IMPAIRED HEPATIC FATTY ACID SYNTHESIS: A POTENTIAL MECHANISM OF THE REDUCED GROWTH PHENOTYPE OF CYSTIC FIBROSIS KNOCKOUT MICE

by

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# TABLE OF CONTENTS

**LIST OF TABLES**  
6

**LIST OF FIGURES**  
7

**ABSTRACT**  
9

**INTRODUCTION AND SPECIFIC AIMS**  
10

**BACKGROUND AND SIGNIFICANCE**  
13

  I. The Growth Phenotype of Cystic Fibrosis  
   13

  II. Known Causes of the Growth Impairment  
   14
     a. Growth Hormones and Factors  
     b. Energy Imbalance  
   16

  III. The Use of Mouse Models to Study Cystic Fibrosis  
   19

  IV. The Role of Fat Metabolism  
   20

  V. Hepatic *de novo* Fatty Acid Synthesis  
   21

  VI. Lipogenic Enzyme Activity  
   24

  VII. The Role of Hepatocytes in the Lipid Metabolism of CF Mice  
   26

  VIII. Significance for Genetic Counseling  
   26

**RESEARCH DESIGN AND METHODS**  
28

  I. Animals and Husbandry  
   28

  II. Measurement of Hepatic *de novo* Fatty Acid Synthesis  
   29
     a. Basis of Methodology  
   29
b. Biological Experiments

c. \(^{2}\text{H}\)-Labeling of Body Water

d. \(^{2}\text{H}\)-Labeling of Newly Made Fatty Acids

e. Analysis of \textit{de novo} Fatty Acid Synthesis

III. Western Blotting

IV. Data Analysis

RESULTS

I. Characterization of \textit{Cftr}^{\text{tm1Unc}} Fatty Acid Metabolism

a. \textit{De novo} Fatty Acid Synthesis of \textit{Cftr}^{\text{tm1Unc}} Mice

b. Expression of Major Lipogenic Enzymes

i. Liver Tissue

ii. Adipose Tissue

II. Characterization of Hepatocyte \textit{Cftr} Knockout Mice Fatty Acid Metabolism

a. \textit{De novo} Fatty Acid Synthesis of Hepatocyte Knockout Mice

b. Expression of Major Lipogenic Enzymes

i. Liver Tissue

DISCUSSION

I. Significance of \textit{Cftr}^{\text{tm1Unc}} Data

II. Altered Adiponectin Levels

III. Hepatocyte Tissue-Specific \textit{Cftr} Knockouts
LIST OF TABLES

Table 1 - Summary of CF Adipose and Liver Western Blot Data 45

Table 2 - Summary of Hepatocyte Knockout Adipose and Liver Western Blot Data 48

Table 3 - Experimental Subjects 65
LIST OF FIGURES

Figure 1 - FEV$_1$ Percent Predicted vs. BMI Percentile in Children 6-20 Years 14
Figure 2 - Growth Curve for Tissue-Specific Neuronal $Cftr$ Knockouts 15
Figure 3 - Pathogenesis of the Energy Imbalance in Cystic Fibrosis 17
Figure 4 - Growth Curve for Tissue-Specific Intestinal Epithelial $Cftr$ Knockouts 19
Figure 5 - Weight of Inguinal White Adipose Tissue 20
Figure 6 - The Fractional Synthesis Rates (FSR) of the Fatty Acids Palmitate and Stearate 22
Figure 7 - Endogenous and Exogenous Fat and Energy Metabolism 23
Figure 8 - CF $de$ novo Fatty Acid Synthesis of Palmitate 36
Figure 9 - CF $de$ novo Fatty Acid Synthesis of Stearate 37
Figure 10 - CF Liver ACC-1 Expression 38
Figure 11 - CF Liver AMPk Expression 39
Figure 12 - CF Adipose ACC-1 Expression 41
Figure 13 - CF Adipose AMPk Expression 42
Figure 14 - Liver and Adipose Western Blot Images 43
Figure 15 - Hepatic and $de$ novo Fatty Acid Synthesis in Tissue-Specific Knockout Mice 45
Figure 16 - Hepatocyte Knockout ACC-1 Expression 46
Figure 17 - Hepatocyte Knockout Liver AMPk Expression 47
Figure 18 - Hepatocyte Knockout Liver Western Blot Images 48
Figure 19 - Model of the Potential Effects of ACC-1 and AMPk on Adipose and Liver 51
Figure 20- Cre/LoxP System for Generating a Tissue-Specific Knockout

Figure 21- Cre/LoxP Crossings for Generating a Tissue-Specific Knockout
Impaired Hepatic Fatty Acid Synthesis: A Potential Mechanism of the Reduced Growth Phenotype of Cystic Fibrosis Knockout Mice

Abstract

by

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Cystic Fibrosis is a genetic disease that results in poor growth manifesting as a reduced fat mass. This study characterized the de novo fatty acid synthesis of both Cftr-null mice and hepatocyte specific Cftr knockouts to determine if this is a potential mechanism resulting in this reduced fat mass. De novo synthesis rates of palmitate and stearate were measured in the liver via isotope labeling of water and mass spectrometry. In addition, the expression of ACC-1 and AMPk were determined via western blotting. Results indicated decreased hepatic fat synthesis in CF males compared to CF females and controls. Enzyme expression results revealed differences in hepatic ACC-1 and AMPk activity levels in both CF genders, which were overall inconclusive. Hepatocyte knockouts showed no difference in palmitate or stearate synthesis and a significant increase in male AMPk expression. Overall, this study supports the notion of an impaired lipid metabolism in CF mice.
INTRODUCTION AND SPECIFIC AIMS

Cystic Fibrosis (CF) is an autosomal recessive genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (Cftr) gene. Cftr encodes a cAMP-regulated chloride channel, and when defective, leads to a wide range of clinical features. Of particular interest is impaired growth, which manifests as both reduced linear growth and decreased fat mass. The current paradigm suggests two disease consequences, pancreatic insufficiency and intestinal malabsorption, as the potential sources of this growth phenotype. Both of these features lead to reduced uptake of dietary fats and consequently fat soluble vitamins, which may lead to the overall poor nutritional status of an individual with CF [Moskowitz et al., 2008].

However, recent studies have suggested that there are other origins of this phenotype beyond pancreatic insufficiency and intestinal malabsorption. For example, homozygous ∆F508 and Cftr-null mice are reported to exhibit fat malabsorption and poor growth still occurs even when the exocrine pancreas is predominantly functional and no pancreatic insufficiency is observed [Bijvelds et al., 2005]. In addition, when Cftr is selectively inactivated in the intestinal epithelium of mice, the animals experience normal fat absorption and nearly normal growth [Hodges et al., unpublished data]. These data suggest that even though pancreatic insufficiency and intestinal malabsorption may be contributors to this phenotype, there are other mechanisms playing a role as well.
Preliminary studies have shown that hepatic fatty acid synthesis of CF mice is diminished in comparison to wild-type mice, suggesting that the liver is not synthesizing enough fat for storage in adipose tissue, which may lead to a reduced total fat mass [Bederman et al., unpublished data]. As this could be an additional factor leading to poor growth, it is important to investigate the mechanism underlying diminished fatty acid synthesis. The purpose of this study was to further characterize this impaired lipid synthesis phenotype in Cfr-null mice, as well as to identify the cell types responsible for this phenotype.

Overall, this study’s main goals were to quantify hepatic fatty acid synthesis between CF and non-CF mice, and then to identify the source(s) of any differences observed. Specifically, this study investigated whether differences in fatty acid synthesis are a direct effect caused from losing Cfr in the liver, or an indirect effect from the absence of Cfr in other tissues. This study examined and compared two different strains of mice: Cfr-null mice and hepatocyte tissue-specific Cfr knockout mice. This line of tissue-specific knockout mice was used to help delineate the role of hepatocytes in fatty acid synthesis as it pertains to CF. For instance, hepatocytes, the predominant site of fatty acid synthesis, may be unable to synthesize the normal amount of fatty acids due to the absence of Cfr. To address these questions, the aims of this study were to:

**AIM 1)** characterize liver fatty acid synthesis in Cfr-null mice by:

i. determining the rate of *de novo* palmitate and stearate synthesis;
ii. determining the relative expression of two major lipogenic enzymes, AMPk and ACC-1.

**AIM 2)** characterize liver fatty acid synthesis in hepatocyte tissue-specific *Cftr* knockout mice by:

i. determining the rate of *de novo* palmitate and stearate synthesis;

ii. determining the relative expression of two major lipogenic enzymes, AMPk and ACC-1.

The fatty acid synthesis of *Cftr*-null mice was expected to be reduced, considering preliminary data had already suggested this to be the case. Hepatocyte knockouts were not predicted to affect fatty acid synthesis since the few studies that have examined Cftr in the liver suggested that hepatocytes do not express Cftr [Kinnman et al., 2000; Cohn et al., 1993].
BACKGROUND AND SIGNIFICANCE

The Growth Phenotype of Cystic Fibrosis:

Cystic Fibrosis (CF) is the most common life-shortening recessive genetic disease in the Caucasian population, and is caused by reduced or absent cAMP-dependent chloride channel (cystic fibrosis transmembrane conductance regulator or Cftr) activity. This absent or malfunctioning chloride channel is the basis for multiple clinical manifestations, such as exocrine pancreatic insufficiency, intestinal malabsorption, liver disease, reduced fertility and chronic pulmonary disease—which is the main cause of early mortality [Moskowitz et al., 2008].

Another major phenotypic consequence of CF is poor growth. According to the 1997 CF Foundation Patient Registry Report, 28% of all patients affected with CF were less than the 10th percentile for height and 33% were less than the 10th percentile for weight, indicating that impaired growth is relatively common among the CF population [CF Patient Registry report, 1997]. However, not only is poor growth a common feature, but it is also a good predictor of future pulmonary function. Research has shown that both poor growth and nutrition at a young age are associated with a worsened pulmonary function later in life [Zemel et al., 2000; Konstan et al., 2003]. In addition, a higher body mass index (BMI) strongly correlates with a higher forced expiratory volume (FEV$_1$), a measure of lung function (Figure 1) [CF Patient Registry Report, 2008]. Another study found that individuals with a higher body mass index (BMI) also have fewer pulmonary
Infections, increased respiratory muscle strength and an overall slower deterioration of pulmonary function [Shephard et al., 1980].

**Figure 1.** Shows greater pulmonary function, measured by forced expiratory volume (FEV), as a result of an increase in body mass index (BMI) for both males and females between the ages of 6-20 years, affected with CF. The vertical black line indicates the 50th percentile BMI for CF individuals. Taken from the CF Foundation's 2008 Patient Registry Report.

**Known Causes of the Growth Impairment:**

Growth Hormones and Factors:

Current knowledge on poor growth in CF supports the notion of two underlying, interconnected causes: the deficiency of growth hormones and growth factors and an unfavorable energy balance. Growth hormones are produced in the pituitary and bind to hepatic cell surface receptors to stimulate the secretion of insulin-like growth factor 1, or IGF-1. IGF-1 subsequently promotes the development of the long bones [Widmaier, Raff and Strang, 2006]. In CF, a decrease in growth hormone (GH) and growth factors, such as IGF-1, has been shown to result from chronic malnutrition and inflammation [Hankard et al., 2002]. In general, studies have suggested that nutrition plays a role in the regulation of IGF-1 production; as a result, chronic malnourishment in CF leads to lower levels of IGF-1 [Underwood et al., 1994]. In addition, the overabundance of circulating
inflammatory cytokines produced to ward off pulmonary infections also reduces the levels of IGF-1 [De Benedetti et al., 1997].

To further understand the correlation between CF, poor growth and growth hormones/factors, Cftr was selectively inactivated in neuronal tissue, including hypothalamic cells [Hodges et al., unpublished data]. The subsequent growth of the neuronal knockout mice was reduced compared to their wild-type controls (Figure 2). However, this difference was most likely attributable to the linear shortening of the CF mice rather than a reduction in fat mass, considering these mice did not have a reduced body mass index as found for CF mice. In other words, the neuronal knockout mice and their wild-type control mice appeared proportionate in their amount of total fat mass given their differing body lengths [Hodges et al., unpublished data].
Energy Imbalance:

While deficiencies of growth hormone and growth factors are important, this study instead focused on a second cause of growth impairment: an abnormal energy balance. In patients with CF, an imbalance in energy can originate from three major factors: (1) energy expenditure, (2) energy uptake and (3) energy losses [Pencharz et al., 2000]. These three factors are closely intertwined with one another, and can cause an imbalance when they deviate from the normal requirements of a system (Figure 3). For instance, increased energy loss and expenditure quickly depletes energy sources, requiring additional energy uptake in order to foster proper growth. As a result, it is a well accepted practice to have individuals with CF on high-calorie diets that exceed the normal caloric intake by 20-50% [Pencharz et al., 2000]. These added calories are thought to be very important since they replenish much of the energy depleted from excess loss and expenditure.

Research has shown that individuals with CF have a resting energy expenditure roughly 9-25% above that predicted for subsequent body weights when compared to healthy controls [Buchdahl et al., 1988; Pencharz et al., 1998, Shepherd et al., 1988]. One study that examined the possible mechanisms behind this issue hypothesized that pulmonary overexertion from difficult breathing in advanced lung disease may raise metabolic rates [Pencharz, Hill and Archibald, 1994]. It should be noted that this mechanism would not explain reduced growth in the early ages of CF subjects.
Energy loss is also a well-recognized cause of this energy imbalance, and is primarily attributed to malabsorption and maldigestion caused by pancreatic insufficiency and dysfunctional intestinal epithelium, respectively [Pencharz et al., 2000]. A nonfunctioning pancreas is unable to secrete enzymes, such as lipase, that aid in the intestinal absorption of fat molecules. These enzymes form membrane-soluble chylomicron lipoproteins that transport fats from the intestines into the blood stream. When enzyme concentrations are reduced, lipids are less capable of being absorbed into the body and these high energy molecules are lost and excreted within the stools.

Unfortunately, even with the standard treatment of pancreatic enzyme replacement therapy (PERT) this remedy does not completely correct the growth impairment—it only improves the phenotype. Heights and weights are not comparable to unaffected individuals, even when pancreatic enzymes are administered over an extended period of time [Benebdeslam et al., 1998; Stettler et al., 2000]. For instance, one study
showed that the maximum amount of fat absorbed when undergoing PERT is approximately 82% for CF patients, which is still 13% lower than unaffected individuals [Brady et al., 2006]. Therefore, lipid malnutrition is not completely corrected. Similar findings are seen in CF mice since they are smaller in size even when they display adequate pancreatic lipase, bicarbonate secretion and biliary bile salt secretion [Bijvelds et al., 2005]. These data suggest that pancreatic insufficiency alone does not explain the growth phenotype, and that intestinal epithelial dysfunction may play an important role in fat malabsorption.

In fact, research has suggested that defective Cftr chloride channels in the intestinal epithelium may contribute to this observed energy imbalance. Intestinal epithelial dysfunction may result in the malabsorption of lipids via a variety of mechanisms including a dysfunctional outer-epithelium, defective intracellular lipid transport proteins and/or impaired lipid esterification and lipoprotein synthesis and secretion [Peretti et al., 2005]. For instance, ileal hypertrophy, villous atrophy and a thick mucus layer may all lead to the defective absorption of lipids into the intestinal epithelium. Cftr may also play a role in the activity or synthesis of intracellular lipid transport proteins, and may also be involved in secretion of these lipoproteins into the adjacent lymph ducts [Peretti et al., 2005; Gusarova et al., 2003].

However, when Cftr is selectively inactivated within intestinal epithelial cells of mice, the final resulting growth phenotype is essentially comparable to that of wild-type mice, although these animals are still prone to intestinal obstruction (Figure 4).
Additionally, when \( C\text{ftr} \) is selectively inactivated in every cell of the body except intestinal epithelial cells, the resulting phenotype is comparable to that of CF mice, and with no incidence of obstruction [Hodges et al., unpublished data]. Thus, these data suggest that there are other mechanisms beyond growth hormone deficiencies, pancreatic insufficiency or intestinal epithelial dysfunction contributing to the growth impairment seen in both CF patients and mouse models.

**The Use of Mouse Models to Study Cystic Fibrosis:**

This study used the mouse as a model organism to study the growth phenotype in CF, as research has shown that CF mice have many features similar to humans affected with CF. Even though mice do not display the pulmonary phenotype to the extent of the human form of CF, they do experience severe intestinal disease, poor growth, vas deferens dysfunction and liver disease. \( C\text{ftr} \)-null mice are also pancreatic sufficient; and therefore, do not exhibit significant dietary lipid malabsorption, which most humans with CF experience [Scholte et al., 2004; Bijvelds et al., 2005]. CF mouse models also lack in

![Figure 4. Growth curve for tissue-specific intestinal epithelium \( C\text{ftr} \) knockouts (lower blue line). Between days 15-35, poor growth manifests, but eventually catches back up to WT around day 35. Asterisks represent significant differences [Hodges et al., unpublished data].](image-url)
both length and weight when compared to wild-type mice, and thus are exceptionally similar to humans in regards to the growth phenotype [Rosenberg et al., 2006]. More specifically, CF mice are on average 20-30% smaller than healthy controls [Snouwaert et al., 1992]. Unpublished data from Bederman and colleagues have shown that CF mice have significantly reduced fat mass, which was exhibited when subcutaneous white adipose tissue collected from the inguinal region of CF mice was weighed and compared to that of wild-type controls (Figure 5) [Bederman et al., unpublished data].

**Figure 5.** Weight of inguinal white adipose tissue collected from CF and control mice. CF mice exhibit nearly four times less inguinal fat mass than a wild-type litter mate [Bederman et al, unpublished data].

**The Role of Fat Metabolism:**

As previously discussed, a great deal of research on the pathogenesis of reduced fat mass in CF has been conducted thus far, and in general, the mechanisms involve a defect in the absorption and/or the trafficking and transport of exogenous lipids. To date, little research has been conducted on the effects of endogenous lipid metabolism in the pathogenesis of CF. In other words, could the absence of Cftr result in a defect of lipid metabolism?
Recent studies have shown that patients with CF have altered essential fatty acid (EFA) levels characterized by increased arachidonic acid, an inflammatory promoter, and decreased docosahexanoic acid or DHA, an inflammatory inhibitor [Strandvik et al., 2004; Freedman et al., 2007]. This altered EFA profile is thought to up-regulate inflammatory pathways, thereby contributing to the pathogenesis of the pulmonary disease. This change in EFA levels has also been proven to be a primary defect in fat metabolism as a result of losing Cftr (rather than a secondary effect from EFA malabsorption). This was exemplified when Anderson and his colleagues demonstrated that cultured airway epithelial cells (or cells not exposed to secondary mechanisms such as intestinal fat malabsorption) exhibited the same EFA profile as an in vivo EFA profile [Anderson et al., 2008].

However, these prior studies have only focused on the association of altered EFA levels and CF pulmonary manifestations. As of yet, there are no published studies that have investigated whether defective fat metabolism affects the storage of triglycerides and ultimately the weight of an organism with CF. Therefore, the purpose of this study was to investigate whether there was a significant underlying defect in fat synthesis preventing the proper amount of fat to be stored in adipose cells.

**Hepatic de novo Fatty Acid Synthesis:**

Preliminary data using mass spectrometry to measure hepatic *de novo* fatty acid synthesis has shown that CF mice synthesize 20% and 25% less of the fatty acids palmitate and stearate, respectively, than wild-type controls (Figure 6) [Bederman et al.,
unpublished data]. These data propose that since CF mice synthesized less palmitate and stearate in the liver, this could essentially add to the energy imbalance, therefore resulting in a reduced total fat mass.

![Figure 6. The fractional synthesis rates (FSR) of the fatty acids palmitate and stearate.](image)

This concept of a reduced total fat mass can best be understood by reviewing the pathways of fat metabolism (Figures 7A and 7B). During the absorption of food particles, also known as the “absorptive state”, amino acids and glucose are sent to hepatocytes and are either used as a substrate for energy production, or are transformed into fatty acids or glycerol, which together form triglycerides and are subsequently stored in adipose tissue (Figure 7A). Once the intestines have completely absorbed all of the dietary particles, the “post-absorptive state” begins and energy production is stimulated. The stored triglycerides break down into fatty acids and glycerol; the fatty acids are transported to other body tissues including the liver, where they undergo beta-oxidation for energy production. The glycerol is transported to the liver, where glycerol and glycogen form glucose for energy production (Figure 7B). Overall, if hepatic fatty acid synthesis were truly impaired, then theoretically, fewer triglycerides would be available for storage in adipose tissue.
Figure 7. Endogenous and Exogenous Fat and Energy Metabolism (A) Fat synthesis and storage pathways of liver and adipose tissue occurs immediately after the intestinal absorption of dietary particles; (B) Fat and glucose catabolic pathways of liver and adipose tissue occurs after the complete absorption of dietary particles. Adapted from Widmaier, Raff and Strang, 2006.
**Lipogenic Enzyme Activity:**

As an additional method to determine if reduced fatty acid synthesis contributes to the CF growth profile, biochemical pathways and intermediates that comprise fat metabolism were examined to identify the rate-limiting steps. Understanding these pathways should aid in the identification of key regulators of fat production. A survey of the literature suggested a panel of such enzymes; however, this study focused on the relative expression of the two main enzymes that regulate hepatic fat metabolism: acetyl-coA carboxylase 1 (ACC-1) and AMP-activated protein kinase (AMPk).

Overall, the majority of fatty acid metabolism occurs in the liver, and is predominantly regulated by the ratio of adenosine triphosphate (ATP) to adenosine monophosphate (AMP). When ATP is hydrolyzed by cellular kinases, the energy of the phosphate bonds is used to fuel cellular processes. The hydrolysis reaction produces AMP, a low energy molecule. Sufficient cellular ATP concentrations ([ATP]>[AMP]) promote energy storage in the form of fatty acids that are subsequently provided to adipocytes for storage as fat (processes represented in Figure 7A).

In situations where the ATP supply is insufficient, the elevated AMP concentration is sensed by the cell and thus promotes the activity of AMPk. AMPk serves as the master sensor of the energy balance (the ATP to AMP ratio) within a cell. AMP binds to the γ-domain of AMPk which induces a conformational change that activates the kinase activity of AMPk. One of the many roles of active AMPk is to phosphorylate (or inhibit) the action of ACC-1, an enzyme that catalyzes the
carboxylation of acetyl-coA to malonyl-coA, the basic unit needed for the elongation of fatty acid chains. When ACC-1 is inhibited, fatty acid synthesis is suppressed and beta-oxidation is stimulated, producing more ATP for energy (process represented in Figure 7B). In this situation, less fat would be available for fatty acid synthesis and subsequent fat storage. If this were the case for an extensive period of time, the result would be a smaller, thinner, CF-like phenotype. When there is an overabundance of ATP, the reverse of this process occurs; AMPk is inhibited, allowing ACC-1 to form malonyl coA for fatty acid synthesis [Voet, Voet and Pratt, 2001].

Based on these energy metabolism mechanisms, it is logical to assume that a decrease in fat synthesis is due to either overactive or higher amounts of AMPk, or underactive or lower amounts of ACC-1. Evidence suggests that the latter assumption is most likely. The previously mentioned preliminary data that showed CF mice to have lower rates of fat synthesis compared to WT, also showed CF mice to have higher rates of cholesterol synthesis [Bederman et al., 2009]. AMPk inhibits both fat and cholesterol synthesis; and therefore, if AMPk were to be overactive or higher in concentration, cholesterol synthesis should also be decreased. On the other hand, underactive ACC-1 could be allowing for the buildup of acetyl-coAs. Since acetyl-coA is the basic starting material for not only fatty acid, but also cholesterol synthesis, any excess acetyl-coAs may be shunted down the cholesterol synthesis pathway, thus potentially increasing cholesterol synthesis.
The Role of Hepatocytes in the Lipid Metabolism of CF Mice:

The purpose of the second aim of this study was to characterize hepatic fatty acid synthesis in hepatocyte tissue-specific Cftr knockouts. Since hepatocytes are the cells responsible for the majority of the liver’s metabolic processes, hepatocyte-specific Cftr inactivation was used to determine whether losing Cftr in the liver alters fat synthesis. Yet, these knockout mice were not expected to have an altered rate of synthesis since their growth does not differ from that of control mice [unpublished data from the Drumm laboratory]. Secondly, Cftr expression studies suggest that hepatocytes do not express Cftr [Kinnman et al., 2000; Cohn et al., 1993]. However, these studies indirectly examined Cftr function by measuring mRNA and protein levels, and therefore may have been unable to detect lower concentrations of either gene product. As a result, hepatocytes were still important to evaluate for any Cftr-related properties.

Significance for Genetic Counseling:

The mechanisms that underlie poor growth in an individual with CF are not fully understood. Treatments used to improve growth are only partially successful, suggesting that there are other underlying causes that have yet to be determined. This study aimed to identify whether diminished de novo fatty acid synthesis is one of the potential causes of a decrease in fat mass, ultimately leading to a smaller individual with a reduced BMI. Since a reduced weight or BMI indicates a poorer prognosis for a patient with CF, understanding and investigating other origins of this problem is extremely important and may allow for further developments in treatment approaches. If specific therapies can be developed that target the causes of poor growth, ultimately improving BMI ratios, then
CF patients may have better lung function and an overall longer lifespan. Findings from this research will also further characterize the phenotype and pathophysiology of CF, which will open doors for additional expansion and exploration of CF disease pathogenesis.

This study is not only beneficial to the field of CF research, but is beneficial to the field of clinical genetics and genetic counseling as well. Since genetic counselors help to facilitate patients’ understanding of the basis of their genetic disease and also provide anticipatory guidance to families, it becomes very important to comprehend the risk factors and mechanisms underlying disease phenotypes. Genetic counselors should be aware of possible contributing mechanisms of poor growth in CF, since these mechanisms could possibly play a future role in their patients’ treatment and prognoses.
RESEARCH METHODS AND DESIGN

Animals and husbandry:

The CF mice used in this study carry the $Cftr^{tm1Unc}$ allele (Snouwaert et al., 1992), which produces no functional Cftr protein. The allele was made congenic by backcrossing with C57BL/6J mice for greater than 15 generations. Controls for the $Cftr^{tm1Unc}$ mice were C57BL/6J mice. The hepatocyte tissue-specific knockout mice were generated by Cre-mediated recombination of a “floxed” $Cftr$ allele, described by Hodges et al., 2008. Mice carrying Cre expressed from the albumin promoter were crossed with mice carrying the floxed $Cftr$ allele to induce hepatocyte-specific $Cftr$ inactivation. Cre-mediated recombination of the floxed $Cftr$ allele creates an exon 10 deletion of the $Cftr$ gene, rendering the Cftr protein nonfunctional. The tissue-specific knockouts used in this study are not congenic; thus, control mice for the tissue-specific knockouts consisted of littermates not carrying a Cre transgene. For a more detailed explanation of this method, see Appendix I.

All mice were housed in sterilized microisolator cages within the Health Sciences Animal Facility (HSAF) of the Animal Resource Center (ARC) at Case Western Reserve University. Housing conditions as well as diet were the same for both knockout and wild-type mice. Housing conditions included a 12 hour light and 12 hour dark schedule, with a consistent temperature of approximately 22 degrees Celsius. Each cage was furnished with corn-cob bedding, which was less likely to be ingested, potentially resulting in intestinal obstruction. All mice were maintained on sterilized water and a standard Harlan
Teklad irradiated chow diet balanced with amino acids, vitamins and minerals. All mice were weaned around 21 days of age. Growth curves were collected on the hepatocyte knockout mice.

All procedures and protocols performed were approved by the Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University. Cage maintenance procedures were conducted under a laminar flow hood within the ARC to avoid any possible contamination. A few procedures, such as mouse sacrificing and tissue harvest, took place outside of the ARC within Dr. Mitchell Drumm’s laboratory. All tissues collected were snap-frozen in liquid nitrogen for preservation and stored in a -80 degree freezer.

All experiments were performed on roughly 3-7 mice of each sex and of each type of knockout mouse, along with their age and gender matched controls. All experimental mice were approximately six weeks of age at the time of experiments. See Appendix II for a list of experimental subjects.

**Measurement of Hepatic de novo Fatty Acid Synthesis:**

**Basis of the Methodology:**

Determination of the fractional fatty acid synthesis rates was based on the incorporation of deuterium from deuterium-enriched body water into C-H bonds of newly made fatty acids. Briefly, after a bolus of $^2$H$_2$O, $^2$H quickly and completely equilibrates
with body water. During de novo fatty acid synthesis, H⁺ are incorporated into newly made C-H bonds of fatty acids via acetyl-CoA, NADPH+H⁺ and from direct water incorporation. Applying the precursor-product relationship and using enrichment of body water as the precursor and enrichment of fatty acids as the product, a fractional synthesis rate is determined as the product/precursor ratio.

**Biological Experiments:**

Newly synthesized fatty acids were identified by metabolic labeling with deuterium (²H) and two saturated fatty acids, palmitate and stearate, were subsequently analyzed (these experiments were conducted as described previously by Bederman et al., 2006]. All mice were given a bolus of deuterated water (20 µl/g body weight of 0.09 g NaCl in 1,000 ml of 99% deuterated water) by interperitoneal injection and then maintained with 5% deuterated drinking water for approximately 24 hours. In order to ensure the accuracy of the injection, mice were sedated by a brief exposure (~10-15 seconds) to Isoflurane. All mice soon regained consciousness with no apparent adverse side effects. All mice were fed ad libitum. Mice were euthanized by exsanguination under anesthesia for blood collection and by cervical dislocation for tissue harvest. The following samples were collected: epididymal fat pads, liver and blood. Adipose and liver tissues were snap-frozen in liquid nitrogen, weights recorded, and stored at -80°C until future analyses.
$^2$H-Labeling of Body Water:

The deuterium labeling of body water was determined by the exchange of $^2$H with the hydrogen ions in acetone. An aliquot of plasma (20µL) was reacted with 4 µL of 10 N NaOH and 4 µL of 5% solution of acetone in acetonitrile overnight at room temperature. Next, acetone was extracted by adding 600µL of chloroform and dried by the addition of sodium sulfate. Samples were subsequently mixed, centrifuged and 100 µL chloroform aliquot was transferred to a GC-MS vial. The acetone isotopic enrichment was analyzed using an Agilent 5973N-MSD equipped with an Agilent 6890 GC system, and a DB-17MS capillary column (30m x 0.25mm x 0.25µm). The mass spectrometer was operated in the electron impact mode (70eV). Selective ion monitoring of mass to charge ratios ($m/z$) 58 and 59 was performed using a dwelling time of 10ms per ion.

$^2$H-Labeling of Newly Made Fatty Acids:

Adipose tissue triglycerides were hydrolyzed using 1 N KOH (70% ethanol/water) for 2 hours at 90°C. Samples were then acidified using 150 µL of 12N HCl and extracted twice with 4 ml of hexane. Combined hexane extracts were evaporated and reconstituted in 2 ml of hexane. A 20 µL aliquot was evaporated to dryness and fatty acids were converted to their trimethylsilyl derivatives by adding bis (trimethylsilyl) trifluoroacetamide + 10% trimethylchlorosilane (BSTFA) and incubated for 1 hour at room temperature.

Liver triglycerides were extracted by adding 19 parts of chloroform:methanol mixture (2:1, vol/vol) as well as Folch wash, and were then homogenized. Samples were
then centrifuged and the supernatant layer was collected. Next, 0.4 µL of water was added and centrifuged for 10 minutes at 4000 rpm. The lower chloroform layer was removed and evaporated to dryness. Samples were reconstituted in 2 mL of hexane. A 500 µL aliquot was evaporated to dryness and triglycerides were hydrolyzed using 1 N KOH (70% ethanol/water) for 2 hours at 90ºC. Samples were then acidified using 150 µL of 12N HCl and extracted twice with 4 ml of hexane. Combined hexane extracts were evaporated and reconstituted in 2 ml of hexane. A 20 µL aliquot was evaporated to dryness and fatty acids were converted to their trimethylsilyl derivatives by adding bis(trimethylsilyl) trifluoroacetamide + 10% trimethylchlorosilane (BSTFA) and incubated for 1 hour at room temperature.

**Analysis of de novo Fatty Acid Synthesis:**

All fatty acids were analyzed as their TMS derivatives using gas chromatography-mass spectrometry. The $^2$H enrichment of palmitate and stearate was determined by selective ion monitoring (SIM) of $m/z$ 313-317 and 341-345, or palmitate and stearate, respectively. Integrated peak areas were corrected for natural isotopic abundance using a correction matrix. Fractional synthesis rates were calculated using the following equation:

$$\frac{\text{Total } ^2\text{H labeling fatty acid}}{\text{^2H labeling body water} \times n} \times 100$$

Where $n$ is the number of exchangeable hydrogens (assumed to equal 22). These rates were compared between CF and wild-type mice of each sex.
Western Blotting:

Proteins were detected and quantified by performing western blotting on protein samples extracted from liver and adipose tissue [Manson et al, 2008]. Antibodies against AMPk (62kDa enzyme), pAMPk, ACC-1 (~280kDa enzyme) and pACC-1 were obtained from Cell-Signaling Technologies (Danvers, MA). The secondary antibody, anti-rabbit IgG, was purchased from Santa Cruz Biotechnology. Protein samples were extracted from approximately 0.1 to 0.5 grams of adipose or liver tissue via grinding the samples and freeze/thawing them in lysis buffer (50mM Tris, pH 7.5, 1% Triton X-100, 100mM NaCl, 50mM NaF, 10µg/ml of pepstatin and leupeptin as well as 200µM Na$_3$VO$_4$). Samples were microcentrifuged at 4°C at ~14,000rpm for 10 minutes, and supernatant was subsequently extracted and quantified using a Versamax microplate reader (Molecular Devices Corporation, Sunnyvale, California).

Proteins were separated using SDS-PAGE with each sample well containing ~40µg of protein on a 6-12% acrylamide gel. Protein samples were transferred to an Immobilon-P membrane (purchased from Millipore, Billerica, MA) for 30 minutes at 15V. The blots were blocked overnight in 10% evaporated milk plus PBST (138mM NaCl, 15mM Na$_2$HPO$_4$, 1.5mM KCl, 2.5mM KH$_2$PO$_4$ and 0.1% Tween 20) at 4°C. All AMPk, pAMPk, ACC-1 and pACC-1 blots were incubated in a 1:1000 dilution of primary antibody in PBST plus 10% evaporated milk at room temperature for 1 hour. Blots were washed three times for five minutes each in PBST, and incubated in a 1:3000 dilution of secondary IgG antibody (conjugated to horseradish peroxidase) plus PBST for 1 hour at room temperature. Blots were washed again in PBST for three times, each 5
minutes. All blots were visualized using a SuperSignal chemiluminescent substrate (Thermo-Scientific, Rockford, IL) and the VersaDoc imaging system (Bio-Rad Laboratories, Hercules, CA). Densitometry software on the VersaDoc imaging system allowed for the quantification of protein expression (Quantity One; Bio-Rad).

Subsequent adipose and liver western blotting data were analyzed using two different methods for all wild-type and CF mice. The first analysis compared only the relative quantities of each enzyme between CF and wild-type mice, ultimately yielding the total enzymatic pool, or how much enzyme is available for phosphorylation and either activation (AMPk) or inactivation (ACC-1). This analysis was completed by measuring the enzyme’s total volume (INT*mm²) for each sample, which was subsequently calculated by multiplying the enzyme’s signal intensity by a set area surrounding each band. The enzyme’s volume should theoretically correspond to the concentration of that specific enzyme within the sample. The second analysis compared the total phosphorylated form of AMPk and ACC-1 to the already measured total concentration of AMPk and ACC-1. This allowed for the determination of how much of the total pool of the enzyme is active or inactive. AMPk is active when phosphorylated, and ACC-1 in inactive when phosphorylated.

**Data Analysis:**

Student’s t-tests were performed on all experimental data, with a p-value of 0.05 as the chosen threshold for statistical significance. The null hypothesis for the *de novo* fatty acid synthesis data was rejected if the fatty acid synthesis levels measured in CF
mice were different (less or greater) than that of controls; and therefore a two-tailed t-test was performed for all fatty acid synthesis analyses. The results from this analysis provided directionality for the western blotting analyses. The null hypothesis for the blotting studies was accepted if the total enzyme concentration and enzyme activity was unchanged, or if the opposite occurred than what would be predicted from the fatty acid synthesis conclusions. A one-tailed t-test was thereby performed on all western blotting analyses.
RESULTS

Characterization of Cftr<sup>tm1Unc</sup> Fatty Acid Metabolism:

De novo Fatty Acid Synthesis of Cftr<sup>tm1Unc</sup> Mice:

Over a period of 24 hours, the percent newly synthesized palmitate and stearate in CF mice were different than age and gender-matched wild-type controls. Palmitate synthesis was decreased by 50% in CF males (n= 5; p=1.4 × 10<sup>-3</sup>), and by nearly 25% in females (n=4; p=0.065) compared to age and gender matched controls (n=5 males, 5 females) (Figure 8). Palmitate synthesis also differed between CF males and females, with females producing nearly 40% more palmitate than males (p=4.7 × 10<sup>-3</sup>).

Figure 8. CF de novo Synthesis of Palmitate. Shows the percent of newly synthesized hepatic palmitate (FSR%) over the course of a 24 hour time period. Approximately 53% of the total concentration of hepatic palmitate was newly synthesized in WT males, 27% for CF males, 60% for WT females and 45% for CF females. P-values are shown amongst the comparison lines. Figure courtesy of Dr. Bederman.
Stearate synthesis was reduced by 40% in CF males (n=5; p=6.5 × 10^{-3}), and by approximately 28% in females (n=4; p=0.13) compared to age and gender-matched controls (n=5 males, 5 females) (Figure 9). Once again, there was a significant gender difference in stearate production, with females synthesizing approximately 40% more stearate than males (p=5.4 × 10^{-3}).

![Percent New Synthesis of Stearate](image)

**Figure 9. CF de novo Synthesis of Stearate.** Shows the percent of newly synthesized hepatic stearate (FSR%) over the course of a 24 hour time period. Approximately 44% of the total concentration of hepatic stearate was newly synthesized in WT males, 26% for CF males, 59% for WT females and 43% for CF females. P-values are shown among the comparison lines. Figure courtesy of Dr. Bederman.

**Expression of Major Lipogenic Enzymes:**

*Liver Tissue*-  
For female *Cftr^{tm1Un}* mice there was a significant difference in the total ACC-1 expression, but not for the inactive form of ACC-1. Female CF mice had approximately 40% less ACC-1 expression than age and gender matched controls (n=5; p=8.2 × 10^{-3}) (Figure 10A). There was no significant difference in the amount of inactive ACC-1;
however, female CF mice tended to have a higher amount of the inactive form than controls (n=5; p=0.059) (Figure 10B). Male Cfrtm1Unt exhibited no statistically significant difference in the total ACC-1 expression (nCF=7 and nWT=5; p=0.30) (Figure 10A). The level of inactive ACC-1, although not statistically significant, did have a tendency to be higher in male CF mice compared to controls (CF n=7 and WT n=5; p=0.075) (Figure 10B). A gender difference between CF males and females was also noted, as males had, on average, 50% higher total ACC-1 expression and a roughly 30% higher level of inactive ACC-1 than females.

Figure 10. CF Liver ACC-1 Expression. Values for CF males and females are normalized to their respective controls, and are presented as “relative quantity”, or a ratio between CF and WT expression, with WT mice mean expression set to a value of 1. P-values are shown among the comparison lines. (A) Represents the average total hepatic ACC-1 concentration (B) Shows the average ratio of the phosphorylated ACC-1 concentration to the total ACC-1 concentration.
Female CF mice exhibited no statistically significant difference in total AMPk or active AMPk expression as there was enormous variation among the CF mice (n$_{CF}$=5, n$_{WT}$=5; p=0.24 and p=0.30 respectively) (Figure 11A). Nevertheless, female CF mice tended to have both higher total AMPk expression as well as an increased amount of active AMPk (Figure 11A and B). In addition, male CF mice total AMPk expression was significantly higher than controls (n$_{CF}$=7, n$_{WT}$=5; p=2.8 × 10$^{-2}$) (Figure 11A). However, the amount of active AMPk was not significantly different between CF and wild-type control mice (CF n=7, WT n=5; p=0.34) (Figure 11B). In addition, there was also another gender-specific trend in that male CF mice had, on average, 38% more total AMPk than CF females.

![Liver AMPk](image)

**Figure 11. CF Liver AMPk Expression.** Values for CF males and females are normalized to their respective controls, and are presented as “relative quantity”, or a ratio between CF and WT expression, with WT mice mean expression set to a value of 1. P-values are shown among the comparison lines. (A) Represents the average total hepatic AMPk concentration (B) Shows the average ratio of the phosphorylated AMPk concentration to the total AMPk concentration.
Overall, in liver tissue, female CF mice exhibited a statistically significant decrease in total ACC-1 expression. Additionally, there was a trend in both female and male CF mice towards a higher amount of phosphorylated, or inactive, ACC-1. And while male CF mice also exhibited significantly higher levels of total AMPk than controls, the amount of phosphorylated, or active, AMPk was equivalent to controls (Figure 14 and Table 1).

**Adipose Tissue**

For female Cafr\textsuperscript{tm1Un}c mice there was a significant difference in ACC-1 expression. Female Cafr\textsuperscript{tm1Un}c mice had nearly 40% less total ACC-1 expression than wild-type controls (p=3.8 × 10^{-4}) and inactive ACC-1 was approximately 44% less in female CF mice (n\textsubscript{CF}=3, n\textsubscript{WT}=3; p=1.8 × 10^{-3}) (Figure 12A and B). For male Cafr\textsuperscript{tm1Un}c mice, there was a statistically significant difference in total ACC-1, but not for inactive ACC-1. The total ACC-1 expression was nearly 70% greater in CF-null mice compared to wild-type mice (n\textsubscript{CF}=4, n\textsubscript{WT}=4; p=2.1 × 10^{-3}) (Figure 12A). However, the levels of inactive ACC-1 were statistically equivalent, even though CF mice tended to have more inactive ACC-1 than controls (n\textsubscript{CF}=4, n\textsubscript{WT}=4; p=0.13) (Figure 12B).
There were no significant differences in the AMPk expression between female CF and wild-type mice. Female CF mice showed extreme variability in AMPk levels and as a result, there was no overall significant difference in total AMPk expression compared to wild-types ($n_{CF}=3$, $n_{WT}=3$; $p=0.28$) (Figure 13A). Conversely, there tended to be a slight difference in active AMPk levels ($n_{CF}=3$, $n_{WT}=3$; $p=6.8 \times 10^{-2}$); however, this difference was not statistically significant as the CF mice showed immense variability in the amount of active AMPk (Figure 13B). Male $Cftr^{tm1Une}$ mice and wild-type controls showed no
difference in either AMPk expression. CF-null males tended to have less total AMPk and more active AMPk; however, neither were statistically significant as wild-type controls had vastly inconsistent levels of AMPk in the adipose (n_{CF}=4, n_{WT}=4; p=0.15 and p=0.14, respectively) (Figure 13A and B).

![Figure 13. CF Adipose AMPk Expression.](image)

Values for CF males and females are normalized to their respective controls, and are presented as “relative quantity”, or a ratio between CF and WT expression, with WT mice average expression set to a value of 1. P-values are shown among the comparison lines. (A) Represents the average total AMPk concentration in adipose tissue (B) Shows the average ratio of the phosphorylated AMPk concentration to the total ACC-1 concentration.
Overall, in inguinal adipose tissue, female CF mice exhibited a reduction in the total ACC-1 expression and a subsequent increase in the inactive form of ACC-1 compared to wild-type controls. Female AMPk expression data were not statistically different; however, female CF mice tended to have more active AMPk than controls. Male CF mice had significantly more total ACC-1 expression, yet exhibited an equal amount of inactive ACC-1 when compared to controls (Figure 14 and Table 1).

**Figure 14. Liver and Adipose Western Blot Images.** Displays actual western blots completed on female and male CF and wild-type mice. ACC-1, pACC-1, AMPk and pAMPk signals were visualized using the VersaDoc imaging system.
In general, there was no observed statistically significant difference in palmitate and stearate synthesis between the hepatocyte knockouts and wild-types. The liver palmitate synthesis tended to be somewhat lower in male hepatocyte knockouts than male wild-types; however, this was not significant \((n_{KO}=6, n_{WT}=5; p_{liver}=0.28, p_{adipose}=0.30)\) (Figure 15). In addition, there were no significant differences in male hepatocyte knockout stearate synthesis \((p=0.73)\), female hepatocyte knockout liver palmitate \((n_{KO}=6, n_{WT}=7; p_{liver}=0.74, p_{adipose}=0.92)\) and stearate synthesis \((p=0.87)\) (Figure 15).

However, there was a significant difference in fatty acid synthesis between hepatocyte knockout males and females. Female knockouts produced, on average, 28-

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**TABLE 1: SUMMARY OF ADIPOSE AND LIVER WESTERN BLOT DATA**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Sex</th>
<th>Total ACC-1</th>
<th>Phosphorylated ACC-1</th>
<th>Total AMPk</th>
<th>Phosphorylated AMPk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Female</td>
<td>↓ ((p=8.2 \times 10^{-3}))</td>
<td>= ((p=0.059))</td>
<td>= ((p=0.24))</td>
<td>= ((p=0.30))</td>
</tr>
<tr>
<td></td>
<td>Males</td>
<td>= ((p=0.30))</td>
<td>= ((p=0.075))</td>
<td>↑ ((p=0.028))</td>
<td>= ((p=0.36))</td>
</tr>
<tr>
<td>Adipose</td>
<td>Females</td>
<td>↓ ((p=1.8 \times 10^{-3}))</td>
<td>↑ ((p=3.8 \times 10^{-4}))</td>
<td>= ((p=0.28))</td>
<td>= ((p=0.068))</td>
</tr>
<tr>
<td></td>
<td>Males</td>
<td>↑ ((p=2.1 \times 10^{-3}))</td>
<td>= ((p=0.13))</td>
<td>= ((p=0.14))</td>
<td>= ((p=0.15))</td>
</tr>
</tbody>
</table>

A comprehensive table displaying the levels of each enzyme measured for CF mice in comparison to age and gender-matched controls. Highlighted arrows are statistically significant differences, whereas non-highlighted arrows are approaching significance, and equal signs are neither significant nor approaching significance. P-values are listed below each symbol within the parentheses.

**Characterization of Hepatocyte Cfr Knockout Mice Fatty Acid Metabolism:**

*De novo* Fatty Acid Synthesis of Hepatocyte Knockout Mice:

In general, there was no observed statistically significant difference in palmitate and stearate synthesis between the hepatocyte knockouts and wild-types. The liver palmitate synthesis tended to be somewhat lower in male hepatocyte knockouts than male wild-types; however, this was not significant \((n_{KO}=6, n_{WT}=5; p_{liver}=0.28, p_{adipose}=0.30)\) (Figure 15). In addition, there were no significant differences in male hepatocyte knockout stearate synthesis \((p=0.73)\), female hepatocyte knockout liver palmitate \((n_{KO}=6, n_{WT}=7; p_{liver}=0.74, p_{adipose}=0.92)\) and stearate synthesis \((p=0.87)\) (Figure 15).

However, there was a significant difference in fatty acid synthesis between hepatocyte knockout males and females. Female knockouts produced, on average, 28-
32% more palmitate (p=0.056) and stearate (p=0.046) in the liver (Figure 15). This gender difference in fatty acid synthesis was not seen in wild-type mice.

![Hepatic Lipid synthesis](image)

**Figure 15.** Hepatic *de novo* fatty acid synthesis in liver *Cftr* knockout mice. Illustrates the percent fractional synthesis rate (FSR) of liver palmitate and stearate. P-values are shown above each comparison line.

**Expression of Major Lipogenic Enzymes:**

*Liver Tissue:*

Both female and male hepatocyte knockouts did not show a significant difference in either the total concentration of ACC-1 or the level of inactive ACC-1 (Females: n_{KO}=4, n_{WT}=4; p=0.40, p=0.07, respectively; Males: n_{KO}=4, n_{WT}=4; p=0.061, p=0.28, respectively) (Figure 16A and B). There were also no apparent gender differences.
Female hepatocyte knockouts did not show a significant difference in AMPk expression \((n_{\text{KO}}=4, n_{\text{WT}}=4; p=0.32 \text{ and } p=0.31, \text{ respectively})\) (Figure 17A and B). On the contrary, male hepatocyte knockouts did show a significant difference in AMPk expression as they had nearly 50\% less total AMPk than controls \((n_{\text{KO}}=4, n_{\text{WT}}=4; p=1.8 \times 10^{-3})\), and on average, approximately 60\% less than female liver-knockouts (Figure 17A). In addition, male knockouts had close to 27\% more active AMPk than controls \((n_{\text{KO}}=4,\)
n_{WT}; p=0.015) and on average, 40% more than female knockouts (Figure 17B, Table 2 and Figure 18).

Figure 17. Hepatocyte Knockout Liver AMPk Expression. Values for CF males and females are normalized to their respective controls, and are presented as “relative quantity”, or a ratio between CF and WT expression, with WT mice average expression set to a value of 1. P-values are shown among the comparison lines. (A) Represents the average total hepatic AMPk concentration (B) Shows the average ratio of the phosphorylated AMPk concentration to the total AMPk concentration.
TABLE 2: SUMMARY OF ADIPOSE AND LIVER WESTERN BLOT DATA

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Sex</th>
<th>Total ACC-1</th>
<th>Phosphorylated ACC-1</th>
<th>Total AMPk</th>
<th>Phosphorylated AMPk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Female</td>
<td>= (0.40)</td>
<td>= (0.07)</td>
<td>= (0.32)</td>
<td>= (0.31)</td>
</tr>
<tr>
<td></td>
<td>Males</td>
<td>= (0.061)</td>
<td>= (0.28)</td>
<td>↓ (0.0018)</td>
<td>↑ (0.015)</td>
</tr>
</tbody>
</table>

A comprehensive table displaying the levels of each enzyme measured for hepatocyte knockout mice in comparison to age and gender-matched controls. Highlighted arrows are statistically significant differences, whereas non-highlighted arrows are approaching significance, and equal signs are neither significant nor approaching significance. P-values are listed below each symbol within the parentheses.

**Figure 18. Hepatocyte Knockout Liver Western Blot Images.** Displays actual western blots completed on female and male hepatocyte knockout and wild-type mice. ACC-1, pACC-1, AMPk and pAMPk signals were visualized using the VersaDoc imaging system.
DISCUSSION

**Significance of Cftr\(^{tm1Unc}\) Data:**

As stated previously, there currently exist three well understood factors that lead to an energy imbalance in cystic fibrosis, including an abnormality in: (1) energy expenditure, (2) energy uptake and (3) energy losses. Energy losses, mostly contributing to pancreatic insufficiency and intestinal malabsorption, increase the need for greater energy uptake, which is further complicated by anorexia, vomiting and chronic lung disease, and thereby increases the body’s global energy expenditure. This process in turn creates a vicious cycle which boosts the body’s need for energy. Nevertheless, while research is available on each of these causes, as well as the efficiency of current medical regimens used to combat these issues, it is evident that there are most likely other underlying factors that also contribute to this energy imbalance that have yet to be explored. This study aimed to identify a potential fourth factor contributing to this energy imbalance: reduced fat synthesis. Is there an intrinsic defect in fat synthesis and beta-oxidation that prevents subsequent fat storage? Overall, the data from this study support the notion of this impaired lipid metabolism.

Analysis of hepatic *de novo* fatty acid synthesis revealed that male CF mice synthesize less saturated fatty acids in the liver than age and gender-matched controls. Moreover, there was a strong gender effect in CF mice, with males displaying, on average, a 40% decrease in hepatic fatty acid synthesis compared to females. These sex-specific differences were not unexpected, as there are a number of discrepancies between
sexes noted in the phenotypes of individuals with CF. For instance, young girls affected with CF were shown to have a lower ‘percent height-appropriate body weight’ (\%haBW) than boys [Zemel et al, 2000]. In addition, women with CF-related diabetes seem to a more severe disease course and significantly shorter lifespan than men with CF-related diabetes [Milla et al, 2005]. These are just a few of the many examples of sex-specific differences observed in CF.

Interestingly, the ACC-1 and AMPk expression results do not appear to support these fat synthesis results. Even though ACC-1 and AMPk activity also suggest the presence of an impaired fat metabolism, the pattern of expression in adipose versus liver and in males versus females is not straightforward or easily interpretable. Among male and female CF mice, ACC-1 expression in the liver and adipose showed the greatest difference compared to AMPk.

In the liver of female CF mice, the proportion of inactive ACC-1 was greater (less total ACC-1= lower proportion of active ACC-1), potentially reducing the total amount of fat synthesized. Male CF mice also tended to have more inactive ACC-1, although not statistically significant. In adipose tissue, female CF mice had greater levels of inactive ACC-1, again potentially resulting in less fatty acid chain elongation in adipose. Conversely, males had a higher proportion of active ACC-1 (more total ACC-1= higher proportion of active ACC-1), suggesting that these mice are synthesizing more fatty acids in adipose (Figure 19).
As for AMPk expression, male CF mice had a greater proportion of active AMPk (more total AMPk = higher proportion of active AMPk), suggesting increased beta-oxidation in the liver. There were no other significant differences observed for AMPk expression, including all data for CF females (Figure 19).

Figure 19. Model of the potential effects of ACC-1 and AMPk on adipose and liver. FAS - fatty acid synthesis, FAO - fatty acid oxidation.

In summary, these data suggest that fat metabolism is impaired in the CF mouse. Yet, what is ultimately responsible for these changes? This is a difficult question to answer since the interplay between adipose and liver is extremely complex and involves various hormones and signaling pathways that ultimately regulate energy metabolism. Out of these numerous pathways, there are most likely countless mechanisms that could potentially lead to this altered fat metabolism. Moreover, strong gender discrepancies
have been published in regard to other CF pathophysiology, which adds to the complexity of characterizing fat metabolism. However, there is one model which may be the most worthy of further investigation: elevated adiponectin levels.

Elevated Adiponectin Levels:

Adipokines are adipose-derived cytokines, or cell-to-cell signaling proteins produced and secreted by adipocytes. Adiponectin, a type of adipokine, directly targets both liver and adipose tissue and ultimately assists in the regulation of glucose uptake and fat metabolism. This hormone is up-regulated in response to weight loss or after short periods of fasting, and stimulates hepatic fatty acid oxidation and glucose uptake as well as inhibits glucose production and fatty acid synthesis [Karbowska and Kochan, 2006]. A study performed by Yamauchi et al., has suggested that adiponectin mediates hepatic AMPk phosphorylation via increasing the cellular concentration of AMP. The higher proportion of AMPk in turn phosphorylates, or inactivates, ACC-1 ultimately silencing fatty acid synthesis and initiating fat oxidation [Yamauchi et al, 2002].

Furthermore, a study conducted by Moriconi et al. has shown that adiponectin levels are increased in patients with cystic fibrosis [Moriconi et al., 2006]. This study compared 24 adults affected with CF to 24 healthy controls matched for BMI, age and sex. Results revealed, on average, a 40% increase in serum adiponectin concentrations in patients with CF (p=0.01), even after adjusting for known confounding variables. The authors suggest that adiponectin could be a marker of energy deficiency, as it is also
significantly higher in patients suffering from anorexia nervosa [Brichard et al., 2003; Housova et al., 2005].

In consideration of the present study, perhaps high serum levels of adiponectin elevate the hepatic intracellular AMP concentration, thereby increasing the activity of AMPk and reducing the activity of ACC-1, eventually diminishing hepatic fatty acid synthesis and increasing beta-oxidation. Additionally, it is possible that this process is also occurring in adipocytes. Studies have shown that adipocytes house adiponectin receptors, and therefore, adiponectin may play a direct role in fat metabolism regulation of adipose tissue [Karbowska and Kochan, 2006]. In general, if hepatocytes are not synthesizing enough lipids for storage in the adipose, then the resulting mouse or individual will have a smaller lean body mass. Additionally, it is possible that since CF males exhibit less fatty acid synthesis in the liver, that they have higher levels of circulating adiponectin than females.

If this were to be the mechanism responsible for the reduced de novo fatty acid synthesis observed, one would expect to see high levels of activated AMPk and high levels of inactivated ACC-1. These data support this model in that female CF mice had higher levels of inactivated ACC-1 in both liver and adipose tissue. Additionally, male CF mice were shown to have higher levels of activated hepatic AMPk. However, there were other patterns observed in the ACC-1 and AMPk expression data that does not support this model. For example, there were no significant differences in AMPk expression in adipose or liver of CF females. In addition, even though males displayed
higher levels of activated hepatic AMPk, these levels are based upon a higher net expression of total AMPk; in other words, males had a higher total AMPk expression; and therefore, a higher total proportion of phosphorylated AMPk compared to controls. CF males also displayed lower levels of inactivated ACC-1 in adipocytes compared to controls, suggesting that they should hypothetically be producing more fat for storage in adipose. As a result of these discrepancies, further research needs to be performed in order to better interpret these findings.

**Hepatocyte Tissue-Specific Knockouts:**

In summary, this model can provide some explanation of the observed data. Yet, there still exist many discrepancies between male and female CF fat metabolism in both adipose and liver. In order to further elucidate these data, fatty acid synthesis as well as enzyme expression were analyzed in mice with Cftr missing only in the liver. If this model held true, one would not expect any change in fat synthesis since adiponectin is mostly produced and secreted by adipose, not hepatocytes. However, the data from the present study indicate no difference in *de novo* fatty acid synthesis between hepatocyte knockouts and controls. While these results are not unanticipated, considering some studies suggest that hepatocytes do not express *Cftr*, enzyme expression analysis yielded higher levels of AMPk activity in CF males compared to control males. Either this result occurred by random chance, or this suggests that the liver should not be discarded as a potential contributor to the observed defect in fat metabolism. In other words, high AMPk activity could signify that the liver is undergoing higher levels of beta-oxidation, another process of fat metabolism that was not directly measured as part of this study.
Overall, further research will need to be performed on hepatocyte-tissue specific knockouts to clarify these findings. Perhaps measuring beta-oxidation intermediates and/or products, or perhaps performing more AMPk western-blot analyses on a larger sample size of males would help elucidate the role of hepatocytes in the CF lipid metabolism phenotype. Nevertheless, this data has shown that losing Cftr in the liver does not lead to the same biochemical profile or phenotype observed in the Cftr-null mice. Therefore, we can rule out the liver as a causative tissue and instead suggest that this reduced fat metabolism is a secondary effect resulting from the loss of Cftr in other tissues.
CONCLUSIONS

Summary of Findings:

In summary, these data support the notion of a fourth mechanism, or a reduced fat synthesis, that may potentially contribute to the global energy deficit responsible for the reduced lean body mass phenotype observed in CF mice. Overall, there were significant differences noted in both de novo fatty acid synthesis as well as lipogenic enzyme expression. However, these differences varied among CF males and females, as males synthesized considerably less fatty acids than females in the liver. In addition, both males and females had differing levels of ACC-1 and AMPk expression compared to one another, making these results very complicated to interpret. In addition, these results also conflicted with the de novo fatty acid synthesis findings and were not simply explained by other potential mechanisms of fat metabolism regulation.

In order to further elucidate these findings, fatty acid synthesis and lipogenic enzyme expression were examined in hepatocyte tissue-specific knockout mice. No differences were noted in the de novo fatty acid synthesis of both CF males and females; however, male CF mice had significantly elevated AMPk activity compared to both male controls and CF females, suggesting that perhaps we cannot discount the liver as a minor contributor to the reduced fat metabolism in CF. As the above findings indicate a highly complex regulation of CF lipid metabolism, additional studies will need to be performed to further clarify the role of the liver and adipose, as well as the underlying pathways and mechanisms involved in this intrinsic metabolic defect.
Implications for Genetic Counseling and the Field of CF Research:

As stated previously, it is important for genetic counselors to comprehend the phenotypes of the diseases for which they counsel families. Since genetic counselors and geneticists are responsible for providing patients and their families with important information relating to disease manifestations, treatment, management, prognosis and anticipatory guidance, these healthcare providers should recognize the underlying mechanisms and risk factors responsible for CF manifestations, including the mechanisms contributing to poor growth. Understanding these mechanisms may also give insight into the efficacy of current treatment approaches with regard to attempts to restore the energy balance.

In addition, understanding the fundamental causes of a reduced body mass in CF is extremely important as a low BMI has been associated with worsened pulmonary function. More importantly, understanding these mechanisms can even set the stage for future therapies. For instance, perhaps a medication could be developed that increases fatty acid synthesis and subsequent triglyceride storage within the body, such as in adipose or lung tissue. Perhaps this would improve pulmonary function, leading to a healthier individual with a longer lifespan.

Study Limitations:

One of the major weaknesses of this study was the small sample size. Some of the ACC-1 and AMPk enzyme data were approaching statistical significance, with p-values close to 0.05. Therefore, perhaps the differences in enzyme expression were too slight to
detect with only 3-7 subjects. Instead, a larger sample size may be able to detect more subtle differences, and thus give more insight into the mechanism of the defect in fat metabolism.

A second major limitation of the study involves the method of signal analysis used in the western-blotting procedure. The Versadoc QuantityOne tool used to detect and analyze the antibody signal was designed to measure the resulting intensity, or darkness, of the band after a set exposure time. In other words, this form of analysis relies on the intensity of the bands as a measurement of the total enzyme signal emitted. However, if a signal is very strong, it may be possible that the Versadoc did not completely capture the entire signal if the resulting band has reached its maximum intensity or darkness. As a result, some signals may be under-detected, which could ultimately skew results.

In addition, this study only investigated the fat metabolism of six-week-old mice. Considering metabolism in general varies at different ages, it would be important to investigate these strains at different ages. Also, another limitation of this study is that the hepatocyte knockout mice were not on a congenic background, and were therefore subjected to genetic variability in the form of different CF modifier genes.

**Directions for Future Research:**

In order to compensate for the limitations described above, the following studies should be performed in the future. First, *de novo* fatty acid synthesis and lipogenic
enzyme analysis should be performed on a greater number of $Cftr^{tm1Unc}$ as well as hepatocyte knockout male and female mice. This may allow for the detection of more subtle differences in ACC-1 and AMPk expression. Secondly, an ELISA (enzyme-linked immunosorbent assay) should also be performed in conjunction with the western-blots. ELISAs are designed to capture the complete signal emitted by the antibody. Nevertheless, ELISAs also have a limitation in that they capture signal emitted from artifactual bands; and therefore, ELISAs may over-represent the actual signal emitted from the enzyme. Thus, both ELISA and western blot data together should give a more accurate and appropriate range of enzyme expression. Third, fatty acid synthesis and ACC-1/AMPk enzyme expression should be examined in mice of various ages in order to gain more insight as to when the defect in fat metabolism appears and if this defect is consistent throughout the lifespan of the mice.

In order to further elucidate the mechanism underlying the lipid synthesis defect and its potential relationship to a reduced BMI, de novo fatty acid synthesis and lipogenic enzyme expression should be measured in adipose tissue-specific $Cftr$ knockouts. $Cftr$ has been shown to be expressed in adipocytes [unpublished data from the Drumm laboratory]; therefore, losing $Cftr$ in adipose may directly impact fat metabolism in both the liver and adipose tissue, perhaps even via the adiponectin model explained previously. The subsequent growth of adipose tissue-specific knockouts has not been shown to differ significantly from controls [unpublished data from the Drumm laboratory]. However, it is possible that there exist subtle differences in weight which may involve measuring a larger sample size of mice in order to detect. Nonetheless, it is
still worthwhile to investigate the adipose as a possible candidate responsible for altered lipid metabolism.

In addition, future studies should also investigate the lipid synthesis profiles of neuronal plus adipose tissue-specific Cftr double-knockouts. First of all, neuronal knockouts have already been shown to have reduced growth that primarily manifests as skeletal shortening [Hodges et al., 2009]. Secondly, the interplay between adipose and neuronal tissue (specifically the hypothalamus and pituitary) is an extremely complex process that involves multiple signaling pathways that regulate energy homeostasis [Widmaier, Raff, Strang, 2006]. Thus, perhaps Cftr needs to be absent in both neuronal tissue as well as in adipocytes in order for the resulting mice to display a reduced BMI in the form of reduced linear growth as well as reduced fat mass.

Lastly, to gain more insight into the possible explanatory models discussed previously, it may be worthwhile to measure the levels of circulating adiponectin in Cftr knockout mice to see if adiponectin levels are elevated as observed in CF patients. In addition, the intracellular concentration of fatty acid synthesis intermediates acetyl coA and malonyl coA should also be measured as this would provide a direct measurement of ACC-1 enzyme activity, since ACC-1 catalyzes the reaction of acetyl coA to malonyl coA. If any of these levels are significantly altered in CF mice, especially males, this may provide an explanation for the observed reduction in fat synthesis.
APPENDIX 1

Generation of Conditional Null Mice

The Cre/LoxP system is a new technology for generating mice with a gene knockout in exclusive tissues of the body. The basis of this technique involves the site specific excision of a fragment of the studied gene, rendering the gene dysfunctional within a certain tissue. This process involves the generation of three strains of mice: (1) a strain with excisable sites located within the significant gene (loxP sites), (2) a strain with a separate gene encoding an enzyme that excises these sites (Cre recombinase) and (3) a strain that includes both these genetic components (the actual knockouts).

The first strain used in developing Cftr tissue-specific knockouts was previously generated and available for use in this study. This strain was developed using targeted mutagenesis of mouse embryonic stem cell lines, two LoxP sites were positioned around exon 10 of Cftr, targeting this section of the gene for excision [Hodges et al., 2008]. LoxP sites are small 34 base-pair repetitive DNA sequences that can undergo nonhomologous recombination with one another, creating a deletion within the gene [Kos, 2004]. This nonhomologous recombination is fostered by Cre recombinase (Cre), a bacteriophage enzyme that catalyzes recombination or excision between the two repetitive sequences. When LoxP sites are positioned in the same direction, Cre catalyzes the excision of the intervening DNA sequence along with one of the LoxP sites. In order to express Cre recombinase within a designated tissue, a transgenic mouse line must be generated that is composed of both the isolated Cre gene and an upstream mouse tissue-specific promoter,
limiting Cre expression solely to the tissue of interest [Kos, 2004]. The transgenic Cre mouse used to generate the hepatocyte knockouts was Albumin Cre, since albumin is a liver-specific protein, and thus an ideal gene in which to position the Cre gene.

To generate a tissue-specific Cftr knockout, a strain of mice containing both the Cre and LoxP genetic components was developed. More specifically, Cre expression within the specific tissue will excise exon 10 of Cftr, resulting in a dysfunctional gene located exclusively within the tissue of interest (Figure 20).

Figure 20. Cre/LoxP system for generating a tissue-specific knockout. (a) The Cre recombinase gene, positioned near a tissue-specific promoter, is continually transcribed and translated for use within the specific tissue. (b) Cre recombinase catalyzes the excision of exon 10. (c) A deleted exon 10 allele results, forming a tissue-specific Cftr knockout. Adapted from Hodges et al., 2008.
Mice containing both the albumin cre transgene and the targeted $Cfr$ gene were generated through a series of crosses. The first cross included a homozygous LoxP mouse with a heterozygous tissue-specific Cre mouse (Figure 22). In order to create a full $Cfr$ knockout mouse, there needs to be two LoxP sites on both $Cfr$ genes. Yet only one Cre transgene is needed for adequate levels of Cre recombinase. Some strains, such as the albumin Cre strain, have both viable homozygotes and hemizygotes; however, other Cre strains may have non-viable Cre homozygotes. This is because in some cases, the transgenes are positioned in unknown locations within the genome, and as a result, the presence of two transgenes may cause a recessive disease.

The resulting F1 generation from cross 1 had a total of two different Cre/LoxP genotype combinations: LoxP+/−Cre−/− and LoxP+/− Cre+/−. One of these F1 LoxP and Cre heterozygote (LoxP+/− Cre+/−) was bred to the original LoxP homozygote (LoxP+/+, Cre−/−) in a second cross resulting in offspring with many possible combinations of LoxP/Cre genotypes, the most important being that which contained both Cre and LoxP genetic components (Figure 21). From this F2 generation, the mice with a LoxP+/+Cre+/− genotype were true knockouts and were used as experimental subjects. Wild-type controls were mice with both LoxP targeted alleles, with no Cre transgene (LoxP+/+Cre−/−).
Figure 21. Cre/LoxP crossings for generating a tissue-specific knockout. (a) Cross 1 involves a LoxP homozygote mouse and Cre hemizygote mouse. The resulting F1 prodigy shown in red will be bred in cross 2 (b) Cross 2 involves one LoxP +/- and Cre+/- and one LoxP +/+ and Cre -/- mice. The resulting F2 mice shown in red are the experimental knockouts, and the offspring in black bold are the experimental wild-type controls.
## APPENDIX 2

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REFERENCES


Hodges, Craig. Preliminary data on conditional *Cftr* neuronal knockouts. Case Western Reserve University: 2009.

Hodges, Craig. Preliminary data on conditional *Cftr* intestinal epithelial knockouts. Case Western Reserve University: 2009.


