THE ROLE OF DIETARY ARACHIDONIC ACID AND DOCOSAHEXAENOIC ACID IN PREVENTING THE PHENOTYPE OBSERVED WITH HIGHLY UNSATURATED FATTY ACID DEFICIENCY

BY

MANUEL ROQUETA RIVERA

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Food Science and Human Nutrition
with a concentration in Human Nutrition
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2010

Urbana, Illinois

Doctoral Committee:

Professor Sharon Donovan, Chair
Associate Professor Manabu Nakamura, Director of Research
Professor Wanda Haschek-Hock
Professor Rex Hess
Professor Mariangela Segre

ABSTRACT

The physiological roles of highly unsaturated fatty acids (HUFA), mainly arachidonic acid (AA, 20:4ω6) and docosahexaenoic acid (DHA, 22:6ω3), are not completely understood. In order to study specific functions for AA and DHA, a delta-6 desaturase knockout (D6D-/-) mouse was created. D6D is a key enzyme in synthesizing HUFA from the precursor dietary essential fatty acids, linoleic acid (LA, 18:2ω6) or α-linolenic acid (ALA, 18:3ω3). By disrupting D6D expression, LA and ALA provided in the diet will not be metabolized to HUFA. Phenotype of the D6D-/- mouse is therefore specific to lack of AA and/or DHA and consists of ulcerative dermatitis, male infertility, gastrointestinal ulcers, and hepatic lipidosis. New insight on specific AA and DHA roles was established through dietary prevention of HUFA deficiency phenotype.

The absence of a D6D isozyme had to be assessed before further characterizing HUFA roles with the D6D-/- mouse model. The presence of a D6D isozyme would interfere with the creation of HUFA deficiency. The primary D6D isozyme candidate was *Fads3* gene due to its increased gene expression in D6D-/- liver and homology to the *Fads2* gene that encodes for D6D. Cloning and transfection of *Fads3* into cultured HEK293 cells confirmed lack of D6D activity (**Chapter 3**).

The order of appearance of D6D-/- phenotype due to HUFA deficiency had yet to be determined. A D6D-/- time course study (**Chapter 4)** characterized the mouse at different ages in order to follow sequence of HUFA deficiency pathology. The amount of HUFA in D6D-/- at weaning was comparable to control mouse indicating the presence of HUFA stores that most likely result from HUFA passed on from the mother. Subsequent HUFA depletion with age correlated with severity of D6D-/- phenotype. Male infertility, gastrointestinal erosions, and

hepatic lipidosis are the first observed HUFA deficiency phenotype to appear at 6 weeks of age, followed by impaired antibody response at 9 weeks, and ulcerative dermatitis by 21 weeks of age.

HUFA supplementation studies helped determine specific roles for AA and DHA in preventing HUFA deficiency phenotype. Hepatic lipidosis was prevented by either AA or DHA (**Chapter 5**). AA essentiality was specific to skin and gastrointestinal function since DHA supplementation was unsuccessful in preventing ulcerative dermatitis or gastrointestinal ulcers (**Chapter 6**). DHA essentiality was specific to male reproduction as indicated by full restoration of spermatogenesis, sperm counts, and sperm motility (**Chapter 7**). The role of DHA in spermatogenesis is related to acrosome biogenesis, a process which relies on vesicle fusion (**Chapter 8**). The immune system (**Chapter 9**) was further characterized following up on splenomegaly and thymic atrophy observations of the first characterization of the D6D-/-. HUFA deficiency results in decreased antibody response indicating essentiality for HUFA in immune function.

In summary, these studies showed for the first time a specific requirement for AA in skin, and of DHA in male reproduction. The mechanism behind DHA requirement in male fertility has been linked to acrosome biogenesis. Future research done with the D6D-/- mouse model will help develop hypothesis on other potential mechanisms behind the essentiality of AA and DHA. Understanding how HUFA maintain tissue homeostasis will help in the development of treatments for diseases that result from an altered essential fatty acid metabolism.

ACKNOWLEDGEMENTS

This dissertation would not have been possible without the support of many people. I would like to thank my adviser, Manabu Nakamura, for his mentorship and encouragement in the process of working towards this degree. I would also like to thank my committee members, Sharon Donovan, Wanda Haschek, Rex Hess, and Mariangela Segre, for their guidance and feedback. Histological evaluations would not have been possible without the expertise of Wanda Haschek and Sandeep Akare. The studies on male fertility and electron microscopy images were achieved with the technical advice of Rex Hess. I am also grateful of Mariangela Segre's support in researching the immune system of the delta-6 desaturase knockout mouse model and for allowing me to use her lab for most of these studies. Mayandi Sivaguru was also of great help in providing advice for immunohistochemistry techniques. I would also like to acknowledge the financial support provided by the CONACyT fellowship. Finally, I would like to express my gratitude to my family and friends whose support and words of encouragement contributed to the successful completion of this work.

TABLE OF CONTENTS

ABBREVIATIONS

AA	arachidonic acid (20:4 n-6)
ALA	α-linolenic acid (18:3 n-3)
CD	cholesta-3,5-diene
D5D	delta-5 desaturase
D6D	delta-6 desaturase
D8D	delta-8 desaturase
DGAT	diacylglycerol-acyl transferase
DHA	docosahexaenoic acid (22:6 n-3)
DPA	docosapentaenoic acid (22:5 n-6)
EFA	essential fatty acids
ELOVL2	elongase enzyme
EPA	eicosapentaenoic acid (20:5 n-3)
FADS2	fatty acid desaturase 2 (D6D gene)
FADS3	fatty acid desaturase 3
FAS	fatty acid synthase
GI	gastrointestinal
HUFA	highly unsaturated fatty acids
IL2	interleukin protein
KLH	keyhole limpet hemocyanin
LA	linoleic acid (18:2 n-6)
LOX	lipoxygenase
LPS	lipopolysaccharide
MTP	microsomal transfer protein
MUFA	monounsaturated fatty acids

PAS	periodic acid-Schiff
PG[X]	prostaglandin
PPAR	peroxisome proliferator activated receptor
PUFA	polyunsaturated fatty acids
SCD	stearoyl Co-A desaturase (delta-9 desaturase)
SNARE	soluble NSF-attachment receptor
SREBP-1c	sterol regulatory binding protein 1c
TG	triglyceride
TNFα	tumor necrosis factor
TPN	total parenteral nutrition
VLCPUFA	very long chain polyunsaturated fatty acids

CHAPTER 1

INTRODUCTION

In the 1930's, dietary essential fatty acids (EFA) were discovered after rats fed a fat-free diet developed pathology reversed by supplementation of the polyunsaturated fatty acids linoleic acid (LA; 18:2 ω6) and α-linolenic acid (ALA 18:3 ω3) (1). Depletion of these dietary EFA results in the classic deficiency symptoms of dermatitis, stunted growth, and reproductive problems. Between the 1940's and 1970's, the importance of EFA was further demonstrated in humans with infants on fat-free formula and adults on fat-free total parenteral nutrition which also developed the classic EFA deficiency symptoms (2). Dermatitis and growth retardation have been attributed solely to LA and ALA deficiency; however the functional importance may not fall entirely on these EFA since they are metabolized to the respective highly unsaturated fatty acids (HUFA), arachidonic acid (AA; 20:4 ω6) and docosahexaenoic acid (DHA; 22:6 ω3) which also participate in physiological processes. It is well established that AA is a precursor to eicosanoids (3), such as prostaglandins and leukotrienes, which act as signaling molecules in a variety of tissues, modulating cell behavior in processes related to inflammation, reproduction, bone metabolism, and immune function. DHA is present in large amounts in brain, retina, and testis and is a HUFA attributed a membrane structural role, providing fluidity to cellular membranes for cell signaling (4).

Despite our current knowledge of AA and DHA, the physiological roles of HUFA are still not completely elucidated. Attributing specific physiological roles to each HUFA and elucidating the mechanism behind their function requires an *in vivo* experimental model of HUFA deficiency. A major limitation in the traditional approach of creating HUFA deficiency is the concurrent depletion of the dietary EFA precursors, LA and ALA. The deficiency symptoms

in this case would not be specific to AA and DHA. Studying AA functions is compromised by stunted growth due to LA deficiency complicating research done on other physiological processes such as reproduction. DHA deficiency studies result in compensatory synthesis of a structurally similar ω6 HUFA, docosapentaenoic acid (DPA; 22:5 ω6) (5), which may mask physiological functions of DHA.

To overcome this obstacle, the delta-6-desaturase knockout (D6D-/-) mouse was created (6,7). D6D is a key enzyme in synthesizing HUFA from the dietary precursor EFA. By disrupting D6D expression, LA and ALA provided in the diet will not be metabolized to HUFA resulting in a state of deficiency specific to the lack of AA and/or DHA. Advantages of using the D6D-/- mouse to elucidate specific AA and DHA functions are therefore: a) the physiological role of AA can be studied without concurrent LA deficiency symptoms of growth retardation and disruption of skin water barrier (1); b) DHA functions can be determined without the compensatory synthesis of the D6D product, docosapentaenoic acid (DPA, 22:5 ω6).

New insight into the role of HUFA was achieved with an initial characterization of the D6D-/- mouse (6,7). Despite the presence of LA and ALA, pathology persisted including male infertility, liver lipidosis, gastrointestinal ulcers, and ulcerative dermatitis (6). Determining specific roles for AA and DHA in each of these physiological processes require further characterization of the D6D-/- mouse. The requirement of HUFA in male reproduction, for example, has been inferred due to the high amounts of AA and DHA in testis, however, the mechanism of HUFA function for male fertility has not been determined. The D6D-/- demonstrated that HUFA deficiency resulted in disruption of spermatogenesis (6,7). Further characterizing the disrupted stage of spermatogenesis in D6D-/- will help elucidate the

mechanism by which HUFA participate in sperm formation and could have implications in treating male infertility.

The general objective of this research is to further characterize the D6D-/- mouse in order to elucidate specific physiological functions of AA and DHA and their underlying mechanisms. In order to achieve this objective, the research is divided into three parts: a) establishing a time course of D6D-/- pathology, b) determining specific HUFA physiological functions through dietary prevention of D6D-/- pathology, c) and elucidating the mechanism behind HUFA requirement for male fertility.

REFERENCES

(1) Burr, G. O., M. M. Burr. 1930. On the nature and role of the fatty acids essential in nutrition. *J Biol Chem* 86**:** 587-621.

(2) Holman, R. T. 1998. The slow discovery of the importance of omega 3 essential fatty acids in human health. *J Nutr* 128**:** 427S-433S.

(3) Samuelsson, B. 1983. From studies of biochemical mechanism to novel biological mediators: prostaglandin endoperoxides, thromboxanes, and leukotrienes. Nobel Lecture, 8 December 1982. *Bioscience Reports* 3**:** 791-813.

(4) Niu, S. L., D. C. Mitchell, S. Y. Lim, Z. M. Wen, H. Y. Kim, N. Salem Jr, and B. J. Litman. 2004. Reduced G protein-coupled signaling efficiency in retinal rod outer segments in response to n-3 fatty acid deficiency. *J. Biol. Chem.* 279**:** 31098-31104.

(5) Neuringer, M., W. E. Connor, D. S. Lin, L. Barstad, and S. Luck. 1986. Biochemical and functional effects of prenatal and postnatal omega-3 fatty acid deficiency on retina and brain in rhesus monkeys. *Proceedings of the National Academy of Sciences of the United States of America* 83**:** 4021-4025.

(6) Stroud, C. K., T. Y. Nara, M. Roqueta-Rivera, E. C. Radlowski, P. Lawrence, Y. Zhang, B. H. Cho, M. Segre, R. A. Hess, J. T. Brenna, W. M. Haschek, and M. T. Nakamura. 2009. Disruption of FADS2 gene in mice impairs male reproduction and causes dermal and intestinal ulceration. *J. Lipid Res.* 50**:** 1870-80.

(7) Stoffel, W., B. Holz, B. Jenke, E. Binczek, R. H. Gunter, C. Kiss, I. Karakesisoglou, M. Thevis, A. A. Weber, S. Arnhold, and K. Addicks. 2008. Delta6-Desaturase (FADS2) deficiency unveils the role of omega3- and omega6-polyunsaturated fatty acids. *EMBO J.* 27**:** 2281–2292.

-

CHAPTER 2

REVIEW OF LITERATURE

Classification of fatty acids

Fatty acid functionality is determined by the physical and chemical properties that result from its chemical structure. The structure of fatty acids consists of a hydrocarbon chain with a carboxyl and methyl end (**Figure 2.1**). This chain varies in length and in the number of double bonds within their chemical structure. Depending on the presence or absence of double bonds within the chemical structure, fatty acids can be classified into two major categories: saturated or unsaturated fatty acids.

Saturated fatty acids do not have double bonds and are major components of cellular membranes. They participate in energy production through beta-oxidation or in energy storage as components of triglycerides in adipose. Palmitic acid (16:0) and stearic acid (18:0) are the most common saturated fatty acids (**Figure 2.1**) and can be synthesized in the liver from acetyl coenzyme-A (**Figure 2.3**).

Unsaturated fatty acids have one or more double bonds within their structure and can be further classified into monounsaturated or polyunsaturated (**Figure 2.1**). Monounsaturated fatty acids (MUFA) have one double bond. Oleic acid (18:1n9) is the most common MUFA and, like saturated fatty acids, is an important building block of cellular membranes. Oleic acid is synthesized from stearic acid after the addition of a double bond by a desaturase enzyme known as stearoyl-CoA desaturase (SCD-1) (**Figure 2.3**).

Polyunsaturated fatty acids (PUFA) have two or more double bonds and also increase fluidity of cellular membranes in addition to being involved in eicosanoid signaling and gene regulation. PUFA can be identified with an omega nomenclature based on the position of the

double bond in reference to the methyl end of the hydrocarbon chain. For example, omega-3 fatty acids have a double bond positioned three carbons from the methyl end, while omega-6 fatty acids have a double bond six carbons from the methyl end. The omega-6 and omega-3 PUFA are termed essential fatty acids (EFA) due to their requirement in the diet. Specifically, omega-6 linoleic acid (LA; 18:2n6) and omega-3 α-linolenic acid (ALA; 18:3n3), cannot be synthesized by humans due to the lack of a desaturase that would insert a double bond at either carbon 3 or 6 from the methyl end of the structure. LA and ALA can be consumed in the diet from plant oil sources such as corn or canola, respectively. In order to obtain the full benefit of LA and ALA, once consumed, these PUFA are metabolized into highly unsaturated fatty acids (HUFA), which are physiologically essential fatty acids of at least 20 carbons in length and a minimum of four double bonds. PUFA metabolism to HUFA requires desaturase and elongase enzymes (**Figure 2.3)**. Delta-5 (D5D) and delta-6 desaturases (D6D) are the desaturases in PUFA metabolism that insert double bonds at a specific position within the fatty acid structure. The role of elongases is chain elongation through addition of carbons. The main HUFA produced from LA is omega-6 arachidonic acid (AA; 20:4n6) while from ALA we obtain the omega-3 docosahexaenoic acid (DHA; 22:6n3). AA and DHA can be considered as physiologically essential fatty acids (**Figure 2.2**).

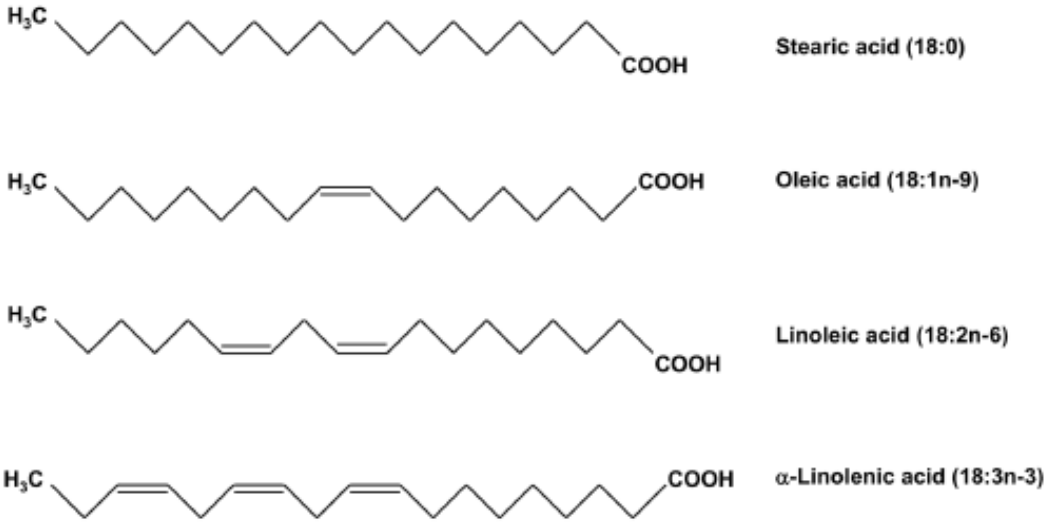

Figure 2.1 *Chemical structure of saturated, monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA).*

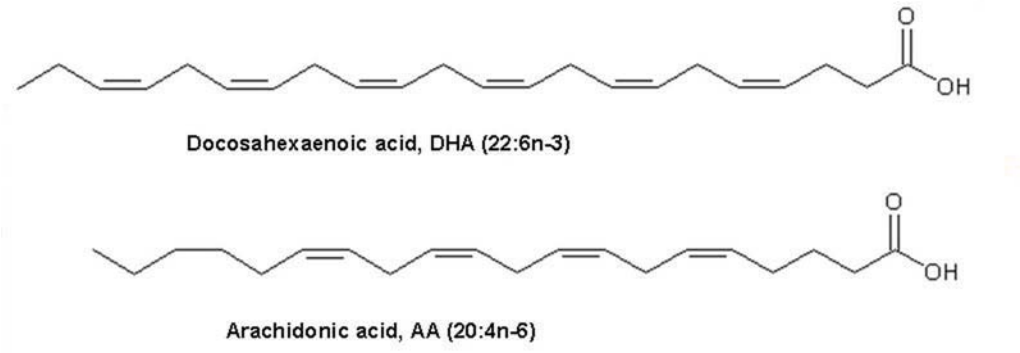

Figure 2.2 *Chemical structure of highly unsaturated fatty acids (HUFA).*

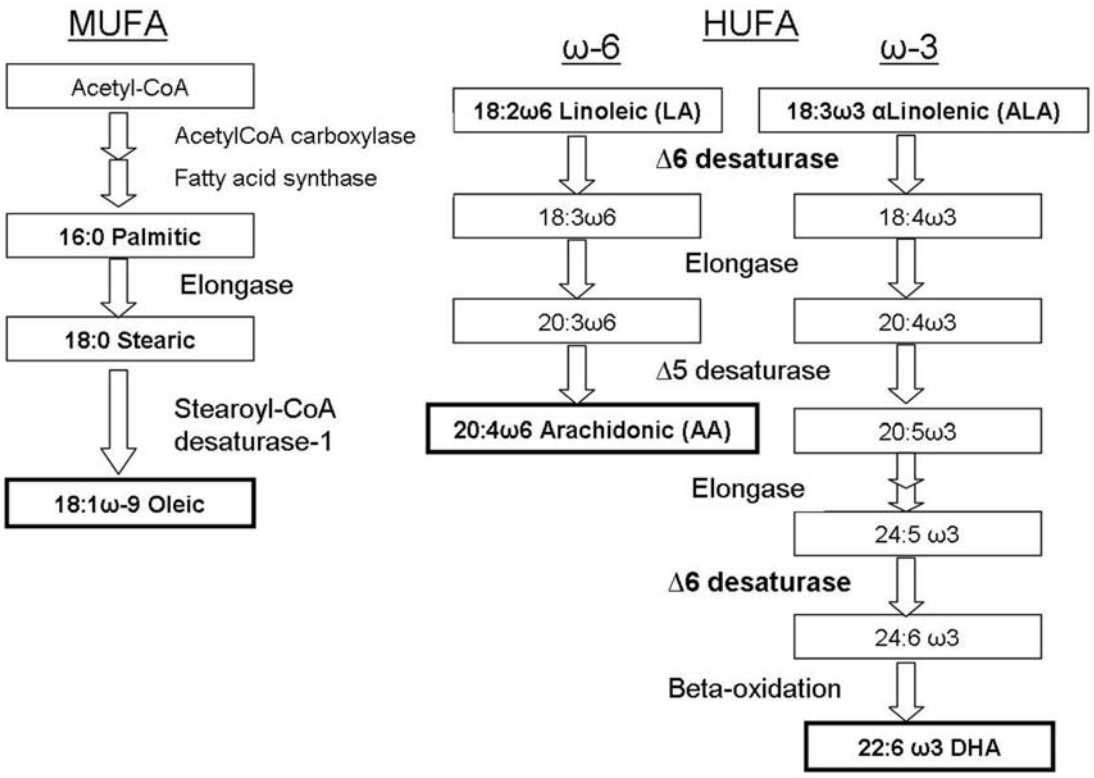

Figure 2.3 *Synthesis of monounsaturated (MUFA) oleic acid (left) and of highly unsaturated fatty acids (HUFA) arachidonic acid (AA) and docosahexaenoic acid (DHA).*

Discovery of essential fatty acids and their functions

The discovery of essential fatty acids (EFA) dates from 1929 when recently weaned rats fed a fat free diet developed dermatitis and growth retardation; fatty livers and testicular degeneration were also observed (1). Neither saturated fatty acids nor MUFA alone were successful in treating these deficiency symptoms (2). Fat sources containing PUFA omega-6 LA were able to restore skin function and growth. Omega-3 ALA treatments showed improvements in fat deficiency symptoms but did not completely cure them (3). These studies demonstrated for the first time the dietary requirement of PUFA for proper physiological function of skin, liver,

and reproductive organs. The specific PUFA for proper function of each tissue was yet to be determined.

A fat free diet study with mice further supported the essentiality of PUFA by reversal of EFA deficiency symptoms through corn oil supplementation containing 60% linoleic acid (4). The mouse EFA deficiency symptoms included dry skin, dermatitis, infertility, and fatty liver, similar pathology to that seen in fat deficient rats. Within this study, another group of mice began the fat-free diet at mature age with a starting body weight of 20 grams. In these mice, observable EFA deficiency symptoms did not occur until 8 months on the fat-free diet. The delay in appearance of EFA deficiency symptoms in mature mice was explained by higher PUFA storage levels in adipose since the EFA deficient diet was initiated at adult age. When a skin injury was induced in these mature mice, wound healing was impaired compared to controls, suggesting a chronic condition of EFA deficiency in which insufficient PUFA stores resulted in delayed tissue repair.

In the 1950s, essentiality of EFA in humans was first studied in infants fed milk of different fat composition and LA levels (5). Infants fed low fat milk presented dry skin and diarrhea after a month. Biochemical evidence of EFA deficiency defined as a ratio of omega-9 mead acid (20:3n9) to omega-6 AA of more than 0.2 was present in the lipid profile of these infants. In absence of EFA, the liver synthesizes mead acid (20:3n9) in place of AA (20:4n6) (**Figure 2.4**). Incorporating LA to the low fat milk corrected serum lipid profiles increasing AA and decreasing mead acid while deficiency symptoms were reversed by two weeks. AA supplementation also corrected skin problems although required up to five weeks to be successful. Saturated fatty acid supplementation did not correct the skin dryness nor did it modify the unsaturated serum lipid profiles.

In the 1970's, the essentiality of PUFA became more apparent with several cases of EFA deficiency due to fat-free total parenteral nutrition (TPN) or intravenous feeding. A 77-year-old female became EFA deficient after receiving fat free TPN due to a resection of the small bowel (6); the patient developed dermatitis within a month. Fat-free TPN given to infants of less than 6 months of age presented an EFA deficient serum lipid profile with a decrease of AA and an increase in mead acid; one infant fed fat-free TPN for 4.5 months presented dermatitis and a very high mead acid to AA ratio of 18; serum profiles were reversed with LA supplementation (7). When on fat-free TPN, newborns were shown to become EFA deficient by the first week of life as reflected by lipid plasma measurements (8). In 1975, three cases of adult EFA deficiency were documented due to fat free TPN; patients developed dermatitis as early as 46 days; LA treatment corrected skin abnormalities (9). Up to this point, all cases of EFA deficiency were treated with success using omega-6 LA. In 1982, LA was incorporated to TPN lipid emulsions with either sunflower or safflower oil. However, the use of safflower oil based TPN resulted in the first human case of omega-3 deficiency. The deficiency was explained due to lack of omega-3 ALA in safflower oil. A 6-year-old girl on safflower oil TPN presented abnormalities in her nervous system and vision. Her serum lipid profiles suggested omega-3 deficiency with only 34% of control values. The switch to sunflower oil lipid emulsions, which contains omega-3 ALA, corrected the deficiency symptoms (10,11). Omega-3 deficiency reflected in plasma levels was also reported in elderly patients fed by gastric tube (12).

LA is a major component of fat emulsions for TPN since soybean oil is currently used a fat source to prevent EFA deficiency. TPN made with fish oil, which is high in HUFA, has also been effective in preventing EFA deficiency. The fish oil based emulsion known as Omegavens (Fresenius Kabi, Bad Homburg, Germany) was developed as an alternative to the soybean oil

preparation. In 2005, a TPN patient allergic to soybean oil developed EFA deficiency due to fat-free TPN and was successfully treated with the fish oil emulsion despite concerns of low LA levels in Omegavens (13). Omegavens is being considered a potential alternative to avoid long term TPN associated liver diseases which are thought to be attributed to the high LA content in soybean oil based TPN (14). The safety and efficacy of TPN with a fish oil based emulsion were tested on 18 infant patients with cholestasis which were previously on soybean oil TPN (15); the patients did not develop essential fatty acid deficiency and reversal of cholestasis was achieved. Several cases have shown that fish oil, used as a sole fat source in TPN, can successfully prevent EFA deficiency (16), demonstrating the essentiality of HUFA and not just LA.

The essentiality of HUFA, AA and DHA, was shown in a human case of impaired HUFA synthesis which resulted in corneal ulcerations, impaired growth, and skin abnormalities (17). Impaired HUFA synthesis in this patient was demonstrated by culturing her skin fibroblasts and evaluating conversion of ^{14}C-labeled omega-6 LA to AA, and from ^{14}C-labeled omega-3 ALA to DHA. Both omega-6 and omega-3 pathways in the patient's skin fibroblasts were disrupted due to delta-6 desaturase (D6D) deficiency as determined by low levels of D6D mRNA and a decrease in HUFA production (17). D6D is the rate limiting enzyme in HUFA synthesis and this case demonstrates that disrupted D6D activity impairs physiological function of tissues despite presence of LA and ALA. This reiterates the importance of HUFA in maintaining tissue function.

The role of AA is well established as being a precursor to eicosanoids which are lipid signaling mediators produced by cells to regulate functions related to inflammation and homeostasis of tissues. In 1964, eicosanoids known as prostaglandins were first identified as AA metabolites (18). Later on, thromboxanes and leukotrienes were also confirmed to be AA

metabolites (19). AA is cleaved from membrane phospholipids by phopholipase and is then metabolized by prostaglandin H2 synthase, also known as cyclooxygenase, into an intermediate prostaglandin (PG) H2. PGH2 is further metabolized into various PG and thromboxanes (20). The type of eicosanoid produced depends on the cell type and tissue. The functions of each eicosanoid depend on the prostanoid receptor to which it binds. These functions have been characterized through the use of knockout mice for each prostanoid receptor (21). Pain and inflammation are physiological processes in which PGI2 and PGE2 are involved (22). PGD2 and E2 participate in immune responses such as allergy and chemotaxis of lymphocytes. PGF2α induces uterus contractions. Bone remodeling is stimulated by PGE2 secreted from osteocytes. Arachidonic acid is therefore involved in the physiological processes of many tissues through the production of its metabolites.

Docosahexaenoic acid is a major fatty acid in phospholipids of brain and retina, and is considered to have a membrane structural role which allows proper function of these tissues. Studies of omega-3 deficiency created by removing the DHA precursor ALA from the diet, has given insight into the possible role of this HUFA in retina and brain. In retina, DHA is in high concentration within the rod photoreceptors of the retina and is important for vision. Visual acuity was reduced by half in rhesus monkeys fed an ALA deficient diet for 8 weeks; DHA was almost depleted at 9% of control plasma phospholipids (23). A specific physiological role for ALA for vision has not been established, other than being the precursor for DHA. Due to DHA accumulation in retina, it is this HUFA and not its precursor which is considered physiologically essential for vision. A possible function for DHA in vision is as modulator of retinoid trafficking in the visual cycle of the retina; DHA was shown to interact with the bovine interphotoreceptor retinoid binding protein favoring delivery of 11-cis-retinal to photoreceptor cells for rhodopsin

restoration (24). The electroretinogram, which evaluates retinal function, is delayed in third generation omega-3 deficient rodents, further demonstrating the importance of omega-3 PUFA for vision (25). These results were explained by inefficient G protein signaling for rhodopsin activation using DHA deficient rodent rod outer segments (26). In relation to brain, omega-3 deficiency rodent studies with up to 87% decrease in omega-3 brain fatty acids show impaired learning and cognitive performance, including spatial learning in a water maze (27), olfactory based learning (28), and a reference memory test with the Barnes circular maze (29).

It is likely that in order to obtain the full benefits of dietary PUFA, LA and ALA must be metabolized to their respective HUFA, AA and DHA. The mechanisms behind AA and DHA functions, as well as their roles in other tissues, have yet to be entirely explained. Many studies of EFA deficiency suggest certain roles for AA and DHA, however, concurrent depletion of the HUFA precursors, LA and ALA, makes it difficult to assign specific roles for each HUFA. An approach to study HUFA functions would be creating specific HUFA deficiency without depleting precursor dietary essential fatty acids. This would require disrupting PUFA metabolism and HUFA synthesis.

Delta-6 desaturase and HUFA synthesis

Research on PUFA metabolism began in the 1950's and 60's with individual measurements of plasma and tissue fatty acids from animals fed LA or ALA. LA was proven to be the precursor to AA while ALA was shown to be precursor to a different set of HUFA of pentaenoic and hexaenoic nature (30), establishing the existence of two separate PUFA metabolic pathways for omega-6 and omega-3 fatty acids. The LA metabolic pathway was further elucidated by feeding rats ^{14}C-labeled LA and isolating its metabolites, which included

AA (31). The use of gas chromatography allowed distinguishing fatty acids by length and number of double bonds. This technique drove the progress of understanding PUFA metabolism with studies of rats fed different levels of LA and ALA indicating competition between omega-3 and omega-6 metabolic pathways for production of HUFA. Increasing the dose of omega-6 LA without changing the omega-3 ALA dose increased omega-6 metabolite levels, such as AA, while suppressing omega-3 fatty acid synthesis from ALA (32). A similar study was done feeding different levels of ALA at constant LA resulting in an increase in omega-3 fats in tissues, while suppressing HUFA synthesis derived from LA (33). In vitro desaturation of LA and ALA using rat liver microsomes also showed competition between PUFA substrates indicating higher affinity towards omega-3 ALA for desaturation (34). The competition between metabolic routes suggested that the omega-6 and omega-3 pathways share the same enzymes for HUFA synthesis. Based on the fatty acid products analyzed by gas chromatography and derived from LA and ALA, it was clear that the enzymes involved in HUFA synthesis would need to elongate and desaturate the fatty acid structure.

In the omega-6 pathway (**Figure 2.4**), the sequence of events starting from LA (18:2n6) to AA (20:4n6) consist of desaturation by D6D at carbon 6 of LA (18:2n6) to make 18:3n6, followed by an elongation resulting in 20:3n6, and a second desaturation by D5D at carbon 5 to make AA (20:4n6). Tissue culture studies suggested that D6D and D5D are two separate desaturase systems by using transformed cell lines lacking D6D activity, unable to make AA from LA , but successful when incubated with the omega-6 intermediate dihomo-gamma linolenic acid (DGLA; 20:3n6) (35). This indicated that a separate enzyme D5D was involved in conversion of the omega-6 intermediate DGLA (20:3n6) to AA (20:4n6). The cloning and expression of the D6D and D5D genes confirmed the role of these enzymes within HUFA

synthesis (36,37); D6D being specific to LA (18:2n6) and ALA (18:3n3), the first step in HUFA synthesis, while D5D acts upon 20:3n6 and 20:4n3, precursors to AA and eicosapentaenoic acid (EPA) respectively.

In the omega-3 pathway (**Figure 2.4**), HUFA synthesis of DHA (22:6n3) begins with ALA (18:3n3) desaturation by D6D to make 18:4n3, which is elongated to 20:4n3. D5D desaturation results in EPA (20:5n3). Two subsequent elongation steps make 24:5n3. D6D desaturation then forms 24:6n3 which is beta-oxidized in peroxisomes to form the HUFA 22:6n3 or DHA (38). In cases of omega-3 deficiency, AA or 20:4n6 can be further desaturated by D6D to form DPAn6 (22:5n6) (**Figure 2.4**).

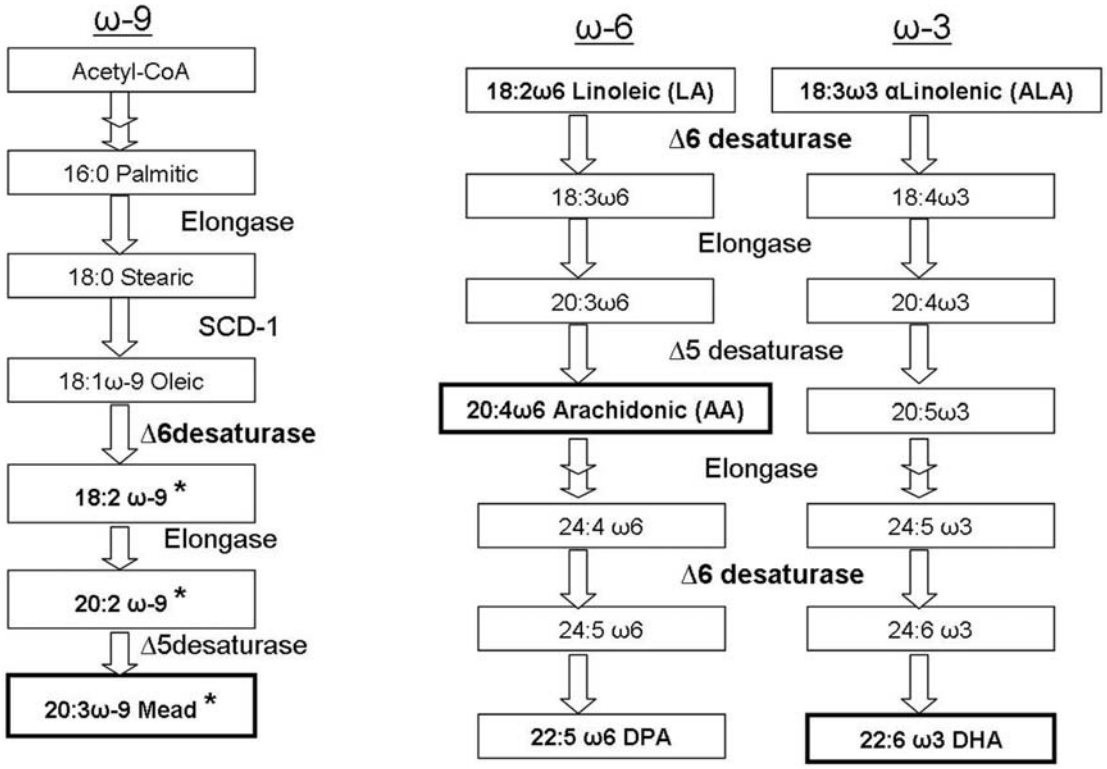

Figure 2.4 *Synthesis of highly unsaturated fatty acids. In omega-3 deficiency, docosapentaenoic acid (DPA) is synthesized. In essential fatty acid deficiency*, mead acid is synthesized from omega-9 oleic acid.*

In summary, the D6D enzyme is critical for HUFA synthesis as the rate limiting enzyme involved in the first and last desaturation steps of the metabolic pathways for synthesis of HUFA: AA, DHA and DPAn6. D6D is described as a front-end desaturase with two membrane spanning domains and an amino-terminal cytochrome b5 binding domain (36,39). This enzyme is located in the membrane of endoplasmic reticulum and its expression occurs in most tissues including liver, adrenal gland, brain, testis, skin, heart, and lung.

D6D is an enzyme of interest in developing a strategy to further study the physiological roles of HUFA. A traditional experimental design to study the physiological roles of EFA is to deplete a whole group of PUFA from the diet making it difficult to attribute deficiency symptoms to a certain fatty acid. Specific functions of AA, for example, could not be studied because its depletion would also require the elimination of its precursor LA, which can readily be converted in mammals to AA with the D6D enzyme. The best approach to study HUFA functions is by creating specific HUFA deficiencies without depleting dietary essential fatty acid precursors, LA and ALA. Based on our knowledge of HUFA synthesis, this can be achieved by disrupting the rate limiting enzyme D6D with the creation of a D6D-/-mouse. A D6D-/- mouse could be fed LA and ALA but would not synthesize HUFA due to absence of D6D expression. Any deficiency symptoms and pathology that occurs in this -/- would be attributed specifically to the lack of HUFA (**Figure 2.5**).

Studying HUFA functions with the D6D -/- mouse model

The *Fads2* gene is found on mouse chromosome 19, has 12 exons and encodes for the 444 amino acid peptide of D6D. There is 87% homology between the mouse and human D6D aminoacid sequences (36). *Fads2* forms a gene cluster with two other desaturase genes, *Fads1*

and *Fads3* (39). The *Fads1* encodes for the D5D enzyme, while the role of the *Fads3* protein product has not been known. A D6D-/- mouse was created by targeted disruption of the *Fad2* gene (40,41). *Fads2*/D6D expression was disrupted by using a targeting vector consisting of a 1.9 kb neomycin resistant gene that replaced a 1.3kb region of the *Fads2* promoter region and exon1. This vector was delivered by electroporation to 129S6/SvEvTac mouse embryonic stem cells. These stem cells were then added to multi-cellular C57BL/6J mouse blastocysts which are implanted to pseudo-pregnant C57BL/6J surrogate females. Offspring with an agouti coat (chimeras) were bred with C57BL/6J wild type females to generate heterozygous offspring with the mutant gene.

The initial characterization of the D6D -/- mixed strain (129S6/SvEvTac and C57BL/6J) mice consisted in providing a diet with sufficient dietary LA and ALA but depleted of HUFA for a period of 4 months at which point phenotype specific to HUFA deficiency was characterized (41). D6D-/- mice presented skin and gastrointestinal tract ulceration, male infertility, altered immune system, and hepatic lipidosis (41). The D6D-/- established essentiality for HUFA in skin, reproduction, gastrointestinal tract, and liver which previously could only be inferred due to inability to create specific HUFA deficiency.

Hepatic lipidosis due to HUFA deficiency

Fatty liver is common in EFA deficiency (42), however it was not clear whether the dietary essential PUFA, LA and ALA, are required in maintaining liver lipid homeostasis, or if this role corresponds to their respective HUFA products, AA and DHA. Liver triglyceride (TG) accumulation occurs in the D6D-/- mouse (41), despite presence of LA and ALA, indicating the requirement for HUFA and not the precursors LA and ALA in modulating TG levels in liver.

Hepatic lipidosis can occur due to increased lipogenesis. Gene expression of lipogenic genes is reduced with PUFA treatments which include HUFA or their EFA precursors, LA and ALA. Since LA and ALA can be metabolized into HUFA it is not clear which group of fatty acids are responsible for downregulation of lipogenic genes. Stearoyl-CoA desaturase (SCD-1) involved in synthesis of MUFA is suppressed by LA and ALA treatments, as well as by their corresponding HUFA products, AA and EPA, in rat primary hepatocytes (43). Rats fed menhaden fish oil, rich in omega-3 PUFA, showed suppressed lipogenesis with reduced expression of fatty acid synthase (FAS), when compared to rats fed saturated tripalmitin (44). Hepatic steatosis can be reversed with PUFA supplementation through downregulation of lipogenic genes, FAS and SCD-1, as shown in ob/ob mice (45). Specifically, omega-3 fatty acid supplementation reduces liver lipid droplet size in mice fed high fat diets (46) and with essential fatty acid deficiency (47). Fish oil-based TPN, rich in omega-3 DHA, also effectively prevented hepatic steatosis in humans (16). The D6D-/- hepatic lipidosis suggests a specific role for HUFA in regulating lipogenesis which cannot be replaced by their precursors LA and ALA.

In addition to the decreased expression of lipogenic genes, increased fatty acid oxidation would also favor prevention of hepatic lipidosis. PUFA bind and activate transcription factor PPARα (48), which upregulate genes of fatty acid oxidation (49). Activation of PPARα can also lead to an increased secretion of apo-B100, an essential component of VLDL (50), the TG rich lipoprotein secreted from liver.

In D6D-/- mice, HUFA deficiency results in altered lipid homeostasis in liver despite presence of LA and ALA. The specific role of HUFA in lipid homeostasis is yet to be elucidated. Due to the role of PUFA in regulating gene expression of lipid metabolism, HUFA

may be required in modulating lipogenesis, fatty acid oxidation, or lipid secretion, in order to prevent lipid accumulation in liver.

AA essentiality for skin physiology

EFA deficiency studies attributed skin pathology to lack of LA, but not to HUFA deficiency. LA supplementation can prevent the common essential fatty acid deficiency symptoms of excessive skin water loss and dermatitis (3). The explanation behind the skin disorders due to an essential fatty acid deficient diet is related to an inefficient epidermal water barrier (51). The skin water barrier results from the structure and composition of the upper most layer of the skin known as the stratum corneum. The stratum corneum consists of layers of corneocytes and intercellular lipids forming a barrier to moisture. The lipids in the stratum corneum are comprised of ceramides, free fatty acids, and cholesterol. Ceramides are the lipid class responsible for providing proper skin water barrier. LA is an essential component of skin acyl-ceramides involved in proper formation of the lamellar layers of the permeability barrier. In EFA deficiency, oleic acid unsuccessfully substitutes LA in skin ceramides resulting in a defective hydrophobic lipid matrix. This was demonstrated in EFA deficient rodents where epidermal water loss was prevented only after linoleate or arachidonate supplementation, but not by oleate supplementation (52). The effectiveness of AA supplementation in preventing skin water loss was explained by retroconversion to LA which was shown in vivo by tracking metabolism of radiolabeled (U-^{14}C) AA in EFA deficient rats; isolated epidermal ceramides formed [^{14}C]-labeled LA (53).

HUFA deficiency in D6D-/- results in scratching behavior which leads to ulcerative dermatitis around neck and ears (41). Ulcerative dermatitis in D6D -/- was unexpected due to

presence of LA. This indicates HUFA is essential for a skin physiological function. The mechanism behind the skin physiological role of HUFA is not entirely clear.

The role of AA in skin is likely to be as precursor to signaling molecules such as prostaglandins (PG) and monohydroxy fatty acids. Monohydroxy fatty acids have a central role in differentiation and inflammatory processes in the skin (54). AA may be acted upon by 15-lipooxygenase (15-LOX) forming the monohydroxy fatty acid, 15-hydroxyeicosatetraenoic acid (15-HETE). 15-HETE has been shown to have anti-inflammatory properties, improving psoriasis vulgaris (55) by reducing skin leukotriene B4 formation. The monohydroxy fatty acid derived from LA, 13- hydroxyoctadecaenoic acid (13-HODE), has antiproliferative properties in skin by modulating protein kinase C (56). The omega-3 DHA is not normally present in skin and DHA topical application on skin of guinea pigs results in epidermal hyperprofileration due to increase in protein kinase C activity (57). Skin hyperproliferation can be reversed by LA-derived 13-HODE topical treatments (58); treatment with AA-derived 15-HETE was not reported. The main skin PG synthesized from AA in human and mouse skin are PGE2 and PGD2 (59). These prostaglandins exert their function based on the prostanoid receptor to which they bind. PGE2 has four types of receptors: EP1 through EP4, while PGD2 has DP1 and DP2. PGD2 is of particular interest in studying D6D-/- skin pathology due to its antipuritic effect and potential involvement in skin repair. PGD2 through its DP1 receptor was shown to decrease scratching behavior in the Nc/Nga mouse model of acrodermatitis (60). Similar to the D6D-/-, Nc/Nga mice present excessive scratching behavior that leads to skin ulceration. Topically applied AA and PGD2 to Nc/Nga mice reduced scratching behavior allowing skin recovery from a lesion (61). Silencing of the cyclooxygenase-1 gene in skin by RNA interference resulted in increased scratching behavior of the Nc/Nga mice, further demonstrating the importance of AA-

derived PG in skin physiology (62). The D6D-/- mouse model confirmed AA essentiality for skin function, with partially restored PGD2 and prevention of dermatitis through AA supplementation (41) .

PUFA are activators of PPAR, therefore, another possible mechanism of AA in contributing to proper skin structure formation may be as a ligand to transcription factor PPARγ. PPARγ activation favors keratinocyte differentiation and lamellar body biogenesis which are important processes for stratum corneum or skin barrier development (63). Keratinocytes differentiate to corneocytes which are the main cell type in the stratum corneum. Lamellar bodies are formed within keratinocytes and are then secreted providing lipids and enzymes required for lamellar layer formation characteristic of the skin barrier (64). Free fatty acids cleaved from phospholipids present in lamellar lipid bodies are important for barrier homeostasis (65) as demonstrated by interference with free fatty acid release through topical application of phospholipase A2 inhibitors on mouse skin. This leads to a perturbed structure of the skin barrier and epidermal hyperplasia (65). Lamellar bodies which deliver these fatty acids are vesicles derived from Golgi and their proper secretion and formation relies on a SNARE complex (66). HUFA have been shown to be required for SNARE complex formation (67) suggesting another possible role for AA in skin function for proper lamellar granule formation, which is essential in delivering components for skin barrier function.

Role of HUFA in gastrointestinal physiology

Intestinal ulceration was reported in the initial characterization of the D6D-/- (41). Protection and repair of the GI tract is mediated by inflammatory mediators which include PG, making AA a major player as a precursor. A reduction in PG production due to lack of AA is a

potential mechanism behind the intestinal pathology in the D6D-/-. The importance of PG for intestinal protection has been well established through the use of non-steroidal anti-inflammatory drugs which block PG synthesis and disrupt the mucosal layer of the intestine leading to lesions and ulcerations of the GI (68). PG influence several factors related to protection of the intestinal epithelium. PGE2 through its EP3 and EP4 receptor, for example, stimulates mucosal bicarbonate secretion for protection against acidic conditions (69). Mucin secretion is also modulated by PGE2 providing a protective barrier for the mucosal layer (70). PG regulation of intestinal blood flow to the mucosa protects the epithelium by diluting toxins (71). Production of PG mainly occurs in the subepithelial tissue or lamina propria (72), which is the site of lymphoid tissue and immune cells, and therefore may have an immunomodulatory function.

The role of DHA in intestine is considered anti-inflammatory through modulation of cytokine responses of the intestinal immune system. The use of fish oil or omega-3 supplementation has been an area of focus for treating GI inflammatory disorders such as Crohn's disease and colitis (73,74). Overproduction of inflammatory cytokines is considered to be a factor in inflammatory intestinal disorders. Fish oil decreases inflammatory cytokine production in patients with Crohn's disease (75) and reduces small intestine inflammation in SAMP1/Yit mice by inhibiting inflammatory cell recruitment to the mucosa (76). Permeability defects after exposure to inflammatory cytokines were corrected by DHA *in vitro* through restoration of tight junction and intestinal epithelial barrier functionality (77). DHA downregulates gene expression of inflammatory cytokines by inhibiting activation of transcription factor NFkB (78,79), which is involved in gene expression of IL1β, IL6, and TNFα. Therefore, studies of omega-3 PUFA functions in intestine have focused on the treatment of inflammatory disorders but their essentiality for intestinal function has not been evaluated. The

D6D-/- mouse model allows the study of a possible DHA functional role in maintaining intestinal homeostasis in absence of AA.

Modulation of the immune system by HUFA

The immune system can be divided into two functional systems: innate and adaptive. The innate immune system is the first line of defense with macrophage and neutrophils involved in destroying pathogens through phagocytosis or release of toxic substances. The adaptive immune system consists of lymphocytes, mainly T and B-cells, developing a highly specific acquired defense against a foreign protein or antigen through the production of antibodies. HUFA are present in immune cells and the fatty acid composition of macrophage, granulocytes, and lymphocytes can be modified according to the fatty acid composition of the diet. These fatty acid modifications impact immune cell function.

One of the earliest studies to demonstrate the impact of PUFA supplementation on immune function consisted of experimental allergic encephalomyelitis in guinea pigs showing diminished clinical signs and reduced lymphocyte proliferation *in vitro* with LA supplementation (80); LA is metabolized to AA, so the effect may not be specific to LA. Several studies indicate reduced lymphocyte proliferation with *in vitro* or *in vivo* supplementation of HUFA in rodents (81,82). Cultured lymphocytes from rat showed highest inhibition of lymphocyte proliferation when treated with HUFA, AA or EPA (81); HUFA inhibitory activity of lymphocyte proliferation was reversed with saturated palmitate or myristate treatments. Rodents supplemented with omega-3 DHA rich-menhaden fish oil or with omega-6 gamma linolenic rich-evening primrose oil, showed decreased lymph node-derived lymphocyte proliferation when compared to mice fed saturated rich hydrogenated coconut oil (82); fish oil fed rats specifically

showed decreased expression of IL-2 receptor on spleen and thymic lymphocytes (82). Human peripheral blood lymphocytes also showed decreased proliferation *in vitro* with HUFA treatments when compared to saturated fatty acid and MUFA treatments (83-85). A mechanism by which HUFA inhibit lymphocyte proliferation can be through modification of membrane lipid raft composition. Lipid rafts are sites for protein activation and cell signaling required for immunological synapse of the T-cell receptor and antigen presenting cells (86). Increased membrane fluidity in lipid rafts due to HUFA presence displaces signaling proteins, interfering with T-cell signaling and activation, required for differentiation and proliferation. A HUFA EPA-treated Jurkat T-cell line and peripheral blood T lymphocytes showed displacement from lipid raft of a protein known as LAT or link to activation of T-cells; phosphorylation of LAT was blocked with HUFA treatment when compared to saturated fatty acid treated cells (87,88). Therefore, lipid rafts are sites in which HUFA may induce their immunosuppressive effects. Inflammatory cytokine production is decreased with EPA and DHA supplementation as demonstrated in lymphocytes (85)(89), macrophages (90), and peripheral blood mononuclear cells (91). A decrease in inflammatory cytokines would also contribute to immune function modulation by HUFA. In addition to immunosuppressive properties, HUFA are involved in resolving inflammation as precursors to lipid mediators of inflammation. Molecules such as DHA-derived resolvins and AA-derived lipoxins allow an appropriate inflammatory and resolution process to restore tissue homeostasis after injury. This resolution process may be compromised with HUFA deficiency. The kinetics of the inflammatory process and its resolution has been characterized in a mouse peritonitis model using zymosan as the inducer (92). An inflammatory process initiates with recruitment of neutrophils for host defense against invading microorganisms. This initial phase is characterized by pro-inflammatory signals such

as cytokines and prostaglandins. AA-derived leukotriene B4 production is involved in neutrophil recruitment and peaks at 4 hours of zymosan injection. Maximum neutrophil infiltration occurs at 12 hours coinciding with DHA conversion to resolving mediators, such as resolvins and maresins, occuring between 12 and 20 hours (92). This period is known as the resolution phase consisting of neutrophil clearance. Halting recruitment of inflammatory cells as well as clearance of neutrophils from the inflammatory site is critical for the return to tissue homeostasis after injury or infection. Neutrophils and monocytes from humans receiving omega-3 HUFA supplementation show decreased chemotaxis suggesting a role for HUFA in modulating immune cell recruitment (93). Neutrophil apoptosis and machrophage phagocytosis of apoptotic cells contribute to inflammatory cell clearance. The docosanoid resolvins derived from DHA play a role in resolution of inflammation (94). DHA-derived maresins stop neutrophil infiltration and induce phagocytosis of apoptotic neutrophil in culture, while AA-derived PGE2 and D2 do not enhance this phagocytosis (95). In addition to phagocytosis of apoptotic neutrophils, DHA derived lipid mediators may contribute to resolution by modulating signaling pathways required for neutrophil apoptosis. NFkB is a transcription factor which plays an important role in regulating granulocyte apoptosis (96). Inhibition of NFkB favors neutrophil apoptosis. DHA deficiency may result in lack of NFkB inhibition resulting in impaired apoptosis which would delay resolution.

In a state of EFA deficiency, adaptive immunity evaluated in mice showed a low response to antigen which could be restored with LA-rich corn oil supplementation (97). EFA deficiency in mice also results in impaired T-cell function as indicated by low delayed type hypersensitivity, while mice fed corn oil or fish oil had greater hypersensitivity (98). Neutrophil functional response was also shown to be impaired in EFA deficient rats (99) and humans (100).

Essential fatty acid (EFA) deficiency leads to impaired immune function, but this may be secondary to growth retardation characteristic of LA deficiency (2).

Evaluation of the essentiality of HUFA for immune function can be achieved with the D6D-/- mouse which does not present growth retardation. The D6D-/- mouse presents spleen pathology consisting of myeloid hyperplasia (41) suggesting an altered immune system. Peritoneal macrophage of HUFA deficient D6D-/- mice presented increased cholesterol biosynthesis and decreased paraoxynase-2 expression, a protein with antioxidant activity (101). AA supplementation of D6D-/- decreased macrophage cholesterol biosynthesis and increased paraoxynase-2 expression suggesting specific role of AA in macrophage function and prevention of atherogenesis (101). This study demonstrates requirement of HUFA in maintaining adequate immune cell function. Further characterization of D6D-/- is required to elucidate other HUFA roles within macrophage and the immune system.

Role of HUFA in male fertility

The requirement of fats for male fertility was demonstrated in the 1930's (1) with rats fed a fat free diet with testicular degeneration; matings of these mice with females on chow diet had an extremely low breeding success rate. Rats receiving a diet deficient in LA and ALA for 15 weeks had low epididymal sperm concentration (102), however, this may be secondary to growth retardation characteristic of LA deficiency (2). The fact that D6D is highly expressed in mouse testis (103) indicates that this tissue is actively synthesizing HUFA. Rat testis has a dramatic increase of DPAn6, a HUFA derived from AA, during the sexual maturation stage of the animals (104). The HUFA, DHA, is more abundant than DPAn6 in the fatty acid composition of human sperm (105). These HUFA are in high levels within membrane phospholipids of mature

spermatozoa (106). The importance of HUFA in fertility is further implied with the observation of lower DHA phospholipid and fatty acids levels in sperm of infertile men when compared to healthy individuals (107). The HUFA deficiency state of the D6D-/- results in disrupted sperm formation indicating a requirement for these fatty acids in fertility and spermatogenesis (40, 41).

Spermatogenesis occurs within the epithelium of seminiferous tubules contained in the testis. Within these polarized tubules, there is a basal and apical side. The basal side contains germ cells known as spermatogonia which go through different cell stages making their way towards the apical or luminal side of the tubule, until formation of spermatozoa. These stages are characterized by cell types that include primary spermatocytes, secondary spermatocytes, round spermatids, and spermatozoa. In the first stage, spermatogonia go through mitosis to form primary spermatocytes. These cells then go through meiosis forming haploid secondary spermatocytes with half the genetic material of the spermatogonia. A second meiosis results in round spermatids. Round spermatids then go through spermiogenesis, a series of structural changes which include head elongation and tail formation that result in spermatozoa. The spermatozoa are then released into the lumen reaching the epididymis where maturation continues. The epididymis, in its cauda region, plays a role in storage of spermatozoa.

The Sertoli cell is essential for spermatogenesis by constantly interacting with germ cells from all stages, providing support and nutrition. Several studies demonstrate an active role for the Sertoli cell in HUFA synthesis suggesting it to be a source of AA and DHA for germ cells throughout spermatogenesis. Primary culture studies of isolated rat Sertoli cells demonstrate synthesis of AA from ^{14}C-labeled LA (108). The Sertoli cell is the primary location of 22 carbon HUFA synthesis as shown in rat testes injected with ^{14}C-labeled AA (109). Higher DPA production was achieved in Sertoli cells versus germ cells. Isolated human germ cell fractions

produced almost no DHA and DPA when incubated with ^{14}C-labeled substrates, further demonstrating a role for Sertoli cells in HUFA synthesis (110). Gene expression of D5D and D6D are limited to Sertoli cells isolated from rat testis (111). The Sertoli cell therefore is the site of HUFA production; however, the fact that germ cells are richer in HUFA despite their lack of HUFA synthesis suggests lipid transport from the Sertoli cell. High HUFA content and active synthesis in testis, as well as fatty acid composition of sperm has been well established, but the exact role of HUFA in spermatogenesis has not been elucidated.

The role of AA in male fertility is most likely as a precursor to DPAn6 and to eicosanoids. Eicosanoids can be synthesized by isolated sperm cells incubated with AA (112). Research of the role of eicosanoids in male fertility has been focused on their impact on mature sperm physiology but not on spermatogenesis. PG are required for sperm motility as demonstrated by inhibition of PG synthesis in rats (113) and mice (114). PG of the E series is shown to enhance calcium influx into cytoplasm of mature sperm which is essential for the acrosome reaction in fertilization (115). In addition to sperm motility and acrosome reaction, PG have an impact on steroid synthesis (116).

AA and DHA are precursors to further elongated PUFA known as very long chain polyunsaturated fatty acids (VLCPUFA), such as 28:4n6 (AA derived) and 30:5n6 (DPA derived), which are incorporated into membrane sphingolipids. Sphingomyelins and ceramides in the head of mammalian spermatozoa contain VLCPUFA as acyl groups and are suggested to participate in sperm capacitation (117).

HUFA analysis of cell types throughout the different stages of spermatogenesis may provide information on the potential functional role of HUFA. In mice, an increase of DPA from 2% to 20% and of DHA from 4 to 14% of total fatty acids was observed from the preleptotene

spermatocyte stage to the condensed spermatid stage with an eventual HUFA decrease in mature sperm to 8-9% (118). In mature sperm, DHA is abundant in tail and due to its high unsaturation is considered to provide motility (119). Phospholipid DHA can also be found in sperm head (120), suggesting a DHA functional role other than membrane fluidity that has yet to be determined.

The D6D male -/- infertility results from disrupted spermiogenesis (40,41), the last stage of spermatogenesis in which round spermatids are elongated to form spermatozoa. The spermiogenesis stage is therefore of particular interest to study HUFA functions. Spermiogenesis consists of 16 steps in which essential components for proper sperm function are acquired (121). Acrosome is formed during the first steps of spermiogenesis as a cap-like structure on the nucleus of the spermatid. The acrosome is a vesicle that contains enzymes, such as acrosin, needed for the acrosomal reaction during fertilization. Acrosome biogenesis occurs prior to the steps of nuclear elongation and flagellum formation, essential morphological changes for sperm motility. Globozoospermia is the formation of round headed shaped sperm and is associated with lack of acrosome formation (122). The globozoospermia mouse models such as PICK1-/-(123), Hrb-/- (124) and Golgi-associated PDZ- and coiled-coil motif-containing protein (GOPC) -/- (125) are acrosome-deficient and all present globozoospermia. The Hrb protein is required for fusion of Golgi-derived proacrosomal vesicles during acrosome biogenesis, while GOPC participates in vesicle trafficking from Golgi to acrosome (123). A potential role of HUFA in fertility could be in acrosome biogenesis for fusion of pro-acrosomal granules derived from Golgi.

Role of HUFA in vesicle fusion

Vesicle fusion is required for several biological processes including exocytosis, release of neurotransmitters, neurite outgrowth, and membrane repair. The soluble NSF-attachment receptor (SNARE) complex is a four helical structure that drives the union of opposing membranes and is an important component of the vesicle fusion process (126). This SNARE complex consists of proteins such as, syntaxin and synaptobrevin, which are on opposing membranes and interact to achieve vesicle fusion. Syntaxin exists in an inactive and closed conformation with its N-terminal folded back over a SNARE motif and requires activation into an open conformation for SNARE assembly (127). In brain, AA activates syntaxin (128) allowing SNARE complex formation consisting of syntaxin-3, SNAP25, and synaptobrevin interactions (129). Neuroendocrine cells showed increased neurite outgrowth, a process that requires SNARE complex, when treated with AA; DHA was shown to efficiently substitute for AA for SNARE assembly *in vitro* as well as in a neurite outgrowth assay of cultured PC12 neuroendocrine cells (129). In retina, rhodopsin delivery by Golgi-derived vesicles requires SNARE components which are regulated by DHA (130). Similar to brain and retina, testis are rich in HUFA and also have SNARE components. Syntaxin, synaptobrevin, and SNAP have been identified in spermiogenesis during acrosome biogenesis in mouse (131), however the role of HUFA in regulating SNARE components in male fertility has not been demonstrated. SNARE complex formation is most likely required for fusion of proacrosomal vesicles during acrosome formation. The mechanism behind proacrosomal vesicle fusion may parallel that seen in neuroendocrine cells and retina, where HUFA activate syntaxin to favor SNARE assembly.

SUMMARY

The study of HUFA and their physiological roles has been limited due to the traditional approach of creating HUFA deficiency by depletion of LA (18:2n6) and/or ALA (18:3n3), precursors to HUFA: AA (20:4n6) and DHA (22:6n3). New insight into the role of HUFA is established with the D6D-/- mouse model. Male infertility, GI ulcers, liver lipid homeostasis, and dermatitis occur with AA and DHA deficiency despite presence of their corresponding EFA precursors LA and ALA. The functionality of skin, GI, liver, and testis relies on HUFA and not just the dietary EFA. Specific HUFA roles within these tissues have been discovered. In skin, LA was considered the functional PUFA by maintaining skin barrier function, however ulcerative dermatitis in the D6D-/- mouse model and its prevention with AA supplementation demonstrated essentiality for AA in skin as well. In male fertility, DHA and not AA fully restored D6D-/- spermatogenesis indicating a specific function for DHA in sperm formation.

Further research is required to fully elucidate the mechanism behind the essentiality of HUFA in tissues. The role of HUFA may be as precursor to signaling molecules such as eicosanoids; as structural components of membranes; as regulators of gene expression; or as activators of vesicle fusion machinery. Understanding HUFA physiological roles could have implications in developing treatments for diseases where HUFA metabolism may be altered.

REFERENCES

(1) Burr, G. O., M. M. Burr. 1929. A new deficiency disease produced by the rigid exclusion of fat from the diet. *Journal of Biological Chemistry* 82: 345-67.

(2) Burr, G. O., M. M. Burr. 1930. On the nature and role of the fatty acids essential in nutrition. *J Biol Chem* 86: 587-621.

(3) Burr, G. O., M. M. Burr, and E. S. Miller. 1932. On the fatty acids essential in nutrition, III. *J. Biol. Chem.* 97: 1-9.

(4) Decker, A. B., D. L. Fillerup, and J. F. Mead. 1950. Chronic Essential Fatty Acid Deficiency in Mice: Five Figures. *J. Nutr.* 41: 507-521.

(5) Wiese, H. F., A. E. Hansen, and D. J. D. Adam. 1958. Essential Fatty Acids in Infant Nutrition: I. Linoleic Acid Requirement in Terms of Serum Di-, Tri- and Tetraenoic acid Levels. *J. Nutr.* 66: 345-360.

(6) Collins, F. D., A. J. Sinclair, J. P. Royle, D. A. Coats, A. T. Maynard, and R. F. Leonard. 1971. Plasma lipids in human linoleic acid deficiency. *Nutr. Metab.* 13: 150-167.

(7) Paulsrud, J. R., L. Pensler, C. F. Whitten, S. Stewart, and R. T. Holman. 1972. Essential fatty acid deficiency in infants induced by fat-free intravenous feeding. *Am. J. Clin. Nutr.* 25: 897-904.

(8) Friedman, Z., A. Danon, M. T. Stahlman, and J. A. Oates. 1976. Rapid onset of essential fatty acid deficiency in the newborn. *Pediatrics* 58: 640-649.

(9) Riella, M. C., J. W. Broviac, M. Wells, and B. H. Scribner. 1975. Essential fatty acid deficiency in human adults during total parenteral nutrition. *Ann. Intern. Med.* 83: 786-789.

(10) Holman, R. T. 1998. The slow discovery of the importance of omega 3 essential fatty acids in human health. *J Nutr* 128: 427S-433S.

(11) Holman, R. T., S. B. Johnson, and T. F. Hatch. 1982. A case of human linolenic acid deficiency involving neurological abnormalities. *Am J Clin Nutr* 35: 617-23.

(12) Bjerve, K. S., I. L. Mostad, and L. Thoresen. 1987. Alpha-linolenic acid deficiency in patients on long-term gastric-tube feeding: estimation of linolenic acid and long-chain unsaturated n-3 fatty acid requirement in man. *Am. J. Clin. Nutr.* 45: 66-77.

(13) Gura, K. M., S. K. Parsons, L. J. Bechard, T. Henderson, M. Dorsey, W. Phipatanakul, C. Duggan, M. Puder, and C. Lenders. 2005. Use of a fish oil-based lipid emulsion to treat essential fatty acid deficiency in a soy allergic patient receiving parenteral nutrition. *Clin. Nutr.* 24: 839-847.

(14) Chen, W. J., S. L. Yeh, and P. C. Huang. 1996. Effects of fat emulsions with different fatty acid composition on plasma and hepatic lipids in rats receiving total parenteral nutrition. *Clin. Nutr.* 15: 24-28.

(15) Gura, K. M., S. Lee, C. Valim, J. Zhou, S. Kim, B. P. Modi, D. A. Arsenault, R. A. Strijbosch, S. Lopes, C. Duggan, and M. Puder. 2008. Safety and efficacy of a fish-oil-based fat emulsion in the treatment of parenteral nutrition-associated liver disease. *Pediatrics* 121: e678-86.

(16) Le, H. D., J. A. Meisel, V. E. de Meijer, K. M. Gura, and M. Puder. 2009. The essentiality of arachidonic acid and docosahexaenoic acid. *Prostaglandins Leukot. Essent. Fatty Acids* 81: 165-170.

(17) Williard, D. E., J. O. Nwankwo, T. L. Kaduce, S. D. Harmon, M. Irons, H. W. Moser, G. V. Raymond, and A. A. Spector. 2001. Identification of a fatty acid delta6-desaturase deficiency in human skin fibroblasts. *J Lipid Res* 42: 501-8.

(18) Nutrition classics. Biochimica et Biophysica Acta 90:207-10, 1964. The enzymic formation of prostaglandin E2 from arachidonic acid. Prostaglandins and related factors 32. Sune Bergstrom, Henry Danielsson and Bengt Samuelsson. 1981. *Nutr. Rev.* 39: 313-315.

(19) Samuelsson, B. 1983. From studies of biochemical mechanism to novel biological mediators: prostaglandin endoperoxides, thromboxanes, and leukotrienes. Nobel Lecture, 8 December 1982. *Bioscience Reports* 3: 791-813.

(20) Smith, W. L., D. L. DeWitt, and R. M. Garavito. 2000. Cyclooxygenases: structural, cellular, and molecular biology. *Annu. Rev. Biochem.* 69: 145-182.

(21) Narumiya, S., G. A. FitzGerald. 2001. Genetic and pharmacological analysis of prostanoid receptor function. *J. Clin. Invest.* 108: 25-30.

(22) Murata, T., F. Ushikubi, T. Matsuoka, M. Hirata, A. Yamasaki, Y. Sugimoto, A. Ichikawa, Y. Aze, T. Tanaka, N. Yoshida, A. Ueno, S. Oh-ishi, and S. Narumiya. 1997. Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature* 388: 678-682.

(23) Neuringer, M., W. E. Connor, C. Van Petten, and L. Barstad. 1984. Dietary omega-3 fatty acid deficiency and visual loss in infant rhesus monkeys. *J Clin Invest* 73: 272-6.

(24) Chen, Y., L. A. Houghton, J. T. Brenna, and N. Noy. 1996. Docosahexaenoic acid modulates the interactions of the interphotoreceptor retinoid-binding protein with 11-*cis*-retinal. *Journal of Biological Chemistry* 271: 20507-20515.

(25) Weisinger, H. S., J. A. Armitage, B. G. Jeffrey, D. C. Mitchell, T. Moriguchi, A. J. Sinclair, R. S. Weisinger, and N. Salem Jr. 2002. Retinal sensitivity loss in third-generation n-3 PUFA-deficient rats. *Lipids* 37: 759-765.

(26) Niu, S. L., D. C. Mitchell, S. Y. Lim, Z. M. Wen, H. Y. Kim, N. Salem Jr, and B. J. Litman. 2004. Reduced G protein-coupled signaling efficiency in retinal rod outer segments in response to n-3 fatty acid deficiency. *J. Biol. Chem.* 279: 31098-31104.

(27) Moriguchi, T., R. S. Greiner, and S. N. Jr. 2000. Behavioral deficits associated with dietary induction of decreased brain docosahexaenoic acid concentration. *J Neurochem* 75: 2563-73.

(28) Greiner, R. S., T. Moriguchi, B. M. Slotnick, A. Hutton, and N. Salem. 2001. Olfactory discrimination deficits in n-3 fatty acid-deficient rats. *Physiol Behav* 72: 379-85.

(29) Fedorova, I., N. Hussein, C. Di Martino, T. Moriguchi, J. Hoshiba, S. Majchrzak, and N. Salem Jr. 2007. An n-3 fatty acid deficient diet affects mouse spatial learning in the Barnes circular maze. *Prostaglandins Leukot. Essent. Fatty Acids* 77: 269-277.

(30) Widmer, C.,Jr, R. T. Holman. 1950. Polyethenoid fatty acid metabolism; deposition of polyunsaturated fatty acids in fat-deficient rats upon single fatty acid supplementation. *Arch. Biochem.* 25: 1-12.

(31) Steinberg G., W. H. Slaton Jr, D. R. Howton, and J. F. MEAD. 1957. Metabolism of essential fatty acids. V. Metabolic pathway of linolenic acid. *J. Biol. Chem.* 224: 841-849.

(32) Mohrhauer, H., R. T. Holman. 1963. The Effect of Dose Level of Essential Fatty Acids Upon Fatty Acid Composition of the Rat Liver. *J Lipid Res* 58: 151-9.

(33) Rahm, J. J., R. T. Holman. 1964. Effect of Linoleic Acid Upon the Metabolism of Linolenic Acid. *J Nutr* 84: 15-9.

(34) Brenner, R. R., R. O. Peluffo. 1966. Effect of saturated and unsaturated fatty acids on the desaturation in vitro of palmitic, stearic, oleic, linoleic, and linolenic acids. *J. Biol. Chem.* 241: 5213-5219.

(35) Mathers, L., M. J. Bailey. 1975. Enzyme deletions and essential fatty acid metabolism in cultured cells. *J. Biol. Chem.* 250: 1152-1153.

(36) Cho, H. P., M. T. Nakamura, and S. D. Clarke. 1999. Cloning, expression, and nutritional regulation of the mammalian delta-6 desaturase. *J Biol Chem* 274: 471-477.

(37) Cho, H. P., M. Nakamura, and S. D. Clarke. 1999. Cloning, expression, and fatty acid regulation of the human delta-5 desaturase. *J. Biol. Chem.* 274: 37335-37339.

(38) Sprecher, H., D. L. Luthria, B. S. Mohammed, and S. P. Baykousheva. 1995. Reevaluation of the pathways for the biosynthesis of polyunsaturated fatty acids. *Journal of Lipid Research* 36: 2471-2477.

(39) Nakamura, M. T., T. Y. Nara. 2004. Structure, function, and dietary regulation of delta6, delta5, and delta9 desaturases. *Annu Rev Nutr* 24: 345-76.

(40) Stoffel, W., B. Holz, B. Jenke, E. Binczek, R. H. Gunter, C. Kiss, I. Karakesisoglou, M. Thevis, A. A. Weber, S. Arnhold, and K. Addicks. 2008. Delta6-Desaturase (FADS2) deficiency unveils the role of omega3- and omega6-polyunsaturated fatty acids. *EMBO J.* 27: 2281–2292.

(41) Stroud, C. K., T. Y. Nara, M. Roqueta-Rivera, E. C. Radlowski, P. Lawrence, Y. Zhang, B. H. Cho, M. Segre, R. A. Hess, J. T. Brenna, W. M. Haschek, and M. T. Nakamura. 2009. Disruption of FADS2 gene in mice impairs male reproduction and causes dermal and intestinal ulceration. *J. Lipid Res.* 50: 1870-80.

(42) Alfin-Slater, R. B., S. Bernick. 1958. Changes in tissue lipids and tissue histology resulting from essential fatty acid deficiency in rats. *Am. J. Clin. Nutr.* 6: 613-624.

(43) Landschulz, K. T., D. B. Jump, O. A. MacDougald, and M. D. Lane. 1994. Transcriptional control of the stearoyl-CoA desaturase-1 gene by polyunsaturated fatty acids. *Biochem. Biophys. Res. Commun.* 200: 763-768.

(44) Clarke, S. D., M. K. Armstrong, and D. B. Jump. 1990. Dietary polyunsaturated fats uniquely suppress rat liver fatty acid synthase and S14 mRNA content. *Journal of Nutrition* 120: 225-31.

(45) Sekiya, M., N. Yahagi, T. Matsuzaka, Y. Najima, M. Nakakuki, R. Nagai, S. Ishibashi, J. Osuga, N. Yamada, and H. Shimano. 2003. Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression. *Hepatology* 38: 1529-1539.

(46) Nemoto, N., S. Suzuki, H. Kikuchi, H. Okabe, S. Sassa, and S. Sakamoto. 2009. Ethyl-eicosapentaenoic acid reduces liver lipids and lowers plasma levels of lipids in mice fed a high-fat diet. *In Vivo* 23: 685-689.

(47) Alwayn, I. P., C. Andersson, B. Zauscher, K. Gura, V. Nose, and M. Puder. 2005. Omega-3 fatty acids improve hepatic steatosis in a murine model: potential implications for the marginal steatotic liver donor. *Transplantation* 79: 606-608.

(48) Kliewer, S. A., S. S. Sundseth, S. A. Jones, P. J. Brown, G. B. Wisely, C. S. Koble, P. Devchand, W. Wahli, T. M. Wilson, J. M. Lenhard, and J. M. Lehmann. 1997. Fatty acids and eicosanoids regulate gene expression through direct interaction with peroxisome proliferator-activated receptors alpha and gamma. *Proceedings of the National Academy of Sciences of the United States of America* 94: 4318-4323.

(49) Leone, T. C., C. J. Weinheimer, and D. P. Kelly. 1999. A critical role for the peroxisome proliferator-activated receptor alpha (PPARalpha) in the cellular fasting response: the PPARalpha-null mouse as a model of fatty acid oxidation disorders. *Proc Natl Acad Sci U S A* 96: 7473-7478.

(50) Linden, D., K. Lindberg, J. Oscarsson, C. Claesson, L. Asp, L. Li, M. Gustafsson, J. Boren, and S. O. Olofsson. 2002. Influence of peroxisome proliferator-activated receptor alpha agonists on the intracellular turnover and secretion of apolipoprotein (Apo) B-100 and ApoB-48. *J. Biol. Chem.* 277: 23044-23053.

(51) Elias, P. M., B. E. Brown. 1978. The mammalian cutaneous permeability barrier: defective barrier function is essential fatty acid deficiency correlates with abnormal intercellular lipid deposition. *Lab. Invest.* 39: 574-583.

(52) Hansen, H. S., B. Jensen. 1985. Essential function of linoleic acid esterified in acylglucosylceramide and acylceramide in maintaining the epidermal water permeability barrier. Evidence from feeding studies with oleate, linoleate, arachidonate, columbinate and alpha-linolenate. *Biochim Biophys Acta* 834: 357-63.

(53) Hansen, H. S., B. Jensen, and P. von Wettstein-Knowles. 1986. Apparent in vivo retroconversion of dietary arachidonic to linoleic acid in essential fatty acid-deficient rats. *Biochim. Biophys. Acta* 878: 284-287.

(54) Ziboh, V. A. 1996. The significance of polyunsaturated fatty acids in cutaneous biology. *Lipids* 31 Suppl: S249-53.

(55) Fogh, K., H. Sogaard, T. Herlin, and K. Kragballe. 1988. Improvement of psoriasis vulgaris after intralesional injections of 15-hydroxyeicosatetraenoic acid (15-HETE). *J. Am. Acad. Dermatol.* 18: 279-285.

(56) Ziboh, V. A., C. C. Miller, and Y. Cho. 2000. Significance of lipoxygenase-derived monohydroxy fatty acids in cutaneous biology. *Prostaglandins Other Lipid Mediat.* 63: 3-13.

(57) Mani, I., L. Iversen, and V. A. Ziboh. 1998. Upregulation of nuclear PKC and MAP-kinase during hyperproliferation of guinea pig epidermis: modulation by 13-(S)-hydroxyoctadecadienoic acid (13-HODE). *Cell. Signal.* 10: 143-149.

(58) Cho, Y., V. A. Ziboh. 1994. 13-Hydroxyoctadecadienoic acid reverses epidermal hyperproliferation via selective inhibition of protein kinase C-beta activity. *Biochem. Biophys. Res. Commun.* 201: 257-265.

(59) Hammarstrom, S., J. A. Lindgren, C. Marcelo, E. A. Duell, T. F. Anderson, and J. J. Voorhees. 1979. Arachidonic acid transformations in normal and psoriatic skin. *J. Invest. Dermatol.* 73: 180-183.

(60) Arai, I., N. Takano, Y. Hashimoto, N. Futaki, M. Sugimoto, N. Takahashi, T. Inoue, and S. Nakaike. 2004. Prostanoid DP1 receptor agonist inhibits the pruritic activity in NC/Nga mice with atopic dermatitis. *Eur. J. Pharmacol.* 505: 229-235.

(61) Honma, Y., I. Arai, Y. Hashimoto, N. Futaki, M. Sugimoto, M. Tanaka, and S. Nakaike. 2005. Prostaglandin D2 and prostaglandin E2 accelerate the recovery of cutaneous barrier disruption induced by mechanical scratching in mice. *European Journal of Pharmacology* 518: 56-62.

(62) Inoue, J., S. Yotsumoto, T. Sakamoto, S. Tsuchiya, and Y. Aramaki. 2005. Changes in immune responses to antigen applied to tape-stripped skin with CpG-oligodeoxynucleotide in NC/Nga mice. *Pharm. Res.* 22: 1627-1633.

(63) Mao-Qiang, M., A. J. Fowler, M. Schmuth, P. Lau, S. Chang, B. E. Brown, A. H. Moser, L. Michalik, B. Desvergne, W. Wahli, M. Li, D. Metzger, P. H. Chambon, P. M. Elias, and K. R. Feingold. 2004. Peroxisome-proliferator-activated receptor (PPAR)-gamma activation stimulates keratinocyte differentiation. *J. Invest. Dermatol.* 123: 305-312.

(64) Fartasch, M. 2004. The epidermal lamellar body: a fascinating secretory organelle. *J. Invest. Dermatol.* 122: XI-XII.

(65) Mao-Qiang, M., M. Jain, K. R. Feingold, and P. M. Elias. 1996. Secretory phospholipase A2 activity is required for permeability barrier homeostasis. *J. Invest. Dermatol.* 106: 57-63.

(66) Sprecher, E., A. Ishida-Yamamoto, M. Mizrahi-Koren, D. Rapaport, D. Goldsher, M. Indelman, O. Topaz, I. Chefetz, H. Keren, T. J. O'brien, D. Bercovich, S. Shalev, D. Geiger, R. Bergman, M. Horowitz, and H. Mandel. 2005. A mutation in SNAP29, coding for a SNARE protein involved in intracellular trafficking, causes a novel neurocutaneous syndrome characterized by cerebral dysgenesis, neuropathy, ichthyosis, and palmoplantar keratoderma. *Am. J. Hum. Genet.* 77: 242-251.

(67) Davletov, B., E. Connell, and F. Darios. 2007. Regulation of SNARE fusion machinery by fatty acids. *Cell Mol. Life Sci.* 64: 1597-1608.

(68) Vane, J. R. 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat. New Biol.* 231: 232-235.

(69) Aihara, E., Y. Nomura, Y. Sasaki, F. Ise, K. Kita, and K. Takeuchi. 2007. Involvement of prostaglandin E receptor EP3 subtype in duodenal bicarbonate secretion in rats. *Life Sci.* 80: 2446-2453.

(70) Tani, S., M. Okuda, R. Morishige, and T. Tanaka. 1997. Gastric mucin secretion from cultured rat epithelial cells. *Biol. Pharm. Bull.* 20: 482-485.

(71) Hoffmann, P., V. E. Eysselein, J. M. Zeeh, F. Procaccino, J. Kao, F. Iwata, and F. W. Leung. 1999. Epidermal growth factor increases basal mucosal blood flow in the rat colon, a prostaglandin dependent effect. *Eur. J. Gastroenterol. Hepatol.* 11: 1305-1310.

(72) Lawson, L. D., D. W. Powell. 1987. Bradykinin-stimulated eicosanoid synthesis and secretion by rabbit ileal components. *Am. J. Physiol.* 252: G783-90.

(73) Belluzzi, A., C. Brignola, M. Campieri, A. Pera, S. Boschi, and M. Miglioli. 1996. Effect of an enteric-coated fish-oil preparation on relapses in Crohn's disease. *N. Engl. J. Med.* 334: 1557-1560.

(74) Stenson, W. F., D. Cort, J. Rodgers, R. Burakoff, K. DeSchryver-Kecskemeti, T. L. Gramlich, and W. Beeken. 1992. Dietary supplementation with fish oil in ulcerative colitis. *Ann. Intern. Med.* 116: 609-614.

(75) Trebble, T. M., N. K. Arden, S. A. Wootton, P. C. Calder, M. A. Mullee, D. R. Fine, and M. A. Stroud. 2004. Fish oil and antioxidants alter the composition and function of circulating mononuclear cells in Crohn disease. *Am. J. Clin. Nutr.* 80: 1137-1144.

(76) Matsunaga, H., R. Hokari, C. Kurihara, Y. Okada, K. Takebayashi, K. Okudaira, C. Watanabe, S. Komoto, M. Nakamura, Y. Tsuzuki, A. Kawaguchi, S. Nagao, and S. Miura. 2009. Omega-3 polyunsaturated fatty acids ameliorate the severity of ileitis in the senescence accelerated mice (SAM)P1/Yit mice model. *Clin. Exp. Immunol.* 158: 325-333.

(77) Li, Q., Q. Zhang, M. Wang, S. Zhao, G. Xu, and J. Li. 2008. N-3 Polyunsaturated Fatty Acids Prevent Disruption of Epithelial Barrier Function Induced by Proinflammatory Cytokines. *Mol. Immunol.* 45: 1356-1365.

(78) Lo, C. J., K. C. Chiu, M. Fu, R. Lo, and S. Helton. 1999. Fish oil decreases macrophage tumor necrosis factor gene transcription by altering the NF kappa B activity. *J. Surg. Res.* 82: 216-221.

(79) Novak, T. E., T. A. Babcock, D. H. Jho, W. S. Helton, and N. J. Espat. 2003. NF-kappa B inhibition by omega -3 fatty acids modulates LPS-stimulated macrophage TNF-alpha transcription. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 284: L84-9.

(80) Meade, C. J., J. Mertin, J. Sheena, and R. Hunt. 1978. Reduction by linoleic acid of the severity of experimental allergic encephalomyelitis in the guinea pig. *J. Neurol. Sci.* 35: 291-308.

(81) Calder, P. C., J. A. Bond, S. J. Bevan, S. V. Hunt, and E. A. Newsholme. 1991. Effect of fatty acids on the proliferation of concanavalin A-stimulated rat lymph node lymphocytes. *Int. J. Biochem.* 23: 579-588.

(82) Yaqoob, P., E. A. Newsholme, and P. C. Calder. 1994. The effect of dietary lipid manipulation on rat lymphocyte subsets and proliferation. *Immunology* 82: 603-610.

(83) Zurier, R. B., R. G. Rossetti, C. M. Seiler, and M. Laposata. 1999. Human peripheral blood T lymphocyte proliferation after activation of the T cell receptor: effects of unsaturated fatty acids. *Prostaglandins Leukot. Essent. Fatty Acids* 60: 371-375.

(84) Soyland, E., M. S. Nenseter, L. Braathen, and C. A. Drevon. 1993. Very long chain n-3 and n-6 polyunsaturated fatty acids inhibit proliferation of human T-lymphocytes in vitro. *Eur. J. Clin. Invest.* 23: 112-121.

(85) Khalfoun, B., G. Thibault, M. Lacord, Y. Gruel, P. Bardos, and Y. Lebranchu. 1996. Docosahexaenoic and eicosapentaenoic acids inhibit human lymphoproliferative responses in vitro but not the expression of T cell surface activation markers. *Scand. J. Immunol.* 43: 248-256.

(86) Horejsi, V. 2003. The roles of membrane microdomains (rafts) in T cell activation. *Immunol. Rev.* 191: 148-164.

(87) Shaikh, S. R., M. Edidin. 2006. Polyunsaturated fatty acids, membrane organization, T cells, and antigen presentation. *Am. J. Clin. Nutr.* 84: 1277-1289.

(88) Zeyda, M., G. Staffler, V. Horejsi, W. Waldhausl, and T. M. Stulnig. 2002. LAT displacement from lipid rafts as a molecular mechanism for the inhibition of T cell signaling by polyunsaturated fatty acids. *J. Biol. Chem.* 277: 28418-28423.

(89) Verlengia, R., R. Gorjao, C. C. Kanunfre, S. Bordin, T. M. de Lima, E. F. Martins, P. Newsholme, and R. Curi. 2004. Effects of EPA and DHA on proliferation, cytokine production, and gene expression in Raji cells. *Lipids* 39: 857-864.

(90) Weldon, S. M., A. C. Mullen, C. E. Loscher, L. A. Hurley, and H. M. Roche. 2007. Docosahexaenoic acid induces an anti-inflammatory profile in lipopolysaccharide-stimulated human THP-1 macrophages more effectively than eicosapentaenoic acid. *J. Nutr. Biochem.* 18: 250-258.

(91) Vedin, I., T. Cederholm, Y. Freund Levi, H. Basun, A. Garlind, G. Faxen Irving, M. E. Jonhagen, B. Vessby, L. O. Wahlund, and J. Palmblad. 2008. Effects of docosahexaenoic acid-rich n-3 fatty acid supplementation on cytokine release from blood mononuclear leukocytes: the OmegAD study. *Am. J. Clin. Nutr.* 87: 1616-1622.

(92) Bannenberg, G. L., N. Chiang, A. Ariel, M. Arita, E. Tjonahen, K. H. Gotlinger, S. Hong, and C. N. Serhan. 2005. Molecular circuits of resolution: formation and actions of resolvins and protectins. *J. Immunol.* 174: 4345-4355.

(93) Schmidt, E. B., K. Varming, J. O. Pedersen, H. H. Lervang, N. Grunnet, C. Jersild, and J. Dyerberg. 1992. Long-term supplementation with n-3 fatty acids, II: Effect on neutrophil and monocyte chemotaxis. *Scand. J. Clin. Lab. Invest.* 52: 229-236.

(94) Serhan, C. N., M. Arita, S. Hong, and K. Gotlinger. 2004. Resolvins, docosatrienes, and neuroprotectins, novel omega-3-derived mediators, and their endogenous aspirin-triggered epimers. *Lipids* 39: 1125-1132.

(95) Serhan, C. N., R. Yang, K. Martinod, K. Kasuga, P. S. Pillai, T. F. Porter, S. F. Oh, and M. Spite. 2009. Maresins: novel macrophage mediators with potent antiinflammatory and proresolving actions. *J. Exp. Med.* 206: 15-23.

(96) Rossi, A. G., J. M. Hallett, D. A. Sawatzky, M. M. Teixeira, and C. Haslett. 2007. Modulation of granulocyte apoptosis can influence the resolution of inflammation. *Biochem. Soc. Trans.* 35: 288-291.

(97) DeWille, J. W., P. J. Fraker, and D. R. Romsos. 1979. Effects of essential fatty acid deficiency, and various levels of dietary polyunsaturated fatty acids, on humoral immunity in mice. *J. Nutr.* 109: 1018-1027.

(98) Piegari, M., A. F. Salvador, S. E. Munoz, M. A. Valentich, and A. R. Eynard. 2001. Delayed-type hypersensitivity and humoral immunity modulation by dietary lipids in a murine model of pulmonary tumorigenesis induced by urethan. *Exp. Toxicol. Pathol.* 53: 181-186.

(99) Gyllenhammar, H., B. Ringertz, W. Becker, J. Svensson, and J. Palmblad. 1986. Essential fatty acid deficiency in rats: effects on arachidonate metabolism, generation of cyclooxygenase products and functional responses in neutrophils. *Immunol. Lett.* 13: 185-189.

(100) Cleland, L. G., M. J. James, S. M. Proudman, M. A. Neumann, and R. A. Gibson. 1994. Inhibition of human neutrophil leukotriene B4 synthesis in essential fatty acid deficiency: role of leukotriene A hydrolase. *Lipids* 29: 151-155.

(101) Rosenblat, M., N. Volkova, M. Roqueta-Rivera, M. T. Nakamura, and M. Aviram. 2009. Increased macrophage cholesterol biosynthesis and decreased cellular paraoxonase 2 (PON2) expression in Delta6-desaturase knockout (6-DS KO) mice: Beneficial effects of arachidonic acid. *Atherosclerosis* .

(102) Aaes-Jorgensen, E., G. Holmer. 1969. Essential fatty acid-deficient rats. I. Growth and testes development. *Lipids* 4: 501-506.

(103) Matsuzaka, T., H. Shimano, N. Yahagi, M. Amemiya-Kudo, T. Yoshikawa, A. H. Hasty, Y. Tamura, J. Osuga, H. Okazaki, Y. Iizuka, A. Takahashi, H. Sone, T. Gotoda, S. Ishibashi, and N. Yamada. 2002. Dual regulation of mouse {Delta}5- and {Delta}6-desaturase gene expression by SREBP-1 and PPAR{alpha}. *J. Lipid Res.* 43: 107-114.

(104) Davis, J. T., R. B. Bridges, and J. G. Coniglio. 1966. Changes in lipid composition of the maturing rat testis. *Biochem. J.* 98: 342-346.

(105) Lenzi, A., L. Gandini, V. Maresca, R. Rago, P. Sgro, F. Dondero, and M. Picardo. 2000. Fatty acid composition of spermatozoa and immature germ cells. *Mol. Hum. Reprod.* 6: 226-231.

(106) Rejraji, H., B. Sion, G. Prensier, M. Carreras, C. Motta, J. M. Frenoux, E. Vericel, G. Grizard, P. Vernet, and J. R. Drevet. 2006. Lipid remodeling of murine epididymosomes and spermatozoa during epididymal maturation. *Biol Reprod* 74: 1104-13.

(107) Gulaya, N. M., V. M. Margitich, N. M. Govseeva, V. M. Klimashevsky, I. I. Gorpynchenko, and M. I. Boyko. 2001. Phospholipid composition of human sperm and seminal plasma in relation to sperm fertility. *Arch. Androl.* 46: 169-175.

(108) Coniglio, J. G., J. Sharp. 1989. Biosynthesis of [14C]arachidonic acid from [14C]linoleate in primary cultures of rat Sertoli cells. *Lipids* 24: 84-85.

(109) Beckman, J. K., J. G. Coniglio. 1980. The metabolism of polyunsaturated fatty acids in rat Sertoli and germinal cells. *Lipids* 15: 389-394.

(110) Retterstol, K., T. N. Tran, T. B. Haugen, and B. O. Christophersen. 2001. Metabolism of very long chain polyunsaturated fatty acids in isolated rat germ cells. *Lipids* 36: 601-606.

(111) Saether, T., T. N. Tran, H. Rootwelt, B. O. Christophersen, and T. B. Haugen. 2003. Expression and regulation of delta5-desaturase, delta6-desaturase, stearoyl-coenzyme A (CoA) desaturase 1, and stearoyl-CoA desaturase 2 in rat testis. *Biol. Reprod.* 69: 117-124.

(112) Roy, A. C., S. S. Ratnam. 1992. Biosynthesis of prostaglandins by human spermatozoa in vitro and their role in acrosome reaction and fertilization. *Mol. Reprod. Dev.* 33: 303-306.

(113) Ratnasooriya, W. D., R. M. Wadsworth. 1987. Effect on fertility in rats of epididymal administration of indomethacin. *Int. J. Fertil.* 32: 152-156.

(114) Balaji, T., M. Ramanathan, and V. P. Menon. 2007. Localization of cyclooxygenase-2 in mice vas deferens and its effects on fertility upon suppression using nimesulide: a preferential cyclooxygenase-2 inhibitor. *Toxicology* 234: 135-144.

(115) Shimizu, Y., A. Yorimitsu, Y. Maruyama, T. Kubota, T. Aso, and R. A. Bronson. 1998. Prostaglandins induce calcium influx in human spermatozoa. *Mol. Hum. Reprod.* 4: 555-561.

(116) Mohn, C. E., J. Fernandez-Solari, A. De Laurentiis, J. P. Prestifilippo, C. de la Cal, R. Funk, S. R. Bornstein, S. M. McCann, and V. Rettori. 2005. The rapid release of corticosterone from the adrenal induced by ACTH is mediated by nitric oxide acting by prostaglandin E2. *Proc. Natl. Acad. Sci. U. S. A.* 102: 6213-6218.

(117) Furland, N. E., E. N. Maldonado, P. A. Aresti, and M. I. Aveldano. 2007. Changes in lipids containing long- and very long-chain polyunsaturated fatty acids in cryptorchid rat testes. *Biol. Reprod.* 77: 181-188.

(118) Grogan, W. M., W. F. Farnham, and B. A. Szopiak. 1981. Long chain polyenoic acid levels in viably sorted, highly enriched mouse testis cells. *Lipids* 16: 401-410.

(119) Connor, W. E., D. S. Lin, D. P. Wolf, and M. Alexander. 1998. Uneven distribution of desmosterol and docosahexaenoic acid in the heads and tails of monkey sperm. *J. Lipid Res.* 39: 1404-1411.

(120) Zalata, A. A., A. B. Christophe, C. E. Depuydt, F. Schoonjans, and F. H. Comhaire. 1998. The fatty acid composition of phospholipids of spermatozoa from infertile patients. *Mol. Hum. Reprod.* 4: 111-118.

(121) Yan, W. 2009. Male infertility caused by spermiogenic defects: lessons from gene knockouts. *Mol. Cell. Endocrinol.* 306: 24-32.

(122) Holstein, A. F., C. Schirren, and C. G. Schirren. 1973. Human spermatids and spermatozoa lacking acrosomes. *J. Reprod. Fertil.* 35: 489-491.

(123) Xiao, N., C. Kam, C. Shen, W. Jin, J. Wang, K. M. Lee, L. Jiang, and J. Xia. 2009. PICK1 deficiency causes male infertility in mice by disrupting acrosome formation. *J. Clin. Invest.* 119: 802-812.

(124) Kang-Decker, N., G. T. Mantchev, S. C. Juneja, M. A. McNiven, and J. M. van Deursen. 2001. Lack of acrosome formation in Hrb-deficient mice. *Science* 294: 1531-1533.

(125) Yao, R., C. Ito, Y. Natsume, Y. Sugitani, H. Yamanaka, S. Kuretake, K. Yanagida, A. Sato, K. Toshimori, and T. Noda. 2002. Lack of acrosome formation in mice lacking a Golgi protein, GOPC. *Proc. Natl. Acad. Sci. U. S. A.* 99: 11211-11216.

(126) Fasshauer, D., R. B. Sutton, A. T. Brunger, and R. Jahn. 1998. Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. *Proc. Natl. Acad. Sci. U. S. A.* 95: 15781-15786.

(127) Rizo, J., T. C. Sudhof. 2002. Snares and Munc18 in synaptic vesicle fusion. *Nat. Rev. Neurosci.* 3: 641-653.

(128) Rickman, C., B. Davletov. 2005. Arachidonic acid allows SNARE complex formation in the presence of Munc18. *Chem. Biol.* 12: 545-553.

(129) Darios, F., B. Davletov. 2006. Omega-3 and omega-6 fatty acids stimulate cell membrane expansion by acting on syntaxin 3. *Nature* 440: 813-817.

(130) Mazelova, J., N. Ransom, L. Astuto-Gribble, M. C. Wilson, and D. Deretic. 2009. Syntaxin 3 and SNAP-25 pairing, regulated by omega-3 docosahexaenoic acid, controls the delivery of rhodopsin for the biogenesis of cilia-derived sensory organelles, the rod outer segments. *J. Cell. Sci.* 122: 2003-2013.

(131) Ramalho-Santos, J., R. D. Moreno, G. M. Wessel, E. K. Chan, and G. Schatten. 2001. Membrane trafficking machinery components associated with the mammalian acrosome during spermiogenesis. *Exp. Cell Res.* 267: 45-60.

-

CHAPTER 3

Fads3 IS NOT A DELTA-6-DESATURASE ISOZYME FOR LINOLEIC ACID

ABSTRACT

Delta-6 desaturase (D6D) is a key enzyme for the synthesis of highly unsaturated fatty acids (HUFA). D6D gene expression and activity was measured in D6D knockout (-/-) liver to verify that the gene was knocked out. Gene expression of D6D was absent; however, desaturation activity remained as indicated by the conversion of ^{14}C-labeled 18:2 n-6 to 18:3. A possible explanation for this conversion is the presence of a D6D isozyme. *Fads3* is a gene that holds close sequence homology to the D6D gene but the function of its gene product is not known. *Fads3* may code for a D6D isozyme since this gene is upregulated in D6D -/- liver. The hypothesis was tested by transfecting the *Fads3* gene into 293-HEK cells incubated with a D6D substrate, 18:2 n-6. *Fads3* was efficiently expressed by cells as confirmed by real time PCR, however, D6D activity was not observed as indicated by the absence of 18:3 fatty acid in culture. Another potential role for *Fads3* is as a delta-8 desaturase (D8D) which would allow HUFA synthesis of 20:4 n-6 in absence of D6D. 20:2 n-6 fatty acid treatments of *Fads3* transfected cells did not result in D8D activity. In conclusion, *Fads3* is not a D6D isozyme, at least for the desaturation step of 18:2 n-6 to 18:3, and it does not have D8D activity.

INTRODUCTION

The *Fads3* gene can be found on the same chromosome as desaturases involved in highly unsaturated fatty acid (HUFA) synthesis, forming a gene cluster with delta-5 (D5D) and delta-6 desaturase (D6D) in humans (1). *Fads3* shares conserved structural domains with D5D and D6D which include an N-terminal cytochrome b5-like domain and a C-terminal fatty acid desaturase domain, therefore, *Fads3* may potentially encode for a desaturase. The putative human *Fads3* gene product consists of 445 aminoacids and presents high sequence homology to D6D, sharing 62% identity (2). The function of the protein encoded by *Fads3* however is not known.

Initial characterization of the D6D-/- suggested the presence of a D6D isozyme. Despite absence of D6D gene expression in D6D-/- liver, desaturation activity remained at 20% of +/+ levels as determined by incubating liver microsomes with ^{14}C-labeled linoleic acid and quantifying desaturation products (3). The formation of a supposed D6D product prompted to check liver gene expression for potential D6D isozymes such as *Fads3* and *Fads6*, which present homology to D6D. Only *Fads3* showed upregulation in liver D6D-/- (3) suggesting possible compensation for lack of HUFA synthesis. *Fads3* was therefore considered a strong D6D isozyme candidate.

In order to study the role of HUFA with the D6D-/- mouse, it is imperative to confirm lack of a D6D isozyme and disrupted HUFA synthesis. The objective of this study is to determine if mouse *Fads3* is a D6D isozyme by measuring D6D activity in *Fads3* transfected HEK293cells incubated with linoleic acid, a D6D substrate. The potential role of Fads3 as a D8D enzyme was also evaluated.

METHODS

Mouse *Fads3* cloning

a) Plasmid extraction from pFLCI Fads3 clone

E. coli DH10B cells, transformed with ampicillin-resistant plasmid containing the *Fads3* gene (RIKEN clone A730007D17), were grown overnight at 37 ºC on agar plates with ampicillin. The next day, a single colony was picked from the plate in order to inoculate a starter culture of 5ml LB medium. After a 12 hour incubation period at 37 ºC in a shaker (300rpm), plasmids were extracted (QIAGEN Miniprep). Enzyme digestion followed with restriction enzyme *Sfi*. The digestion products were run on an agarose gel by electrophoresis and the band corresponding to the *Fads3* insert was cut out and extracted (QIAquick gel extraction kit).

b) Addition of restriction sites to FADS3 insert by PCR

In order to clone the *Fads3* insert into the mammalian expression vector, compatible restriction sites were required. Restriction sites for Xho and BamH1 were chosen since the *Fads3* sequence does not contain either Xho1 or BamH1 site according to Biology Workbench 3.2. The BamHI and Xho sites were added to the 5' and 3' end of the *Fads3* cDNA insert by PCR. Primer designed to add a Bamh1 restriction site to *Fads3* was: Forward- 5'-GGATCCATGGGCGGTGTCGGGGAGCCCGGAGGGG-3'; primer to add Xho restriction site on other end of *Fads3* was: Reverse-5'-CTCGAGGTGTCTGGGTTGCTTTTTATATAAAAAT. The underlined regions represent the restriction site region. These sites allow using the corresponding enzymes to digest the *Fads3* cDNA from the plasmid and proper insertion into the

mammalian expression vector. PCR was run in order to obtain a *Fads3* with the newly added restriction sites.

c) Cloning Fads3 into pGEM-T vector and sequence confirmation

The PCR product of *Fads3* was run on an agarose gel by electrophoresis to confirm *Fads3* insert size (1.2kb). This PCR product was then extracted from the gel and ligated to a pGEM T-vector. Ligation to the pGEM-T vector is an intermediate step that facilitates subsequent ligation to a mammalian vector.

Competent *E. coli* cells were transformed with the pGEM T-vector containing the *Fads3* insert with the desired Xho and BamH1 restriction sites. These cells were spread on agar plates and incubated overnight at 37 ºC. Colonies were chosen from the plate and used in a PCR reaction that would amplify the *Fads3* insert. The PCR product was run on an agarose gel, the band corresponding to *Fads3* was then extracted from the gel, digested using Xho and BamH1 enzymes, and sequenced using BigDye3.1. Sequencing was done at the University of Illinois Core Sequencing Facility using an ABI 3730XL capillary sequencer. Once a colony containing the correct sequence for *Fads3* was found, it was used to start a culture for a large scale extraction of the plasmid (QIAGEN Maxi plasmid kit).

d) Cloning Fads3 into pcDNA3.1 mammalian vector

The plasmid was extracted from the culture and digested using Xho and BamH1 restriction enzymes. The resulting *Fads3* insert was run on a gel, extracted, and ligated to the mammalian vector pcDNA3.1 using a T4 DNA ligase (Promega). Competent cells were transformed with this plasmid and grown on an agar plate. Colonies were chosen for a PCR insert check. Once a correct sequence was confirmed using BigDye3.1, the colony was used to

start a culture for a large scale extraction of the plasmid (QIAGEN Maxi plasmid kit). The *Fads3* insert in the pcDNA3.1 mammalian vector was then used for transfection into HEK-293 cells.

Quantification of the *Fads3* mRNA level by real-time PCR

In a separate experiment, RNA was extracted from *Fads3* transfected cells using Trizol. cDNA was prepared using random primers and Superscript III reverse transcriptase. *Fads3* and D6D gene expression were measured by Real-time quantitative PCR, using SYBR Green fluorescent dye (Applied Biosystems) with mouse *Fads3* primers, forward 5'-AACCCCTGTTGATTGGAGAGCTA -3', reverse 5'- GCTCGGAAGTCCTCGATCAG -3'; human D6D primers, forward 5'-CTGCCAACTGGTGGAATCATC -3', reverse 5'-ACAAACACGTGCAGCATGTTC-3'.

D6D and D8D activity of FADS3 transfected HEK-293 cells

HEK-293 cells were distributed to 60mm cell culture dishes from a 1.5 x 10^6 cells/ml suspension. 1.5 x 10^6 cells were added to each plate with a final volume of 3 ml complete medium (DMEM 10% FBS) and incubated overnight at 37 ºC. The plasmids were transfected in duplicate. Plasmids used were pcDNA3.1, empty vector used as a negative control; pD6D or pD5D, as a positive desaturase control; p*Fads3*.

Transfection into HEK-293 cells was achieved using Lipofectamine 2000 (Invitrogen). Plasmid (8ug) and lipofectamine were mixed at a 1 to 5 ratio in serum free medium. Different ratios were tested and the 1 to 5 ratio gave the highest expression of transfected plasmid. Transfection mix was added to plated cells and incubated for 5 hours at 37 ºC. Media was then replaced with complete media (10% FBS) and cells were incubated overnight.

Transfected cells were treated with either 18:2 n-6 or 20:2n6 bound to BSA at a final concentration of 150μM. A set of *Fads3* transfected cells treated with BSA alone were used as a negative control. Cells were incubated for 24 hours. Total lipids were extracted from treated cells using the Folch method (4). In order to obtain quantifiable fatty acids, extractions from duplicates were pooled. Fatty acid methyl esters were prepared with 3M methanolic-HCl and analyzed by HP5890 gas chromatography (Agilent Technologies, Wilmington, DE) using a 30 m × 0.25 mm Omegawax capillary column (Supelco). Program used was 175 ºC for 5 minutes, followed by a 5 ºC increase per minute until a final temperature of 195 ºC for 50 minutes.

RESULTS

Fads3 cloning

Like in human, mouse *Fads3*, D6D, D5D form a gene cluster in the 19th mouse chromosome. An alignment comparison of mouse *Fads3* and D6D/Fads2 resulted in 73% identity. The mouse *Fads3* presents the same conserved structural domains of D5D and D6D, which consist of a cytochrome b5-like domain and a membrane FADS like domain. The putative mouse *Fads3* protein results in 449 amino acids, similar in length to the 445 amino acids of human *Fads3*. *Fads3* cloning into the mammalian vector was successful as confirmed by sequencing.

Blast alignment comparison of mouse Fads3 and D6D results in 73% identity

GENE ID: 56473 Fads2 | fatty acid desaturase 2 [Mus musculus]
gb|AF126798.1|AF126798 Mus musculus delta-6 fatty acid desaturase mRNA, complete cds
Length=1508

```
 Score =  411 bits (222),  Expect = 2e-110
 Identities = 984/1337 (73%), Gaps = 111/1337 (8%)
 Strand=Plus/Plus

Query  257   CCCATCTTCCGCTGGGAGCAGA-TCCGCCAGCATGACCTAC-CAGGCGACAAGTGGCTGG  314
             |||| |||||| |||||| ||| || |  ||||  |||| | ||  ||||  |||||| |
Sbjct  129   CCCACCTTCCGTTGGGAGGAGATTCAG-AAGCACAACCTGCGCA-CCGACCGGTGGCTCG  186

Query  315   TCATCGAGCGC-CGTGTCTACGACATCAGCC-GCTGGGCACAGCGGCACCCAGGGGG-TA  371
             ||||||| |||  | |||||| || | | ||   ||| | ||||||||||| |||||  |
Sbjct  187   TCATCGACCGCAAG-GTCTACAACGTTA-CCAAATGGTCCCAGCGGCACCCGGGGGGCCA  244

Query  372   GCCGCCTCATCGGCCACCA--CGGTGCGGAGGACGCCACGGATGCCTTCCACGCCTTCCA  429
              |||  ||||||| ||| |  ||| |  || || || ||||||||||||  ||||||||
Sbjct  245   -CCGTGTCATCGGACACTATTCGG-G-AGAAGATGCTACGGATGCCTTCCGTGCCTTCCA  301

Query  430   CCAAGATCTCCATTTTGTGCGCAAGTTCCTGAAACCCCTGTTGATTGGAGAGCTAGCCCC  489
              |  || ||  | || ||| |||||||| |||| |||||| ||||||| ||||| |||||
Sbjct  302   TCTGGACCTGGACTTCGTGGGCAAGTTCTTGAAGCCCCTGCTGATTGGTGAGCTGGCCCC  361

Query  490   AGAGGAACCCAGCCAGGATGGAGCTCAGAATGC-C-CAGCTGATCGAGGACTTCCGAGCC  547
             |||||| ||||||| |||  | |  || || || | ||| | | |||||||||| | |||
Sbjct  362   AGAGGAGCCCAGCCTGGACCGTG-GCA-AAAGCTCTCAGATCACCGAGGACTTCAGGGCC  419

Query  548   TTG-CGCCAGGCAGCTGAAGACATGAAGCTGTTTGAAGCTGATACCA-CT-TTCTT-TGC  603
              ||  |  || | ||||| |||||||| || ||  || |  | |||| || ||||| |
Sbjct  420   CTGAAG-AAGACTGCTGAGGACATGAACCTCTTCAAAAC-CA-ACCACCTGTTCTTCT-T  475

Query  604   ACTCCTGCTGGGCCACATCCT-GGCTATGG--AGTTGTTGGCCTGGCTT-ATCATCTACC  659
              ||||| |||  ||||||| | | | ||||  ||   || |||||| || ||| ||| |
Sbjct  476   TCTCCTCCTGTCCCACATCATCGTC-ATGGAAAG-CCTT-GCCTGG-TTCATCCTCT-CG  530

Query  660   T-CTTGGGCCCTGGCTGGGTGTCCAGTATCCT--T-GCTGCC-CTGATCCTGGCCATCTC  714
             | ||| ||| |||||||| | |||  || |||  |  | |||  || |||| || | |||
Sbjct  531   TACTTCGGCACTGGCTGGAT-TCC--TACCCTCGTCACAGCCTTTG-TCCTCGCTACCTC  586

Query  715   TCAGGCCCAGTGCTGG-TGTCTGCAACATGATCTA-GGTCATGC-TTCCATCT-TCACTA  770
             |||||||||  ||||| || |||||||||||| ||| || ||  | |||  ||| | |  |
Sbjct  587   TCAGGCCCA-AGCTGGATGGCTGCAACATGA-CTATGGCCA-CCTTTCTGTCTAT-AAGA  642

Query  771   AGTCCAGGTGGAACCATGTGGCCCAGC-AGTTCGTGATGGGGCAGTTGAAAGGCT-TTTC  828
             | ||||  |||||||| || | ||| | |||| || || || || || ||||| |   ||
Sbjct  643   AATCCATATGGAACCACGTTGTCCA-CAAGTTTGTCATTGGCCACTT-AAAGGGTGCCTC  700

Query  829   CGCCCACTGGTGGAATTTCCGCCACTTCCAGCACCATGCCAAACCCAACATCTTCCACAA  888
              ||| ||||||||||   ||| || ||||| |||||||||| ||||||||||||||||||
Sbjct  701   AGCCAACTGGTGGAACCACCGACATTTCCAACACCATGCCAAGCCCAACATCTTCCACAA  760

Query  889   AGACCCAGATGT-GACTGTC-GCACCTGTCTTCCTCCTGGGGGAGT--CA-TCTGTGGAG  943
              ||||| ||  |  |  | | |||  ||| ||  |||| || ||||  ||  |  | |||
Sbjct  761   GGACCCGGACATAAAGAGCCTGCA--TGTGTTTGTCCTTGGCGAGTGGCAGCCCCTTGAG  818

Query  944   TATGGCAAGAAGAAAC-GCAGATACCTGCCCTACAACCACCAGCATCTATACTTCTTCCT  1002
             |||||||||||||| | | | |||||||||||||||||||||||||  ||||||||||||
Sbjct  819   TATGGCAAGAAGAAGCTG-AAATACCTGCCCTACAACCACCAGCATGAATACTTCTTCCT  877

Query  1003  GATTGGCCCTCCGCTGCTCA-CCTTGGTGAACTTTGAAGTTGAAAATCTGGCGT-AC-AT  1059
             ||| || || |||||||||| || |  || || ||  |||   | |||   | | || ||
Sbjct  878   GATCGGACCGCCGCTGCTCATCCCT-ATGTAC-TTCCAGTACCAGATC-ATCATGACAAT  934

Query  1060  GCTGGTG-TGCATGCAGTGGACGGACTT-GCTGTGGGCTGCCAGTTTCTACTCCCGC-TT  1116
             | |   |  ||| | | |||  |||||| ||| |||||   ||| | ||| |   || ||
```

```
Sbjct  935   GAT-CAGCCGCAGGGACTGGGTGGACTTGGCT-TGGGCCATCAGCTACTA-T-ATGCGTT  990

Query  1117  T-TTCTTGTCCTACTCTCCC-TTCTATGGTGCCA-C-TGGGA-CACT-G--CTCCTCTTT  1168
              | ||||   |||||  |||| |||||  | | || | ||||| | || |   |||||
Sbjct  991   TCTTCTACACCTAC-ATCCCTTTCTA-CG-G-CATCTTGGGAGCCCTGGTTTTCCTC-AA  1045

Query  1169  GTTGCTGTCAGGGTGCTGGAGAGCCACTGGTTCGTGTGGATCACGCAGATGAACCACATC  1228
               ||  | ||||| | ||||||||||||||||| |||||| |||| ||||||||||| |
Sbjct  1046  CTT--TATCAGGTTCCTGGAGAGCCACTGGTTTGTGTGGGTCACACAGATGAACCACCTT  1103

Query  1229  CCCAAGGAGATTGGCCATGAAAAGC-ATCGGGACTGG-GCAAGC-TCTCAGCTGGCAGCC  1285
                || ||||||||  | |||  | | | |||||||||  | |||  | ||||||||||||
Sbjct  1104  GTCATGGAGATTGATCTTGATCA-CTACCGGGACTGGTTC-AGCAGC-CAGCTGGCAGCC  1160

Query  1286  ACCTGCAATGTGGAACCTTCGC-TCTTCATTGACTGGTTCAGCGGGCACCTCAATTTCCA  1344
              ||||||||||||||  |  || | ||||||| ||||||||||||||||||||||||||||
Sbjct  1161  ACCTGCAATGTGGAGCAGTC-CTTCTTCAATGACTGGTTCAGCGGGCACCTCAATTTCCA  1219

Query  1345  GATTGAGCACCACCTCTTCCCCACGATGCCAAGGCACAAC-TAC-CGGAGGGTGGCCCCC  1402
              |||||||||||||||||||||||| |||||| | |||||| | | |  ||  || ||||
Sbjct  1220  GATTGAGCACCACCTCTTCCCCACTATGCCACGTCACAACCTGCACA-AGATTG-CCCCA  1277

Query  1403  CTGGTCAAGGCGT-TCTGCGCCAAGCACGGCCTACACTACGAGGTGAAGCCTTTCCTCAC  1461
              ||||| ||| | | |||||||||||||| ||| |  | ||| ||| |||||| || || |
Sbjct  1278  CTGGTGAAGTC-TCTCTGCGCCAAGCATGGCATTGAATACCAGGAGAAGCCGTTGCTGAG  1336

Query  1462  CGCTCTGGTGGATATCATCGGGTCC-CTGAAGAAGTCTGGCGA-CATCTGGCTGGATGCA  1519
               || ||| | || ||  | | || | |||||||||||||| || | | ||||||||||
Sbjct  1337  GGCCCTGATCGACATTGT-GAGTTCACTGAAGAAGTCTGGGGAGC-TGTGGCTGGATGCT  1394

Query  1520  TACCTCCATCAATGAAG  1536
              ||||||||| |||||||
Sbjct  1395  TACCTCCATAAATGAAG  1411
```

Mouse *Fads3* consists of 449 aminoacids

"MGGVGEPGGGPGPREGPAPLGAPLPIFRWEQIRQHDLPGDKWLV

IERRVYDISRWAQRHPGGSRLIGHHGAEDATDAFHAFHQDLHFVRKFLKPLLIGELAP

EEPSQDGAQNAQLIEDFRALRQAAEDMKLFEADTTFFALLLGHILAMELLAWLIIYLL

GPGWVSSILAALILAISQAQCWCLQHDLGHASIFTKSRWNHVAQQFVMGQLKGFSAHW

WNFRHFQHHAKPNIFHKDPDVTVAPVFLLGESSVEYGKKKRRYLPYNHQHLYFFLIGP

PLLTLVNFEVENLAYMLVCMQWTDLLWAASFYSRFFLSYSPFYGATGTLLLFVAVRVL

ESHWFVWITQMNHIPKEIGHEKHRDWASSQLAATCNVEPSLFIDWFSGHLNFQIEHHL

FPTMPRHNYRRVAPLVKAFCAKHGLHYEVKPFLTALVDIIGSLKKSGDIWLDAYLHQ"

Sequence confirmation of *Fads3* clone in mammalian vector

The following forward and reverse sequences using BigDye 3.1 and the ABI 3730XL capillary sequencer, confirmed that the correct sequence of the *Fads3* clone (highlighted) was used for transfections.

FAD3 m13F-21 Forward

annnnaggcgaattgggcccgacgtcgcatgctcccggccgccatggcggccgcgggaat
 X X G E L G P T S H A P G R H G G R G N
tcgattggatccatgggcggtgtcggggagcccggaggggggacccgggccgcgggagggg
 S I G S M G G V G E P G G G P G P R E G
cccgcaccgctgggggcgcccctacccatcttccgctgggagcagatccgccagcatgac
 P A P L G A P L P I F R W E Q I R Q H D
ctaccaggcgacaagtggctggtcatcgagcgccgtgtctacgacatcagccgctgggca
 L P G D K W L V I E R R V Y D I S R W A
cagcggcacccaggggtagccgcctcatcggccaccacggtgcggaggacgccacggat
 Q R H P G G S R L I G H H G A E D A T D
gccttccacgccttccaccaagatctccattttgtgcgcaagttcctgaaacccctgttg
 A F H A F H Q D L H F V R K F L K P L L
attggagagctagccccagaggaacccagccaggatggagctcagaatgcccagctgatc
 I G E L A P E E P S Q D G A Q N A Q L I
gaggacttccgagccttgcgccaggcagctgaagacatgaagctgtttgaagctgatagc
 E D F R A L R Q A A E D M K L F E A D S
actttctttgcactcctgctgggccacatcctggctatggagttgttggcctggcttatc
 T F F A L L L G H I L A M E L L A W L I
atctacctcttgggccctggctgggtgtccagtatccttgctgccctgatcctggccatc
 I Y L L G P G W V S S I L A A L I L A I
tctcaggcccagtgctggtgtctgcaacatgatctaggtcatgcttccatcttcactaag
 S Q A Q C W C L Q H D L G H A S I F T K
tccaggtggaaccatgtggcccagcagttcgtgatggggcagttgaaaggctttccgcc
 S R W N H V A Q Q F V M G Q L K G F S A
cactggtggaatttccgccacttccagcaccatgccaaacccaacatcttccacaaagac
 H W W N F R H F Q H H A K P N I F H K D
ccagatgtgactgtcgcacctgtcttcctcctgggggagtcatctgtggagtatggcaag
 P D V T V A P V F L L G E S S V E Y G K
aagaaacgcagatacctgccctacaaccaccagcatctatacttcttcctgattggccct
 K K R R Y L P Y N H Q H L Y F F L I G P
ccgctgctcaccttggtgaactttgaagttgaaaatctggcgtacatgctggtgtgcatg
 P L L T L V N F E V E N L A Y M L V C M
cagtggacggacttgctgtgggctgccagtttctactccgctttttcttgtctactctcc
 Q W T D L L W A A S F Y S A F S C L L S
cttnctatggtgccactggganactgctcctctttgttgctgtcagggtgctggaaancn
 L X M V P L G X C S S L L L S G C W K X
actggtttcgtgtggatcacncaaatgaaccacatnccccaagnaaantggnccatgaaa
 T G F V W I X Q M N H X P Q X X W X M K
aaacattngggactgggcaaanctttnanctggn
 K H X G L G K X X X W X

FAD3 m13R-48 Reverse

```
nnantttcntgatnggggcagttgaaaanggnttttcccgccccnnctggnggaattttn
  X  F  X  D  X  G  S  -  K  X  X  F  P  A  X  X  X  G  I  X
ccgccatttnccaggcacccatgccaaaaccccaacnttttttccacaaagaccccagatg
 P  P  F  X  R  H  P  C  Q  N  P  N  X  F  P  Q  R  P  Q  M
tgactgtcgccccttnttttnctncntgggggggagttcatntgtggagtatggcnagnaga
 -  L  S  P  X  F  X  X  X  G  G  V  H  X  W  S  M  X  X  R
aaacgcagatacctgcccttacaaaccacccagcatttatacttttttcctgattggccc
 K  R  R  Y  L  P  L  Q  T  T  Q  H  L  Y  F  F  P  D  W  P
ntccgctgctcacctttggtgaactttgaagttgaaaatctggcgtacatgctggtgtgc
 X  R  C  S  P  L  V  N  F  E  V  E  N  L  A  Y  M  L  V  C
atgcagtggacggacttgctgtgggctgccagtttctactcccgcttttttcttgtcctac
 M  Q  W  T  D  L  L  W  A  A  S  F  Y  S  R  F  F  L  S  Y
tctcccttctatggtgccactgggacactgctcctctttgttgctgtcagggtgctggag
 S  P  F  Y  G  A  T  G  T  L  L  L  F  V  A  V  R  V  L  E
agccactggttcgtgtggatcacgcagatgaaccacatccccaaggagattggccatgaa
 S  H  W  F  V  W  I  T  Q  M  N  H  I  P  K  E  I  G  H  E
aagcatcgggactgggcaagctctcagctggcagccacctgcaatgtggaaccttcgctc
 K  H  R  D  W  A  S  S  Q  L  A  A  T  C  N  V  E  P  S  L
ttcattgactggttcagcgggcacctcaatttccagattgagcaccacctcttccccacg
 F  I  D  W  F  S  G  H  L  N  F  Q  I  E  H  H  L  F  P  T
atgccaaggcacaactaccggagggtggcccccctggtcaaggcgttctgcgccaagcac
 M  P  R  H  N  Y  R  R  V  A  P  L  V  K  A  F  C  A  K  H
ggcctacactacgaggtgaagcctttcctcaccgctctggtggatatcatcgggtccctg
 G  L  H  Y  E  V  K  P  F  L  T  A  L  V  D  I  I  G  S  L
aagaagtctggcgacatctggctggatgcatacctccatcaatgaaggcagcacccttat
 K  K  S  G  D  I  W  L  D  A  Y  L  H  Q  -  R  Q  H  P  Y
gggtggtgaaggggtaagggtccagactccagcagtcaccgagtcactgggcaagattga
 G  W  -  R  G  K  G  P  D  S  S  S  H  R  V  T  G  Q  D  -
cagtcatcctcacccccctgctggggcagcctgtctgcccgcctggtcctgctggctttcc
 Q  S  S  S  P  P  A  G  A  A  C  L  P  A  W  S  C  W  L  S
tcagtcctcagcattttctggtcagcaatcctatggccatggcacatgtccaacaggacc
 S  V  L  S  I  F  W  S  A  I  L  W  P  W  H  M  S  N  R  T
agggtggagggaacatacttaaaatgacacggtgagcatagcatgttttcctagagcaag
 R  V  E  G  T  Y  L  K  -  H  G  E  H  S  M  F  S  -  S  K
aactggaaggaaaacttgttatttttatataaaaacaacccagacacctcgagaatcact
 N  W  K  E  N  L  L  F  L  Y  K  N  N  P  D  T  S  R  I  T
agtgaattcgcggccgcctgcaggtcgaccatatgggagagctcccaacgcgttggatgc
 S  E  F  A  A  A  C  R  S  T  I  W  E  S  S  Q  R  V  G  C
atagcttgagtattctatagtgtcacctaaatagctggcgnatnttcttt
 I  A  -  V  F  Y  S  V  T  -  I  A  G  X  X  S
```

Fads3 gene expression of transfected cells

Fads3 mRNA abundance was measured in HEK293 cells transfected at different ratios of *Fads3* plasmid (µg) to lipofectamine (µl) to determine optimum experimental conditions for *Fads3* expression. A ratio of 1 to 5, DNA to lipofectamine, resulted in the highest *Fads3* expression overcoming any D6D expression present in the cell line (**Table 3.1**).

Fads3 is not a D6D isozyme

HEK293 cells transfected with an empty vector, a D6D plasmid, or a *Fads3* plasmid were analyzed for total fatty acids (**Figure 3.1**). Empty vector transfected HEK293 cells did not desaturate 18:2n6 as indicated by absence of D6D product 18:3n6. Despite overexpression of *Fads3,* HEK293 cells transfected with *Fads3* plasmid did not desaturate 18:2n6 as indicated by absence of 18:3n6, showing a fatty acid profile same as the empty vector trasfected cells. Transfection with D6D plasmid resulted in synthesis of 18:3n6, as well as a four time reduction in 18:2n6 substrate and a five time increase in 20:3n6 product when compared to *Fads3* transfected and empty vector transfected cells.

Fads3 does not have D5D or D8D activity

HEK293 cells transfected with an empty vector, a D5D plasmid, or a *Fads3* plasmid were analyzed for total fatty acids (**Figure 3.2**). Empty vector transfected HEK293 cells did not desaturate 20:2n6 as indicated by absence of desaturase product. Despite overexpression of *Fads3,* HEK293 cells transfected with *Fads3* plasmid did not desaturate 20:2n6 as indicated by no increase in desaturase products, either of D5D (20:3 n-5,11,14) or of D8D which would have made 20:4 n-6. *Fads3* transfected cell fatty acid profile was similar to that of empty vector transfected cells. Transfection with D5D plasmid resulted in synthesis of 20:3 n-5,11,14, as well as a reduction in 20:2n6 substrate.

DISCUSSION

Despite overexpression of *Fads3* in HEK293 cells, incubation with the D6D substrate 18:2n6 did not result in synthesis of the D6D product 18:3n6. The lack of D6D activity demonstrates that *Fads3* is not a D6D isozyme, at least for the first step in HUFA synthesis. The lack of an isozyme in the D6D-/- mouse was further demonstrated in a separate study where [U-13 C] labeled 18:2n6 was given by oral gavage to D6D-/- mice in order to track its metabolism (3). The -/- liver was analyzed and no radiolabeled D6D products were detected including arachiconic acid. This demonstrates the lack of a D6D isozyme in the -/- as well as complete disruption of omega-6 HUFA synthesis. The omega-3 metabolic pathway however was not evaluated.

An alternative route for the synthesis of HUFA, 20:4n6, from 18:2n6 in absence of D6D, would require delta-8 desaturation. 18:2n6 would be elongated to 20:2n6 which would then be desaturated at carbon 8 to form 20:3 (n-8,11,14). This would bypass the D6D step. The D8D product, 20:3 (n-8,11,14), could then be desaturated by D5D to form the HUFA, 20:4n6. In this study, we demonstrate that *Fads3* does not function as a D8D at least for the omega-6 pathway. Recently, *Fads2*, which encodes for D6D was also shown to have D8D activity for the substrates 20:2n6 and 20:3n3, with preference towards the omega-3 fatty acid substrate (5).

The function of *Fads3* may be as a desaturase for substrates other than omega-6 fatty acids. In omega-3 HUFA synthesis, D6D participates in two steps: desaturation of 18:3n3, and desaturation of 24:5n3 which becomes 24:6n3 and then beta-oxidized to 22:6n3 or DHA (6,7). *Fads3* may be specific to a fatty acid as in the case of a D5D isozyme in salmon that has 63% identity to D5D/FADS1 and specifically desaturates omega-3 fatty acids (8). A similar situation

may occur with *Fads3* and D6D which have 73% identity in mouse. The function of *Fads3* may also be specific to certain tissues. Eight *Fads3* alternative transcripts have been detected in baboon tissues showing differential expression (9). Certain transcripts had higher expression in immune tissues while other dominated in brain or heart suggesting potential tissue specific roles. The presence of several transcripts indicates more than one gene product for *Fads3*. Three protein isoforms were confirmed in rat and mouse which were also differentially distributed in tissues (10). Despite low *Fads3* expression in rat liver when compared to D6D, a *Fads3* isoform was abundant in liver supporting its potential role in lipid metabolism (10).

Although the *Fads3* gene function has not been determined, we can conclude it is not involved in synthesis of omega-6 HUFA. The residual AA and DHA in D6D-/- tissues at 5 months of age are likely transferred from the mother to the pup during pregnancy until weaning.

TABLES AND FIGURES

Table 3.1 *Fads3* mRNA abundance of *Fads3* transfected HEK293 cells at different DNA to lipofectamine ratios (2^-ΔCt).

DNA(ug)/ Lipofectamine (ul)	FADS3 mRNA	D6D mRNA
1 to 0.5	0.016	0.009
1 to 2.5	0.030	0.008
1 to 5	**1.867**	0.007

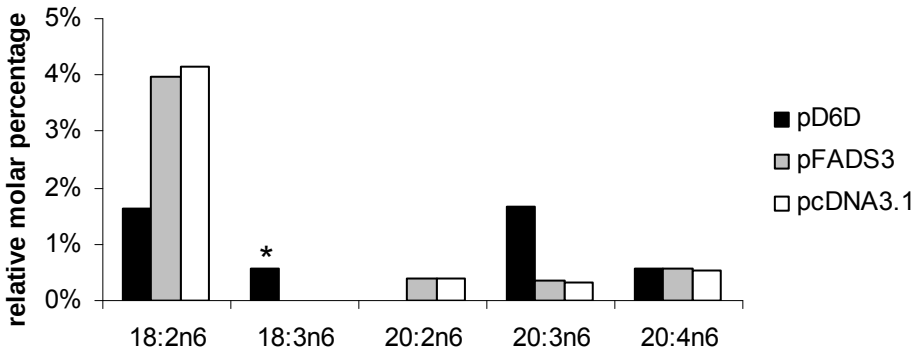

Figure 3.1 *Total fatty acid analysis of 18:2n6 treated HEK293 cells transfected with plasmid Fads3 (pFADS3), plasmid delta-6 desaturase (pD6D), or an empty vector (pcDNA3.1); pooled from duplicates. *Only pD6D transfected cells showed D6D activity as indicated by 18:3n6 production.*

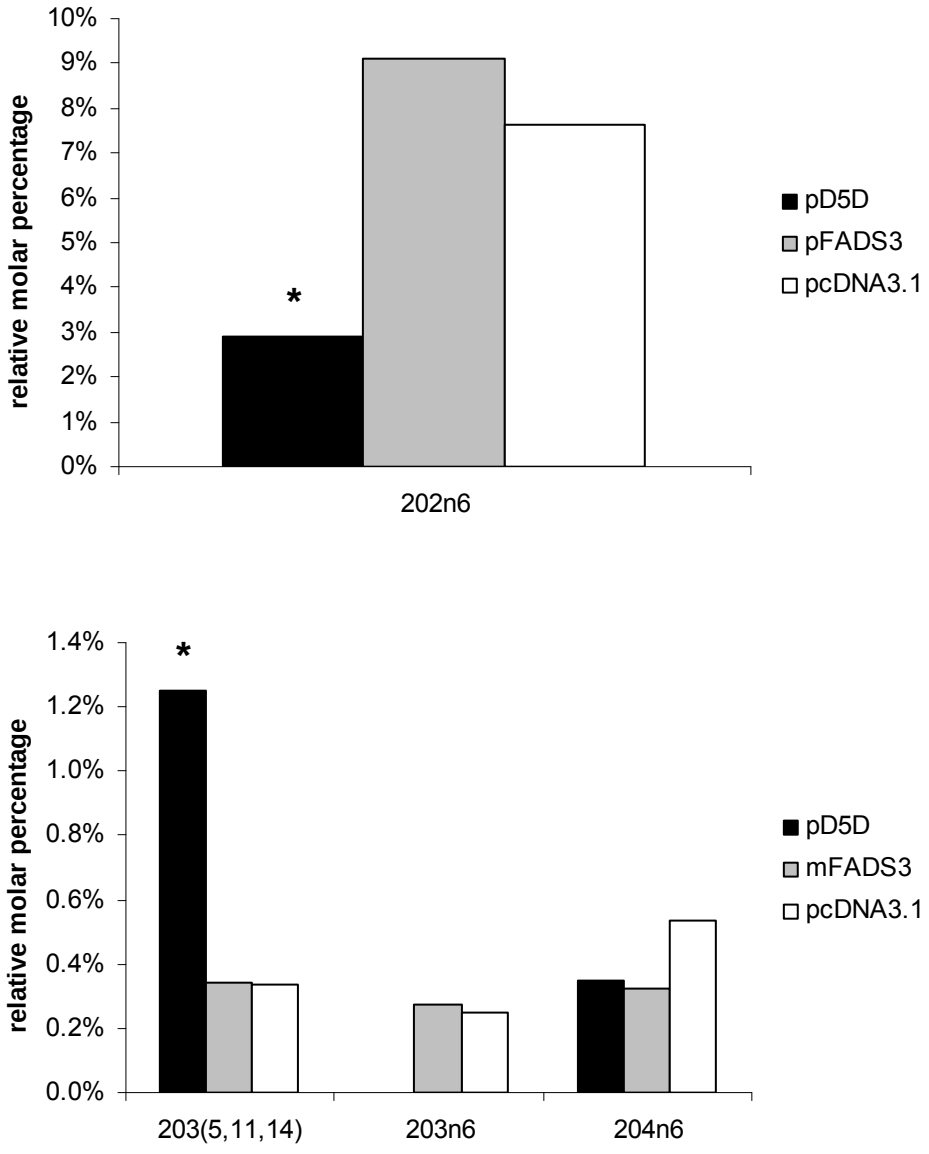

Figure 3.2 *Total fatty acid analysis of 20:2n6 treated HEK293 cells transfected with plasmid Fads3 (pFADS3), plasmid delta-5 desaturase (pD5D), or an empty vector (pcDNA3.1); pooled from duplicates. *Only pD5D transfected cells showed desaturase activity as indicated by decrease in 20:2n6 substrate and increased production of 20:3(n-5,11,14).*

REFERENCES

(1) Nakamura, M. T., T. Y. Nara. 2004. Structure, function, and dietary regulation of delta6, delta5, and delta9 desaturases. *Annu Rev Nutr* 24: 345-76.

(2) Marquardt, A., H. Stohr, K. White, and B. H. Weber. 2000. cDNA cloning, genomic structure, and chromosomal localization of three members of the human fatty acid desaturase family. *Genomics* 66: 175-83.

(3) Stroud, C. K., T. Y. Nara, M. Roqueta-Rivera, E. C. Radlowski, P. Lawrence, Y. Zhang, B. H. Cho, M. Segre, R. A. Hess, J. T. Brenna, W. M. Haschek, and M. T. Nakamura. 2009. Disruption of FADS2 gene in mice impairs male reproduction and causes dermal and intestinal ulceration. *J. Lipid Res.* 50: 1870-80.

(4) Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 226: 497-509.

(5) Park, W. J., K. S. Kothapalli, P. Lawrence, C. Tyburczy, and J. T. Brenna. 2009. An alternate pathway to long-chain polyunsaturates: the FADS2 gene product Delta8-desaturates 20:2n-6 and 20:3n-3. *J. Lipid Res.* 50: 1195-1202.

(6) Sprecher, H., D. L. Luthria, B. S. Mohammed, and S. P. Baykousheva. 1995. Reevaluation of the pathways for the biosynthesis of polyunsaturated fatty acids. *Journal of Lipid Research* 36: 2471-2477.

(7) Marzo, I., M. A. Alava, A. Pineiro, and J. Naval. 1996. Biosynthesis of docosahexaenoic acid in human cells: evidence that two different Δ6-desaturase activities may exist. *Biochimica et Biophysica Acta* 1301: 263-272.

(8) Hastings, N., M. K. Agaba, D. R. Tocher, X. Zheng, C. A. Dickson, J. R. Dick, and A. J. Teale. 2004. Molecular cloning and functional characterization of fatty acyl desaturase and elongase cDNAs involved in the production of eicosapentaenoic and docosahexaenoic acids from alpha-linolenic acid in Atlantic salmon (Salmo salar). *Mar. Biotechnol. (NY)* 6: 463-474.

(9) Park, W. J., K. S. Kothapalli, H. T. Reardon, L. Y. Kim, and J. T. Brenna. 2009. Novel fatty acid desaturase 3 (FADS3) transcripts generated by alternative splicing. *Gene* .

(10) Pedrono, F., H. Blanchard, M. Kloereg, S. D'andrea, S. Daval, V. Rioux, and P. Legrand. 2009. The fatty acid desaturase 3 (Fads3) gene encodes for different FADS3 protein isoforms in mammalian tissues. *J. Lipid Res.* . -

CHAPTER 4

TIME COURSE PATHOLOGY

OF DELTA-6 DESATURASE KNOCKOUT (D6D -/-) MOUSE

ABSTRACT

A D6D-/- mouse was created to study the physiological roles of highly unsaturated fatty acids (HUFA). The D6D-/- mouse is unable to synthesize HUFA, therefore, any pathology present is due to HUFA deficiency. Externally, pathology occurs around 5 months of age with ulcerative dermatitis. Further histological evaluation at 5 months showed gastrointestinal (GI) ulcers, infertility, hepatomegaly, and altered immune system. A time course characterization of the D6D-/- at 3, 6, 9,12, and 16 weeks of age was done in order to determine the sequence in which HUFA deficiency phenotype appears, as well as to measure HUFA levels in -/- at weaning since HUFA stores may explain the delay in appearance of dermatitis until 5 months of age. A total of 44 fully backcrossed C57/bl/6J male and female mice of +/+ and -/- genotype were distributed among the 5 time points. At 3 weeks of age, the -/- mice have liver and testis HUFA levels comparable to wild type and no liver pathology. Male infertility, hepatic lipidosis, and GI lesions are the first evidence of HUFA deficiency that appear at 6 weeks of age correlating with a significant decrease in testis and liver HUFA. In conclusion, the D6D-/- mice present HUFA stores at weaning most likely provided prenatally in milk from the mother. An increase in severity of HUFA deficiency phenotype occurs as the -/-mice age and are further depleted of arachidonic acid (AA) and docosahexaenoic acid (DHA).

INTRODUCTION

Initial characterization of -/- at 5 months of age showed residual AA and DHA in tissues such as liver, brain, and testis. The synthesis of these HUFA by a D6D isozyme, potentially *Fads3,* was hypothesized. However, the presence of an isozyme was ruled out by an *in vivo* analysis with ^{13}C-labeled 18:2 n-6 showing halted synthesis of 20:4 n-6 and by testing the activity of *Fads3* (1). I therefore hypothesized that HUFA are transferred from the mother to the pups at birth and are depleted as the mice age. Pathology would appear as tissues become deficient in HUFA, and HUFA stores at birth could delay appearance of dermatitis in D6D-/- which occurs until 5 months of age. In addition to dermatitis at 5 months of age, a histological evaluation of D6D-/- tissues shows gastrointestinal ulcers, disrupted spermatogenesis, liver lipidosis, and spleen myeloid hyperplasia (1), however the age and HUFA levels at which each deficiency phenotype occurs has not been established. A time course study of D6D-/- pathology will help determine which tissues are first to be impaired by HUFA deficiency. This information will help in the design of future experiments using the D6D-/- by providing time points of HUFA deficiency symptoms for different tissues.

In this study we determined the order of appearance of the known D6D-/- pathology due to HUFA deficiency as well as quantify HUFA depletion in D6D-/- liver and testis at different time points, from weaning until 3 months of age.

METHODS

Experimental design

All animal work was approved by the University of Illinois Institutional Animal Care and Use Committee. Mice used in the experiment were produced by breeding both wild type (+/+) and heterozygous (+/-) males and females of pure C57BL/6J strain fed a standard rodent chow. At weaning, a total of 17 male mice (8 +/+, 9 -/-) and 15 female mice (8+/+, 7 -/-) were distributed in groups of different euthanasia time points (3, 6, 9, and 12 weeks of age). Groups consisted of 4 mice per genotype for each time point. In a separate experiment, 6 +/+ and 6 -/- were euthanized at 16 weeks of age. All mice received the AIN93G diet which is a purified, nutritionally adequate diet that contains sufficient linoleic acid and α-linolenic acid, but no D6D products (2). Mice were single housed at weaning and received the diet until their corresponding time point for euthanasia. All mice were injected with 100 µl of a T-cell dependant antigen (KLH) 8 days prior to euthanasia.

Tissue collection and histology

At each time point of euthanasia, mice were weighed, bled from the retro-orbital vein, and euthanized by carbon dioxide inhalation. A portion of the blood collected (500 µl) was diluted in 50 µl of anticoagulant 0.5M EDTA and used for a complete blood count. The other portion of blood collected was left at room temperature for sera collection.

Tissues collected at euthanasia were liver, spleen, gastrointestinal tract, and testis. Liver, spleen, testis were removed and weighed. The entire gastrointestinal tract was cassetted for histological examination as a swiss roll preparation in order to allow evaluation of entire tract. Half the liver was frozen for fatty acid and RNA analysis while the other half was fixed in 10%

neutral buffered formalin. Left testis was frozen for fatty acid and RNA analysis while right testis was fixed in Davidson's fixative and transferred to 10% neutral buffered formalin after 24 hours. Tissues were trimmed, processed, and paraffin embedded. Sections were cut at 3 microns and stained with hematoxylin and eosin for histological evaluation. At 3 weeks, only liver was examined histologically. In subsequent time points, all tissues were examined.

Fatty acid and triglyceride (TG) analysis

Total lipids were extracted from liver and testis using the Folch method (3). C17:0 phosphatidylcholine and triacylglycerol were added as internal standards to liver while testis only received C17:0 phosphatidylcholine. Liver lipid fractions of phospholipid and triglyceride were obtained by thin layer chromatography with a mixture of petroleum ether, ethyl ether, and acetic acid at 80:20:1. Total lipids were analyzed in testis. Liver and testis fatty acids were methylated with methanolic HCl (Supelco, Bellefonte, PA) at 75ºC for 90 minutes. Fatty acid methyl esters were identified by HP5890 gas chromatography (Agilent Technologies, Wilmington, DE) using a 30 m x 0.25 mm Omegawax capillary column (Supelco) at 180ºC for 2 minutes followed by a 3ºC gradient until a final temperature of 230ºC for 35 minutes.

Gene expression

RNA was analyzed with a slight modification of a method previously described (4). Testis was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA), and total RNA extracted. MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA), along with random hexamers, were used to synthesize cDNA. Real-time quantitative PCR, using SYBR Green fluorescent dye (Applied Biosystems) was used to analyze RNA relative to a ribosomal RNA L7a. Oligonucleotides used for real-time quantitative PCR were SCD-1-F,

GTCATCGTTTGTGGTCAGATACT, SCD1-R, GCCGGCATGATGATAGTCAGT, DGAT-2-F, GTGGCTGCAGGTCGGGTTCC, DGAT-2-R, GACGGCACCTGTGGGACAGC, MTP-1-F, TGCCATGCAAAATAGCGGTCACA, MTP-1-R, TAGCCCACGCTGTCTTGCGG, D5D-F, CCAGCTTTGAACCCACCAA, D5D-R, CATGAGGCCCATTCGCTCTA, ELOVL2-F, GCTAATGGCATGACGGACAA , ELOVL-2-R, GTTCCCCGGCACTTCATTT.

Antibody response to a T-cell dependant antigen

Mice were challenged with one hundred μg of a soluble T-cell dependant antigen, keyhole limpet haemocyanin (KLH), injected intraperitoneally eight days before termination. Serum was collected and assayed for antibodies specific to KLH by ELISA on plates coated with antigen KLH.

Peripheral blood neutrophil counts

Blood was collected in EDTA and submitted to the University of Illinois Diagnostic Laboratory for neutrophil quantification.

Statistical Analyses

Significant differences between +/+ and -/- groups were determined by Student's t-test.

RESULTS

Time course pathology of D6D-/-

Delta-6 desaturase knockout (D6D-/-) tissues were evaluated at 3, 6, 9, and 12 weeks of age. First signs of HUFA deficiency were observed at the 6 week time point, which included decreased spermatogenesis, mild hepatic lipidosis, and gastrointestinal erosions. Only liver was

evaluated at the previous time point of 3 weeks and did not show lipidosis. Histological evaluation of tissues at subsequent time points indicates D6D-/- pathology becomes more apparent as mice age (**Table 4.1**). At 9 weeks of age, the -/- immune system is unable to produce antibody at the same level as +/+ when challenged with an antigen. At 12 weeks of age, hepatic lipidosis is marked and gastrointestinal erosions become ulcers. By 16 weeks of age, mild neutrophilia occurs before ulcerative dermatitis which appears between 18 and 20 weeks of age (1). Despite pathology occurring by 6 weeks of age, body and tissue weights, as well as organ to body ratios, do not differ statistically as HUFA are depleted(**Table 4.2 and 4.3**). It is until 16 weeks of age, when liver weight is significantly higher in D6D-/- versus +/+.

Hepatic lipidosis occurs by 6 weeks of age in D6D -/-

Liver HUFA are depleted rapidly from 3 to 6 weeks of age. D6D -/- liver AA and DHA levels are at 27.6% and 19% of +/+ (**Figure 4.1**). This drop in hepatic HUFA molar percentage coincides with the appearance of mild hepatic lipidosis at 6 weeks of age. Lipidosis was confirmed by measuring liver triglycerides. An increase of TG from 5.45 to 22.53 μg/mg liver occurs at 6 weeks of age, parallel to the severe drop in liver HUFA levels (**Figure 4.2**). By 12 weeks of age, liver AA and DHA have decreased 88% when compared to +/+. Liver TG are significantly higher in -/- at 27.51 μg/mg liver when compared to +/+ which only has 6.2 μg/mg liver. According to histological evaluation, the lipidosis becomes more severe with age as HUFA are further depleted. At 16 weeks of age, D6D-/- have severe hepatic lipidosis (**Figure 4.3**).

Liver gene expression also changes with HUFA depletion (**Figure 4.4**). Elongase-2 and D5D gene expression increases in all -/- as early as 3 weeks of age and is maintained at high

expression in D6D-/- when compared to +/+. SCD1 gene expression is significantly higher ($p<0.05$) only at 12 weeks of age.

Disrupted spermatogenesis occurs by 6 weeks of age in D6D -/-

In addition to hepatic lipidosis, impaired male fertility is also present by 6 weeks of age, The first time point examined shows testicular pathology of disrupted spermatogenesis. Rate of HUFA depletion in testis is similar to liver. Testis HUFA analysis at 6 weeks indicates AA is 67% less than +/+ while the sum of DPAn6 and DHA are 62% less than +/+. The depletion of these HUFA continues as the mice age and by 12 weeks, HUFA are 91% less than +/+ (**Figure 4.5**).

Gene expression of elongase-2 (ELOVL2) and delta-5 desaturase (D5D) was measured in testis with D5D increased expression, although not significant, in -/- testis. ELOVL2 did not change in expression.

Gastrointestinal (GI) lesions appear by 6 weeks of age in D6D -/-

The third HUFA deficiency phenotype to appear by 6 weeks of age is the presence of erosions in the ileocolic region. In more severe cases, inflammation extended through the intestinal wall into the mesentery. As indicated by liver and testis HUFA analysis, the amount of AA depleted by 6 weeks of age is significant. A threshold of AA deficiency in GI may have been reached compromising the GI protection provided by AA-derived prostaglandins. As the mice age, AA is further depleted and the lesions progress to ulcers by 12 weeks of age.

Immune system deficiency in D6D -/- by 9 weeks of age

The immune system was evaluated by challenging the mouse with a T-cell dependant antigen and measuring the amount of antibody produced and present in serum. At 9 weeks, -/- mice had a significantly lower antibody response ($p<0.05$) compared to +/+. The low antibody response persists as the mice age (**Figure 4.6**).

Peripheral blood neutrophils increase in D6D -/- by 16 weeks age

The majority of the -/- mice show mild neutrophilia (**Figure 4.7**) by 16 weeks of age, becoming severe once dermatitis appears at approximately 21 weeks of age. The increase in number of neutrophils may be secondary to an inflammatory state caused by GI ulcers since neutrophilia does not occur before 16 weeks of age.

DISCUSSION

The D6D -/- mouse at 3 weeks of age has a considerable amount of HUFA in liver with 11 times more AA and 8 times more DHA than -/- at 4 months of age. This confirms the hypothesis that D6D -/- mice receive a certain amount of HUFA from the mother during pregnancy and before weaning delaying appearance of D6D-/- pathology tissue due to HUFA deficiency. Interestingly, despite HUFA supply by the mother, 3 week old +/+ mice still had significantly more liver HUFA than -/- mice of the same age indicating active HUFA synthesis from +/+ pups as an important contributor to liver AA and DHA at that time point. In -/-, HUFA are rapidly depleted once mice are separated from the mother at 3 weeks of age. By 6 weeks of age, liver HUFA levels dropped significantly by more than half. The period of 3 weeks to 6 weeks of age is a period of growth in which mice nearly double their weight. This growth period

may have contributed to the accelerated depletion of HUFA. The accelerated rate of HUFA depletion by 6 weeks of age coincides with first signs of HUFA deficiency in liver, as well as in gastrointestinal tract and testis. At this time point, HUFA deficiency phenotype consist of mild hepatic lipidosis with 3 times more TG than +/+ and early lesions in the gastrointestinal tract.

Male D6D-/- mice are infertile due to disrupted spermatogenesis and inability to impregnate female as demonstrated in a separate study (5). In this study, we show disrupted spermatogenesis occuring in D6D-/- as early as 6 weeks of age. There are two important 22 carbon length HUFA in testis, DHA (22:6n3) and DPA (22:5n6). It is the depletion of the sum of DHA and DPA molar percentages which correlates with testis pathology. In +/+, DPAn6 represents 4.74% of total fatty acids in testis while DHA is 2.95%; combined these HUFA represent around 7.7% of fatty acids in testis. In 6 week old infertile -/-, DPAn6 is already depleted while DHA is present at 2.22%, a total percentage below the 7.7% observed in +/+. A separate study showed DHA supplementation at 0.2% fully restoring spermatogenesis in -/- and raising the total percentage of 22 carbon length HUFA to 7.33%, all contributed by DHA since DPAn6 remained depleted (5). This indicates that DHA and DPA are interchangeable for spermatogenesis. Therefore, as long as total percentage of 22 carbon length HUFA is approximately 7%, mice should be fertile. It is also worth noting HUFA are not completely depleted in D6D-/- testis by 2 months of age as suggested by Stoffel et al (6).

It is long known that essential fatty acid deficiency results in hepatic lipidosis (7). In the case of the D6D-/-, we can attribute hepatic lipidosis specifically to the lack of AA and DHA, since PUFA, LA and ALA, were provided in diet and present in liver. LA and ALA alone were not sufficient to prevent accumulation of TG in D6D-/- liver. The mechanism behind the essentiality of AA and DHA in maintaining liver lipid homeostasis has not been determined.

Increased expression of lipogenic genes was hypothesized, however, the SCD-1 gene in D6D-/- liver only shows a slight increase in expression until 12 weeks of age. SCD-1 cannot explain lipidosis. Measurements of other lipogenic genes involved in triglyceride synthesis is required to determine if increased lipogenesis is the underlying cause of lipidosis in D6D-/-.

The initial observation of a possible altered immune system in the 21 week old D6D-/- consisted of thymic atrophy and splenomegaly accompanying severe dermatitis (1). Thymic atrophy was not present in any of the time points in the current study when animals were terminated before manifestation of ulcerative dermatitis. Therefore, thymic atrophy is most likely secondary to development of severe dermatitis. Splenic myeloid hyperplasia appears at around the same time as gastrointestinal ulceration, therefore spleen pathology may be secondary to intestinal inflammation. Before 16 weeks of age, increase in neutrophil numbers are not observed, however, an impaired ability to produce antibody in response to a T-cell dependent antigen challenge occurs by 9 weeks of age. We can conclude that HUFA deficiency interferes with the development of an adaptive immune response for antibody production independent of other D6D-/- pathologies.

In summary, this study established that D6D-/- pathology appears as HUFA are depleted in tissues. By 6 weeks of age, HUFA deficiency symptoms are already present in liver, testis, and intestine, while skin function is not impaired until approximately 21 weeks of age. These deficiency symptoms are specific to AA and/or DHA and not their corresponding fatty acids precursors, LA and ALA. HUFA supplementation studies of either AA or DHA will indicate if there is specific HUFA essentiality for each tissue function or if both HUFA are required to reverse D6D-/- pathology of hepatic lipidosis, gastrointestinal ulcers, male infertility, and dermatitis. The mechanisms behind HUFA essentiality in these tissues is yet to be elucidated.

TABLES AND FIGURES

Table 4.1 *Time course of delta-6 desaturase knockout (D6D-/-) phenotype due to highly unsaturated fatty acid deficiency (male n=2-3; female n=2).*

Age (weeks)	D6D -/- phenotype
6	Decreased spermatogenesis Hepatic lipidosis - mild (+)* GI erosions (+)
9	Low antibody response
12	Hepatic lipidosis - marked (++) GI erosion/ulceration at ileocolic junction (++)
16	Splenic myeloid hyperplasia-mild to moderate Neutrophilia-mild Initial signs of skin lesions
18-20	Ulcerative dermatitis

*Lipidosis not present at 3 weeks.

Table 4.2 *Body and tissue weights of -/- mice from 3 to 16 weeks of age.*

Age	Body (g)		Liver (g)		Testis (mg)		Spleen (mg)	
Weeks	+/+	-/-	+/+	-/-	+/+	-/-	+/+	-/-
3	10.4±0.07	7.8±1.6	nd	nd	nd	nd	nd	Nd
6	17.8±4.3	17.6±1.5	1±0.002	0.86±0.05	73±14	86±13	53±2	47±7
9	20±6.6	15.3±3.2	0.97±0.01	1.05±0.18	100±2	89±19	55±6	41±4
12	20.2±5.9	18.1±3.4	0.93±0.09	0.92±0.12	106±2	83±32	69±1	75±6
16	26.1±1	24.6±3.5	0.95±0.1	1.2±0.19*	102±6	91±10	72±8	75±18

*Mean ± SD. *statistically different by Student's t-test ($p < 0.05$), compared to +/+. Male n=2-3; female n=2. nd=not determined*

Table 4.3 *Tissue to body weight ratios of -/- mice from 6, 9, 12, and 16 weeks of age.*

Age	Liver		Testis		Spleen	
Weeks	+/+	-/-	+/+	-/-	+/+	-/-
6	4.1±1%	4.9±0.3%	0.35±0.01%	0.46±0.01%	0.3±0.05%	0.27±0.1%
9	3.6±0.8%	4.9±0.6%	0.41±0.04%	0.5±0.1%	0.29±0.1%	0.26±0.1%
12	3.4±0.8%	3.7±0.9%	0.43±0.02%	0.4±0.1%	0.37±0.08%	0.36±0.03%
16	3.6±1%	4.87±1%*	0.4±0.03%	0.37±0.04%	0.27±0.03%	0.3±0.07%

*Mean ± SD. *statistically different by Student's t-test ($p < 0.05$), compared to +/+. Male n=2-3; female n=2.*

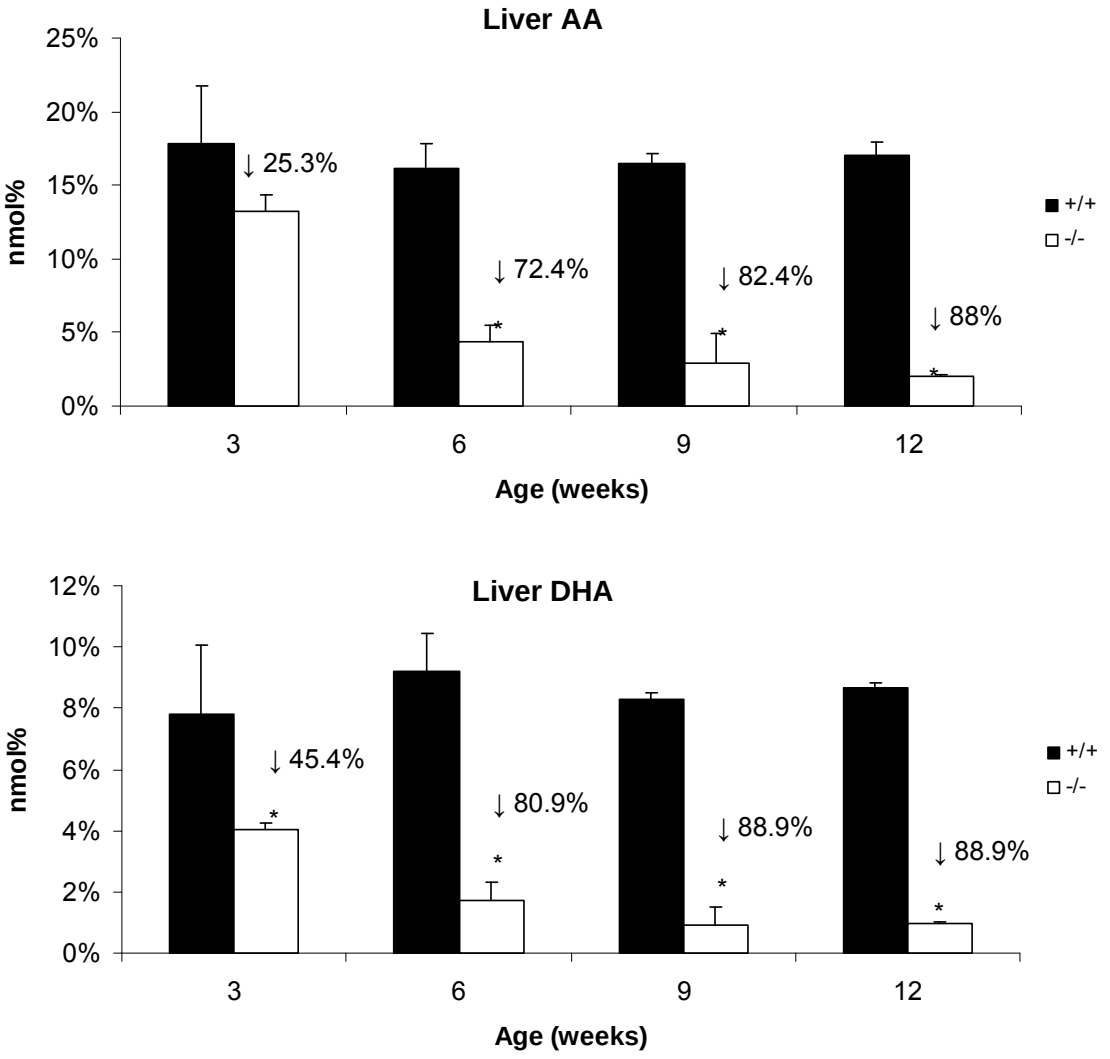

Figure 4.1 *Liver HUFA depletion in D6D -/- mouse at 3, 6, 9, and 12 weeks of age. Mean±SD. Male n=2-3; female n=2. * p<0.05, Student's t-test, +/+ compared to -/- within age group.*

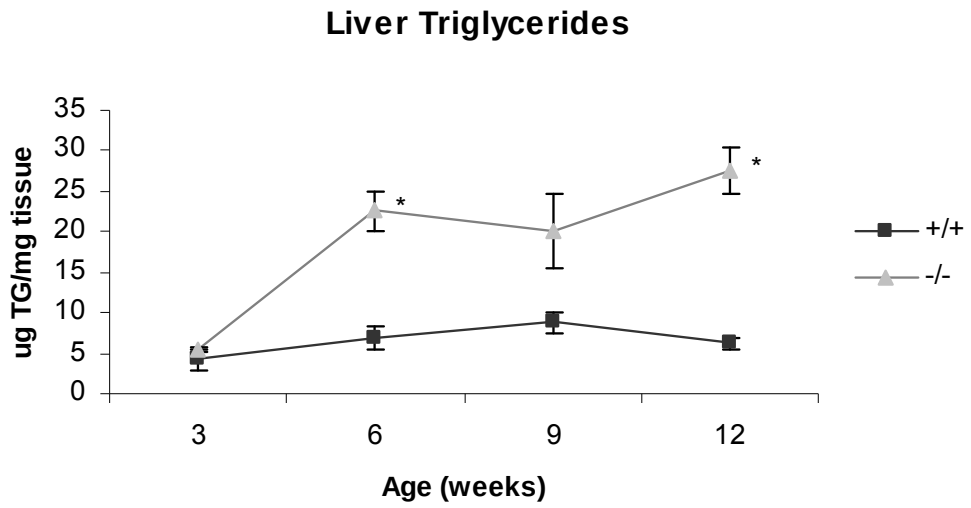

Figure 4.2 *Liver triglycerides accumulate in D6D-/- as HUFA are depleted. Mean±SD. Male n=2-3; female n=2. * p<0.05, Student's t-test, +/+ compared to -/- within age group.*

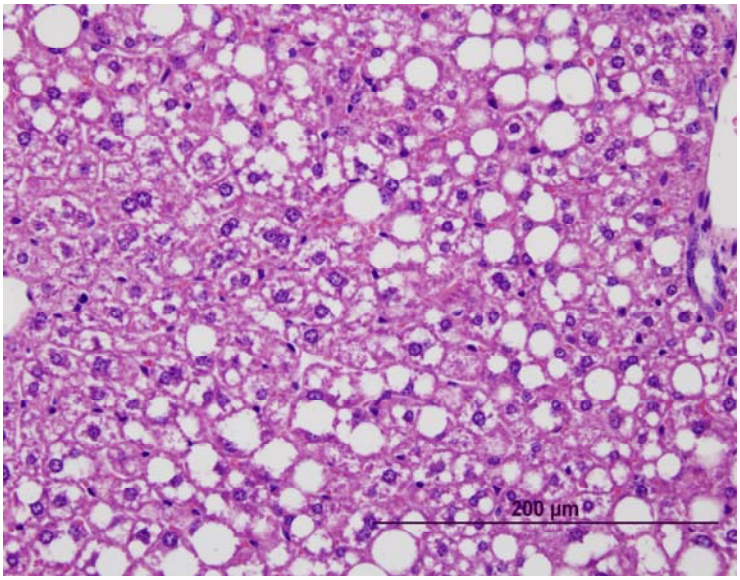

Figure 4.3 *Liver histology (hematoxylin and eosin). Hepatic lipidosis in 16 week old -/- mouse with accumulated lipids as indicated by large vacuoles (macrovesicular lipidosis).*

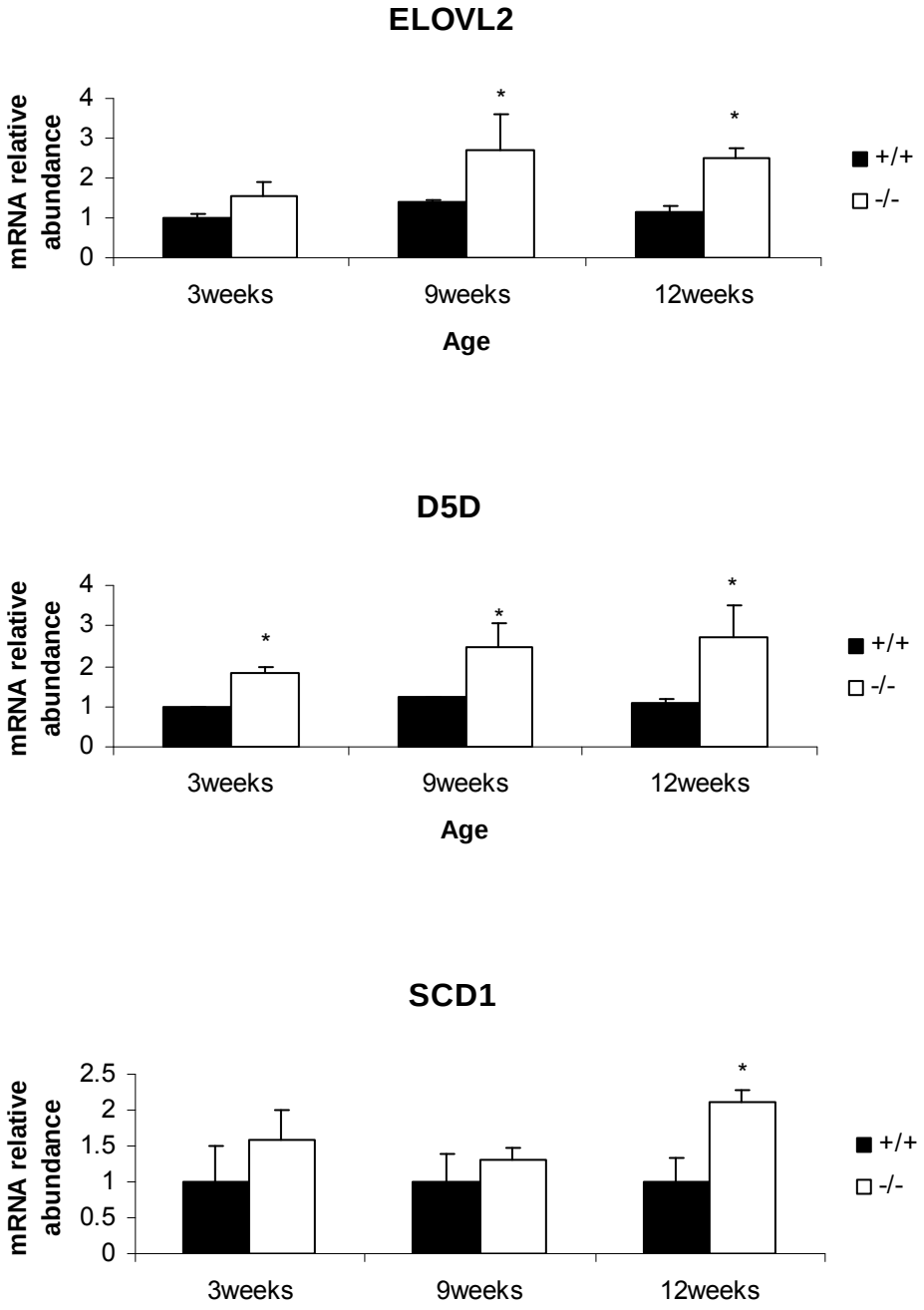

Figure 4.4 *Liver gene expression for lipogenic genes. Mean±SD. Male n=2-3; female n=2. * p<0.05, Student's t-test, +/+ compared to -/- within age group.*

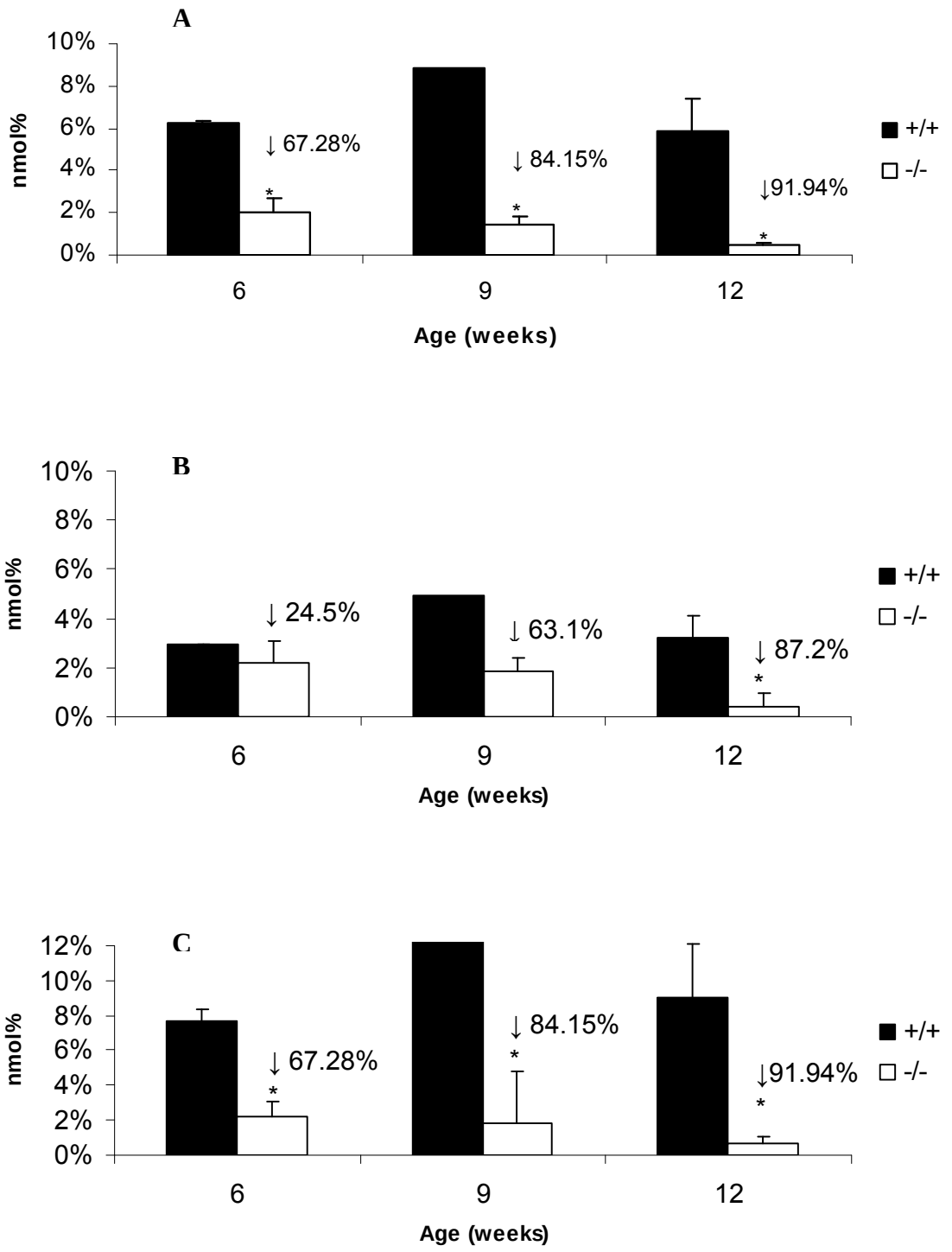

Figure 4.5 *Testis HUFA depletion. A) arachidonic acid (AA); B) docosahexaenoic acid (DHA); C) DHA+docosapentaenoic acid. Mean±SD (n=2-3). * p<0.05, Student's t-test.*

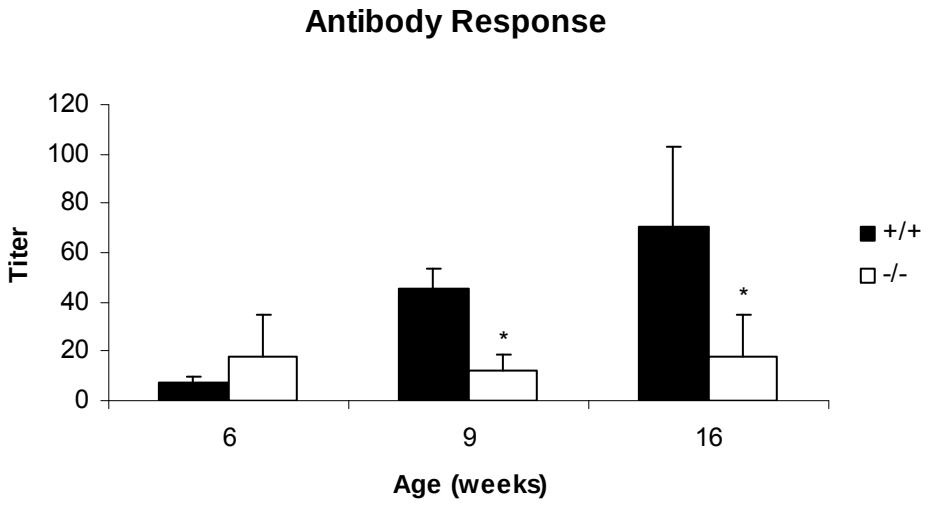

Figure 4.6 *Antibody response to a T-cell dependant antigen, KLH, at 6, 9 and 16 weeks of age. Mean±SD. n=2-3 males, n=2 females for 6 and 9 weeks, n=6 males for 16 weeks.*
** p<0.05, Student's t-test, +/+ compared to -/-.*

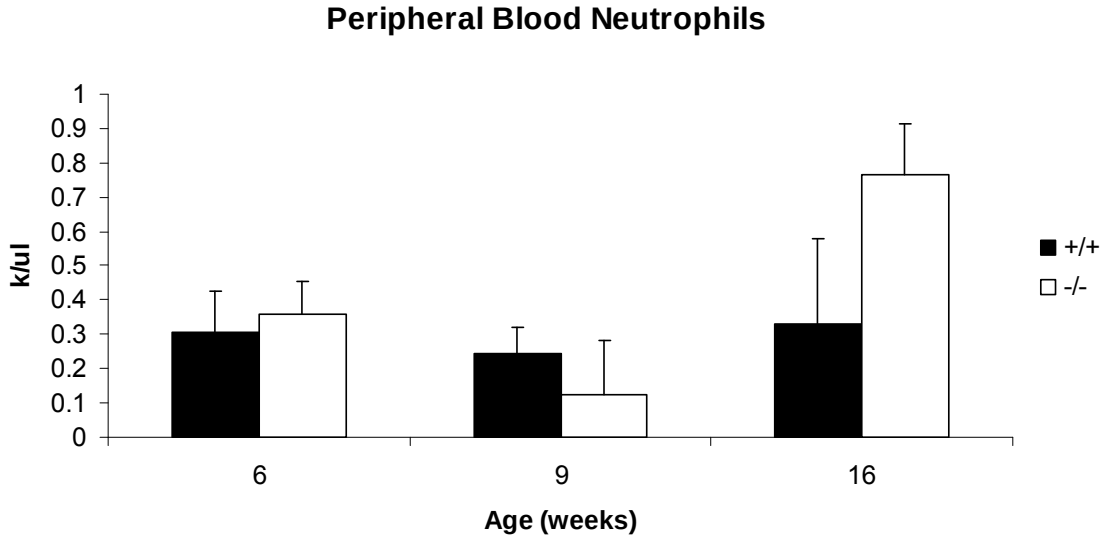

Figure 4.7 *Peripheral blood neutrophils in D6D -/-. Mean±SD. n=2-3 males, n=2 females for 6 and 9 weeks, n=6 males for 16 weeks. * p<0.05, Student's t-test, +/+ compared to -/-.*

REFERENCES

(1) Stroud, C. K., T. Y. Nara, M. Roqueta-Rivera, E. C. Radlowski, P. Lawrence, Y. Zhang, B. H. Cho, M. Segre, R. A. Hess, J. T. Brenna, W. M. Haschek, and M. T. Nakamura. 2009. Disruption of FADS2 gene in mice impairs male reproduction and causes dermal and intestinal ulceration. *J. Lipid Res.* 50: 1870-80.

(2) Reeves, P. G., F. H. Nielsen, and G. C. Fahey. 1993. AIN-93 purified diets for laboratory rodents: Final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* 123: 1939-1951.

(3) Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 226: 497-509.

(4) Koo, H. Y., M. A. Wallig, B. H. Chung, T. Y. Nara, B. H. Cho, and M. T. Nakamura. 2008. Dietary fructose induces a wide range of genes with distinct shift in carbohydrate and lipid metabolism in fed and fasted rat liver. *Biochim. Biophys. Acta* 1782: 341-348.

(5) Roqueta-Rivera, M., C. K. Stroud, W. M. Haschek, S. J. Akare, M. Segre, R. S. Brush, M. P. Agbaga, R. E. Anderson, R. A. Hess, and M. T. Nakamura. 2010. Docosahexaenoic acid supplementation fully restores fertility and spermatogenesis in male delta-6 desaturase knockout mice. *J. Lipid Res.* 51: 360-7.

(6) Stoffel, W., B. Holz, B. Jenke, E. Binczek, R. H. Gunter, C. Kiss, I. Karakesisoglou, M. Thevis, A. A. Weber, S. Arnhold, and K. Addicks. 2008. Delta6-Desaturase (FADS2) deficiency unveils the role of omega3- and omega6-polyunsaturated fatty acids. *EMBO J.* 27: 2281–2292.

(7) Alfin-Slater, R. B., S. Bernick. 1958. Changes in tissue lipids and tissue histology resulting from essential fatty acid deficiency in rats. *Am. J. Clin. Nutr.* 6: 613-624.

(8) Kobayashi, T., D. Zadravec, and A. Jacobsson. 2007. ELOVL2 overexpression enhances triacylglycerol synthesis in 3T3-L1 and F442A cells. *FEBS Lett.* 581: 3157-3163.

-

CHAPTER 5

DIETARY ARACHIDONIC ACID AND DOCOSAHEXAENOIC ACID PREVENT HEPATIC LIPIDOSIS IN DELTA-6 DESATURASE KNOCKOUT (D6D -/-) MOUSE

ABSTRACT

The D6D-/- mouse presents highly unsaturated fatty acid (HUFA) deficiency symptoms in liver by 6 weeks of age. Disrupted HUFA synthesis results in hepatic lipidosis which is characterized by large lipids vacuoles in cytoplasm of hepatocytes. The severity of liver pathology increases as the D6D-/- ages and becomes more depleted of HUFA. Liver triglyceride (TG) levels increase with HUFA depletion. To determine which HUFA is essential for liver lipid homeostasis, a diet supplemented with either 0.2% (w/w) arachidonic acid (AA) or docosahexaenoic acid (DHA) was fed to wild type (+/+) and -/- males at weaning until 16 weeks of age (n=3-5). Both dietary AA and DHA supplementation are capable of preventing hepatic lipidosis, maintaining liver triglycerides at +/+ levels. Accumulation of liver TG cannot be explained by changes in gene expression of lipogenic enzymes. In conclusion, HUFA are essential in liver lipid homeostasis. Future studies are required to determine a potential role of HUFA in lipid secretion for hepatic TG homeostasis.

INTRODUCTION

Hepatic lipidosis is characterized by accumulation of lipids in cytoplasm of hepatocytes. Increased lipogenesis is one of many factors which can contribute to lipidosis (1). PUFA of the omega-3 and omega-6 family are known to downregulate expression of lipogenic genes while saturated and monounsaturated fatty acids are not capable of inhibiting fatty acid synthesis (2). Reversal of hepatic steatosis can be achieved with PUFA supplementation. The leptin deficient *ob/ob* mouse, for example, develops hepatic lipidosis which can be ameliorated through dietary supplementation of fish oil (3). Downregulation of genes involved in *de novo* fatty acid synthesis, such as fatty acid synthase (FAS) and stearoyl coenzyme-A desaturase (SCD-1), was observed in PUFA-supplemented *ob/ob* mice when compared to their non-supplemented counterparts. In a separate study, rats fed menhaden fish oil also showed suppressed lipogenesis with reduced expression of FAS, when compared to rats fed saturated tripalmitin (4). PUFA also suppress the lipogenic genes involved in HUFA synthesis: D6D and delta-5 desaturase (D5D) (5,6). Both omega-6 and omega-3 PUFA achieve lipogenic suppression by decreasing nuclear content of transcription factor SREBP1c (7,8) which targets lipogenic genes such as SCD1 and FAS for upregulation (9).

Deficiency of essential fatty acids, LA and ALA, leads to hepatic lipidosis (10). However, liver triglyceride (TG) accumulation occurs in a specific HUFA deficient state with the D6D-/- mouse (11), indicating a requirement for HUFA and not the precursors LA and ALA in modulating TG levels in liver. It is not clear if both HUFA are needed to maintain liver function or if either AA or DHA alone can specifically prevent lipidosis. These HUFA may have a role in maintaining liver lipid homeostasis through regulation of lipogenic genes.

The objective of this study is to compare dietary supplementation of AA and DHA in reversing liver pathology and TG accumulation, as well as measure changes in lipogenic gene expression.

METHODS

Animal study

All animal work was approved by the University of Illinois Institutional Animal Care and Use Committee. Mice used in the experiment were produced by breeding heterozygous males and females of mixed strain (129S6/SvEvTac/C57BL/6J) fed a standard rodent chow. At weaning, a total of 30 male mice, 9 wild type (+/+), 9 heterozygote (+/-), and 12 D6D null (-/-) were distributed to receive one of the following diets: control diet (AIN93G), AIN93G supplemented with 0.2 w/w % AA, or AIN93G with 0.2 w/w % DHA. The AIN93G diet is a purified, nutritionally adequate diet that contains sufficient linoleic acid and α-linolenic acid, but no D6D products (12). DHASCO and ARASCO oils (Martek Biosciences, Columbia, MD) were used for supplementation of DHA and AA, respectively. All dietary groups consisted of 3 mice with exception of the AA supplemented -/- (n=4) and DHA supplemented -/- (n=5). Mice were single housed at weaning and received the diet until four months of age.

Tissue collection and histology

Animals were euthanized by carbon dioxide inhalation at 4 months of age. Liver was removed and weighed; half the liver was then frozen for fatty acid and RNA analysis while the other half was fixed in 10% neutral buffered formalin. Fixed liver was trimmed, processed, and paraffin embedded. Sections were cut at 3 microns and stained with hematoxylin and eosin for

histological evaluation. Frozen sections were prepared from formalin fixed liver and stained with oil-red-O for evaluation of lipid.

Gene expression

RNA was analyzed with a slight modification of a method previously described (13). Liver was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA), and total RNA extracted. MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA), along with random hexamers, were used to synthesize cDNA. Real-time quantitative PCR, using SYBR Green fluorescent dye (Applied Biosystems) was used to analyze RNA relative to a ribosomal RNA L7a. Oligonucleotides used for real-time quantitative PCR were: SCD-1-F, GTCATCGTTTGTGGTCAGATACT, SCD1-R, GCCGGCATGATGATAGTCAGT, DGAT-2-F, GTGGCTGCAGGTCGGGTTCC, DGAT-2-R, GACGGCACCTGTGGGACAGC, MTP-1-F, TGCCATGCAAAATAGCGGTCACA, MTP-1-R, TAGCCCACGCTGTCTTGCGG, D5D-F, CCAGCTTTGAACCCACCAA, D5D-R, CATGAGGCCCATTCGCTCTA, ELOVL2-F, GCTAATGGCATGACGGACAA , ELOVL-2-R, GTTCCCCGGCACTTCATTT.

Fatty acid extraction and analysis

Total lipids were extracted from liver using the Folch method (14). C17:0 phosphatidylcholine and triacylglycerol were added as internal standards to liver. Liver lipid fractions of phospholipid and triglyceride were obtained by thin layer chromatography with a mixture of petroleum ether, ethyl ether, and acetic acid at 80:20:1. Total lipids were analyzed in testis. Liver and testis fatty acids were methylated with methanolic HCl (Supelco, Bellefonte, PA) at 75ºC for 90 minutes. Fatty acid methyl esters of phospholipids and triacyglycerols were identified by HP5890 gas chromatography (Agilent Technologies, Wilmington, DE) using a 30

m x 0.25 mm Omegawax capillary column (Supelco) at 180ºC for 2 minutes followed by a 3ºC gradient until a final temperature of 230ºC for 35 minutes.

Statistical analysis

Statistical analysis with Statview version 5.01 for Windows was conducted using one-way ANOVA with Fisher's PLSD post test and Student's t-test. Data were presented as mean ± SD; $p < 0.05$ was considered as statistically significant.

RESULTS

Both dietary AA and DHA can prevent hepatic lipidosis in D6D -/-

Liver absolute weight and liver to body weight ratios were increased only in the non-supplemented -/- AIN mice, while HUFA supplemented -/- had liver weights similar to +/+ (**Table 5.1**). Macrovesicular hepatic lipidosis occured only in -/- without HUFA supplementation, consisting of large vacuoles of accumulated lipids in cytoplasm of hepatocytes (**Figure 5.1A**). Individual supplementation of either AA or DHA at 0.2% (w/w) of diet prevented hepatic lipidosis in -/-, restorting hepatocyte morphology to +/+ (**Figure 5.1A**). Oil red stain confirmed the presence of accumulated neutral lipids in vacuoles of D6D-/- hepatocytes (**Figure 5.1B**).

Hepatic HUFA phospholipids restored with supplementation

Fatty acids in liver phosphoslipids of -/- with or without HUFA supplementation were compared (**Figure 5.2**). In non-supplemented -/-, oleic acid and LA were significantly higher when compared to +/+ while AA and DHA were nearly depleted; a D5D product unique to -/-, 20:3 (n7, 11, 14), increased in amount.

Dietary AA reduced LA in -/- to +/+ levels while dietary DHA was not as effective in doing this. AA supplementation restored hepatic phospholipid AA in -/- to 23% of fatty acids, significantly above the 15% of AA in +/+. DHA supplementation in -/- raised phospholipid DHA to 12.7% of total fatty acids compared to 7.8% in +/+. The 20:3 (n7, 11, 14) fatty acid, unique to -/- mice, was present in non-supplemented -/- at 7.7%, while HUFA supplementation significantly decreased this fatty acid to 0.7% in the AA supplemented -/-, and to 1.2% in DHA supplemented -/-.

Liver TG levels maintained at +/+ level

Non-supplemented -/- mice showed significantly increased TG amounts in liver ($p<0.05$) at 79.5 μg/mg while +/+ only had 12.1 μg/mg. Dietary HUFA supplementation was capable of maintaining TG levels closer to +/+ level at 18.8 μg/mg for AA supplemented -/- and at 19.1 μg/mg for DHA supplemented -/-. There was no significant difference in liver TG amounts between HUFA supplemented -/- and +/+ mice.

Liver gene expression of lipogenic enzymes are not all increased with HUFA deficiency

Gene expression of lipogenic enzymes (**Table 5.2**) significantly increased ($p<0.05$) for D5D (2.5x) and ELOVL2 (1.8x) in non-supplemented -/- compared to +/+. AA supplemented -/- maintained high gene expression for D5D (2.2x) and ELOVL2 (2.1x), while DHA supplementation decreased D5D and ELOVL2 expression to +/+ levels. SCD-1 did not reach significant difference, however average gene expression in -/- was slightly higher. Genes involved in TG synthesis, MTP-1 and DGAT-2, were not significantly different in non-supplemented -/-.

DISCUSSION

HUFA deficiency results in hepatic lipidosis as demonstrated previously in the initial characterization of the D6D-/- (11), as well as in the D6D-/- pathology time course study (**Chapter 4**) where HUFA depletion parallels severity of hepatic lipidosis. The present study demonstrates the ability of dietary HUFA in preventing hepatic accumulation of TG. Both AA and DHA supplementation successfully prevented formation of large lipid droplets of TG in D6D-/- liver and restored respective HUFA levels of AA and DHA.

Hepatic lipidosis in D6D-/- could not be explained by changes in lipogenic gene expression. The diacylglycerol acyltransferase-2 (DGAT2) enzyme participates in TG synthesis by esterifying fatty acids into TG. Overexpression of liver DGAT-2 results in steatosis (15) while suppression of DGAT-2 reverses diet-induced lipidosis in rats (16). Increased DGAT-2 gene expression was not observed in D6D-/- liver. SCD-1 synthesizes the main monounsaturated fatty acid, oleic acid, providing substrate for esterification into TG and its expression also did not significantly increase in D6D-/- liver. Microsomal triglyceride transfer protein (MTP) participates in TG packaging into VLDL for secretion (20). Gene expression for this protein was not changed with HUFA deficiency.

Delta-5 desaturase (D5D) and elongase-2 (ELOVL-2) participate in fatty acid desaturation and elongation (17) of PUFA for HUFA synthesis. D5D synthesizes AA from 20:3n6; or eicosapentaenoic acid (EPA; 20:5n3) from 20:4n3. ELOVL2 substrates are most likely the 20 carbon PUFA, AA (20:4n6) and EPA (20:5n3) (18), which are precursors to other HUFA, docosapentaenoic acid (DPA; 22:5ω6) and DHA, respectively. Both D5D and ELOVL2 had increased expression in D6D-/- liver; most likely as a compensatory mechanism to lack of HUFA in tissue. Increased hepatic expression of these genes has been previously demonstrated

in an omega-3 PUFA deficient state in rat (19). In D6D-/-, increased expression of D5D and ELOVL2 does not explain hepatic lipidosis since AA supplementation was not able to downregulate these genes yet was effective in preventing liver pathology. Only DHA supplementation was capable of decreasing D5D and ELOVL2 expression back to +/+ level. Although not related to hepatic lipidosis, this data provides new insight on gene expression regulation of D5D and ELOVL2. Little is known about the regulation of ELOVL2. Failed downregulation of D5D and ELOVL2 genes in AA supplemented -/- may indicate specific regulatory function of DHA (22:6n3), omega-6 DPA (22:5n6), or their corresponding 24 carbon products, which are near depletion in AA supplemented -/-. DPAn6 is structurally similar to DHA and therefore may participate in feedback regulation of D5D and ELOVL2 in absence of DHA.

The underlying cause of D6D-/- hepatic lipidosis could not be explained by changes in lipogenic gene expression. An alternative mechanism involves altered lipoprotein secretion. Microsomal triglyceride transfer protein (MTP) is a critical protein for TG packaging into VLDL (20). MTP is present in endoplasmic reticulum where VLDL is assembled. VLDL is then transported through Golgi. MTP is also present within the trans-Golgi network where it colocalizes with SNARE protein (21). SNARE proteins are involved in fusion of transport vesicles and target membranes. The SNARE components syntaxin-5, SNAP23 and VAMP4 have also been identified in liver cytosolic lipid droplets (22). HUFA are required for SNARE complex formation (23). HUFA deficiency in D6D-/- therefore may disrupt SNARE complex formation within the VLDL formation process which could eventually interfere with TG secretion from liver favoring hepatic steatosis.

In summary, the role of HUFA in maintaining lipid homeostasis is yet to be elucidated. Further research is needed to elucidate the mechanism behind the essentiality of HUFA in TG homeostasis.

TABLES AND FIGURES

Table 5.1 *Liver absolute and relative weights of +/+ and -/- groups fed HUFA supplemented and non-HUFA supplemented diets.*

Liver	Genotype	AIN	AA	DHA
Absolute weight (grams)	+/+	0.95±0.1	1.01±0.1	1.1±0.2
	-/-	1.2±0.2	1.03±0.19	1.03±0.15
Relative to body weight	+/+	3.65±0.4%	3.9±0.4%	4.1±0.03%
	-/-	4.86±0.2%*	3.94±0.5%	4.24±0.5%

*Mean ± SD. n=3-5. *p<0.05, compared to +/+ with Student's t-test. AIN, AIN93G diet without HUFA supplementation. AA, 0.2% arachidonic acid supplemented AIN93G diet. DHA, 0.2% docosahexaenoic acid supplemented AIN93G diet.*

Table 5.2 *Liver lipogenic gene expression of +/+ and -/-.*

Gene	+/+ AIN	-/- AIN	-/- AA	-/- DHA
d5d	1±0.6	2.5±1 *	2.2±1	1.5±0.5
elovl2	1±0.5	1.8±0.01*	2.1±0.6*	1.4±0.3
scd-1	1±0.6	1.4±0.5	1.5±1.5	1.2±0.9
dgat-2	1±0.1	0.7±0.3	0.67±0.4	1.31±0.2
mtp-1	1±0.1	1±0.4	0.7±0.05	1.12±0.3

*Mean ± SD. n=3-5. *p<0.05, compared to +/+ with Student's t-test. AIN, AIN93G diet without HUFA supplementation. AA, 0.2% arachidonic acid supplemented AIN93G diet. DHA, 0.2% docosahexaenoic acid supplemented AIN93G diet.*

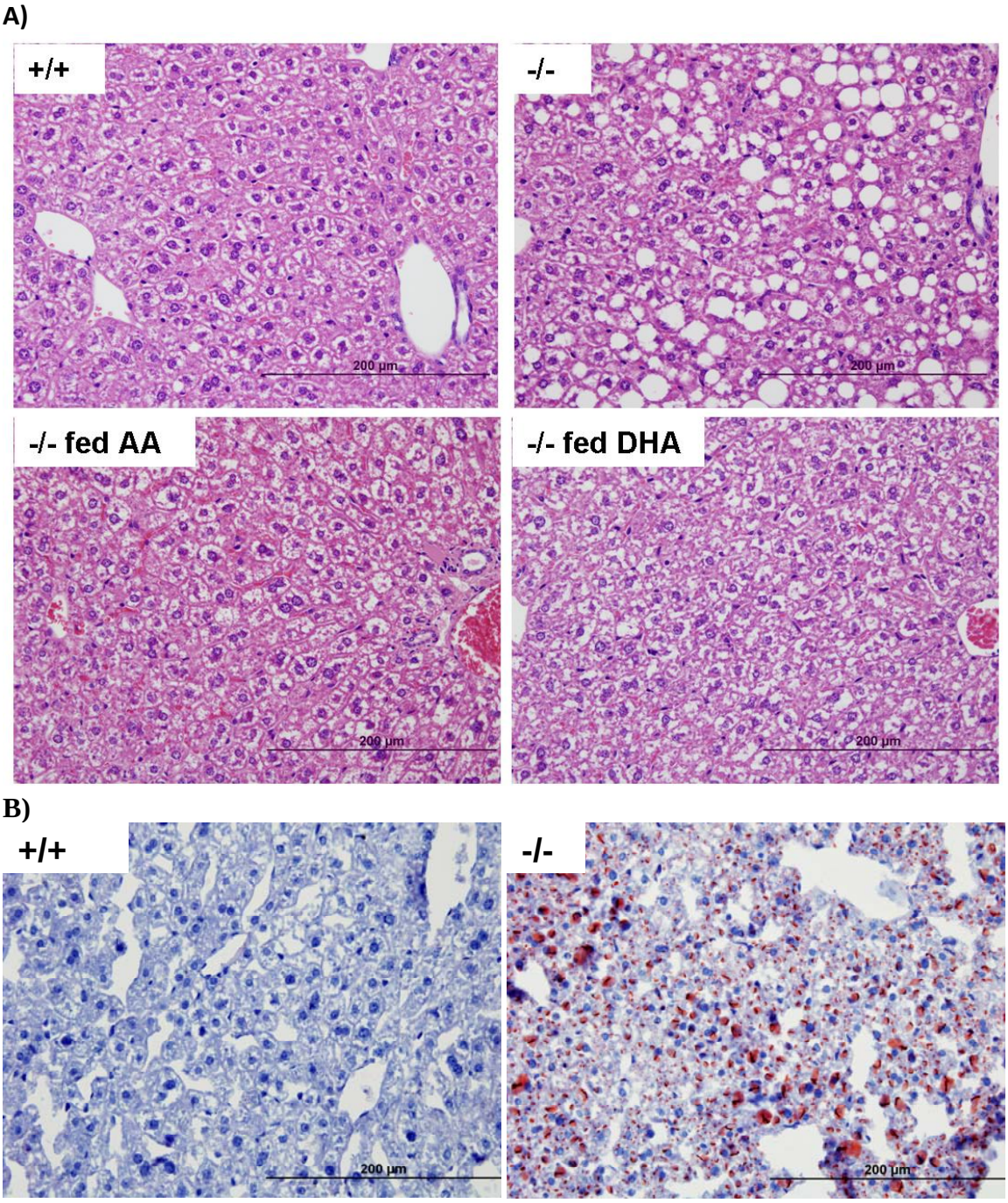

Figure 5.1 A) *Liver histology (hematoxylin and eosin). 16 week old wild type mice (+/+) show normal hepatocyte morphology while -/- hepatocytes have large vacuoles containing due to lipid accumulation (macrovesicular lipidosis). AA and DHA supplementation prevent lipidosis in -/- showing similar hepatocyte morphology to +/+.* ***B)*** *Oil red O stain confirms accumulation of neutral lipids in D6D-/- liver.*

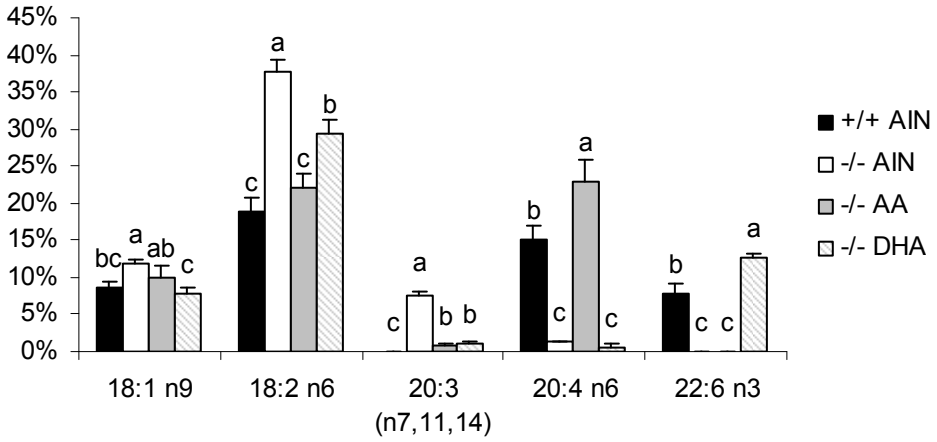

Figure 5.2 *Molar percentage of fatty acids in liver phospholipids of +/+ and -/- mice. AIN, AIN93G diet without HUFA supplementation. AA, 0.2% arachidonic acid supplemented AIN93G diet. DHA, 0.2% docosahexaenoic acid supplemented AIN93G diet. Mean±SD. n=3-5. Groups with different letters are significantly different ($p<0.05$) by one-way ANOVA followed by Fisher's PLSD.*

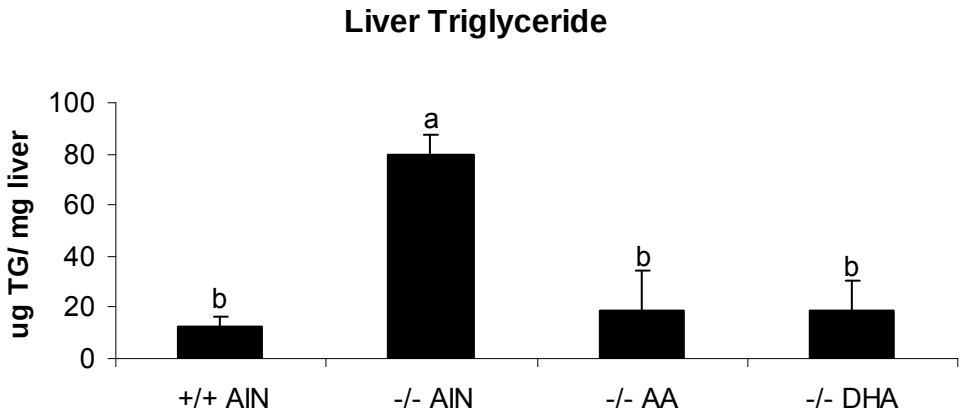

Figure 5.3 *Triglyceride quantification of +/+ and -/- liver. AIN, AIN93G diet without HUFA supplementation. AA, 0.2% arachidonic acid supplemented AIN93G diet. DHA, 0.2% docosahexaenoic acid supplemented AIN93G diet. Mean±SD. n=3-5. Groups with different letters are significantly different ($p<0.05$) by one-way ANOVA with Fisher's PLSD.*

REFERENCES

(1) Postic, C., J. Girard. 2008. The role of the lipogenic pathway in the development of hepatic steatosis. *Diabetes Metab.* 34: 643-648.

(2) Clarke, S. D., D. B. Jump. 1994. Dietary polyunsaturated fatty acid regulation of gene transcription. *Annual Review of Nutrition* 14: 83-98.

(3) Sekiya, M., N. Yahagi, T. Matsuzaka, Y. Najima, M. Nakakuki, R. Nagai, S. Ishibashi, J. Osuga, N. Yamada, and H. Shimano. 2003. Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression. *Hepatology* 38: 1529-1539.

(4) Clarke, S. D., M. K. Armstrong, and D. B. Jump. 1990. Dietary polyunsaturated fats uniquely suppress rat liver fatty acid synthase and S14 mRNA content. *Journal of Nutrition* 120: 225-31.

(5) Cho, H. P., M. Nakamura, and S. D. Clarke. 1999. Cloning, expression, and fatty acid regulation of the human delta-5 desaturase. *J. Biol. Chem.* 274: 37335-37339.

(6) Cho, H. P., M. T. Nakamura, and S. D. Clarke. 1999. Cloning, expression, and nutritional regulation of the mammalian delta-6 desaturase. *J Biol Chem* 274: 471-477.

(7) Kim, H. J., M. Takahashi, and O. Ezaki. 1999. Fish oil feeding decreases mature sterol regulatory element-binding protein 1 (SREBP-1) by down-regulation of SREBP-1c mRNA in mouse liver. A possible mechanism for down-regulation of lipogenic enzyme mRNAs. *J Biol Chem* 274: 25892-25898.

(8) Xu, J., M. T. Nakamura, H. P. Cho, and S. D. Clarke. 1999. Sterol regulatory element binding protein-1 expression is suppressed by dietary polyunsaturated fatty acids: a mechanism for the coordinate suppression of lipogenic genes by polyunsaturated fats. *Journal of Biological Chemistry* 274: 23577-23583.

(9) Worgall, T. S., S. L. Sturley, T. Seo, T. F. Osborne, and R. J. Deckelbaum. 1998. Polyunsaturated fatty acids decrease expression of promoters with sterol regulatory elements by decreasing levels of mature sterol regulatory element-binding protein. *Journal of Biological Chemistry* 273: 25537-25540.

(10) Alfin-Slater, R. B., S. Bernick. 1958. Changes in tissue lipids and tissue histology resulting from essential fatty acid deficiency in rats. *Am. J. Clin. Nutr.* 6: 613-624.

(11) Stroud, C. K., T. Y. Nara, M. Roqueta-Rivera, E. C. Radlowski, P. Lawrence, Y. Zhang, B. H. Cho, M. Segre, R. A. Hess, J. T. Brenna, W. M. Haschek, and M. T. Nakamura. 2009. Disruption of FADS2 gene in mice impairs male reproduction and causes dermal and intestinal ulceration. *J. Lipid Res.* 50: 1870-80.

(12) Reeves, P. G., F. H. Nielsen, and G. C. Fahey. 1993. AIN-93 purified diets for laboratory rodents: Final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* 123: 1939-1951.

(13) Koo, H. Y., M. A. Wallig, B. H. Chung, T. Y. Nara, B. H. Cho, and M. T. Nakamura. 2008. Dietary fructose induces a wide range of genes with distinct shift in carbohydrate and lipid metabolism in fed and fasted rat liver. *Biochim. Biophys. Acta* 1782: 341-348.

(14) Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 226: 497-509.

(15) Monetti, M., M. C. Levin, M. J. Watt, M. P. Sajan, S. Marmor, B. K. Hubbard, R. D. Stevens, J. R. Bain, C. B. Newgard, S. Farese RV, A. L. Hevener, and R. V. Farese Jr. 2007. Dissociation of hepatic steatosis and insulin resistance in mice overexpressing DGAT in the liver. *Cell. Metab.* 6: 69-78.

(16) Choi, C. S., D. B. Savage, A. Kulkarni, X. X. Yu, Z. X. Liu, K. Morino, S. Kim, A. Distefano, V. T. Samuel, S. Neschen, D. Zhang, A. Wang, X. M. Zhang, M. Kahn, G. W. Cline, S. K. Pandey, J. G. Geisler, S. Bhanot, B. P. Monia, and G. I. Shulman. 2007. Suppression of diacylglycerol acyltransferase-2 (DGAT2), but not DGAT1, with antisense oligonucleotides reverses diet-induced hepatic steatosis and insulin resistance. *J. Biol. Chem.* 282: 22678-22688.

(17) Jakobsson, A., R. Westerberg, and A. Jacobsson. 2006. Fatty acid elongases in mammals: their regulation and roles in metabolism. *Prog. Lipid Res.* 45: 237-249.

(18) Horton, J. D., N. A. Shah, J. A. Warrington, N. N. Anderson, S. W. Park, M. S. Brown, and J. L. Goldstein. 2003. Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. *Proc. Natl. Acad. Sci. U. S. A.* 100: 12027-12032.

(19) Igarashi, M., K. Ma, L. Chang, J. M. Bell, and S. I. Rapoport. 2007. Dietary n-3 PUFA deprivation for 15 weeks upregulates elongase and desaturase expression in rat liver but not brain. *J. Lipid Res.* 48: 2463-2470.

(20) Leung, G. K., M. M. Veniant, S. K. Kim, C. H. Zlot, M. Raabe, J. Bjorkegren, R. A. Neese, M. K. Hellerstein, and S. G. Young. 2000. A deficiency of microsomal triglyceride transfer protein reduces apolipoprotein B secretion. *J. Biol. Chem.* 275: 7515-7520.

(21) Swift, L. L., M. Y. Zhu, B. Kakkad, A. Jovanovska, M. D. Neely, K. Valyi-Nagy, R. L. Roberts, D. E. Ong, and W. G. Jerome. 2003. Subcellular localization of microsomal triglyceride transfer protein. *J. Lipid Res.* 44: 1841-1849.

(22) Bostrom, P., L. Andersson, M. Rutberg, J. Perman, U. Lidberg, B. R. Johansson, J. Fernandez-Rodriguez, J. Ericson, T. Nilsson, J. Boren, and S. O. Olofsson. 2007. SNARE proteins mediate fusion between cytosolic lipid droplets and are implicated in insulin sensitivity. *Nat. Cell Biol.* 9: 1286-1293.

(23) Darios, F., B. Davletov. 2006. Omega-3 and omega-6 fatty acids stimulate cell membrane expansion by acting on syntaxin 3. *Nature* 440: 813-817.

-

CHAPTER 6

AA SUPPLEMENTATION, BUT NOT DHA, PREVENTS ULCERATIVE DERMATITIS AND GASTROINTESTINAL ULCERS IN DELTA-6 DESATURASE KNOCKOUT MOUSE[1]

ABSTRACT

In order to study arachidonic acid (AA) functions without depleting precursor linoleic acid (LA), a delta-6 desaturase knockout (D6D-/-) mouse was produced. Previous studies have shown that the main D6D-/- pathology is severe ulcerative dermatitis manifested in 100% of -/- mice around 5 months of age despite abundant LA in tissues, a fatty acid essential for skin barrier function. In addition to skin pathology, gastrointestinal (GI) ulcers are also observed in -/- possibly due to AA deficiency. The main purpose of this study was to evaluate the prevention of skin and gastrointestinal HUFA deficiency symptoms by AA supplementation. Wild type (+/+) and -/- received either a non-supplemented AIN93G diet (AIN), an AA supplemented AIN diet at 0.2% or 0.4% (n=3 per group), or a 0.2% (w/w) DHA supplemented AIN diet. AA supplemented -/- groups at both doses did not develop dermatitis or GI ulcers, even at 8 months of age, while 50% of the DHA -/- group developed skin lesions and 100% developed GI ulcers at 4 months of age. Total skin lipid extracts showed significantly lower AA in non-supplemented -/- fed AIN, whereas skin AA was restored in AA supplemented -/- to 129.5% of +/+ levels. Skin prostaglandin (PG) D2, a modulator of skin reaction to irritants, was decreased to 11.6% of +/+ ($p<0.05$) in the non-supplemented -/-. AA supplementation partially restored -/- skin PGD2 to 40.5% of +/+. In conclusion, dietary AA but not DHA was able to prevent dermatitis and

[1] Part of this study has been published in: Stroud et al, 2009, J. Lipid Res. 50: 1870-80 (7).

gastrointestinal ulcers which demonstrates AA essentiality in skin and GI function possibly through production of prostaglandins.

INTRODUCTION

The role of PUFA in skin physiology has been attributed mainly to omega-6 linoleic acid (LA), a dietary essential fatty acid which cannot be synthesized *de novo* by mammals. Common skin essential fatty acid deficiency symptoms are excessive skin water loss and dermatitis (1). Skin pathology due to an essential fatty acid deficient diet is a result of an inefficient epidermal water barrier (2). LA supplementation prevents this pathology as an essential component of skin barrier ceramides. Ceramides are required for lipid lamellar layer formation of the stratum corneum, the upper most layer of the skin, essential for moisture retention.

Despite the presence of LA in the diet, the D6D-/- mouse still develops a skin disorder characterized by ulcerative dermatitis with excessive scratching behavior. This would indicate that AA may have an essential role for skin function, possibly as a precursor to prostaglandins. Prostaglandins (PG) are signaling molecules that can modulate a localized tissue response at low concentrations. Prostaglandin D2 (PGD2), for example, is required for skin repair and has been shown to have an antipruritic effect on mice with acrodermatitis, reducing scratching behavior to allow skin recovery from a lesion (3). Therefore, PGD2 is a molecule of interest in studying the etiology of skin D6D-/- pathology.

Prostaglandin (PG) are also essential in maintaining gastrointestinal integrity as demonstrated through use of NSAID, which block PG synthesis resulting in gastric and intestinal ulceration (4). AA deficiency in D6D-/- would decrease levels of PG required for GI function explaining the appearance of gastrointestinal lesions as early as 6 weeks of age (**Chapter 4**).

The essentiality of DHA in GI function is less clear. The objective of this study is to determine the essentiality of AA and DHA in preventing dermatitis and gastrointestinal ulcers through dietary supplementation and creation of specific deficiencies of each HUFA.

METHODS

Animals

All animal work was approved by the University of Illinois Institutional Animal Care and Use Committee. C57BL/6J-129S6/SvEvTac mixed strain male and female mice of +/+ and -/- genotype were used.

AA supplementation at 0.4% (w/w) of diet

21-day old +/+ and -/- females (n=3) were fed *ad libitum* AIN93G diet with and without supplementation of 0.4% (w/w) AA (ARASCO, Martek Biosciences, Columbia, MD). Water was given *ad libitum.* Body weight was measured every 2 days. Animals without HUFA supplementation were euthanized at 4 months of age while the AA supplemented mice were euthanized at 8 months of age by CO_2 inhalation. Skin samples from both time points were obtained for PGD2 measurement and for histological evaluation. Skin samples were also collected for fatty acid analysis. The entire gastrointestinal tract was cassetted for histological examination as a swiss roll preparation in order to allow evaluation of entire tract.

AA or DHA supplementation at 0.2% (w/w) of diet

21-day old +/+ and -/- males (n=3) were fed *ad libitum* AIN93G diet with and without supplementation of 0.2% (w/w) DHA (DHASCO, Martek Biosciences, Columbia, MD) or 0.2% AA (ARASCO, Martek Biosciences, Columbia, MD). Water was given *ad libitum.* Animals

were euthanized at 4 months of age by CO_2 inhalation. Skin samples and gastrointestinal tract were obtained for histological evaluation.

Fatty acid analysis

Total lipids were extracted from spleen using the Folch method (5). C17:0 phosphatidylcholine was added as an internal standard. Fatty acids extracts were methylated with methanolic HCl (Supelco, Bellefonte, PA) at 75ºC for 90 minutes. Fatty acid methyl esters were identified by HP5890 gas chromatography (Agilent Technologies, Wilmington, DE) using a 30 m x 0.25 mm Omegawax capillary column (Supelco) at 180ºC for 2 minutes followed by a 3ºC gradient until a final temperature of 230ºC for 35 minutes.

Skin PGD2 assay

Prostaglandin extraction from skin was based on the method by Sugimoto et al (6). A skin sample was removed from the back of the mouse (intrascapular region), weighed, and minced on ice. Tissue was then homogenized in ice-cold phosphate buffered saline, containing 10μM indomethacin. An equal volume of acetone was added to the sample, mixed, and incubated on ice for 5 minutes. Precipitate was removed by centrifugation at 2000 g for 10 minutes at 4°C. Supernatants containing prostaglandin were blown down with nitrogen and resuspended in enzyme immunoassay buffer. Methoximation of samples was performed and PGD2 was measured using the PGD2-Mox EIA kit (Cayman Chemical, Ann Arbor, MI).

Statistical Analyses

Significant differences between +/+ and -/- groups were determined by Student's t-test.

RESULTS

Dietary AA, but not DHA, prevents ulcerative dermatitis in D6D-/-

A 0.4% AA supplementation study was done to evaluate prevention of dermatitis in D6D-/-. In this study, non-supplemented D6D-/- body weight plateaus by 60 days (**Figure 6.1**) on diet. In contrast to non-supplemented -/-, AA supplemented -/- body weight does not plateau nor does ulcerative dermatitis develop 60 days beyond the usual time point of skin lesion appearance in non-supplemented -/-. A second HUFA supplementation study confirmed dietary AA prevention of dermatitis in 100% -/- at a lower dose of 0.2%. In order to determine if the omega-3 DHA can also prevent dermatitis in -/-, a 0.2% DHA supplemented -/- group was also included. However, dietary DHA did not prevent dermatitis successfully as skin lesions appeared in 50% of the mice by 4 months of age similar to non-supplemented -/- mice.

Skin PGD2 levels in -/- partially restored with dietary AA supplementation

Skin PGD2 in non-supplemented -/- mice was significantly lower ($p<0.05$) with only 11.6% of wild type (**Figure 6.2**). AA supplementation partially restored levels of -/- skin PGD2 to 53.8% of AA supplemented +/+ and 40.5% of non-supplemented +/+. This increase was significantly different versus non-supplemented -/- ($p<0.05$).

Skin AA levels restored in -/- with dietary AA supplementation

Skin AA (20:4 n6) was significantly lower in non-supplemented -/- mice ($p<0.05$), while supplementation increased AA levels to 129% of +/+ (**Figure 6.3**). The skin fatty acid profile (**Figure 6.4**) of non-supplemented -/- mice unexpectedly showed lower levels of linoleic acid

(18:2 n6), a major component of skin ceramides. Supplementation was able to restore the -/- skin fatty acid profile of linoleic acid and arachidonic acid to +/+ levels.

Dietary AA but not DHA prevents gastrointestinal ulcers in -/-

Histological evaluation of gastrointestinal tract (**Figure 6.5**) indicated presence of ulcers with loss of mucosa and infiltration of inflammatory cells primarily in ileocolic junction and sometimes in duodenum of non-supplemented -/- mice. When supplemented with AA, GI pathology was not observed. DHA supplementation however was not capable of preventing GI pathology showing ulceration similar to non-supplemented -/-.

DISCUSSION

Dietary supplementation of AA, but not of DHA, is capable of preventing ulcerative dermatitis in 100% of -/- mice. Both dose levels of 0.2% and 0.4% AA supplemented AIN93G diets (w/w) were successful in maintaining skin physiology in D6D-/-. Based on previous studies (7), it is approximately at 120 days of age when D6D-/- mice fed a HUFA deficient diet develop initial signs of skin pathology. AA supplemented -/- mice showed no signs of skin lesions throughout the 200 days on the diet. HUFA analysis indicates skin AA levels were significantly higher in supplemented -/- when compared to +/+ suggesting availability of AA for PG production required for proper skin physiology. PGD2 is one of the main PG synthesized from AA in human and mouse skin (8). PGD2 exerts its function through binding to a prostanoid receptor. PGD2 bindng to DP1 receptor decreases scratching behavior in the Nc/Nga mouse model of acrodermatitis (9). Similar to the D6D-/-, Nc/Nga mice present excessive scratching behavior that leads to skin ulceration. Therefore, PGD2 is of particular interest in studying D6D-/- skin pathology due to its antipuritic effect and potential involvement in skin

repair. Skin PGD2 levels were increased in AA supplemented D6D-/- and no characteristic scratching behavior was observed. Despite skin PGD2 being only partially recovered in comparison to +/+, the amount restored was enough to prevent skin pathology. Both AA and PGD2 are effective in treating skin pathology in Nc/Nga mice. Topically applied AA and PGD2 to Nc/Nga mice reduced scratching behavior allowing skin recovery from a lesion (10). Silencing of the cyclooxygenase-1 gene in skin by RNA interference resulted in increased scratching behavior of the Nc/Nga mice, further demonstrating the importance of AA-derived PG in skin physiology (11). PG production was also most likely restored in GI since AA supplemented -/- did not develop ulcers. PG are important for maintaining GI epithelial integrity. PGE2 for example stimulates mucosal bicarbonate secretion to protect the GI against acidic conditions (12).

In -/- tissues, such as liver and testis, fatty acid substrates to D6D accumulate significantly above +/+ levels. LA increases up two times that of +/+ in liver (7). The skin fatty acid profile of non-supplemented -/- mice unexpectedly showed significantly lower levels of LA (18:2 n6), a major component of skin ceramides. Low levels of LA in skin may indicate altered ceramide composition within the skin barrier. AA supplementation successfully restores LA back to +/+ levels in the D6D-/-. A possible mechanism of AA in contributing to proper skin structure formation may be as a ligand to transcription factor PPARγ. Keratinocytes differentiate to corneocytes which are the main cell type in the stratum corneum. PPARγ activation favors keratinocyte differentiation and lamellar body formation which are important processes for stratum corneum or skin barrier formation (13).

Lamellar bodies are formed within keratinocytes and are then secreted providing lipids and enzymes required for lamellar layer formation characteristic of the skin barrier (14).

Lamellar bodies are vesicles derived from Golgi and their proper secretion and formation relies on a SNARE complex (15). HUFA have been shown to be required for SNARE complex formation (16) suggesting another possible role for AA in skin function through proper lamellar granule formation which is essential in delivering components for skin barrier function.

DHA supplementation was not able to prevent skin pathology or GI ulcers in D6D-/- further demonstrating the essentiality of AA in these tissues. AA-derived PG are essential in maintaining gastrointestinal integrity as demonstrated through use of NSAIDS, which block PG synthesis resulting in gastrointestinal ulceration (4). AA deficiency in D6D-/- would decrease levels of PG required for GI function possibly explaining the appearance of gastrointestinal lesions as early as 6 weeks of age (**Chapter 4**). DHA supplementation may accelerate depletion of AA in skin and GI, similar to AA depletion of liver where HUFA analysis indicates higher depletion of hepatic AA in DHA supplemented -/- when compared to non-supplemented -/- mice (**Chapter 5**). Accelerated depletion of AA may have an impact on the appearance phenotype specific to AA deficiency.

FIGURES

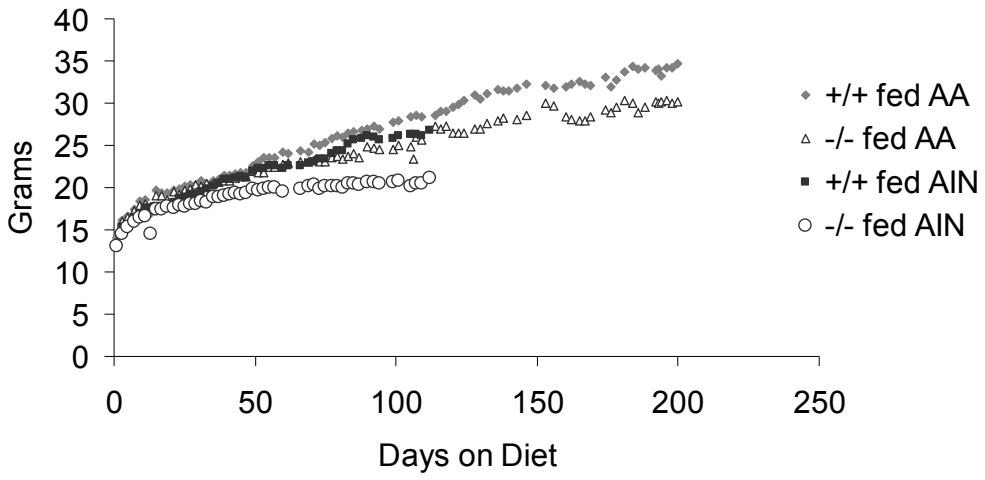

Figure 6.1 *Average body weigh of mice fed AIN93G diet with and without 0.4% AA supplementation for 200 and 112 days respectively, n=3. Previous studies show skin lesions in -/- by 120 days on a diet deficient of HUFA. No dermatitis was present in AA-supplemented -/- at 200 days.*

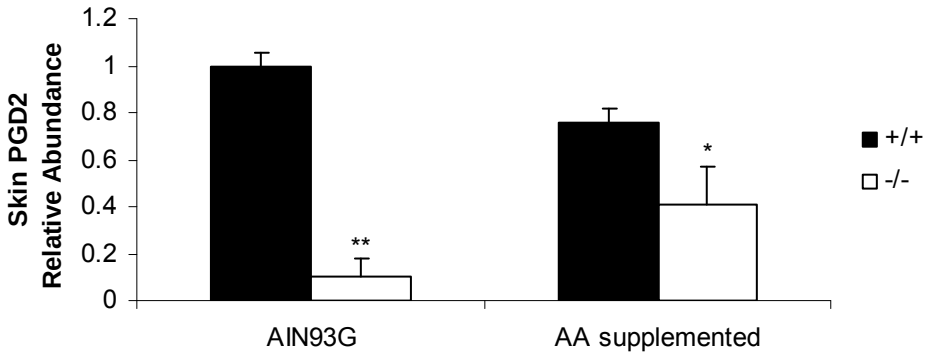

Figure 6.2 *Skin prostaglandin (PG) D2 levels relative to wild type (+/+) mouse. Non-supplemented (AIN93G) -/- have a 90% decrease in PGD2. AA supplemented -/- skin has partially restored PGD2 levels. Mean±SD. n=3. * $p < 0.05$, ** $p < 0.001$ by Student's t-test.*

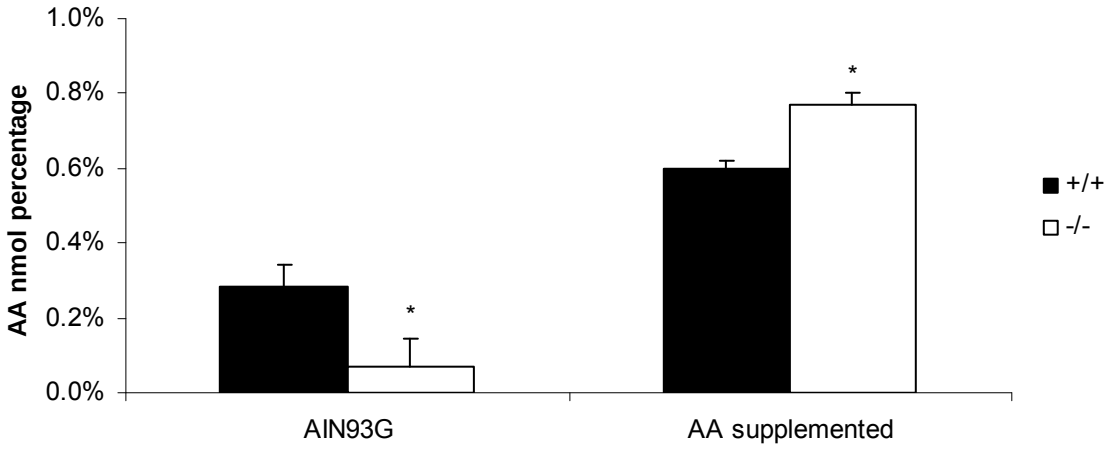

Figure 6.3 *Skin arachidonic acid (AA) molar percentages of wild type and D6D -/- mice; Mean±SD. n=3. *p<0.05, Student's t-test, +/+ compared to -/-.*

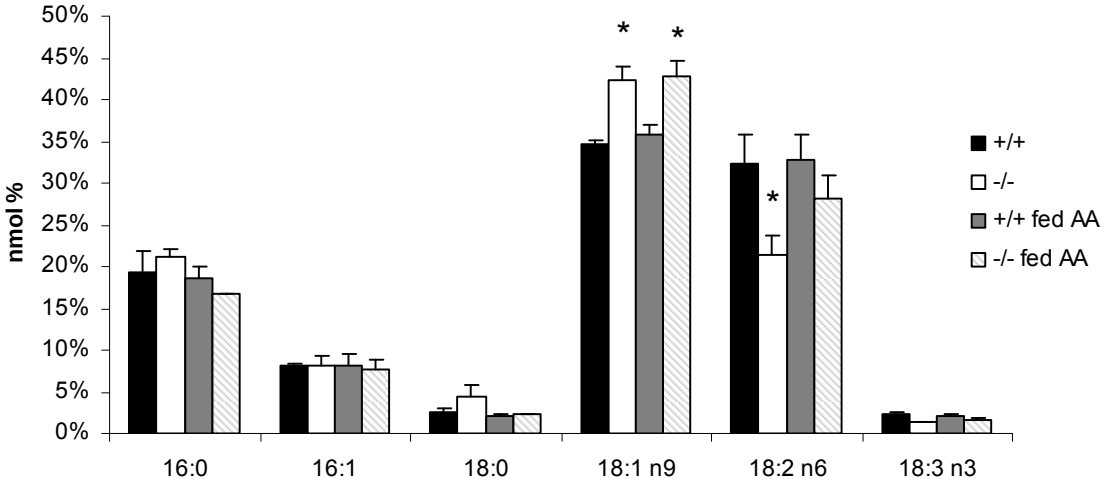

Figure 6.4 *Skin fatty acid molar percentages of wild type and D6D -/- mice on 0.4% AA supplemented and non-supplemented diets. Mean±SD. n=3. * $p<0.05$, Student's t-test. +/+ compared to -/-.*

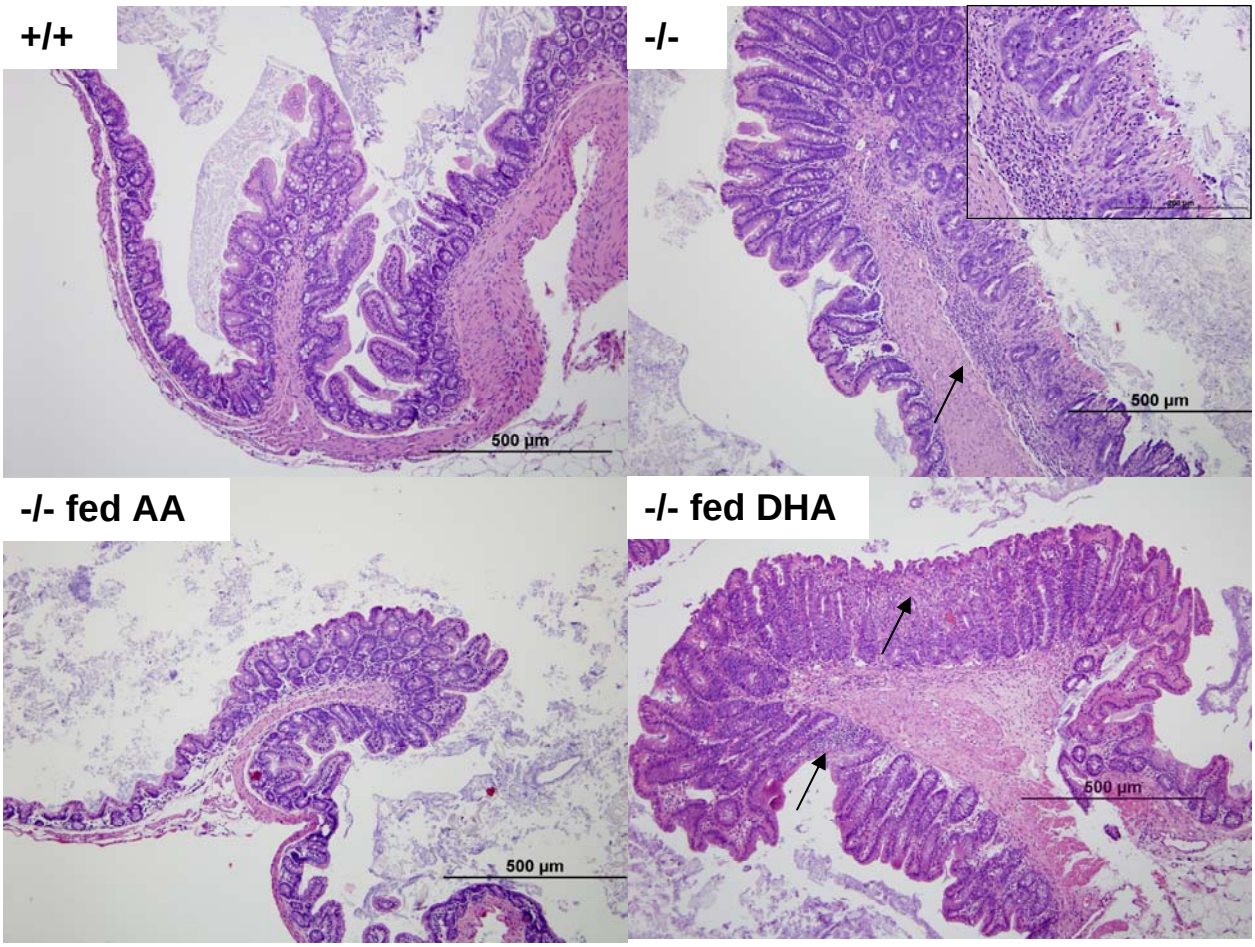

Figure 6.5 *Ileocolic junction (hematoxylin and eosin). Wild type mice (+/+) present normal celluar morphology of mucosa and serosa. D6D-/- mice present loss of mucosa with inflammatory cells in serosa (arrow, inset). AA supplemented -/- do not present ulceration, unlike DHA supplemented -/- which has similar pathology to non-supplemented D6D-/- (arrow).*

REFERENCES

(1) Burr, G. O., M. M. Burr. 1930. On the nature and role of the fatty acids essential in nutrition. *J Biol Chem* 86: 587-621.

(2) Wertz, P. W., D. C. Swartzendruber, W. Abraham, K. C. Madison, and D. T. Downing. 1987. Essential fatty acids and epidermal integrity. *Arch. Dermatol.* 123: 1381-1384.

(3) Takaoka, A., I. Arai, M. Sugimoto, N. Futaki, T. Sakurai, Y. Honma, and S. Nakaike. 2007. Role of scratch-induced cutaneous prostaglandin D production on atopic-like scratching behaviour in mice. *Exp. Dermatol.* 16: 331-339.

(4) Vane, J. R. 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat. New Biol.* 231: 232-235.

(5) Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 226: 497-509.

(6) Sugimoto, M., I. Arai, N. Futaki, Y. Honma, T. Sakurai, Y. Hashimoto, and S. Nakaike. 2007. Putative mechanism of the itch–scratch circle: Repeated scratching decreases the cutaneous level of prostaglandin D2, a mediator that inhibits itching. *Prostag Leukotr Ess* 76: 93-101.

(7) Stroud, C. K., T. Y. Nara, M. Roqueta-Rivera, E. C. Radlowski, P. Lawrence, Y. Zhang, B. H. Cho, M. Segre, R. A. Hess, J. T. Brenna, W. M. Haschek, and M. T. Nakamura. 2009. Disruption of FADS2 gene in mice impairs male reproduction and causes dermal and intestinal ulceration. *J. Lipid Res.* 50: 1870-80.

(8) Hammarstrom, S., J. A. Lindgren, C. Marcelo, E. A. Duell, T. F. Anderson, and J. J. Voorhees. 1979. Arachidonic acid transformations in normal and psoriatic skin. *J. Invest. Dermatol.* 73: 180-183.

(9) Arai, I., N. Takano, Y. Hashimoto, N. Futaki, M. Sugimoto, N. Takahashi, T. Inoue, and S. Nakaike. 2004. Prostanoid DP1 receptor agonist inhibits the pruritic activity in NC/Nga mice with atopic dermatitis. *Eur. J. Pharmacol.* 505: 229-235.

(10) Honma, Y., I. Arai, Y. Hashimoto, N. Futaki, M. Sugimoto, M. Tanaka, and S. Nakaike. 2005. Prostaglandin D2 and prostaglandin E2 accelerate the recovery of cutaneous barrier disruption induced by mechanical scratching in mice. *European Journal of Pharmacology* 518: 56-62.

(11) Inoue, E., J. Yamauchi. 2006. AMP-activated protein kinase regulates PEPCK gene expression by direct phosphorylation of a novel zinc finger transcription factor. *Biochem Biophys Res Commun* 351: 793-9.

(12) Aihara, E., Y. Nomura, Y. Sasaki, F. Ise, K. Kita, and K. Takeuchi. 2007. Involvement of prostaglandin E receptor EP3 subtype in duodenal bicarbonate secretion in rats. *Life Sci.* 80: 2446-2453.

(13) Mao-Qiang, M., A. J. Fowler, M. Schmuth, P. Lau, S. Chang, B. E. Brown, A. H. Moser, L. Michalik, B. Desvergne, W. Wahli, M. Li, D. Metzger, P. H. Chambon, P. M. Elias, and K. R. Feingold. 2004. Peroxisome-proliferator-activated receptor (PPAR)-gamma activation stimulates keratinocyte differentiation. *J. Invest. Dermatol.* 123: 305-312.

(14) Fartasch, M. 2004. The epidermal lamellar body: a fascinating secretory organelle. *J. Invest. Dermatol.* 122: XI-XII.

(15) Sprecher, E., A. Ishida-Yamamoto, M. Mizrahi-Koren, D. Rapaport, D. Goldsher, M. Indelman, O. Topaz, I. Chefetz, H. Keren, T. J. O'brien, D. Bercovich, S. Shalev, D. Geiger, R. Bergman, M. Horowitz, and H. Mandel. 2005. A mutation in SNAP29, coding for a SNARE protein involved in intracellular trafficking, causes a novel neurocutaneous syndrome characterized by cerebral dysgenesis, neuropathy, ichthyosis, and palmoplantar keratoderma. *Am. J. Hum. Genet.* 77: 242-251.

(16) Davletov, B., E. Connell, and F. Darios. 2007. Regulation of SNARE fusion machinery by fatty acids. *Cell Mol. Life Sci.* 64: 1597-1608.

-

CHAPTER 7

DHA SUPPLEMENTATION, BUT NOT AA, FULLY RESTORES MALE FERTILITY AND SPERMIOGENESIS IN D6D -/-[1]

ABSTRACT

Delta-6 desaturase knockout mice (-/-) are unable to synthesize highly unsaturated fatty acids (HUFA): arachidonic acid (AA), docosahexaenoic acid (DHA), and docosapentaenoic acid (DPAn6). The -/- males exhibit infertility and arrest of spermatogenesis at late spermiogenesis. To determine which HUFA is essential for spermiogenesis, a diet supplemented with either 0.2% (w/w) AA or DHA was fed to wild type (+/+) and -/- males at weaning until 16 weeks of age (n=3-5). A breeding success rate of DHA-supplemented -/- was comparable to +/+. DHA-fed -/- showed normal sperm counts and spermiogenesis. Dietary AA was less effective in restoring fertility, sperm count and spermiogenesis than DHA. Testis fatty acid analysis showed restored DHA in DHA-fed -/-, but DPAn6 remained depleted. In AA-fed -/-, AA was restored at the +/+ level, and 22:4n6, an AA elongated product, accumulated in testis. Cholesta-3,5-diene was present in testis of +/+ and DHA-fed -/-, whereas it diminished in -/- and AA-fed -/-, suggesting impaired sterol metabolism in these groups. Expression of spermiogenesis marker genes was largely normal in all groups. In conclusion, DHA was capable of restoring all observed impairment in male reproduction, whereas 22:4n6 formed from dietary AA may act as an inferior substitute for DHA.

[1]This study has been published: Roqueta-Rivera et al., 2010, J. Lipid Res. 51: 360-7

INTRODUCTION

Delta-6-desaturase (D6D) is the first and rate limiting enzyme for highly unsaturated fatty acid (HUFA) synthesis that consists of a series of elongation and desaturation reactions (1). The dietary essential fatty acids 18:2n-6 (linoleic acid) and 18:3n-3 (α-linolenic acid) are substrates for D6D and precursors of physiologically important HUFA such as 20:4n-6 (arachidonic acid, AA), 22:5n6 (docosapentaenoic acid, DPAn6), and 22:6n3 (docosahexaenoic acid, DHA). D6D is also required for the final desaturation step for the synthesis of DPAn6 and DHA.

These HUFA are present in high concentration in testes and sperm of mammals. DPAn6, a HUFA derived from AA, dramatically increases in rat testes during the sexual maturation stage (2). In mice, AA, DPAn6, and DHA are abundant in membrane phospholipids of round spermatids (3) and mature mouse spermatozoa (4), suggesting an important role for these fats for proper spermatogenesis. In humans, DHA is the main HUFA in sperm (5). DHA is specifically high in the sperm tail when compared to the sperm head in monkeys (6), implying a role of DHA in sperm tail function. AA may also have a role in male fertility as a precursor to eicosanoids. Prostaglandin E2, for example, has been shown to increase sperm motility (7), while inhibition of cycloxygenase-2 in mouse vas deferens results in a decrease of sperm motility and fertility (8).

In addition to the presence of HUFA in mammalian testis and spermatozoa, there are also very long chain polyunsaturated fatty acids (VLCPUFA) that contain C26-C38 hydrocarbon chains (9-11). These VLCPUFA are elongation products of the C20 and C22 chain HUFA (12). These VLCPUFA are incorporated mainly into sphingomyelin and ceramides in the sperm head (9,10). These sphingolipids are suggested to be involved with capacitation of sperm (13,14).

In the study reporting discovery of essential fatty acids, testicular degeneration and a low breeding success rate were among the deficiency symptoms in rats fed a fat free diet (15). A later study reported that rats receiving a diet deficient in all essential fatty acids had a lower epididymal sperm concentration (16). However, these previous studies were unable to demonstrate the essentiality of HUFA for male fertility because these animals also had severe growth retardation and dermatitis. In order to deplete tissue HUFA in these studies, D6D enzyme substrates, linoleic acid and α-linolenic acid, were eliminated as well as all products from the diet. However, linoleic acid is required for skin water barrier function (17). Thus, deficiency of linoleic acid resulted in severe growth retardation and dermatitis, confounding the study into the physiological roles of HUFA, including male fertility (15,18).

To overcome this confounding problem, we and others created mice with the D6D gene disabled (19,20). The D6D knockout (-/-) mouse is unable to synthesize HUFA, thus allowing us to specifically create AA deficiency without depleting tissue linoleic acid, or to create DPAn6 and DHA deficiency without depleting tissue AA. The D6D-null mouse developed intestinal ulcers and severe dermatitis by 5 months of age despite an adequate supply of linoleic acid and α-linolenic acid from diet (19). Moreover, the male -/- mouse became infertile before manifestation of dermatitis. Histology of the D6D -/- mouse revealed disrupted spermiogenesis, the last stage of spermatogenesis in which spermatids develop to spermatozoa (19,20). Although the essentiality of HUFA in spermiogenesis and male fertility has been demonstrated by these studies, the specific role of each HUFA for spermatogenesis has not been elucidated. Thus, the objective of this study was to determine if dietary AA and DHA can restore spermatogenesis in the D6D-null mouse, and to elucidate the role of these HUFA in spermiogenesis.

METHODS

Animal study

All animal work was approved by the University of Illinois Institutional Animal Care and Use Committee. Mice used in the experiment were produced by breeding heterozygous males and females of mixed strain (129S6/SvEvTac/C57BL/6J) fed a standard rodent chow. At weaning, a total of 30 male mice, 9 wild type (+/+), 9 heterozygote (+/-), and 12 D6D null (-/-) were distributed to receive one of the following diets: control diet (AIN93G), AIN93G supplemented with 0.2 w/w % AA, or AIN93G with 0.2 w/w % DHA. The AIN93G diet is a purified, nutritionally adequate diet that contains sufficient linoleic acid and α-linolenic acid, but no D6D products (21). DHASCO and ARASCO oils (Martek Biosciences, Columbia, MD) were used for supplementation of DHA and AA, respectively. All dietary groups consisted of 3 mice with exception of the AA supplemented -/- (n=4) and DHA supplemented -/- (n=5). Mice were single housed at weaning and received the diet until four months of age.

Male fertility

Fertility was evaluated by breeding single housed males with either a +/+ or +/- female for 4 days at four different time points: 6, 9, 12 and 15 weeks of age; different females were used at each time point; at least 12 mating attempts were done per dietary group for each genotype. Copulatory behavior was confirmed in all mice. The percentage of successful matings as indicated by pregnant females and viable litters was noted.

Tissue collection and histology

Animals were euthanized by carbon dioxide inhalation at 4 months of age. Left testis and left epididymis were removed and weighed; left testis was then frozen for HUFA and RNA analysis while left epididymis was used for sperm collection from cauda. Right testis and epididymis were fixed in Davidson's fixative and transferred to 10% neutral buffered formalin after 24 hours. Tissues were trimmed for paraffin embedding. Sections were cut at 3 microns and stained with hematoxylin and eosin for histological evaluation.

Sperm count and motility

The cauda epididymis was cut with a surgical blade, minced with small scissors and placed in 2 ml of dmKBRT buffer at 37°C for 15 minutes. The dmKBRT buffer contained 120 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 10 mM $NaHCO_3$, 0.36 mM $NaH_2PO_4H_2O$, 1.2 mM $MgSO_4$, 5.6 mM glucose, 1.1 mM Na pyruvate, 25 mM TAPSO, 18.5 mM sucrose, and 6 mg/ml BSA. The sperm cell suspensions were then observed using an inverted microscope to record sperm motility. Epididymal sperm counts were done by hemocytometer from epididymal sperm in 2 ml of dmKBRT buffer.

Gene expression

RNA was analyzed with a slight modification of a method previously described (22). Testis was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA), and total RNA extracted. MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA), along with random hexamers, were used to synthesize cDNA. Real-time quantitative PCR, using SYBR Green fluorescent dye (Applied Biosystems) was used to analyze RNA relative to a ribosomal RNA L7a. Oligonucleotides used for real-time quantitative PCR were: mTISP69-F,

5’CGGACGCTCAGGTTAACTTGA 3’, mTISP69-R, 5’CCACAGGAACCCCAAGCA 3’, mTISP50-F, 5’ACCTTTGGCATCAAGCATTC 3’, mTISP50-R, 5’GCACATTTCTGTGGGAGGAT 3’, mAkap3-F, 5’CGCAAAGACCTGGAGAAAAG 3’, mAkap3-R, 5’-ACCTTTTCGTTGGTCCACTG-3’, mCttn-F, 5’AGGTGCCATCTGCCTATCA 3’, mCttn-R, 5’TCTCGGCTTCTGCCTTCC 3’, mTnp1-F, 5’ATGTCGACCAGCCGCAAGC 3’, mTnp-R, 5’CCACTCTGATAGGATCTTTGG 3’, mSfap-F, 5’AGAAGGGAAGCTCAGATCCA 3’, mSfap-R, 5’AGAAGGGAAGCTCAGATCCA 3’

Fatty acid extraction and GC-MS analysis

Total lipids were extracted from frozen testis according to the method of Folch et al (23). VLCPUFA methyl esters were prepared with a slight modification of a method previously described (12). A mixture of pentadecanoic acid (15:0), heptadecanoic acid (17:0), heneicosanoic acid (21:0), pentacosanoic acid (25:0), and heptacosanoic acid (27:0) was added as an internal standard to the lipid extracts from the testis. The extracts were derivatized to fatty acid methyl esters (FAME) with HCl in methanol at 85°C overnight. After extracting with hexane, FAME were separated on thin layer chromatography plates with hexane:ether (80:20) to remove cholesterol. Absolute ethanol was added to the scraped bands, which was then sonicated for 10 min. FAME were extracted with hexanes after adding water. Gas chromatography-mass spectrometry (GC-MS) analysis was performed as previously described (12).

Statistical analysis

Statistical analysis with Statview version 5.01 for Windows was conducted using one-way ANOVA with Fisher’s PLSD post test (**Table 7.1 and Fig. 7.1B**) and the Wilcoxon sum

rank test (**Figure 7.1A**). Data were presented as mean ± SD; $p < 0.05$ was considered as statistically significant.

RESULTS

Fertility, sperm counts and motility were restored by dietary DHA

One of three non-supplemented -/- males was able to impregnate a female at the first time point of 6 weeks of age. Beyond six weeks of age, all non-supplemented -/- males (AIN) failed to impregnate females of +/+ or +/- genotype. The rate of successful matings (**Figure 7.1A**) for non-supplemented -/- (8%) was significantly lower ($p<0.05$) when compared to non-supplemented +/+ (67%) and +/- (50%). Both dietary AA and DHA supplemented -/- had significantly higher success rates, 38% and 61% , respectively ($p<0.05$) than the non-supplemented -/- (**Figure 7.1A**).

Wild type (+/+) and heterozygote (+/-) mice from all dietary groups presented normal total sperm numbers stored in epididymis (**Figure 7.1B**). On the other hand, non-supplemented -/- animals showed a drastic decrease in sperm count (18% of +/+). Spermatozoa in non-supplemented -/- had abnormal morphology with a condensed rounded head (globozoospermia, **Figure 7.3B inset**). DHA supplementation to -/- males fully restored sperm count ($11.62 \pm 3.0 \times 10^6$) to wild type levels, while AA supplementation only partially restored sperm counts ($2.62 \pm 1.2 \times 10^6$) (**Figure 7.1B**). Epididymal sperm was motile in all AA and DHA supplemented -/- animals, while sperm in -/- animals fed the control diet completely lacked motility.

DHA was required for sperm head elongation and flagellum formation

Testis and epididymis weights did not differ among groups in either absolute weight or in % weight relative to body weight. Testis histology showed all stages of spermatogenesis in +/+ and +/- genotypes regardless of the dietary treatments (**Figure 7.2A**). Consistent with our previous study (19), all animals of non-supplemented -/- had disrupted spermatogenesis specifically at Step 9 of spermiogenesis where round spermatids are elongated. Spermatogonia, spermatocytes, and round spermatids were present, while elongated spermatids and spermatozoa were absent (**Figure 7.2B**). AA supplementation partially restored spermatogenesis (**Figure 7.2C**), while DHA supplemented -/- show all stages of spermatogenesis from spermatogonia to spermatozoa (**Figure 7.2D**).

All +/+ mice had spermatozoa in the lumen of the epididymis (**Figure 7.3A**). Non-supplemented -/- epididymis contained mostly sloughed round spermatids and spermatocytes, cells from an earlier stage of spermatogenesis than spermatozoa (**Figure 7.3B**). A closer examination revealed that the few spermatozoa present in the epididymis of -/- exhibited globozoospermia (**Figure 7.3B, inset**). Partial restoration of spermatogenesis by AA supplementation is indicated by a mix of mature spermatozoa, spermatocytes and round spermatids present in the epididymal lumen (**Figure 7.3C**). DHA supplemented -/- group presented only spermatozoa in epididymal lumen (**Figure 7.3D**), the same as in the +/+ (**Figure 7.3A**).

Expression of genes analyzed were largely unchanged in -/-

Spermatogenesis did not proceed successfully beyond the round spermatid phase (Step 9 of spermiogenesis) in -/- males, therefore, we measured gene expression of late spermiogenesis

markers in testis (**Table 7.1**). There was a 45% decrease in sperizin (Znrf4, TISP69) RNA in -/- males of all dietary groups. Two other genes that encode sperm flagellar proteins, Shippo1 (Odf3, TISP50) and A-kinase anchoring protein (Akap3), had a mild (20%) but statistically significant decrease in RNA expression in -/- fed the non-supplemented diet. Other spermiogenesis markers analyzed were transition protein 1 (Tnp1), cortactin (Cttn), and sperm flagellum associated protein (Sfap1), all of which were normally expressed in -/- with the non-supplemented diet. These results suggest that a gene expression sequence still proceeds to the spermiogenesis stage in the -/- .

Testis DHA and cholesta-3,5-diene were restored by dietary DHA

Testis fatty acid analysis (**Table 7.2**) shows significant changes due to genotype and dietary treatments. As shown in the previous study (19), non-supplemented -/- presented low levels of DHA (35% of +/+), AA (8% of +/+), near depletion of DPAn6, and accumulation of 20:3Δ7,11,14, a product of linoleic acid desaturated by delta-5 desaturase and elongated to 20 carbons (**Table 7.2**). As expected, AA supplementation to -/- males restored AA to the level of +/+ animals, but not DHA and DPAn6, whereas 22:4n6 and 24:4n6, elongation products of AA, accumulated in the AA supplemented -/- group. Testis DHA was restored in -/- receiving DHA supplementation, while AA in testis was low, similar to non-supplemented -/-. DPAn6 was nearly depleted in -/- (**Table 7.2**), indicating that DPAn6 is dispensable for spermatogenesis and male fertility in presence of sufficient DHA.

The very long chain polyunsaturated fatty acids (VLCPUFA, $C \geq 26$) in testis of all +/+ groups were elongation products of DPAn6 (26:5n6, 28:5n6, 30:5n6), and a possible elongation product of DHA (30:6n3), all of which were quantitatively minor (**Figure 7.4A**). In non-supplemented -/-, these VLPUFAs became undetectable, except for 30:6n3, while an AA-

elongated product, 26:4n6, appeared. AA supplemented -/- had AA-elongation products 28:4n6 and 30:4n6, but did not present the common DPAn6-elongated VLPUFAs found in +/+. The DHA supplemented -/- lacked n6 VLPUFAs, however, 28:5n3, 28:6n3 and 30:6n3 were present. VLPUFA analysis revealed presence of a large peak at 49 min in +/+ and DHA supplemented -/- (**Figure 7.4A**). The peak diminished in non-supplemented and AA supplemented -/-. The major species in the peak was identified as cholesta-3,5-diene (CD, **Figure 7.4B**), a dehydration product of the alcohol group at the 3 position of cholesterol.

DISCUSSION

In this study, we determined the effects of dietary AA and DHA on the fertility and spermatogenesis of the D6D-null males. Supplementing 0.2% DHA alone was able to fully restore male fertility, spermiogenesis, sperm morphology, and sperm count in -/- males. This restoration occurred despite very low AA and near depletion of DPAn6, the major HUFA present in +/+ males. Considering the variable ratios of DPAn6 and DHA among species (24), DPAn6 and DHA may be interchangeable for sperm function, and therefore DPAn6 is dispensable for sperm function in mice as long as sufficient DHA is present. It was a little unexpected that DHA supplementation alone can fully restore male reproduction and spermiogenesis because of proposed roles of AA as eicosanoid precursor in male reproductive function (7,8). Although the underlying mechanism is yet to be elucidated, there could be a functional redundancy that might compensate for the lack of eicosanoids. Alternatively, the residual AA present in the DHA supplemented group might be sufficient as a precursor of eicosanoids for male reproduction.

In AA supplemented -/- mice, sperm counts and a breeding success rate were partially restored even though the testis DHA and DPAn6 were as low as the non-supplemented -/-. This

partial rescue is unlikely to be due to the restoration of testis AA in the AA supplemented -/- considering DHA supplemented -/- had full recovery with AA levels similar to non-supplemented -/-. On the other hand, there was an accumulation of the AA elongated products 22:4n6 and 24:4n6 in testis of AA supplemented -/-. Thus, 22:4n6 or 24:4n6 may have acted as a substitute for DHA and DPAn6 although these AA-elongated fatty acids do not seem as effective as DPAn6 or DHA in restoring fertility.

Yet to be elucidated is the mechanism underlying the loss of spermatogenesis in the -/- and the remarkable restoration by dietary DHA. In monkey sperm, 99% of DHA is present in flagella (6). Thus, the failure of spermatogenesis at a late stage of spermiogenesis may be at least in part due to lack of DHA and DPAn6 for structural components of the flagellar membrane phospholipids. However, the impairment of spermatogenesis is not limited to tail formation. It extends to globozoospermia and possible impairment of sterol metabolism. Globozoospermia is a rare form of infertility in humans, characterized by a rounded sperm head (25). The Jackson Laboratory lists 39 mutant mouse strains under globozoospermia (http://www.jax.org), suggesting multiple causes of this abnormality. Mammalian sperm heads contain ceramides and sphingomyelins with high percentages of VLPUFAs (9,10). Loss of VLPUFA in non-supplemented -/- may play a role in the impaired sperm head function and structure.

Another important finding of this study is decreased cholesta-3,5-diene (CD) in the lipid extract of non-supplemented and AA-supplemented -/-, and restoration by dietary DHA. Because of the paucity of literature on CD, it is unclear if CD is present in testis, or if it is derived from sterols during sample processing, although presence of CD in cornea has been reported (26). Whichever the case, our data indicate an impairment of sterol metabolism in -/- that was restored by DHA supplementation. Desmosterol (24-dehydrocholesterol), an

intermediate metabolite of the last step of cholesterol synthesis, is not present in large quantity in most tissues. However, free desmosterol is the major sterol present in flagella of monkey sperm followed by free cholesterol and cholesteryl esters (6,27). Furthermore, the major impairment in the hormone sensitive lipase-null mouse is a complete loss of spermatogenesis (28), similar to our D6D-null mice. Because hormone sensitive lipase is the only esterase that can hydrolyze cholesteryl ester, a loss of the enzyme resulted in accumulation of cholesteryl ester in Sertoli cells and loss of spermatogenesis (28), indicating essentiality of cholesterol in spermatogenesis.

Sperizin is a protein highly expressed in spermatids and may act as ubiquitin ligase (29,30). A study reported complete abolition of sperizin RNA in the testis of D6D-null mice, and suggested an arrest of the gene expression sequence at the spermiogenesis stage (20). However, in our study, sperizin showed only a mild decrease in the -/- animals of all three dietary groups, which displayed a drastic difference in spermiogenesis, excluding sperizin as the cause of impaired spermatogenesis. Moreover, several other markers specific to spermiogenesis showed largely normal expression in -/- animals fed different diets including genes that encode proteins in sperm flagella such as Shippo1 (31), Akap3 (32), and Sfap1(33). Thus, our RNA analysis suggests that there is no general arrest of gene expression sequence at the spermiogenesis stage although it is possible that expression of specific genes may be affected by DHA deficiency.

In conclusion, this study demonstrated that DHA supplementation to D6D-null male mice restored spermatogenesis and fertility in the absence of DPAn6 and low AA in testis, while dietary AA was much less effective. The accumulation of 22:4n6 in the AA supplemented -/- testis suggests that 22:4n6 may act as a lesser substitute for DPAn6 or DHA in spermiogenesis. Cholesta-3,5-diene was detected in testis lipid extract from +/+ and DHA-supplemented -/-,

whereas it greatly decreased in non-supplemented and AA-supplemented -/-, suggesting impairment of sterol metabolism in the latter groups. The expression of spermiogenesis marker genes in -/- animals was largely normal. The mechanism underlying the loss of spermatogenesis in the -/- and the rescue by dietary DHA is yet to be elucidated.

TABLES AND FIGURES

Table 7.1 *Spermiogenesis-specific gene expression markers in testis at 16 weeks of age.*

		relative mRNA expression			
Name	**Description/function**	**+/- AIN**	**-/- AIN**	**-/- AA**	**-/- DHA**
Shippo1 (Odf3, TISP50)	Flagellum structural component	1 ±0.06[a]	0.81 ±0.06[b]	0.96 ±0.14[ab]	1.01 ±0.1[a]
Sperizin (Znrf4, TISP69)	Proteasome-mediated degradation of spermatid proteins ?	1 ±0.03[a]	0.55 ±0.11[b]	0.63 ±0.19[b]	0.63 ±0.02[b]
A-kinase anchoring protein (Akap3)	Flagellum component	1 ±0.11[a]	0.80 ±0.12[b]	0.84 ±0.07[b]	0.88 ±0.03[ab]
Cortactin (Cttn)	Spermatid-Sertoli cell interaction	1 ±0.06	1.25 ±0.20	1.08 ±0.16	1.06 ±0.09
Transition protein-1 (Tnp1)	Compaction of sperm head	1 ±0.16	0.93 ±0.28	0.93 ±0.17	1.10 ±0.15
Sperm flagellum associated protein (Sfap1)	Flagellum structural component	1 ±0.11	1.03 ±0.04	nd	nd

Mean ± SD. Groups without a common letter differ by Fisher's PLSD after one-way ANOVA, $p<0.05$; nd=not determined. +/+ AIN, wild type fed AIN93G diet; -/- AIN, knockout fed AIN93G; -/- AA, knockout with 0.2% AA supplementation; -/- DHA, knockout with 0.2% DHA supplementation.

Table 7.2 *Total fatty acid analysis of testis from 16 week old delta-6-desaturase knockout mice with and without HUFA supplementation.*

	Relative molar percentage			
HUFA	**+/+ AIN**	**-/- AIN**	**-/- AA**	**-/- DHA**
20:3(Δ7,11,14)	nd	6.59	0.83	0.35
20:4n6	8.55 ±3.65	0.68	10.45	0.79 ±0.53
22:4n6	1.07 ±0.43	0.31	6.33	0.1 ±0.07
22:5n6	8.49 ±3.88	0.06	0.05	0.01 ±0.01
22:6n3	5.49 ±2.52	1.91	1.43	7.33 ±4.58
24:4n6	0.35 ±0.16	1.306	13.61	0.29 ±0.19

Mean ± SD. Groups without SD were pooled. +/+ AIN, wild type fed AIN93G diet; -/- AIN, knockout fed AIN93G; -/- AA, knockout with 0.2% AA supplementation; -/- DHA, knockout with 0.2% DHA supplementation; nd=not detected.

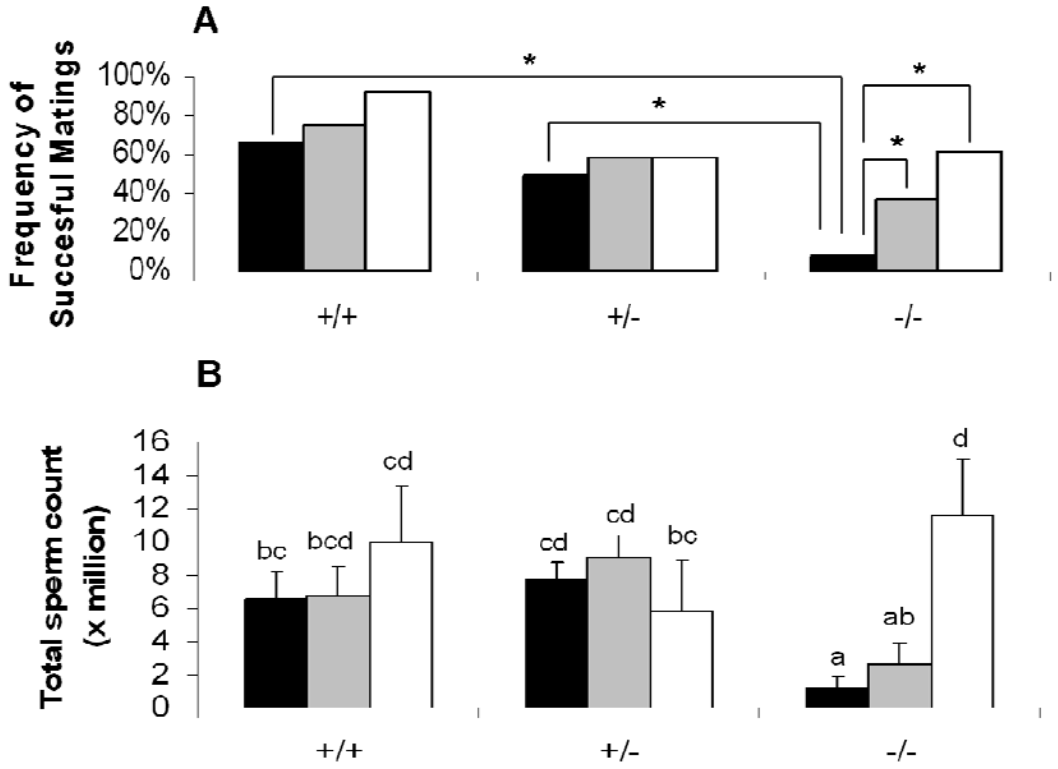

Figure 7.1 ***(A)*** *Fertility of +/+, +/-, and -/- male mice fed AIN93G diet (black), 0.2% AA supplemented (gray), or 0.2% DHA supplemented (white) to the AIN93G diet (n=3-5) . Fertility is expressed as frequency of matings resulting in impregnated females and a viable litter; 12 to 18 attempts per group. * p<0.05 by Wilcoxon rank sum test.* ***(B)*** *Total mature sperm count of epididymis from 16 week-old male mice. Mean ± SD. Groups without a common letter are statistically different by Fisher's PLSD after one-way ANOVA (p<0.05).*

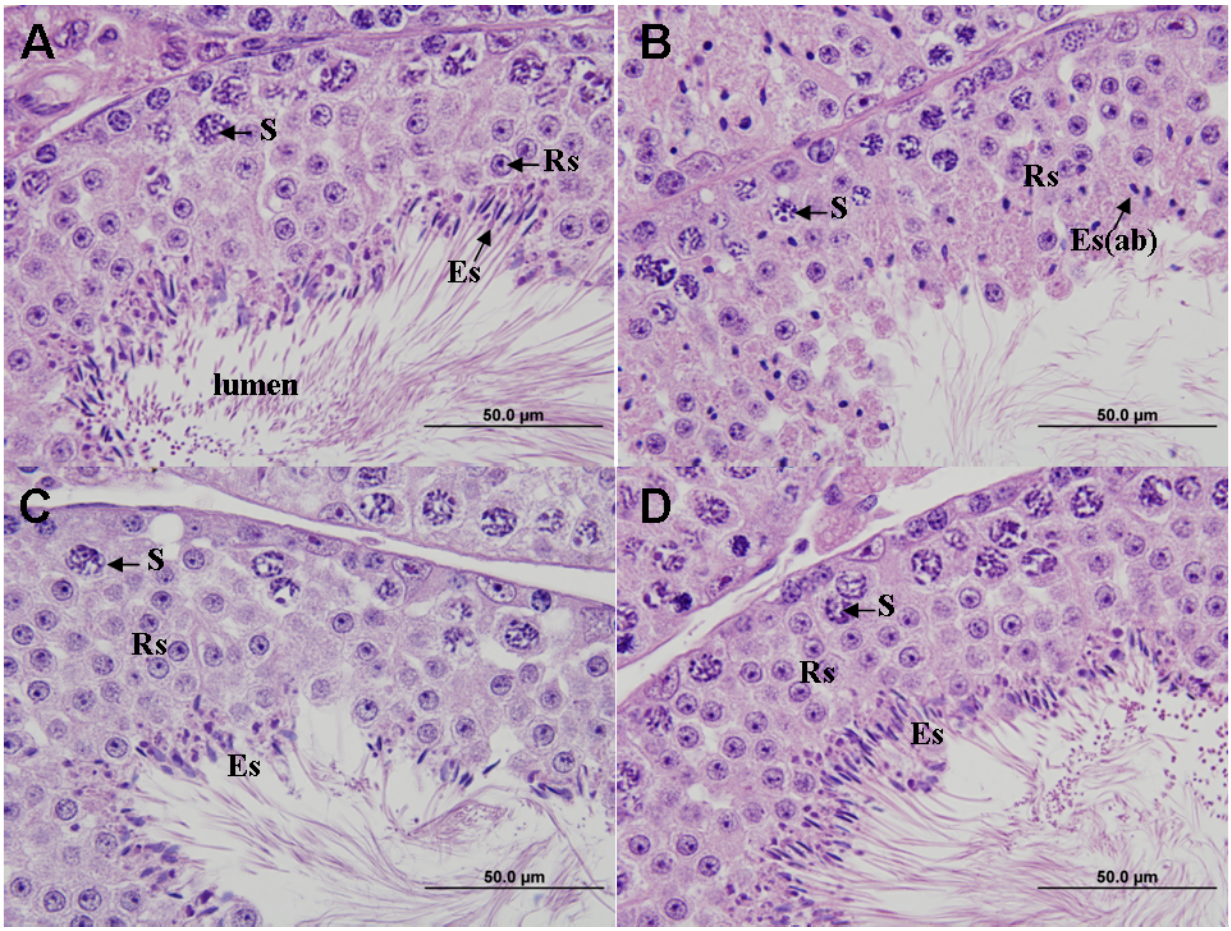

Figure 7.2 *Testis histology of +/+ and -/- male mice fed AIN93G diet with or without 0.2% AA, or 0.2% DHA supplementation from weaning until 16 weeks of age.* ***A.*** *Seminiferous tubule in testis of +/+ without supplementation showing stage VII of spermatogenesis with normal elongated spermatids.* ***B.*** *Seminiferous tubule from non-supplemented -/- shows impairment of spermiogenesis, with abnormal elongation of spermatid head.* ***C.*** *AA supplementation to -/- partially restores spermatogenesis.* ***D.*** *DHA supplementation to -/- completely restores spermatogenesis showing no difference when compared to +/+. H & E stain. S, spermatocytes. Rs, round spermatids. Es, elongated spermatids. Es(ab), abnormal elongated spermatids.*

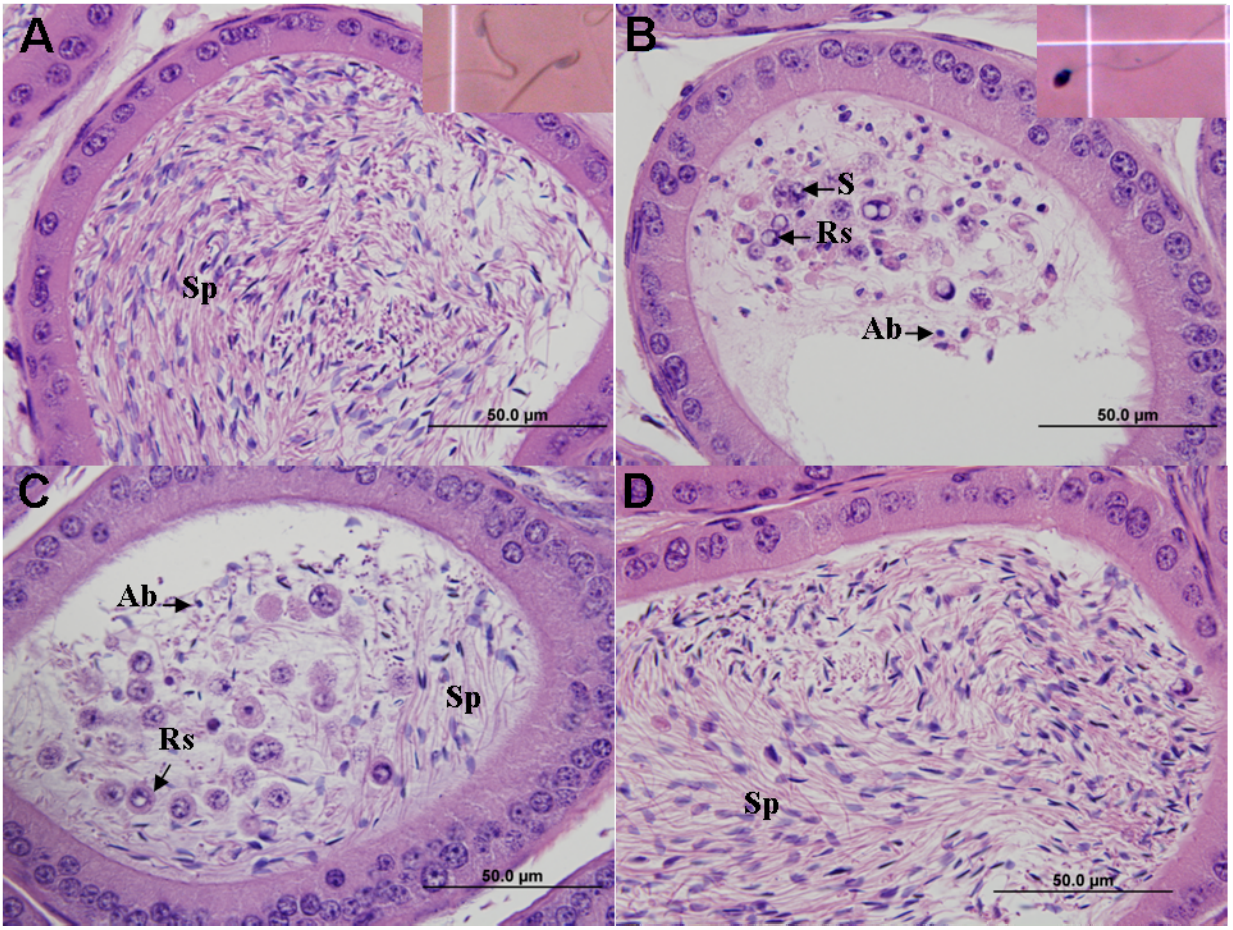

Figure 7.3 *Epididymis histology of +/+ and -/- male mice fed AIN93G diet with or without 0.2% AA, or 0.2% DHA supplementation from weaning until 16 weeks of age.* ***A.*** *Epididymis of +/+ without supplementation contains viable, mature spermatozoa (Sp), with normal heads and tails. Inset: sperm showing normal morphology.* ***B.*** *Epididymis of non-supplemented -/- contains sloughed round spermatids (Rs) and spermatocytes (S) and abnormal rounded head sperm (Ab). Inset: globozoospermia (rounded head sperm),* ***C.*** *AA supplemented -/- epididymis shows a combination of sloughed round spermatids (Rs), some abnormal rounded head sperm (Ab), as well as spermatozoa (Sp), with normal morphology.* ***D.*** *DHA supplemented -/- epididymis has only spermatozoa (Sp) and shows no difference compared to +/+. H & E stain.*

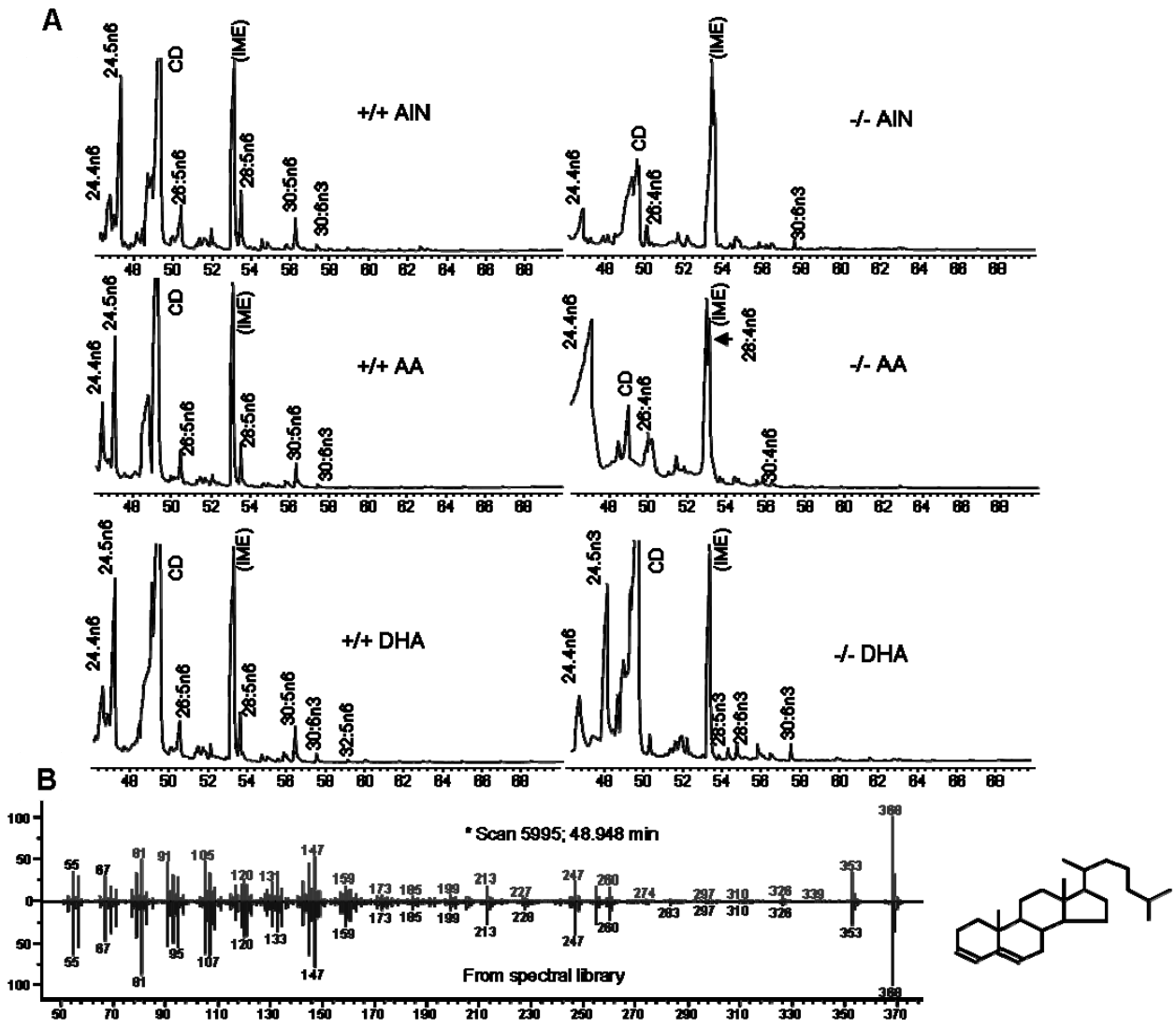

Figure 7.4 *A*. *Very long chain polyunsaturated fatty acid (VLPUFA) analysis of testis at 16 weeks of age by gas chromatography-mass spectrometry. +/+ AIN, wild type fed AIN93G diet; +/+ AA, wild type with 0.2% AA supplementation; +/+ DHA, wild type with 0.2% DHA supplementation; -/- AIN, knockout fed AIN93G; -/- AA, knockout with 0.2% AA supplementation; -/- DHA, knockout with 0.2% DHA supplementation; IME, isocholesteryl methyl ether (normalizer); CD, cholesta-3,5-diene.* ***B****. Mass spectra of the peak at 48.9 minutes of +/+ testis sample matches reference spectra (NIST Mass Spectral Library, Agilent Technologies) of cholesta-3,5-diene (chemical structure shown at the right).*

REFERENCES

(1) Nakamura, M. T., T. Y. Nara. 2004. Structure, function, and dietary regulation of delta6, delta5, and delta9 desaturases. *Annu. Rev. Nutr.* 24: 345-76.

(2) Davis, J. T., J. G. Coniglio. 1967. The effect of cryptorchidism, cadmium and anti-spermatogenic drugs on fatty acid composition of rat testis. *J. Reprod. Fertil.* 14: 407-413.

(3) Grogan, W. M., W. F. Farnham, and B. A. Szopiak. 1981. Long chain polyenoic acid levels in viably sorted, highly enriched mouse testis cells. *Lipids* 16: 401-410.

(4) Rejraji, H., B. Sion, G. Prensier, M. Carreras, C. Motta, J. M. Frenoux, E. Vericel, G. Grizard, P. Vernet, and J. R. Drevet. 2006. Lipid remodeling of murine epididymosomes and spermatozoa during epididymal maturation. *Biol Reprod* 74: 1104-13.

(5) Lenzi, A., L. Gandini, V. Maresca, R. Rago, P. Sgro, F. Dondero, and M. Picardo. 2000. Fatty acid composition of spermatozoa and immature germ cells. *Mol. Hum. Reprod.* 6: 226-231.

(6) Connor, W. E., D. S. Lin, D. P. Wolf, and M. Alexander. 1998. Uneven distribution of desmosterol and docosahexaenoic acid in the heads and tails of monkey sperm. *J. Lipid Res.* 39: 1404-1411.

(7) Colon, J. M., F. Ginsburg, J. B. Lessing, C. Schoenfeld, L. T. Goldsmith, R. D. Amelar, L. Dubin, and G. Weiss. 1986. The effect of relaxin and prostaglandin E2 on the motility of human spermatozoa. *Fertil. Steril.* 46: 1133-1139.

(8) Balaji, T., M. Ramanathan, and V. P. Menon. 2007. Localization of cyclooxygenase-2 in mice vas deferens and its effects on fertility upon suppression using nimesulide: a preferential cyclooxygenase-2 inhibitor. *Toxicology* 234: 135-144.

(9) Furland, N. E., S. R. Zanetti, G. M. Oresti, E. N. Maldonado, and M. I. Aveldano. 2007. Ceramides and sphingomyelins with high proportions of very long-chain polyunsaturated fatty acids in mammalian germ cells. *J. Biol. Chem.* 282: 18141-18150.

(10) Furland, N. E., G. M. Oresti, S. S. Antollini, A. Venturino, E. N. Maldonado, and M. I. Aveldano. 2007. Very long-chain polyunsaturated fatty acids are the major acyl groups of sphingomyelins and ceramides in the head of mammalian spermatozoa. *J. Biol. Chem.* 282: 18151-18161.

(11) Robinson, B. S., D. W. Johnson, and A. Poulos. 1992. Novel molecular species of sphingomyelin containing 2-hydroxylated polyenoic very-long-chain fatty acids in mammalian testes and spermatozoa. *J. Biol. Chem.* 267: 1746-1751.

(12) Agbaga, M. P., R. S. Brush, M. N. Mandal, K. Henry, M. H. Elliott, and R. E. Anderson. 2008. Role of Stargardt-3 macular dystrophy protein (ELOVL4) in the biosynthesis of very long chain fatty acids. *Proc. Natl. Acad. Sci. U. S. A.* 105: 12843-12848.

(13) Gadella, B. M., M. Lopes-Cardozo, L. M. van Golde, B. Colenbrander, and T. W. Gadella Jr. 1995. Glycolipid migration from the apical to the equatorial subdomains of the sperm head plasma membrane precedes the acrosome reaction. Evidence for a primary capacitation event in boar spermatozoa. *J. Cell. Sci.* 108 (Pt 3): 935-946.

(14) Cross, N. L. 2000. Sphingomyelin modulates capacitation of human sperm in vitro. *Biol. Reprod.* 63: 1129-1134.

(15) Burr, G. O., M. M. Burr. 1929. A new deficiency disease produced by the rigid exclusion of fat from the diet. *J. Biol. Chem.* 82: 345-67.

(16) Leat, W. M., C. A. Northrop, F. A. Harrison, and R. W. Cox. 1983. Effect of dietary linoleic and linolenic acids on testicular development in the rat. *Q. J. Exp. Physiol.* 68: 221-231.

(17) Hansen, H. S., B. Jensen. 1985. Essential function of linoleic acid esterified in acylglucosylceramide and acylceramide in maintaining the epidermal water permeability barrier. Evidence from feeding studies with oleate, linoleate, arachidonate, columbinate and alpha-linolenate. *Biochim Biophys Acta* 834: 357-63.

(18) Aaes-Jorgensen, E., G. Holmer. 1969. Essential fatty acid-deficient rats. I. Growth and testes development. *Lipids* 4: 501-506.

(19) Stroud, C. K., T. Y. Nara, M. Roqueta-Rivera, E. C. Radlowski, P. Lawrence, Y. Zhang, B. H. Cho, M. Segre, R. A. Hess, J. T. Brenna, W. M. Haschek, and M. T. Nakamura. 2009. Disruption of FADS2 gene in mice impairs male reproduction and causes dermal and intestinal ulceration. *J. Lipid Res.* [Epub ahead of print].

(20) Stoffel, W., B. Holz, B. Jenke, E. Binczek, R. H. Gunter, C. Kiss, I. Karakesisoglou, M. Thevis, A. A. Weber, S. Arnhold, and K. Addicks. 2008. Delta6-Desaturase (FADS2) deficiency unveils the role of omega3- and omega6-polyunsaturated fatty acids. *EMBO J.* 27: 2281–2292.

(21) Reeves, P. G., F. H. Nielsen, and G. C. Fahey. 1993. AIN-93 purified diets for laboratory rodents: Final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.* 123: 1939-1951.

(22) Koo, H. Y., M. A. Wallig, B. H. Chung, T. Y. Nara, B. H. Cho, and M. T. Nakamura. 2008. Dietary fructose induces a wide range of genes with distinct shift in carbohydrate and lipid metabolism in fed and fasted rat liver. *Biochim. Biophys. Acta* 1782: 341-348.

(23) Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* 226: 497-509.

(24) Bieri, J. G., E. L. Prival. 1965. Lipid composition of testes from various species. *Comp Biochem Physiol* 15: 275-82.

(25) Dam, A. H., I. Feenstra, J. R. Westphal, L. Ramos, R. J. van Golde, and J. A. Kremer. 2007. Globozoospermia revisited. *Hum. Reprod. Update* 13: 63-75.

(26) Cenedella, R. J., L. L. Linton, and C. P. Moore. 1992. Cholesterylene, a newly recognized tissue lipid, found at high levels in the cornea. *Biochem. Biophys. Res. Commun.* 186: 1647-1655.

(27) Lin, D. S., W. E. Connor, D. P. Wolf, M. Neuringer, and D. L. Hachey. 1993. Unique lipids of primate spermatozoa: desmosterol and docosahexaenoic acid. *J. Lipid Res.* 34: 491-499.

(28) Osuga, J., S. Ishibashi, T. Oka, H. Yagyu, R. Tozawa, A. Fujimoto, F. Shionoiri, N. Yahagi, F. B. Kraemer, O. Tsutsumi, and N. Yamada. 2000. Targeted disruption of hormone-sensitive lipase results in male sterility and adipocyte hypertrophy, but not in obesity. *Proc. Natl. Acad. Sci. U. S. A.* 97: 787-792.

(29) Fujii, T., K. Tamura, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, K. Yomogida, H. Tanaka, Y. Nishimune, H. Nojima, and Y. Abiko. 1999. Sperizin is a murine RING zinc-finger protein specifically expressed in Haploid germ cells. *Genomics* 57: 94-101.

(30) Pickart, C. M. 2001. Mechanisms underlying ubiquitination. *Annu. Rev. Biochem.* 70: 503-533.

(31) Egydio de Carvalho, C., H. Tanaka, N. Iguchi, S. Ventela, H. Nojima, and Y. Nishimune. 2002. Molecular cloning and characterization of a complementary DNA encoding sperm tail protein SHIPPO 1. *Biol. Reprod.* 66: 785-795.

(32) Morales, C. R., S. Lefrancois, V. Chennathukuzhi, M. El-Alfy, X. Wu, J. Yang, G. L. Gerton, and N. B. Hecht. 2002. A TB-RBP and Ter ATPase complex accompanies specific mRNAs from nuclei through the nuclear pores and into intercellular bridges in mouse male germ cells. *Dev. Biol.* 246: 480-494.

(33) Baek, N., J. M. Woo, C. Han, E. Choi, I. Park, H. Kim do, E. M. Eddy, and C. Cho. 2008. Characterization of eight novel proteins with male germ cell-specific expression in mouse. *Reprod. Biol. Endocrinol.* 6: 32.

CHAPTER 8

HIGHLY UNSATURATED FATTY ACIDS ARE REQUIRED FOR ACROSOME BIOGENESIS IN SPERMATOGENESIS

ABSTRACT

The delta-6 desaturase knockout (D6D-/-) male mice are unable to synthesize highly unsaturated fatty acid (HUFA) and become infertile as result of disrupted spermiogenesis, the final stage in sperm formation where round spermatids become spermatozoa. Dietary HUFA prevents infertility in D6D-/- with DHA fully restoring spermiogenesis while AA supplementation is less effective. To elucidate the mechanism underlying the essential role of HUFA in spermiogenesis, acrosome biogenesis in D6D-/- mice was examined by histological comparison of testis from wild type (+/+) and D6D-/- mice fed HUFA deficient diet. Acrosome was absent in non-supplemented -/- round spermatids as indicated by periodic acid-Schiff stain while HUFA supplementation of either AA or DHA restored periodic acid-Schiff stain. Immunofluorescence using an acrosomal marker, acrosin, showed the characteristic cap-like acrosome structure in +/+, while D6D-/- presented acrosin as a dot-like pattern suggesting impaired vesicle fusion which is required for acrosome biogenesis. DHA supplementation restored cap-like staining of acrosin while fragmented acrosome was observed with dietary AA. Electron microscopy confirmed formation of Golgi-derived proacrosomal vesicles in D6D-/- which never fuse to develop acrosome, further supporting immunohistochemistry data. In conclusion, DHA restores D6D-/- fertility as an essential component of acrosome biogenesis, most likely providing an adequate lipid environment for proacrosomal vesicle fusion.

INTRODUCTION

The highly unsaturated fatty acids (HUFA), arachidonic acid (AA) and docosahexaenoic acid (DHA), are physiologically essential for the proper function of many tissues, however, the mechanisms behind their essentiality are not fully understood. A delta-6-desaturase knockout (D6D-/-) mouse was created to study HUFA function and underlying mechanisms behind HUFA deficiency symptoms (1). The D6D-/- lacks the enzyme to synthesize HUFA, allowing for HUFA deficiency without depletion of essential fatty acid precursors. One of the main HUFA deficiency symptoms in male D6D-/- mice is infertility (1). In testis, DHA is in high concentrations, but its exact role for fertility has not been established.

The D6D-/- males display arrest of spermatogenesis at the spermiogenesis stage, resulting in infertility. During spermiogenesis, round spermatids acquire structural components and go through morphological changes in order to develop into spermatozoa with an elongated head and motile tail. This process is interrupted with HUFA deficiency as indicated in the D6D-/- male which develops abnormal round head sperm morphology (2), a condition known as globozoospermia (3). Dietary supplementation of omega-3 DHA is able to prevent infertility and globozoospermia in D6D-/-, restoring normal breeding success rates and sperm counts (2). AA supplementation, however, only partially restores fertility parameters, suggesting a specific role for DHA in proper sperm formation (2).

Similar to the D6D-/- mouse, there are other mouse models which are infertile due to globozoospermia, such as the Hrb (4) and GOPC (5) deficient mice. Globozoospermia in these mice is accompanied by a complete loss of acrosome. The acrosome is a vesicle formed on the nuclear envelope of round spermatids during spermiogenesis. Absence of an acrosome results in

round headed sperm (6). Impaired acrosome biogenesis is therefore hypothesized to occur in the D6D-/- due to HUFA deficiency.

The objective of this study is to elucidate the mechanism underlying an essential role of HUFA in spermiogenesis by examining acrosome biogenesis in D6D-/- mice.

METHODS

Animals and diets

All animal work was approved by the University of Illinois Institutional Animal Care and Use Committee. Mice used in the experiment were produced by breeding heterozygous males and females fed a standard rodent chow. Mice were mixed strain (129S6/SvEvTac/C57BL/6J) for immunohistochemistry and pure strain (C57BL/6J) for electron microscopy. At weaning, wild type (+/+) and D6D null (-/-) mice were distributed to receive one of the following diets: control diet (AIN93G), AIN93G supplemented with 0.2 w/w % AA, or AIN93G with 0.2 w/w % DHA. The AIN93G diet is a purified, nutritionally adequate diet that contains sufficient linoleic acid and α-linolenic acid, but no AA or DHA (7). DHASCO and ARASCO oils (Martek Biosciences, Columbia, MD) were used for supplementation of DHA and AA, respectively. Dietary groups consisted of 3-5 mice. Mice were single housed at weaning and received the diet until four months of age.

Histology

Testis were excised and fixed in Davidson's fixative and transferred to 10% neutral buffered formalin after 24 hours. The testes were trimmed and embedded in paraffin. Sections were cut at 3 microns and stained with hematoxylin/eosin and with periodic acid Schiff (PAS).

Immunohistochemistry

Paraffin sections at 5 microns were deparaffinized and rehydrated. Antigen retrieval was carried out with 10mM citrate buffer in water bath at 95ºC for 40 minutes. Samples were then permeabilized in 1% Triton-X, blocked with Image iT FX Signal Enhancer (Invitrogen #I36933), and incubated with 2% primary antibody overnight at room temperature. After washing, sections were incubated with 1% secondary fluorescent antibodies for three hours at room temperature. Finally, samples were treated with 0.5 μg/ml of Hoescht 33342 (Invitrogen # H21492) for nuclear staining during 5 minutes at room temperature. Imaging was done with a Zeiss LSM 710 Confocal Microscope. Three dimensional rendering of deconvoluted immunohistochemistry images was done using Imarisx64 version 7 (Saint Paul, MN), with background subtraction based on fluorescence intensity threshold.

The following antibodies were used: rabbit polyclonal anti-acrosin (Santa Cruz Biotechnology, Inc.; sc-67151), mouse monoclonal anti-actin (Abcam Inc.; ab40864), and Golgi marker mouse monoclonal antibody to 58k Golgi protein (Abcam Inc.; ab27043). Secondary fluorescent antibodies used were anti-rabbit Alexa 488 (Invitrogen # A11008) and anti-mouse Alexa 647 (Invitrogen #A21235).

Electron microscopy

Adult male mice (C57BL/6J) of +/+ and -/- genotype fed AIN93G diet until 16 weeks of age were injected intraperitoneallly with heparin (125 IU/kg body weight) 15 minutes prior to euthanasia, then anesthetized with pentobarbital (30μg/g body weight), perfused with PBS, and fixed with 5% glutaraldehyde in 0.05M sodium cacodylate buffer (pH 7.2-7.4) through the left ventricle. Testes were diced into small pieces, postfixed in osmium tetroxide/potassium

ferrocyanide, dehydrated, and embedded in epoxy-resin. One-micrometer sections were cut and stained with toluidine blue for high resolution light microscopy examination of spermiogenesis. Ultramicrotome sections at 70nm were prepared and stained for electron microscopy. Imaging was done with a Hitachi H600 electron microscope.

RESULTS

HUFA deficiency results in disrupted acrosome formation

HUFA deficient D6D-/- mice have disrupted spermiogenesis at stage of acrosome biogenesis. In +/+ mice, acrosome was detected by PAS stain as a purple-magenta color (**Figure 8.1 arrows**) on nuclear envelope of round spermatids as well as around the elongated sperm head (**Figure 8.1A-B**). PAS stain was absent in non-supplemented D6D-/- mice (**Figure 8.1C-D**) therefore lacking acrosome as well as developed elongated sperm head. Both AA (**Figure 8.1E-F**) and DHA supplemented -/- mice (**Figure 8.1G-H**) present PAS stain, indication of restored acrosome.

Spermiogenesis disrupted at Golgi phase due to HUFA deficiency

Spermiogenesis can be divided into four phases: Golgi, cap, acrosomal, and maturation (8). Acrosome biogenesis occurs during the Golgi and cap phases. The initial Golgi phase consists of sorting and packaging of acrosomal components into vesicles known as proacrosomal granules. These granules are transported from Golgi to the nuclear envelope of round spermatids where several proacrosomal vesicles and granules then fuse to form a single acrosomal granule (9,10). Subsequently the acrosome continues to grow in size as Golgi-derived granules continue to provide acrosomal components such as the protease acrosin (11,12). Acrosome biogenesis

proceeds with a cap phase in which the acrosomal granule is flattened over half the nucleus forming a cap-like structure. Differentiation of round spermatid into elongated spermatid and spermatozoa occurs during the acrosomal and maturation phase.

Nuclear stain shows all stages of spermatogenesis from spermatogonia to elongated heads of spermatozoa in +/+ (**Figure 8.2**). Immunohistochemistry shows acrosin, a protein contained within acrosomal vesicles, specifically in the spermiogenesis stage of round spermatids (**Figure 8.2**). Acrosin staining forms the characteristic acrosome cap-like structure attached to nuclear envelope of round spermatids. Golgi stain can be seen above the acrosomal structure.

Nuclear stain for non-supplemented D6D-/- show stages of spermatogenesis up to round spermatid but lack elongated spermatids confirming globozoospermia (**Figure 8.2**). Acrosin is present as several small vesicles near nuclear envelope and within cytosol, failing to form the acrosomal cap-like structure (**Figure 8.2**). DHA supplementation restored normal localization of acrosin in D6D-/- as indicated by the characteristic cap-like structure on round spermatids; elongated sperm heads are also observed (**Figure 8.2**).

Actin is associated to the acrosomal structure (13) and involved in trafficking of proacrosomal vesicles to the docking site on nucleus, also known as the acroplaxome (14). Actin is also a component of Sertoli cell ectoplasmic specializations which interact with round spermatid during acrosome biogenesis (14). This was indicated with actin immunofluorescence showing Sertoli cell specializations in a crescent shape above round spermatids in +/+ and all D6D-/- mice (**Figure 8.3**). Thus, HUFA deficiency does not disrupt the Sertoli-round spermatid interaction. The acrosin cap-like structure is present below the actin stain in +/+ and HUFA supplemented -/- (**Figure 8.3**). HUFA deficient D6D-/- however lack this acrosin cap-like

structure and only show a dot-like pattern near nucleus and in cytosol, representative of vesicles. Three dimensional rendering of images seen in Figure 8.3 were obtained for acrosin and actin staining. In +/+, acrosomal caps are observed as a continous acrosin stain while -/- mice present scattered acrosin which do not form a cap (**Figure 8.4**). AA supplemented -/- presents an arc-like acrosin stain from a top view; however a side view indicates a fragmented acrosomal cap (**Figure 8.5**). DHA supplementation was more effective presenting a continous acrosomal cap similar to +/+ (**Figure 8.5**). Disrupted acrosome biogenesis in D6D-/- cannot be attributed to altered Sertoli cell interactions with round spermatids since actin is not mislocalized in non-supplemented -/-. Impaired proacrosomal vesicle fusion is a more likely explanation as indicated by electron microscopy.

Golgi-derived proacrosmal vesicles do not fuse or form an acrosomal granule attached to nucleus in HUFA deficient mice

Electron microscopy evaluation of round spermatids confirmed lack of acrosome formation in D6D-/- mice. Golgi-derived proacrosomal vesicles and granules are formed in both +/+ (**Figure 8.6B)** and -/- **(Figure 8.7B**) but only +/+ forms a single acrosomal granule (**Figure 8.6C-D**) attached to the nuclear envelope. This acrosomal granule flattens over the nuclear envelope forming a cap-like structure (**Figure 8.6E-F**) which eventually becomes the acrosome (**Figure 8.6G-H**). Proacrosomal vesicles and granules are present in D6D-/- and can be seen between Golgi and nuclear envelope (**Figure 8.7A-B**), but do not fuse to form a single acrosomal structure confirming immunohistochemistry data and indicating disruption of spermiogenesis at step 2 of the initial Golgi phase. In a later step of spermiogenesis, the -/- spermatid nuclear envelope is flattened (**Figure 8.7C-D**) but does not have the single acrosomal granule attached as in +/+. The acroplaxome, docking site of proacrosomal vesicles, can be identified as a darkened

region on the nuclear envelope (**Figure 8.7E-F**) and is present in both +/+ and -/-; however, acrosome is not attached in D6D-/-. Several fragmented small vesicles are present on top of D6D-/- nucleus where acrosome should be present (**Figure 8.7F**). In addition to lack of acrosome, the -/- spermatid also presents abnormal nuclear indentations (**Figure 8.7G**).

DISCUSSION

Globozoospermia is a type of male infertility characterized by round headed sperm lacking acrosome (3,6). The acrosome is a vesicle on the nuclear envelope of round spermatids which forms prior to a series of morphological changes which include elongation of spermatid head and formation of tail (8). Disruption of acrosome formation is therefore associated with failed sperm head elongation which leads to globozoospermia. In this study, we demonstrate HUFA essentiality for acrosome development, as HUFA deficient D6D-/- completely lack acrosome. The mechanisms underlying disrupted acrosome formation have been studied in mice deficient in proteins that participate in acrosome biogenesis. These proteins include GOPC, Hrb, and PICK1 which are involved in the trafficking of proacrosomal vesicles from Golgi to nuclear envelope and their subsequent fusion for acrosome biogenesis. GOPC encodes for the Golgi associated PDZ and coiled-coil motif containing protein which is present in the trans-Golgi network and participates in vesicle trafficking of granules from Golgi to nucleus (5). PICK1 encodes for protein interacting with C kinase 1 and associates with GOPC favoring vesicle budding from Golgi as well as participating in vesicle trafficking (15). Hrb encodes for HIV1-Rev binding protein which is present in the cytosolic surface of proacrosomal vesicles (4). Hrb, GOPC, or PICK1 deficiency results in impaired proacrosomal vesicle fusion.

The HUFA deficient D6D-/- mice present similar acrosomal alterations to those seen in

Hrb, GOPC, and PICK1-/- mice with disrupted acrosome biogenesis in step 2 of spermiogenesis during the initial Golgi phase. Electron microscopy of D6D-/- round spermatids confirms the presence of proacrosomal vesicles and granules between Golgi and nucleus. The docking site for proacrosomal vesicles on the nuclear envelope, known as acroplaxome (14), is present in the D6D-/- round spermatid, however, docking and fusion to form the single acrosomal granule does not occur. This further supports the hypothesis of impaired vesicle fusion suggested by acrosin immunofluorescence in D6D-/-, represented by dot-like pattern in cytosol and near nucleus, but lacking an acrosomal cap-like structure. These spermiogenic defects, also observed in GOPC, Hrb, and PICK1-/-mice, suggest a potential mechanism of HUFA requirement for vesicle fusion in acrosome formation.

Vesicle fusion relies on the formation of a soluble NSF-attachment receptor (SNARE) complex consisting of a four helical structure that drives the union of opposing membranes (16). HUFA have been shown to interact with components of the SNARE complex vesicle fusion in neuroendocrine cells and retina, which similar to testis are also rich in DHA. In brain, DHA favors hippocampus neuronal development and synaptic function (17), which relies on neurite outgrowth and the SNARE complex (18). In retina, DHA optimizes the visual transduction system (19) and modulates vesicle fusion for rhodopsin delivery to retinal outer segments (20). SNARE proteins have also been identified in testis (21) however their interaction with HUFA for acrosome formation has not been elucidated.

This SNARE complex consists of proteins such as, syntaxin and synaptobrevin, which are on opposing membranes and interact to achieve vesicle fusion. Syntaxin exists in an inactive and closed conformation and requires activation into an open conformation for SNARE assembly (22). AA activates syntaxin (23) allowing SNARE complex formation consisting of syntaxin-3,

SNAP25, and synaptobrevin interactions and favoring neurite outgrowth (18). DHA was shown to efficiently substitute for AA for SNARE assembly *in vitro* (18). In retina, rhodopsin delivery by Golgi-derived vesicles requires SNARE components which are regulated by DHA (20).

Syntaxin, synaptobrevin, and SNAP have also been identified in mouse testis in acrosome biogenesis (21), however the role of HUFA in regulating SNARE components in male fertility has not been demonstrated. SNARE complex formation is most likely required for fusion of proacrosomal vesicles during acrosome formation. Similar to neuroendocrine cells, free HUFA may be required to activate syntaxin to favor SNARE assembly. DHA supplementation was more effective than AA in restoring acrosome, which could suggest a specific role for this HUFA in SNARE complex assembly for acrosome biogenesis.

In conclusion, we demonstrated essentiality of HUFA for acrosome biogenesis in restoring D6D-/- fertility. The most likely mechanism behind impaired acrosomal biogenesis in HUFA deficiency is impaired proacrosomal vesicle fusion as seen in Hrb, GOPC, and PICK1-null mouse models of globozoospermia, which are disrupted at the same spermiogenesis stage as D6D-/-. Further research into the interaction of HUFA with acrosomal SNARE components may have implications in developing treatments for certain cases of male infertility.

FIGURES

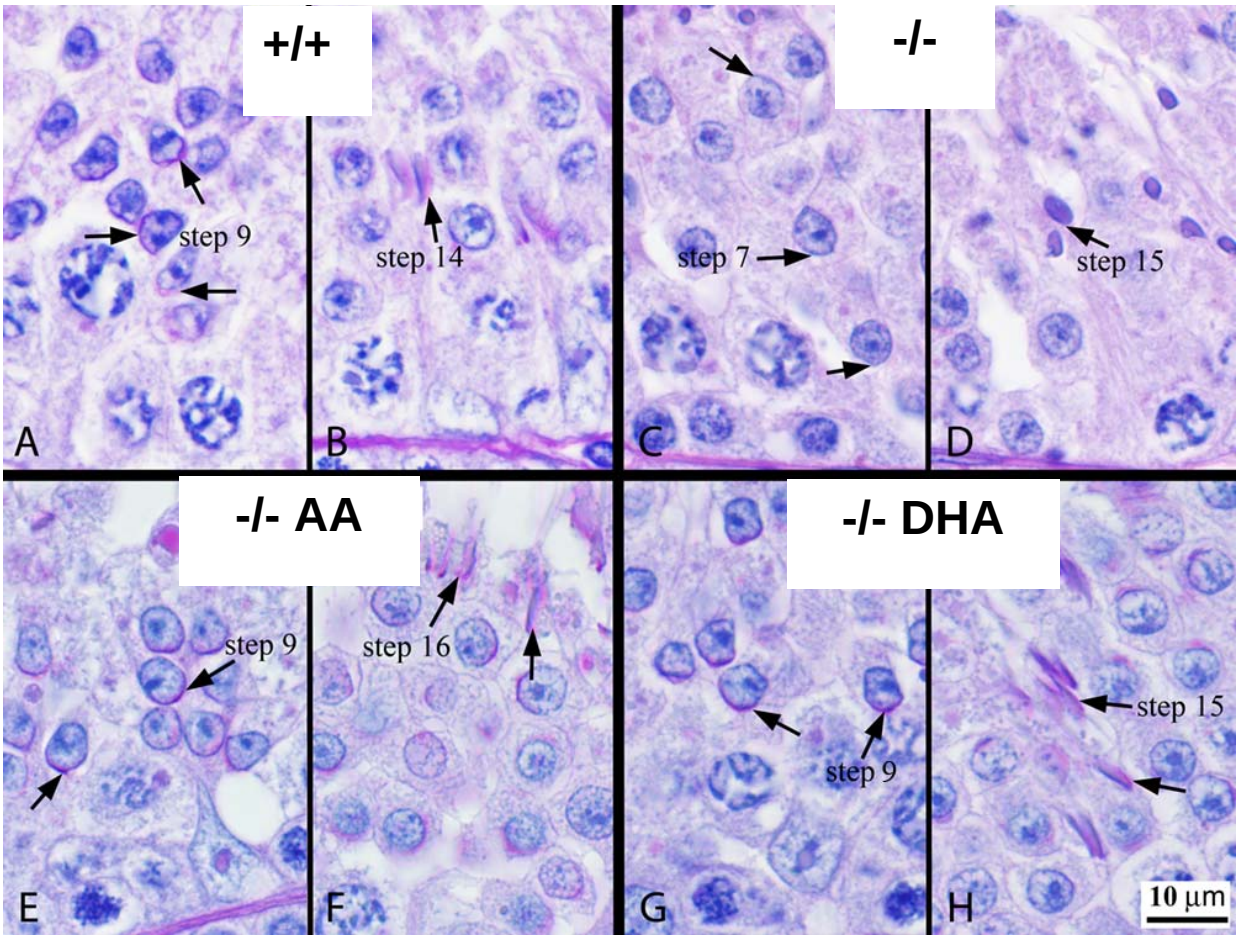

Figure 8.1 *Histology of testis stained with periodic acid-Schiff (PAS) to detect acrosome in wild type (+/+) and delta-6 desaturase knockout (-/-). Only HUFA supplemented -/- mice fed either arachidonic acid (-/-AA) or docosahexaenoic acid (-/-DHA) present positive PAS staining. Arrows indicate acrosome staining absent in HUFA deficient D6D-/-.*

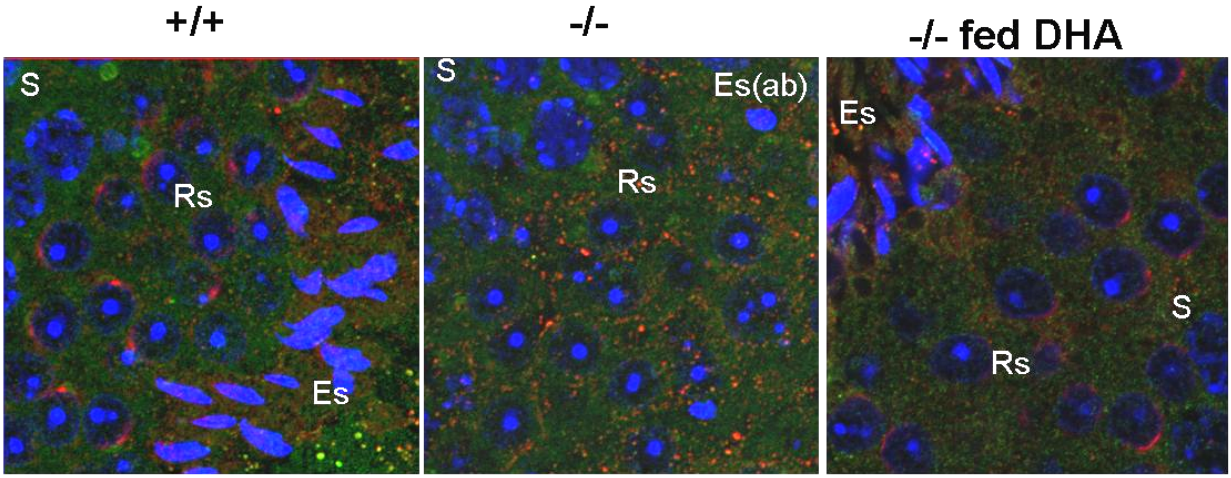

Figure 8.2 *Immunohistochemistry of acrosin (red), Golgi (green), nuclei (blue). +/+ mouse presents acrosin as cap-like structure on nuclear envelope of round spermatids (Rs) with Golgi above acrosin; elongated spermatids (S) are present. D6D-/- presents acrosin as dot-like pattern, but does not form cap-like structure indicating failed acrosome formation; spermatids to not develop elongated head and are abnormal (Es(ab)). DHA supplementation restores acrosin cap-like staining as well as formation of Es.*

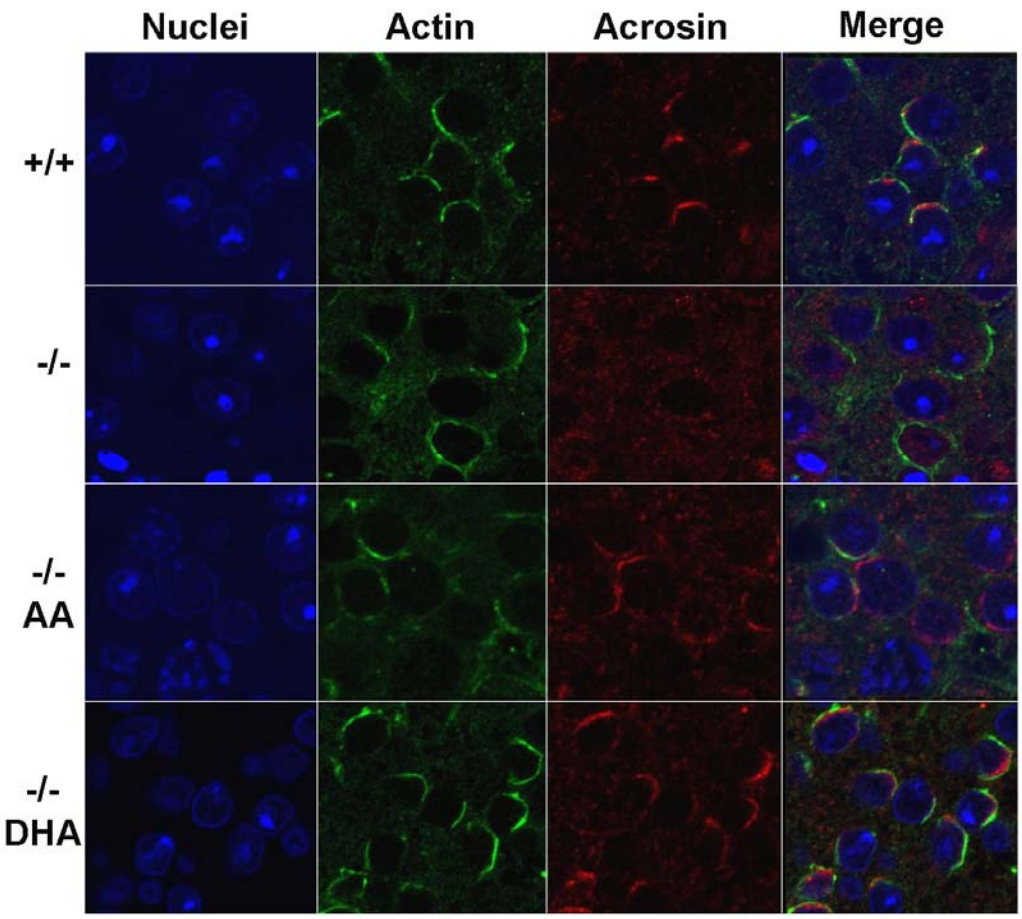

Figure 8.3 *Immunohistochemistry of nuclei (blue), Golgi (green), acrosin (red). The acrosomal marker acrosin stains as a cap-like structure on nuclear envelope of round spermatids in +/+ and DHA supplemented -/- mice (DHA-/-); D6D-/- lacks acrosomal structure with acrosin dispersed near nucleus; AA supplementation restores acrosomal cap-like structure although in fewer cells and in some cases seems fragmented. Actin is not disturbed by HUFA deficiency.*

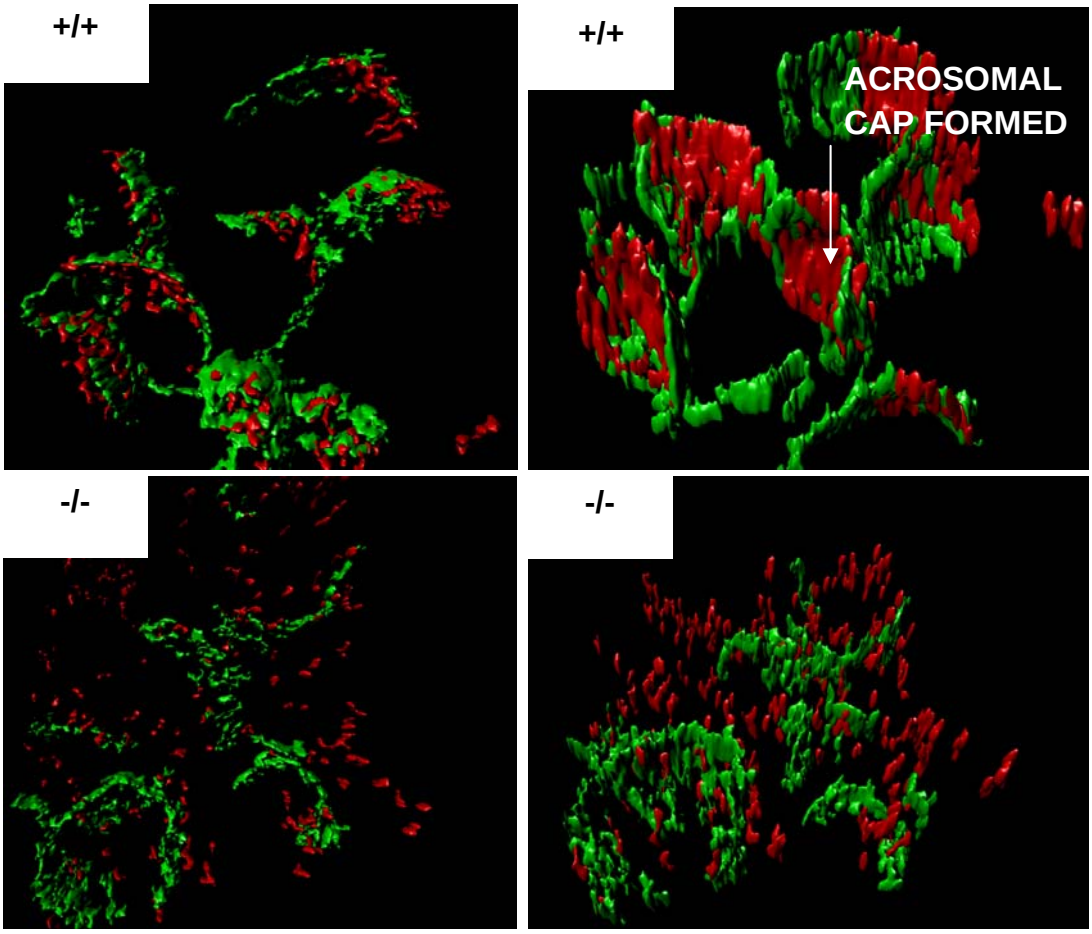

Figure 8.4 *Immunohistochemistry. Three dimensional rendering of acrosin (red) and actin (green) stain of wild-type (+/+) and knockout (-/-) round spermatids. Left column shows a top view with acrosin as arc-like structure in +/+, while dispersed acrosin is in non-supplemented -/-. Right column is a side view of acrosomal cap structures in +/+ which are not formed in -/-.*

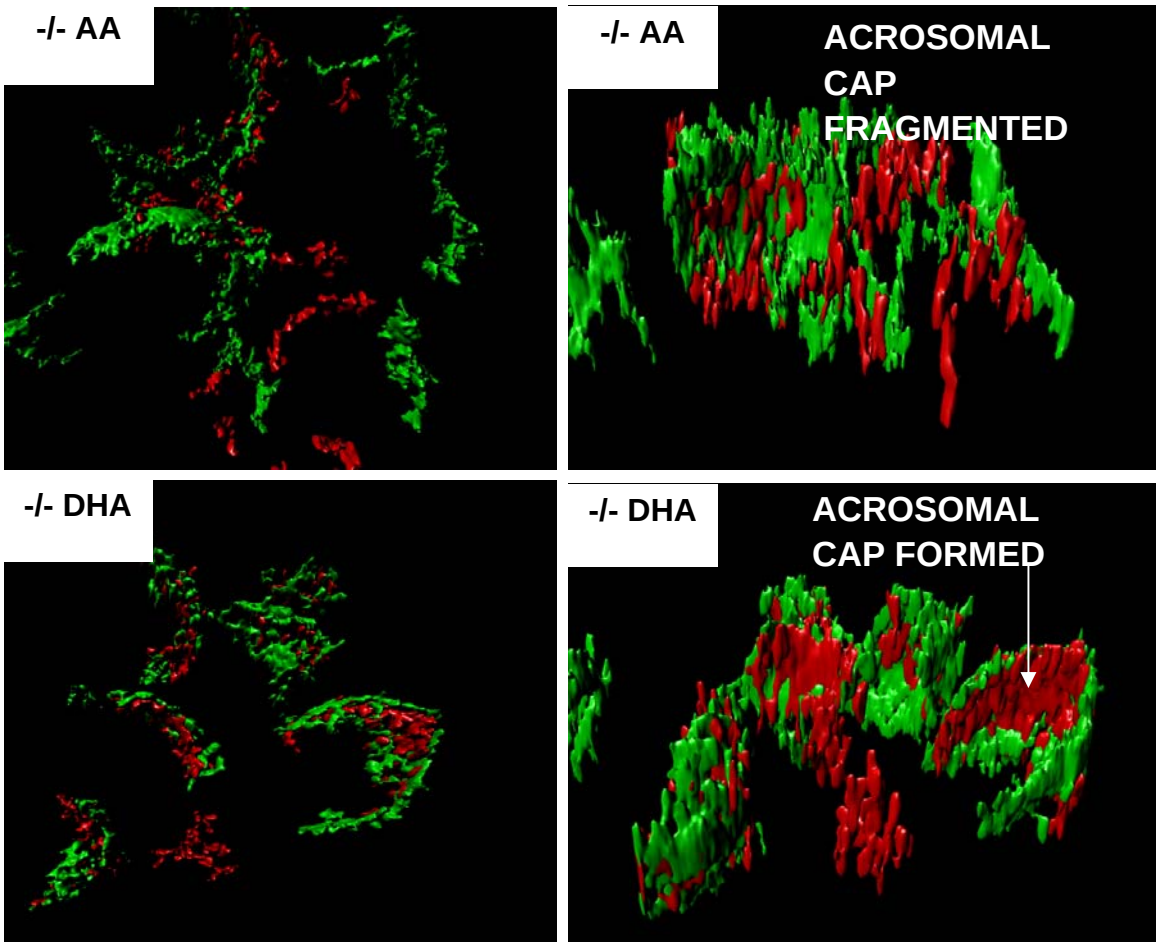

Figure 8.5 *Immunohistochemistry. Three dimensional rendering of acrosin (red) and actin (green) stain of round spematids from knockout (-/-) mice supplemented with AA or DHA. Left column shows a top view with acrosin as arc-like structures in both AA and DHA -/-. Right column is a side view of acrosomal cap structures which are fragmented with AA supplementation while DHA supplementation fully restores cap.*

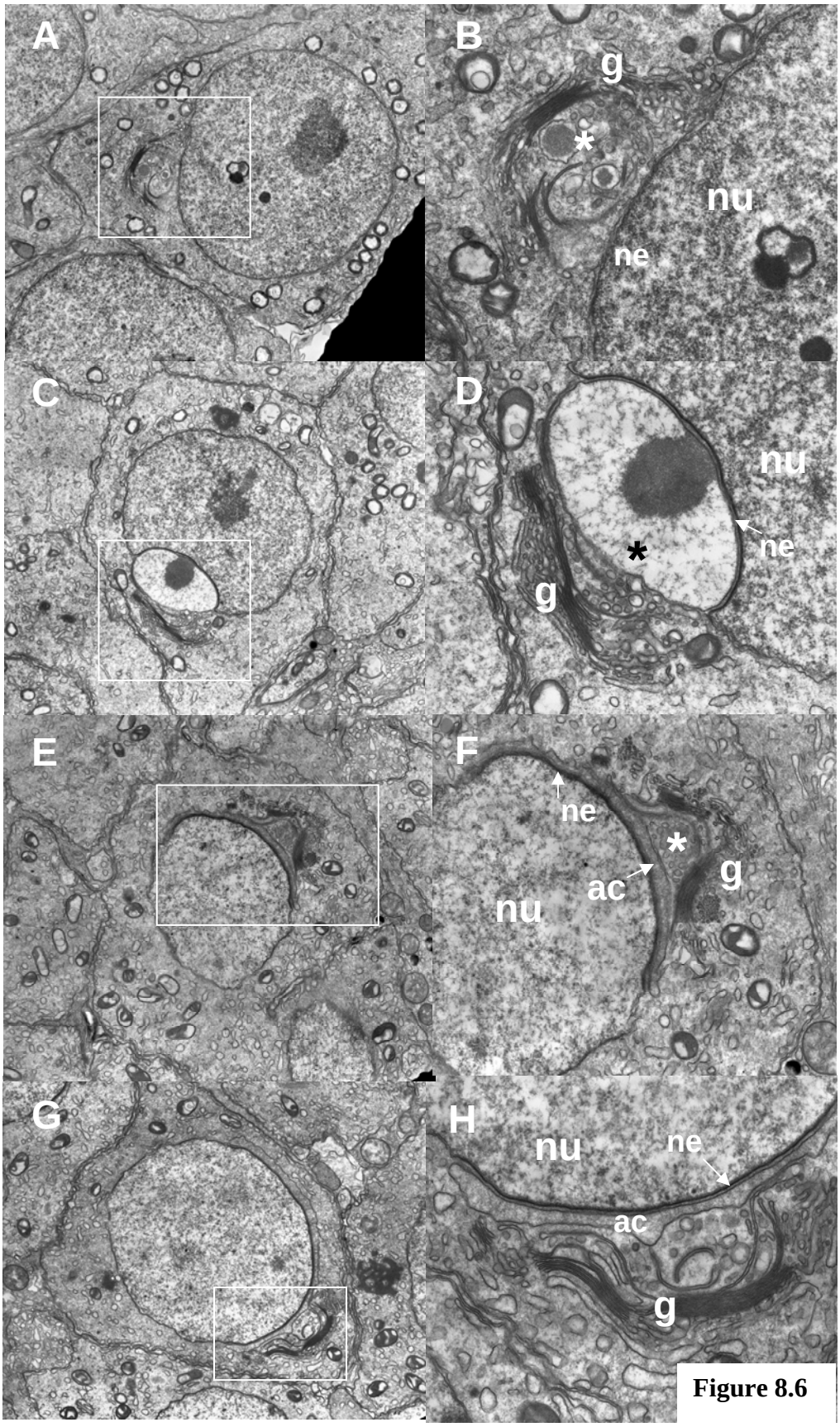

Figure 8.6

Figure 8.6 *Electron microscopy images of acrosome formation in +/+ testis. (A) Golgi phase round spermatid. (B) Higher magnification view of boxed region in A showing Golgi-derived proacrosomal vesicles and granules (*) before attaching to nuclear envelope. (C) Round spermatid with single acrosomal vesicle attached to nuclear envelope. (D) Higher magnification of boxed region in C showing attached vesicle (*). (E) Cap phase round spermatid with flattened acrosomal vesicle on darkened nuclear envelope or acroplaxome. (F) Higher magnification of boxed region in E showing vesicles (*) between Golgi and acrosomal cap. (G) Acrosome formed on +/+ round spermatid. (H) Higher magnification of G boxed region. n=nucleus, ne=nuclear envelope, g=Golgi, ac=acrosome.*

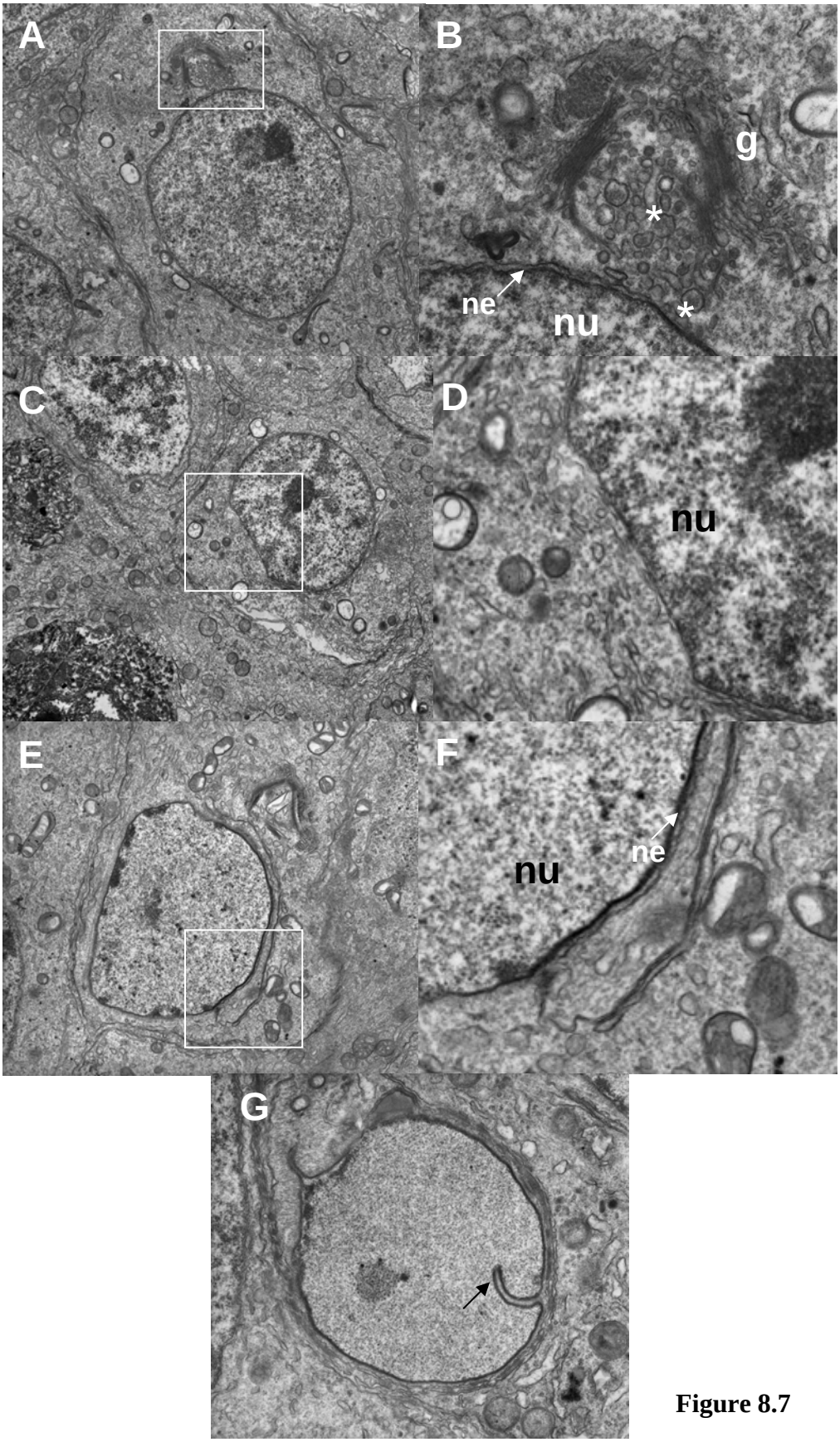

Figure 8.7

Figure 8.7 *Electron microscopy images of failed acrosome biogenesis in D6D-/- testis. (A) Golgi phase round spermatid. (B) Higher magnification view of boxed region in A with Golgi-derived proacrosomal vesicles and granules (*) before attaching to nuclear envelope. (C) Round spermatid with flattened region of nuclear envelope without acrosomal vesicle indicating disruption at Golgi phase. (D) Higher magnification of boxed region in C. (E) Round spermatid with darkened nuclear envelope acroplaxome but no attached acrosome. (F) Higher magnification of boxed region in E. (G) Round spermatid without acrosome and with nuclear indentation (black arrow). n=nucleus, ne=nuclear envelope, g=Golgi.*

REFERENCES

(1) Stroud, C. K., T. Y. Nara, M. Roqueta-Rivera, E. C. Radlowski, P. Lawrence, Y. Zhang, B. H. Cho, M. Segre, R. A. Hess, J. T. Brenna, W. M. Haschek, and M. T. Nakamura. 2009. Disruption of FADS2 gene in mice impairs male reproduction and causes dermal and intestinal ulceration. *J. Lipid Res.* 50: 1870-80.

(2) Roqueta-Rivera, M., C. K. Stroud, W. M. Haschek, S. J. Akare, M. Segre, R. S. Brush, M. P. Agbaga, R. E. Anderson, R. A. Hess, and M. T. Nakamura. 2010. Docosahexaenoic acid supplementation fully restores fertility and spermatogenesis in male delta-6 desaturase knockout mice. *J. Lipid Res.* 51: 360-7.

(3) Holstein, A. F., C. Schirren, and C. G. Schirren. 1973. Human spermatids and spermatozoa lacking acrosomes. *J. Reprod. Fertil.* 35: 489-491.

(4) Kang-Decker, N., G. T. Mantchev, S. C. Juneja, M. A. McNiven, and J. M. van Deursen. 2001. Lack of acrosome formation in Hrb-deficient mice. *Science* 294: 1531-1533.

(5) Yao, R., C. Ito, Y. Natsume, Y. Sugitani, H. Yamanaka, S. Kuretake, K. Yanagida, A. Sato, K. Toshimori, and T. Noda. 2002. Lack of acrosome formation in mice lacking a Golgi protein, GOPC. *Proc. Natl. Acad. Sci. U. S. A.* 99: 11211-11216.

(6) Dam, A. H., I. Feenstra, J. R. Westphal, L. Ramos, R. J. van Golde, and J. A. Kremer. 2007. Globozoospermia revisited. *Hum. Reprod. Update* 13: 63-75.

(7) Reeves, P. G., F. H. Nielsen, and G. C. Fahey. 1993. AIN-93 purified diets for laboratory rodents: Final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* 123: 1939-1951.

(8) Leblond, C. P., Y. Clermont. 1952. Definition of the stages of the cycle of the seminiferous epithelium in the rat. *Ann. N. Y. Acad. Sci.* 55: 548-573.

(9) Ramalho-Santos, J., R. D. Moreno, G. M. Wessel, E. K. Chan, and G. Schatten. 2001. Membrane trafficking machinery components associated with the mammalian acrosome during spermiogenesis. *Exp. Cell Res.* 267: 45-60.

(10) Toshimori, K. 2009. Dynamics of the mammalian sperm head: modifications and maturation events from spermatogenesis to egg activation. *Adv. Anat. Embryol. Cell Biol.* 204: 5-94.

(11) Escalier, D., J. M. Gallo, M. Albert, G. Meduri, D. Bermudez, G. David, and J. Schrevel. 1991. Human acrosome biogenesis: immunodetection of proacrosin in primary spermatocytes and of its partitioning pattern during meiosis. *Development* 113: 779-788.

(12) Huang, W. P., H. C. Ho. 2006. Role of microtubule-dependent membrane trafficking in acrosomal biogenesis. *Cell Tissue Res.* 323: 495-503.

(13) Halenda, R. M., P. Primakoff, and D. G. Myles. 1987. Actin filaments, localized to the region of the developing acrosome during early stages, are lost during later stages of guinea pig spermiogenesis. *Biol. Reprod.* 36: 491-499.

(14) Kierszenbaum, A. L., L. L. Tres, E. Rivkin, N. Kang-Decker, and J. M. van Deursen. 2004. The acroplaxome is the docking site of Golgi-derived myosin Va/Rab27a/b- containing proacrosomal vesicles in wild-type and Hrb mutant mouse spermatids. *Biol. Reprod.* 70: 1400-1410.

(15) Xiao, N., C. Kam, C. Shen, W. Jin, J. Wang, K. M. Lee, L. Jiang, and J. Xia. 2009. PICK1 deficiency causes male infertility in mice by disrupting acrosome formation. *J. Clin. Invest.* 119: 802-812.

(16) Fasshauer, D., R. B. Sutton, A. T. Brunger, and R. Jahn. 1998. Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. *Proc. Natl. Acad. Sci. U. S. A.* 95: 15781-15786.

(17) Cao, D., K. Kevala, J. Kim, H. S. Moon, S. B. Jun, D. Lovinger, and H. Y. Kim. 2009. Docosahexaenoic acid promotes hippocampal neuronal development and synaptic function. *J. Neurochem.* 111: 510-521.

(18) Darios, F., B. Davletov. 2006. Omega-3 and omega-6 fatty acids stimulate cell membrane expansion by acting on syntaxin 3. *Nature* 440: 813-817.

(19) Litman, B. J., S. L. Niu, A. Polozova, and D. C. Mitchell. 2001. The role of docosahexaenoic acid containing phospholipids in modulating G protein-coupled signaling pathways: visual transduction. *J Mol Neurosci* 16: 237-42; discussion 279-84.

(20) Mazelova, J., N. Ransom, L. Astuto-Gribble, M. C. Wilson, and D. Deretic. 2009. Syntaxin 3 and SNAP-25 pairing, regulated by omega-3 docosahexaenoic acid, controls the delivery of rhodopsin for the biogenesis of cilia-derived sensory organelles, the rod outer segments. *J. Cell. Sci.* 122: 2003-2013.

(21) Ramalho-Santos, J., R. D. Moreno. 2001. Targeting and fusion proteins during mammalian spermiogenesis. *Biol. Res.* 34: 147-152.

(22) Rizo, J., T. C. Sudhof. 2002. Snares and Munc18 in synaptic vesicle fusion. *Nat. Rev. Neurosci.* 3: 641-653.

(23) Rickman, C., B. Davletov. 2005. Arachidonic acid allows SNARE complex formation in the presence of Munc18. *Chem. Biol.* 12: 545-553.

-

CHAPTER 9

PRELIMINARY IMMUNE SYSTEM CHARACTERIZATION OF DELTA-6 DESATURASE KNOCKOUT (D6D -/-) MOUSE

ABSTRACT

Highly unsaturated fatty acids (HUFA) are important components of membrane phospholipids of immune cells and are also precursors to signaling molecules which modulate immune function. The D6D-/- is unable to synthesize HUFA providing a model to further characterize the physiological roles of arachidonic acid (AA) and docosahexaenoic acid (DHA) in the immune system. Certain immune parameters of D6D-/- mice were characterized including an *in vivo* antibody response and cytokine production of splenocytes and macrophages. HUFA deficiency results in decreased antibody production ($p<0.05$). Splenocytes and macrophages are involved in the process leading to antibody formation and were therefore quantified and characterized by flow cytometry as well as in culture. Although, D6D-/- splenocyte counts, as well as CD3+, CD4+, and CD8+ T-cell subpopulations did not differ from +/+, a decrease in IL2 production was observed in HUFA deficient males. D6D-/- preliminary data suggests a decrease in F4/80+ peritoneal macrophage and increase for CD11b+ peritoneal cells 4 days after an inflammatory challenge with thioglycollate. In summary, the D6D-/- indicates HUFA essentiality for immune function, specifically antibody production, which cannot be prevented by the presence of the HUFA precursors LA and ALA. More work is required to elucidate the mechanism behind the requirement of HUFA in establishing an adequate immune response.

INTRODUCTION

The main highly unsaturated fatty acids (HUFA) present in tissues are arachidonic acid (AA) and docosahexaenoic acid (DHA) (1). AA and DHA can be found in membrane phospholipids of immune cells and are known to modulate immune function (2). AA participates in regulating immunity as a precursor to a variety of prostaglandins and leukotrienes, which are synthesized by immune cells such as monocytes, macrophages, mast cells, and neutrophils (3). Prostaglandin E2 (PGE2) is a major player in inflammation after tissue injury, increasing vasodilation and vascular permeability as well as participating in resolving inflammation. Leukotrienes participate in the inflammatory process favoring leukocyte chemotaxis and modulating neutrophil activity (4). DHA on the other hand is considered anti-inflammatory, decreasing inflammatory cytokine production (5) and driving resolution of inflammation as a precursor to docosanoids (6). Immune cell functionality is sensitive to membrane HUFA levels which can be modified based on the composition of fatty acids consumed in the diet (7). It is therefore important to further elucidate the role of HUFA in immune function.

The D6D-/- mouse is an experimental model of HUFA deficiency which can be used to determine what aspects of the immune system have an absolute requirement for AA and DHA. The D6D-/- is unable to synthesize HUFA becoming AA and DHA deficient which leads to ulcerative dermatitis at 21 weeks of age (8). The immune system plays an active role in the initial inflammatory condition of skin lesions and its subsequent repair. HUFA derived signaling molecules participate in this process recruiting immune cells to the site of injury and resolving inflammation to achieve a return to tissue homeostasis. As mentioned previously, AA and DHA are known to modulate immunity and inflammation. A state of HUFA deficiency in D6D-/- may

therefore disrupt a proper inflammatory response and subsequent tissue repair, allowing skin lesions to persist. In addition to skin pathology, atrophy of thymus and enlargement of spleen was observed in D6D-/- mice with dermatitis (8). Spleen and thymus are important organs of the immune system; therefore, an impaired immune system due to HUFA deficiency was hypothesized. It was important to take into account the presence of ulcerative dermatitis and its possible contribution to spleen and thymus D6D-/- pathology. Thus, in order to establish if the D6D-/- immune system is altered due to HUFA deficiency, immune function parameters needed to be tested before the development of dermatitis in D6D-/-.

In this study, immune function of D6D-/- was evaluated by measuring the ability of the mouse to develop antibody in response to a T-cell dependant antigen as well as attempting to characterize splenocytes, thymocytes, and macrophage. Both spleen and thymus are sites in which several types of immune cells interact to develop an adequate antibody response. Macrophages and dendritic cells participate as antigen presenting cells interacting with T-cells. T-cells then differentiate into helper T-cells which are needed for activation of B-cells to drive antibody production. Several steps involving different types of immune cells are required to produce antibody.

The essentiality of HUFA in the immune system was confirmed in the D6D-/- mice which all present impaired antibody production (**Chapter 4**). The mechanism behind the HUFA requirement for adaptive immune response is yet to be determined. Impairment of functionality may occur in any cell involved in the process leading up to antibody production, which include lymphocytes and macrophages. In order to determine the role of HUFA in antibody production, D6D-/- T-cell populations were characterized by flow cytometry and interleukin-2 (IL-2) production of cultured splenocytes was quantified. Peritoneal macrophages were also attempted

to be characterized by flow cytometry and functionality was evaluated in culture by measuring tumor necrosis factor alpha (TNFα) production upon stimulation with lipopolysaccharide (LPS). Preliminary data indicates a requirement for HUFA in maintaining an adequate immune response, specifically antibody production; however more studies are required to establish the underlying mechanism behind this essentiality.

METHODS

Animal studies

All animal work was approved by the University of Illinois Institutional Animal Care and Use Committee. C57BL/6J-129S6/SvEvTac mixed strain male and female mice of +/+ and -/- genotype were used for all assays except peritoneal macrophage studies, which used pure strain C57BL/6J.

Diets

At weaning, +/+ and -/- females (n=3) and males (n=3-6) were given AIN93G diet and water *ad libitum*.. Animals were euthanized at 4 months of age. A HUFA supplemented group (n=3) of female +/+ and -/- were fed AIN93G diet with 0.4% (w/w) AA (ARASCO, Martek Biosciences, Columbia, MD) from weaning until 8 months of age.

Tissue collection and histology

At euthanasia, mice were weighed, bled from the retro-orbital vein and then euthanized by carbon dioxide inhalation. Blood collected was left at room temperature to allow clotting and subsequently refrigerated for clot shrinking in order to collect sera used for the antibody response assay. Sera was frozen until needed.

Tissues collected at euthanasia were spleen and thymus, which were removed and weighed. Splenocytes and thymocytes were collected from male and female mice. Briefly, spleen and thymus were teased with forceps in Petri dish with one milliliter RPMI1640 medium, complete with 10% fetal bovine serum, which was then transferred to 9ml of RPMI1640 to obtain cell suspensions which were spun down, washed, and resuspended to a final concentration of $2x10^7$ cells per milliliter of RPMI1640. These cell suspensions were used for thymocyte and splenocyte counts by hemacytometer, for splenocyte cytokine production assay, and for splenocyte flow cytometry.

In a separate study, half the spleen was frozen for HUFA analysis while the other half was fixed in 10% neutral buffered formalin for histological evaluation. Tissues were trimmed for paraffin embedding. Sections were cut at 3 microns and stained with hematoxylin and eosin for histological evaluation. Bone marrow was collected from femur with phosphate buffered saline using a syringe. A cytospin slide was prepared from the bone marrow cell suspension and evaluated for myeloid and erythroid precursors.

Spleen HUFA analysis

Total lipids were extracted from spleen using the Folch method (9). C17:0 phosphatidylcholine was added as an internal standard. Fatty acids extracts were methylated with methanolic HCl (Supelco, Bellefonte, PA) at 75ºC for 90 minutes. Fatty acid methyl esters were identified by HP5890 gas chromatography (Agilent Technologies, Wilmington, DE) using a 30 m x 0.25 mm Omegawax capillary column (Supelco, Bellefonte, PA) at 180ºC for 2 minutes followed by a 3ºC gradient until a final temperature of 230ºC for 35 minutes.

Antibody response to a T-cell dependant antigen

Mice were challenged with 100 micrograms of a soluble T-cell dependant antigen, keyhole limpet haemocyanin (KLH), injected intraperitoneally eight days before termination. Serum was collected at euthanasia and assayed for antibodies specific to KLH by ELISA, as described previously with slight modifications (10). Briefly, 96 well Immulon 2HB microtiter assay plates (Thermo scientific #3455, Pittsburgh, PA) were coated with KLH (10microgram/milliliter solution in coating buffer), 50 microliters per well. Plates were incubated overnight at 4ºC. Wells were blocked the next morning for 1 hour at room temperature with 100 microliters of PBS with 0.5% bovine serum albumin and tween20. Wells are washed three times with PBS tween. Serial dilution of +/+ and D6D-/- sera samples were distributed in wells and incubated for 2 hours at room temperature. Wells were then washed five times and incubated for 1 hour with enzyme horseradish peroxidase conjugated to goat anti-mouse (H+L) at a dilution of 1:4000. Wells were then washed seven times. The developing reagent, 100 microliters of substrate ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]- diammonium salt) was added to each well. Color development was measured with spectrophotomer at 450nm. Serum titer was defined as the dilution of sample giving a measurement equal to 25% of a maximum reponse to KLH obtained from a positive control.

Splenocytes

a) Flow cytometry

Splenocytes ($1x10^6$ cells) were stained with anti-mouse fluorescent antibodies: APC-hamster anti-mouse CD3-epsilon (clone 145-2c11; BD bioscience, San Jose, CA), FITC conjugated anti-mouse CD4 (clone YTS191.1; Immunotech), and PE conjugated anti-mouse

CD8α (clone KT15; Beckman Coulter; Miami, FL) in order to evaluate T-cell populations. Staining was done as described previously with slight modifications (11). Briefly, cell suspensions of 50 microliters ($1x10^6$ splenocytes) were washed with one milliliter PBS azide in 10% fetal bovine serum. Cells were spun down (1500rpm for 5 minutes) and supernatant removed. Ten microliters of each labeled antibody were added to cells, incubated for 30 minutes, and then washed twice with 1ml of PBS azide10% fetal bovine serum. Cells were resuspended with 450 microliters of PBS azide, fixed with 50 microliters of 1% paraformaldehyde, and kept on ice until evaluation with flow cytometry was done on a BD Biosciences LSR II flow cytometer.

b) Cytokine production

Splenocytes ($2x10^6$ per milliliter) in complete RPMI1640 are distributed in a 24-well plate (BD falcon tissue-culture treated polystyrene, flat-bottom #353047, San Jose, CA) at 0.4 milliliter per well; and stimulated with 0.4 milliliter concavalin-A (2.5μg/ml). Supernatants were collected after 48 hours of incubation at 37ºC and assayed by ELISA to quantify cytokine production of IL-2 according to instructions of BD OptEIA Set Mouse IL-2 kit (BD Biosciences, #2614kI San Jose, CA).

Peritoneal macrophage

a) Flow cytometry

Peritoneal lavage cell suspensions ($2x10^6$ per milliliter), from mice challenged with thioglycollate four days prior to euthanasia, were treated with lysis buffer to remove red blood cells. $1x10^6$ cells were stained with Alexa Fluor 488 conjugated anti-mouse F4/80 macrophage pan marker (ebioscience clone BM8, San Diego, CA) and PE conjugated anti-mouse CD11b

(ebioscience clone M1/70, San Diego, CA). Briefly, cells were washed with PBS, incubated with 2 microliters of blocking antibody CD16/32 for 5 minutes on ice, and then incubated with 2 microliters of fluorescent antibody for 30 minutes in the dark on ice. Cells were washed twice and resuspended in 500 microliters of PBS and transferred to 5 milliliter polystyrene round bottom tubes with cell strainer cap (BD Falcon #352235, San Jose, CA) to eliminate potential clumps. Flow cytometry was done on an iCyt flow cytometer.

b) Macrophage cytokine and nitric oxide production

From +/+ and -/- cell suspensions at $2x10^6$cells/ml, peritoneal macrophages were distributed in a 96 well plate at $1x10^5$cells/well. Cells were incubated overnight at 37ºC. The following day, cells were treated by triplicate either with medium, interferon-gamma (IFNγ), LPS, or a combination of IFNγ and LPS. After 48 hour incubation with treatment, supernatants were collected and assayed for nitric oxide and TNFα production (Mouse Ready-SET-Go ELISA Kit, ebioscience #887324, San Diego, CA). Nitric oxide assay is based on the Griesse reaction in which nitrite is quantified by color development at absorbance of 550nm. Cells attached to plate were measured by Cyquant cell proliferation assay (Invitrogen add #C7026, Carlsbad, CA).

Statistical Analyses

Significant differences between groups were determined by Student's t-test.

RESULTS

Spleen histology indicates myeloid hyperplasia in D6D-/- mouse before dermatitis

HUFA deficiency in D6D-/- results in ulcerative dermatitis at 21 weeks of age and is accompanied by splenomegaly and thymic atrophy (8). Since the severe dermatitis may exacerbate spleen and thymus pathology, D6D-/- mice immune parameters were evaluated before development of dermatitis at 16 weeks of age. D6D-/- spleen and thymus weights as well as cell counts were not significantly different from +/+ in both males and females (**Tables 9.1 and 9.2**). However, histology of D6D-/- spleen showed myeloid hyperplasia, an increase in red pulp (**Figure 9.1**), and significantly higher ($p<0.05$) number of neutrophils (**Table 9.3**). This data fits with bone marrow evaluation of myeloid precursors where a significant increase ($p<0.05$) of the myeloid to erythroid ratio was observed in D6D-/- (**Table 9.3**). Splenic myeloid hyperplasia is prevented with AA supplementation at 0.4% (**Figure 9.1**).

Spleen of D6D-/- is depleted of HUFA which can be restored with supplementation

AA (20:4 n-6) represents an important molar percentage in the +/+ spleen at 17% (**Figure 9.2A**). Non-supplemented D6D-/- spleen AA (20:4 n6) was depleted and non detectable (**Figure 9.2A**), while supplementation restored AA to 20.31% (**Figure 9.2B**). Spleen LA (18:2 n6) accumulated in the non-supplemented D6D-/- due to the absence of D6D enzyme. LA accumulation in D6D-/- was 177% of +/+. With AA supplementation, LA no longer accumulates in D6D-/- and is restored to +/+ levels. OA (18:1n9) also accumulated in D6D-/- at 173% of +/+; with AA supplementation OA was restored to +/+ levels. DHA depletion occurred in D6D-/- spleen with and without AA supplementation.

Antibody response is impaired in D6D-/- mouse

Antibody response, to the T-cell dependent antigen keyhole limpet hemocyanin (KLH), was compromised in 100% of 16 week old D6D-/- mice; both male and female (**Figure 9.3**). Titer of male +/+ was at 75.41±10.82 while D6D-/- was significantly lower ($p<0.05$) at 18.87±7.08. Female +/+ titer was at 58.56±32.87 while D6D-/- was at 15.37±5.14.

Spleen T-cell population and cytokine production is normal in D6D-/-

Spleen T-cells were characterized due to impaired T-cell dependant antibody response. T-cell CD3, CD4, and CD8 positive cell populations were characterized in spleen by flow cytometry (**Figure 9.4**). T-cells positive for CD3, a pan marker for subset of T-cells, was used to gate a population and determine percentages of CD3+CD4+ and CD3+CD8+ cells. Two well defined populations were observed in spleen. No significant differences were observed between cell percentages of +/+ and D6D-/-. T-cell functionality was tested by measuring cytokine production after a mitogen challenge in culture. The IL-2 cytokine is produced by T-cells for lymphocyte differentiation. Significant differences ($p<0.05$) were observed in IL-2 cytokine production in male mice, with +/+ producing 42.6 pg/ml versus 20.6 in D6D-/-; female mice however did not present a significant difference (**Table 9.4).**

Preliminary macrophage characterization of D6D-/- indicates differences in phenotype.

D6D-/- mice were submitted to an inflammatory challenge with a thioglycollate intraperitoneal injection 4 days prior to euthanasia (n=3). Peritoneal cells were collected and characterized. No significant differences were observed in number of peritoneal cells collected between +/+ and D6D-/-; $1.3x10^7 \pm 0.64$ cells from +/+ and $1.1x10^7 \pm 0.26$ from -/-. Flow cytometry evaluation of peritoneal cells from +/+ and -/- (n=2 respectively) by forward and side scatter showed a defined macrophage population in +/+; in -/-, this population was less defined

and with a higher forward scatter, indication of larger cell morphology (**Figure 9.5**). Population of cells containing macrophage was gated (circled region) based on F4/80 macrophage pan marker. Staining with the mature macrophage marker of F4/80 indicated a lower percentage of mature macrophage cells in D6D-/- (**Figure 9.6 and 9.8**). F4/80+ cells in +/+ were 87.18±1.45% while D6D-/- was at 75.72±3.18% of gated cells. The CD11b marker, a component of the complement receptor was higher in D6D-/- (**Figure 9.7 and 9.8**). CD11b+ cells were at 28.56±3.86% while D6D-/- was at 53.44±16.79% of gated cells.

Peritoneal cells from +/+ and -/- mice (n=3) were cultured and adhered macrophage were characterized for nitric oxide and TNFα production after stimulation with IFNγ and LPS, or a combination of both (**Figure 9.9**). Adherence was significantly lower in D6D-/- macrophage stimulated with LPS. Nitric oxide production was similar between +/+ and -/-. TNFα production was statistically higher ($p<0.05$) in D6D-/- when stimulated with IFNγ and LPS. TNFα production of +/+ was 1.1±0.1 ng/1000cells while D6D-/- produced 1.5±0.1 ng/1000cells.

DISCUSSION

This study characterized some of the immune parameters of D6D-/- at 16 weeks of age prior to the appearance of any skin pathology. The objective was to determine if the immune system is altered due to HUFA deficiency before development of dermatitis. D6D-/- mice with dermatitis at 21 weeks of age present splenomegaly and thymic atrophy (8). Before dermatitis, 16 week old D6D-/- mouse spleen and thymus weights, as well as splenocyte and thymocyte counts, were not significantly different from +/+ (**Table 9.1 and 9.2**) which suggests splenomegaly and thymic atrophy are most likely secondary to ulcerative dermatitis.

Although splenomegaly was not observed, closer histological evaluation of D6D-/- spleen indicated an increase in red pulp due to myeloid hyperplasia. The spleen is a lymphoid tissue composed of defined regions of red and white pulp. Neutrophils and red blood cells accumulate in the red pulp while lymphocytes predominate in the white pulp. The increase in red pulp in D6D-/- spleen correlates with increased neutrophil counts and bone marrow myeloid precursor cells (**Table 9.3)**. A possible explanation behind the increase of neutrophils may be related to D6D-/- pathology within the gastrointestinal tract (GI). GI lesions appear in D6D-/- by 6 weeks of age (**Chapter 4**). The severity of these lesions increased as the mice age and as the D6D-/- mice become further depleted of HUFA. Recruitment of inflammatory cells occurs in response to the presence of these lesions. Therefore an increased demand of neturophil production would occur to compensate for the persistent inflammatory condition in the intestine. HUFA may therefore be required for neutrophil homeostasis as well as resolving inflammation. The main HUFA in spleen is AA, at ~20% of total fatty acids. AA becomes depleted in D6D-/- spleen of 16 week old mice. Dietary AA restores this HUFA in D6D-/- spleen and prevents myeloid hyperplasia, demonstrating the importance of this fatty acid for spleen function. AA supplementation also prevents gastrointestinal ulcers (**Chapter 5**) further indicating a physiological role for AA in neutrophil homeostasis and tissue repair. The functional role of AA in this case is most likely as a precursor to signaling molecules such as prostaglandins and leukotrienes which have both been shown to modulate neutrophil chemotaxis (12), which is a critical process for inflammation and its resolution.

The observed pathology in spleen may impact its other functions within the immune system such as antibody production. Successful antibody production involves several immune cell types. Foreign proteins must first be ingested by cells of the innate immune system such as

macrophage and dendritic cells. The foreign protein or antigen is processed and then presented to T-cells as an MHC class II complex. T-cells recognize the MHC complex and then differentiate into CD4+ T-helper cells which activate B-cells to drive their transformation into plasma cells for antibody production. The white pulp of spleen is the site of B and T cell interactions required to develop this adaptive immune response (13). A decrease in white pulp in D6D-/- may therefore result in impaired antibody production.

We quantified antibody produced in response to a foreign soluble protein antigen, keyhole limpet hemocyanin (KLH) in D6D-/-. This antigen induced a T-cell dependant antibody response which was significantly decreased in 100% of HUFA deficient D6D-/- mice (**Figure 9.3**). This suggests an impaired immune system which may result from inefficient lymphocyte function. Splenocyte functionality was therefore assessed by measuring IL-2 cytokine production. IL-2 plays an important role in T-cell proliferation and differentiation into T-helper cells. Cytokine production of IL-2 was only significantly different in male D6D-/- (**Table 9.4**). The average amount of IL2 produced in female D6D-/- was lower compared to +/+, although did not reach significant difference. An increase in sample size may be required to reach a statistical difference for female mice. A decrease in IL-2 production may explain an impaired antibody response since this could disrupt T-cell differentiation into CD4+ T-helper cells which participate in the process leading up to antibody production. The T-cell and antigen presenting cell immunological synapse which leads to T-cell activation relies on membrane interaction and proper localization of proteins within lipid rafts. Fatty acid composition of lipid rafts as well as recruitment of signal transduction proteins can be modified by PUFA feeding in rodents (14,15). Specific HUFA deficiency may result in inadequate localization of signaling proteins within lipid rafts which then interferes with proper T-cell activation and IL-2 production.

Further splenocyte cytokine production quantification is required to obtain a better idea on how HUFA deficiency is altering T-cell functionality. The IL-4 cytokine would be an ideal end point since it is secreted by T-cells for B-cell activation.

In order to determine if HUFA deficiency modified T-cell subpopulations as a result of lower IL-2 production, flow cytometry was used to quantify CD3, CD4, CD8 receptors on splenocytes. CD3 is a marker for mature T-cell lymphocytes. CD4 is present on T-helper cells which activate B-cells to drive antibody production. CD8 is a marker for cytotoxic T-cells. Flow cytometry quantification of these populations resulted in the same scattergram profile (**Figure 9.4**) and population percentages between +/+ and D6D-/- spleen. Therefore, impaired antibody response in D6D-/- cannot be explained by altered expression of T-cell population markers in spleen.

Another immune cell which participates in the process leading up to antibody production is the macrophage. The macrophage plays an important role in antibody production through phagocytosis of foreign proteins and contributes as an antigen presenting cell. The macrophage is also involved in neutrophil homeostasis, which may be altered in D6D-/- as indicated by increased spleen neutrophils, peripheral blood neutrophil counts, and myeloid precursors in bone marrow. Macrophage is critical for neutrophil clearance by phagocytosis of apoptotic neutrophils resulting in a cascade of events leading up to neutrophil homeostasis (16). Macrophage phagocytosis of apoptotic neutrophils inhibits interleukin-23 (IL-23) production which signals bone marrow to stop myeloid precursor cell production and release into circulation (17). Impaired macrophage functionality may therefore lead to D6D-/- pathology of increased neutrophil counts and impaired antibody response.

Due to the potential role of macrophage in the development of D6D-/- pathology, preliminary data was gathered characterizing this immune cell. Peritoneal lavage cell suspensions containing macrophage after an inflammatory challenge of thioglycollate, were characterized by flow cytometry. Differences in forward and side scatter, which denote size and complexity of the cell, were observed in the D6D-/-. A less defined macrophage population was present in D6D-/- (**Figure 9.5**). The marker for macrophages F4/80 was decreased in D6D-/- (**Figure 9.6**), while the marker for CD11b (**Figure 9.7**) was increased. CD11b, together with CD18, form the complement receptor which participates in both chemotaxis and phagocytosis. These flow cytometry results may indicate a different stage of macrophage differentiation within the D6D-/-, 4 days post-inflammatory challenge. A time course study evaluating immune cell populations would help determine if HUFA deficiency alters the differentiation process of the macrophage, as well as the appearance of immune cell populations after an inflammatory challenge.

Peritoneal macrophages were cultured to evaluate their functionality. Macrophage adherence to plate was lower ($p<0.05$) in D6D-/- when cells were challenged with a 48 hour treatment of LPS. An AA requirement for adherence has been previously demonstrated (18). Macrophage cytokine production of TNFα was significantly increased ($p<0.05$) in D6D-/- when cells were treated with a combination of IFNγ and LPS. TNFα is an inflammatory cytokine produced mainly by macrophage and is involved in NFKb activation, stimulation of phagocytosis, and chemoattraction of neutrophils. The increase in TNFα production in D6D-/- parallels the increase in CD11b marker in the -/- which is also involved in chemotaxis and phagocytosis. DHA downregulates TNFα production (19), therefore, the lack of HUFA may

result in dysregulated inflammatory cytokine production by immune cells which could also partially explain increased neutrophil counts and altered macrophage phenotype.

Macrophage phagocytosis is a key function in the innate immune system; however, it also plays an important role in antibody production and neutrophil homeostasis. The role of PUFA in modulating phagocytosis has been previously shown in cultured macrophage treated with PUFA resulting in increased phagocytosis of unopsinized zymosan particles when compared to saturated fatty acid treatments (20)(21). Phospholipase A2, involved in the release of AA from membrane, is required for phagocytosis (22). The mechanism behind the requirement of AA in phagocytosis was proposed to be in membrane fusion of vesicles for pseudopod formation. Macrophage pseudopods consist of membrane projections that engulf the target particles prior to endocytosis. With phospholipase A2 inhibition, intracellular accumulation of vesicles occurs possibly due to lack of fusion with the forming pseudopod, resulting in lower phagocytic acitivity; AA treatment was shown to override phospholipase A2 inhibition reducing the number of intracellular vesicles (22). This mechanism is similar to the AA requirement in neurite outgrowth within neuroendocrine cells, which also relies on membrane fusion and extension (23). Interestingly, preliminary work with D6D-/- suggests only slight differences in peritoneal macrophage phagocytosis of opsonized zymosan and sheep red blood cells when compared to +/+. It is important to note a limitation of using cell culture for immune function characterization. Fetal calf serum added to cell culture medium contains 10% AA; the presence of this fatty acid in the medium may change the cell fatty acid profile of HUFA deficiency achieved *in vivo*, allowing lymphocytes to regain function. A cell culture system with serum free media may be preferable to evaluate immune function *ex vivo*.

Despite the limitation of arachidonic acid in fetal bovine serum used in cell culture, the impact of HUFA deficiency on macrophage lipid metabolism was demonstrated using the D6D-/- (24). Macrophage cholesterol biosynthesis is increased while antioxidant paraoxynase activity is decreased in peritoneal macrophages collected from D6D-/-; both parameters can be restored to +/+ levels with AA supplementation (24). An alteration in macrophage lipid metabolism by HUFA deficiency may therefore also contribute to alteration in immune functionality.

In summary, HUFA deficiency results in impaired antibody response which is indicative of an altered adaptive immune system. Spleen histology indicates a decrease in white pulp which may explain impaired antibody production. Although spleen cell counts were not different between +/+ and D6D-/-, we did not quantify individual immune cell populations such as B-cells. Increased spleen red pulp consisting mainly of higher counts of neutrophils, as well as increased myeloid precursors in bone marrow may be secondary to inflammation within GI or a result of altered neutrophil homeostasis. The macrophage is involved in both antibody production and neutrophil homeostasis through phagocytosis; therefore further characterization of D6D-/- macrophage functionality is necessary. This preliminary immune system characterization of the D6D-/- will help develop future studies to give insight on specific roles of AA and DHA for proper immune function.

TABLES AND FIGURES

Table 9.1 *Male spleen and thymus weights and cell counts of 16 week old mice.*

Males	Spleen		Thymus	
	+/+	-/-	+/+	-/-
Absolute weight (mg)	69±6	142±42	19±4	27±8
Cells ($x10^8$)	1.22±0.16	1.58±0.46	0.39±0.2	0.4±0.13

Mean ± SD. n=4-6.

Table 9.2 *Female spleen and thymus weights and cell counts of 16 week old mice.*

Females	Spleen		Thymus	
	+/+	-/-	+/+	-/-
Absolute weight (mg)	91±28	125±43	22±5	25±6
Cells ($x10^8$)	1.27±0.18	1.46±.77	0.83±0.1	0.58±0.52

Mean ± SD. n=3.

Table 9.3 *Spleen neutrophil count and bone marrow myeloid to erythroid (M:E) ratio in 16 week old D6D -/- mice. The spleen neutrophil count is an average counted in 10 fields using high power magnification.*

	Spleen Neutrophils (average/HPF 100x)		**Bone Marrow M:E ratio**	
	+/+	-/-	+/+	-/-
Male	2.8±1	7.9±2.6*	1.9±0.4	3.1±0.5*
Female	2.5±1	11.9±5.5*	1.3±0.4	2.2±0.3*

*Mean ± SD. *statistically different by Student's t-test ($p < 0.05$), compared to +/+. Male n=2-3; female n=2*

Table 9.4 *Spleen lymphocyte IL-2 cytokine production (picograms per ml) when stimulated with concavalin-A.*

	IL2 (pg/ml)	
	+/+	-/-
Males	42.6±8	20.6±10.5*
Females	27.5±4.4	21.9±9.7

*Mean ± SD. *Statistically different by Student's t-test ($p < 0.05$), compared to +/+. Male n=4-6; female n=2.*

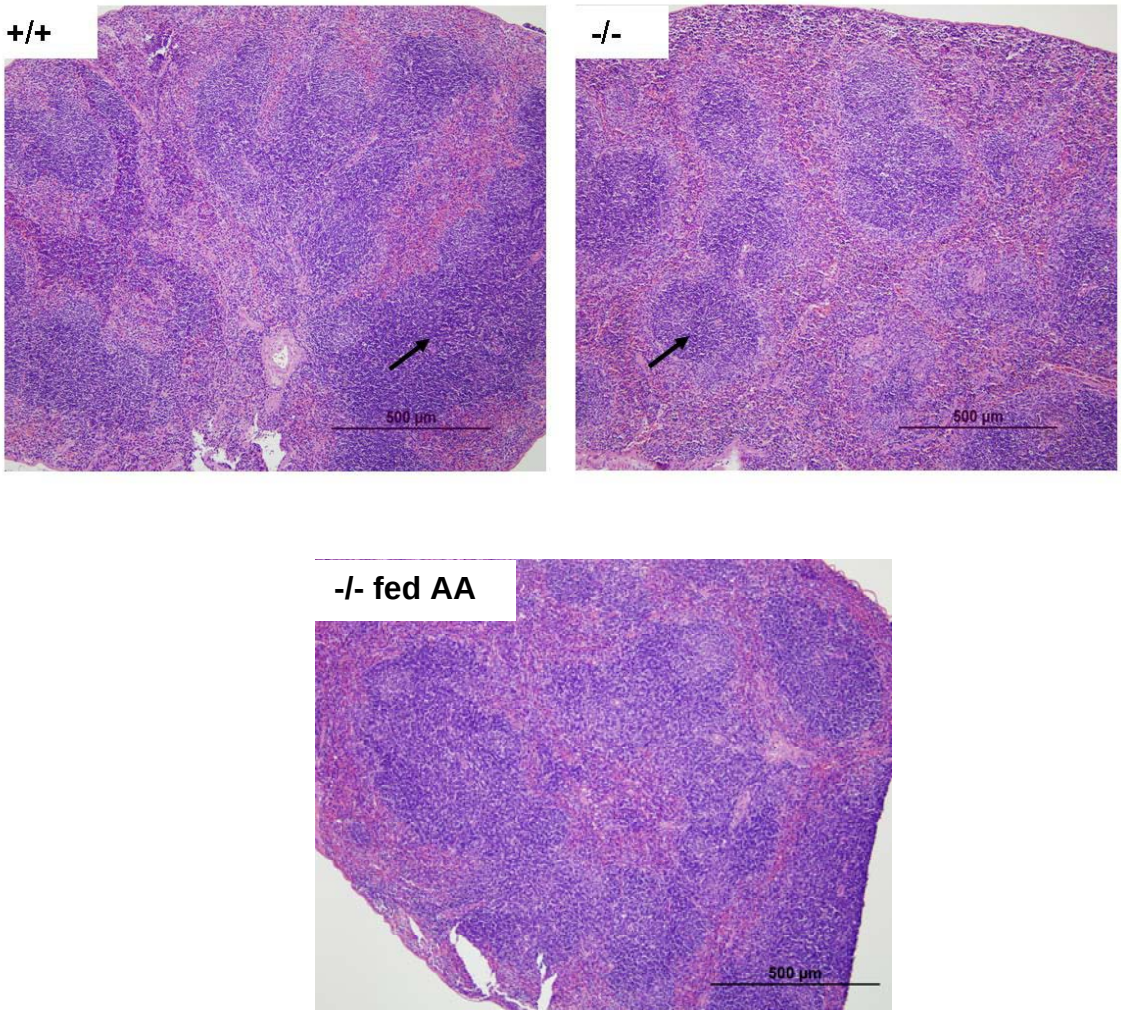

Figure 9.1 *Spleen histology (hematoxylin and eosin). Wild type mouse (+/+) shows normal distribution of red pulp and white pulp (arrows). D6D-/- has decreased white pulp (arrows). AA supplemented -/- shows normal distribution of white pulp similar to +/+.*

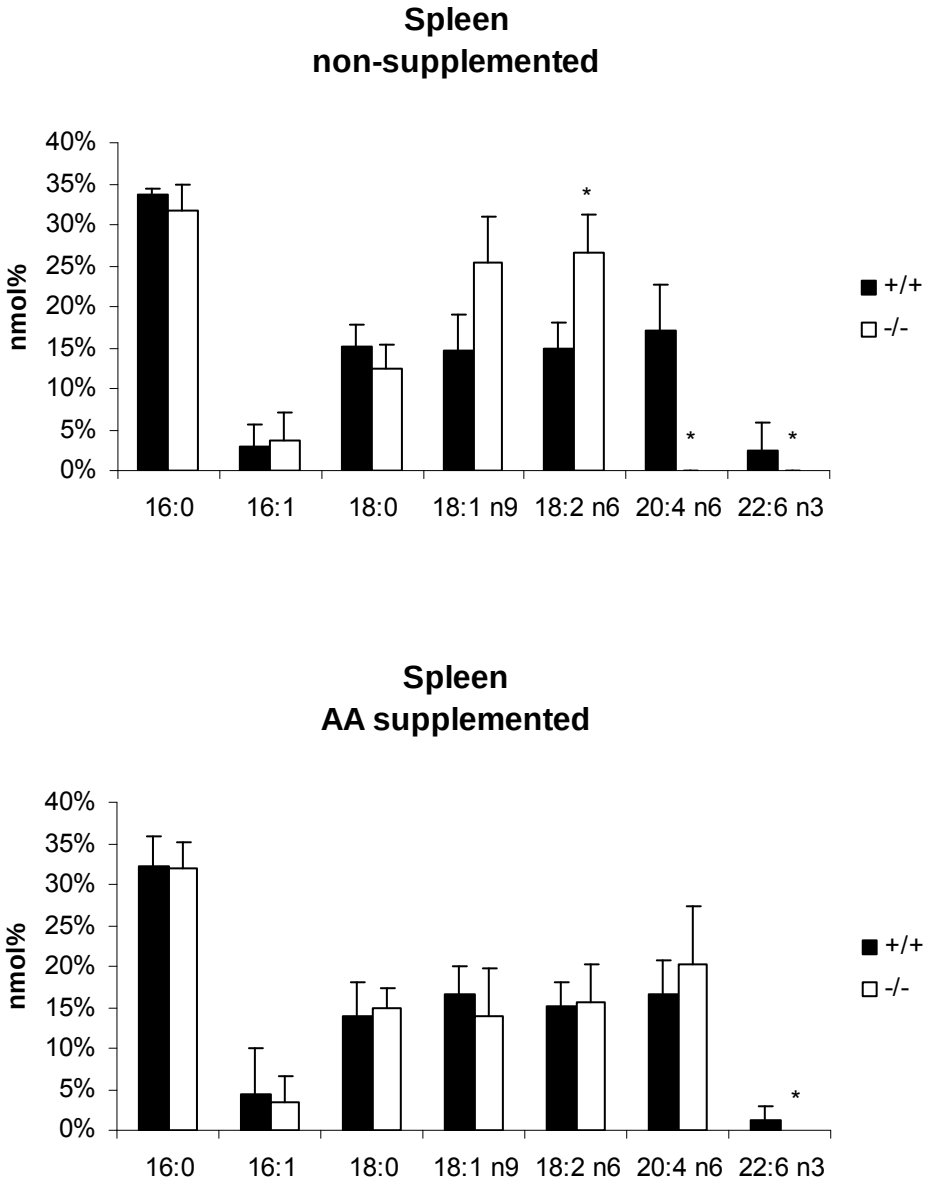

Figure 9.2 *D6D-/- spleen fatty acid analyses of female +/+ and -/- mice (n=3) with and without 0.4% (w/w) AA supplementation. Values represented as mean ± SD. n=3. **=p<0.05 as determined by Student's t-test, comparing +/+ versus -/-.*

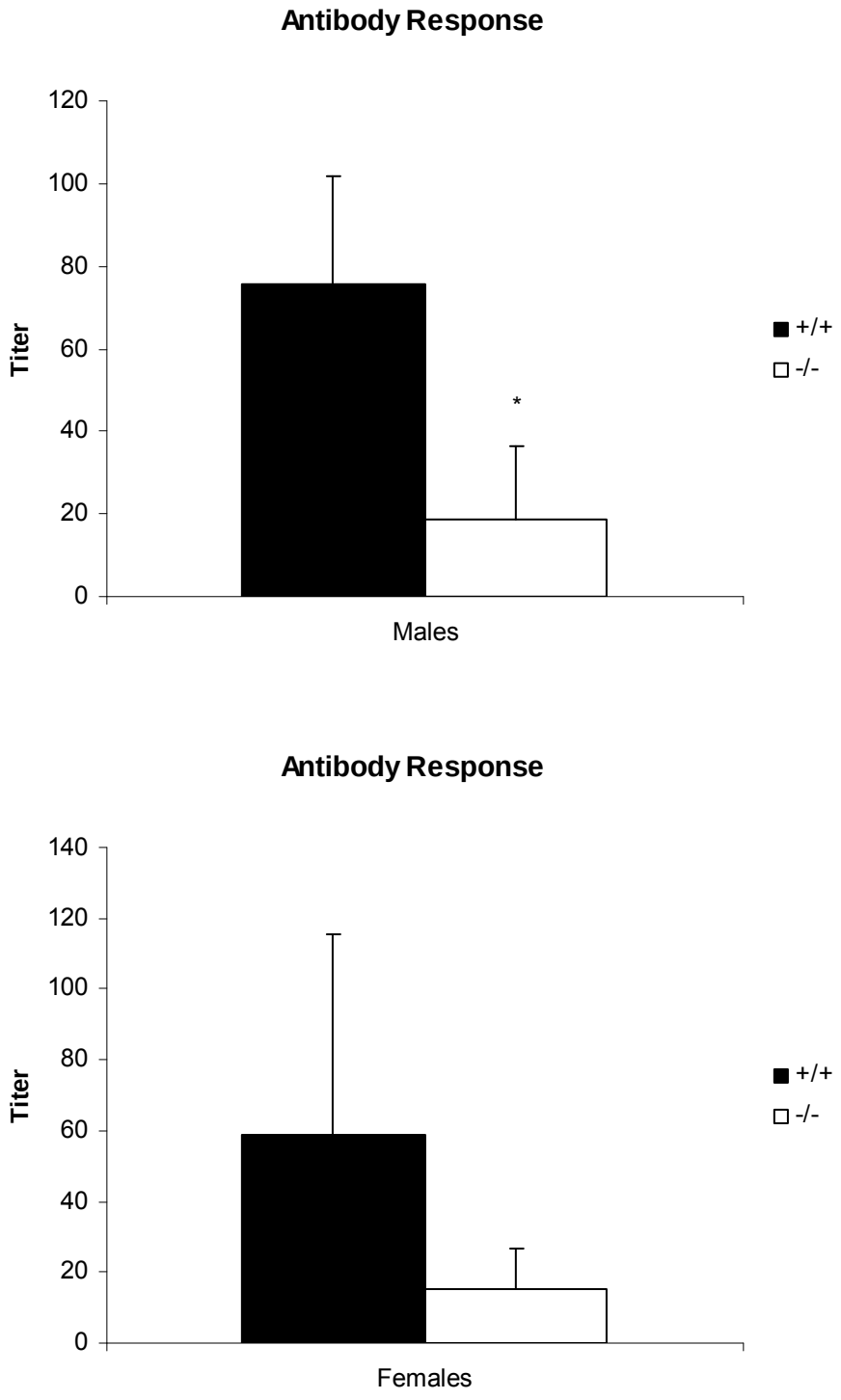

Figure 9.3 *Antibody response to a T-cell dependant antigen of KLH in (A) male (n=4-6) and (B) female (n=3) 16 week old mice. Mean ± SD. *= p<0.05 as determined by Student's T-test.*

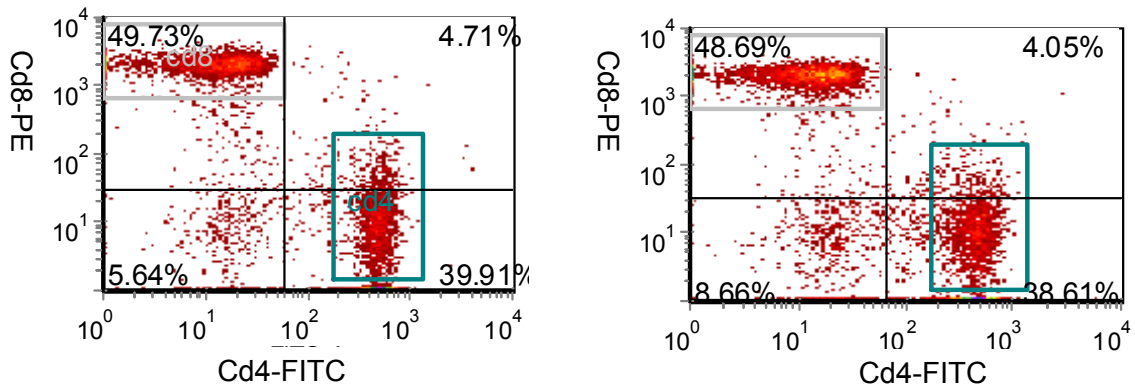

Figure 9.4 *A typical scattergram of spleen CD3 positive cells gated for CD4 and CD8 T-cell populations in +/+ and D6D-/- (n=3).*

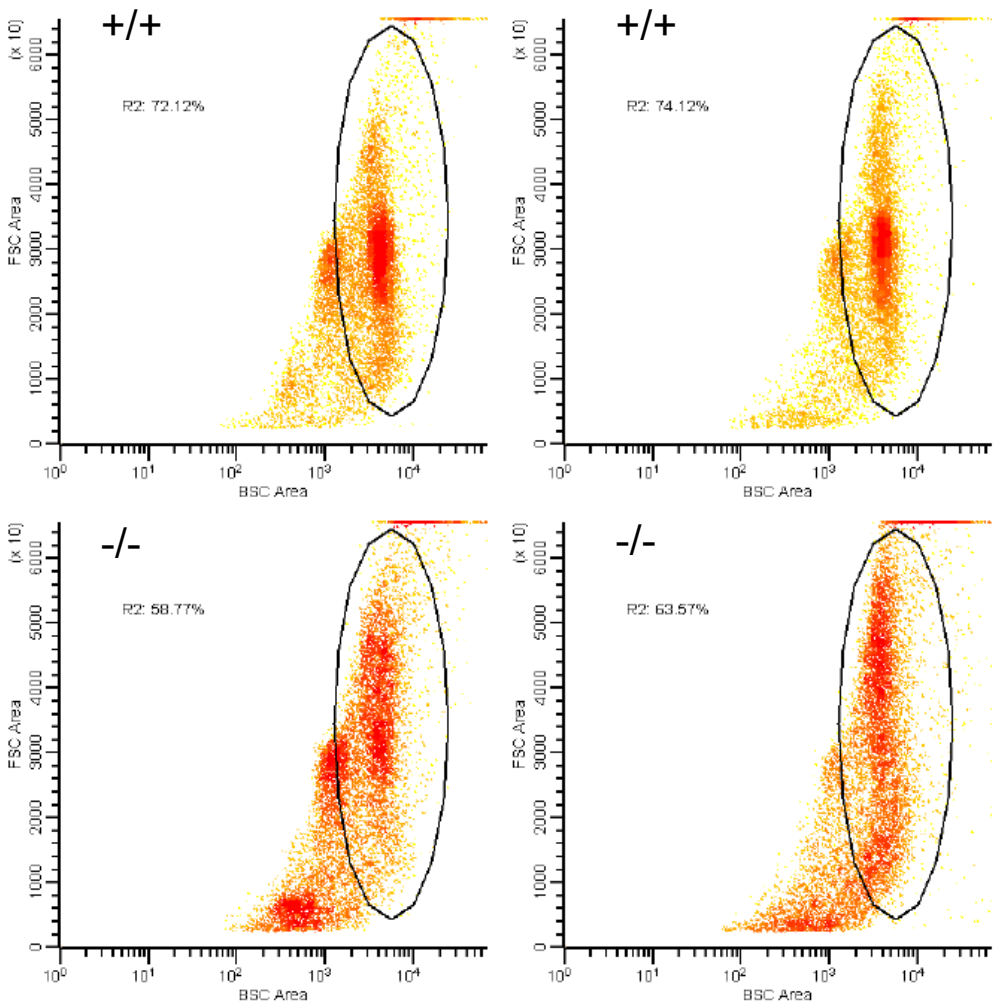

Figure 9.5 *Flow cytometry scattergram of +/+ and D6D-/- peritoneal cells 4 days post-injection with thioglycollate (n=2). Macrophage population is contained in encircled region. Population not defined in -/-.*

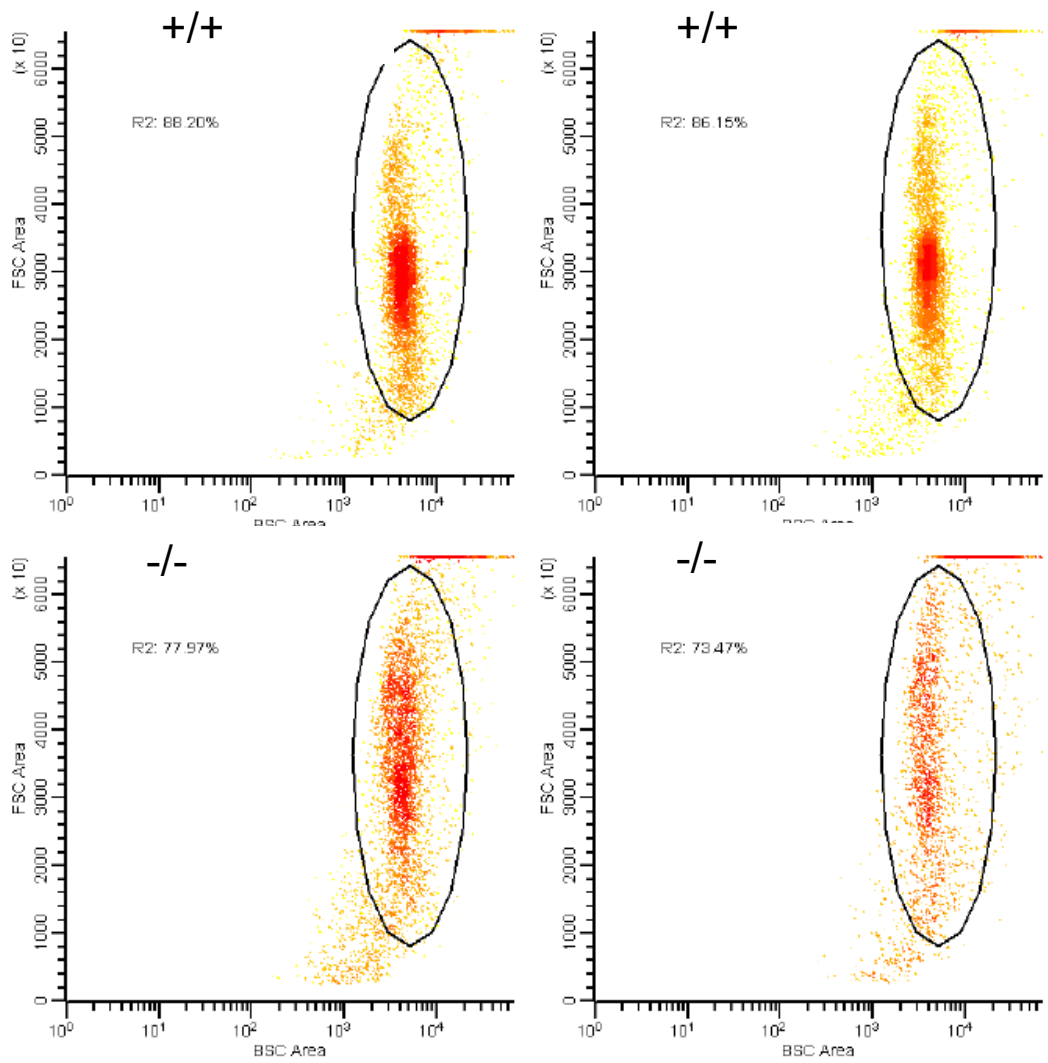

Figure 9.6 *Flow cytometry scattergram of +/+ and D6D-/- peritoneal cells positive for mature macrophage marker F4/80 (n=2). F4/80+ cells in D6D-/- have a less defined population and higher forward scatter, indication of larger size.*

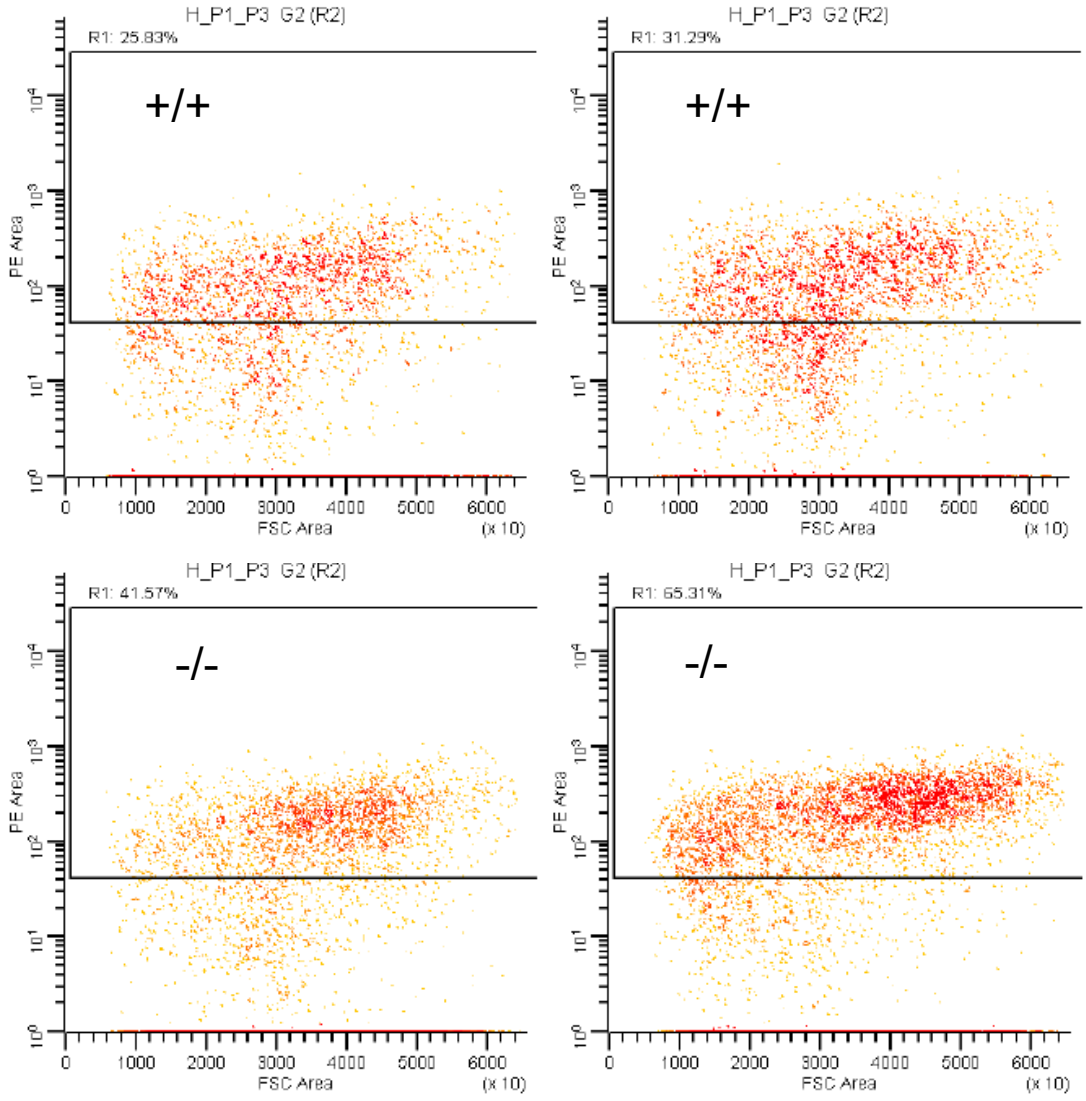

Figure 9.7 *Flow cytometry scattergram of +/+ and -/- (n=2) peritoneal cells positive for Cd11b, component of the complement receptor present of macrophage. Cd11b positive cells are in a higher percentage in D6D-/-.*

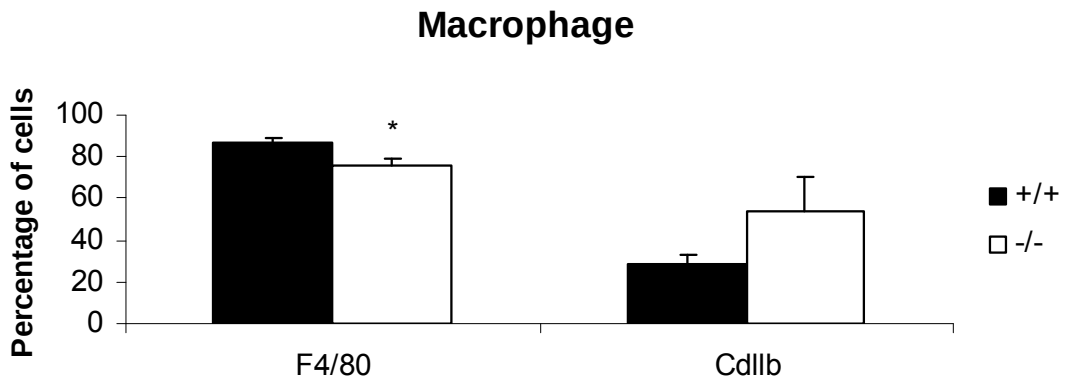

Figure 9.8 *Preliminary flow cytometry percentages of +/+ and -/- peritoneal cells positive for F4/80 or Cd11b. Mean±SD. n=2. *=p<0.05 statistically different according to Student's t-test.*

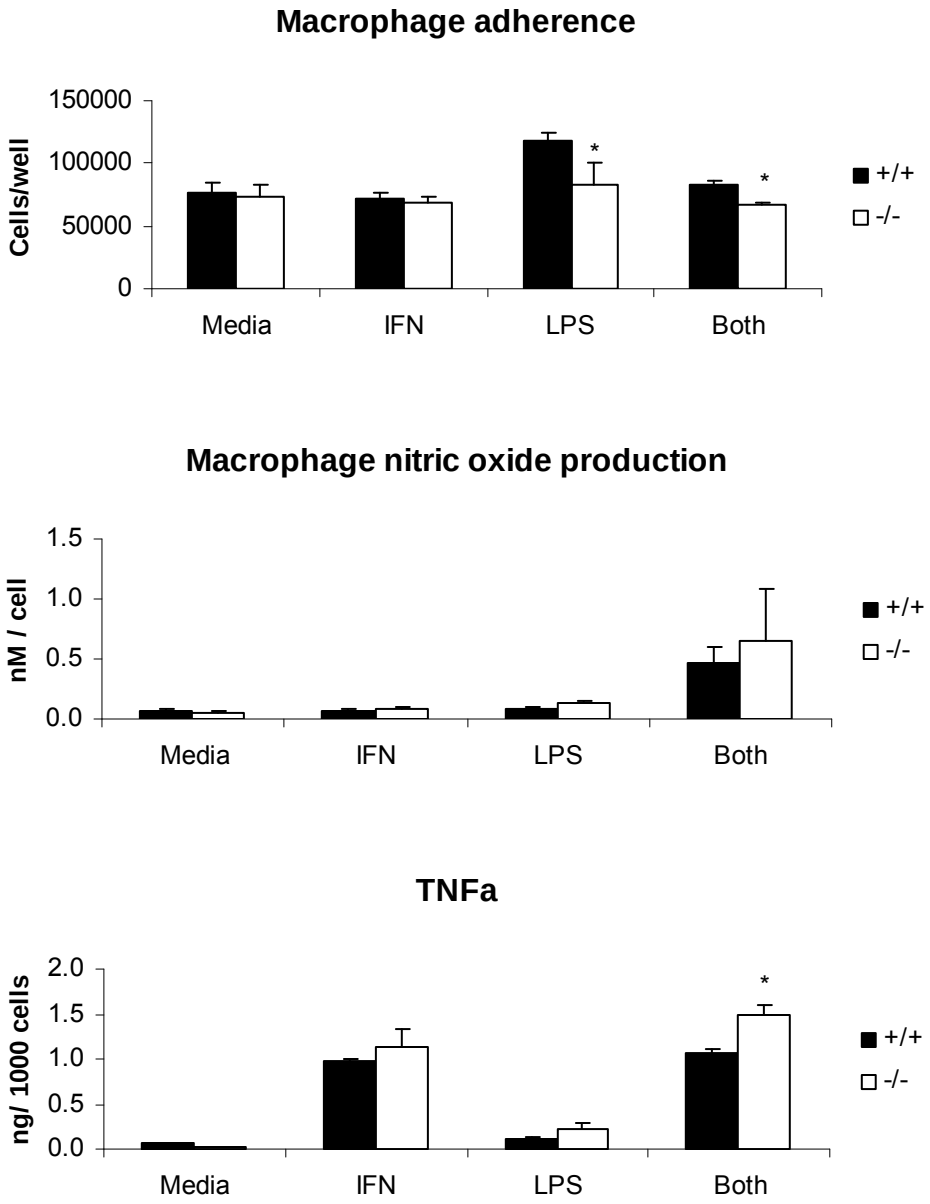

Figure 9.9 A*dherence, nitric oxide and TNFα production of cultured +/+ and -/- peritoneal macrophage (n=3) after a 48 hour treatment of either 10% FBS, IFNγ, LPS, or a combination of IFNγ and LPS (both). Mean±SD. *=p<0.05 statistically different according to Student's t-test.*

REFERENCES

(1) Nakamura, M. T., H. P. Cho, J. Xu, Z. Tang, and S. D. Clarke. 2001. Metabolism and functions of highly unsaturated fatty acids: an update. *Lipids* 36: 961-964.

(2) Calder, P. C. 2001. Polyunsaturated fatty acids, inflammation, and immunity. *Lipids* 36: 1007-24.

(3) Funk, C. D. 2001. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 294: 1871-5.

(4) Samuelsson, B. 1983. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science* 220: 568-575.

(5) Verlengia, R., R. Gorjao, C. C. Kanunfre, S. Bordin, T. M. de Lima, E. F. Martins, P. Newsholme, and R. Curi. 2004. Effects of EPA and DHA on proliferation, cytokine production, and gene expression in Raji cells. *Lipids* 39: 857-864.

(6) Serhan, C. N., R. Yang, K. Martinod, K. Kasuga, P. S. Pillai, T. F. Porter, S. F. Oh, and M. Spite. 2009. Maresins: novel macrophage mediators with potent antiinflammatory and proresolving actions. *J. Exp. Med.* 206: 15-23.

(7) Li, Q., M. Wang, L. Tan, C. Wang, J. Ma, N. Li, Y. Li, G. Xu, and J. Li. 2005. Docosahexaenoic acid changes lipid composition and interleukin-2 receptor signaling in membrane rafts. *J. Lipid Res.* 46: 1904-1913.

(8) Stroud, C. K., T. Y. Nara, M. Roqueta-Rivera, E. C. Radlowski, P. Lawrence, Y. Zhang, B. H. Cho, M. Segre, R. A. Hess, J. T. Brenna, W. M. Haschek, and M. T. Nakamura. 2009. Disruption of FADS2 gene in mice impairs male reproduction and causes dermal and intestinal ulceration. *J. Lipid Res.* 50: 1870-80.

(9) Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 226: 497-509.

(10) Wu, P. J., E. H. Greeley, L. G. Hansen, and M. Segre. 1999. Immunological, hematological, and biochemical responses in immature white-footed mice following maternal Aroclor 1254 exposure: A possible bioindicator. *Arch. Environ. Contam. Toxicol.* 36: 469-476.

(11) Greeley, E. H., J. M. Ballam, J. M. Harrison, R. D. Kealy, D. F. Lawler, and M. Segre. 2001. The influence of age and gender on the immune system: a longitudinal study in Labrador Retriever dogs. *Vet. Immunol. Immunopathol.* 82: 57-71.

(12) Bannenberg, G. L., N. Chiang, A. Ariel, M. Arita, E. Tjonahen, K. H. Gotlinger, S. Hong, and C. N. Serhan. 2005. Molecular circuits of resolution: formation and actions of resolvins and protectins. *J. Immunol.* 174: 4345-4355.

(13) Kraal, G. 1992. Cells in the marginal zone of the spleen. *Int. Rev. Cytol.* 132: 31-74.

(14) Fan, Y. Y., D. N. McMurray, L. H. Ly, and R. S. Chapkin. 2003. Dietary (n-3) polyunsaturated fatty acids remodel mouse T-cell lipid rafts. *J. Nutr.* 133: 1913-1920.

(15) Fan, Y. Y., L. H. Ly, R. Barhoumi, D. N. McMurray, and R. S. Chapkin. 2004. Dietary docosahexaenoic acid suppresses T cell protein kinase C theta lipid raft recruitment and IL-2 production. *J. Immunol.* 173: 6151-6160.

(16) Nathan, C. 2006. Neutrophils and immunity: challenges and opportunities. *Nat. Rev. Immunol.* 6: 173-182.

(17) Liu, Z., W. Long, D. A. Fryburg, and E. J. Barrett. 2006. The Regulation of Body and Skeletal Muscle Protein Metabolism by Hormones and Amino Acids. *J. Nutr.* 136: 212S-217.

(18) Lefkowith, J. B., M. Rogers, M. R. Lennartz, and E. J. Brown. 1991. Essential fatty acid deficiency impairs macrophage spreading and adherence. Role of arachidonate in cell adhesion. *J. Biol. Chem.* 266: 1071-1076.

(19) Endres, S., R. Ghorbani, V. E. Kelly, K. Georgilis, G. Lonnemann, J. W. M. Vander Meer, J. G. Cannon, T. S. Rogers, M. S. Klempner, P. C. Weber, E. J. Schaefer, S. M. Wolff, and C. A. Dinarello. 1989. The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. *New England journal of Medicine* 320: 265-271.

(20) Calder, P. C., J. A. Bond, D. J. Harvey, S. Gordon, and E. A. Newsholme. 1990. Uptake and incorporation of saturated and unsaturated fatty acids into macrophage lipids and their effect upon macrophage adhesion and phagocytosis. *Biochem. J.* 269: 807-814.

(21) Calder, P. C., R. Albers, J. M. Antoine, S. Blum, R. Bourdet-Sicard, G. A. Ferns, G. Folkerts, P. S. Friedmann, G. S. Frost, F. Guarner, M. Lovik, S. Macfarlane, P. D. Meyer, L. M'Rabet, M. Serafini, W. van Eden, J. van Loo, W. Vas Dias, S. Vidry, B. M. Winklhofer-Roob, and J. Zhao. 2009. Inflammatory disease processes and interactions with nutrition. *Br. J. Nutr.* 101 Suppl 1: S1-45.

(22) Lennartz, M. R., A. F. Yuen, S. M. Masi, D. G. Russell, K. F. Buttle, and J. J. Smith. 1997. Phospholipase A2 inhibition results in sequestration of plasma membrane into electronlucent vesicles during IgG-mediated phagocytosis. *J. Cell. Sci.* 110 (Pt 17): 2041-2052.

(23) Darios, F., B. Davletov. 2006. Omega-3 and omega-6 fatty acids stimulate cell membrane expansion by acting on syntaxin 3. *Nature* 440: 813-817.

(24) Rosenblat, M., N. Volkova, M. Roqueta-Rivera, M. T. Nakamura, and M. Aviram. 2009. Increased macrophage cholesterol biosynthesis and decreased cellular paraoxonase 2 (PON2) expression in Delta6-desaturase knockout (6-DS KO) mice: Beneficial effects of arachidonic acid. *Atherosclerosis* .

CHAPTER 10

SUMMARY AND FUTURE RESEARCH

The general objective of this research was to elucidate specific physiological roles of AA and DHA and their underlying mechanisms. In order to achieve this objective, the research was divided into three parts: a) establishing a time course of D6D-/- pathology, b) determining specific HUFA physiological functions through dietary prevention of D6D-/- pathology, c) and elucidating the mechanism behind HUFA requirement for male fertility.

In chapter 4, a time course study of D6D-/- pathology was done to explain the delay in appearance of HUFA deficiency phenotype, specifically ulcerative dermatitis which occurs by 21 weeks of age. This study demonstrates the presence of HUFA stores in D6D-/- at weaning or 3 weeks of age, indicating HUFA is provided by the mother. These HUFA stores are rapidly depleted after weaning up to 6 weeks of age at which point the rate of depletion slows down. The period of accelerated depletion coincides with the first observed HUFA deficiency phenotype of hepatic lipidosis and disrupted spermatogenesis. In future studies, avoiding these HUFA stores in D6D-/- before weaning could help in accelerating HUFA depletion in tissue such as brain, which is abundant in HUFA and still at approximately 85% of +/+ at 5 months of age. This has been an important limitation in researching the physiological role of HUFA in brain function.

Specific functions for each HUFA were determined by preventing D6D-/- phenotype through dietary supplementation of either arachidonic acid (AA) or docosahexaenoic acid (DHA) (**Table 10.1**). In chapter 5, the dietary essential fatty acids linoleic and α-linolenic acid alone could not modulate liver lipid homeostasis resulting in hepatic lipidosis in D6D-/-. In this case, either AA or DHA supplementation was sufficient to prevent liver pathology. Therefore, essentiality in liver does not seem to be specific to either HUFA. The mechanism behind HUFA

requirement in preventing accumulation of liver triglyceride is yet to be elucidated. This study indicates increased lipogenesis does not seem to play a role in this pathology. Future studies could focus on inefficient lipid secretion due to HUFA deficiency considering the presence of SNARE components during the VLDL secretion process and recent evidence of HUFA interactions with SNARE complex.

In chapter 6, an essential requirement for AA in skin function was demonstrated through prevention of D6D-/- ulcerative dermatitis by AA supplementation but not dietary DHA. Previously, linoleic acid was considered the main polyunsaturated fatty acid required for skin physiology; however, its presence did not prevent D6D-/- skin pathology. Prevention of gastrointestinal ulceration was also specific to AA. The role of AA in skin and gastrointestinal tract is most likely related to prostaglandin production. Further research is required to determine which prostaglandins maintain epithelial integrity and how this is achieved. The trigger for excessive scratching behavior in D6D-/- prior to ulcerative dermatitis is yet to be elucidated.

In chapter 7, a specific role for DHA was determined for male fertility. The role for HUFA in male reproduction was previously only inferred, however, specific HUFA deficiency in the D6D-/- mouse allowed attribution of requirement for DHA in spermatogenesis since AA was not as successful in restoring all fertility parameters. Chapter 8 demonstrates a novel role for DHA in acrosome biogenesis, an important process in sperm cell maturation. Acrosome formation relies on vesicle fusion. Future research will consist in identifying vesicle fusion SNARE proteins which participate in the development of the acrosome and determine if DHA drives the formation of SNARE complex during spermiogenesis. It is also important to elucidate why AA fails to completely restore fertility parameters, as well as to identify specific steps that were rescued by its supplementation.

Several studies have demonstrated a role for HUFA in modulating the immune system through diet. Chapter 10 shows that HUFA not only modulate immune function but are essential in the immune system, specifically in the development of an antibody response to a T-cell dependant antigen. The underlying mechanism behind impaired antibody response due to HUFA deficiency is yet to be elucidated. Decreased white pulp of D6D-/- spleen may be a focus area of future research since this is the site of immune cell interactions in the development of antibody. Challenging the mouse with a T-cell independent antigen would also help elucidate which immune cells are compromised by HUFA deficiency. Macrophage from D6D-/- were shown to have altered metabolism with increased cholesterol synthesis and decreased paraoxynase activity. Further macrophage characterization, specifically in relation to phagocytosis, would be important to elucidate a role for HUFA in immune function. Preliminary work suggests impaired phagocytosis. This process relies on SNARE components for pseudopod formation and would most likely require HUFA. Neutrophil homeostasis would be another area of research to study HUFA function, considering the observed spleen myeloid hyperplasia, neutrophilia, and increase in bone marrow myeloid precursors, although this may be secondary to gastrointestinal ulceration.

In all, the two key findings in this research were AA essentiality in skin and a specific requirement for DHA in male fertility, specifically in acrosome biogenesis through vesicle fusion. Future research with the D6D-/- model will help elucidate the mechanism behind HUFA essentiality in testis as wells as other tissues, such as liver, skin, and the immune system. Further comprehension of the role of HUFA would help in the development of treatments for diseases that result from altered HUFA metabolism.

SUMMARY TABLE

Table 10.1 *Summary of delta-6 desaturase knockout (D6D-/-) phenotype reversed by highly unsaturated fatty acid (HUFA) supplementation.*

	Supplementation	
D6D-/- phenotype	**AA (20:4n6)**	**DHA (22:6n3)**
Dermatitis	Prevented	Not prevented
Gastrointestinal ulcers	Prevented	Not prevented
Hepatic lipidosis	Prevented	Prevented
Male infertility	Partially restored	Fully restored

CURRICULUM VITAE

Manuel Roqueta-Rivera

Urbana, IL 61801 · (217) 841-0047 · mroquet2@illinois.edu

EDUCATION

Ph.D. Human Nutrition (Nutritional Biochemistry)
with Certificate in Business Administration
University of Illinois, Urbana (May 2010) GPA: 3.97/4.0.
B.S. Biochemical Engineering (Food Science)
Instituto Tecnológico de Durango, Mexico (2005) GPA: 4.0/4.0

SKILLS

Leadership, Communication and Problem-Solving

- Designed and lead animal and cell culture studies on the role of omega-3 and omega-6 fatty acids in modulating skin physiology, male fertility, and immune function.
- Experimental assay troubleshooting
  (cell culture, gas chromatography, flow cytometry, gene expression, PCR).
- Supervised maintenance of lab equipment (gas chromatography).
- Well developed written and oral communication skills (fluent in English and Spanish); basic speaking and reading knowledge of French.
- Effectively presented research results (oral and poster) to both technical and non-technical audiences at conferences; Procter and Gamble research award finalist at the international Experimental Biology conference, San Diego 2008.
- Wrote and edited animal protocols for IACUC approval, revised lab protocols, published articles in peer-reviewed journals.
- Collaborated with national and international universities.
- Supervised undergraduates and international scholars assisting in lab research.
- Head teaching assistant for FSHN101, an introductory food science and human nutrition course at the University of Illinois.

Technical Skills

- Lipid biology: tissue lipid extraction, fatty acid analysis by thin layer / gas chromatography,
- Molecular Biology: tissue gene expression by RT-PCR, cloning and overexpression of genes, DNA sequencing, RNA extraction, PCR, ELISAs.
- Cell biology: Hepatocyte/macrophage/lymphocyte culture, cytokine measurements, flow cytometry, immunohistochemistry.
- Microbiology: bacterial culture; isolation and enumeration of microorganisms.
- Animal: colony management, genotyping, dissection, tissue collection.
- Computer skills: MS office, Statistical Analysis (SAS).

PROFESSIONAL EXPERIENCE

Doctoral Student, University of Illinois(UIUC), Urbana, 2005-2010.

- Dissertation: **"The role of dietary arachidonic acid and DHA in preventing the phenotype observed with highly unsaturated fatty acid deficiency".**
- Studied role of DHA in male reproduction.
- Studied role of arachidonic acid in skin physiology.
- Immune system characterization of the delta-6-desaturase knockout mouse (D6D -/-).
- 3 years experience in transgenic mouse breeding.
- Characterized rate of omega fatty acid depletion and sequence of D6D-/- pathology.
- Cloned the FADS3 gene and tested its activity by transfecting liver cells.

Teaching Assistant, Introduction to Food Science, UIUC, 2008–2010.

- Directed a team of teaching assistants in a 700-student food science introductory course.
- Organized exams, grading of papers, and lead review sessions.

PUBLICATIONS AND ABSTRACTS

- **Roqueta-Rivera M,** Stroud CK, Haschek WM, Akare SJ, Segre M, Brush RS, Agbaga MP, Anderson RE, Hess RA, Nakamura MT. Docosahexaenoic acid supplementation fully restores fertility and spermatogenesis in male delta-6 desaturase-null mice. J Lipid Res. 2010 Feb;51(2):360-7.
- Stroud CK, Nara TY, **Roqueta-Rivera M**, Radlowski EC, Lawrence P, Zhang Y, Cho BH, Segre M, Hess RA, Brenna JT, Haschek WM, Nakamura MT. Disruption of FADS2 gene in mice impairs male reproduction and causes dermal and intestinal ulceration. J Lipid Res. 2009 Sep;50(9):1870-80.
- Rosenblat M, Volkova N, **Roqueta-Rivera M**, Nakamura MT, Aviram M. Increased macrophage cholesterol biosynthesis and decreased cellular paraoxonase 2 (PON2) expression in Delta6-desaturase knockout (6-DS KO) mice: Beneficial effects of arachidonic acid. Atherosclerosis. 2009 Dec 1.
- Dietary arachidonic acid prevents ulcerative dermatitis and partially restores skin prostaglandin in delta-6-desaturase (D6D) knockout mouse. Abstract submitted to Experimental Biology, San Diego, CA, April 2008 (Poster and Oral) and ISSFAL conference, Kansas City, MO, May 2008 (Poster). **Roqueta-Rivera M**, Chad K. Stroud, Mariangela Segre, Wanda M. Haschek, Manabu T. Nakamura.

AWARDS

- Food Science and Human Nutrition Outstanding PhD Student, UIUC, 2010
- National Council Science and Technology (CONACyT) Fellowship (Mexico), 2005-2008
- Holmes Merit Award in Nutrition, UIUC, 2009
- American Society for Nutrition, Procter and Gamble Research Award Finalist, EB 2008
- Nutritional Sciences Symposium, UIUC, 1st Place Oral Competition, 2008
- Strunk Fellowship Award, UIUC, 2008
- Perkins Lipid Research Award, UIUC, 2008
- International Society Study of Fatty Acids/Lipids (ISSFAL) New Investigator Award, 2008
- Holmes Merit Award in Nutrition, UIUC, 2007