INVESTIGATING THE LOW ADIPOSITY OF CYSTIC FIBROSIS MICE

by

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Abstract

by

JEANNIE KLAVANIAN

Background: Cystic fibrosis (CF) patients and mice have low amounts of adipose tissue. The purpose of this study was to determine whether this adipose tissue deficiency is due to fewer adipocytes, smaller adipocytes, or both.

Methods: Studies were performed on F508del (Cfrtm1kth) mice and wild-type (WT) littermates. Gonadal fat pads were excised and weighed. DNA and triglycerides were extracted from fat pads and quantified using PicoGreen and colorimetric assays. Cell number and density were estimated from histological sections of CF and WT adipose tissue.

Results: CF mouse adipocytes in inguinal fat pads were found to be similar in number between CF and non-CF samples, as both had comparable DNA content, but histological assessment showed that CF cells were clearly smaller than non-CF adipocytes. CF adipocytes had less triglyceride when normalized to DNA content.
Conclusions: CF mice have smaller adipocytes but similar total number of adipocytes compared to WT mice.

INTRODUCTION AND RESEARCH AIMS

Cystic Fibrosis (CF) is the most common lethal autosomal recessive inherited disorder in the Caucasian population. The disorder is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene (37), which encodes an anion channel that regulates fluid and electrolyte balance through chloride permeability and downregulation of sodium transport across epithelial tissue (12, 47). Chloride, sodium, and water channels function on a chemical gradient. The concerted action of ion channels and transporters maintain the fluid balance across an epithelium and in the airway and other tissues. These processes keep the mucus layer appropriately hydrated, allowing cilia on the apical cell surface to beat and accomplish transport of mucus from the distal to proximal airways. Mutations in the CFTR gene prevent chloride permeation across the apical membrane of epithelia through CFTR, disrupting transepithelial ion transport and fluid flow. Specifically, chloride secretion is impaired with an accompanying increase in sodium absorption, which causes water to be absorbed across the epithelium. This results in a dehydrated surface epithelium conducive to the formation of sticky mucus that can damage different organs. It is of major concern when this thick, sticky mucus accumulates in the lungs, impeding the clearance of bacteria by cilia, resulting in chronic infection and
inflammation. This causes progressive deterioration of lung tissue, reducing pulmonary function in humans with CF and eventually leading to death (47).

CFTR mutations affect a number of body systems in addition to the respiratory tract. The majority of CF patients exhibit exocrine pancreatic insufficiency at birth (37). Pancreatic insufficiency results from the destruction of pancreatic acinar cells responsible for the secretion of pancreatic enzymes for the breakdown of nutrients in the small intestine. As a result, CF patients exhibit growth retardation from severe malnutrition (41). To treat pancreatic insufficiency and alleviate malnutrition, patients are placed on Pancreatic Enzyme Replacement Therapy (PERT). Despite PERT and adequate nutritional intake, patients continue to exhibit some degree of steatorrhea, low body weight, inadequate height, and low Body Mass Index (BMI) (58).

Studies show that although the proportion of underweight individuals with CF has decreased over the decades (38), the average height (48, 38) and weight (49) of patients is still below the average of non-CF populations, even with nutritional intervention and enzyme supplementation. This persistence of malnutrition and growth retardation is not fully understood, and suggests that there are additional contributing factors to poor growth aside from exocrine pancreatic insufficiency and poor nutrition. This is of concern because there is a significant correlation between severity of growth retardation and severity of lung disease in CF patients (21, 30). Specifically, the greater the growth retardation in a patient, the poorer the prognosis is because of an increased risk for pulmonary disease (31). This
relationship, although not fully understood, is important when evaluating clinical interventions for CF patients.

Recent research includes elucidating factors contributing to growth retardation in CF in addition to pancreatic enzyme insufficiency and increased dietary energy requirements. The focus of the research presented here deals specifically with the adipose tissue deficit observed in CF patients. Low adipose stores have been demonstrated previously in CF patients (36, 50) compared to the general population. Adipose tissue is important in energy homeostasis and is essential for proper storage and utilization of energy. The deficit of adipose tissue, even in the presence of high fat diets and pancreatic enzyme supplementation, is suggestive of an imbalance of metabolism. Thus, the fact that patients with CF have low levels of adipose tissue leads to the following scientific question: In CF patients, is the lack of adipose cell storage a result of altered adipocyte size, adipocyte number, or both?

The purpose of this study was to characterize the nature of deficient fat stores in CF patients. To investigate this difference, adipose tissue was studied in mice with the functional absence of CFTR, specifically those homozygous for the F508del mutation. See appendix I for complete list of experimental mice. CF mice share multiple phenotypic manifestations with CF patients, such as severe intestinal disease; however their housing in microisolator cages prevents most spontaneous lung infections and their minimal exocrine pancreatic involvement would not classify them as insufficient (45, 44). Despite these difference, CF mice have significantly lower body weight, length, BMI, and adipose tissue even with normal dietary lipid absorption and a normal (4) or high fat diet diet (5),
thereby providing a good model to study the adipose tissue deficit present in CF patients without the confounding effects of chronic infection or pancreatic insufficiency.

Bederman, et al. recently found that de novo lipogenesis was decreased in CF mice (4). They also revealed that hepatic triglyceride (TG) content was lower in CF adipose tissue as compared to WT adipose tissue. Based on this, it was hypothesized that CF mice have smaller adipocytes resulting from less lipid deposition.

In order to address these questions, the aims of this study were to:

1. Determine if there is a difference in the size, quantity, and/or volume of adipocytes in CF mice and WT mice adipose tissue that can be observed histologically.

2. Determine DNA content from adipose tissue from a cohort of CF and WT mice to determine adipocyte concentration within each tissue sample.

3. Compare triglyceride levels in CF and WT mouse adipose tissue to investigate differences in size of adipocytes.

This study compared the adipose tissue of CF mice to WT mice molecularly and visually. Determining adipocyte quantity among tissue samples allows for the measurement of adipocyte size. Determining the size and quantity of adipose cells in CF mice compared to
WT mice can help determine the functionality and effectiveness of those cells in acquiring and releasing fat in adipose cells. CF and WT mice were compared visually by looking at the histology of CF and WT mouse adipocytes using hematoxylin and eosin (H&E) staining, and comparing adipocyte size and morphology microscopically. CF and WT mouse tissue were compared molecularly by taking similar amounts of adipose tissue from CF and WT mice and comparing DNA content to overall adipose tissue weight. Since DNA weight stays constant from cell to cell, DNA concentration within adipose tissue quantifies the number of adipose cells within a tissue sample. In order to further analyze adipocyte content, TGs were quantified and normalized to adipocyte concentrations to understand TG content within adipocytes. Values were compared between CF and WT mice. Possible outcomes and implications of this study are demonstrated in Figure 1.

**Figure 1: Possible outcomes of adipose tissue investigation.** (A) CF mice have more adipocytes per gram of adipose tissue. Since CF mice have less adipose tissue in their fat pad, this would indicate that CF mice have smaller adipocytes and a similar or less number of total adipocytes than WT mice. (B) CF mice have fewer adipocytes present per gram of adipose tissue, which would indicate that, CF mice have fewer total adipocytes in their fat pad that are larger than WT mouse adipocytes. (C) CF mice have fewer adipocytes present per gram of adipose tissue, which would indicate fewer total adipocytes that are relatively the same size as WT mouse adipocytes.
BACKGROUND AND SIGNIFICANCE

Cystic Fibrosis: Disease Characteristics

Cystic Fibrosis (CF) is the most common autosomal recessive disorder present amongst Caucasians. The disease has an incidence of 1/2500 and a carrier frequency of 1/25 among people of European ethnicity (2). The disease results from mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR) (37) with over 1800 mutations identified (8). These mutations result in a variable phenotype, even among patients with identical mutations. The most common mutation is a deletion of phenylalanine codon 508, referred to as F508del. Approximately 87% of people with CF have at least one copy of F508del, and 47% of people with CF have 2 copies (9).

The CFTR protein is expressed in various tissues throughout the body. Primarily, it functions as a cAMP-regulated chloride channel that, when absent, alters regulation of other ion channels, specifically sodium and water channels (37). CFTR normally controls the transport of chloride ions across epithelia in order to maintain hydration on surface epithelia and ducts that line the airways, intestinal tract, and other organs (20). When CFTR is not functioning properly, chloride ions cannot permeate the epithelium thorough CFTR, and sodium is hyper-absorbed into the
cell. This causes water to move through the cell, dehydrating the luminal surface, which is conducive to the build-up of a thick mucus coat (see Figure 2). This mucus can damage organs and collect in the lungs where it fosters bacterial infections that can be lethal (47). Many secretory organs are affected in CF, including the pancreas, the upper and lower respiratory tracts, biliary system, male genitalia, intestine, and sweat glands. CFTR is also expressed in cells other than epithelial, such as lymphocytes and cardiac myocytes, and functions as a chloride channel within intracellular compartments, such as the endoplasmic reticulum (52).

The disease typically manifests in early childhood, usually in the form of chronic respiratory problems (37). This progressive lung disease is the most prominent indicator of CF, and deterioration of lung tissue with eventual pulmonary failure is commonly the cause of mortality and morbidity in patients. However, there is much variability of clinical manifestations among patients, and the disease is characterized by a number of symptoms in addition to pulmonary disease (51). CF can present as early as birth, or as late as adulthood. Male patients are azoospermic as a result of bilateral absence of the vas deferens. Infants can have meconium ileus, protracted jaundice, abdominal calcifications, failure to thrive, hypoproteinemia, cholestasis, abdominal distention, staphylococcus aureus pneumonia, vitamin A deficiency, hemolytic anemia, and intestinal atresia. Children can have nasal polyposis, steatorrhoea, rectal prolapse, distal intestinal obstruction, chronic pancreatitis, and liver disease. Adolescents and adults can develop allergic bronchopulmonary aspergillosis, pansinusitis, bronchiectasis, haemoptysis, portal
hypertension, and delayed puberty (51). Poor growth, which is the focus of this research, is very common and is the result of a multitude of factors.

**Growth retardation in Cystic Fibrosis patients**

Historically, failure to thrive and poor growth were major contributors of death in children with CF before treatment options, such as enzyme replacement therapy, became available. Patients with CF typically exhibit reduced and delayed growth in both weight accretion and linear growth. Lai, et al analyzed the heights and weights of 13,116 children with CF in the 1993 National CF Patient Registry and reported the mean and median height-for-age and weight-for-age in children with CF to be in the 30\textsuperscript{th} and 20\textsuperscript{th} percentile, respectively. This study also demonstrated that 20\% of these children were less than the 5\textsuperscript{th} percentile in height-for-age or weight-for-age (32). Another study examined 25 children with CF ages 5-10 and compared them to a control group of equal weight, age, and sex (50). They reported a slower growth rate in boys with CF compared to those without CF in regard to height, fat-free mass, and fat mass, even with comprehensive nutritional care. A reduction of fat and fat-free mass was also shown in a study involving 143 children and adolescents aged 2-18 with CF as compared to healthy controls (1).

Given advances in treatment, there has been a change in average body composition in CF patients in the last 20 years. The average BMI (kg/m\textsuperscript{2}) in patients with CF increased from 20.7 in 1990 to 22.3 in 2011 (49) and the proportion of underweight patients (<18.5 BMI) has decreased from 20.6\% before 1990 to 11.1\% in 2011. These numbers show that the CF population is improving when it comes to growth. However, a recent study by Woestenenk
et al (2013), demonstrated that mean height and weight are still below the average in CF patients compared to the general population.

**Gaining Weight Is Associated With the Best Prognosis**

Difficulties gaining weight are of major concern because of the correlation shown between poor growth and poor pulmonary function in CF patients. Specifically, a patient’s degree of underweight is inversely correlated with survival (31). Patients who are pancreatic sufficient, thus able to break down and absorb fat more efficiently, have significantly better pulmonary function than their counterparts with steatorrhea (21). Furthermore, Konstan et al. (30) demonstrated that even at 3 years of age, patients with CF have significant clinical evidence of lung disease and substantial growth retardation and that both of these factors are independent predictors of pulmonary function at age 6. For this reason, treatment largely focuses on helping patients maintain a healthy weight.

Still unknown regarding the correlation between lung function and growth is which component is the cause and which component is the effect. In other words, is poor growth contributing to the advancing pulmonary disease, or are more severe pulmonary problems hindering growth? Certain medications that have improved lung function have not been as effective at improving weight gain in patients (42), and having a healthy BMI does not always indicate better pulmonary function. If a contributing factor of poor growth can be identified, then those data can aid in determining the degree of contribution towards pulmonary function.
Causes of Poor Growth in Patients with CF

Impaired growth has been primarily attributed to malnutrition, malabsorption, inadequate intake of macronutrients, and excessive energy expenditure (22).

Malabsorption

Malabsorption occurs in patients as a result of exocrine pancreatic insufficiency, intestinal dysfunction, and biliary tract disease. Pancreatic insufficiency is prevalent in 85-90% of patients with CF (14) and results in reduced absorption of dietary nutrients, particularly triglycerides (TGs). Lipid malabsorption leads to steatorrhea, loss of fat-soluble-vitamins, and loss of essential fatty acids. Normally, the pancreas secretes pancreatic enzymes that contribute to lipolysis of TGs, resulting in intestinal absorption. Pancreatic insufficiency describes low to no secretion of pancreatic enzymes resulting in impaired lipolysis and decreased absorption of dietary fat, leading to steatorrhea. This is a primary cause of poor growth and malnutrition in untreated patients. Pancreatic insufficiency is treated by pancreatic enzyme replacement therapy (PERT), which is essential to help patients achieve optimal growth and nutrition status. Before supplementation was initiated, patients could excrete as much as 80% of fat intake. With the supplementation, 10-20% of dietary fat is still being malabsorbed in many patients, but this is a marked improvement (58).

CF patients also have a reduction of pancreatic and duodenal bicarbonate secretion, resulting in lower (by 1-2 units) duodenal pH. A normal acidic intestinal pH of 1.5-7, depending on the stage of digestion, is important for adequate digestion and absorption of fat, since an alkaline environment promotes digestive enzyme activity. Bicarbonate is
needed to neutralize acid from the stomach. When compared to non-CF controls, patients with CF reportedly have longer periods of pH levels below 4.0 in the postprandial period, and less time above a pH of 5.8. This is problematic because lipase is irreversibly destroyed at a pH of 4.0 and the coating of enzyme supplements dissolve at a pH of 5.8. Moreover, a correlation exists between postprandial pH levels and the degree of fat absorption in CF patients who are treated with PERT. The addition of bicarbonate to pancreatic enzymes results in a slight increase in the average fat absorption in CF patients. While these results are not significant enough to warrant implementation of this modified drug to patients with CF, this disrupted duodenal bicarbonate secretion is thought to play a role in poor fat absorption in the presence of PERT (43).

Intestinal disease is present in a number of CF patients and is illustrated by intestinal obstruction and meconium ileus, which is thought to have an effect on proper nutritional absorption (16). Additionally, CF mice have been shown to have severe intestinal disease (45). Hodges et al. (23) found that absence of CFTR in the intestinal epithelium of mice causes gut obstruction but does not impair growth. Additionally, when CFTR function was restored to intestinal epithelium, obstruction was prevented, but growth remained low. Although intestinal dysfunction is thought to contribute to low growth, these data illustrate that this contribution is small.

*Inadequate intake of nutrients*

Low-fat diets, decreased appetite, anorexia, and metabolic disease are all factors that contribute to inadequate intake of nutrients. Nutritional rehabilitation has become standard
protocol in patients with CF. The goal is to identify patients with CF as early as possible, preferably through newborn screening, and start nutritional interventions and pancreatic supplementation in order to promote better long-term growth outcomes (41). Nutritional intervention consists of a high-energy high-fat diet, composed of 35%-40% calories from fat in order to meet the increased energy demands, illustrated later, in CF patients. Fat-soluble vitamin supplements are also recommended (27). With improvements in medical management, CF patients have better growth, but intestinal fat malabsorption is still present to varying degrees in patients (58).

*Excessive energy expenditure*

In order for an individual to have a constant weight, there must be an energy balance, and energy intake must equal energy expenditure. The fact that some CF patients still struggle to maintain a healthy body weight, regardless of PERT and nutritional support, implies an imbalance between dietary intake and energy expenditure. Studies do indeed show that CF patients exhibit an elevated resting energy expenditure compared to those without CF, which is found to be negatively correlated with pulmonary function and nutritional status (53). Elevated resting energy expenditure has also been shown in mice with *CFTR* mutations (11). An elevated resting energy expenditure in combination with the low lean tissue mass and impaired exercise capacity (46) is contradictory since lean tissue mass is positively correlated with resting energy expenditure. This suggests an intrinsic problem in adipose tissue and muscle tissue function, which are both key in supporting energy homeostasis. This is further supported by the fact that CF mice do not respond to nutritional replenishment even with high energy diets (5). The intake of dietary energy substances,
such as carbohydrates, lipids, and proteins, are not being stored as energy reserves. Therefore, CF patients and mice not only are unable to take in enough energy, they also are utilizing more energy at rest, creating a negative energy balance.

**Characteristics of adipose tissue**

White adipose tissue (WAT) plays a large role in energy homeostasis within organisms. The primary function of WAT is to store and supply energy when needed via lipogenesis and lipolysis, respectively. WAT uses its buffering capacity to allow adipose cell expansion and adipose cell shrinkage during energy storage and energy use, respectively. The expansion of adipose tissue can happen either by cells increasing in size (hypertrophy), or number (hyperplasia). Adipose cells can expand to store excess fatty acids, or shrink by hydrolyzing stored TGs (26). The buffer capacity is illustrated by low circulating triglycerides in the adipose tissue postprandially (1-2 mg in a mouse), compared with the bulk of dietary TG intake (100-200 mg in a mouse) being deposited in adipose tissue. This inhibits excessive lipid uptake by non-adipose tissue, which could potentially lead to lipotoxicity (19).

In healthy human adults, aged and malfunctioning adipose cells are replaced by new differentiating ones, keeping total adipocyte number constant. During development, WAT expansion is driven mostly by hyperplasia, whereas in adulthood, expansion is mainly attributed to hypertrophy. Adipose cells exhibit a wide range in their volumes, with some adipocyte diameters differing by 10-fold. This variability is due to its primary function of storage. The larger the adipose cell, the more lipid it can store (26).
The physical characteristics of adipose cells impacts cell function. Relationships between adipose tissue morphology and subsequent physiological effects have been reported. Arner et al. (3) looked at subcutaneous adipocyte size and morphology in people with BMI’s ranging from 18-60 kg/m². They found a curve-linear correlation between fat mass and adipocyte volume, which supports the concept that adipose tissue growth can be attributed to both an increase in preexisting cell volume and the generation of new adipocytes. The authors also found that subjects with hypertrophy generated fewer adipocytes over time, which resulted in their existing adipocytes to accumulate more lipids than the adipocytes of patients that were in a hyperplastic state.

Differences in environmental conditions and genetic factors are shown to also have an impact on adipocyte gene expression and metabolism. Jernas, et al. (2006) used microarray analysis to examine differences between populations of large and small cells (25). They found several genes, including leptin, expressed at much higher levels in large cells, which demonstrate that hypertrophy can significantly alter gene expression which can have an effect on adipose cell function. This supports previous studies that show the enlargement of subcutaneous abdominal adipocytes is associated with lipid mobilization and increased glucose metabolism, and is also an independent predictor of insulin resistance and type 2 diabetes (55). The fact that genetic expression within an adipocyte can alter adipocyte function is important to be aware of when considering causes of differences in adiposity.
These studies demonstrate that having an understanding of different adipocyte morphological features can help in predicting what could cause varied adipose tissue morphology and what the implications could be in terms of body metabolic functioning.

**Studies on Adipose Tissue Storage and Lipid Metabolism in CF Patients**

Since adipose tissue plays an important role in energy homeostasis in individuals, it is reasonable to predict that there is an adipose tissue defect in CF patients, as many studies have supported the presence of an energy imbalance. Adipose tissue stores are shown to be low in CF patients when compared to non-CF populations. Newby et al. studied the body composition of eight adults with CF and found low amounts of lean and fat tissues, averaging 8.3 and 2.7 kg less tissue than controls, respectively (36). In addition to low fat storage, other defects in lipid transport and deposition have been shown to be dysfunctional. Sources and pathways for lipid deposition are illustrated in Figure 3.

**Figure 3: Adipose storage sources.**
Dietary carbohydrate and fat intake contribute to overall fat storage within an individual. Dietary fat is broken down in the intestines, and carried through the body’s circulation, and deposited in the adipocytes. Dietary carbohydrates are carried directly to fat cells for storage, or to the liver to undergo de novo lipogenesis where the newly made lipids are deposited in adipose tissue.
Studies on metabolic characteristics of CF patients show a reduction in intestinal uptake and solubilization of long chain fatty acids (28), low levels of essential fatty acids in circulating pool of lipids (17), and a defect in insulin-mediated suppression of adipose tissue lipolysis (33). Collectively, these studies suggest that adipose tissue energy imbalance is caused by decreased lipid uptake and increased TG breakdown. Elevated TG breakdown, or lipolysis, is a contributing factor of elevated resting energy expenditure, which, as illustrated earlier, is seen in CF patients and mice.

In 2002, Figueroa and colleagues showed that 16% of CF patients had higher circulating TG levels (hypertriglyceridemia) and lower cholesterol concentrations than the population means in the United States (18). Normally, TG elevation is associated with obesity, but this correlation has not been observed in CF patients. These increased TG levels were thought to be due to excessive hepatic TG synthesis, decreased TG clearance, or a combination of both. CF patients who have hypertriglyceridemia have also been shown to have an increased insulin level (24). Insulin plays an important role in lipid metabolism by activating lipoprotein lipase, which is necessary to transport TGs into the adipose cell, as well as upregulating hormone sensitive lipolysis, which is key in lipolysis (33). Patients have also been shown to have elevated leptin levels, which is counterintuitive, because leptin is normally present in blood serum in direct correlation to the amount of adipose tissue present, with larger cells secreting more leptin (1).

These studies demonstrate decreased uptake of lipids, decreased absorption of dietary fats, increased adipose tissue breakdown, and an increase in circulating TGs in patients. These
data support the presence of a physiological difference in the adipose tissue between CF patients and the non-CF population.

**CF mouse models**

CF mice have proven to be a good model to study cystic fibrosis. This work focuses on mice with the F508del mutation in *CFTR*. Unlike humans, F508del mice lack pulmonary complications and their pancreatic insufficiency is mild (44). Additionally, they exhibit a more severe intestinal phenotype than CF patients. Similar to humans, they have severe intestinal disease and obstruction that can lead to complications and early death. F508del mice are found to have significantly lower body weight, length, BMI, and adipose tissue even with normal food intake and dietary lipid absorption (4). They also exhibit increased energy expenditure (11). Absence of CFTR in the intestinal epithelium of mice causes gut obstruction, but does not impair growth. Additionally, when CFTR function is restored to the intestinal epithelium, obstruction was prevented, but growth remained low. These findings suggest that problems with growth are not entirely dependent on food consumption, degree of pancreatic sufficiency, and intestinal dysfunction, making these mice an ideal model to study additional factors contributing to growth retardation.

Lipid metabolic abnormalities have been seen in CF mice as well. Bederman, et al. found that de novo lipogenesis was decreased in CF mice (4). They also revealed that hepatic TG content was lower in CF adipose tissue as compared to WT adipose tissue, which could be attributed to either lower rates of adipose tissue lipogenesis, or reduced deposition of hepatic de novo triglycerides. The observed increase of circulating TGs in CF patients,
combined with the low amount of hepatic TG deposition in adipose cells in CF mice suggests a defect in adipocytes’ ability to sequester fat.

Summary

In summary, previous research has demonstrated that patients and mice with CF have low adipose stores and an abnormal metabolic profile. These findings support the hypothesis that there is an underlying morphological difference in the adipose tissue of CF patients that causes this atypical adipose tissue profile. The purpose of this study was to elucidate whether low levels of adipose tissue in CF mice is due to fewer adipose cells, smaller adipose cells, or a combination of these possibilities. Further understanding of the adipose tissue difference that is present in CF adipose tissue, and whether it is a quantitative or a qualitative problem, will help in understanding how to most effectively treat the low adipose stores shown to be present in CF patients.

Relevance to genetic counseling

The topic of cystic fibrosis is extremely important in the field of genetic counseling, as it is one of the most common genetic disorders present in the US. Genetic counselors are involved in counseling patients and families for the disorder in a number of different settings, such as preconception counseling, prenatal diagnosis, newborn screening, and counseling for late-onset forms. Genetic counselors assist patients and families in understanding the underlying mechanism of CF, its inheritance, as well as the management of the disorder, which includes treatment of manifestations and screening for possible future manifestations. It is important for genetic counselors to be aware of the potential
mechanisms contributing to poor growth so that they can provide accurate information and guidance for patients and families.

It is anticipated that this research will provide empiric data to elucidate one of the causes of poor weight gain, a major clinical symptom in CF patients. If the basis for the prevalent growth failure seen in CF patients can be determined, then treatment can be better tailored to treat this common manifestation. Since treatment options constitute a large part of the information given to patients by genetic counselors, results from this study may provide important empirical evidence for genetic counselors and other health professionals to improve patient care.
Low Adiposity in Cystic Fibrosis Mice is Due to Smaller, not Fewer Adipocytes

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Abstract

**Background:** Cystic fibrosis (CF) patients and mice have low amounts of adipose tissue. The purpose of this study was to determine whether this adipose tissue deficiency is due to fewer adipocytes, smaller adipocytes, or both.

**Methods:** Studies were performed on F508del (Cftr<sup>tm1kth</sup>) mice and wild-type (WT) littermates. Gonadal fat pads were extracted and weighed. To understand adipocyte content, DNA and triglycerides were extracted from fat pads and quantified using a PicoGreen assay and colorimetric assay, respectively. Sections of CF and WT adipose tissue were viewed histologically to observe adipocyte size.

**Results:** CF mouse adipocytes were shown by histology to be smaller than non-CF adipocytes. Excised inguinal fat pads from CF animals had similar total DNA content to non-CF animals when normalized to tissue size and was consistent with histological estimate that CF adipocytes are roughly half the size of non-CF cells. Also consistent with the above observations is that triglyceride content per adipocyte, normalized to DNA, is lower in CF adipose tissue.

**Conclusions:** CF mice have smaller adipocytes but similar total number of adipocytes compared to WT mice.
Introduction

Cystic Fibrosis (CF) is a genetic disorder caused by the absence or reduction of functional Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) (37). Mutations in CFTR negatively affect multiple body systems, resulting in pulmonary disease, exocrine pancreatic insufficiency, growth retardation, and other manifestations. Growth retardation, present in the majority of untreated patients, is thought to be caused by multiple factors, including malnutrition, exocrine pancreatic insufficiency, elevated energy expenditure, and reduced intestinal absorption (41). Treatment for growth retardation has largely focused on early nutritional intervention and Pancreatic Enzyme Replacement Therapy (PERT) (17, 41). Although average growth and body weight have improved in patients undergoing these treatments, low body weight and growth are still present in CF individuals receiving nutritional interventions and PERT (49, 56, 1). This is of concern because of the correlation demonstrated between poor growth and poor pulmonary function in patients with CF (31). Individuals with CF that are able to maintain a healthy weight and body mass index (BMI) have better pulmonary function later in life, which in turn positively affects survival rate (30).

Recent research goals include elucidating factors contributing to growth retardation in CF. The focus of this research specifically concentrates on the adipose tissue deficit. Low adipose stores have been demonstrated previously in CF patients (36, 50) compared to the general population. Adipose tissue is important in energy homeostasis and is essential for proper storage and utilization of energy. It is the main store of energy as lipids for an organism. It can expand to store excess fatty acids in the form of triglycerides (lipogenesis),
or shrink by hydrolyzing stored triglycerides (lipolysis). The WAT in individuals can expand in adipocyte size (hypertrophy) and quantity (hyperplasia) in the presence of a positive energy imbalance in order to store surplus energy (26). Low adiposity, even in the presence of high fat diets and PERT (36), is suggestive of an intrinsic difference of adipose tissue function. Additionally, studies show that CF patients exhibit elevated resting energy expenditure compared to those without CF (53). Elevated resting energy expenditure has also been shown in mice with CFTR mutations (11). This abnormal energy profile in combination with the low adipose storage further suggests an intrinsic problem in adipose tissue function, since it is key in supporting energy homeostasis. The fact that patients with CF have low levels of adipose tissue leads to the following scientific question: In CF patients, is the lack of adipose cell storage a result of altered adipocyte size or adipocyte number?

The purpose of this study was to directly answer the question of cell volume vs. cell number. Bederman, et al. recently found that de novo lipogenesis was decreased in CF mice (4). They also revealed that hepatic triglyceride (TG) content was lower in CF adipose tissue as compared to WT adipose tissue. Based on this, it was hypothesized that CF mice have smaller adipocytes resulting from less lipid deposition. To investigate this difference, DNA content and triglyceride content in adipose tissue was determined in mice with the functional absence of CFTR. Adipocytes were also histologically examined. F508del mice share certain manifestations with CF patients, however they have no spontaneous lung infection and their pancreatic insufficiency is less severe (45, 44). Despite this difference, CF mice are found to have significantly lower body weight, length, BMI, and adipose tissue
even with normal food intake and dietary lipid absorption (4), thereby providing a good model to study the adipose tissue deficit present in CF patients.

**Methods**

**Mouse models**

CF mice used in this study were homozygous for the F508del mutation ($Cftr^{tm1kth}$) (57). Littermates homozygous for the wild-type $CFTR$ allele (designated “WT”) were used as controls. Mice were fed standard chow and maintained at half strength laxative Colyte to prevent intestinal obstruction. Mice were housed at standard conditions with a 12h light and 12h dark cycle at 22°C. The cohort consisted of 13 WT mice and 12 CF mice which were sacrificed at age 6-8 weeks. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Case Western Reserve University.

**Fat pad isolation**

Adipose tissue was surgically removed from the gonadal fat pad, weighed, and sectioned into 3-4 fragments. One fragment was used for histological examination and the remaining fragments were weighed separately and subjected to tissue digestion, DNA extraction, and glycerol quantification. The rationale for using multiple assays on the same fat pad was to provide confidence in the study protocol. Adipose tissue used for histology was immediately fixed in 10% formalin for 12 hours, washed for 4-5 hours, and then embedded in paraffin. The samples used for DNA extraction were frozen in -80°C before analysis.
**Histology**

Tissue paraffin blocks were sectioned at 4 µm and stained using hematoxylin and eosin (H&E). The slides were imaged at 40x magnification using a slide scanner provided by the Imaging Core Facility of the Department of Genetics and Genome Sciences at Case Western Reserve University. High resolution images were analyzed using VisioPharm software to view and count adipocytes. Two researchers individually counted adipocytes for each sample. The researchers were blinded to the sample genotypes and chose three random views of a particular section of a sample to count the total number of adipocytes in that area. Counts were normalized to the magnification and area of field used and adipose cells were averaged between the three separate counts per sample. CF and WT average adipocyte counts were statistically compared using a student’s t-test. Two WT samples and two CF sample did not stain, leaving 11 WT samples and 10 CF samples for analysis.

**DNA extraction**

DNA was extracted from adipose tissue samples using a modified protocol based on previously published methodology (7). Briefly, tissue sample of a known weight was placed in 600 µl of digestion buffer (100 mM NaCl, 10 mM Tris Cl, pH 8, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml proK). The sample was homogenized and an equal volume of Phenol:Chloroform:Isoamyl alcohol solution (vol/vol 25:24:1) was added. The aqueous phase was removed and transferred to a 2 ml Eppendorf tube and DNA was precipitated by the addition of 1/10th volume of 3 M NaOAc and 2X volume of 100% EtOH. DNA was pelleted by centrifugation at 14,000 rpm for 10 minutes, supernatant was removed, and pellet was washed with 70% EtOH and centrifuged for 10 minutes. Lastly, the DNA pellet
was dried and suspended in 50 µl of H2O.

**DNA quantification**

DNA solution was diluted 10X and the concentration was determined using Quant-It PicoGreen dsDNA Assay Kit (Invitrogen) according to the manufacturer’s instructions. Results were normalized using a standard curve of stock DNA. Tissue specific DNA concentrations were determined by normalizing DNA (mg) content to sample adipose tissue weight. This concentration was then extrapolated and multiplied by the entire fat pad weight in order to determine total DNA content per fat pad for WT (N=12) and CF (N=13) samples. These values were averaged for each genotype and statistical significance was determined using a student’s t-test.

**Triglyceride quantification**

Following the aqueous layer removal described previously, the organic phase, containing proteins, lipids, and RNA, was removed and chloroform was allowed to evaporate. After evaporation, 0.5 mL of KOH was added and triglycerides were hydrolyzed for 45 minutes at 80°C, and then allowed to evaporate to dryness. HCl was added to neutralize the sample, and 1 mL of chloroform was added. The sample was vortexed and centrifuged for 5 minutes at 14,000 rpm. Samples were diluted 100-fold and a colorimetric assay was performed to determine the concentration of glycerol in the sample. The colorimetric assay was executed by adding 20 ul of our sample to 180 ul of glycerol reagent in a clear bottom 96-well plate. Results were normalized using a standard curve. A TG is composed of three fatty acids and a glycerol unit, therefore glycerol amount is representative of triglyceride amount within a
sample. TG values were compared to DNA levels to determine TG amount relative to adipocytes. Statistical significance was determined using a student’s t-test. One sample was an outlier and was omitted, and one sample did not extract correctly, leaving 13 WT mice and 10 CF mice.

**Results**

**Characterization of mice**

CF mice were found to weigh less than age-matched WT mice (13.7±.6 vs 17.8±0.6 g; p=0.00015; Fig. 1A). Extracted gonadal fat pads of CF mice weighed one-third of WT mouse fat pads (0.043±0.006 vs 0.128±0.015 g; p<0.0001; Fig. 1B). The gonadal fat pad as a percentage of body weight was calculated and CF mice were found to have smaller fat pad weights as a proportion of total body weight (0.314±0.044 vs 0.702±0.064 %; p<0.0001; Fig. 1C). These findings demonstrate lower body weights in CF mice, a significant proportion of which is due to decreased amounts of adipose tissue.

**Histology**

Fig. 2A and Fig. 2B show sections of CF and WT mouse adipose tissue. When observing these sections microscopically, CF mice appeared to have smaller adipose cells than WT mice. To confirm this, CF and WT mouse adipose cell numbers were totaled and compared. CF mice on average had significantly more adipose cells per mm² compared to WT mice (1616.78± 177 vs 2553.3±168 cells/mm²; p=0.0005; Fig. 2C), demonstrating ratio of 1.7:1. This number of adipose cells per section of tissue in CF mice compared to WT mice two dimensionally. This technique was carried out independently by two separate researchers, with both researchers obtaining similar differences in adipocyte number, thereby providing
confidence in these results. The number of cells/volume was derived from the observed cells/area count. CF mice were estimated to have 2.1 times more adipocytes per volume compared to WT mice (65009.71±10538 vs. 129017.8±14104 cells/mm³; p=.0005; Fig. 2D), giving a CF to WT cell ratio of 2.1:1.

**DNA concentrations**

The histological studies performed suggested CF mouse adipose tissue contains an increase in cell number, and a decrease in cell size. This finding was further investigated by determining DNA concentrations for WT and CF mouse adipose tissue. Since DNA content stays constant between cells, DNA concentration is representative of the number of adipose cells in a sample, i.e. a higher concentration of DNA represents a higher concentration of adipocytes in that tissue. CF mice had greater than twice the concentration of DNA per gram of adipose tissue than WT mice (0.45±0.07 vs 0.19±0.04 mg DNA/g tissue; p=0.0023; Fig. 3A), indicating more cells within that tissue. More cells per mg of tissue in CF mice suggest that these cells are smaller in size, which supports the histological observation described earlier. Total DNA content per fat pad was calculated by multiplying the concentration of DNA per gram of tissue by total tissue weight within a fat pad. CF and WT mice had similar amounts of total DNA per fat pad (0.016±0.003 vs 0.022±0.004 mg; p=0.376; Fig. 3B). These data demonstrate that, since CF and WT mouse fat pads have similar total DNA content, the total number of adipocytes is comparable. These values for DNA concentrations in mouse adipose tissue agree with the values of previous studies quantifying DNA in adipose tissue (40).

**Triglyceride content**
In order to confirm if CF adipose cells are in fact smaller, as the DNA and histological studies indicate, TG levels were analyzed in CF and WT mouse adipose tissue and normalized to DNA levels. CF adipose tissue was found to have significantly less mg TG per mg of DNA than WT mice (49.4±9.2 vs 76.2±9.6 mg TG/mg DNA; p=0.038; Fig. 4A), indicating less TG content per adipocyte. A lower concentration of TGs per adipocyte in CF mice indicates a smaller overall adipocyte, which supports the histological observations and molecular data. TG concentration was calculated per gram of adipose tissue, and was not found to be significantly different between CF and WT adipose tissue (3.40±0.61 vs 5.66±1.57 mg/g; p=0.21; Fig. 2B), as expected. Total TG content per fat pad was also calculated, and CF mice were found to have almost a 2.5 times less TG content per fat pad (0.173±0.03 vs 0.419±0.08 mg; p=0.009; Fig. 4C), which was expected due to the finding that CF fat pads weighed approximately one-third of WT fat pads. These data showed that CF mice have fewer TGs per fat pad, and fewer TGs per adipocyte. Since CF and WT mouse fat pads have similar numbers of cells, these data support the hypothesis that the deficit in fat in CF mice results from smaller adipocytes.
Figure 1: Mouse weight and inguinal fat pad weight are altered in CF mice. (A) CF mice on average weighed less than WT mice, (B) Inguinal fat pads on average weighed less in CF mice compared to WT mice, (C) CF mice had a lower gonadal fat pad weight as a percentage of body weight compared to WT mice.

Figure 2: Adipose cell morphology compared using histology. (A) Section of H&E stained WT mouse adipose tissue, (B) section of H&E stained CF mouse adipose tissue, (C) average cell counts/area of WT and CF mice, (D) average cell counts/volume of WT and CF mice.
Figure 3: DNA concentration in CF and WT adipose tissue was calculated. (A) CF mice have a two times higher concentration of DNA per gram of adipose tissue than WT mice. (B) CF and WT mice have similar concentrations of DNA in their whole gonadal fat pad.

Figure 4: Triglyceride concentration was calculated and normalized to DNA content and tissue amount. (A) CF mice have a less TG content per unit of DNA, demonstrating that CF mice have less TG content per adipocyte compared to WT mice. (B) CF mice have similar levels of total TG content per gram of tissue and (C) 2.5 times less total TG content relative to total fat pad.
Discussion

Rationale and Results

Understanding the process of fat metabolism and storage in CF patients is essential for health management and treatment options. Although PERT and nutritional intervention have improved growth indices, the fact that many patients are still below the average for weight and height suggests that there are other factors contributing to growth retardation that have yet to be elucidated.

This project was aimed at further understanding the mechanism of growth retardation in CF patients, with a specific focus on adipose tissue. Adipose deficits have previously been reported in CF patients and mice to contribute to the overall growth retardation observed (36, 4). The goal of this research was to further characterize the adipose tissue deficit seen in CF mice (F508del). Here, it was hypothesized that low adiposity in CF patients and mice is due to smaller adipose cell size. CF mice were found to have over double the amount of adipocytes present per unit adipose tissue, but had relatively similar numbers of total adipocytes in their gonadal fat pads.

For the analysis of body composition, it was found that CF mice had significantly lower body weight, lower gonadal fat pad weight, and less fat as a proportion of total body weight compared to WT mice, which agrees with previous growth findings of F508del mice (4). CF and WT adipose tissue was observed histologically and CF adipocytes appeared smaller compared to WT adipocytes. These microscopically viewed adipocytes were quantified independently by two researchers, and both found that CF mice had approximately twice
the amount of adipocytes as WT mice. The number of cells/volume was derived from the observed cells/area count. CF mice were found to have 2.1 times more adipocytes per volume compared to WT mice (922.25±135.82 vs. 1892.035±188.91 cells/mm$^3$; $p=.0005$), giving a CF to WT cell ratio of 2.1:1. This ratio is similar to the ratio obtained from DNA studies which showed CF mice had 2.4 times higher concentration of DNA per unit of adipose tissue in CF mice. A higher concentration of DNA is representative of a higher number of adipocytes within an area of tissue, which is indicative of smaller adipocytes.

When normalized to the entire gonadal fat pad, CF and WT mice had comparable DNA content, indicating a similar number of adipocytes per fat pad. Since CF fat pads weighed less than WT fat pads but had a similar number of adipocytes, the reduced amount of adipose tissue must be the result of smaller adipocytes in CF mice. Adipose tissue triglyceride (TG) content was measured for CF and WT mice using a glycerol colorimetric assay and showed a lower concentration of TGs per unit of DNA in CF adipose tissue, demonstrating that CF adipocytes have fewer stored TGs, further supporting smaller sized adipocytes present in CF mice. These data are consistent with the study hypothesis that CF mice have smaller adipocytes when compared to WT mice. Due to the similarity in disease manifestation between CF mice and CF patients in regard to the growth phenotype, it is expected that CF patients will also exhibit smaller adipocytes.

**Proposed Mechanisms**

When considering the cause of less lipid content in adipose tissue, it is important to consider the sources of TG that lead to deposition in the adipocyte, as well as the mechanisms that contribute to lipid uptake and breakdown within the adipocyte. A
reduction in lipids stored in adipocytes could be caused by [i] low delivery of dietary lipids, [ii] decreased breakdown of extracellular TG via lipoprotein lipase (LPL), [iii] impaired internalization of free fatty acids and TG synthesis or [iv] increased breakdown of intracellular lipids by hormone sensitive lipase (HSL). Few studies have analyzed the impairment of lipid metabolism in CF mice (4, 33, 6, 54, 28, 18, 11).

Sources of TG’s include dietary lipids that are absorbed in the intestines and carried by chylomicrons to adipocytes, de novo lipogenesis (DNL) in the adipocyte, and hepatic DNL from dietary carbohydrates. Bederman et al. recently investigated mechanisms of low adipose tissue deposition in F508del mice and reported that hepatic DNL was decreased and that CF mice had fewer de novo fatty acids deposited in their adipose tissue than WT mice (4). This suggests a possible mechanism for decreased adipose stores being an impairment of DNL. They also reported normal absorption of dietary TGs; however, this was based on fecal fat content and not intestinal lipolysis. Bijvelds et al. found some evidence of altered lipolysis of dietary TGs in CFTR null mice (6), but other studies of CFTR null mice and F508del mice had no significant change in lipolysis (54). Additionally, CF patients taking PERT have been found to have impairment of intestinal uptake of fatty acids (28), demonstrating that results are still inconclusive regarding normal dietary TG absorption in CF humans and mice.

Circulating TGs are internalized to an adipocyte by the action of specific proteins and hormones. Lipoprotein lipase (LPL) is an enzyme located on the adipocyte that is responsible for breaking down chylomicrons and VLDL’s to allow for fatty acid
deposition. Insulin and glucose both increase adipose tissue LPL activity, which explains
the increase in LPL activity during feeding and a decrease in activity during fasting (33).
A study looking at plasma TG levels showed that 16% of CF patients had higher circulating
TG levels, which could be the result of excessive hepatic TG synthesis, decreased TG
clearance, or a combination of both (18). Since TG synthesis in the liver is decreased in CF
mice (4), decreased TG clearance is a possible explanation. Although plasma TG levels
have not been determined in F508del mice, decreased activity of LPL, or decreased
response to insulin or glucose could account for decreased adipocyte TG deposition and
increased plasma TG levels. Future studies investigating the functioning of LPL would help
determine if lipids are being broken down and further internalized in the adipocyte.

Intracellular lipolysis is the breakdown of intracellular TGs by HSL in order to supply an
organism with energy. HSL is upregulated by epinephrine and downregulated by insulin.
Our finding of fewer TG per adipocyte in CF mice could be the cause of increased lipolytic
rate resulting in increased availability of free fatty acids for oxidation. CF patients have
increased energy expenditure as a result of increased energy demand (53). This has also
been demonstrated in CF mice (11). Additionally, CF patients have a defect in insulin-
mediated suppression of adipose tissue lipolysis (33). These data support the idea that
increased energy demand could cause an increase in lipolysis of TG to supply the body
with energy, which could explain this study’s finding of smaller adipocytes.

Additionally, Cystic Fibrosis Related Diabetes (CFRD) has been a complication of cystic
fibrosis that has become more prevalent as life expectancy improves in patients (15). The
main defect in CFRD appears to be delayed and impaired insulin secretion (35); however, insulin resistance has been observed in some patients as well, but other findings have been unable to confirm this (15). These mixed data regarding the insulin defect make the precise etiology CFRD unclear. However, insulin resistance, if present, could result in increased TG lipolysis and decreased TG lipogenesis, which could also be a contributing factor to the small adipose stores in CF patients, as well as the CFRD seen in some patients. Future studies investigating the adipocyte response to insulin and epinephrine would be helpful in determining if the adipocyte is responding properly to internal signals.

Clinical implications

Current nutritional intervention for CF patients consists of a high-fat high-energy (HFHE) diet, composed of 35%-40% calories from fat in order to meet the increased energy demands and achieve a healthy weight (27). Although this technique has been effective, growth retardation is still seen in patients prescribed HFHE diets, and patients are not meeting their expected growth potential (49). Additionally, pancreatic sufficient CF mice that are on a HFHE diet were severely growth retarded (5). This persistent growth retardation in the presence of a HFHE diet combined with this study’s data showing smaller adipocytes suggests that lipids may not be entering the cell effectively. If CF mice are not effectively sequestering TGs, there could be implications for other health conditions, such as lipotoxicity. Lipotoxicity describes abnormally high levels of lipid deposition in non-adipose tissues, such as skeletal muscle, kidney, heart, liver, pancreas, and others, potentially leading to cardiac disease, insulin resistance, and other metabolic diseases. If this is occurring, dysfunction in lipid storage ability could be problematic for CF patients who are consuming HFHE diets. Additionally, proper nutritional therapy is an important
part of the management of diabetes in both CF and non-CF patients with diabetes. This nutritional therapy in non-CF patients typically consists of a normal energy diet, consisting of 30% fat, 10-20% protein, and 50-60% carbohydrates (15). This diet is not recommended for patients with CF, whose current nutritional recommendations include HFHE diets in order to maintain a healthy weight.

The benefit of understanding contributing factors of growth retardation in CF patients also includes the positive correlation seen between patient BMI and better pulmonary function. Although it is not clear which, if either, factor is causative, understanding the mechanism of low growth in patients may assist in elucidating this information. Recently, the medication Ivacaftor has been shown to be successful in improving pulmonary function and CFTR activity in patients with the G551D mutation in CFTR (13). An improvement in weight gain was also demonstrated, with children on Ivacaftor gaining 2.8 kg more than children on placebo when drug was administered for 48 weeks. It is difficult to determine from these data whether the improved lung function could be causing this better weight gain, whether the better weight gain helped to improve lung function, or whether they are independent of each other. However, other medications that have improved lung function have not been as effective at improving weight gain in CF patients (42), suggesting that the improved lung function does not directly result in improved weight gain. Studies have shown CFTR expression in adipose tissue (39); however, the functional role is still not clear. Future studies investigating what is causing this improvement in weight in patients taking this medication could help determine whether this is an effect of restored CFTR activity in WAT. Additionally, future studies to determine the mechanism for adipose tissue
expansion in patients taking Ivacaftor, and whether it is allowing lipids to be better absorbed and utilized, are warranted.

**Conclusions**

In conclusion, findings from this study demonstrate that functioning CFTR plays a role in adipose tissue function. This study provides novel evidence that low adiposity in CF mice is caused by small adipocyte size. This result is important in guiding future research to determine the mechanism of low adiposity of CF patients and mice. A better understanding of lipid metabolism in CF patients may eventually change our current nutritional recommendations for these patients.
DISCUSSION

Rationale and Results

Understanding the process behind fat metabolism and storage in CF patients is essential for health management and treatment options. Although PERT and nutritional intervention have improved growth indices, the fact that many patients are still below the average for weight and height suggests that there are other factors contributing to growth retardation that have yet to be elucidated.

This project was aimed at further understanding the mechanism of growth retardation in CF patients, with a specific focus on adipose tissue. Adipose deficits have previously been reported in CF patients and mice to contribute to the overall growth retardation observed (36, 4). The goal of this research was to further characterize the adipose tissue deficit seen in CF mice (F508del). It was hypothesized that low adiposity in CF patients and mice was due to smaller adipose cell size.

Using three experimental assays, it was demonstrated that CF mice have smaller adipocytes when compared to WT mice, which is consistent with the study hypothesis. Due to the similarity in disease manifestation between CF mice and CF patients, it is expected that CF patients will also exhibit smaller adipocytes.

This finding of smaller adipocytes in CF mice is supported by all three assays. H&E staining is a validated way to observe cells in a two dimensional format. These microscopically viewed adipocytes were quantified independently by two researchers, and
both found that CF mice had double the amount of cells per section of adipose tissue. The fact that both researchers found similar proportions provides confidence to the methodology used. The limitation of this methodology is that it only allows for adipocytes to be viewed on a 2-D section, and not as a whole. Since adipocytes typically have some irregularity in shape, and are generally not a perfect circle, a 2-D image is not completely reflective of the adipocyte size differences.

The number of cells/volume was derived from the observed cells/area count. CF mice were found to have 2.1 times more adipocytes per volume compared to WT mice, giving a CF to WT cell ratio of 2.1:1. This ratio is similar to the ratio obtained from DNA studies (2.4:1), and would be a better representation of actual difference in cell count than the ratio obtained from our initial cells/area count. However, as adipose tissue is heterogeneous in nature, it is difficult to ascertain whether this is a true representation of adipocyte ratio.

An anecdotal observation was also made that CF tissue appeared to have more mitotic cells than WT. While this was not quantified, it would potentially explain why the DNA content of CF tissue is slightly higher than that predicted by cell volume. Moreover, if mouse adipose tissue is more actively creating new cells, it could be speculated that old adipose cells are being recycled more quickly. Additional CF adipose tissue histological work should be aimed at determining whether adipocytes are proliferating and going through apoptosis normally.

DNA concentration within tissue is considered an appropriate way to determine cell
number. In this study, DNA analysis showed double the concentration of DNA in adipose tissue of CF mice. The DNA concentrations for mouse adipose tissue were in a similar range as the DNA concentrations obtained in previous research for mouse adipose tissue (40). Since DNA content stays constant between cells, a higher concentration of DNA is representative of a higher number of adipocytes within an area of tissue, which is indicative of smaller adipocytes. When normalized to the entire gonadal fat pad, CF and WT mice had comparable concentrations of DNA, implying similar numbers of adipocytes per fat pad. Since CF fat pads weighed less than WT fat pads but had a similar number of adipocytes, these data support the conclusion that the reduced amount of adipose tissue must be the result of smaller adipocytes in CF mice.

Adipose tissue triglyceride (TG) content was determined for CF and WT mice using a glycerol colorimetric assay. Previous studies have illustrated that this methodology is an appropriate technique to quantify triglycerides. TGs are composed of one unit of glycerol and three units of fatty acids, therefore one mole of glycerol is equal to one mole of triglyceride. Glycerol was measured instead of fatty acids because adipose tissue can have free fatty acids that are separate from TGs. Typically, there is no free glycerol in adipocytes, making it a valid indicator of TG content. As the intent of this was to analyze lipid content of adipocytes, glycerol measurements were most appropriate. TG amount was normalized to DNA content in adipose tissue. There was a lower concentration of TGs per unit of DNA in CF adipose tissue, demonstrating that CF adipocytes have fewer stored TGs, further supporting smaller sized adipocytes present in CF mice.
Proposed Mechanisms and Future Research

White adipose tissue (WAT) plays an important role in energy homeostasis in individuals. It is the main store of energy as lipids for an organism. It can expand to store excess fatty acids in the form of TGs (lipogenesis), or shrink by hydrolyzing stored TGs (lipolysis). The WAT in individuals can expand in adipocyte size and quantity. This process, known as remodeling, involves both hypertrophy (increase in size) and hyperplasia (increase in number) of adipocytes in the presence of a positive energy imbalance in order to store surplus energy (26).

When considering the cause of less deposition of lipid content into WAT, it is important to consider the sources of TG that will lead to deposition in the adipocyte (Figure 3), as well as the mechanisms that contribute to lipid uptake and breakdown within the adipocyte (Figure 4). A reduction in lipids stored in adipocytes could be caused by [i] low delivery of dietary lipids, [ii] decreased breakdown of extracellular TG via lipoprotein lipase (LPL), [iii] impaired internalization of free fatty acids and TG synthesis or [iv] increased breakdown of intracellular lipids by hormone sensitive lipase (HSL). Few studies have analyzed the impairment of lipid metabolism in CF mice (4, 33, 6, 54, 28, 18, 11).
Low delivery of dietary lipids

Sources of TG’s include dietary lipids that are absorbed in the intestines and carried by chylomicrons to adipocytes, de novo lipogenesis (DNL) in the adipocyte, and hepatic DNL from dietary carbohydrates. Bederman et al. recently investigated mechanisms of low adipose tissue deposition in F508del mice and reported that hepatic DNL was decreased and that CF mice had fewer de novo fatty acids deposited in their adipose tissue than WT mice (4). This suggests a possible mechanism for decreased adipose stores being an impairment of DNL. They also reported normal absorption of dietary TGs; however, this was based on fecal fat content and not intestinal lipolysis. Bijvelds et al. found some evidence of altered lipolysis of dietary TGs in CFTR null mice (6), but other studies of CFTR null mice and F508del mice had no significant change in lipolysis (54). Additionally, CF patients on PERT have been found to have impairment of intestinal uptake of fatty acids.
(28), demonstrating that results are still inconclusive regarding normal dietary TG absorption in CF humans and mice. Future studies investigating the delivery of dietary lipids to the adipocyte would be helpful in further clarifying if lipid transport pathways are functioning appropriately.

*Decreased breakdown of extracellular lipids by lipoprotein lipase*

Circulating TGs are internalized to an adipocyte by the action of specific proteins and hormones. Lipoprotein lipase (LPL) is an enzyme located on the adipocyte that is responsible for breaking down chylomicrons and VLDL’s to allow for fatty acid deposition. Insulin and glucose both increase adipose tissue LPL activity, which explains the increase in LPL activity during feeding, and a decrease in activity during fasting (33). A study examining plasma TG levels showed that 16% of CF patients had higher circulating TG levels, which could be the result of excessive hepatic TG synthesis, decreased TG clearance, or a combination of both (18). Since hepatic TG synthesis is shown to be decreased in CF mice (4), decreased TG clearance is a possible explanation. Although plasma TG levels have not been determined in F508del mice, decreased activity of LPL, or decreased response to insulin or glucose could account for decreased adipocyte TG deposition and increased plasma TG levels. Future studies investigating the functioning of LPL would help determine if lipids are being broken down and further internalized in the adipocyte. Additionally, further evaluation of the adipocytes response to insulin and glucose may help determine whether those two substances are being recognized and used by the adipocyte appropriately.
Impaired internalization of free fatty acids and TG synthesis

After chylomicrons and VLDLs are broken down and free fatty acids enter the adipocyte, other factors are involved in allowing TGs to be synthesized from those free fatty acids. Currently there are no known existing relevant data regarding CF and this process.

Increased breakdown of intracellular lipids by hormone sensitive lipase (HSL)

Intracellular lipolysis is the breakdown of intracellular TGs by HSL in order to supply an organism with energy. HSL is upregulated by epinephrine and downregulated by insulin, as shown in figure 3. The finding of fewer TG per adipocyte in CF mice in this study could be the cause of increased lipolytic rate resulting in increased availability of free fatty acids for oxidation. CF patients have indeed been shown to have increased energy expenditure as a result of increased energy demand (53). This has also been demonstrated in CF mice (11). Additionally, CF patients have been previously shown to have a defect in insulin-mediated suppression of adipose tissue lipolysis (33). These data support the idea that increased energy demand could cause an increase in lipolysis of TG to supply the body with energy, which could explain our finding of smaller adipocytes.

Additionally, Cystic Fibrosis Related Diabetes (CFRD) has been a complication of cystic fibrosis that has become more prevalent as life expectancy improves in patients. The main defect in CFRD appears to be delayed and impaired insulin secretion, however insulin resistance has been observed in some patients as well (15). These mixed data regarding the insulin defect make the actual nature unclear. However, insulin resistance, if present, could result in increased TG lipolysis and decreased TG lipogenesis, which could also be a
contributing factor to the small adipose stores in CF patients, as well as the CFRD seen in some patients.

Future studies investigating the adipocyte response to insulin and epinephrine in CF mice would be helpful in determining if the adipocyte is responding properly to external signals. An increased response to epinephrine could result in increased breakdown of TGs in the adipocyte, whereas a decreased sensitivity to insulin could cause the cell to also break down TGs more quickly.

**Additional findings and implications**

Interestingly, unpublished studies by this research team have shown that when comparing adipocyte number in older CF and WT mice, aged 7-23 weeks, using solely DNA concentration and histological examination, there is no significant difference in adipocyte number. Additionally, the researchers have also shown that as CF mice age, their weights tend to be more similar to aged WT mice, suggesting that something could occur as mice age that allows them to store fat more efficiently. Alternatively, these data could not be the result of an altered physiological mechanism in aged CF mice. CF mice that are able to survive to older ages typically present with a less severe phenotype. The fact that CF mice that are older have similar weights to WT mice may be the result of more severely affected mice dying before they reach an older age, therefore leaving healthier CF mice that have similar growth indices to WT mice. Further metabolic studies comparing young CF mice to old CF may be helpful in understanding this difference.
Clinical implications

Current nutritional intervention for CF patients consists of a high-energy high-fat (HFHE) diet, composed of 35%-40% calories from fat in order to meet the increased energy demands and achieve a healthy weight (27). Although this technique has been effective and allowed many patients to maintain a healthy weight, there is still growth retardation seen in patients prescribed HFHE diets, and patients are not meeting their expected growth potential (49). Additionally, pancreatic sufficient CF mice that are on a HFHE diet were shown to be severely growth retarded (5). This persistent growth retardation in the presence of a HFHE diet combined with this study’s data showing smaller adipocytes suggests that lipids may not be entering the cell effectively. If lipids are not entering the adipocyte appropriately, the question arises regarding where these lipids are deposited. Dysfunction of lipid storage can lead to lipotoxicity, i.e. lipid deposit in non-adipose tissues, such as skeletal muscle, kidney, heart, liver, pancreas, and others, potentially leading to cardiac disease, insulin resistance, and other metabolic diseases. If this occurs, dysfunction in lipid storage ability could be problematic for CF patients who are consuming HFHE diets.

Additionally, proper nutritional therapy is an important part of the management of diabetes in both CF and non-CF patients with diabetes. This therapy in non-CF patients typically consists of a normal energy diet, consisting of 30% fat, 10-20% protein, and 50-60% carbohydrates (15). This dietary regime, however, is not an option for CF patients, whose current recommendations include HFHE diets.

The benefit of understanding contributing factors of growth retardation in CF patients also includes the positive correlation seen between patient BMI and better pulmonary function.
Although it is not clear which, if either, factor is causative of the other, understanding the mechanism of poor growth in patients may assist in elucidating this information. Recently, the medication Ivacaftor has been shown to be successful in improving pulmonary function in patients with the G551D mutation in CFTR (13). There has also been an improvement in weight gain, with children on Ivacaftor gaining 2.8 kg more than children on placebo when drug was administered for 48 weeks. It is difficult to speculate from this data whether the improved lung function could be causing this better weight gain, whether the better weight gain helped to improve lung function, or whether they are independent of each other. However, other medications that have improved lung function have not been as effective at improving weight gain in CF patients (42), suggesting that the improved lung function does not directly result in improved weight gain.

If Ivacaftor is acting specifically on epithelial CFTR, the fact that growth retardation improves in addition to lung function suggests that there is a tissue specific function of CFTR on WAT. Studies have shown CFTR expression in adipose tissue (39), however the functional role is still not clear. Future studies investigating the basis for this improvement in weight in patients taking this medication could provide information as to whether this is an effect of restored CFTR activity in WAT. Additionally, future studies may help determine the mechanism for adipose tissue expansion in patients taking Ivacaftor, and whether it allows lipids to be better absorbed and utilized.
CONCLUSIONS:

Summary of Findings

The findings of this study demonstrate that CFTR plays a larger role in adipose tissue function than previously thought. This study provides novel evidence that low adiposity in CF mice is caused by small adipocyte size. This result is important in guiding future research to determine the mechanism of low adiposity of CF patients and mice. A better understanding of lipid metabolism in CF patients may eventually change our current nutritional recommendations for these patients.

Care for CF patients has greatly improved since the disease’s initial discovery. The lifespan of patients, which at one point did not exceed childhood, now extends well into the 40s and 50s (37). The earlier CF is diagnosed, the earlier treatment can begin, which correlates with better survival. However, even with better treatment, patients still have difficulty gaining weight and reaching normal growth parameters. For this reason, a better understanding of growth retardation is needed.

Study Limitations:

This study analyzed the adipose tissue deficit in a single genotype of CF mice. There are numerous Cfr genotypes, which can result in mild to severe CF phenotypes in mice and humans. It is unknown whether this finding of smaller adipocytes would also be present in mice of different genotypes and phenotypes. Another limitation of this study was the gender distribution used as there was a higher female to male mouse ratio in the mice
(17:8). When separating the mice by gender and making the comparisons as described above, no statistical difference was found between males and females. However since the F508del phenotype slightly differs between male and female mice, it would be important to study a cohort of mice with more equally distributed sexes in order to better assess potential differences.

Finally, only fat tissue in the gonadal fat pad was assessed. The gonadal fat pad is an ideal study model for two main reasons: 1. technically, it is easy to remove the entire gonadal fat pad because it is encapsulated, 2. fat typically deposits in the visceral region initially, making it a valid representation of fat deposition. Subcutaneous adipose tissue was not analyzed; however it would be important to know if there are any adipocyte differences between the two depots.

**Implications for the Field of Genetic Counseling and Cystic Fibrosis Research**

As discussed before, genetic counselors commonly counsel patients and families about cystic fibrosis in a number of different settings, such as preconception counseling, prenatal diagnosis, newborn screening, and counseling for late-onset forms. Genetic counselors help the patient and families understand the underlying mechanism of CF, its inheritance, as well as the management of the disorder, which includes treatment of manifestations and screening for possible future manifestations. Thus, in order to provide accurate information, it is important that genetic counselors and health professionals understand the potential mechanisms contributing to poor growth.
This research provides information regarding the growth differences seen in CF patients compared to the general population. Although these data cannot immediately be applied directly to patient care, this research paves the way for future research investigating the mechanisms involved in low adiposity in patients with CF, which could eventually alter treatment recommendations for patients. Since treatment availability constitute a large part of the information given to patients by genetic counselors, these study results may assist genetic counselors, researchers, and other health professionals to improve patient care.

**Direction for Future Research**

Numerous CF studies have sought to characterize the mechanisms behind growth retardation in CF patients, primarily because of the improved respiratory prognosis seen in patients with normal growth indices. However, few studies have analyzed the adipose tissue deficit present. This research demonstrates that CF mice have the same number of adipocytes as WT mice that are unable to store lipids properly. Further research to investigate the functionality of various mechanisms, mentioned previously, underlying this impaired deposition of lipids into the adipocyte will be beneficial.

The mechanisms underlying lipid storage is complex, leaving many potential reasons for why CF mice have inadequate fat storage. Functional studies on CF mouse adipocytes will be helpful in characterizing potential defects in lipolysis and lipogenesis at the adipocyte. These functional studies include analyzing adipocyte response to insulin, epinephrine, and other lipid metabolism signaling molecules. Finally, expression of certain proteins could be assessed, including LPL and HSL, which are key in lipogenesis and lipolysis, respectively.
### Appendix 1

<table>
<thead>
<tr>
<th>Mouse</th>
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<th>Genotype</th>
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<tbody>
<tr>
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References


