3 fatty acids) versus 20% corn oil (~12% of energy from LA) were associated with significantly reduced pancreatic lesions [3]. Similarly, Roebuck and colleagues found that 20% corn oil (~12% of energy from LA) was associated with enhanced perimental pancreatic carcinogenesis in rats [85]. The anti-tumorigenic effect of n-3 PUFAs demonstrated in these studies point to the opposing role of n-6 and n-3 PUFAs as potent oncogenic precursors based on dietary factors alone. While n-3 PUFAs are known for their anti-inflammatory properties [86-87] , concluding n-6 PUFAs as carcinogenic promoters based on the doses used in these studies may not be accurate. In order to better establish the relationship between diet and disease in a rodent model, a properly scaled design that includes appropriate doses of nutrients, allometrically based on human DRIs, must first be implemented in the basic design.

The amount of LA in background dietary designs among animal research is highly variable. As such, the translation of data from rodents to humans of poorly designed experimental models has contributed to uncertainty among the literature, noted by many of these

studies. Therefore, designing rodent diets considering allometric scaling factors and the inclusion of all human equivalent dietary components may better pinpoint LA's specific role in cardiovascular disease, inflammation, and carcinogenesis.

1.4 Animal Model Research

Rodent Dietary Models

Currently, there are three main rodent diets used by research intervention studies. These diets include AIN76A: 5% corn oil (CO) (59% LA, 6.6% of energy); AIN93G: 7% soybean oil (SBO) (54% LA, 8.3% of energy); and AIN93M: 4% SBO (54% LA, 4.8% of energy) [88] **(Figure 4).** While these diets do provide most of the essential fatty acids, they appear to be inconsistent in levels of LA (based on % of energy). This is of particular interest in investigating the proposed role of LA-derived foods on enhanced risk for chronic and acute diseases. Notably, current human consumption of LA is held respectively at ~6 % of energy (7-10 % of energy) [10], but most rodent dietary intervention studies do not use human equivalent LA doses in the background diet. Rodent dietary designs utilizing an inappropriate dose of LA (i.e. deficient or excessive) that does not correspond to human consumption or designs lacking all essential fatty acids may potentially heighten or exaggerate the responses in tissues [10].

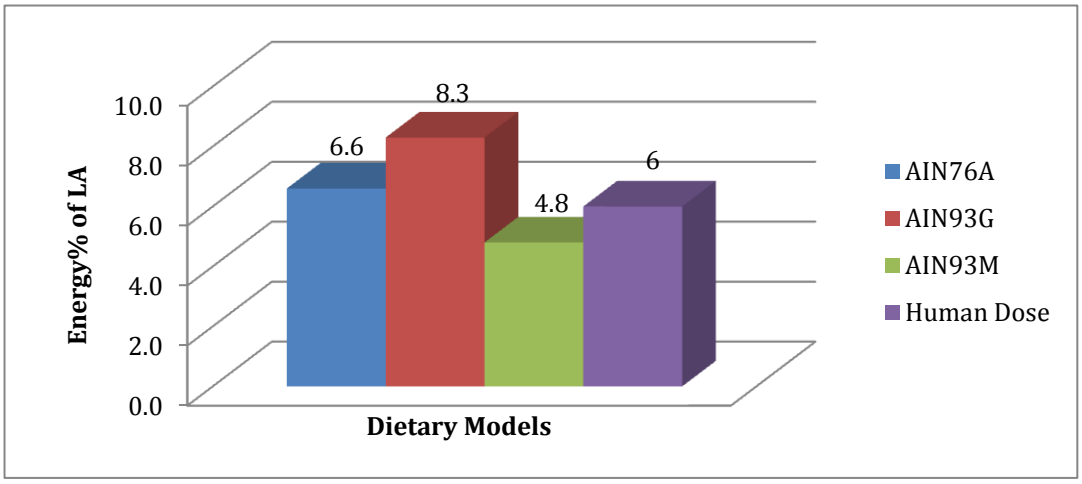


Figure 4.The discrepancy of LA doses of current rodent diets based on % of energy from LA compared to a human equivalent dose containing 6% energy from LA.

Rodent Designs Supplemented with LA

A collection of rodent studies interested in the response of LA supplementation on tissue fatty acid composition strikingly outline the variability in animal model experimental design **(Figure 5).** The array of varying initial and supplemented doses of LA, portray the inconsistency of animal dietary design. Additionally, the comparison to a human equivalent dose of ~6% of energy denotes the improper dosing in the background diet for interspecies comparisons. Rucker and Storms in 2002, propose that direct extrapolation to an adult human based on doses given to rodents may be in error by a factor of 10 or higher [89]. Consequently, the overestimation of nutrients for a smaller animal may lead to toxicities when extrapolated to a larger animal [89]. Therefore, using a standard scientific rationale for dosing may provide more consistent translatable findings.

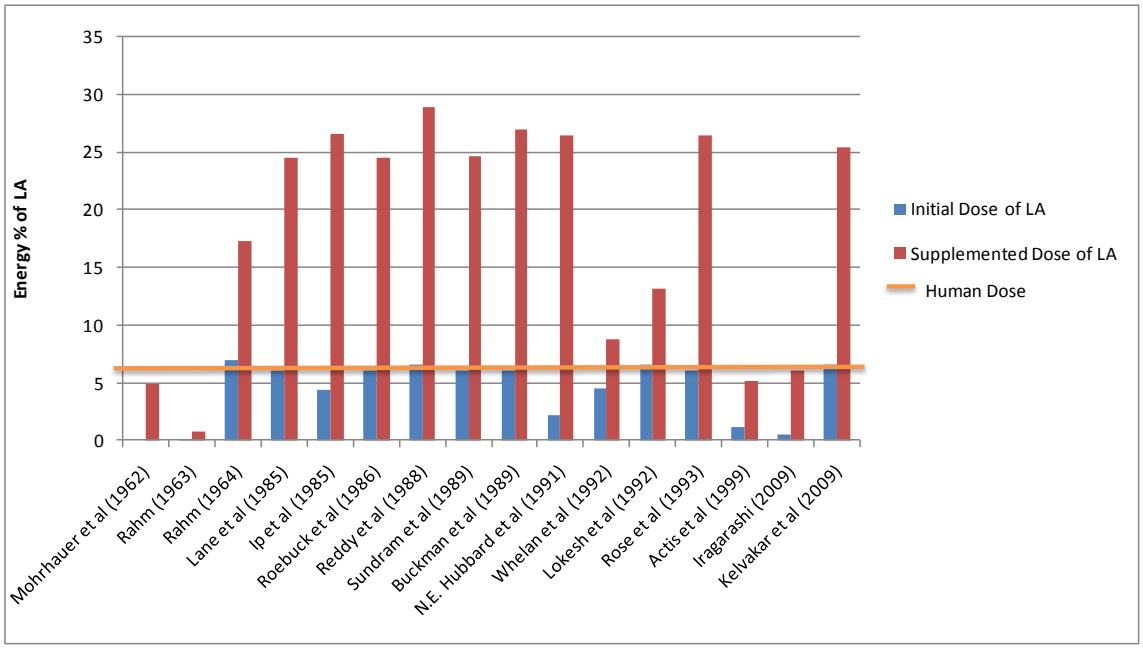


Figure 5. Comparison of a human equivalent dose (6% energy from LA) to initial and supplemented doses of LA in rodent dietary studies.

Table 3. The comparison of background and supplemented doses of % energy from LA and the rationale for experimental design are displayed among various rodent dietary studies.

Rodent Dietary Designs					
Study	**Background Energy% LA**	**Supplemented Energy% LA**	**Background Source of PUFA**	**Intended Outcome**	**Rationale**
Mohrhauer et al (1962)	0	0.009, 0.02, 0.05, 0.10, 0.18, 0.32, 0.61, 1.26, 1.79, 4.87	Fat-Free	To show the influence of various levels of LA, AA, and ALA esters upon FA composition in liver lipids of rats.	Based on previous studies comparing animals fed fat-free diets with those fed single levels of essential fatty acids.
Mohrhauer et al (1963)	0	0.08 up to 0.73 but Supplemented ALA at differing ratios	Fat-Free	To compare to a previous study feeding LA and ALA singly	Intake levels were chosen in the range of unstated ratios.
Rahm et al (1964)	0	0.28, 0.97, 1.97, 3.87, 7.57, 12.42, 17.26	Fat-Free	Determine the inhibition of linolenate metabolism by linoleate.	None
Lane et al (1985)	~5% Fat- Chow Diet (Wayne Lab Blox)- Distribution not indicated	6, 24.6 (Based on AIN76A)	Corn Oil	To demonstrate whether a diet high in PUFAs (corn oil) would enhance or promote DMBA induced mouse mammary tumorigenesis in mice.	Based the % energy from other studies showing that high corn oil diets change the FA composition in mammary cells.
Ip et al (1985)	~20% w/w Synthetic Fat Diet (~44% en)	4.4, 9.0, 14.3, 26.6	Coconut oil, Corn oil	To determine the requirement of essential fatty acid for di-methylbenz(a)anthracene-induced mammary tumorigenesis.	Based on other studies investigating LA as a tumor-promoter.
Roebuck et al (1986)	6 (AIN diet not specified)	2.5, 24.5	Corn oil, Coconut oil	To examine if the effect of high levels of unsaturated fats selectively stimulate carcinogen-induced foci.	Previous investigation found the high fat diets are comparable in their effects on rat growth of pancreatic lesions.
Reddy et al (1988)	6 (Based on AIN76A)	28.9 (CO group), Additional groups with CO+ Menhaden Oil	Corn oil, menhaden oil (EPA, DHA, AA, LA, oleic acid, palmitic acid)	To investigate the efficacy of varying amounts of mehaden oil and corn oil on colon carcinogenesis for determination of optimal dietary levels.	Based on previous studies looking at the combination of corn oil and mehaden oil.
Sundram et al (1989)	Rodent Chow (No specification)	24.6 (CO group), 23.8 (SBO group)	Corn oil, Soybean oil, Crude palm oil, Deodorized palm oil, Metabisulfite treated palm oil	To investigate the effect of different high palm oil diets on mammary carcinogenesis in female rats treated with DMBA.	None
Buckman et al (1989)	3 (Purina Mouse Chow)	12	Mainly safflower oil, Triolein, Coconut oil	To examine whether oleate, when added to a diet that promotes metastasis (12% enLA) would modulate the growth and metastasis of line 4526 mouse mammary tummors.	Based on studies in which mice were fed 12% energy from LA, they observed significantly more tumors.
N.E. Hubbard et al (1991)	Stock Diet (Purina Mouse Chow)	2.2, 13.2, 26.4	8:0, 10:0, 12:0, 14:0, 16:0, 16:1n-7, 18:0, 18:1n-7, n-9, 18:2n-6	To test whether increasing the dietary level of oleic acid can alter the level of 18:2n-6 -enhanced metastasis.	Based on a previous study, suggesting that 12% w/w (26.4% energy) from LA induced lung metastasis.
Whelan et al (1992)	4.4 (Prolab Chow Diet)	7.5	High oleic sunflower, Tripalmitin, 18:1n-9 (Oleic Acid Ethyl Ester)	To evaluate the effects of dietary AA on hepatic tissue FA composition compared to results with those obtained from matched animals on LA diets.	Based on previous studies looking at dietary AA while controlling for other FA's.
Lokesh et al (1992)	6	12	Corn oil, 14:0, 16:0, 16:1, 18:0, 18:1n-9	To investigate the use of lipids with different FA compositions as a potential fat source for parenteral and enteral diets.	Oils were selected for their wide differences in FA compositons. No rationale for doses.
Rose et al (1993)	6 (Based on AIN76A)	4.4, 17.6, 26.4	Safflower Oil, Coconut Oil	To determine the effect of 3 different levels of dietary LA intake on the growth of MDA-MB-435 cancer cells in mammary fat pads of nude mice.	Based on previous studies looking at high -fat diets on the growth of MDA-MB-435 human breast cancer cell line.

Rodent vs. Man

Comparing rodent data to human clinical trials results in an observable difference. In comparing the % change of AA with increasing LA (% of energy) between 8 rodent dietary studies [2, 21, 33, 62, 90-93], delineates the inconsistent response of AA when LA is provided at doses of 0.73% to 27% of energy **(Figure 6).** The differences in the background dietary composition of these 8 rodent models explain the erratic data. The % change in AA plasma phospholipids with increasing LA (% of energy) in human studies does not appear to portray the same LA to AA relationship observed in rodents **(Figure 7).** Data obtained from humans indicate a weak correlation between both variables. This intriguing finding is indicative of the disconnection between rodent and human data. The large response on tissue AA fluctuation in the rodent models is likely the source for heightened fear governing LA conversion to AA in tissues. This gap in interspecies translation leads to the importance of an established rodent dietary model. Formulating an appropriate dietary design for comparison to human clinical trials will hopefully result in more comparable findings.

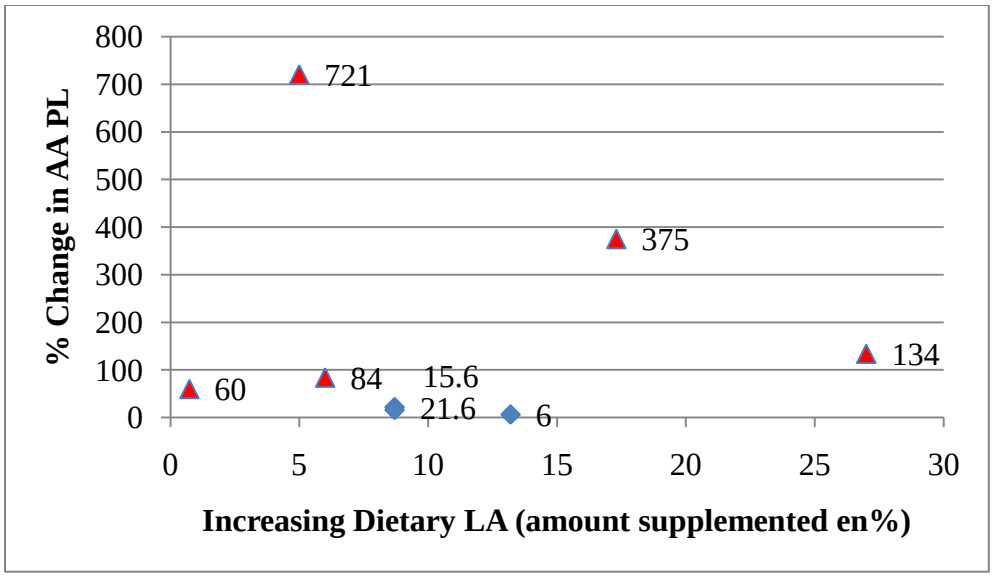


Figure 6. Rodent data comparing the % change in AA with increasing LA (% of energy) between 8 studies [2, 21, 33, 62, 90-93]. The data represents an inconsistent response of AA when LA is provided at doses of 0.73% to 27% of energy. The red triangles indicate a significant value.

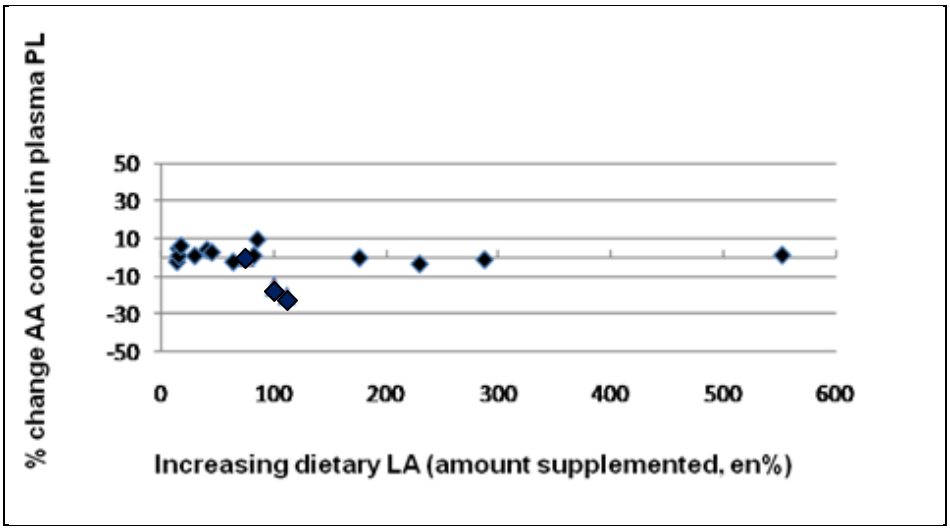


Figure 7. Human data comparing the % change in AA (plasma phospholipids) with increasing LA. The positive dose responsive relationship between LA and AA observed in rodents does not appear to exist in humans. The red triangles indicate a significant value. Derived from Brian Rett's Thesis, 2011.

1.5 RELEVANT DOSING FOR ANIMAL MODELS: A SCALING APPROACH

Introduction

An appropriate dietary design in animal models requires proper extrapolation between species. Supplemental doses in rodent models must meaningfully represent normal consumption by humans. For proper use of animal models in nutritional interventions, a certain degree of thoughtfulness should precede the design. If animal models are to truly predict biological responses in humans, we first must examine the paradigm of interspecies comparison.

History of Allometric Scaling

Basal metabolic rate (BMR) was first proposed for describing allometric relationships between body mass and metabolic rate of both animals and humans [94]. BMR represents the minimal cost of living or energy turnover at constant body temperatures [94-95]. In 1838, Sarrus and Rameaux were the first to suggest a rationale for interspecies comparison. Their proposed theory, the Surface Law, suggested that heat loss and production based on different-sized species should be related to surface area rather than body mass [94]. This proportionality of BMR and surface area raised to the 2/3-power was accepted well into the 20^{th}-century [96]. In 1916, Krogh suggested an empirical approach whereby BMR was related to a 0.73 power function of body mass [94-95]. Krogh's discovery led Max Kleiber in 1932, to show that metabolic rate is proportional to its ¾-power not to body weight alone [97]. This theory is based on the following equation:

$$M = 70W^{3/4}$$

Where M is the metabolic rate in kilocalories per day and W is body mass in kilograms. This three-quarter power scaling of BMR is a model of comparative physiology well-accepted by many researchers for its widespread scaling implications [96]. In pursuit of understanding this power scaling exponent, West et al [98] proposed a set of principles detailed by the process of natural selection and sustained "hierarchical branching networks." Scaling variation observed at intracellular levels and sub-cellular compartments (mitrochondria) tested in mammalian species, all proposed the quarter-power allometric scaling feature reflecting constraints inherent in genetics and energy expenditure into circulation [98].

Three-quarter power scaling is one approach for interspecies comparison that may be implemented into current research of nutritional animal model designs. Currently there is no clear standardization of nutrient comparisons in animal research. Typical doses are formulated based on bodyweight or relative doses that maximize the intended results. Therefore, the aim of this project is to establish a mathematical model for interspecies evaluation, in particular (n-6) PUFA levels, for appropriate extrapolation to humans. In order to devise a mathematical model for widespread implementation in research, this model must include several components: it should linearize the relationship between rodent and human data; should be applicable to the general range of (n-6) PUFA studies; be tested on its ability to yield similar biological effects in animal models and humans; and be straightforward to use.

Body weight has previously been used as a nutritional extrapolation approach, but according to Rucker and Storms, direct extrapolation from a mouse to an adult human may be in error by a factor of 10 or more [89]. The misunderstanding in human equivalent doses (HED) has left a gap in allometric dose translation [99]. Therefore, Rucker and colleagues proposed

interspecies comparison is best expressed as a measure of metabolic body size or food intake [89]. Rucker's point is further supported in homeothermic animals, showing that mineral requirements are similar across species when expressed as measure of energy intake [89]. With these findings, we propose a model based on consideration of daily energy consumption in rodents and humans as a means of dose standardization.

1.6 Implication in Rodent Dietary Design

When designing an experiment it is crucial to establish scientific rationale for experimental design. If the overall goal of nutritional intervention studies is to translate the results to people, a standardized diet appropriate for interspecies comparison must be formulated. The following proposal hopes to provide more consistent findings and a well-recognized background dietary standard for all research paradigms.

1.7 Research Objectives

Specific Aims

Dietary fat has been correlated with the incidence of chronic and acute conditions that are potentially modifiable [63, 100]. More specifically, the concern for n-6 PUFA overconsumption of LA appears to arise from rodent experimental models that lack appropriate design for comparison to humans. The primary aim of this study is to address the disconnection between experimental models for dietary comparison and provide a more suitable model for nutrient extrapolations between species.

Our *overall goal* is to formulate a rodent dietary model with an appropriate background HED of LA that will equate human equivalent responses based on changes in tissue AA. This dietary framework will provide a comparative model for nutritional interventions and therefore appropriate translational research. ***The objective of this application,*** **which is the next step toward achieving our overall goal, is to determine the level of LA supplementation that will mimic human equivalent responses based on changes in tissue AA**. *The central hypothesis*, based on a mathematical model for allometric scaling, will formulate an appropriate dose of LA based on energy consumption that will equate human equivalent responses. *The rationale underlying the proposed research* comes from a preliminary study in our laboratory looking at dietary supplementation of n-3 fatty acids, α- linolenic acid (ALA) and eicosapentanoeic acid (EPA) on tissue concentration in rodents. It was found that an increased amount of ALA was required in the background rodent diet to mimic human responses. When conducting interspecies comparisons, it was found that scaling nutrients in standardized rodent diets based on differences in energy intake extrapolated most nutrients to human intakes based on the DRIs. Similarly, the

same should hold true when testing the mathematical model for allometric scaling on n-6 PUFAs.

We will test our central hypothesis and achieve the overall objects of this application by pursing the following *two specific aims*:

1. Determine the extent to which supplementation of HEDs of LA to a human equivalent background diet will mimic human responses in tissue AA.

   Working hypothesis: If the model for allometric scaling is formulated correctly, the response in rodents should be similar to the responses in humans at equivalent doses.
2. Determine the extent to which supplementation of AA at HEDs to a human equivalent background diet will mimic human responses in tissue AA.

   Working hypothesis: If the model for allometric scaling is formulated correctly the response in rodents should be similar to the responses in humans at the same dose.

The proposed research is *innovative* because there is currently no standardization in rodent dietary models or a precise scientific rationale for experimental design. *We expect this approach to demonstrate that:* 1) the mathematical model for interspecies allometric scaling appropriately equates human equivalent responses and 2) rodent diets should be based on differences in energy consumption. Application of this knowledge is expected to provide translational research for interspecies comparison.

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Part II

Allometric scaling of dietary linoleic acid on changes in tissue arachidonic acid using human equivalent diets in mice

Disclosure

This article has been submitted to Nutrition & Metabolism for review.

Author's Contributions: Kylie Weldon conducted the research and co-wrote the manuscript, and Jay Whelan formulated and designed the research, co-wrote the manuscript and had final responsibility for all parts of the manuscript.

Abstract

It is hypothesized that dietary linoleic acid (LA) promotes chronic and acute diseases by enriching tissues with arachidonic acid (AA), its downstream metabolite. However, levels of LA in rodent diets are notoriously erratic making interspecies comparisons unreliable. Therefore, the ability to extrapolate the biological effects of dietary LA from experimental rodents to humans necessitates an allometric scaling model that is rooted within a human equivalent context. To determine the physiological response of dietary LA on tissue AA, a mathematical model for extrapolating nutrients based on energy was designed to mimic human equivalent doses. *C57BL/6J* mice were divided into 9 groups fed a background diet equivalent to that of the US diet with supplemental doses of LA or AA. Changes in the phospholipid fatty acid compositions were monitored in plasma and erythrocytes and compared to data from humans supplemented with equivalent doses of LA or AA. Increasing dietary LA had little effect on tissue AA, while supplementing diets with AA significantly increased tissue AA levels, recapitulating results from human trials. Thus, interspecies comparisons for dietary LA between rodents and humans can be achieved when rodents are provided human equivalent doses based on differences in metabolic activity as defined by energy consumption.

List of abbreviations

AA, arachidonic acid; ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; DRI, dietary reference intake; EFA, essential fatty acid; EPA, eicosapentaenoic acid; HED, human equivalent dose; LA, linoleic acid; PUFA, polyunsaturated fatty acid

Manuscript

2.0 Introduction

As surrogates for human inquisition, animal models reside at the core of medical innovation. Through careful environmental control, these genetically similar models facilitate therapeutic advancements in the magnitude of human disease. Rodent dietary composition is of particular interest in the field of nutrition research as it provides a way to assess the translational ability of individual dietary constituents, through appropriate dosing of nutrients, to physiological effects observed in humans consuming similar levels of nutrients.

Dietary profiles of n-6 polyunsaturated fatty acid (PUFA), linoleic acid (LA) and the relationship to chronic and acute diseases, in both rodents and humans, appears to lie in tissue enrichment of downstream metabolite, arachidonic acid (AA) [1-3]. It is hypothesized that dietary metabolism of AA produces bioactive compounds called eicosanoids that are positively correlated with the appreciation of tissue AA [4]. While the relationship of AA and eicosanoids is well established, the response of dietary LA on changes in tissue levels of AA, within the context of a human equivalent diet, remains inconclusive.

The inconsistent use of n-3 and n-6 essential fatty acids (EFAs) in the background of rodent diets is pervasive in the literature [5-10]. These EFAs are important components of the Western diet and can impact the AA phospholipid pool when absent or provided at insufficient quantities in the diet. Despite suggestions otherwise, a systematic review of the human literature reports that increases in dietary LA does not appear to significantly modify AA levels in phospholipids of plasma/serum or erythrocytes when supplemented to standard Western diets [11]. Therefore, if precise physiological nutrient translation of fatty acids is desired, it may be

important for dietary aspects of the rodent model to bear firm resemblance to human dietary components.

This study was designed to necessitate a standard for allometric scaling in animal dietary design particularly regarding the relationship of dietary LA and tissue AA. This is the first study to examine the physiological response of dietary LA provided at human equivalent supplemental doses within the context of a Western background diet, based on a percentage of energy (i.e., metabolic activity), on changes in plasma/serum or erythrocyte AA phospholipids. We further investigated the potential contribution of dietary AA on tissue AA content within the context of a Western-type diet. This mathematical model for allometric scaling should better equate interspecies translation and accommodate the differences in metabolic disparity between rodents and humans.

2.1 Methods

Animals

Sixty-two *C57BL/6J* male mice (Harlan Laboratory, Indianapolis, IN), 6-7 weeks of age, were, randomly assigned to nine dietary groups; 5-7 animals per group housed 2-3 animals per cage. They were housed in a temperature controlled room with a 12 hr light-dark cycle. Prior to sacrifice, animals were fasted overnight. All animal procedures were approved by the University of Tennessee Animal Care and Use Committee in accordance with NIH guidelines.

Diets

All animals were maintained on a control diet for one week prior to being transferred to one of the experimental diets or maintained on the control diet. The control diet was based on a US17 Monsanto diet with slight modifications in macronutrient distributions **(Table 1)**. The diet was designed to mimic the Western diet with the following distribution (% of energy): protein 16%, carbohydrates 50% and lipids 34% (Research Diets, New Brunswick, NJ) [12]. Within the lipid fraction, saturated, monounsaturated and polyunsaturated fats were designed to be provided at 13%, 14% and 7% of energy, respectively. The polyunsaturated fats LA, ALA, AA and EPA+DHA were provided at 6%, 0.6%, 0.07% and 0.1% of energy, respectively. These levels are similar to those suggested in the literature for humans on a Western diet [12-13] and/or supported by the DRIs for median daily intakes [14]. AA and EPA+DHA were provided as ethyl esters (NuChek Prep, Elysian, MN). Experimental diets remained isocaloric and were formulated using the control diet as the background diet containing LA at ~6% of energy with additional adjustments in LA content (-2%, +2%, +4%, +6%, +8% of energy) with the addition (or subtraction) of sunflower oil (70% w/w LA) at the expense of cocoa butter, palm and trisun oils. The diets supplemented with AA were adjusted at the expense of cocoa butter.

Water and food were provided ad libitum for 21-25 days. Fresh diets were provided daily and uneaten food was discarded to minimize oxidation prior to consumption. Fatty acid analysis of the diets is presented in **(Table 2)**.

Fatty acid analysis

Animals were randomized on a 5 day sacrifice cycle. Following 21-25 days on the experimental and control diets, 0.5-1.0 ml of whole blood was collected via cardiac puncture

under anesthesia (isofluorane inhalation) using a tuberculin syringe with a 25 gauge needle containing an anticoagulant (3.8% trisodium citrate). Whole blood was centrifuged at 660 x g for 4 min at room temperature for separation of plasma and erythrocytes, where each fraction was subjected to lipid extraction. Three ml of chloroform-methanol (1:2, v/v) were added to each fraction, and lipids were extracted with chloroform (1 ml) plus saline (1 ml), followed by chloroform (1 ml) (2x). The pooled chloroform extracts were evaporated and resuspended in a small amount of chloroform (~25 µL), and phospholipids were separated via thin layer chromatography (TLC) using HPTLC plates precoated with silica gel 60 (Merck, Darmstadt, Germany) using a chloroform-methanol (8:1, v/v) solvent system. The phospholipids were recovered from the TLC plates and saponified in 0.5 N NaOH and in the presence of BF_3 in methanol at 86°C. Fatty acids were extracted with equal volumes of hexane (2x) and evaporated under nitrogen. Fatty acid methyl esters were resuspended in hexane and analyzed by gas chromatography with a Hewlett-Packard 5880 gas chromatograph (Rochester, NY) using a DB23 capillary column (0.25 mm x 30 m) (J and W Chromatography, Folsom, OH) with hydrogen as the carrier gas, with temperature programming from 160°C to 250°C at 3.5°C/min The internal standard 1,2 diheptadecanoyl-sn-glycero-3-phosphocholine (17:0) (Avanti Polar Lipids, Alabaster, AL) was added to each sample prior to lipid extraction. The fatty acid methyl esters were identified by comparing the retention times with those of known standards (NuChek Prep, Elysian, MN).

Statistical analysis

Phospholipid fatty acid content in plasma and erythrocytes were compared across treatment groups using a one-way analysis of variance (ANOVA), followed by Tukey's Honestly

Significant Difference (HSD) post-hoc test to determine significant differences between groups. All data was tested for normality using Kolmogorov-Simimov test (K-S test) and Shaprio-Wilk tests, homogeneity by Levene's Test of Equality of Variance, and for outliers using boxplot tests. The data was evaluated by SPSS 18 statistical package (University of Tennessee, Knoxville, TN). Data was considered significant at $p < 0.05$.

2.2 Results

Food intake and weight gain were not statistically different between dietary groups (data not shown).

Fatty acid composition of plasma phospholipids.

The composition of oleic acid and LA in plasma phospholipids tended to reflect differences in dietary levels of these fatty acids; however, much of these effects were not statistically significant **(Table 3).** The dietary group with the lowest levels of LA and highest levels of oleic acid (group 1) had the lowest levels of LA and highest levels of oleic acid in the plasma phospholipids, respectively. The levels of AA did not change in any of the groups with increasing or decreasing levels of dietary LA **(Table 4).** DHA levels were not different among groups, with the exception of group 5. When AA was supplemented to the diets, tissue AA levels progressively increased in a dose responsive manner at the expense of LA, but tissue DHA levels did not change. A summary of the effects of LA and AA supplementation are provided in **(Figure 1 and Figure 2)**, respectively.

Fatty acid composition of erythrocyte phospholipids

The composition of oleic acid and LA in the phospholipids of erythrocytes reflected differences in dietary levels of these fatty acids where LA supplementation significantly increased LA in the tissues **(Table 5 and Figure 3).** Levels of dihomo-gamma-linolenic acid (20:3n-6) and AA were unaffected by changes in dietary LA. Similarly, DHA content in erythrocytes were unaffected by changes in LA intake. When AA was supplemented in the diets, tissue AA content progressively increased primarily at the expense of LA, but reductions in dihomo-gamma-linolenic acid were also observed **(Table 6 and Figure 4)**. DHA levels were not reduced with increasing levels of dietary AA.

2.3 Discussion

Animal models are not intended to replace humans, but be a substitute that is often better controlled for and better able to answer narrow research questions that could not be done, on a practical basis, with humans. A common challenge faced by nutrition researchers who are interested in interspecies comparisons is identifying an appropriate background diet and appropriate doses for supplemented nutrients. In order to make these choices, studies have to be performed that can demonstrate human equivalent responses to ensure translation between species. Without these fundamental studies, no guidelines can be formulated governing scientific justification for dosing when extrapolation to humans is desired. Currently, no guidelines exist for appropriate dosing of dietary PUFAs for experimental models (i.e., mice, rats) as they relate to humans and their intakes. As such, the overall objective of this research was to determine the extent to which supplementation of human equivalent doses of LA and AA had on changes in

tissue AA content within the context of a Western-type diet using a common experimental rodent model and comparing these results to humans. This is the first known study of its kind.

Interspecies comparisons were pioneered by Max Kleiber where he described the non-linear relationship between metabolic rate and body mass as it relates to allometric scaling with interspecies comparisons. This pioneer in the field of animal energetics introduced the concept that the relationship between metabolic rate and body weight could be linearized with the following equation: Metabolic rate = a(Body Weight)$^{0.75}$ (where "a" is a proportionality constant) [15]. More recently, Rucker and Storms (2002) elegantly described the pitfalls of using differences in body weight as a means of making interspecies extrapolations for micronutrients because of these non-linear relationships [16]. They addressed the appropriateness of several mathematical approaches to extrapolate nutrient intake between mice and humans and suggested food (energy) intake rather than body weight should be used to extrapolate nutrients for interspecies comparisons [16-17]. Interestingly, when this concept was applied to a variety of standardized semi-purified diets (i.e., AIN76A, AIN93G, AIN93M), extrapolations of the nutrients based on energy differentials mimic human equivalent doses. This provided the scientific justification for the background diet and doses used in this study.

With this in mind, we generated a "human equivalent" background diet where the macronutrient composition mimicked that of the human diet when based on energy and evaluated the impact of dietary LA (4%-14% of energy) and AA (0.08%-1.35% of energy) on changes in tissue AA levels in plasma and erythrocyte phospholipids. These amounts translate into human equivalent levels of 9-31 g/d and 0.18-2.7 g/d of LA and AA, respectively, and are within those ranges reported in the DRIs for humans and/or used in clinical trials [11, 14]. Importantly, as opposed to rodent diets that selectively provide only one or two of the essential

fatty acids (EFAs) (i.e., corn oil or soybean oil based diets), our background diet contained all the major n-6 and n-3 PUFAs found in the human diet (i.e., LA, ALA, AA and long chain n-3 PUFA). This is critical as all of these fatty acids are found in the Western diet and can have an impact on tissue AA levels. For this reason, there has been great interest placed upon n-6 PUFA metabolism, particularly when all EFAs are sufficiently provided in the diet at human equivalent levels.

The rodent model has been the superior target for investigation of specific fatty acids and downstream metabolites on tissue fatty acid composition since 1963 when Mohrhauer and Holman explored the metabolism of dietary EFAs [7]. In this classic and highly cited paper, rodents were initially fed a fat-free diet (i.e. with the exclusion of all EFAs) prior to supplementation of LA (ethyl linoleate) up to 5% from energy, where a 721% increase in liver AA composition was observed in the highest doses. Other studies recapitulated these earlier results when LA was provided to a background diet that lacked nearly all or completely all n-3 and n-6 PUFAs [9-10]. When LA was increased from nearly 0% of energy to 6-7% of energy in rodents, liver AA composition increased 84-173% [6, 8]. Increasing LA from 6% of energy to 27% of energy (or a human equivalent dose of 59 g/d), resulted in a 134% increase in tissue AA composition [5]. The addition of LA at supra-physiological doses (i.e., 17.3% of energy) from a background diet containing 7% LA, increased AA content by 375% when no other EFAs were provided [10]. However, when more moderate levels of LA were supplemented to a diet containing human equivalent levels (i.e., 6.6% to 13.2% of energy), AA content in liver phospholipids increased a modest 6% [8]. Notably, tissues have a requirement for unsaturated fatty acids for structural function and to help maintain membrane fluidity. When animals are fed a diet that exclusively contains a single PUFA (i.e., LA), its selective and robust conversion to a

more highly unsaturated form is not surprising. These findings underscore the differential impact of dietary LA in rodent diets on changes in tissue AA content when the background diet is devoid of LA and/or other PUFAs, or providing LA at doses approaching pharmacological levels.

What is an appropriate background diet in rodents and what is an appropriate dose of LA that has translational ability to humans? This would be dependent upon the human literature; that is, what is the effect of LA on changes in tissue AA in individuals consuming a typical Western diet? The DRI for LA is 12g/d and 17g/d for women and men, respectively (approximately 6% of energy) [14]. In a recent review of the literature, decreasing LA content in the diet up to 90% or increasing the levels up to 550% was not associated with changes in AA content in the phospholipid pools of plasma/serum or erythrocytes [11]. It is not unreasonable to think, that with a background diet containing LA, ALA, AA, and long-chain n-3 PUFAs (i.e., EPA and DHA) at typical intakes, modifying LA levels may not influence tissue AA levels in these populations. Hence, in order to establish a human equitable response of dietary LA on tissue AA composition in the rodent model, it seems best accomplished when all EFAs are present in the diet, especially for results that are expected to translate proportionally to humans.

Increasing LA from 0% to 2% of energy replete tissue pools of n-6 PUFAs by increasing AA phospholipid concentrations [18]. Intake of LA above 2-3% of energy in humans is not reportedly accompanied by an increase in AA content in plasma or erythrocyte phospholipids [11, 19-24], results consistent with our data. Poor conversion rates in humans would account for these results where the estimated fractional conversion of LA to AA in adults was between 0.3% and 0.6% [25]. In rodents, tracer kinetic analysis demonstrated greater efficiency of C20 fatty

acids in conversion to downstream end-products relative to C18 precursors [26]. This would imply that feedback inhibition of Δ-6 desaturase, the rate limiting step in the conversion of LA to AA, may be responsible. Likewise, the present study reports no significant alteration in plasma/serum or erythrocyte AA phospholipids at the lowest supplemental dose of LA (4% of energy) or the highest supplemental dose (14% of energy). These results are supported by prior rodent dietary studies supplementing LA at similar levels (6.8% and 8.7% of energy) to a background diet already containing LA (4.5% of energy) [27-28]. Hence, supplementation of HEDs of LA to a background rodent diet consisting of all EFAs found in the human diet, (including LA and AA) more accurately correspond to the changes in tissue fatty acid composition of AA in humans consuming similar levels [11, 19-21, 24].

Additionally, our data demonstrates the observable inverse relationship of dietary AA on changes in tissue LA within the rodent model. When AA was supplemented to rodents consuming a Western-like diet, tissue AA content increased in a dose dependent manner, suggesting the lack of changes with LA supplementation was not due to saturation of AA in the phospholipid pools analyzed. Likewise, when dietary AA was provided to rodents (mice, hamsters) at 1.5-4% of energy, AA content in hepatic phospholipids increased 21-80%; [12, 27-29]. Similar results were observed in intestines, macrophages, lung, heart, spleen, kidneys, testes and platelets [12, 27-28]. These results are comparable to humans [11]; however, the response in rodents is more modest than that observed in humans supplemented with AA [11]. Our highest supplemented dose of 1.35% of energy (or a HED of 3 g/d) increased phospholipid AA levels in plasma/serum by 40%, while providing a dose of 0.75-1.5 g/d in humans increased tissue AA content by ~85% [30-31] with a maximum change of 136% at a dose of 6 g/d [32]. Rodents, compared to humans, have higher requirements for the more highly unsaturated fatty acid DHA

in their tissue phospholipids [33]. This may preclude the need for higher levels of AA in tissues, accounting for the more modest effects observed in rodents following AA supplementation. Of importance, these changes in AA content were always at the expense of tissue LA, suggesting that dietary AA targets the same phospholipid pool occupied by LA [12, 27-28]. This inverse relationship between tissue levels of AA and LA following AA supplementation is supported by human clinical data [30]. The inverse is not always true. While some studies suggest an inverse relationship exists between tissue LA and AA levels when LA is supplemented in the diet [11, 20, 23], these studies are in the minority.

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Conclusion

Currently there are no guidelines providing assistance as to how to formulate a human equivalent diet for rodents to improve translation of data to that of humans. The overall objective of this research was to test a theoretical model for allometric scaling based on energy differences between species. We chose the relationship between dietary LA and its effects on tissue AA content as a testable hypothesis. We wanted to determine the extent to which supplementation of human equivalent doses of LA and AA had on changes in tissue AA content within the context of a Western-type diet using a common experimental rodent model. We proposed that providing animals a background diet that mimicked the Western diet with regards to macro- and micronutrients and fatty acid profiles, and supplementing LA at human equivalent doses, we could observe a human equivalent response with regard to changes in AA levels in plasma/serum and erythrocyte phospholipids. Our results recapitulated those in humans and provide support for the concept that allometric scaling between species for dietary LA can be accomplished based on energy and metabolic differences. It is important to note that these results cannot be extrapolated to all tissues.

APPENDIX

Table 4. Composition of the diets

	Dietary Groups								
	1	2	3	4	5	6	7	8	9
	-2%[1]		+2%	+4%	+6%	+8%	+0.23%	+0.45%	+1.36%
Diet	LA	Control	LA	LA	LA	LA	AA	AA	AA
	g/100g								
Protein	17.4	17.4	17.4	17.4	17.4	17.4	17.4	17.4	17.4
Carbohydrate	54.5	54.5	54.5	54.5	54.5	54.5	54.5	54.5	54.5
Lipid	17.1	17.1	17.1	17.1	17.1	17.1	17.1	17.1	17.1
	g/kg								
Casein	150	150	150	150	150	150	150	150	150
L-Cysteine	3	3	3	3	3	3	3	3	3
Corn Starch	295	295	295	295	295	295	295	295	295
Maltodextrin 10	75	75	75	75	75	75	75	75	75
Sucrose	100	100	100	100	100	100	100	100	100
Cellulose	50	50	50	50	50	50	50	50	50
Cocoa Butter, Deodorized	41.6	37.5	33.3	29.1	25.0	21	36.9	35.9	32.1

Table 4. Continued

	Dietary Groups								
	1	2	3	4	5	6	7	8	9
	-2%[1]		+2%	+4%	+6%	+8%	+0.23%	+0.45%	+1.36%
Diet	LA	Control	LA	LA	LA	LA	AA	AA	AA
	g/100g								
Flaxseed Oil	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Palm Oil, Bleached, Deodorized	58.4	52.5	46.8	41.1	35.3	29.5	52.5	52.5	52.5
Safflower Oil, USP	15.6	28.5	28.5	28.5	28.5	28.5	28.5	28.5	28.5
Trisun Extra	30	27	24.1	21.1	18.2	15.2	27	27	27
Sunflower Oil	-	-	12.8	25.7	38.5	51.3	-	-	-
Arachidonic Acid, Ethyl Ester	0.35	0.35	0.35	0.35	0.35	0.35	0.97	1.93	5.8
Eicosapentaenoic Acid, Ethyl Ester	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Docosahexaenoic Acid, Ethyl Ester	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24

[1]% change with regards to energy

Abbreviations: AA, arachidonic acid; LA, linoleic acid

Table 5. The fatty acid composition of the diets

	Dietary Groups								
	1	2	3	4	5	6	7	8	9
	-2%[1]		2%	4%	6%	8%	0.23%	0.45%	1.36%
Fatty Acids	LA	Control	LA	LA	LA	LA	AA	AA	AA
12:0	0.47[2]	0.42	0.39	0.34	0.27	0.26	0.42	0.43	0.42
14:0	0.69	0.69	0.59	0.56	0.48	0.43	0.63	0.65	0.66
16:0	26.48	24.69	22.66	20.63	18.71	16.8	24.62	24.31	23.71
16:1	0.17	0.1	0.15	0.14	0.09	0.09	0.15	0.16	0.16
18:0	13.13	11.39	11.19	10.08	9.52	8.5	11.69	11.29	10.54
18:1n-9	42.44	40.44	38.44	36.62	35.00	33.06	40.11	39.79	39.00
18:2n-6	13.24	18.93	23.23	28.24	32.5	37.39	19.01	19.05	19.08
18:3n-3	1.78	1.79	1.77	1.8	1.77	1.8	1.78	1.84	1.83
20:0	0.58	0.54	0.52	0.49	0.48	0.44	0.55	0.55	0.51
20:1	0.14	0.17	0.15	0.15	0.17	0.14	0.16	0.17	0.14
20:4n-6	0.21	0.21	0.22	0.22	0.2	0.22	0.55	1.11	3.32

Table 5. Continued

	Dietary Groups								
	1	2	3	4	5	6	7	8	9
	-2%[1]		2%	4%	6%	8%	0.23%	0.45%	1.36%
Fatty Acids	LA	Control	LA	LA	LA	LA	AA	AA	AA
20:5n-3	0.09	0.03	0.08	0.07	0.06	0.1	0.05	0.09	0.06
22:0	0.31	0.31	0.34	0.37	0.42	0.46	0.31	0.29	0.30
22:6n-3	0.26	0.29	0.27	0.28	0.29	0.31	0.3	0.27	0.27

[1]% change with regards to energy

[2]g/kg diet

Abbreviations. LA, linoleic acid; AA, arachidonic acid

Table 6. The fatty acid composition of plasma phospholipids from mice fed linoleic acid supplemented diets

	Dietary Groups					
Fatty Acid	1 -2% LA[1]	2 Control	3 +2% LA	4 +4% LA	5 +6% LA	6 +8% LA
16:0	33.54 ± 0.93[2]	34.40 ± 0.80	33.60 ± 1.03	33.25 ± 0.96	35.29 ± 1.15	32.87 ± 0.88
18:0	13.68 ± 0.55	13.41 ± 0.31	13.07 ± 0.56	13.84 ± 0.61	13.97 ± 0.66	13.49 ± 0.54
18:1n-9	12.50 ± 0.50	11.69 ± 0.65	9.11 ± 0.64	9.23 ± 0.70	10.29 ± 1.26	10.10 ± 1.18
18:2n-6	17.80 ± 0.28[a]	19.22 ± 0.42[ab]	20.58 ± 0.44[ab]	21.86 ± 0.93[b]	22.15 ± 1.53[b]	22.46 ± 1.27[b]
20:4n-6	13.41 ± 0.40	12.74 ± 0.50	13.94 ± 0.84	13.39 ± 0.63	11.77 ± 0.75	13.10 ± 0.63
22:6n-3	9.07 ± 0.98[a]	8.54 ± 0.56[ab]	9.09 ± 0.60[a]	8.43 ± 0.59[ab]	6.53 ± 0.79[b]	7.99 ± 0.49[ab]

[1]%change with regards to energy

[2]Relative abundance (mol%) presented as mean ± SEM

[ab]Means with the same superscript within the same row are not statistically different at $p < 0.05$, Tukey's honestly significant difference.

Abbreviations: LA, linoleic acid

Table 7. The fatty acid composition of plasma phospholipids from mice fed arachidonic acid supplemented diets

	Dietary Groups			
	2	7	8	9
Fatty Acid	Control	+0.23% AA[1]	+0.45% AA	+1.36% AA
16:0	34.40 ± 0.87[2]	33.62 ± 0.82	33.71 ± 1.07	34.76 ± 0.81
18:0	13.41 ± 0.34	13.25 ± 0.42	13.34 ± 0.53	12.82 ± 0.36
18:1n-9	11.69 ± 0.72	11.80 ± 0.83	11.69 ± 0.96	11.53 ± 0.94
18:2n-6	19.22 ± 0.46[a]	18.88 ± 0.59[ab]	17.04 ± 0.28[bc]	15.47 ± 0.41[c]
20:4n-6	12.74 ± 0.54[a]	14.21 ± 0.66[ab]	15.94 ± 0.81[bc]	17.93 ± 1.00[c]
22:6n-3	8.54 ± 0.55	8.52 ± 0.52	8.28 ± 0.51	7.51 ± 0.45

[1]%change with regards to energy

[2]Relative abundance (mol%) presented as mean ± SEM

[abc]Means with the same superscript within the same row are not statistically different at $p < 0.05$, Tukey's honestly significant difference.

Abbreviations: AA, arachidonic acid

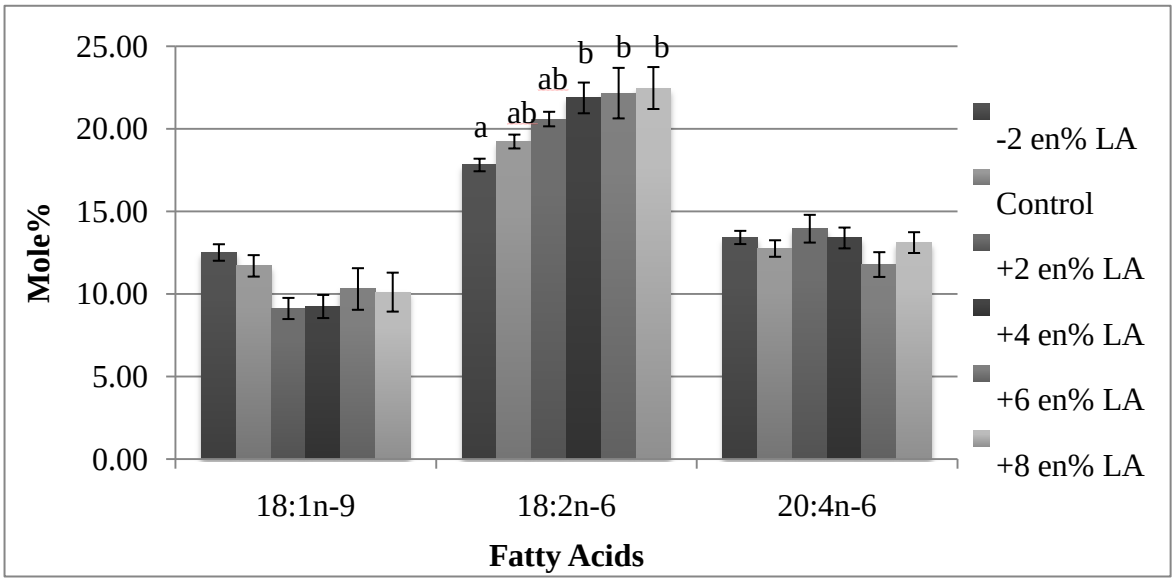


Figure 8. Effects of increasing/decreasing dietary linoleic acid on changes in plasma/serum phospholipid fatty acid concentration. Mice were fed background diets that mimicked the composition of a Western diet with increasing or decreasing levels (% change, based on energy) of linoleic acid. The data (mol%) is presented as mean ± SEM. Means with the same superscript within the same row (i.e., individual fatty acid) are not statistically different at ($p<0.05$). Abbreviations: LA, linoleic acid

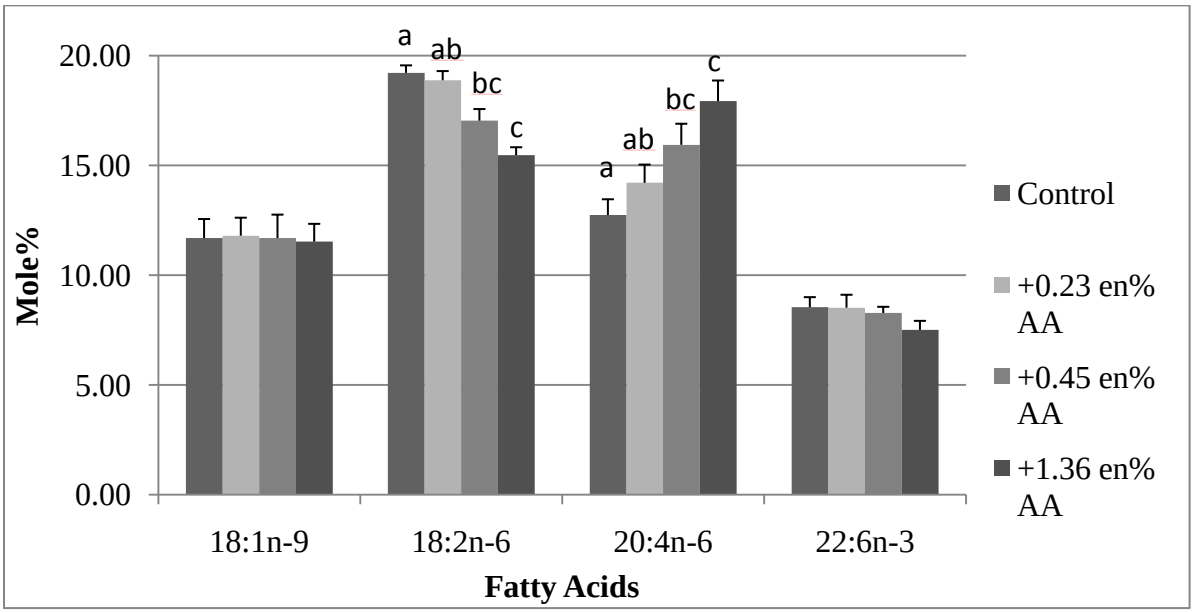


Figure 9. Effects of increasing dietary arachidonic acid on changes in plasma/serum phospholipid fatty acid concentration. Mice were fed background diets that mimicked the composition of a Western diet with increasing levels (% change, based on energy) of arachidonic acid. The data (mol%) is presented as mean ± SEM. Means with the same superscript within the same row (i.e., individual fatty acid) are not statistically different at ($p<0.05$). Abbreviations: AA, arachidonic acid

Table 8. The fatty acid composition of erythrocytes phospholipids from diets supplemented with linoleic acid

	Dietary Groups					
Fatty Acid	1 -2% LA[1]	2 Control	3 +2% LA	4 +4% LA	5 +6% LA	6 +8% LA
16:0	34.19 ± 0.66[2]	34.80 ± 0.63	34.40 ± 0.48	32.81 ± 0.69	34.67 ± 0.62	33.57 ± 0.71
18:0	13.77 ± 0.30	14.09 ± 0.52	15.45 ± 0.43	14.73 ± 0.45	14.18 ± 0.30	15.35 ± 0.57
18:1n-9	17.57 ± 0.40[a]	16.63 ± 0.46[a]	14.50 ± 0.34[b]	14.61 ± 0.21[b]	14.48 ± 0.28[b]	14.09 ± 0.31[b]
18:2n-6	10.69 ± 0.13[a]	12.03 ± 0.23[b]	12.23 ± 0.24[b]	13.09 ± 0.48[bc]	13.72 ± 0.28[cd]	14.52 ± 0.12[d]
20:3n-6	1.18 ± 0.33	1.12 ± 0.04	1.22 ± 0.61	1.19 ± 0.08	1.18 ± 0.03	1.18 ± 0.06
20:4n-6	14.33 ± 0.54	13.60 ± 0.57	14.58 ± 0.45	15.12 ± 0.65	14.56 ± 0.41	13.66 ± 0.74
22:4n-6	1.40 ± 0.05	1.27 ± 0.13	1.44 ± 0.10	1.62 ± 0.13	1.67 ± 0.06	1.51 ± 0.10
22:5n-6	0.40 ± 0.05	0.45 ± 0.02	0.50 ± 0.03	0.58 ± 0.10	0.55 ± 0.02	0.47 ± 0.04
22:5n-3	0.70 ± 0.04	0.64 ± 0.04	0.60 ± 0.03	0.63 ± 0.05	0.60 ± 0.03	0.57 ± 0.05
22:6n-3	5.76 ± 0.34	5.37 ± 0.49	5.43 ± 0.20	5.64 ± 0.36	5.29 ± 0.27	4.99 ± 0.54

[1]%change with regards to energy

[2]Relative abundance (mol%) presented as mean ± SEM

[abcd]Means with the same superscript within the same row are not statistically different at $p < 0.05$, Tukey's honestly significant difference.

Abbreviations: LA, linoleic acid

Table 9. The fatty acid composition of erythrocyte phospholipids from diets supplemented with arachidonic acid

	Dietary Groups			
	2	7	8	9
Fatty Acid	Control	+0.23% AA[1]	+0.45% AA	+1.36% AA
16:0	34.80 ± 0.69[2]	34.08 ± 0.86	34.43 ± 0.91	35.17 ± 0.95
18:0	14.09 ± 0.57	14.56 ± 0.31	14.03 ± 0.37	13.95 ± 0.34
18:1n-9	16.64 ± 0.50	16.49 ± 0.15	15.94 ± 0.29	15.62 ± 0.51
18:2n-6	12.03 ± 0.25^{a}	10.85 ± 0.16^{b}	9.65 ± 0.18^{c}	7.84 ± 0.07^{d}
20:3n-6	1.12 ± 0.05^{a}	0.94 ± 0.01^{b}	0.77 ± 0.02^{c}	0.44 ± 0.01^{d}
20:4n-6	13.60 ± 0.63^{a}	15.13 ± 0.52^{ab}	16.77 ± 0.53^{bc}	18.71 ± 0.93^{c}
22:4n-6	1.27 ± 0.15^{a}	1.54 ± 0.09^{ab}	1.90 ± 0.07^{bc}	2.20 ± 0.11^{c}
22:5n-6	0.45 ± 0.03^{a}	0.49 ± 0.02^{ab}	0.57 ± 0.04^{ab}	0.73 ± 0.05^{c}
22:5n-3	0.64 ± 0.06	0.64 ± 0.03	0.65 ± 0.03	0.55 ± 0.04
22:6n-3	5.37 ± 0.54	5.28 ± 0.23	5.29 ± 0.37	4.78 ± 0.40

[1]%change with regards to energy

[2]Relative abundance (mol%) presented as mean ± SEM

[abcd]Means with the same superscript within the same row are not statistically different at $p < 0.05$, Tukey's honestly significant difference.

Abbreviations: AA, arachidonic acid

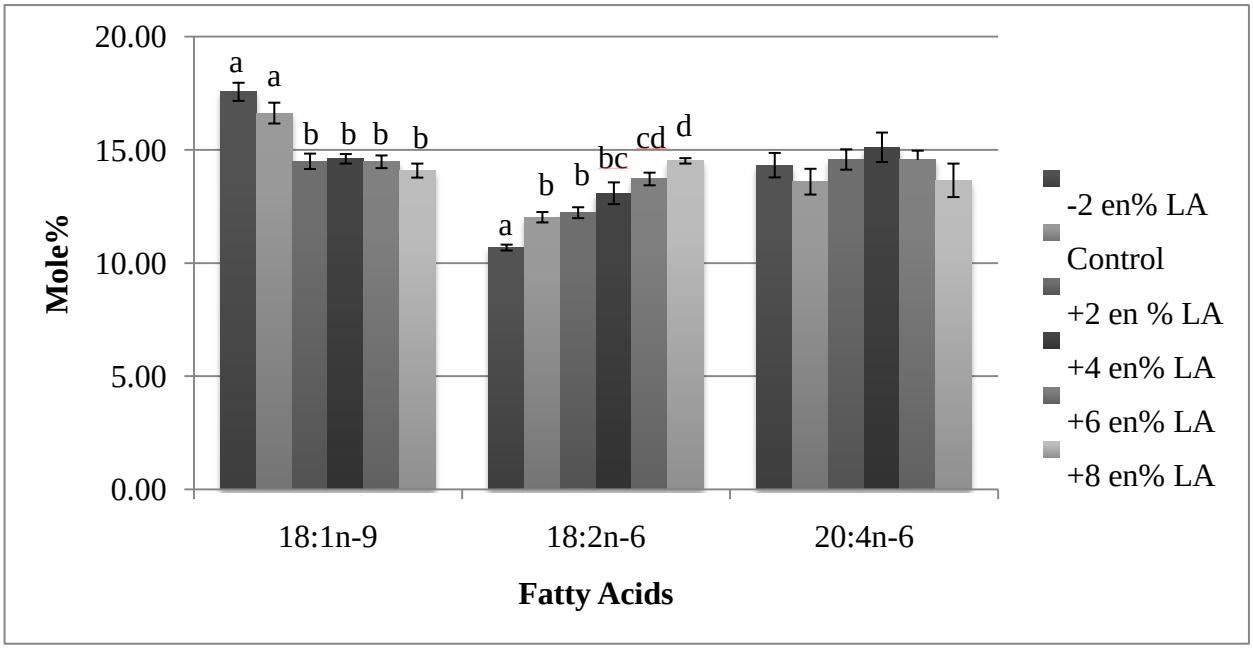


Figure 10. Effects of increasing/decreasing dietary linoleic acid on changes in erythrocyte phospholipid fatty acid concentration. Mice were fed background diets that mimicked the composition of a Western diet with increasing or decreasing levels (% change, based on energy) of linoleic acid. The data (mol%) is presented as mean ± SEM. Means with the same superscript within the same row (i.e., individual fatty acid) are not statistically different at ($p<0.05$). Abbreviations: LA, linoleic acid.

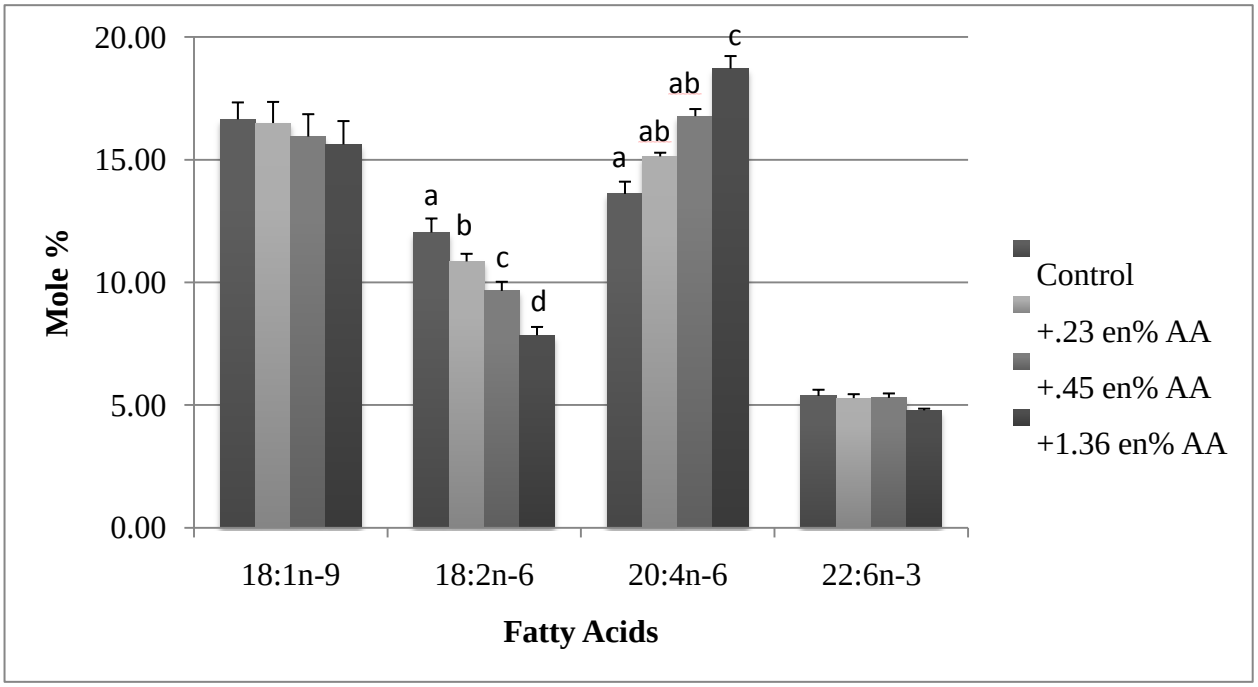


Figure 11. Effects of increasing dietary arachidonic acid on changes in erythrocyte phospholipid fatty acid concentration. Mice were fed background diets that mimicked the composition of a Western diet with increasing levels (% change, based on energy) of arachidonic acid. The data (mol%) is presented as mean ± SEM. Means with the same superscript within the same row (i.e., individual fatty acids) are not statistically different at ($p<0.05$). Abbreviations: AA, arachidonic acid.

Vita

Kylie Alexandra Weldon was born in Jackson, MS on March 9, 1987. She was raised in Beavercreek, OH where she attended Main Elementary (K-3) and St. Luke (4-8). She graduated in 2005 from Beavercreek High School, Beavercreek, OH. Kylie attended The Ohio State University, Columbus, OH (2005-2009) where she received her Bachelor of Science in Dietetics. Kylie is currently pursuing her Master's of Science in Nutrition and the Dietetic Internship at the University of Tennessee, Knoxville.

"Don't be afraid, for I am with you. Don't be discouraged, for I am your God. I will strengthen you and help you. I will hold you up with my victorious right hand"

~ Isaiah 41:10

"Hard things are put in our way, not to stop us, but to call out our courage and strength"

~Unknown Author

"With ordinary talent and extraordinary perseverance, all things are attainable"

~Thomas Foxwell Buxton