Nutrition in Cystic Fibrosis: Macro- and Micronutrients

Nutrition in cystic fibrosis: macro-and micronutrients
Thesis, University of Utrecht, The Netherlands

ISBN 90-9020386-9
Coverphoto Doug Harrington; Fractal Art Copyright © All rights reserved Fractalarts.com
Cover & layout Noenus Design, Soest
Print Print Partners Ipskamp, Enschede

Financial support The printing of this thesis has been made possible with the financial support of Astra Zeneca, Solvay Farma, Tramedico, Bristol-Myers Squibb, Mead Johnson, Nutricia, Roche, Zambon, Cobra Medical, Norgine, Olympus Nederland BV.

Nutrition in Cystic Fibrosis: Macro- and Micronutrients

Voeding bij taaislijmziekte: macro- and micronutrienten

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen
in het Academie Gebouw der Universiteit
op donderdag 16 maart 2006 des middags te 12.45 uur

door Johanna Hermiena (Annemarie) Oudshoorn
geboren op 27 juli 1966 te Rotterdam

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The past decade extensive research has led to a better understanding of CF together with the development of new therapies. It is our ultimate hope to find a cure for this disease. Until this occurs we must ensure that patients are diagnosed early and get the best possible treatment available by referral to specialist units.

Adam Jaffé; Cystic Fibrosis: Review of the Decade, 2001

Aan mijn ouders

Aan Bert, Ruben, Lyanne en Laurens

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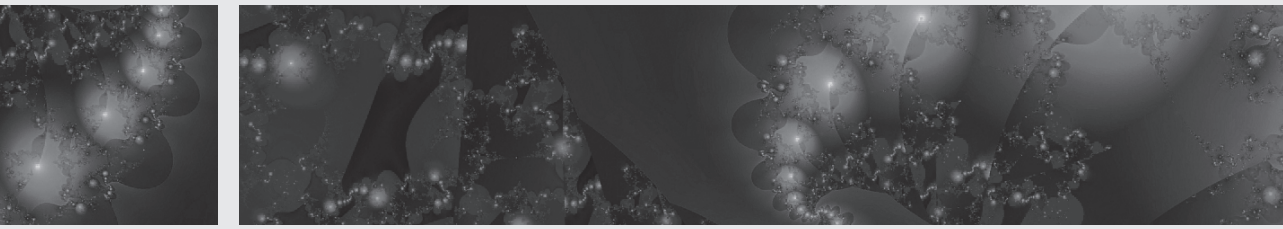


GENERAL INTRODUCTION

Cystic fibrosis (CF) is a disorder characterized by progressive lung disease, pancreatic insufficiency, malnutrition, hepatobiliary disease and elevated sweat electrolyte levels. It is the most common life-threatening autosomal recessive inherited disease in Caucasians. The carrier frequency is 1 in 22-28 in Caucasians, with an incidence of 1 in 2000-3000 (1). It is less frequent in African Americans (1 in 15,300) and considered rare in Southeast Asians. Over the last 50 years, a better understanding of the underlying pathophysiology in combination with a more aggressive antibiotic therapy for lung infections, and appropriate nutritional management with oral pancreatic enzyme replacement therapy, has improved the prognosis for CF markedly, especially the first 20 years of life (figure 1). The mean survival has almost doubled from 20 to 35 years of age in the last 10 to 15 years (2,3).

History

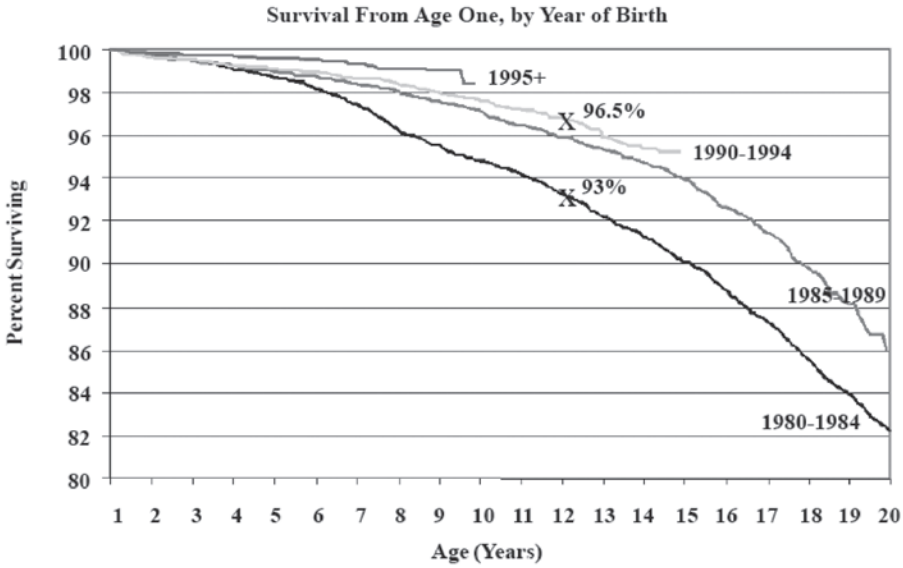
The first detailed pathologic descriptions of exocrine pancreatic disease in childhood appeared in the 1930s from Fanconi and Anderson who separated cystic fibrosis (CF) from celiac disease as an entity with pancreatic and pulmonary components (4). Subsequently, in the early 1950s di Sant'Agnese reported elevated sweat salt concentrations in CF suggesting abnormalities in electrolyte transport as a possible underlying pathology (4). This report is the basis of the currently used quantitative pilocarpine iontophoresis sweat test, which has remained the gold standard for diagnosis of the disease.

The first attempt at localizing the cystic fibrosis-gene by linkage analysis was described in 1968 (5). However it was only in 1985 that the CF gene could be assigned to chromosome 7 (6,7,8). The subsequent positional cloning and sequencing of the gene in 1989 was the result of a combined effort of research groups in Toronto and Michigan (9,10,11).

Aetiology and Pathophysiology

The gene causing CF, called CFTR (cystic fibrosis transmembrane conductance regulator), encodes a protein that is expressed in the apical membrane of epithelial cells in many tissues. The CFTR-protein (Figure 2) functions principally as a cyclic adenosine monophosphate-(cAMP) dependent chloride channel that plays an important role in chloride transport across apical epithelial surfaces. It also appears capable of regulating other ion channels (12). The CFTR-gene consists of 27 exons, spans over 250 kb and encodes the CFTR protein of 1480 amino acids (10). Over 1000 mutations have now been described (12,13). These mutations are located throughout the entire coding region of the gene as well as the promoter region, but are most commonly found in the nucleotide binding domains (NBD) and regulatory (R) domain. The most common mutation is ΔF508 and affects 66 % of the CF chromosomes worldwide (14). It is most frequent in northern Europeans (70-80%), less frequent in southern Europeans (50-55%), and is found in only a minority of Africans and Asians affected by this disease (15,16). The mutations in the CFTR gene have been classified into five different groups according to the type of mutation. (Figure 3) The mechanisms by which mutations

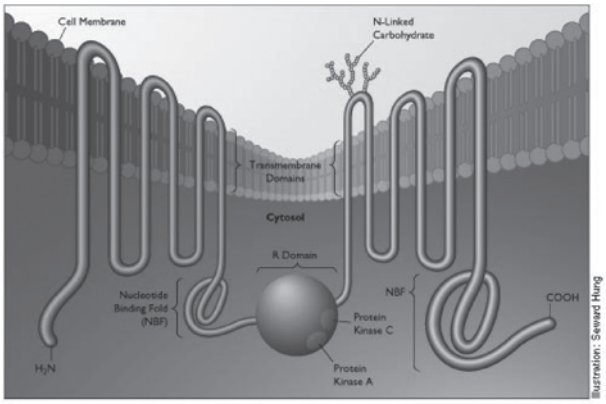
Figure 1.



(Cystic Fibrosis Foundation Patient Registry Annual Data Report 2004 p.4)

Figure 2. CFTR-protein

The amino acid sequence of CFTR auggeats that it consists of two homologous structural units, each including a transmembrane domain and a nucleotide-binding fold, connected by a central regulatory (R) domain rich in phosphorylation sites. Experimental data confirm a transmembrane transport function with likely regulatory roles for adenosine triphosphate (ATP) binding and phosphorylation.



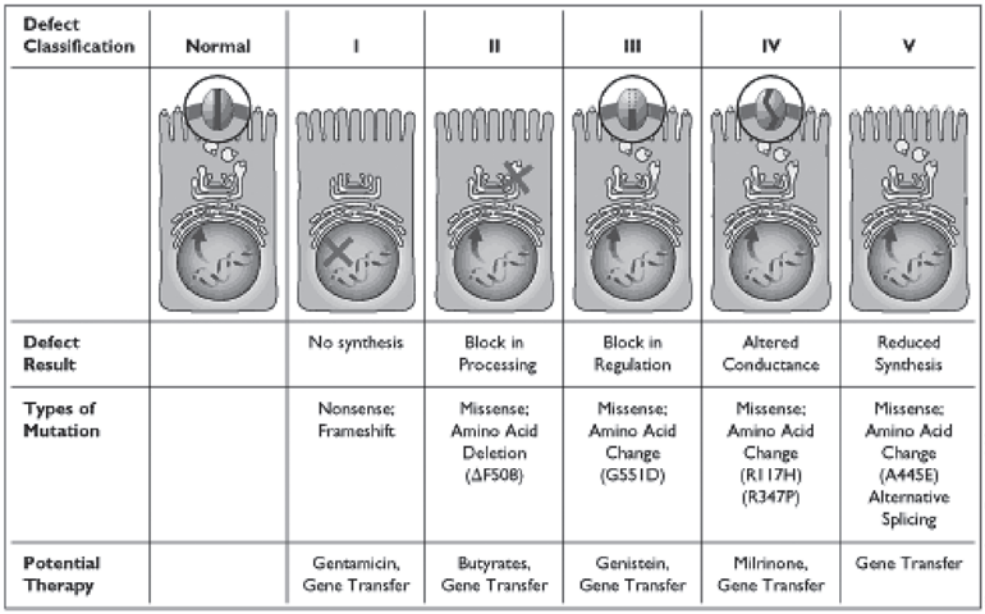
(Moss RB. Cystic fibrosis: Pathogenesis, pulmonary infection, and treatment. Clin Infect Dis 21:839, 1995)
(Hospital Practice. New approaches to cystic fibrosis. Richard B.Moss, Stanford University. http://www.hosppract.com/issues/2001/01/moss.htm)

disrupt CFTR function are (a) defective CFTR production due to premature transcription termination signals resulting in either unstable messenger RNA or an abnormal protein which is rapidly degraded (class I); (b) defective CFTR processing and trafficking to the apical membrane, e.g. ΔF508 (class II); (c) defective regulation of chloride channel function due to mutations in CFTR phosphorylation or ATP-binding sites (class III); (d) defective chloride conductance due to missense mutations in the membrane-spanning domains of CFTR that line the channel (class IV); and (e) reduced levels of normal mRNA transcript and/or protein required for normal function (class V) (17,18)

The CF phenotype is highly heterogeneous among individual patients, even between siblings carrying identical CFTR mutations. This indicates that disease severity is also determined by modifier genes and environmental effects (12,19). Nevertheless, 'severe' mutations (class I, II and III) are those where the absence of functional CFTR correlates well with pancreatic insufficiency (> 95% cases), liver disease (in 40% of patients), young age at diagnosis (usually < 1 year),

Figure 3. Classification of the different mutations in Cystic Fibrosis

The 800 or so genetic mutations associated with cystic fibrosis have been divided into five broad classes based on, their impact on the CFTR transporter molecule. An impressive number of corrective agents, are in or approaching clinical trials; nowever, only gene transfer represents a potential cure. (Adapted from Zialanski and Tsui, 1995)



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high sweat chloride levels (>80 meq/l) and meconium ileus (ca. 20% of cases) (12,20). "Mild mutations" (class IV and V) may still produce a small amount of functional CFTR and are generally associated with pancreatic sufficiency (70-80% cases), may be diagnosed at a later age, have lower sweat chloride levels, no meconium ileus and milder pulmonary disease.
Of particular interest to the present study is the observation that CF patients demonstrate diminished skeletal muscle performance and exercise intolerance. This has been explained by diminished nutritional status and decreased oxygen delivery due to restricted pulmonary function (21). However a *primary* abnormality in muscle metabolism was suggested by results from a ^{31}P-magnetic resonance spectroscopy study of forearm muscle in which CF patients showed a 19-25% diminished efficiency of mitochondrial oxidative ATP synthesis during steady state sub-maximal voluntary contractions (22). This muscle-related abnormality in oxygen metabolism in CF patients was confirmed by Moser et al (23). Peripheral muscle weakness in concomitance with increased oxygen need during exercise and reduced maximal exercise performance was demonstrated in children with CF, even in the absence of diminished pulmonary or nutritional status (24). Taken together, these studies strongly suggest an intrinsic abnormality in skeletal muscle either at the level of the myofilaments or the mitochondria in CF. An abnormality in the myofilaments could be a reduced energetic efficiency of contraction resulting, for example, from a phenotypic shift towards more fast myosin gene expression. At the level of the mitochondria, a reduced efficiency of oxidative ATP synthesis could be the problem. There is some evidence for abnormal mitochondrial function in CF disease in the literature (25-27). In skin fibroblasts of CF patients findings suggestive of an anomaly in the mitochondrial NADH dehydrogenase complex (respiratory chain enzyme complex 1) system have been reported (25). Similarly, studies in fibroblasts and leucocytes reported mitochondrial abnormalities such as increased calcium content and lower NADH dehydrogenase activity compared to controls (26,27).

Improving prognosis

The considerable prognostic improvement in CF during the last decades has been the result of many small steps. However these steps have mainly been made in three areas: *treatment and prevention of pulmonary infections*, optimizing *physical fitness* and *nutrition*.

Approach I: treatment and prevention of pulmonary infections

Aggressive oral or intravenous antibiotics are now given as therapy and to prevent pulmonary infections, especially in case of *Pseudomonas aeruginosa* colonization. If necessary, antibiotic inhalation therapy with tobramycine or colistine is prescribed (28).
In order to facilitate the expectoration of the thick mucus in CF physiotherapeutic techniques are applied by the patients themselves, and if necessary inhalation therapy is administered. The inhalation medications used are NaCl 0.9%, acetylcysteine, or Pulmozyme®. In case of a bronchospastic component, bronchodilators like salbutamol are taken.

Approach II: optimizing physical fitness

Physical activity as an addition to daily physiotherapy has long been recommended for CF patients to prevent deterioration of lung function, and to improve well-being and physical fitness (29). Exercise training has indeed been shown to be beneficial in children with CF by improving their exercise tolerance and pulmonary function as a result of increased muscle mass, improved aerobic fitness and cardiopulmonary efficiency (30-32). Different long term and intensive short term exercise programs in children and adults improve various aspects of exercise performance (29,31,33-35). However, although short term exercise training is usually followed without any difficulty, continuing a program for long periods of time on a regular basis is a problem for many patients. Especially in children, the essential motivation for durable aerobic exercise is less than in adults. Training programs that correspond more with the daily physical activity of a child, which is characterized by short-term anaerobic activities, will be easier to sustain. If physical fitness could be boosted by for instance a nutritional supplement, perhaps exercise would not have to be followed so stringently.

Approach III: Nutrition

CFTR dysfunction results in thickened pancreatic secretions causing obstruction to the ducts and subsequent destruction and obliteration of acinar tissue, resulting in pancreatic insufficiency (4). Compensating the lack of these digestive enzymes by pancreatic supplements will always leave some residual maldigestion, especially for fat. Traditionally therefore the recommended high caloric intake emphasized a high percentage of carbohydrates. However, in 1988 Corey compared survival, growth, and pulmonary function in CF patients in Boston and Toronto (36). The patients in Boston received the traditional low fat intake, whereas the Toronto patients received high amounts of calories and fat. These different approaches to nutritional guidance and intervention in CF patients resulted in better height and weight, and superior median age of survival in Toronto as compared to Boston (resp.30 and 21 years). So current guidelines for nutritional intake in CF comprise an energy intake of 120-150% of the recommended dietary allowance (RDA) for healthy individuals, a high-normal fat intake of 35-40 energy%, and sufficient amounts of pancreatic enzymes (37-39). So far, little emphasis has been on adequate protein intake as part of improving nutritional status, although moderate to severe protein deficits are seen in everyday practice at the time of diagnosis, and also during the course of the disease. When CF is diagnosed by neonatal screening, the nutritional status, including protein, is generally better in infancy and childhood than when the diagnosis is made after symptoms occur (40). In older CF patients a negative balance between protein synthesis and protein breakdown in children with chronic but stable pulmonary disease is found, which is even more prominent during pulmonary infections (41) This negative balance could be influenced positively by increasing protein intake (3.6-4.2 $g \cdot kg^{-1} \cdot d^{-1}$) during pulmonary exacerbations, and in stable pulmonary disease (42,43). This intake is considerably higher than the current recommended protein intake of 1.0-1.5 $g \cdot kg^{-1} \cdot d^{-1}$ (37).

Antioxidants, a group of micronutrients, are necessary to neutralize surplus of oxygen radicals. Several studies have reported an oxidant-antioxidant imbalance in CF, also known as oxidative stress (44-47). This is due to chronic inflammation and recurrent infections of the lungs of CF patients (48). These infections are the result of the retention of thick, dehydrated mucus in the small airways, ultimately leading to persistent colonization and inflammation. The numerous neutrophils, which are present in chronic inflammation, excrete free radicals, proteases and immune reaction products, causing progressive lung tissue damage and deterioration of lung function. Since the amount of oxidants supersedes the antioxidant capacity in CF (44-47), supplementation with antioxidant micronutrients is an option to improve the antioxidant status.

The different antioxidants can be divided in four classes (44,49):

1. enzymatic antioxidants e.g. the three superoxide dismutases (=SOD) (cytosolic Cu,Zn- SOD; mitochondrial Mn-SOD; extracellular SOD), peroxisomal catalase, glutathione peroxidases localised in cytosol, in mitochondria and extracellularly (dietary selenium is essential for glutathione peroxidase activity;)
2. soluble antioxidants (uric acid, glutathione, albumin, albumin-bound bilirubin, and various heme-binding proteins)
3. nutritional antioxidants (vitamins C and E(α-tocopherol), β-carotene, Coenzyme Q10);
4. metal-binding compounds (transferrin, lactoferrin, ferritin, ceruloplasmin, and albumin).

A disturbance in the oxidant-antioxidant balance remains despite supplementation with the fat soluble vitamins A, E, and D as demonstrated by increased amounts of lipid hydroperoxides (Hpx), Malondialdehyde (MDA), and protein carbonyl levels, superoxide dismutase, and glutathione peroxidase activities (47,50-52). Hpx and MDA are breakdown products of lipid peroxidation. The presence of protein carbonyl groups are the evidence of free radical attacks on proteins.
Due to the emphasis on increased fat intake, the intake of dietary water-soluble antioxidants in foods such as fruits and vegetables, like vitamin C and β-carotene, may also be diminished. In patients with CF various single micronutrient supplementation studies have been performed, using various amounts of α-tocopherol, retinol, ascorbic acid, and carotenoids, and have shown beneficial effects predominantly on this oxidant-antioxidant imbalance (53-56) Vitamin E (α-tocopherol) is an antioxidant primarily responsible for scavenging peroxyl radicals and in addition inhibits the production of superoxide (57,58). β-carotene, a precursor of vitamin A, quenches oxygen radicals and interferes as a chain breaker of radical initiated reactions (57,59). Vitamin C (ascorbate) is a water-soluble antioxidant which scavenges oxidants produced by neutrophils including superoxide anion and hydroxyl radicals, and also hydrogen peroxide and hypochlorous acid. The other side of the coin is that vitamin C in high concentrations and in the presence of excess iron, can also act as a prooxidant (57,60).

Vitamin B2 (riboflavin) is a precursor of intramitochondrial flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which normalizes the activities of flavin-dependent mitochondrial enzymes, acting in the β oxidation (61,62). It also acts as cofactor to glutathione reductase, which keeps glutathione (GSH) in the reduced state (63). Intracellular GSH has radical-trapping properties. Vitamin B2 can therefore be considered an indirect antioxidative vitamin, which aids in the prevention against initiation and propagation of reactive oxygen species. The reduced form of lipid soluble Coenzyme Q10 is an effective antioxidant, which protects directly against lipid peroxidation by scavenging radicals, and indirectly by regeneration of the antioxidants alpha-tocopherol and ascorbate (64-67).
Nutritional supplements are widely used in exercise programs of athletes to increase muscle mass, and exercise performance and endurance (68-70). This could also be the case for CF patients and would reduce the demand for extensive training. As such, this could well constitute a good alternative to optimize muscle strength. This in turn could have a positive influence on exercise performance, exercise tolerance and lung function. Various studies have reported a beneficial effect of vitamin E on physical performance, but later studies report contradictory results (71). The beneficial effects of micronutrients, like vitamin A, C and E, Coenzyme Q10 and selenium in sportsmen can be attributed to a protective effect on exercise-induced oxidative stress (69,72). Besides the antioxidant action of the reduced form of CoQ10, the oxidized form of CoQ10 acts as an essential electron carrier in the mitochondrial ATP synthesis of all body cells (66). Creatine has been reported to increase lean body mass when used in conjunction with resistance training, to enhance power and strength, and to improve performance in high intensity, short-term exercise tasks (73). Since only transiently decreased levels of carnitine have been described in cystic fibrosis, no primary abnormality of carnitine metabolism seems to exist in CF (74,75). Nevertheless, carnitine has important roles in skeletal muscle bioenergetics, and muscle performance can not only be improved in skeletal muscle carnitine deficiency, but also in case of normal muscle carnitine levels, like in athletics (76). Another nutritional supplement used by athletes is taurine, which is a free amino acid abundant in mammalian cells. It plays an important role in numerous metabolic actions, including antioxidation, and modulation of cellular calcium levels (77). In healthy young men taurine supplementation resulted in attenuation of exercise related oxidative stress and improvement of the exercise capacity (78). Additionally, taurine has been reported to have a beneficial effect on the nutritional status of CF patients by enhancement of nutrient absorption in the ileum (79).

Outline of this thesis

In **Part 1/Chapter 1** we investigated the effect of anaerobic training on anaerobic and aerobic performance, lung function, body composition, peripheral muscle strength, and health-related quality of life in children with CF. Anaerobic exercise performance in children with CF has received little attention compared to aerobic performance. Since the nature of a child's daily physical activity includes both aerobic and anaerobic energy metabolism, but is mostly characterized by short-term anaerobic activities, a training program with anaerobic exercise consisting of short bouts of high intensity activities might be an alternative for aerobic exercise.

Part 2 addresses the important role of protein in improving the nutritional status of CF patients. In CF children with chronic but stable pulmonary disease protein breakdown exceeds protein synthesis, which is even more prominent during pulmonary infections. Since this negative balance could be influenced by increasing dietary protein, we investigated the protein need for reversing this balance.

Part 3 is devoted to two clinical studies on micronutrients. Coenzyme Q10 is a lipid soluble antioxidant, and its plasma levels have been reported to be decreased in diseases associated with increased oxidative stress. Since CF patients present with a negative oxidant-anti-oxidant balance resulting from chronic pulmonary inflammation and recurrent infection, we investigated whether the CoQ10 levels were also low in pediatric CF patients. (**Chapter 3.1**) The effects of a mixture of multiple micronutrients are described in **Chapter 3.2**. In patients with CF, various micronutrient supplementation studies have been performed, using various doses of micronutrients with antioxidative action (e.g., α-tocopherol, retinol, ascorbic acid, and carotenoids). Although beneficial effects on the oxidant-antioxidant imbalance were shown, the effect on pulmonary function was marginal. Given that CF patients also present with decreased muscle strength, we investigated whether a mixture of multiple antioxidants, cofactors and muscle fortifying substances would have a significant effect on both pulmonary function and muscle performance.

In search of an explanation for the altered skeletal muscle performance and exercise intolerance documented in human patients we devoted **Part 4** to experimental investigations of CF muscle mechanics and energetics in a transgenic mouse CF model. In **Chapter 4.1** we investigated twitch contraction mechanics and oxygen consumption in isolated, intact hindlimb muscles of delta F508 homozygote mice and control wild type mice to see if a defect at the level of skeletal muscle itself exists. In **Chapter 4.2** we tested in the same experimental model if supplementation of the antioxidant and mitochondrial electron carrier Coenzyme Q10, the antioxidant α-tocopherol, the indirect antioxidant Vitamin B2, and a combination of carnitine and α-lipoic acid would improve muscle performance in CF.

A summary and general discussion of the clinical relevance and some limitations, with suggestions for further research, is given in the last chapter.

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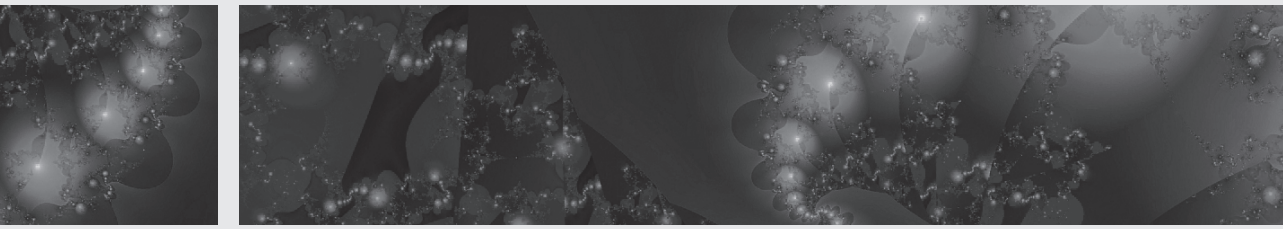

PART 1

Exercise in Cystic Fibrosis: anaerobic exercise

CHAPTER 1

Effects of Anaerobic Training in Children with Cystic Fibrosis

A Randomized Controlled Study

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Chest 2004 Apr;125(4):1299-1305

Abstract

Background: Children's physical activity patterns are characterized by short-term anaerobic activities. Anaerobic exercise performance in children with cystic fibrosis (CF) has received little attention compared to aerobic performance. This study investigated the effects of anaerobic training in children with CF.

Design and methods: Twenty patients were randomly assigned to the training group (TG) [11 patients; mean (± SD) age, 13.6 ± 1.3 years; mean FEV_1, 75.2 ± 20.7% predicted] or the control group (CG) [9 patients; mean age, 14.2 ± 2.1 years; FEV_1, 82.1 ± 19.1% predicted]. The TG trained 2 days per week for 12 weeks, with each session lasting 30 to 45 min. The training program consisted of anaerobic activities lasting 20 to 30 s. The control subjects were asked not to change their normal daily activities. Body composition, pulmonary function, peripheral muscle force, habitual physical activity, aerobic and anaerobic exercise performance, and quality of life were reevaluated at the end of the training program, and again after a 12-week follow-up period.

Results: Patients in the TG significantly improved their anaerobic performance, aerobic performance, and quality of life. No significant changes were seen in other parameters, and no improvements were found in CG. After the follow-up period, only anaerobic performance and quality of life in TG were significantly higher compared to pretraining values.

Conclusions: Anaerobic training has measurable effects on aerobic performance (although not sustained), anaerobic performance, and health-related quality of life in children with CF. Therefore, anaerobic training could be an important component of therapeutic programs for CF patients.

Introduction

Regular aerobic exercise has positive effects on the aerobic capacity of patients with cystic fibrosis (CF) (1,2,3). In addition, higher aerobic fitness has been associated with prolonged survival and quality of life (4,5). Compared to the attention given to aerobic studies, little has been given to anaerobic fitness. Especially lacking are controlled studies on the effects of anaerobic training in children and adolescents with CF. This is somewhat surprising since children's natural activity patterns are characterized by very short vigorous bouts of physical activity, interspersed with varying levels of low-to-moderate intensity. Therefore, physical activity patterns in children may be more suited for a high-intensity anaerobic training program. Several studies (7-12) have shown reduced anaerobic performance in children with CF. In addition, children with CF do not participate in activities with high intensity as much as do healthy control subjects (13).

It has been shown that healthy children's anaerobic performance can be enhanced through participation in structured exercise programs (14,15). Rotstein and colleagues (14) reported an increase in aerobic and anaerobic performance after an anaerobic training program. It is not clear whether anaerobic training can improve anaerobic and aerobic fitness in children with CF. Improvement of anaerobic performance could be important for the daily functioning of children with CF. The aim of this study was therefore to investigate the effects of an anaerobic training program on anaerobic and aerobic performance, lung function, body composition, peripheral muscle strength, and health-related quality of life (HRQOL) of children with CF.

Materials and methods

Subjects

Children with CF were recruited from the Cystic Fibrosis Center at University Medical Center (Utrecht, the Netherlands). Inclusion criteria were as follows: children aged 9 to18 years with a stable clinical condition (ie, no need for oral or IV antibiotic treatment in the 3 months prior to testing); the absence of musculoskeletal disorders; and an FEV_1 of > 30% predicted. Twenty-three patients agreed to participate. Our institutional ethics committee approved the study protocol. Informed consent was obtained from each participant and from his or her parent.

Study Protocol

After the baseline measurements, the tests were repeated within 7 days after the training program was finished, and again 12 weeks later. The study was designed as a randomized controlled trial. After the pretraining tests, the children were randomly assigned by concealed opaque envelopes to either the training group (TG) or the control group (CG). The control

subjects were asked to continue their normal daily activities as well as their physiotherapy regime. The primary researcher was blinded for the experimental condition.

Nutritional Assessment

Anthropometric measurements were made prior to exercise testing. Fat-free mass (FFM) was determined in fasting condition using bioelectrical impedance techniques. Body weight (BW) was measured using a platform beam balance (Mettler; Greifensee, Switzerland) with an accuracy of 0.02 kg. Height was measured with a stadiometer (Holtain; Crymich, UK) with an accuracy of 0.1 cm. Body mass index (BMI) was determined (weight/height^2).

Pulmonary Function Tests

Pulmonary function tests were performed after the inhalation of 800 µg salbutamol via metered-dose inhaler with a spacer in order to rule out important bronchial hyperreactivity. FVC, FEV_1, and forced expiratory flow between 25% and 75% of expiratory vital capacity were obtained from maximal expiratory flow-volume curves (Masterscreen; Jaeger; Wuerzburg, Germany). Residual volume and total lung capacity were measured in a volume-constant body plethysmograph (Masterlab; Jaeger) and the residual volume/total lung-capacity ratios were calculated from the actual values. Values are expressed as the percentage of predicted values (16).

Peripheral Muscle Strength

Isometric muscle force measurements were performed for four muscle groups (ie, shoulder abductors, elbow flexors, hip-extensors, and knee extensors) according to the description of Backman et al (17). Results for peripheral muscle force are presented as the total maximal muscle force (ie, summed maximal force in four muscle groups, since factor analyses showed a single-factor solution [eigenvalue, 2.7; 68% of total variance]).

Exercise Testing

Subsequent anaerobic and aerobic exercise tests were performed on an electronically braked cycle ergometer (Lode Examiner; Groningen, the Netherlands). All subjects were familiar with the different tests and equipment used. During the tests, heart rate was monitored continuously by three-lead ECG (Hewlett-Packard; Amstelveen, the Netherlands) and oxygen saturation by pulse oximetry (200 E; Nellcor; Breda, the Netherlands). Verbal encouragement was given throughout the tests to stimulate maximal performance.

Each subject performed a Wingate anaerobic test (WanT) to assess anaerobic performance (18). The WanT is a valid and reliable test to evaluate short-term anaerobic power in healthy children, and in children with CF and other chronic illnesses (7,11,18,19). The subjects were instructed to start pedaling as fast as possible after a 1-min warm-up against 15-W resistance, while at the same time the full breaking force was applied through an integrated computer

program. Anaerobic performance indexes were reported as mean power (MP [power averaged for > 30 s]) and peak power (PP [highest power during the test]).
After the WanT, the subjects rested for at least 45 min before aerobic fitness was assessed by a standard progressive incremental exercise test. Workload was increased by 15 W at 1-min intervals. The maximal workload (Wmax) was defined as the highest workload maintained during 30 s. Continuous respiratory gas analysis and volume measurements were performed breath by breath with a triple V valveless mouthpiece, and were stored in a computerized exercise system (Oxycon Champion; Jaeger). Internal gas and volume calibrations were made before each test. Measurements taken included oxygen uptake ($\dot{V}O_2$), carbon dioxide production, ventilation, and respiratory exchange ratio (ie, carbon dioxide production/$\dot{V}O_2$). The highest $\dot{V}O_2$ achieved during the last 30 s of exercise was taken as the peak $\dot{V}O_2$ ($\dot{V}O_2$peak) (8). Efforts were considered to be at a maximum level if subjects showed clinical signs of intense effort and were unable to maintain speed at > 50 revolutions per minute (20), and if at least one of the following two criteria were met: (1) cardiac frequency of > 180 beats/min; or (2) maximal respiratory exchange ratio of > 1.0 (21,22). Predicted $\dot{V}O_2$peak values were obtained from an age-matched and gender-matched Dutch reference population (23).

Lactate

Blood samples were drawn 3 min after peak aerobic exercise from an antecubital vein and were collected in tubes (Vacutainer; Becton Dickinson; Franklin Lakes, NJ) and subsequently analyzed (Vitros 250 analyzer; Johnson & Johnson Clinical Diagnostics; Rochester, NY).

Daily Physical Activity

Physical activity was assessed with the habitual activity estimation scale (24). This scale reviews the subject's activity level for 2 weekdays during the previous 2 weeks. The total percentage of time spent being active is presented. The habitual activity estimation scale has been used in studies of children with CF (25,26) and in other studies of children with chronic disease (27).

Quality of Life

Quality of life was measured with a disease-specific HRQOL questionnaire, the CF questionnaire (CFQ). The CFQ consists of a 47-item teen/adult version and a 35-item child version (28,29). The CFQ takes into account the different developmental stages, and makes it possible to monitor the health status and quality of life of patients with CF from 6 years of age throughout adulthood (28,29).

Anaerobic Training Program

The subjects in the TG trained on a individual basis, and the standardized training sessions were led by the children's own physiotherapist. Specific written instructions in the form of a booklet were given to the physiotherapists. The TG trained 2 days per week for 12 weeks.

Each session lasted 30 to 45 min. Guidelines based on a review of anaerobic training studies in children were used (30). The training program consists of eight basic training sessions that were repeated every 4 weeks. The training program is described in more detail at www.chestjournal.org/cgi/content/full/125/4/1299/DC1. Individual scores and changes in training overload were carefully recorded in a logbook. The children were constantly encouraged to exercise at maximal speed.

Statistical Analysis

The data are presented as mean ± SD. All data were tested for normality with the Shapiro-Wilks test. The analysis of variance for repeated measures was used for within-group and between-group comparisons. Between-group comparisons were made with an unpaired t test. Changes within the two groups were analyzed with a two-tailed paired t test. Pearson correlation analyses and linear regression analyses were performed for HRQOL with aerobic and anaerobic indexes. Data were analyzed using the statistical software package (SPSS, version 9.0; SPSS; Chicago, IL).

To achieve a difference in PP per kilogram BW of 10% with an SD of 0.8 W/kg and a statistical power of 80%, it was calculated that eight patients had to be included in each study group.

Results

Twenty-three patients were initially enrolled into the study. Two patients (FEV_1, 37.5 ± 7.8% predicted) in the CG failed to complete the study because of pulmonary exacerbation, and one patient (FEV_1, 105% predicted) in the TG withdrew from the study for practical reasons. The baseline characteristics of the remaining 20 patients are shown in Table 1, and the baseline results for anaerobic and aerobic performance are shown in Table 2. Both exercise tests were well-tolerated by all patients, and all patients fulfilled the criteria for undergoing a maximal aerobic exercise test. Comparisons between groups revealed no significant differences at baseline. The adherence of the TG to the exercise training was excellent. The mean attendance rate at the exercise sessions was 98.1 ± 4.3%. Reasons for absence were holidays and sickness.

Effects of Exercise Training

Body Composition, Pulmonary Function, Muscle Force, and Habitual Physical Activity

At the end of the 12-week training period, a significant within-group increase was found for height (TG, 1.5 ± 0.9 cm [$p < 0.001$]; CG, 1.1 ± 1.0 cm [$p < 0.05$]) and weight (TG, 0.4 ± 0.6 kg [$p < 0.05$]; CG, 0.8 ± 1.0 [$p < 0.05$]). Within-group and between-group comparisons revealed no significant differences for body composition, pulmonary function, peripheral muscle force, and habitual physical activity at the end of the training period.

Table 1. Baseline Characteristics of the TG and the CG*

Characteristics	TG (n = 11)	CG (n = 9)
Age, yr	13.6 ± 1.3	14.2 ± 2.1
Height, cm	155.5 ± 8.2	159.8 ± 8.5
Weight, kg	41.9 ± 5.9	47.7 ± 8.7
BMI, kg/m²	17.2 ± 1.0	18.5 ± 2.3
FFM, kg	27.9 ± 3.4	31.4 ± 4.6
FEV_1, % predicted	75.2 ± 20.7	82.1 ± 19.1
VC, % predicted	85.0 ± 14.0	93.2 ± 15.8
$FEF_{25–75}$, % predicted	54.1 ± 45.3	51.0 ± 30.6
RV/TLC, %	37.2 ± 11.2	32.6 ± 8.7
Isometric muscle force, nm	648 ± 118	668 ± 78
Activity level, %	25.6 ± 13.7	25.9 ± 19.1

* Values given as means ± SD. VC = vital capacity; $FEF_{25–75}$ = forced expiratory flow at 25 to 75% of vital capacity; RV = residual volume; TLC = total lung capacity. p Value was not significant for all between-group comparisons.

Table 2. Baseline Results for Anaerobic and Aerobic Performance*

Variables	TG (n = 11)	CG (n = 9)
Anaerobic performance		
PP		
W	547 ± 178	647 ± 179
W/kg BW	12.8 ± 2.5	13.4 ± 1.6
W/kg FFM	18.7 ± 3.7	20.3 ± 3.2
MP		
W	296 ± 92	365 ± 104
W/kg BW	6.9 ± 1.3	7.7 ± 0.9
W/kg FFM	10.1 ± 1.8	11.4 ± 1.8
Aerobic performance		
$\dot{V}O_2$peak		
mL/min	1677 ± 242	1904 ± 330
mL/kg/min	40.2 ± 4.2	40.7 ± 8.3
mL/kg/FFM	60.1 ± 5.4	60.8 ± 8.4
% predicted	83.1 ± 9.1	84.2 ± 10.4
Wmax, W	140 ± 20	156 ± 26
Lactate, mmol/L	6.9 ± 1.9	9.6 ± 4.0

* Values given as mean ± SD. p Values were not significant for all between-group comparisons.
TG = training group; CG = control group; PP = peak power; BW = body weight; FFM = fat-free mass; MP = mean power; $\dot{V}O_2$peak = peak oxygen uptake; Wmax = maximal workload

Table 3. Effect of Training Program on Anaerobic and Aerobic Performance*

Variables	TG (n = 11)	CG (n = 9)
Anaerobic performance		
ΔPP		
W	66.9 ± 23.8#	– 3.4 ± 53.7
W/kg BW	1.4 ± 0.6#	– 0.3 ± 1.1
W/kg FFM	2.2 ± 1.2#	– 0.6 ± 2.0
ΔMP		
W	36.6 ± 11.8#	– 6.7 ± 29.9
W/kg BW	0.7 ± 0.3#	– 0.3 ± 0.8
W/kg FFM	1.2 ± 0.6#	– 0.4 ± 1.1
Aerobic performance		
Δ$\dot{V}O_2$peak		
mL/min	88 ± 106†	– 48 ± 63
mL/kg/min	1.5 ± 2.6	– 0.6 ± 1.9†
mL/kg FFM/min	1.3 ± 4.6	– 3.2 ± 2.5§
% predicted	4.7 ± 5.6†	– 2.1 ± 2.8
ΔWmax, W	11 ± 14†	– 2 ± 5
ΔLactate, mmol/L	1.8 ± 1.4§	– 1.6 ± 2.9

* Values given as mean ± SD.
#$p < 0.001$; †$p < 0.05$; §$p < 0.01$

TG = training group; CG = control group; ΔPP = change in peak power; BW = body weight; FFM = fat-free mass; MP = change in mean power; Δ$\dot{V}O_2$peak = change in peak oxygen uptake; Wmax = maximal workload

Anaerobic and Aerobic Performance

The changes observed after the 12-week training period for anaerobic and aerobic performance are shown in Table 3. The TG showed significant improvements in absolute PP (11.7%) and MP (12.4%), in PP and MP per kilogram BW (10.9% and 10.1%, respectively), and in PP and MP per kilogram FFM (11.8% and 11.9%, respectively). With respect to aerobic measurements, the TG showed significant improvements in $\dot{V}O_2$peak (5.2 mL/min and 5.7% predicted), Wmax (7.9%), and serum lactate levels (26.1%), while the increase in $\dot{V}O_2$ peak per kilogram FFM (5.2%) was not significant. In the CG, a significant decrease was found for $\dot{V}O_2$peak per kilogram BW (– 1.5%) and per kilogram FFM (– 5.6%), while other parameters were unchanged.

Quality of Life

At the end of the 12-week training period, a significantly higher score was found in the domain of physical functioning in the TG (70.3 ± 13.8 vs 88.4 ± 9.0, respectively; $p < 0.001$),

but no change was found in the CG (83.2 ± 18.5 vs 87.1 ± 17.9, respectively; p = 0.20) or in other quality-of-life domains.
Regression analysis in the TG indicated that the change in PP accounted for 41% of the variance in the physical functioning domain ($p < 0.05$). Changes in MP or $\dot{V}O_2$peak were not independent correlates of changes in quality-of-life scores.

Effects of Follow-up Period
Comparing the pretraining period and the end of the follow-up period, a significant increase was found for mean height (TG, 2.8 ± 1.0 cm [$p < 0.001$]; CG, 2.1 ± 1.2 cm [$p < 0.01$]), weight (TG, 1.7 ± 1.5 kg [$p < 0.01$]; CG, 1.7 ± 1.3 kg [$p < 0.01$]), and FFM (TG, 2.0 ± 2.3 kg [$p < 0.05$]; CG, 1.7 ± 1.2 kg [$p < 0.05$]), but no changes were found for habitual physical activity. However, between-group comparisons of changes observed at the pretraining assessment compared with the end of the follow-up period were not significant. BMI, pulmonary function, peripheral muscle force, and habitual physical activity did not change significantly in both groups.
The TG showed significant higher follow-up levels of absolute PP (54.6 ± 47.7 W; $p < 0.001$) and MP (24.9 ± 73.5 W; $p < 0.01$) when compared with pretraining levels. The increase in the CG was not significant compared to baseline values (PP, 21.7 ± 15.6 [$p = 0.34$]; MP, 12.7 ± 34.4 [$p = 0.31$]). All other anaerobic indexes decreased to baseline values.
With respect to aerobic performance, no significant differences were found in the TG between values at baseline and at the end of follow-up period. The CG showed significantly lower $\dot{V}O_2$peak (BW decrease, 1.5 ± 1.7 mL/kg/min [$p < 0.05$]; FFM decrease, 3.0 ± 1.9 mL/kg/min) and serum lactate levels (decrease, 1.2 ± 1.2 mmol/L; $p < 0.05$) when comparing values in the pretraining period and at the end of the follow-up period. At the end of the follow-up period, the domain of physical functioning in the TG (8.3 ± 8.4; $p < 0.01$) was still significantly higher compared to pretraining values.

Discussion

The aim of this study was to investigate the effects of an anaerobic training program in children with CF. In this single-blind, randomized, controlled study, after a 12-week training period, improvements were observed in anaerobic and aerobic outcome parameters and in HRQOL. In addition, at the end of a 12-week follow-up period most outcome parameters decreased to pretraining values, with the exception of anaerobic performance and HRQOL.
To our knowledge, this is the first study to document that children with CF are able to improve their anaerobic exercise capability through a high-intensity training program. These results are in agreement with those of anaerobic training studies in healthy children (14,15,31). The TG increased their PP by 12.2%, which compares favorably with the results of

the study by McManus and colleagues (32) after an 8-week sprint-running training program performed three times per week in healthy girls (PP, 9.7%). Rotstein and colleagues (14) reported an increase in PP and MP per kilogram BW (14% and 10%, respectively) after a 9-week training program that was performed three times per week. These results are consistent with the increase found in our study (PP/kg BW, 11%; MP/kg BW, 10%). Grodjinovsky and colleagues (15) reported much lower improvements in MP per kilogram BW (3 to 4%) and PP per kilogram BW (4%) after a 6-week anaerobic training program that was performed three times per week in healthy children who were 11 to 13 year old. Although guidelines for anaerobic training in children have not yet been clearly established, (30) the outcomes of these findings suggest that a minimal time period of approximately 8 weeks would be likely to induce a substantial improvement in pediatric anaerobic fitness.

Changes in aerobic exercise capacity usually are associated with specific training programs involving several hours per week at submaximal intensity (33). Although the intensity of our training program may be considered very stressful and the duration of the exercises very brief, anaerobic training resulted in an increase in $\dot{V}O_2$peak (5%). This result is in accordance with those of studies (14,34) in healthy children that have shown an increase in aerobic capacity of 4 to 7% after anaerobic training. These results may be explained by the fact that resynthesis of adenosine triphosphate during high-intensity exercise depends on both aerobic and anaerobic processes (35). Moreover, in children approximately 40% of energy production in the WanT test comes from aerobic metabolism (30). In addition, although an incremental exercise test explores aerobic capacity and is not a valid tool for assessing anaerobic performance (36), the increase in serum lactate values seen in the TG suggests a larger contribution of anaerobic glycolysis during peak aerobic exercise. In other words, improved anaerobic energy metabolism possibly enhanced aerobic power output. This is supported by the finding that during specific aerobic training a decrease is seen in serum lactate concentration (37).

Increased anaerobic performance has been linked to biochemical changes in the muscle of children (31). Fournier and colleagues (31) reported a 21% increase in anaerobic enzyme activity after sprint training. Subsequently, higher serum lactate values in our study provide indirect evidence in support of improvements in biochemical processes associated with anaerobic metabolism as a result of the training program.

Improvements in HRQOL, as measured by the disease-specific CFQ, were seen in the TG. This is consistent with the results of other studies (5,38), which found improvements in quality-of-life scores after aerobic training, as measured with a generic measure (ie, the quality of well-being scale). Furthermore, as shown in our study and in other studies (5,38), changes in HRQOL are related to changes in exercise performance. This emphasizes the need to assess further both HRQOL and exercise performance, which provide valuable information on the multidimensional impact of the disease on patient's quality of life and can make an important contribution to decision making in clinical practice (38,39).

In our study, no positive or adverse effects were seen in pulmonary function, FFM, and peripheral muscle strength due to the training program. As could be expected, no change was found in the amount of habitual physical activity, since we asked the participants not to change their activity level during the study period. Until now, the influence of physical training on pulmonary function has not been established clearly. Several studies (40,41,42) have shown improvements in pulmonary function after aerobic training. In contrast, other studies (1,2,38,43) have failed to detect improvements in pulmonary function. Eventually, all studies show that training is safe for the patients' respiratory condition.

Effects of Follow-up Period

The increases found in the study parameters in the TG decreased to baseline values after the 12-week follow-up period, with the exception of anaerobic performance and HRQOL. Generally, the benefits of exercise disappear if physical activity is discontinued. However, as shown in our study and the studies of others (38,41), benefits due to training seem to continue for some time in patients with CF, regardless of follow-up training sessions.

Clinical Implications of Anaerobic Training

Up to now, anaerobic exercise has received little attention compared to that for aerobic exercise, although many activities in daily life as well as sport activities are both aerobic and anaerobic in nature (8,9). Regular exercise is an important part of treatment in patients with CF. Adherence to exercise programs depends on individual motivation and variation in activities. The children enjoyed our training program, which motivated them to attend 98% of the training sessions. Our anaerobic training program offered the necessary variation to enhance adherence to it. The increase in anaerobic and aerobic performance and in HRQOL after anaerobic training indicates that this type of training can be included in the overall physical rehabilitation of children with mild-to-moderate CF. Ideally, an exercise program for children with CF could be made of aerobic, anaerobic, and strength-training activities. This makes it possible to individually tailor the program according to the preference of the participants, thereby improving the levels of exercise adherence (44).

In conclusion, our results suggest that children with mild-to-moderate CF can enhance their anaerobic and aerobic performance and HRQOL through participation in a structured anaerobic exercise training program. In addition, aerobic performance increases as well. The improvements in anaerobic performance and HRQOL are maintained through a 12-week follow-up period. Therefore, anaerobic training could be an important component of the rehabilitation program of children with CF.

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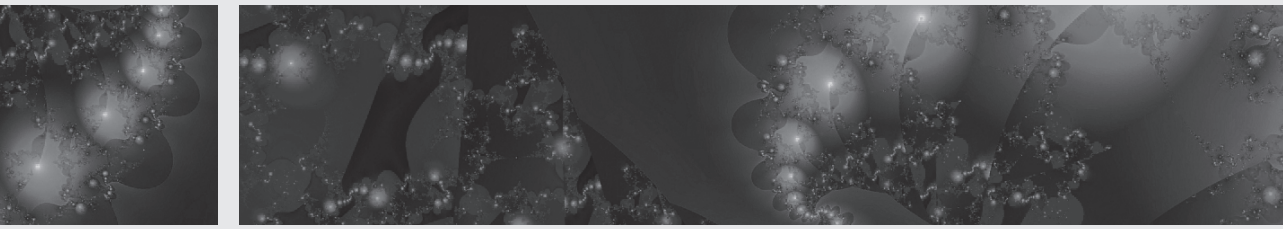

PART 2

Macronutrients in Cystic Fibrosis

CHAPTER 2

Short-term protein intake and stimulation of protein synthesis in stunted children with cystic fibrosis

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Am J Clin Nutr 2005;81:605-10

Abstract

Background: Stunted children with cystic fibrosis (CF) have less net protein anabolism than do children without CF, and the result is retarded growth in the CF patients. It is not known whether protein intake above that recommended by the Cystic Fibrosis Foundation would further stimulate whole-body protein synthesis.

Objective: We studied the effects of 3 amounts of protein intake on whole-body protein synthesis and breakdown by using isotopic infusion of [1-^{13}C]valine and [$^{15}N_2$]urea in children with stable CF who required tube feeding.

Design: In 8 pediatric CF patients, we administered 3 randomly allocated isocaloric diets with normal (NP), intermediate (IP) and high (HP) amounts of protein (1.5, 3 and 5 g·kg^{-1}·d^{-1}, respectively) by continuous drip-feeding during a 4-d period at 6-wk intervals. Each patient acted as his or her own control. On the fourth day of feeding, whole-body protein synthesis and breakdown were measured.

Results: Protein synthesis was significantly higher in the HP group ($\bar{x}$ ± SEM: 1.78 ± 0.07 μmol · kg^{-1} · min^{-1}) than in the IP (1.57 ± 0.08 μmol · kg^{-1} · min^{-1}; *P* = 0.001) and NP (1.37 ± 0.07 μmol · kg^{-1} · min^{-1}; *P* <0.001) groups. There were no significant differences in protein breakdown. Net retention of nitrogen was significantly higher in the HP group (12.93 ± 1.42 μmol · kg^{-1} · min^{-1}) than in the IP (7.61 ± 1.40 μmol · kg^{-1} · min^{-1}; *P* = 0.01) and NP (2.48 ± 0.20 μmol · kg^{-1} · min^{-1}; P < 0.001) groups.

Conclusion: In stunted children with CF requiring tube feeding, the highest stimulation of whole-body protein synthesis was achieved with a short-term dietary protein intake of 5 g · kg^{-1} · d^{-1}.

Introduction

Cystic fibrosis (CF) is the most common life-threatening recessive inherited disease in whites. The carrier frequency is 1 in 22-28 in whites, and the incidence is 1 in 2000-3000. Approximately 30 000 persons are affected in the United States (1). In the past 10-15 y the mean survival of CF patients has increased significantly, from 20 to 34 y, as a result of improved medical care (2). One of the major factors contributing to this improvement is a greater emphasis on optimal nutrition. It has been shown that patients with better nutritional status have improved linear growth and maintain better pulmonary function and exercise tolerance (3-5). In addition, malnutrition has been shown to have adverse effects on respiratory muscle strength, ventilatory drive, and immune defense mechanisms (6,7).

Historically, the literature on nutritional strategies in CF has focused primarily on energy and fat intakes (8-12). In CF patients with a forced expiratory volume in 1 s (FEV_1) > 85% predicted, the CF gene has no significant effect on resting energy expenditure, whereas, in those with FEV_1 < 85% predicted, there is a curvilinear increase in resting energy expenditure (13). For children with CF, an energy intake of 120-150% of the recommended dietary allowance (RDA) for healthy persons is currently recommended, including a high-normal fat intake of 35-40 % of energy (14). It is well known that feeding enhances both whole-body and organ-specific protein synthesis in humans (15). However, there has been little study of the optimal amounts of dietary protein intake in pediatric CF patients.

At the time of a diagnosis of CF, severe protein deficits can already be present, and they result in lean body mass wasting or stunting of linear growth (16). The nutritional status is significantly worse in children diagnosed when symptomatic than in those diagnosed by neonatal screening (17). In general, protein deficits can result from increased protein breakdown, decreased protein synthesis, or both. Holt et al (18) found that children with CF with pulmonary infections have protein synthesis rates that are 50% lower than those in healthy controls and 43% lower than those in children with CF with chronic but stable pulmonary disease (P <0.001). Conversely, in the same study, they found significantly greater whole-body protein breakdown (WBPB) in a group of stable CF patients than in an infected group or control subjects (≈250% and 170%, respectively; P < 0.01) (18). During pulmonary infection, protein synthesis and net protein deposition can be enhanced with short-term nutritional supplementation (19;20). Kien et al (21) studied the effects of enhanced feeding (protein intake: 4 $g \cdot kg^{-1} \cdot d^{-1}$) in stable pediatric CF patients and control subjects after a 12-h fast, and they found a 23% increase in protein synthesis in the fed state in the CF group (P = 0.058) and no change in WBPB. Under the same circumstances, WBPB increased 34% in a healthy control group (P = 0.001), which indicated that feeding may affect protein turnover differently in children with stable CF and in healthy subjects.

We have been unable to identify any controlled studies that have investigated the effects of different dietary protein intakes on protein synthesis and breakdown in chronic and stable pediatric CF patients. The current recommended protein intake is 1.0-1.5 $g \cdot kg^{-1} \cdot d^{-1}$ (22).

The aim of this study was, by using the isotopic infusion of [1-^{13}C]valine and [$^{15}N_2$]urea, to ascertain the dietary protein intake that corresponded to a maximal rate of whole-body protein synthesis in pediatric CF patients with chronic but stable pulmonary disease.

Subjects en Methods

Subjects

Children with a diagnosis of CF, confirmed with the use of a pilocarpine iontophoresis sweat chloride test, a CF gene mutation analysis, or both, were recruited from the pediatric outpatient clinics of 2 academic medical centers and one teaching hospital in the northwest part of the Netherlands. Eight patients were eligible, and all agreed to be enrolled. Inclusion criteria were age 7-12 y, prepubertal status, mild lung disease (FEV_1 > 75%), nasogastric or percutaneous endoscopic gastric feeding tube in situ, and no weight loss during the previous 30 d (23). Exclusion criteria were diabetes mellitus, clinical signs of intercurrent pulmonary infection (ie, tachypnea > 30 breaths/min, increased coughing, or rectal temperature of ≥ 38.5 °C), and use of antibiotics other than chemoprophylaxis ≤ 14 d before the beginning of the protocol. Home medications (ie, bronchodilators, mucolytic agents, or pancreatic enzyme replacement therapy) were continued.

All procedures were explained to the subjects and their parents, and written informed consent was obtained from all subjects. The Medical Ethical Committee of the Academic Medical Center, Amsterdam, approved the study protocol.

Study design and procedures

We conducted a prospective, randomized, single-blinded, crossover trial of 3 isoenergetic diets containing 1.5 [normal-protein (NP)], 3 [intermediate-protein (IP)], and 5 [high-protein (HP)] g protein · kg^{-1} · d^{-1} in children with CF; each patient served as his or her own control subject. Endpoints were whole-body protein synthesis and WBPB and net nitrogen retention obtained by using a stable isotopes technique.

At enrollment, a medical history was obtained and physical examination performed, including height, weight, temperature, and FEV_1, which was measured with a MasterScreen Paed (Viasys Healthcare, Bilthoven, Netherlands). At the start of the study, oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) were continuously measured for 30 min by indirect calorimetry by using a ventilated hood system (Vmax model 2900; SensorMedics, Anaheim, CA). The energy intake of each diet was set at 200% of the patient's individual baseline resting energy expenditure [ie, ≈120% of the recommended dietary allowance RDA for CF (24)]. During a 4-d period, the liquid study diet was administered via a gastric tube 24 h/d with the use of a feeding pump (Kangaroo 324; Sherwood Medical, St Louis). The diets consisted of a mixture of carbohydrate powder (Fantomalt), fat emulsion (Solagen),

protein powder (Protifar; all products of Nutricia, Zoetermeer, Netherlands) and additional household salt dissolved in a body weight-related volume of water. Because of the continuous drip-feeding, the carbohydrate intake was at a constant rate of 5 mg glucose · kg^{-1} · min^{-1}. The protein intake was varied per diet (1.5, 3 and 5 g · kg^{-1} · d^{-1} for the NP, IP, and HP diets, respectively), and the remaining non-protein energy was supplied by fat. In addition, the children were allowed to drink nonenergetic beverages ad libitum. The sequence of the diets was randomly allocated, provided that within the study group all possible sequential combinations were assigned. The patients and their caretakers were blinded to the composition of the formula. On the fourth day, a catheter was inserted antegradely in a deep antecubital vein of each arm. One catheter was used for sampling arterialized blood using a heated (60 °C) hand box. At time –5 min (0825), baseline blood and breath samples were taken for determination of background isotopic enrichments. At time zero, via the second catheter in the opposite arm, primed continuous intravenous infusions of [1-13C]valine at a rate of 0.153 μmol · kg^{-1} · min^{-1} (prime 9.1 μmol · kg^{-1}), and [$^{15}N_2$]urea, at a rate of 0.28 μmol · kg^{-1} · min^{-1} (prime 200 μmol · kg^{-1}), were started, and a prime of $NaH^{13}CO_3$ 4.2% was administered (2.1 μmol · kg^{-1}) (all: Cambridge Isotopes, Cambridge, MA). The isotopes were dissolved in normal saline and infused by using a Perfusor Secura FT infusion device (B Braun Melsungen AG, Melsungen, Germany), sterilized by passage through an 0.20-μm millipore filter (Minisart; Sartorius AG, Göttingen, Germany). All infusions were tested for pyrogenicity by the hospital pharmacy.

After an equilibration period of 330 min of stable isotope infusion, 4 blood samples were collected at 10-min intervals (at 330, 340, 350, and 360 min), immediately stored on ice, and subsequently centrifuged at 1860 x *g* for 10 minutes at 4 °C. After separation, plasma was stored at –20 °C until analysis of isotopic enrichment of valine, α-ketoisovalerate (KIV), and urea (at 360 min). Plasma concentrations of insulin, glucagon, cortisol, catecholamines, and free fatty acids were measured.

During the experiment (8 h), tube feeding of an equivalent of one third of the total daily dietary intake was continued. The entire protocol was repeated twice for each child, so that, at the end of all experiments, each child had been exposed to diets containing 1.5, 3 and 5 g protein · kg^{-1} · d^{-1}. Between these study diets, the children resumed their regular diet for a 6-wk period.

Assays

Valine and urea were isolated on a cation exchange column (AG50W-X8; Bio-Rad Laboratories Inc, Hercules, CA) after precipitation of the plasma proteins with trichloroacetic acid. The proteins were eluted with ammonia and evaporated to dryness. Valine was derivatised to the *N*(*O*,*S*)-methoxycarbonylmethyl derivative as described by Husek (25). After extraction with chloroform, an aliquot was injected into a gas chromatograph-combustion-isotope ratio mass spectrometry system [(IRMS) HP 6890 series GC system; Hewlett-Packard, Palo Alto, CA; and Finnigan Delta^{plus} IRMS; Finnigan-MAT, Bremen, Germany]. Separation was achieved on a CP-SIL 19CB capillary column (25 m x 0.32 mm x 0.2 μm; Varian, Middelburg, Netherlands). After combustion of the GC effluent to carbon dioxide,

the ^{13}C enrichment of valine was measured by IRMS. Data acquisition and delta calculations were performed using the THERMOFINNIGAN ISODAT NT software (version 0.144; Finnigan-MAT). Urea was measured as the *bis*-trimethylsilyl derivative described by Matthews et al (26). Isotopic enrichment was measured on a gas chromatography-mass spectrometry system (HP6890 series GC system and 5973 Mass Selective Detector; Hewlett- Packard) equipped with a J&W Scientific DB 17 capillary column (30 m x 0.25 mm x 0.25 µm; Agilent Technologies, Palo Alto, CA). Selected ion monitoring (ie, electron impact ionization), data acquisition, and quantitative calculations were performed by using CHEMSTATION software (version D.01.02.16; Agilent Technologies, Palo Alto, CA). The *bis*-trimethylsilyl derivative was monitored at a mass-to-charge ratio (*m/z*) of 189 for urea and *m/z* of 191 for [$^{15}N_2$]urea. The ratio of urea tracer to tracee was calculated as described by Patterson et al (27). The [1-^{13}C]α-KIV enrichment was determined in the O-*t*-butyldimethylsilyl-quinoxalinol derivative according to the method of Kulik et al (28). The same gas chromatography-mass spectrometry system was used for the urea analysis. The KIV derivative was monitored at *m/z* 245 for KIV and *m/z* 246 for [1-^{13}C]KIV. KIV tracer:tracee was calculated as described by Patterson et al (27). Plasma insulin concentrations were ascertained by using an enzyme immunoassay on an Immulite analyzer (DPC, Los Angeles) with an intraassay CV of 3.5-6%, an interassay CV of 7.5-9%, and a detection limit of 15 pmol/L. Glucagon was measured by radioimmunoassay (Linco Research, St Charles, MO) with an intraassay CV of 3-5%, an interassay CV of 9-13%, and a detection limit of 15 ng/L. Cortisol was measured by enzyme immunoassay on an Immulite analyzer with an intraassay CV of 2-4%, an interassay CV of 3-7%, and a detection limit of 50 nmol/L. Serum free fatty acids were measured by an enzymatic method (NEFAC; Wako Chemicals GmbH, Neuss, Germany) with an intraassay CV of 2-4%, an interassay CV of 3-6%, and a detection limit of 0.02 mmol/L.
During the final 30 minutes (330-360 min) of isotope infusion, $\dot{V}O_2$ and $\dot{V}CO_2$ were measured by using indirect calorimetry. Concurrent breath samples were taken at 0, 60, 150, 240, 270, 300, 330, and 360 min for enrichment of $^{13}CO_2$ (BreathMAT^{plus}; Finnigan-MAT) to calculate total valine oxidation.

Calculations and statistical analysis

Dietary effects on whole-body protein synthesis (S) were studied by using 2 independent indicators: nonoxidative disposal of valine and net nitrogen retention in the body (29). The rate of appearance (Ra) of valine in plasma and valine oxidation (µmol · kg^{-1} · min^{-1}) were calculated according to standard equations (30-33). To calculate the rate of S, wich is equivalent to the rate of non-oxidative disposal of valine (NODVal; µmol · kg^{-1} · min^{-1}), valine oxidation was subtracted from the rate of appearance of valine (RaVal):

$$S = \text{NODVal} = \text{RaVal} - \text{valine oxidation} \quad (1)$$

Endogenous valine breakdown (*B*) (µmol · kg^{-1} · min^{-1}) was calculated by subtracting exogenously administrated valine from the total RaVal:

$$B = \text{RaVal} - \text{Val(diet)} = \text{endogenous RaVal} \quad (2)$$

Net valine deposition (anabolism) (μmol · kg^{-1} · min^{-1}) in the body was calculated by subtracting *B* from *S*:

$$\textbf{Net valine deposition} = S - B \qquad (3)$$

From the urea data [Ra of urea (RaUrea), net nitrogen (*N*) retention (*S*) was calculated as

$$S = \text{Net } N \text{ retention} = \text{dietary } N - (2 \times \text{RaUrea}) \ \mu\text{mol} \cdot kg^{-1} \cdot min^{-1} \qquad (4)$$

Repeated-measures analysis of variance was used to establish differences in these variables at normal, intermediate and high dietary protein intakes. The analysis was performed by using the mixed procedure of SAS for WINDOWS statistical software (Version 5.0.2195; SAS Institute Inc, Cary, NC) with compound symmetry covariance structure and the restricted maximum likelihood estimation method. $P < 0.05$ was considered significant. Data are presented as means ± SEMs.

Results

Patients

Baseline characteristics of the patients are shown in **Table 1**. The patients were stunted mainly in linear growth (mean SD = –1.1), whereas their weight-for-height was normal (34).

Valine data

The individual data for the rate of appearance of valine and valine oxidation are shown in **Table 2**. Valine kinetics showed a significant, progressive increase of non-oxidative disposal with increasing dietary protein intake of 1.37 ± 0.07 μmol · kg^{-1} · min^{-1} in the NP, 1.57 ± 0.08 in the IP, and 1.78 ± 0.07 in the HP diets (**Table 3**). There was no significant change in the endogenous rate of appearance of valine between the 3 diets.

Urea data

There was a significant increase in urea production (rate of appearance) with increasing dietary protein load: 4.43 ± 0.09 , 7.59 ± 0.73, and 12.38 ± 0.65 μmol · kg^{-1} · min^{-1} in the NP, IP, and HP diets, respectively (Table 2). This increase was significantly less than the increase in protein intake, which resulted in net nitrogen retention with increased protein intakes: 2.48 ± 0.20, 7.61 ± 1.40, and 12.93 ± 1.42 μmol · kg^{-1} · min^{-1} in the NP, IP, and HP diet, respectively (Table 3).

Hormone data

There were no significant differences in the plasma concentrations of insulin, cortisol and free fatty acids between the diets (**Table 4**). Only the plasma concentration of glucagon in the HP diet was significantly different from that in the NP and IP diets (82 ± 4, 65 ± 5, and 71 ± 4 ng · L^{-1}, respectively).

Table 1. Baseline characteristics of patients.

nr	Sex	Age (years)	Height in cm (SD)[1]	Weight in kg (weight for height, SD)[1]	Best FEV_1 (% pred.)
1	M	12.0	149.5 (-1)	40.1 (0.5)	75
2	M	7.9	130.0 (-0.5)	29.5 (1)	106
3	M	11.5	138.0 (-2)	26.7 (-2)	90
4	M	9.2	131.0 (-1.5)	38.1(2)	86
5	F	10.7	136.5 (-1.5)	26.7 (-1)	97
6	F	10.7	137.0 (-1.5)	27.7 (-1)	95
7	M	7.6	133.0 (0.5)	30.7 (1)	95
8	M	9.6	121.0 (-2.5)	25.1 (1)	88
mean		9.5	134.5 (-1.1)	30.6 (0.2)	91.5

[1]Anthropometric data are shown as absolute numbers and standard deviation (SD) scores, according to the Dutch growth charts, generated by the 4th Nationwide Dutch Growth Study 1997 (34); FEV_1, forced expiratory volume in 1 second; % pred, percentage of predicted value.

Table 2. Individual data for rate of appearance (Ra) of valine, valine oxidation, and Ra urea[1]

Pt.no.	Ra Valine ($\mu mol \cdot kg^{-1} \cdot min^{-1}$)			Valine oxidation (% of flux) ($\mu mol \cdot kg^{-1} \cdot min^{-1}$)			Ra Urea ($\mu mol \cdot kg^{-1} \cdot min^{-1}$)		
	NP	IP	HP	NP	IP	HP	NP	IP	HP
1	1.86	2.42	2.82	0.54 (29)	0.84 (35)	1.13 (40)	4.57	11.01	10.93
2	1.73	2.46	3.14	0.49 (28)	0.83 (34)	1.22 (39)	4.64	5.94	13.96
3	2.26	2.70	3.35	0.54 (24)	0.83 (31)	1.30 (39)	4.64	6.68	11.40
4	1.63	1.94	2.54	0.50 (31)	0.81 (42)	1.08 (43)	4.62	4.14	13.83
5	1.97	2.44	3.16	0.52 (26)	0.71 (29)	1.32 (42)	4.14	8.08	8.97
6	1.99	2.22	3.26	0.42 (21)	0.68 (31)	1.39 (43)	3.98	7.67	14.20
7	1.66	2.38	2.55	0.47 (28)	0.90 (38)	1.00 (39)	4.61	9.20	12.27
8	1.77	2.56	2.97	0.45 (25)	0.97 (38)	1.11 (37)	4.23	7.98	13.48
mean	1.86^a	2.39^b	2.97^c	$0.49\ (26)^a$	$0.82\ (34)^b$	$1.19\ (40)^c$	4.43^a	7.59^b	12.38^c
SEM	0.07	0.08	0.11	0.02	0.03	0.05	0.09	0.73	0.65

[1] NP, normal protein diet (1.5 g · kg^{-1} · d^{-1}); IP, intermediate protein diet (3.0 g · kg-1 · d-1); HP, high protein diet (5.0 g · kg^{-1} · d^{-1}). Values in a row under each section heading with different superscript letters are significantly different, $P \leq 0.01$ (repeated-measures ANOVA).

[2] Percentage of flux in parentheses.

Table 3. Relative contribution of non-oxidative disposal of valine and endogenous rate of appearance (Ra) of valine to net valine flux[1]

	NP	IP	HP
Valine flux	1.86^{a}±0.07 (100)	2.39^{b}±0.08 (100)	2.97^{c}±0.11 (100)
Valine oxidation	0.49^{a}±0.02 (26.3)	0.82^{b}±0.03 (34.3)	1.19^{c}±0.05 (40.1)
Non-oxidative disposal	1.37^{a}±0.07 (73.7)	1.57^{b}±0.08 (65.7)	1.78^{c}±0.07 (59.9)
Endogenous Ra Valine	1.26±0.07 (67.7)	1.20±0.11 (50.2)	1.05±0.15 (35.4)
Valine balance	0.11^{a}±0.03	0.37^{b}±0.05	0.73^{c}±0.12
Net N retention	2.48^{a}±0.20	7.61^{b}±1.40	12.93^{c}±1.42

[1] All values are mean ± SEM; percentage of flux in parentheses. NP, normal protein diet (1.5 g · kg^{-1} · d^{-1}); IP, intermediate protein diet (3.0 g · kg^{-1} · d^{-1}); HP, high protein diet (5.0 g · kg^{-1} · d^{-1}). Values in a row with different superscript letters are significantly different, $P \leq 0.01$ (repeated-measures ANOVA).

Table 4. Plasma concentration of hormones and free fatty acids (FFAs)[1]

	diet		
	NP	IP	HP
Insulin (pmol · L^{-1})	86 ± 7	93 ± 9	123 ± 26
Glucagon (ng · L^{-1})	65 ± 5^{a}	71 ± 4^{a}	82 ± 4^{b}
Cortisol (nmol · L^{-1})	173 ± 23	192 ± 42	187 ± 22
FFA (pg · mL^{-1})	0.11 ± 0.01	0.10 ± 0.01	0.09 ± 0.02

[1] All values are mean ± SEM. NP, normal protein diet (1.5 g · kg^{-1} · d^{-1}); IP, intermediate protein diet (3.0 g · kg^{-1} · d^{-1}); HP, high protein diet (5.0 g · kg^{-1} · d^{-1}). Values in a row with different superscript letters are significantly different, $P \leq 0.01$ (repeated-measures ANOVA).

Discussion

The patients in our study were stunted predominantly in linear growth, whereas their weight-for-height was normal. This observation is in agreement with a large cross-sectional survey of >3000 pediatric and adolescent CF patients in the United Kingdom, which showed a progressive, parallel, downward slope of SD curves for both height and weight as compared with the population without CF (35). In addition, during the past 5 y, the patient registry annual data reports (23 000 patients) of the American Cystic Fibrosis Foundation showed, for persons of both sexes aged 0-20 y, an average height at approximately the 30th-35th percentile and weight at the 40th percentile until the age of 8 y, and this was followed by a gradual decline to the 25th-30th percentile; this indicates that most pediatric CF patients were stunted but had normal weight-for-height (36,37).

In this study we showed that, in stunted pediatric CF patients with chronic but stable pulmonary disease, whole-body protein synthesis could be enhanced 30% by increasing dietary protein intake to 5 g · kg^{-1} · d^{-1}. With the HP diet the rate of protein synthesis was significantly greater than that with the currently recommended NP diet. Protein breakdown, expressed as the endogenous rate of appearance of valine, did not change significantly. When reviewing relevant literature on the subject, we were unable to find a relation between either dietary protein or energy intake to WBPB (20,38-40). We therefore concluded that the non-significant trend of a decrease of protein breakdown that we observed cannot be attributed to any dietary changes. The energy intake in the current study (72.6 ± 8.2 kcal · kg^{-1} · d^{-1}) is within the range of that cited in the aforementioned studies (20,38-40).

So far, few studies have addressed the effect of different amounts of protein intake on rates of protein synthesis and breakdown in patients with CF. In all those studies, protein intake was varied simultaneously with total energy intake, so that it remains unclear whether the results can be explained solely by different amounts of dietary protein. One study that compared the acute effects of 2 consecutive amounts of dietary protein intake (3.2 g protein · kg^{-1} · d^{-1} with 76 kcal · kg^{-1} · d^{-1} during 4 days, and 1.6 g protein · kg^{-1} · d^{-1} with 85 kcal · kg^{-1} · d^{-1} during 8 consecutive days) on whole-body protein metabolism in stunted adolescent and young adult CF patients did not find significant changes in protein synthesis or breakdown (20). However, in that study, 2 variables (both protein and energy intake) were altered at the same time, so that their specific relative contributions to the effect remained uncertain. A further confounder was the possibility of a carryover effect, because the study design did not correct for this possibility, and the result was a lack of difference in protein synthesis and breakdown rates. In another study, nocturnal nutritional supplementation with a semi-elemental formula (1.14 g protein · kg^{-1} · $12h^{-1}$, 32 kcal · kg^{-1} · $12h^{-1}$, 70% free amino acids, and 30% small peptides), a nonelemental formula (2.49 g protein · kg^{-1} · $12h^{-1}$, 45 kcal · kg^{-1} · 12h-1, and 100% casein), and a modified form of the nonelemental formula (1.14 g protein · kg^{-1} · $12h^{-1}$, 32 kcal · kg^{-1} · $12h^{-1}$, and 100% casein) was compared (38). Although

no significant changes in either protein synthesis or catabolism between the 3 nocturnal diets were observed in that study, net protein deposition was significantly (40%, $P < 0.05$) greater with the nonelemental formula than with the modified nonelemental formula. However, the supplemental feeding with the protein-rich formula was combined with a higher energy intake than was used with the lower-protein formula.

Several major long-term studies on the effects of 2 different amounts of protein intake in pediatric CF patients have been performed. In a cohort study over 2 consecutive years, Parsons et al (39) compared a nitrogen intake of 464 ± 16 mg · kg^{-1} · d^{-1} (which is equivalent to 2.9 g whole protein · kg^{-1} · d^{-1}) in the first year to a nitrogen intake of 608 ± 18 mg · kg^{-1} · d^{-1} (which is equivalent to 3.8 g whole protein · kg^{-1} · d^{-1}) in the second year. No changes were observed in either whole-body nitrogen flux or protein synthesis rates in the first year. The protein breakdown rates were significantly ($P < 0.05$) lower during the second year, which resulted in greater net protein deposition and greater weight growth velocity ($P < 0.05$). Height velocity was increased nonsignificantly ($P < 0.1$), from a mean SD ± SEM of –1.03 ± 0.54 in the first year to a mean SD ± SEM of +0.26 ± 0.70 in the second year. Energy intake was significantly higher during the second year than during the first year (82 ± 1 and 65 ± 2 kcal · kg^{-1} · d^{-1}, respectively; $P < 0.05$). In an intervention study without control group, in which normal protein feeding via a gastrostomy tube was used to re-feed adolescent and adult CF patients, Vaisman et al found that body composition was restored with concomitant increase in energy expenditure after 1 y, without measurable changes in whole body protein turnover (40). Finally, Shepherd et al (41) conducted a 2-y follow up study that showed that both energy and protein supplementation (≥120% of RDA) in 10 stunted pediatric CF patients induced an initial (first month) but non-sustained 50% stimulation of protein synthesis during nutritional supplementation. Nevertheless, this led to clinically significant catch-up growth in both weight and height within 6 mo, which continued for ≥1 y. In our short-term study, we found that protein synthesis and total-body protein content can be increased in pediatric CF patients by 4 d isocaloric, high-protein intake. In long-term studies, the effect of extra protein will not show itself in protein kinetics measured with stable isotopes, because the changes in lean body mass develop only slowly under those circumstances, and stable isotope measurements in general are too insensitive to pick up the small difference between synthesis and breakdown. Long-term studies of the effects of extra protein should aim at effects on linear growth or body composition. Therefore, it would be very interesting to study long-term effects of isocaloric high-protein intakes on height and body composition in these children in a controlled, prospective study.

In the current study, there were no changes in plasma concentrations of anabolic hormones between the diets. However, the non-significant amino acid-induced increase in plasma insulin concentration might have partly accounted for both the increase of protein synthesis and the decrease of protein breakdown (42). The only significant hormonal change we observed was the increased aminogenic glucagon plasma concentration with the HP diet.

There is evidence that glucagon has an attenuating effect on amino acid-induced increase in nonoxidative disposal of tracer isotopes and hence on the protein synthesis rate (43). Despite this effect, we observed significantly greater protein synthesis with the IP and HP diets. We concluded that the increased protein synthesis rates resulted from increased dietary protein intake.

In studies of valine kinetics, whole-body protein synthesis is calculated by subtracting valine oxidation from total flux. However, body retention of infused ^{13}C can occur either in the form of nonexcreted $^{13}CO_2$, or as a result of fixation in metabolites other than carbon dioxide (44). This body retention of carbon dioxide will lead to an underestimation of valine oxidation, and inversely, to an overestimation of protein synthesis. $^{13}CO_2$ recovery factors obtained in post-absorptive states and euglycemic, hyperinsulinemic clamping studies vary from 0.67 to 0.82 (45,46). Although there were no validated correction factors available for our study, we assumed the abovementioned effect to be equal for the 3 study diets. Moreover, we measured net nitrogen retention in the body as an independent variable of protein metabolism by studying urea kinetics.

The urea data supported the observations with the valine data. With the higher-protein diets, there was also a significant increase of ≈ 200% (3.1-fold rise) in net nitrogen retention in the body, despite the increase in urea production. Because dietary protein can be either oxidized only to urea and carbon dioxide or incorporated into the body, the increased positive nitrogen balance can be considered as an increase of protein synthesis in the body. Hence, these data show that both biochemical pathways (oxidation and incorporation of protein) were simultaneously yet unequally stimulated by increasing dietary protein intake.

In conclusion, our data support the hypothesis that, in stunted pediatric CF patients, short-term protein synthesis and thus net nitrogen retention in the body can be significantly increased by an HP (5 g protein · kg^{-1} · d^{-1}) diet. Whether or not these effects would be sustained with a prolonged diet, and thus leading to catch-up growth in height, requires further investigation.

Acknowledgements

We thank Maruschka P Merkus for her help with the statistical analysis of the data.

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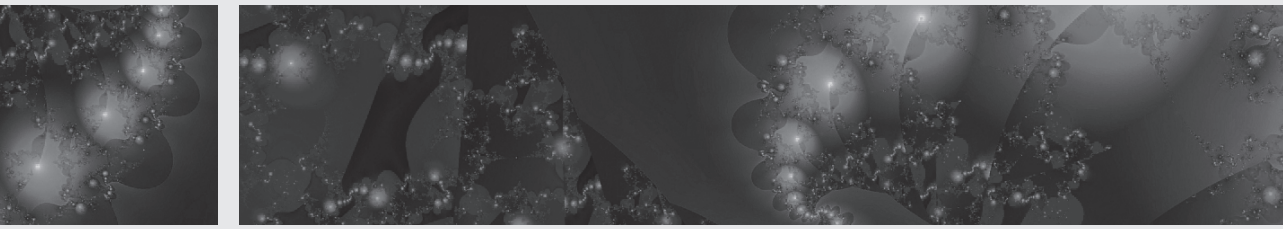

PART 3

Micronutrients in Cystic Fibrosis: human/clinical studies

CHAPTER 3.1

Decreased Coenzyme Q10 concentration in plasma of children with cystic fibrosis

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Submitted to J Pediatr Gastroenterol Nutr

Abstract

Objectives: Coenzyme Q10 (CoQ10) is an effective lipophilic antioxidant and protects against lipid peroxidation by scavenging radicals. Patients with cystic fibrosis generally have fat malabsorption, so we hypothesized that overall plasma CoQ10 concentration in pediatric patients with cystic fibrosis might be diminished. As these patients have increased oxidative stress due to chronic pulmonary inflammation, we also assumed that the oxidized form of CoQ10 might be relatively increased.

Methods: Total plasma CoQ10 levels, as well as the oxidized and reduced form, were measured by HPLC in 30 children with cystic fibrosis (mean FEV_1 predicted 88.5% ± 18.7%) and 30 age-matched controls.

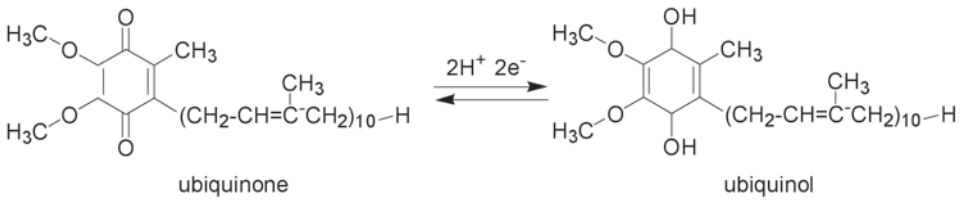
Results: Total plasma CoQ10 levels were significantly lower in the cystic fibrosis group as compared to the control group (respectively 0.87 ± 0.42 μmol/l and 1.35 ± 0.39 μmol/l; $p < 0.001$). When correcting for the lower serum cholesterol in cystic fibrosis patients this difference remained significant: CoQ10/cholesterol ratio (μmol/mol) was 268.8 ± 136.7 and 334.0 ± 102.9 in patients and controls respectively ($P<0.05$). However, the CoQ10 redox status was identical in patients and controls (86.4% ± 7.1% resp 85.4% ± 7.3%).

Conclusion: We found that the overall plasma CoQ10 concentration is lower in cystic fibrosis patients, probably due to fat malabsorption. The CoQ10 redox status was not disturbed, indicating that CoQ10 could still be adequately regenerated in this group of cystic fibrosis patients with mild-to-moderate pulmonary disease.

Introduction

Coenzyme Q10 is present in cellular membranes of lipophilic cell organelles as well as in serum lipoproteins (1,2). As it is part of a redox-couple, an equilibrium exists between oxidized CoQ10 (CoQ10ox; ubiquinone) and reduced CoQ10 (CoQ10red; CoQ10H2; ubiquinol) (Figure 1) (3). Approximately 60% of plasma CoQ10 is associated with low-density lipoproteins (LDL), 25% with high-density lipoproteins (HDL), and 15% with other lipoproteins (2). Hence a strong correlation is found between plasma CoQ10 and LDL, and thus cholesterol concentration (4,5).

Figuur 1. The CoQ10ox (ubiquinone)– CoQ10red (ubiquinol) redox couple.

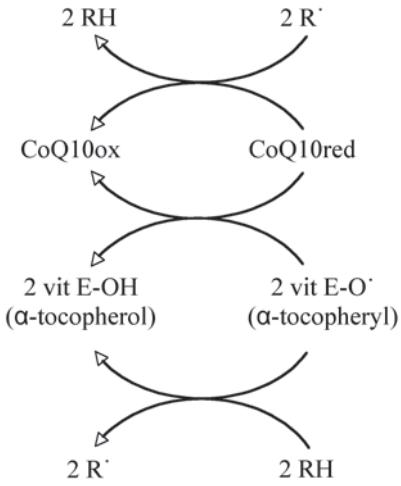


CoQ10 originates both from endogenous synthesis and dietary intake (6,7,8). While plasma levels are significantly influenced by dietary uptake, tissue levels of CoQ10 depend mainly on de novo synthesis (9). CoQ10red acts as a potent antioxidant in mitochondria and lipid membranes by scavenging free radicals, thus efficiently protecting membrane phospholipids and serum LDL from free radical-induced oxidative damage (2,3,6). CoQ10red also recycles oxidized α-tocopheryl back to α-tocopherol (vitamin E; figure 2), thus potentiating its anti-oxidative effect (10,11). In addition CoQ10ox acts as an essential electron carrier in the mito-chondrial ATP synthesis of all body cells (6).

Cystic fibrosis (CF) is an autosomal recessive inherited disease predominantly characterized by chronic pulmonary inflammation and infection in combination with maldigestion and malabsorption due to pancreatic insufficiency (12). These symptoms are the result of a defective Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)-protein, which has an essential role in chloride transport (13). In the lungs the defective CFTR-protein leads to poor hydration of the airway surface lining fluid resulting in thick mucus, defective ciliary clearance and airway plugging, which combination promotes recurrent bacterial infection and chronic inflammation, and thereby induces oxidative stress. The surplus of free oxygen radicals destroys the surrounding lung tissue and contributes to the progressive deterioration of pulmonary function (14,15,16). These reactive oxygen species combined with the impaired absorption of fat soluble antioxidant nutrients, such as α-tocopherol and β-carotene, result in an oxidant-antioxidant imbalance (17,18,19).

We hypothesized that the equilibrium between oxidized CoQ10 and reduced CoQ10 in cystic fibrosis patients might be disturbed. In addition, we assumed that the fat malabsorption characteristic for cystic fibrosis may negatively influence the intestinal uptake of CoQ10, resulting in reduced plasma levels.

Figuur 2. The CoQ10ox (ubiquinone) – CoQ10red (ubiquinol) redox couple and the antioxidant function of CoQ10red. CoQ10red as well as α-tocopherol scavenge free radicals ($R^{\bullet}$) directly. CoQ10red also regenerates α-tocopherol.



Materials and methods

Subjects

Pediatric patients aged 8-18 years-old were enrolled in the study. CF patients in clinical stable condition were recruited from the CF centre of the Wilhelmina Children's Hospital. The control patients consisted of children from the pediatric gastroenterology outpatient clinic as well as children who underwent ENT (ear, nose and throat) or orthopedic surgery. Exclusion criteria were clinical signs of infection and diseases associated with oxidative stress, e.g. cardiac, metabolic, cerebral, neuromuscular or mitochondrial diseases. All CF patients routinely received α-tocopherol supplementation (mean ± SD 120 ± 75 mg). α-tocopherol levels are monitored annually to ascertain the necessary amount of supplementation. This study was approved by the UMC Medical Ethics Committee, and informed consent was obtained from all patients and/or their parents.

Sample collection

Peripheral blood samples were collected in venoject 2 ml heparinized tubes (cholesterol) and 7 ml EDTA tubes (CoQ10 and α-tocopherol). The latter were immediately protected from light and briefly stored on ice and subsequently centrifuged for 10 minutes at 2000 x *g* at 4° C. Plasma specimens were transferred to prelabelled capped polypropylene tubes and stored at -80° C until analysis.

Laboratory assays

Total serum cholesterol was measured by routine clinical dry chemistry and colorimetric methods using cholesterol ester hydrolase and cholesterol oxidase (Dry Chemistry Vitros 950, OrthosClinical Diagnostics).

Prior to extraction and analysis for CoQ10red, CoQ10ox and α-tocopherol concentrations, the frozen plasma samples were thawed in a water bath of ambient temperature. All sample handling was carried out under subdued light to avoid photochemical decomposition. Subsequently 1 ml plasma was transferred into a glass tube and 100 μl of a glutathione solution was added to a final concentration of 0.01 M. This mixture was slowly added to 2 ml of 2-propanol containing Q9 (1.5 mg/L) under continuous agitation, placed for 15 minutes at approximately 4° C, and centrifuged at 2000 x *g* for 15 min at 4° C. 750 μl of the supernatant was injected directly into a HPLC system (quaternary Waters 2690 pump with automated injector and two reversed-phase suplex pkb100 columns (250 x 4.6 mm, 5 μm particle size), preceded by a short precolumn (suplex pkb100, 20 x 4.0 mm, 5 μm particle size); Supelco, Bellefonte, USA). Detection was performed by a Photodiode Array detector (Waters, Milford, USA). Column oven and sample tray temperature were 20° C and 4° C respectively. The system was controlled by Millennium software version 3.05 (Waters, Milford, USA). The mobile phase consisted of ammoniumacetate (7.5 g/L) in methanol (A) / acetonitril (B) / 2-propanol (C) / 50% acetonitril, 50% water (D) with a linear gradient. The flow rate was 1.5 ml/min and run time was 60 minutes.

Quantification of metabolite concentrations

CoQ10red and α-tocopherol were measured simultaneously by high performance liquid chromatography with diode-array detection at 292 nm, while CoQ10ox and Q9 were measured at 275 nm. Concentrations of CoQ10ox and CoQ10red were calculated based on peak areas using internal standardization with Q9. Total CoQ10 was calculated as the sum of CoQ10ox and CoQ10red. CoQ10 is not commercially available as calibrator, and therefore a relative response factor (RRF) for CoQ10red (at 292 nm) compared to CoQ10ox (at 275 nm) was determined. The RRF was established in threefold by reducing a known amount of CoQ10ox using sodium tetrahydroborate, and comparing the response area of the formed CoQ10red (at 292 nm) with the response area of the original solution containing only CoQ10ox (at 275 nm). The RRF was 4.0 ± 0.4 (mean ± sd). α-Tocopherol was quantified by

external standardization. The results for CoQ10 were expressed as molar concentrations (μmol/l). Since lipophilic antioxidants like CoQ10 are carried by circulating lipoproteins in plasma, CoQ10 was also expressed as μmol/mol cholesterol. The CoQ10 redox status was expressed as percentage reduced CoQ10 of total CoQ10.
CoQ10 plasma levels, CoQ10red/CoQ10 ratio (redox status), cholesterol, as well as anthropometric parameters (height, weight, and body mass index (BMI)), were measured or calculated in both patients and controls. In the CF group data for the most recent FEV_1% predicted (forced expiratory volume in 1 second as percentage of predicted) and the fat resorption quotient were also collected.

Statistical analysis
Results are presented as means ± SD. Data were entered in a SPSS database and the differences were tested for significance using an independent Student t-test. The Spearman test was used for calculating the correlation between the CoQ10 levels and FEV_1, plasma cholesterol and α-tocopherol levels, and fat resorption quotient.

Results

Subjects
Thirty pre-adolescent and adolescent children with stable CF and thirty age-matched clinically healthy control subjects were studied. There was no significant difference in age or gender distribution between the CF children and the controls. Although a considerable difference in standard deviation for height ($P<0.01$) was seen, the standard deviation for weight-for-height and BMI were not different (p=0.42 and 0.57 respectively). The patient characteristics are summarized in table 1. Anthropometric data are shown as standard deviation (SD) scores, according to the Dutch growth charts, generated by the 4th Nationwide Dutch Growth Study 1997 (20).

Plasma levels of CoQ10ox and CoQ10red and cholesterol
Total CoQ10 levels in cystic fibrosis patients were significantly lower than in the control group (0.87 ± 0.42 μmol/l versus 1.35 ± 0.39 μmol/l; $P<0.001$). No significant difference was found in the redox status (CoQ10red to total CoQ10 ratio). The cholesterol levels were significantly lower in the CF group ($P<0.001$), as well as the ratio of CoQ10 to cholesterol ($P< 0.05$). α-tocopherol values were similar in both groups, respectively 18.4 ± 6.9 and 21.0 ± 3.3 μmol/l for CF patients and the control group. Values are presented in table 2.
CoQ10 levels between males and females within the study group and the control group were not significantly different. Likewise, between two age groups (7-11 years and 12-18 years), no

significant difference in CoQ10 level was found. CoQ10 plasma levels did not correlate with the severity of the cystic fibrosis when categorized into mild CF (FEV_1% pred. ≥ 80%) and moderate CF (FEV_1% pred. < 80%) (21). In addition the Spearman test showed some correlation between CoQ10 and α-tocopherol (r = 0.4, p = 0.02), but no correlation between either CoQ10 and FEV_1% pred. (r = 0.2, p = 0.2), or CoQ10 and the fat resorption quotient (r = 0.1, p = 0.6).

Table 1. Characteristics of CF patients and controls.

	Cystic Fibrosis (n = 30)	**Controls (n = 30)**
Gender (male/female)	16/14	13/17
Age (yrs)	11.9 ± 2.6	11.3 ± 2.9
Height (in SD)	- 0.99 ± 0.97*	- 0.17 ± 0.97
Weight-for-height (in SD)	- 0.29 ± 0.94	- 0.09 ± 0.95
BMI (kg/m2)	17.1 ± 2.6	17.4 ± 1.6
FEV_1 % predicted	88.5 ± 18.7	not performed
Fat Resorption Quotient (%)	89 ± 7	not performed

Anthropometric data are shown as standard deviation (SD) scores, according to the Dutch growth charts, generated by the 4th Nationwide Dutch Growth Study 1997 (24).
Values are expressed as means ± SD. * p<0.01, independent Student t-test.

Table 2. Plasma values for total CoQ10, CoQ10 redox status (percentage reduced CoQ10 of total CoQ10), cholesterol and CoQ10/cholesterol ratio in CF patients and healthy controls.

	CF	**Controls**
Total CoQ10 (μmol/l)	0.87 ± 0.42†	1.35 ± 0.39
Reduced CoQ10 (μmol/l)	0.76 ± 0.38	1.16 ± 0.35
CoQ10 redox status in %	86.4 ± 7.1	85.4 ± 7.3
Cholesterol (mmol/l)	3.2 ± 0.5 †	4.1 ± 0.5
CoQ10/chol ratio (μmol/mol)	268.8 ± 136.7*	334.0 ± 102.9
α-tocopherol (μmol/l)	18.4 ± 6.9	21.0 ± 3.3

Values are presented as means ± SD. * p < 0.05; † p < 0.001, independent Student t-test.

Discussion

This study clearly shows reduced CoQ10 plasma levels in children with CF, even when corrected for the lower serum cholesterol levels. Interestingly, in our patients with CF the percentage reduced Q10 and oxidized Q10 was not different from controls. This equilibrium is disturbed in other diseases associated with increased oxidative stress, such as coronary heart disease (22), hyperlipidemia (23), neurodegenerative disorders (24,25) and liver diseases (26). Evidently, in our group of CF patients with mild-to-moderate pulmonary disease (mean FEV_1 % predicted 88.5 ± 18.7) reduced CoQ10 could still be adequately regenerated by NADH and NADPH (27).

Malabsorption of lipid soluble vitamins, including the antioxidants beta-carotene and α-tocopherol, is well known in CF, and routine supplementation is therefore advised (28,29). As CoQ10 plasma concentration is also mainly dependent on dietary uptake (8), we suppose that the low concentration we found here is primarily due to the fat malabsorption characteristic for CF patients. Obviously these low plasma Q10 levels could be boosted with adequate supplementation (8,30). Nevertheless, we wonder whether this patient group will benefit, as the CoQ10 redox status was not disturbed.
In summary, we found significantly lower levels of CoQ10 in pediatric patients with cystic fibrosis as compared to age-matched controls, but a normal redox status in these patients with mild-to-moderate pulmonary disease.

Acknowledgements
The authors wish to thank their colleagues of the Department of Metabolic and Endocrine Disorders for their technical support.

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CHAPTER 3.2

Dietary supplementation with a multiple micronutrient mixture: no beneficial effects in pediatric cystic fibrosis

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Submitted to J of Cystic Fibrosis

Abstract

Background: To determine the effect of a multiple micronutrients mixture on lung function, and muscle performance in pediatric cystic fibrosis patients.

Methods: A double-blind, randomized, placebo controlled, cross-over trial was performed in 22 cystic fibrosis patients aged 12.9 ± 2.5 years old with predominantly mild lung disease (predicted forced expiratory volume in 1 second 87.2 ± 17.0 %). Each patient received the micronutrient mixture (ML1) or placebo in random order for three months with a wash-out period of three months. Anthropometric measures, pulmonary function, exercise performance by bicycle ergometry and muscular strength were determined.

Results: Analysis was performed using the paired Student t-test comparing the change in each parameter during ML1 and placebo. No significant difference between the effect of the ML1 and placebo was obtained for any of the parameters studied, although for almost all of the variables studied, the placebo slightly outperformed ML1. Plasma vitamin E and A levels increased during ML1 (16.9 ± 11.6 and 0.09 ± 0.17 µmol/l versus -2.3 ± 7.9 and -0.06 ± 0.23 µmol/l; $P<0.001$ and $P=0.06$, respectively) compared with placebo.

Conclusions: The micronutrient mixture was not superior to placebo with respect to changes in pulmonary function or muscle performance in pediatric CF patients, despite a significant increase in plasma vitamin E concentrations.

Introduction

Cystic fibrosis (CF) is an autosomal recessive inherited disorder primarily characterized by chronic pulmonary infection and inflammation (1,2). During active infection neutrophils and macrophages generate reactive oxygen species (ROS), which are necessary for bacterial killing, but may be harmful when tissue antioxidant capacity is exceeded. Despite routine supplementation with the fat soluble vitamins, such as the antioxidants vitamin E and carotenoids, biochemical markers for a disturbed oxidant-antioxidant balance are indeed present in many CF patients (4-7). A diminished intake of water-soluble antioxidants from fruits and vegetables, such as vitamin C, due to the emphasis on increased fat intake, might contribute to this problem. Therefore, several supplementation studies have been performed in CF patients, using various amounts of α-tocopherol, retinol, ascorbic acid, and carotenoids. These studies have shown beneficial effects on oxidant-antioxidant imbalance, but an inconsistent effect has been reported for pulmonary function (8-12).
Physical exercise programs can improve exercise tolerance and pulmonary function in CF patients, mainly as a result of increased muscle mass (13,14). However, it turned out to be extremely difficult for the participants in these studies to maintain the increased exercise training, and just a few still complied after one year. A nutritional supplement might therefore be a good alternative to optimize muscle strength. As positive effects of creatine, carnitine and taurine on muscle mass and/or performance in athletes have been described (15-17), an increased intake of these components might also be associated with an improved exercise tolerance in CF patients.
Since CF is characterized by both oxidative stress and poor exercise tolerance, we hypothesized that children with CF could benefit from a mixture of micronutrients with antioxidant or muscle fortifying action, as well as water soluble vitamins involved in either mitochondrial function or red blood cell synthesis (18).

Methods

Subjects and study design

Thirteen boys and sixteen girls with CF aged 9.8-18.9 years (mean 13.3 yrs) with predominantly mild pulmonary disease were recruited from the CF centre of the Wilhelmina Children's Hospital, University Medical Centre Utrecht. Exclusion criteria were pulmonary exacerbation and musculoskeletal disorders. The study protocol was approved by the UMC Medical Ethics Committee, and each participant and/or the parents gave informed consent. In a cross-over design each participant received 100 ml of a liquid micronutrient mixture (ML1) for 3 months, or 100 ml placebo with a wash-out period of 3 months. The composition of ML1 is described in table 1.

Table 1. Composition of the micronutrient mixture ML1

		100 ml			100 ml
Energy value	kcal	80	protein	g	6.0
	kJ	335	(casein:whey=1:1)	En%	30
			Carbohydrates	g	7.1
			(lactose, saccharose,	En%	35
			organic acids)		
			Fat (linoleic acid 0.8)	g	3.1
				En%	35
Minerals:			**Vitamins:**		
Na	mg	43	A	µg RE	267
K	mg	300	D	µg	2.0
Cl	mg	81	C (ascorbic acid)	mg	100
Ca	mg	126	E (α-tocopherol)	mg	215
P	mg	73	B1 (thiamine)	mg	10
Mg	mg	8	B2 (riboflavin)	mg	1.2
			B3 (pantothenate)	mg	1.6
			B5 (niacin)	mg NE	8.0
			B6 (pyridoxine)	mg	2.4
			B11 (folate acid)	µg	240
			B12 (cobalamin)	µg	1.2
			Biotin (Vit H)	µg	40
Trace elements:			**Others:**		
Fe	mg	0.4	Carnitine	mg	1200
Zn	mg	6.0	Choline	mg	40
Cu	mg	0.6	Creatine	mg	1200
Mn	mg	1.2	Taurine	mg	1200
F	mg	0.4	Coenzyme Q10	mg	60
Mo	µg	20			
Se	µg	20			
Cr	µg	13			
I	µg	40			

RE;Retinol Equivalent
NE; Niacine Equivalents

Table 2. Clinical characteristics of the 22 subjects, who completed the study.

	Subjects (n=22)
Male : Female	10 : 12
Age (years)	12.9 ± 2.5
Height (m)	1.51 ± 0.12
(in SDS)	(-0.9 ± 1.0)
Weight (kg)	42.2 ± 12.6
(W-for-H in SDS)	(-0.01 ± 1.0)
BMI (kg/m^2)	18.0 ± 3.1
FFM (kg)	28.8 ± 7.8
Skinfolds (sum of 4; in mm)	34.6 ± 18.4
FEV_1 (ml)	2130 ± 584
FEV_1 (% predicted)	87.2 ± 17.0
FVC (ml)	2745 ± 680

W-for-H; Weight for Height, BMI; body mass index, FFM; fat free mass calculated from four standard skinfold measurements, FEV_1; forced expiratory volume in one second, FVC; forced vital capacity

The placebo tasted the same, contained identical amounts of protein, carbohydrates and fats, but lacked the micronutrients. Four female and two male participants dropped out of the study during the first few weeks because of unpleasant taste of the mixture. One boy did not complete the entire study due to the emotional impact of a non-study CF related complication. One participant could not perform the bicycle tests at the measurements after both ML1 and placebo, first because of a soccer injury and secondly because of a broken leg.
Because of small stature two participants performed a standardized treadmill test (endurance) instead of the bicycle tests (19). Finally, 22 patients, with a mean age of 12.9 ± 2.5 years, were available for analysis. The clinical characteristics of the 22 remaining subjects who completed the study are described in table 2.

Nutritional assessment

Anthropometric measurements were acquired prior to exercise testing. Bodyweight was measured using a platform beam balance (Mettler, Greifensee, Switzerland). Height was measured with a stadiometer (Holtain, Crymich, UK). Body mass index (BMI) was calculated as weight/height^2. Body composition, i.e. fat-free mass (FFM), was determined by calculation from the sum of four standard skinfold-thickness measurements (20-22).

Pulmonary function tests

Pulmonary function tests were performed after administration of 800 μg of salbutamol, in order to rule out important bronchial hyperreactivity. Forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV_1) were obtained from maximal expiratory flow-volume curves (Masterscreen, Jaeger/Viasys, Hochberg, Germany). Values are expressed as percent of predicted (pred) values as compared to normal values for age and gender (23).

Peripheral Muscle Strength

Isometric muscle force was measured for six muscle groups on the non-dominant side of the patient, using a hand-held dynamometer (Penny and Giles, Christchurch, UK). Maximal voluntary force of the shoulder abductors, elbow flexors, hip extensors, and knee extensors were measured as described by Backman et al (24). Each muscle group was tested three times, and the highest value obtained was reported.

Exercise testing

Subsequent anaerobic and aerobic exercise tests were performed on an electronically controlled resistance cycle ergometer (Lode Examiner, Lode, Groningen, The Netherlands) as described by Klijn et al (25,26). The Wingate test (WAnT) was used to assess short-term anaerobic power. The WAnT comprises four different parameters: peak power in 30 s (Ppeak), mean power in 30 sec (Pmean), Ppeak per kg FFM, and Pmean per kg FFM. The subjects rested for at least 45 minutes before aerobic fitness was assessed by a standard progressive incremental exercise test (Wmax). All subjects were familiar with the test equipment. Verbal encouragement was given throughout the tests to stimulate maximal performance.

Laboratory assays

Malondialdehyde

In the fasting state, prior to exercise testing, a catheter was inserted in a deep antecubital vein and a venous blood sample was drawn and collected in a heparinized, plastic tube and subsequently processed. Plasma was extracted by centrifugation at 3000 rpm (1500 x *g*) at room temperature, and stored at -20 °C until analysis. Malondialdehyde (MDA) levels were determined fluorimetrically (Fluostar Galaxy, BMG, Germany) by using the TBARS (Thio Barbituric Acid Reactive Substances) method. Plasma samples (0.2 ml) were mixed with 2.0 ml of 0.2% thiobarbaturic acid (TBA) in a 0.1M sodium acetate buffer (pH=3,5) in the presence of buthylated hydroxytoluene to prevent lipid oxidation during the assay. Subsequently the samples were incubated for 60 minutes at 100°C, followed by cooling to room temperature. To the reaction mixture 3.05 ml of 0.6% HCl in butanol was added, vortexed and then cooled to 8°C, followed by centrifugation for 10 minutes at 3000 rpm (1500 x *g*). The fluorescence of the supernatant was then measured at 520nm excitation/550nm emission. The Fluostar created

the calibration line by measuring 7 different inserted calibration samples and hence calculated the MDA concentration of the patient samples.

Vitamin E (α-tocopherol) and A (retinol)
Serum concentrations of α-tocopherol and retinol were determined simultaneously by reversed phase HPLC. After denaturation of serum proteins with ethanol, the fat-soluble vitamins were extracted with hexane, reconstituted in ethanol in dark colored safe-lock tubes and separated and quantified using a reverse-phase column (Supelco, 25 cm x 4.6 mm ID, Supelcosil ™ LC-18, 5 µm, Sigma Aldrich, The Netherlands) and UV detection (separations Model 785A, The Netherlands). Tocopherol-α-acetate was added before the extraction step as an internal standard. Calibration curves were prepared with retinol (Sigma art. no R-7632), α-tocopherol (Sigma art. no T-3251) and Tocopherol-α-acetate (Sigma art. no T-3251) standards. Control sera were obtained from the National Quality Institute for Laboratories (SKML) and were analyzed with each batch of test samples to monitor the reproducibility of the method. Absorptions were measured at three different wavelength (325, 292, 284 nm) for the calculation of the three components. This technique is a standard method in the laboratory of clinical chemistry in our hospital.

Data analysis
Data were analyzed using the Statistical Package for the Social Science (SPSS, version 11.0 for Windows, Chicago, IL). Since a cross-over design was used, a simple paired Student t-test was used. Data are presented as mean ± SD.

Results

The clinical characteristics of the 22 children with CF who completed the study are presented in Table 2. All patients were adolescents with an FEV_1 pred of 87.2 ± 17 % (mean ± SD).
At the end of the intervention period the plasma level of vitamin E had increased significantly (16.9 ± 11.6 versus -2.3 ± 7.9 µmol/l; $P<0.001$) compared with placebo. For vitamin A a higher, though not significant, plasma level was found after the intervention period (0.09 ± 0.17 versus -0.06 ± 0.23 µmol/l; $P=0.06$).
However, for all other parameters no significant difference was found. The change in nutritional parameters, BMI, FFM and skinfolds, was essentially identical in the intervention and the placebo period (Table 3). Also no significant differences were seen in the change of any of the pulmonary function tests under ML1 and placebo. Similar results were seen for peripheral muscle strength, aerobic and anaerobic muscle performance. However, for the pulmonary function tests FEV_1, FVC, as well as for Wmax, VO_2max and the aerobic and anaerobic Ppeak of

WAnT a trend towards significance was found for the placebo. Similarly we found that plasma MDA concentrations decreased during placebo (-0.263 µmol/l ± 0.66 µmol/l), but marginally increased during ML1 (0.023 µmol/l ± 0.61 µmol/l) (P=0.15). Neither mixture nor placebo showed a seasonal effect, nor was the order in which intervention and placebo were given of any effect (no carry-over effect). During the intervention period six pulmonary infections were reported (one intravenous), as opposed to eleven pulmonary infections in the placebo period (four intravenous). This difference was not significant.

Table 3. Changes in nutritional assessment, pulmonary function, peripheral muscle strength, and exercise testing parameters during the micronutrient mixture (ML1) and placebo.

	Change of parameter during ML1	Change of parameter during placebo	P-value
BMI (kg/m²)	0.3 ± 0.6	0.2 ± 0.9	0.46
Sum of 4 skinfolds (mm)	1.6 ± 4.6	2.8 ± 9.8	0.65
FFM calculated from skinfolds (kg)	0.9 ± 1.0	0.6 ± 1.3	0.42
FEV1 (ml)	-76 ± 244	52 ± 192	0.11
FEV1pred (%)	-5.7 ± 11.1	-0.1 ± 9.1	0.15
FVC (ml)	-62 ± 242	35 ± 203	0.14
Muscular strength; sum of all muscles (nm)	-10 ± 135	-46 ± 135	0.41
Ppeak (Watt)	52 ± 100	104 ± 160	0.16
Pmean (Watt)	32.6 ± 49.6	50.3 ± 109.3	0.29
Anaerobic power/kg bodyweight (Watt/kg)	0.8 ± 2.3	1.4 ± 2.4	0.38
Anaerobic power/FFM (Watt/kg LBM)	1.0 ± 2.8	2.3 ± 3.7	0.13
Anaerobic capacity/kg bodyweight (Watt/kg)	0.5 ± 1.0 (0.2)	0.7 ± 1.4	0.42
Anaerobic capacity/kg FFM (Watt/kg LBM)	0.6 ± 1.1	1.1 ± 2.4	0.25
Wmax (Watt)	-3.2 ± 13.8	4.2 ± 17.7	0.19
$\dot{V}O_2$ max (ml/kg/min)	-1.5 ± 3.5	0.3 ± 3.9	0.10
Parameters are given as mean ± SD			

BMI; body mass index, FFM; fat free mass calculated from four standard skinfold measurements, FEV_1; forced expiratory volume in one second, FVC; forced vital capacity
Ppeak; peak power measured by WanT, Pmean; mean power measured by WAnT,
Anpow/kg; (anaerobic) muscle power per kg bodyweight (WAnT), Wmax; cycling maximal workload, $\dot{V}O_2$max; maximal oxygen consumption during bicycle ergometry

Discussion

In this study a mixture of multiple micronutrients, with either muscle fortifying or antioxidant action, showed no beneficial effects on either pulmonary function or muscle performance in CF patients as compared to placebo.

Isometric muscle strength, anaerobic power (WAnT) and endurance (Wmax, $\dot{V}O_2$max) were similar in the micronutrient and placebo group. A similar lack of effect has been reported for triathlon athletes receiving antioxidant supplementation (27). Creatine, however, seems to improve muscular performance, but most effectively in combination with high-intensive training (28,29). Also much higher doses are used (initial loading dose of 20 g/d for 7 d, and a maintenance dose of 3-5 g/d versus 1.2 g/d, respectively) (28). An open-label pilot study in 18 adolescent CF patients showed a significant increase in maximal isometric muscle strength, but no change in lung function after creatine supplementation (30). In this study also higher doses as compared to our study were used (initial loading dosage 12 g/d first 7 d, maintenance dose 6 g/d for 11 weeks). Taurine has been described as an effective free radical scavenger and has been reported to reduce oxidative damage (31,32). A positive correlation between change in taurine concentration and changes in exercise time to exhaustion and maximal workload (Wmax) was reported in healthy young men after taurine supplementation (17). Unfortunately, this intervention study lacked a control group, so taurine might not be as effective as suggested. Supplementation of L-carnitine has been reported to normalize decreased levels induced by high-intensity physical exercise (33,34). So carnitine supplementation may enhance performance in athletes, but healthy, essentially sedentary persons and untrained individuals might not benefit from supplementation.

We did not find any significant changes in pulmonary function. A similar study showed comparable results after giving an antioxidant mixture for two months (12). In this study and ours, interestingly, a non-significant trend towards the opposite was seen, as the decline in FEV_1 and FVC in the intervention group was higher. Moreover, in our study plasma MDA concentration rose in the micronutrient mixture group, and declined in the placebo group, suggesting a shift to a pro-oxidative state when giving the micronutrient combination. Negative effects of antioxidant supplementation, especially vitamin E, have been described by others, and may play a role in our study (18,35-38). Although Wood et al (12). did describe a correlation between an increase in β-carotene plasma levels and an improvement in FVC, as well as a correlation between an increase in plasma selenium concentrations and an increase in FEV_1, this does not imply that exogenous administration of these substances does have the desired beneficial effect on pulmonary function. In our study too an increase in plasma vitamin E correlated with increase in pulmonary function (P=0.04, results not shown). Both our investigations and the study by Wood et al (12). showed a trend towards a negative effect on pulmonary function of a micronutrient mixture consisting either partially or totally of antioxidants. Although it has been thoroughly documented that low levels of antioxidant

molecules such as vitamin E and β-carotene can be normalized in CF patients (8-10), so far no hard evidence has been brought forward that boosting plasma levels of these antioxidants reduces pulmonary deterioration. A study by Renner et al (11). showed that high dose β-carotene supplementation does normalize plasma MDA levels. However no effect on FEV_1 was seen, although the number of days on antibiotics was less in the intervention group.
In conclusion, in the current study we found no effects of a micronutrient mixture on either muscle performance or pulmonary function. Moreover, we here present some evidence that a combination of anti-oxidants might have untoward effects on FEV_1 and FVC, perhaps through disturbing the oxidant-antioxidant balance. Therefore, we believe that no further studies concerning combination supplements are warranted. Nevertheless, careful designed interventions with antioxidants that normalize parameters of oxidative stress, such as β-carotene or vitamin E, might have a place.

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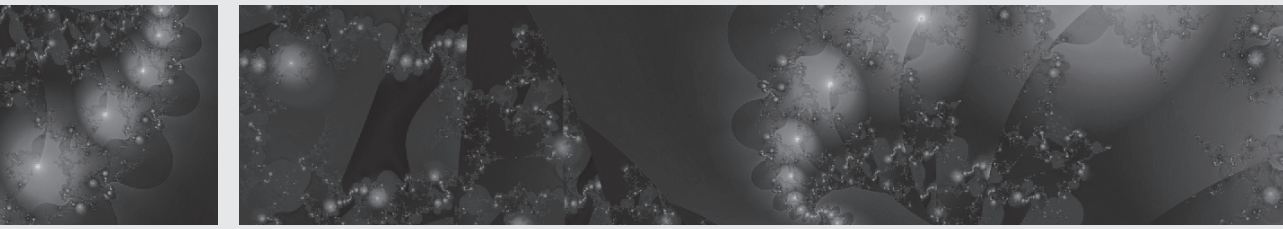

PART 4

Muscle performance and micronutrients in CF: animal model studies

CHAPTER 4.1

Abnormal mechanical and energetic properties of skeletal muscle in a mouse model of cystic fibrosis

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Submitted to American Journal of Respiratory and Critical Care Medicine

Abstract

Rationale: Patients with cystic fibrosis (CF) frequently present with exercise intolerance. The pathophysiology behind this phenomenon is unclear. Evidence has been mounting for an intrinsic abnormality at the level of skeletal muscle itself.

Objectives: To investigate whether skeletal muscle abnormalities exist in a transgenic cystic fibrosis mouse model.

Methods: Twitch contraction mechanics and oxygen consumption of intact extensor digitorum longus (EDL) and soleus muscles isolated from CF and wild type (WT) control mice were simultaneously recorded at various frequencies of electrical stimulation. Mitochondrial enzyme activities were determined in homogenate.

Measurements and main results: Body weight and muscle mass were significantly lower in CF, but isometric twitch force per g muscle was significantly higher in CF compared to WT. During serial stimulation at 2 Hz versus 1 Hz CF EDL muscles were unable to fractionally increase mechanical output (0.04 ± 0.12 versus 0.39 ± 0.04 in WT) and oxygen consumption (0.03 ± 0.07 versus 0.61 ± 0.02 in WT). Oxygen cost per twitch was similar for WT and CF EDL muscles. Qualitative and quantitative differences between the detailed time courses of twitch contraction amplitude and duration at 2 Hz for CF and WT EDL muscles suggested energetic limitation of mechanical performance in CF. Mitochondrial enzyme assays revealed unaltered mitochondrial content of CF EDL muscle.

Conclusions: The results seem to indicate that an apparent, unexplained increased gain of excitation-contraction coupling concomitant with unchanged mitochondrial density in skeletal muscle in CF result in a relatively reduced capacity for ATP free energy homeostasis associated with diminished muscle performance in CF.

Introduction

Cystic fibrosis (CF) is the most common lethal autosomal recessive inherited disease in Caucasians with an incidence of approximately 1 in 2000 to 3000 live births (1). CF is caused by mutations in a single gene on chromosome 7, which encodes for the cystic fibrosis transmembrane conductance regulator (CFTR) protein. The most common gene mutation is the ΔF508 mutation (2). The CFTR protein is a membrane bound chloride channel, which has been extensively studied in epithelial secretory cells lining the respiratory, gastrointestinal, hepatobiliary, pancreatic, and reproductive tracts as well as the sweat glands.

Expression of the CFTR protein has also been found in muscle tissues, e.g. cardiac (5,6) and smooth muscle (7,8) but is unclear whether CFTR is present in skeletal muscle. However, altered skeletal muscle performance and exercise intolerance has been documented in CF patients (9). At first this has been attributed to diminished nutritional status and decreased oxygen delivery due to restricted pulmonary function (10-12). However, evidence for a defect in skeletal muscle itself was found, when a significantly reduced capacity for ATP free energy homeostasis during contractile work was demonstrated in children and adolescents with cystic fibrosis by means of phosphorus-31 nuclear magnetic resonance spectroscopic measurements of adenosine tri-phosphate (ATP) metabolism in exercising forearm muscle (9). It remained unclear whether this altered ATP balance was caused by a lower mitochondrial capacity for ATP synthesis (i.e., ATP supply in energy balance) or a decreased energetic efficiency of muscle contraction (i.e., ATP demand) (10). In a follow-up study, the same group showed that children with CF have peripheral muscle weakness in addition to reduced muscle mass concomitant with reduced energetic efficiency of exercise and reduced maximal exercise performance (13). This hypothesis of a primary defect in skeletal muscle in CF was challenged by Moser and coworkers, but they too concluded that an intrinsic muscle defect must play a role in the diminished exercise performance in CF. (14) Importantly, their results also pointed towards abnormalities in oxygen metabolism in CF.

Evidence for altered capacity of oxidative metabolism in CF disease, specifically abnormalities in mitochondria, has been found in skin fibroblasts of CF patients suggesting an anomaly in the mitochondrial NADH dehydrogenase complex (respiratory chain enzyme complex 1) system (15). Similarly, studies in fibroblasts and leucocytes reported mitochondrial abnormalities as increased calcium content and lower NADH dehydrogenase activity compared to controls (16,17). However, involvement of mitochondria in the exercise intolerance in CF patients has yet to be established. We hypothesized, that the diminished exercise performance might be due to an intrinsic abnormality in oxidative metabolism in muscle. We investigated this problem in a transgenic mouse model of CF disease.

Materials and methods

Contractile function and energetic efficiency of isolated, superfused fast-twitch (extensor digitorum longus, EDL) and slow-twitch (soleus, SOL) muscles of male nine week-old transgenic CFTR-delta F508 (C57BL/6 DF508; CF) mice and sex- and age-matched wild type (C57BL/6; WT) controls (both JAX®mice; Mouse Genome Database, The Jackson Laboratory, Bar Harbor, Maine, USA) were studied.
All experimental procedures were approved by the Committee on Animal Experiments of the University Medical Center Utrecht and complied with the principles of good laboratory animal care and use.

Simultaneous recording of muscle oxygen consumption and force development
Muscles were isolated, fixed, mounted and stimulated according to the method described by Jeneson et al (18) Oxygen consumption and twitch (i.e. single isometric) contraction mechanics were measured simultaneously (high-resolution oxygraph, Oroboros, Innsbruck, Austria; adjustable Harvard Apparatus 60-2995 force transducer, Harvard Instruments Limited, Edenbridge, UK; Grass S88 dual channel stimulator, Astro-med, West Warwick, RI, USA). After 30 minutes of equilibration (to avoid measurements at high chamber oxygen-leak) the measurement protocol started with 10 minutes of rest (to record basal respiratory flux) followed by 10 minutes of serial contraction stimulation at 0.5, 1.0 or 2.0 Hz for EDL, and at 1.5 or 3.0 Hz for SOL muscles. Respiratory fluxes were corrected for chamber oxygen leak by measuring the exponential decay of PO_2 in the oxygraph chamber containing Ringer solution according to Syme (19). Oxygen solubility of 5 % CO_2 – 95 % O_2 equilibrated Ringer medium was calculated according to Haller and oxygen electrode response times were constant at approximately 4 seconds (20). All measurements were performed as randomized paired-experiments with simultaneous measurement of one WT and one CF muscle in a dual-chamber setup.

Citrate Synthase and Respiratory Chain Enzyme Complex 1 assays
The activities of respiratory chain complexes in homogenate of four different EDL muscles of CF as well as WT mice were measured spectrophotometrically: Citrate synthase according to Alp (21) and respiratory chain enzyme Complex 1 according to Fischer (22). Proteins were determined by the method of Lowry (23). To eliminate the possible effect of an increased amount of connective tissue in CF muscle the enzyme activity was calculated per gram muscle as well as per microgram protein.

Data acquisition, analysis and statistics
Oxygraph and force transducer data-acquisition was performed with LabView software (National Instruments, Woerden, The Netherlands). Mechanical performance was analyzed

on a twitch-per-twitch basis with respect to five parameters: rise time, amplitude, area (tension-time integral), relaxation rate and half-width (in msec) using LabView programs. Changes in individual EDL twitch contraction parameters during serial contractions were quantified by scaling to values determined for each parameter in each muscle from serial contractions at 0.5 Hz; this particular duty cycle corresponds to a sub-maximal workload for EDL muscle at which no fatigue occurs over a period of 10 min (18). Absolute muscle respiratory rates and time constants were calculated using Origin 6.0 (Microcal Software Inc., Northampton, MA, U.S.A.). Reported data are presented as arithmetic means ± standard error (SE). Statistical analyses were performed using a Student's unpaired t-test (24). Differences between CF and WT muscle were considered significant if $P<0.05$.

Results

Specific twitch force of CF EDL and SOL muscles

CF mice had significantly lower body weights and hindlimb muscle mass (both EDL and SOL muscle) than WT controls (Table 1). However, specific isometric twitch force (i.e., isometric force (N) produced in a single twitch per g muscle) of intact EDL and SOL muscles isolated from CF mice was significantly higher (1.3 and 1.4 fold, respectively) than WT EDL and SOL muscles (Table 1).

Table 1. Baseline characteristics.

	CF (n=6)	WT (n=6)	p-value
Bodyweight (g)	19.6 ± 1.81	27.6 ± 1.51	0.01
EDL muscle mass (mg)	5.67 ± 0.64	9.25 ± 0.32	<0.01
EDL as % of bodyweight	0.029 %	0.034 %	
EDL twitch force (mN/g muscle)	7.50 ± 0.29	5.96 ± 0.42	0.01
SOL muscle mass (mg)	3.00 ± 0.68	7.43 ± 0.35	<0.01
SOL as % of bodyweight	0.015 %	0.027 %	
SOL twitch force (mN/g muscle)	4.76 ± 0.38	3.48 ± 0.21	0.01

Bodyweight , extensor digitorum longus (EDL) and soleus (SOL) muscle mass, and specific isometric twitch force of both muscles, were measured in cystic fibrosis (CF; n=6) and wild type (WT; n=6) mice. Values are presented as mean ± SE.

Figure 1a. Representative run of repeated isometric contractions (raw data) during 10 minutes stimulation at 0.5 Hz.

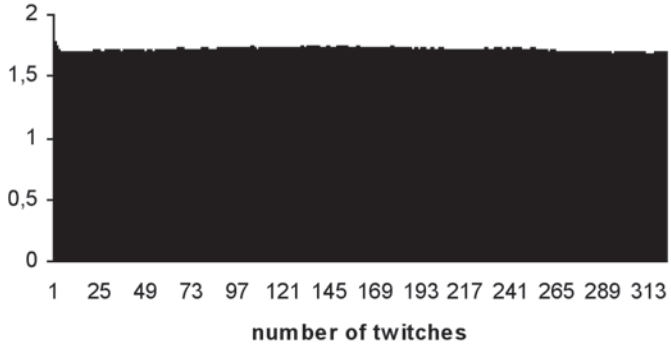


Figure 1b. Single isometric contraction (twitch) with display of amplitude (A; in Volts), tension-time-integral (TTI; area under the curve), and half-width (HW; parameter of twitch duration; in msec)

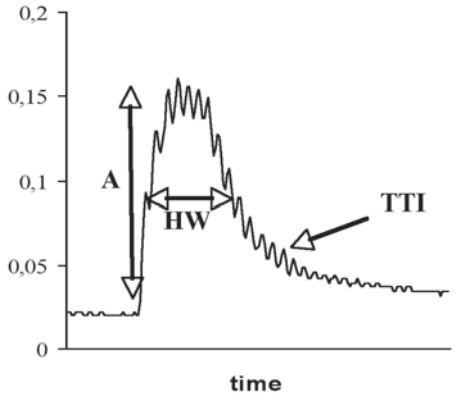


Figure 1c. Typical oxygen tracing (raw data) before, during and after 10 min stimulation at 0.5 Hz of isometric contractions.

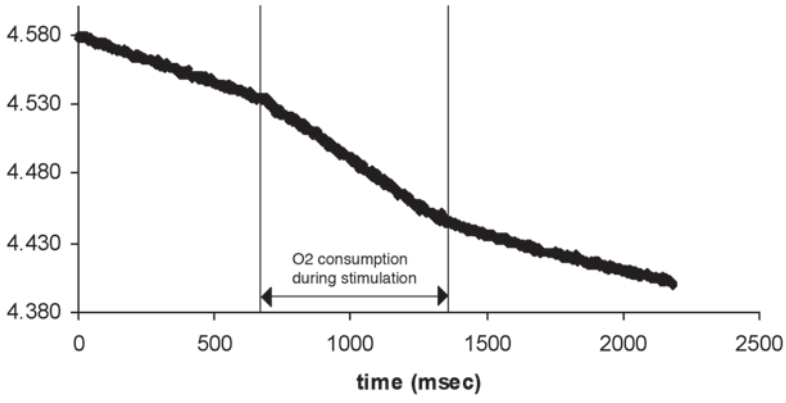


Figure 2a. Total isometric force (in kilonewton per gram extensor digitorum longus [EDL] muscle) generated during 10 minutes of contraction at three stimulation frequencies, e.g. 0.5, 1.0 and 2.0 Hz, in cystic fibrosis (CF; n= 6) and wild type (WT; n=5) mice. * p<0.05, † p=0.08

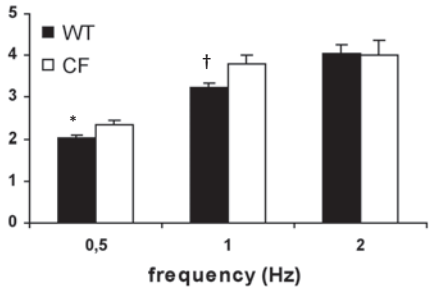
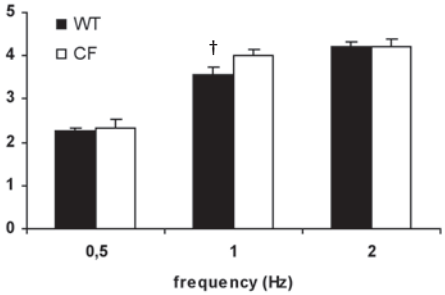


Figure 2b. Comparison of extra oxygen consumed above baseline (in micromol per gram extensor digitorum longus [EDL] muscle) during stimulation at three consecutive frequencies, e.g. 0.5, 1.0 and 2.0 Hz, in cystic fibrosis (CF; n= 6) and wild type (WT; n=5) mice. † p=0.08



EDL mechanics and oxygen consumption during serial twitch contractions

Figure 1 shows raw data of a typical serial stimulation experiment of a CF EDL muscle for 10 minutes at 0.5 Hz. Figure 1a shows a recording of the mechanical output during serial stimulation. One single twitch has been enlarged in figure 1b showing amplitude, half-width (in msec) and tension-time integral (area under the curve). The scaled half-width - the parameter used in this study to evaluate indirectly calcium handling – for this particular twitch contraction would correspond to its half-width divided by the average half-width of contractions 50-100 in Figure 1a (see Methods). A typical oxygen consumption tracing before, during and after serial contractions at 0.5 Hz is displayed in Figure 1 c. During 10 min of serial electrical stimulation at 0.5 Hz and 1.0 Hz there was a higher total tension-time integral produced per gram muscle in CF EDL muscles, which was not the case at 2.0 Hz ($P<0.05$, $P=0.08$ and $P>0.5$, respectively) (Fig. 2a).

Figure 2c. Fractional increase in total isometric force (displayed as tension time integral) produced per gram extensor digitorum longus (EDL) muscle per doubling of the duty cycle from 0.5 to 1.0 Hz, and from 1.0 to 2.0 Hz, during 10 minutes stimulation in cystic fibrosis (CF; n= 6) and wild type (WT; n=5) mice.

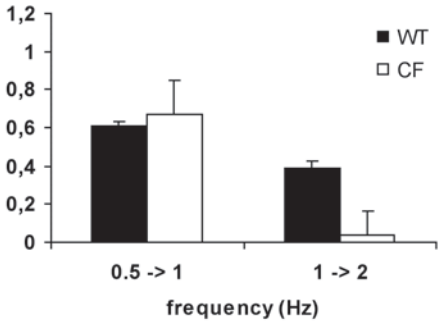
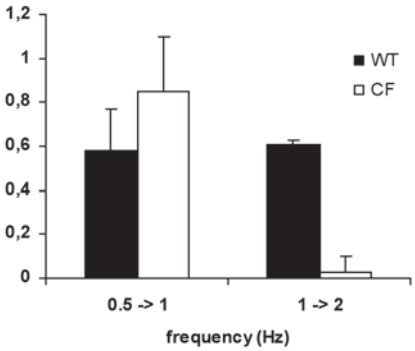


Figure 2d. Fractional increase in total extra oxygen consumed per gram extensor digitorum longus (EDL) muscle per doubling of the duty cycle from 0.5 to 1.0 HZ, and from 1.0 to 2.0 Hz, during 10 minutes stimulation in cystic fibrosis (CF; n= 6) and wild type (WT; n=5) mice.



Concomitant total extra oxygen consumed above basal rate was not different for CF and WT EDL muscles at 0.5 and 2.0 Hz (Fig. 2b). At 1.0 Hz, total extra oxygen consumed by CF muscles tended to be significantly higher than WT (P=0.08; Fig. 2b). Figures 2c and 2d show the fractional increase of total tension-time integral and concomitant total extra oxygen consumption per gram muscle per doubling of stimulation duty cycle over the 0.5-2 Hz range of frequencies studied. Doubling of the stimulation duty cycle from 0.5 to 1.0 Hz caused the same fractional increase of total tension-time integral (0.67 ± 0.18 versus 0.61 ± 0.02, respectively; Figure 1c) and concomitant fractional increase of oxygen consumption (0.85 ± 0.25 versus 0.58 ± 0.19, respectively; NS) (Fig. 2d) in CF and WT EDL muscle. However, subsequent doubling of the stimulation duty cycle from 1.0 to 2.0 Hz caused fractional increment of total tension-time integral and

concomitant extra oxygen consumption only in WT (0.39 ± 0.04 and 0.61 ± 0.02, respectively) but not in CF EDL muscles (0.04 ± 0.12 and 0.03 ± 0.07, respectively) (Figures 2c and 2d).
Total oxygen consumed per g muscle during 10 min serial contractions at 0.5, 1.0 and 2.0 Hz was linearly related to tension-time integral produced per g muscle in both CF and WT EDL (Fig. 3). No significant difference was found between the regressions for each genotype (Fig. 3).
The time course of mechanical performance of CF versus WT EDL muscles during stimulation at 2.0 Hz is shown in Figure 4a. The first 20 sec of contractions have been omitted for clarity of presentation. Normalized force of twitch contraction had attained 100% of its steady-state value (see Methods) for both CF and WT EDL muscles within that time period (Fig. 4a). During the remainder of the protocol, however, CF muscles lost both qualitatively and quantitatively more force than WT. Specifically, residual twitch force at the end of the protocol was 1.5-fold lower than WT (40 + 3% (n=5) versus 60 + 3 % (n=6), respectively; Fig. 4a). Qualitative differences were identified by computing the time derivative of normalized force for each genotype (Fig. 4a, inset). Both at the onset as well as towards the end of stimulation, the rate of fall of normalized twitch force – i.e. the rate of fatigue - was identical for CF and WT muscles (-0.28 and -0.08 %/s, respectively). However, whereas in WT muscles the high initial fatigue rate was rapidly controlled within 35 s after onset of stimulation, in CF muscles this high fatigue rate persisted; only after 110 s did it decline towards WT levels, but notably at a slower rate than WT (Fig. 4a, inset). No such qualitative or quantitative difference in mechanical performance between CF and WT EDL muscles was observed during serial stimulation at 0.5 and 1.0 Hz (data not shown).
Figure 4a shows the time course of the twitch duration of EDL muscles, quantified by the parameter scaled half-width (see Methods), during 9 min of serial stimulation at 2 Hz for WT and CF genotypes. The higher scatter in the CF data compared to WT reflects the dependency of the accuracy of scaled half-width estimation on twitch amplitude. Analogous to the time course of normalized force of twitch contraction, qualitative and quantitative differences in the time course of scaled half-width were found between CF and WT muscles. Analysis of the time-derivative (Fig. 4b, inset) showed that in WT muscles, scaled half-width increased after 30 s of stimulations attaining a constant rate of 2.6 + 0.1 %/min. This rate persisted for 5 min, after which it leveled off. At the end of the protocol, scaled half-width had attained a value of 120 + 11 % (Fig. 4b). In CF muscles, scaled half-width likewise increased shortly after onset of stimulations, but at a much higher rate than in WT (9.2 + 0.3 versus 2.6 + 0.1 %/min, respectively). This high rate persisted for 2 min, after which it transiently dropped to 6 %/min before leveling off to a similar low rate as in WT (Fig. 4b, inset). The increase of scaled half-width at the end of the protocol was twofold higher in CF than in WT (42 + 12% (n=5) versus 20 + 11% (n=6), respectively; Fig. 4b). No such qualitative or quantitative difference in scaled half-width time course was observed between CF and WT EDL muscles during serial stimulation at 0.5 and 1.0 Hz (data not shown).

Figure 3. Energetic efficiency. Relation between the total isometric force produced and the amount of oxygen utilized per gram EDL muscle in cystic fibrosis (CF; n=5, 5 and 4 respectively at the three frequencies) and wild type (WT; four of each frequency) mice at 0.5, 1.0 and 2.0 Hz stimulation.

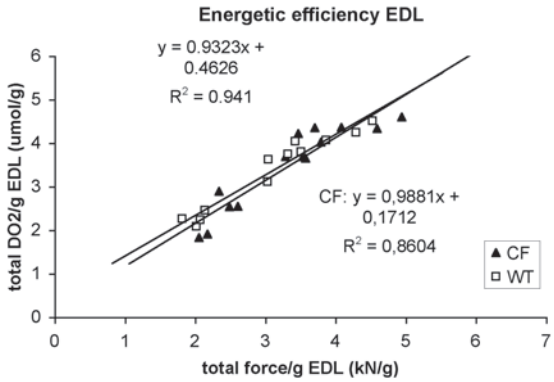


Table 2. Mitochondrial enzyme assays.

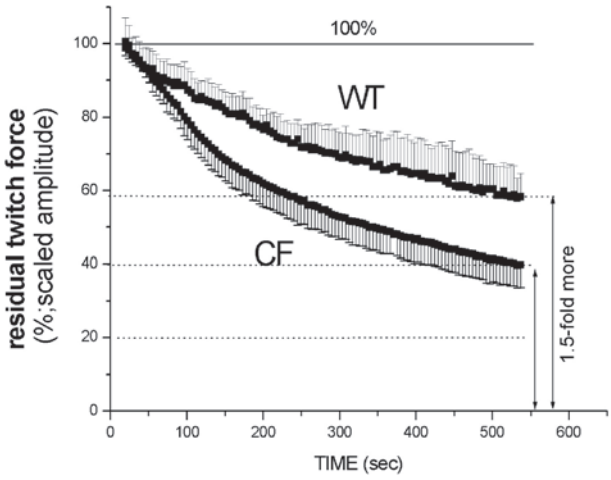
	Activity per gram EDL muscle		Activity per gram protein	
	CF (n=4)	WT (n=4)	CF	WT
Citrate Synthase	19.34 ± 1.98*	27.02 ± 1.07*	0.12 ± 0.02	0.13 ± 0.02
Complex 1	10.19 ± 0.73	10.37 ± 0.58	0.06 ± 0.01	0.05 ± 0.01
C1/CS ratio	0.53 ± 0.03	0.39 ± 0.04		

Values of citrate synthase (CS) and respiratory chain enzyme complex 1 (C1) activities (per gram EDL muscle), corrected values (per gram protein) were measured in EDL homogenate of cystic fibrosis (CF; n=4) and wild type (WT; n=4) mice. Also the C1 to CS ratio as a parameter of mitochondrial density was calculated.
Values are expressed in mmol.ml-1.gram-1and as mean ± SE. * p=0.014

Mitochondrial enzyme activities in CF EDL homogenate

Total and corrected citrate synthase and respiratory chain enzyme complex 1 activities in EDL homogenate for CF and WT genotypes are shown in table 2. Total citrate synthase activity per gram EDL muscle homogenate was significantly lower in CF compared to WT (P<0.05). Total complex 1 activity in homogenate was not different for CF and WT EDL homogenate. After correction for protein content, no difference in citrate synthase or Complex 1 activity was found for CF and WT EDL muscle homogenates. The ratio of complex 1 to citrate synthase activity in EDL homogenate was likewise not significantly different between CF and WT.

Figure 4a. Residual twitch force (in scaled amplitude) of extensor digitorum longus (EDL) muscles of cystic fibrosis (CF; n= 6) and wild type (WT; n=5) mice, contracting at 2.0 Hz for nine minutes.



Data are expressed as mean+SD (WT) and mean–SD (CF)
For clarity of presentation every tenth point is shown.

Inset to figure 4a. Fatigue rate (derivative of scaled force [fig.4a]; see Methods) of extensor digitorum longus (EDL) muscles, of cystic fibrosis (CF; n= 6) and wild type (WT; n=5) mice, contracting at 2.0 Hz for nine minutes.

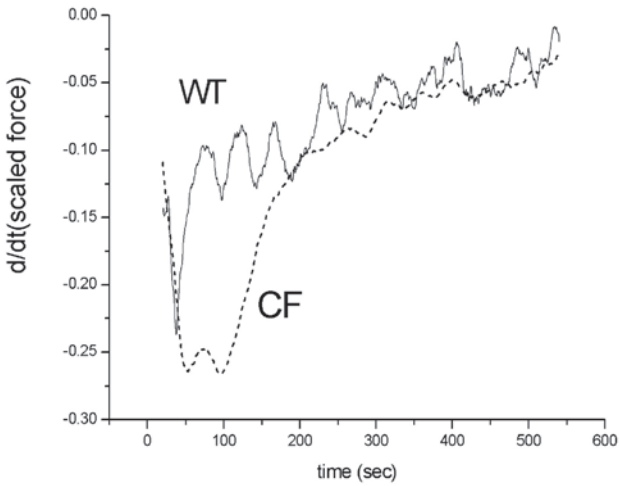
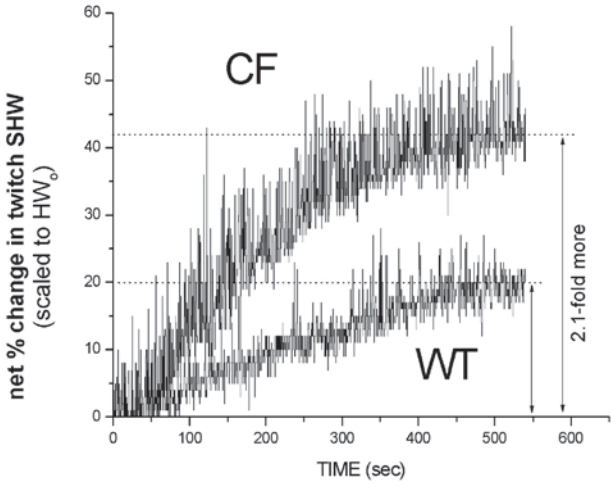
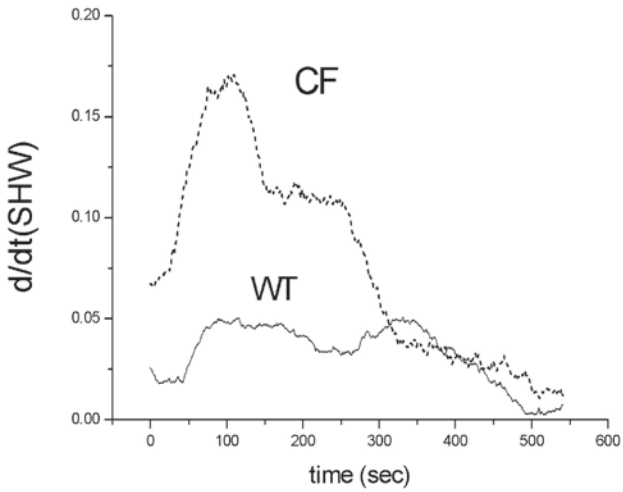


Figure 4b. Net percentage change in twitch duration (displayed as half-width [HW]) of extensor digitorum longus (EDL) muscles, of cystic fibrosis (CF; n= 6) and wild type (WT; n=5) mice, contracting at 2.0 Hz for nine minutes.



Data are expressed as mean ± SD.
For clarity of presentation every tenth point is shown.

Inset to figure 4b. Rate of twitch duration changes (derivative of scaled half-width) of extensor digitorum longus (EDL) muscles, of cystic fibrosis (CF; n= 6) and wild type (WT; n=5) mice, contracting at 2.0 Hz for nine minutes.



Discussion

We have found evidence for energetic limitation of sustained contractile performance of fast-twitch hindlimb muscle in the CF genotype. An unexplained increased gain of excitation-contraction coupling rather than abnormal mitochondrial function appeared to underlie this finding in CF mice. In the following, we shall discuss these findings and how they relate to previous observations on muscle function and energetics in human CF patients.

Specific force of CF EDL and SOL twitch contractions

Absolute isometric force of twitch contraction of EDL and SOL muscles from CF mice was significantly lower than WT because muscle weights in CF were significantly lower than WT (1.7 and 2.2-fold, respectively; Table 1). This result was similar to findings in human CF patients presenting with reduced muscle mass and peripheral muscle weakness (13). Surprisingly, however, *specific* force of isometric twitch contractions of EDL and SOL muscles isolated from CF mice was significantly higher than WT (1.3- and 1.4-fold, respectively; Table 1). The specific force of isometric twitch contraction is determined by two factors: (i) the myosin isoform composition of the muscle, and (ii) the gain of excitation-contraction coupling. Myosin is an actin-based motor protein and consists of two light chains and two heavy chains. The latter is a critical component of the contractile apparatus in mammalian muscle. In mammals two non-muscle, two smooth muscle and eight striated muscle isoforms exist, explaining the functional heterogeneity of slow, mixed and fast fibers (25). A generalized shift in myosin composition of hindlimb muscles towards faster myosin isoforms in transgenic CF mice would therefore explain the result. Different factors, among which alterations in muscle contractile activity and mechanical loading, have been shown to induce changes in the expression patterns of myosin heavy chain isoforms, in proteins regulating excitation-contraction coupling and in the metabolic profile (aerobic-oxidative or glycolytic) (27,28). The pattern of myosin isoform expression correlates very highly with functional parameters that reflect contraction speed, e.g. relaxation rate, generation of force and fatigability (28-30). Muscle fatigue in the light of mechanical performance reflects alterations of the magnitude and kinetics of activation as well as the cross-bridge function (myofibrillar fatigue), and one putative cause is a reduction of Ca^{2+} release upon stimulation as a result of, amongst other factors, inhibited reuptake. In experiments of repeated isometric contractions as ours fatigue is displayed by a decrease of twitch tension and an increase of twitch duration (31). A phenotypic shift towards faster myosin isoforms should, however, also give rise to faster rise time, and decreased energetic efficiency of twitch contractions. We did not find evidence for either (Fig. 3).

Alternatively, an increased gain of excitation-contraction (E-C) coupling in CF skeletal muscle would also explain our observation. E-C coupling is the series of ionic and molecular events whereby membrane depolarization results in contraction of striated muscles.

Contraction is initiated by a depolarization of the T-tubular membrane resulting in a release of calcium from the sarcoplasmatic reticulum (32,33). An increase in E-C coupling may result from increased calcium release from the sarcoplasmic reticulum upon excitation, increased calcium sensitivity of the myofilaments, or decreased damping of the calcium signal due to a lower concentration of cytosolic calcium buffers (e.g. parvalbumin in fast fibers) (34,35). The latter mechanism appears unlikely to be involved here. It would predict a higher fractional increase of the specific force of EDL twitch contraction compared to SOL because EDL muscle contains twofold more fast fibers than SOL muscle in WT (36). Additionally, fast fibers, but not slow fibers, abundantly express cytosolic calcium buffer (36). Instead, we found that specific force of EDL and SOL muscles was increased to the same extent in CF mice (Table 1). Discrimination between increased sarcoplasmic reticulum calcium release upon excitation versus increased myofilament calcium sensitivity in CF as the more likely mechanism involved was not possible on mere basis of the present study. (34,37,38) However, in light of our observations regarding mechanical performance and respiration of EDL muscle during serial stimulations discussed below, it is important to point out that increased sarcoplasmic reticulum calcium release but not increased myofilament calcium sensitivity would be associated with an increased time-averaged calcium integral per excitation-contraction cycle and would therefore likely have resulted in compensatory mitochondrial biogenesis via the calmodulin-pathway to match ATP demand and supply capacity (39). We did not find evidence for increased mitochondrial density in CF muscles (Table 2).

A link between CFTR function and excitation-contraction coupling in skeletal muscle remains elusive. First, CFTR expression and function in this cell type have to be clarified. To date, only a single study has tested CFTR expression in rodent skeletal muscle (7). CFTR gene expression could be observed, albeit at a much lower level than in smooth muscle (see Figure 3 in article by Robert et al) (7). This issue needs to be resolved prior to any speculation on putative mechanistic links between CFTR dysfunction and altered skeletal muscle function observed here.

EDL mechanics and oxygen consumption during serial twitch contractions

The experimental design of our present investigation of energetic limitation of sustained mechanical performance in the CF genotype used prior knowledge of the mechanical and energetic properties of isolated WT EDL and SOL muscles at 20° C. Specifically, mitochondrial oxygen consumption in isolated WT EDL muscle was previously found to be maximally activated during serial stimulation at 1.5 Hz or higher (18). In relation to this, significant fatigue of isolated WT EDL muscle was only found during serial stimulation at 2 Hz, but not at 0.5 or 1 Hz (unpublished data). For WT SOL muscles, we found that maximal mitochondrial respiration could not be attained experimentally using the present set-up (18). Therefore, we did not perform the equivalent set of experiments in CF SOL muscle, but focused only on the EDL.

CF EDL muscles were clearly outperformed by WT muscles during serial stimulation at 2 Hz but not at 0.5 and 1 Hz. Notably, WT but not CF muscles were capable of increasing force output concomitant with oxygen consumption to support contractile energy metabolism over the entire range of stimulation frequencies studies (Figures 2c and 2d). The key question raised by these results was whether the inability of CF muscles to increase oxygen consumption during serial stimulation at 2 Hz above the rate attained during serial stimulation at 1 Hz constituted the cause or merely the result of the higher fatigability. In and by itself, the results on stationary states of mechanical performance and oxygen metabolism (Figures 2a and 2b) could not answer this question. Here, the dynamics of mechanical performance, both with regard to amplitude as well as duration of individual twitch contractions (see Fig. 1) during serial stimulation at 2 Hz provided the necessary additional information (Fig. 4). Notably, the duration of individual twitch contractions of fast-twitch muscle provides indirect information on cytosolic calcium handling (38,40). Specifically, an increase of this parameter during serial stimulation indicates compromised calcium resequestration following an action potential-gated calcium release pulse (38). Calcium resequestration into the sarcoplasmatic reticulum (SR) is catalyzed by ATP free energy-driven SR calcium pumps, the rate of which is sensitive to the cytosolic ATP free energy potential as well as stimulation duty cycle (41). In this study, twitch duration was quantified using the parameter scaled half-width (see Methods). As such, the 3.5-fold higher rate of increase of this parameter at the onset of serial stimulation at 2 Hz in CF compared to WT suggested that the calcium handling ability of WT muscles was superior (Fig. 4b). Notably, in WT muscles the rate of twitch broadening attained a constant, low value (3%/min) within 1 min after onset of stimulations – i.e. coinciding with the time span during which the initial high rate of fatigue was controlled in WT muscle (Fig. 4a, inset). Conversely, the time span of the persisting high initial rate of fatigue (100 s) in CF muscles (Fig. 4a, inset) coincided with the initial high rate of twitch broadening in this muscle genotype (9%/min; Fig. 4b, inset). In addition, the time span during which the high rate of fatigue was finally controlled in CF muscles (100-250 s into the protocol; Fig. 4a, inset) coincided with the second phase of elevated twitch broadening (6%/min) compared to WT (Fig. 4b, inset). According to this interpretation of the data, Figure 4a would predict that, within 1 min after onset of serial contractions at 2 Hz, mitochondrial respiration in WT muscle attained and maintained a rate that exceeded the rate at 1 Hz, whereas in CF muscles this would not be the case, and maximal mitochondrial activation should have been delayed by 1 min compared to WT. Unfortunately, the latter prediction could not be objectified in the oxygen consumption data due to cumulative diffusion delays on the order of 30-45 s between the muscle and the chamber electrode.

Combining the present data on fractional increments of total mechanical output and oxygen consumption (Fig. 2) as well as our previous observation of a reduced capacity for ATP free energy homeostasis in exercising CF forearm muscle (9), the following speculative picture emerges for fast-twitch skeletal muscle function in CF: During contractile workloads

balanced by submaximal mitochondrial respiration rates (0-1 Hz for EDL muscles at 20°C), CF muscles tend to produce more total force per g muscle accompanied by higher respiration rates (tendency for 1 Hz). This appears to be due to a higher gain of excitation-contraction coupling identified in this muscle genotype (Table 1). The elevated potential for contractile ATP utilization in CF muscle is not balanced by a concomitant increase in mitochondrial density (Table 3). At higher contractile workloads (e.g., 2 Hz in the present study), therefore, the higher ATP consumption rate in CF muscle cannot be fully matched by mitochondrial ATP synthesis causing a relatively bigger fall of ATP free energy in CF muscles as compared to WT (9). Finally, this results in stronger inhibition of SR calcium recovery in CF and subsequently higher levels of fatigue.

In conclusion, we propose that EDL muscle function in this mouse model of CF was limited by oxidative insufficiency, in comparison to WT muscles. The apparent inferior matching of oxidative ATP supply and ATP demand capacity in CF muscle identified in the present study did, however, not result from any mitochondrial abnormality in CF suggested previously. (15, 16, 17) (Table 3) Nor did it result from any detectable shift in muscle composition (Fig. 2), but instead from an unexplained apparent increased gain of excitation-contraction coupling. This point can be made quantitatively explicit by comparison of the ratio of citrate synthase activity per gram muscle per unit of twitch force per gram for EDL muscle, whereby citrate synthase activity is taken to be proportional to oxidative ATP supply and contractile force is taken to be proportional to ATP demand. (42)For CF EDL muscle, this ratio is 25% lower than WT (0.016 versus 0.022 μmol*mN/ml, respectively). Future studies will be needed to establish if the present results in this mouse model of CF hold for skeletal muscle in human CF patients. If so, therapeutic strategies aiming to boost mitochondrial ATP synthesis capacity in CF patients (e.g., via dietary supplementation of cofactors) may perhaps improve exercise performance in this group of patients (43).

Acknowledgements
We thank Arie Doornebal for assistance in the data analysis.

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CHAPTER 4.2

Beneficial effects of direct CoQ10 supplementation on mechanical performance of a fast-twitch mouse muscle

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Abstract

Background: Cystic fibrosis (CF) patients have decreased skeletal muscle performance and exercise capacity. Apart from diminished nutritional status and pulmonary function this altered performance seems to be caused by an intrinsic defect in skeletal muscle itself, possibly at the mitochondrial level. In respiratory chain diseases, boosting mitochondrial function through supplementation of micronutrients has proven successful.

Objective: To investigate the effect of direct administration of either vitamin E, vitamin B2, coenzyme Q10, or α-lipoic acid and carnitine to a fast twitch muscle in a transgenic mouse model of CF.

Design: Contractile function and energetic efficiency of 14 isolated, superfused fast-twitch EDL muscles from transgenic CFTR-deltaF508 (CF) mice and 14 wild type FVB controls (WT) were studied before and after direct supplementation of vitamin B2, vitamin E, coenzyme Q10, α-lipoic acid and carnitine.

Results: The CF mice had significantly lower bodyweights (22.5 ± 2.9 and 28.9 ± 7.6, respectively; $P<0.01$) and EDL muscle weights (6.5 ± 0.9 and 8.2 ± 1.9 mg, respectively; $P<0.01$) than the WT control mice. Specific isometric twitch force of intact EDL muscles isolated from CF mice was significantly higher than WT EDL muscles (1.3 fold; $P<0.05$). After direct supplementation of CoQ10, a significant improvement in mechanical performance (isometric twitch contractions during a series of subsequent contractions at 0.5 and 1.0 Hz) of 1.6-fold and 1.5-fold, respectively in WT and CF EDL, was found. No effect was seen after vitamins B2 or E, or a mixture of α-lipoic acid and acetylcarnitine.

Conclusions: Direct supplementation of CoQ10 has beneficial effects on mechanical performance of intact superfused fast-twitch mouse muscle of both wild type and transgenic CFTR-deltaF508. We propose that its effect is the result of directly boosting mitochondrial capacity for ATP synthesis through its role as cofactor in the respiratory chain. Future studies are needed to test this hypothesis as well as to investigate whether these beneficial effects can be maintained when CoQ10 is administered through dietary means in the intact animal.

Introduction

Cystic fibrosis (CF) patients often present with decreased exercise performance. The diminished nutritional status and decreased oxygen delivery due to restricted pulmonary function in CF have long been thought to cause this particular symptom of the disease (1,2,3). However, we have previously reported evidence for an intrinsic defect in skeletal muscle in CF (4). Specifically, we found a significantly reduced capacity for ATP free energy homeostasis during contractile work in children and adolescents with cystic fibrosis using phosphorus-31 nuclear magnetic resonance spectroscopic measurements of ATP metabolism in exercising forearm muscle. (4) In a follow-up study, we showed that pediatric CF patients have peripheral muscle weakness, reduced energetic efficiency of exercise and reduced maximal exercise performance, in addition to reduced muscle mass (5). Moser et al corroborated our findings of an intrinsic problem in muscle in CF patients (6). Importantly, their results too pointed towards abnormalities in oxygen (and thus energy) metabolism (6).

In vitro evidence for pathophysiological alterations in oxidative ATP metabolism in CF, and more specifically for abnormalities in mitochondria, have been found in skin fibroblasts of patients suggesting an anomaly in the mitochondrial NADH dehydrogenase complex (respiratory chain enzyme complex I) system (7). Similarly, studies in fibroblasts and leucocytes from CF patients reported mitochondrial abnormalities such as increased calcium content and lower NADH dehydrogenase activity compared to controls (8,9). In superfused intact fast-twitch muscles isolated from transgenic mice with the delta F508 mutation, the most common CF mutation in man, we found a relatively reduced capacity for oxygen consumption concomitant with diminished mechanical performance (Oudshoorn, this thesis, chapter 4.1).

In the treatment of a number of mitochondrial-linked diseases, especially those involving primary deficiencies of L-carnitine, coenzyme Q10, and cofactor- and vitamin-responsive enzyme defects, boosting mitochondrial function through supplementation of micronutrients has proven to be successful improving tissue function (10,11). Specifically, supplementation of respiratory chain enzyme cofactors such as riboflavin and coenzyme Q10 as well as free radical scavengers such as vitamin E and α-lipoic acid (with or without acetylcarnitine, a direct substrate for the Krebs cycle) have been tested with positive results (12-15). Vitamin B2 (riboflavin; VitB2) is a precursor of intramitochondrial flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which normalizes the activities of flavin-dependent mitochondrial enzymes, acting in the β-oxidation(14,16). It also acts as cofactor to glutathione reductase, which keeps the antioxidant glutathione (GSH) in the reduced state (17). Coenzyme Q10 (CoQ10) in its reduced form (CoQ10red or ubiquinol-10) is an effective lipophilic antioxidant and protects directly against lipid peroxidation by scavenging radicals and indirectly by regeneration of the antioxidants vitamin E and vitamin C (18-21). Alternatively, in the oxidized form

(CoQ10ox; ubiquinone-10) it acts as an essential electron carrier in the respiratory chain in the mitochondrial inner membrane (22). Vitamin E (α-tocopherol; VitE) is a powerful antioxidant and is responsible for scavenging peroxyl radicals and inhibition of the production of superoxide (23,24). Lastly, α-lipoic acid is a mitochondrial cofactor and antioxidant (25).
Based on these considerations, we hypothesized that mitochondrial function and thereby muscle performance in CF can likewise be improved by supplementation of selected respiratory chain enzyme cofactors and protective agents. This hypothesis was tested in an experimental model of CFTR-deficient mouse muscle. Direct effects of the selected micronutrients on contractile performance and oxygen consumption of isolated superfused intact extensor digitorum longus (EDL) muscle of control and transgenic DF508 (CF) mice were studied using mechanical strain measurements and oxygen polarography. We found beneficial effect for CoQ10 for both wild type and CF EDL, but not for VitE and VitB2.

Methods

Animals and muscle preparation

Contractile function and energetic efficiency of 14 isolated, superfused fast-twitch (extensor digitorum longus, EDL) muscles from transgenic CFTR-deltaF508 (FVB/DF508;CF) mice and 14 sex- and age-matched wild type FVB controls (FVB;WT) were studied (both from Erasmus University laboratory, Rotterdam, The Netherlands). All experimental procedures were approved by the Committee on Animal Experiments of the University Medical Center Utrecht and complied with the principles of good laboratory animal care. Mice (aged approximately 25 weeks) were euthanized by cervical dislocation and subsequently weighed. EDL muscles of both hindlimbs were prepared free from the surrounding tissue and ligated at proximal and distal tendons with 5.0 silk suture (Ethicon, Norderstedt, Germany).

Simultaneous recording of muscle oxygen consumption and force development

EDL muscles were isolated, fixed, mounted and stimulated according to the method described by Jeneson (26). Oxygen consumption and twitch (i.e. single isometric) contraction mechanics were measured simultaneously (high-resolution oxygraph, Oroboros, Innsbruck, Austria; adjustable Harvard Apparatus 60-2995 force transducer, Harvard Instruments Limited, Edenbridge, UK; Grass S88 dual channel stimulator, Astro-med, West Warwick, RI, USA) as described by Oudshoorn et al (chapter 4.1). After 30 minutes of equilibration the measurement protocol started with 10 minutes of rest, to record basal respiratory flux, followed by 10 minutes of serial contraction stimulation at 0.5 Hz, and 10 minutes of recovery at 0.015 Hz. Subsequently, in both measuring chambers two different mixtures,

either VitE, VitB2, or CoQ10, were added and left to incubate for half an hour. Thereafter, a consecutive measuring protocol of 10 minutes each at 0.5, 1.0 and 2.0 Hz, followed by a recovery phase at 0.015 Hz for 10 minutes was performed. All measurements of muscle respiration were performed as randomized paired-experiments with simultaneous measurement of either two WT or two CF EDL muscles in a dual-chamber setup. To avoid oxygen limitation of respiration in EDL muscles at 20 ° C all measurements were performed above a PO_2 of 450 Torr (27). Chamber volume (approx. 5.3 ml) and muscle weight (blotted and tendon free) were determined after each experiment.

Direct administration of micronutrient solutions

Ringers solution (116 mM NaCl, 25.3 mM $NaHCO_3$, 4.6 mM KCl, 2.5 mM $CaCl_2$, 1.16 mM $KH2PO_4$, 1.16 mM $MgSO_4$, pH of 7.4) was prepared according to Syme (28) and placed into the measuring chambers to accommodate the EDL muscle. 0.006 ml of an α-tocopherol-TPGS (VitE) solution of 75 mg/ml (WKZ pharmacy preparation for oral administration) was added to 20 ml of Ringers solution to prepare a solution of 0.43 mg/l (0.002 mg/5.3 ml) which exceeds the plasma concentration of Vit.E in humans (7.8-33.2 μmol/l). 0.0055 gram of riboflavin (VitB2)(Fluka Biochemika, Buchs, Switzerland) was added to 10 ml Ringers to attain a solution of 0.01 mg/l (5,5 mg/5.3 ml). For the CoQ10 solution 0.0022 grams of the lipid soluble coenzyme Q10 (Sigma-Aldrich, Steinheim, Germany) was added to 4 ml ethanol 99.8% and subsequently stirred for 30 minutes to attain a solution of 10 mg/l (5.5 mg/5.3 ml). A mixture of α-lipoic acid and acetyl-L-carnitine (hereafter termed lipcar) was prepared by adding 0.00312 gram of each compound to 10 ml Ringers; solution 1.7 mg/30 ml; 0.312 mg/5.3 ml) (Sigma-Aldrich, Steinheim, Germany). Micronutrients were delivered by injection of 0.1 ml of each solution into the chambers and left to incubate for 30 minutes

Data acquisition, analysis and statistics

Oxygraph and force transducer data-acquisition was performed with LabView software (National Instruments, Woerden, The Netherlands). Mechanical performance was analyzed on a twitch-per-twitch basis with respect to five parameters: rise time, amplitude, area (tension-time integral), relaxation time and half-width (HW; in ms) using LabView subroutines. Non-linear curve-fitting analysis of the time-course of mechanical parameters during serial stimulation with respect to initial and steady-state values as well as time-constants was performed using Origin 6.0 (Microcal Software Inc., Northampton, MA, U.S.A.). Absolute muscle respiratory rates were calculated using Origin 6.0. Reported data are presented as arithmetic means ± SD. Statistical analyses were performed using a Student's unpaired t-test. Differences between CF and WT muscle were considered significant if $P<0.05$.

Table 1. Mouse characteristics of FVB/WT and FVB/DF508.

	WT (n=14)	CF (n= 14)
Age (weeks)	26.2 ± 5.4	27.7 ± 5.9
M	7	4
Bodyweight (g)	28.9 ± 7.6	22.5 ± 2.9*
EDL wet weight (mg)	8.2 ± 1.9	6.5 ± 0.9*

Mean ± SD; *P<0.01

Table 2: Mechanical characteristics of EDL muscle contraction during a single twitch, and during steady-state of serial contraction at 0.5 Hz

	WT (n=6)	CF (n= 7)
Single twitch		
rise time (ms)	11.4 ± 2.0	10.9 ± 1.9
relaxation time (ms)	61.6 ± 18.2	53.3 ± 6.8
specific force (N/g)	3.0 ± 0.6	3.8 ± 0.5†
Serial contraction		
rise time (ms)	7.7 ± 0.8	6.7 ± 1.5
relaxation time (ms)	32.3 ± 8.0	33.7 ± 7.4
half-width duration (ms)	36.9 ± 3.0	34.2 ± 4.5

Mean ± SD; †P<0.05

Table 3: Mechanical characteristics of EDL muscle single twitch contraction: FVB WT versus C57BL/6 WT

	FVB WT (n=6)	C57BL/6 WT (n= 6)
specific force (N/g)	3.0 ± 0.6	4.2 ± 0.8†
rise time (ms)	11.4 ± 2.0	12.7 ± 0.3
relaxation time (ms)	61.6 ± 18.2	97.1 ± 25.3†
half-width duration (ms)	36.9 ± 3.0	45.7 ± 2.2*

Mean ± SD; *P<0.01; †P<0.05

Results

Anatomical and mechanical properties of mice and EDL muscles

CF and WT mouse characteristics including age, sex, body and muscle mass are presented in table 1. The FVB/CF mice had significantly lower bodyweights (22.5 ± 2.9 and 28.9 ± 7.6, respectively; $P<0.01$) and EDL muscle weights (6.5 ± 0.9 and 8.2 ± 1.9 mg, respectively; $P<0.01$) than the FVB/WT control mice. Specific isometric twitch force (i.e., isometric force (N) produced in a single twitch per g muscle) of intact EDL muscles isolated from CF mice was significantly higher than WT EDL muscles (1.3 fold; $P<0.05$). No differences were found between CF and WT in either rise or relaxation time of a single twitch or of HW at steady-state during serial contraction at 0.5 Hz (Table 2). Table 3 shows twitch contraction parameters for WT FVB and WT C57BL/6 mice for comparison since our previous study in a CF mouse model was done using the latter strain (Oudshoorn, this thesis (chapter 4.1)). Data for that strain were taken from that study (Oudshoorn, this thesis (chapter 4.1)). Specific force (N/g) of isometric twitch contraction was lower for WT FVB mice than WT C57BL/6 mice. With respect to the kinetics, we found that only the rise time was the same in both groups. Both the relaxation time of a single twitch and the steady-state HW attained during serial contraction at 0.5 Hz were significantly shorter in WT FVB mice.

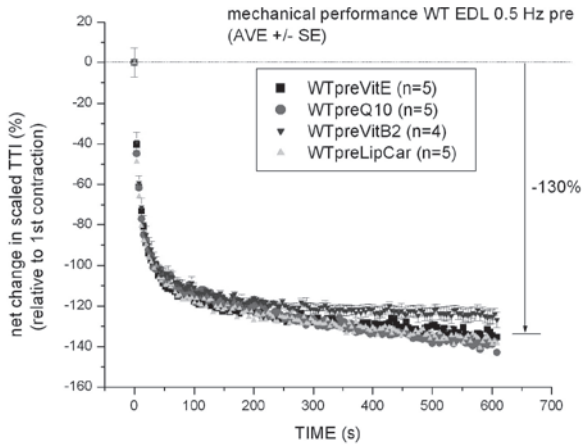
Effects of direct micronutrient supplementation on mechanical performance at 0.5 Hz

Figure 1 shows the net change in scaled tension-time-integral (sTTI) of serial twitch contractions at 0.5 Hz for WT before (pre; figure 1a) and after (post; figure 1b) micronutrient supplementation for each of the tested compounds. Figure 1a shows the previously described steep drop in sTTI directly after onset of stimulation towards a steady-state value attained within 100 s (Ter Veld et al, Pflugers Archives, in press). As can be seen, this effect was the same in each group and highly reproducible between groups. The net drop in sTTI was 130% compared to the first contraction. Figure 1b shows the same data after acute supplementation of the various tested compounds. For the VitB2, VitE and lipcar groups no effect on sTTI was found. However, for the CoQ10 group a substantial improvement in mechanical performance was found: the net drop in sTTI compared to the first contraction was 1.6-fold less than before addition of CoQ10. The same result was observed in the CF groups (Figures 2a and b). Figure 2a shows the time-course of net change in sTTI for the four CF groups before addition of micronutrients. Similarly as in WT, a net drop in sTTI of 120 to 170% compared to the first contraction was found. After supplementation, a 1.5-fold improvement in mechanical performance was found only for the CoQ10 group (Figure 2b).

Effects of direct micronutrient supplementation on twitch half-width (HW)

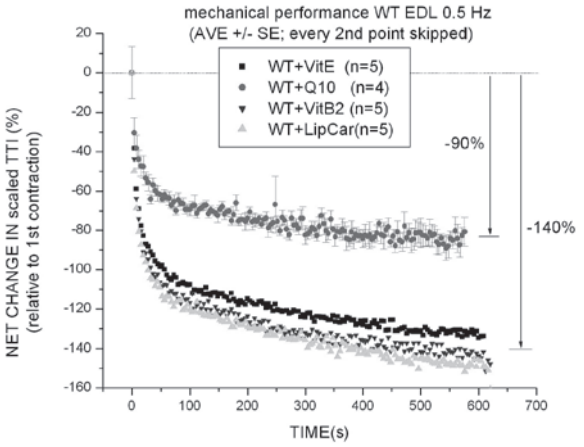
Figure 3 shows the time-course of HW of twitch contraction during serial stimulation at 0.5 Hz before and after addition for each of the micronutrient supplementation experiments

Figure 1a. The time-course of the net change in scaled tension-time integral (TTI) of twitch contractions of wild type EDL muscle stimulated at 0.5 Hz prior to the direct supplementation of vitamin E (VitE), coenzyme Q10 (Q10), vitamin B2 (VitB2), and a-lipoic acid/acetylcarnitine (lipcar) for each of group. The net change was computed by each twitch TTI scaled to the steady state TTI determined at 0.5 Hz (50-100s) and this value was subtracted from the first contraction.



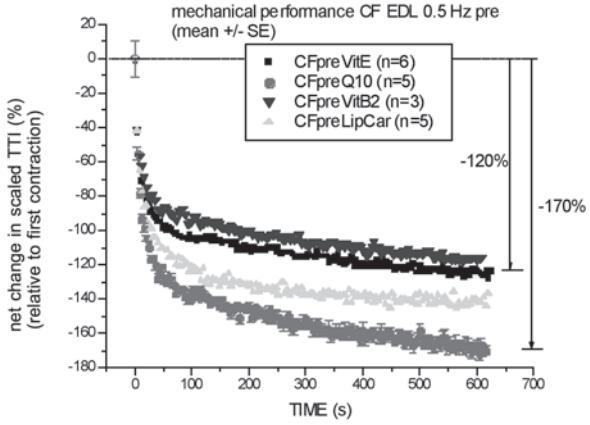
The time-course of the pre-vitB2 group is given as mean±SE and is representative for the SE of the other groups.

Figure 1b. The time-course of the net change in scaled tension-time integral (TTI) of twitch contractions of WT EDL muscle stimulated at 0.5 Hz after the direct supplementation of micronutrients for each of group. Net change in scaled TTI for WT after various additions at 0.5 Hz



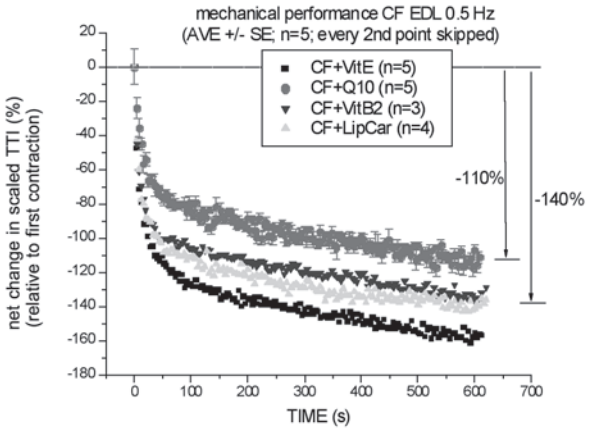
The time-course of the post-CoQ10 group is given as mean±SE and is representative for the SE of the other groups. For clarity every second point is skipped.

Figure 2a. The time-course of the net change in scaled tension-time integral (TTI) of twitch contractions of transgenic CF EDL muscle stimulated at 0.5 Hz prior to the direct supplementation of vitamin E, coenzyme Q10, vitamin B2, and a-lipoic acid/acetylcarnitine for each of group. The net change was computed by each twitch TTI scaled to the steady state TTI determined at 0.5 Hz (50-100s) and this value was subtracted from the first contraction.



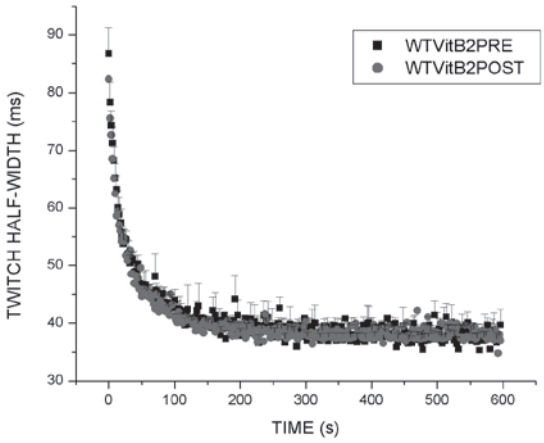
The time-course of the pre-Q10 group is given as mean±SE and is representative for the SE of the other groups.

Figure 2b. The time-course of the net change in scaled tension-time integral (TTI) of twitch contractions of CF EDL muscle stimulated at 0.5 Hz after the direct supplementation of micronutrients for each of group. Net change in scaled TTI for WT after various additions at 0.5 Hz



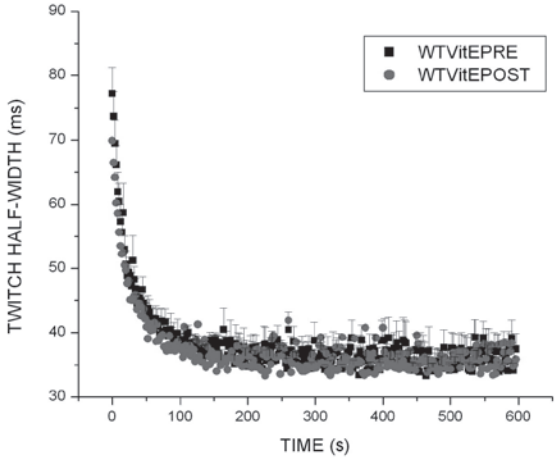
The time-course of the post-Q10 group is given as mean±SE and is representative for the SE of the other groups. For clarity every second point is skipped.

Figure 3a. The time-course of twitch half-width (in ms) of twitch contractions of WT EDL muscle during serial stimulation at 0.5 Hz prior and post direct supplementation of VitB2.



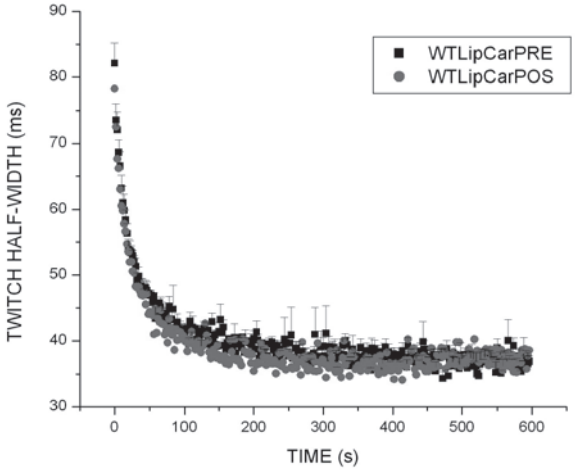
The time-course of the pre-VitB2 group is given as mean + SE and is representative for the - SE of the pre-VitB2 group and the ±SE of the post-VitB2 group.

Figure 3b. The time-course of twitch half-width (in ms) of twitch contractions of WT EDL muscle during serial stimulation at 0.5 Hz prior and post direct supplementation of VitE.



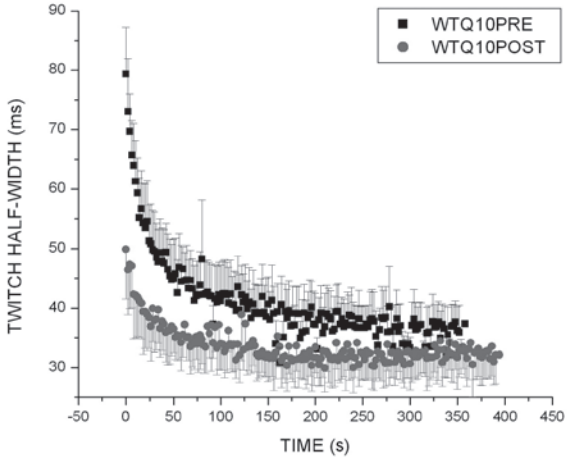
The time-course of the pre-VitE group is given as mean + SE and is representative for the - SE of the pre-VitE group and the ±SE of the post-VitE group.

Figure 3c. The time-course of twitch half-width (in ms) of twitch contractions of WT EDL muscle during serial stimulation at 0.5 Hz prior and post direct supplementation of a-lipoic acid/ acetylcarnitine.



The time-course of the pre-lipcar group is given as mean + SE and is representative for the – SE of the pre-lipcar group and the ± SE of the post-lipcar group.

Figure 3d. The time-course of twitch half-width (in ms) of twitch contractions of WT EDL muscle during serial stimulation at 0.5 Hz prior and post direct supplementation of Q10 (WT group).



For clarity the time-course of the pre-Q10 group is given as mean + SE and the post-Q10 group as - SE.

Figure 4a. The time-course of twitch half-width (in ms) of twitch contractions of CF EDL muscle during serial stimulation at 0.5 Hz prior and post direct supplementation of vitamin E.

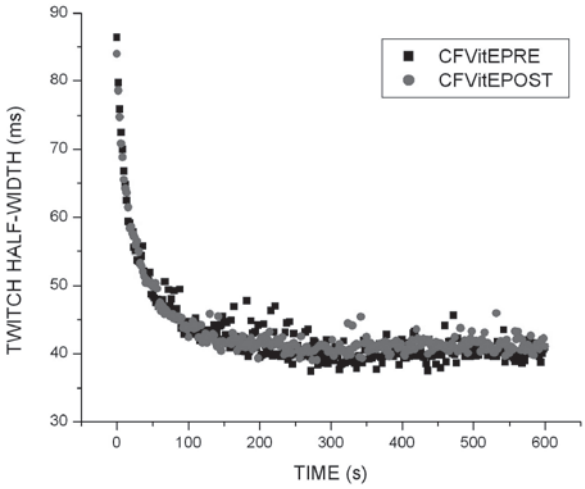
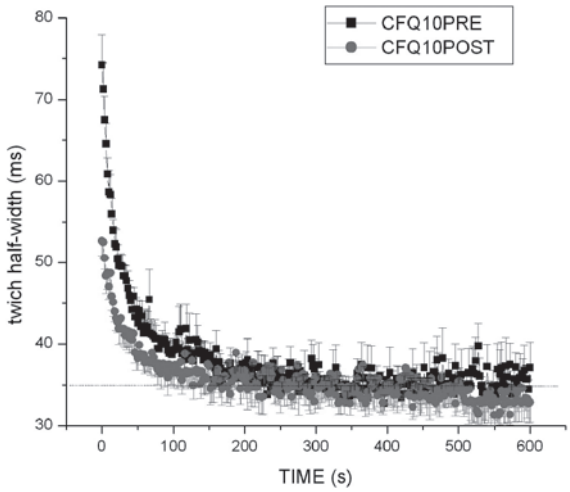


Figure 4b. The time-course of twitch half-width (in ms) of twitch contractions of CF EDL muscle during serial stimulation at 0.5 Hz prior and post direct supplementation of coenzyme Q10.



For clarity the time-course of the pre-Q10 group is given as mean + SE and the post-Q10 group as - SE.

in the WT groups. No effect was found for VitE, VitB2, or lipcar (figures 3a-c). CoQ10 did have a clear and significant effect (figure 3d). The HW of the first contraction (initial HW) post-supplementation of CoQ10 was 1.3-fold shorter than pre (50 ± 17 versus 79 ± 16 ms (mean ± SD; n=4), respectively; $P<0.05$). The same result was found in paired comparison of pre- and post-supplementation for each muscle (data not shown). Twitch HW at steady state was not significantly different between pre- and post (37 ± 9 versus 32 ± 8 ms (mean ± SD; n=4), respectively). The time-course of this parameter during serial stimulation followed a bi-exponential decay function characterized by two time constants (tau1 and tau2) (r2>0.9) and was faster post-supplementation (tau1 8.5 ± 2.8; tau2 62.4 ± 9.8 ms (mean ± SE of regression)) than pre (11.1 ± 1.4 and 106 ± 16 ms (mean ± SE of regression), respectively).
In the CF groups the result was the same. Figure 4a shows an identical time-course of twitch HW pre- and post-supplementation of VitE. A similar negative result was found for VitB2 and lipcar (data not shown). Figure 4b shows the result for supplementation of CoQ10. Analogous to the findings in WT, the HW of the first contraction post-supplementation of CoQ10 was 1.3-fold shorter than pre (53 ± 4 versus 74 ± 8 ms (mean ± SD; n=5), respectively; $P<0.01$). Twitch HW at steady state was not significantly different between pre- and post (35 ± 2 versus 33 ± 3 ms (mean ± SD; n=5), respectively). The kinetics of attaining steady-state with respect to this parameter were only slightly faster post-supplementation (tau1 23.4 ± 3.6; tau2 81.5 ± 6.5 ms versus 12.4 ± 1.2 and 132 ± 43 ms (mean ± SE of regression); post versus pre, respectively).

Effects of direct micronutrient supplementation on mechanical performance at 1 Hz
Figure 5a shows the net change in sTTI of serial twitch contractions at 1 Hz for WT post- micronutrient supplementation for each of the tested compounds. As found for the mechanical performance at 0.5 Hz (Figure 1b), the CoQ10 group outperformed the other groups. The net drop in sTTI after 10 min of contraction at 1 Hz compared to the first contraction in the series was 1.5-fold less in the CoQ10 group than any of the other groups (Figure 5a).
Figure 5b shows the results for the CF groups. A similar result was found as in WT, although the scatter in the CoQ10 group data was large: after six min of contraction at 1 Hz the net drop in sTTI was less for the CoQ10 group than for any of the other groups (Figure 5b).
Figure 5c shows a comparison of the net change in sTTI during 10 min of serial contraction at 1 Hz for the WT and CF VitE supplementation groups. In absence of any measurements prior to micronutrient delivery at this stimulation frequency, this particular comparison provided the best possible information on differences in performance during serial contraction at 1 Hz between WT and CF muscles since supplementation of VitE did not have any effect on net mechanical performance in either group (Figures 1-4). WT EDL muscles outperformed CF muscles 1.5-fold, both with respect to net drop in sTTI after 600 contractions as well as the rate of fatigue after 90 contractions (30% versus 45% and -1.5 versus -2.3 %/min, respectively; Figure 5c).

Figure 5a. The time-course of the net change in scaled tension-time integral (TTI) of twitch contractions of wild type EDL muscle stimulated at 1.0 Hz after the direct supplementation of vitamin E, coenzyme Q10, vitamin B2, and a-lipoic acid/acetylcarnitine for each of group. The net change was computed by each twitch TTI scaled to the steady state TTI determined at 0.5 Hz (50-100s) and this value was subtracted from the first contraction.

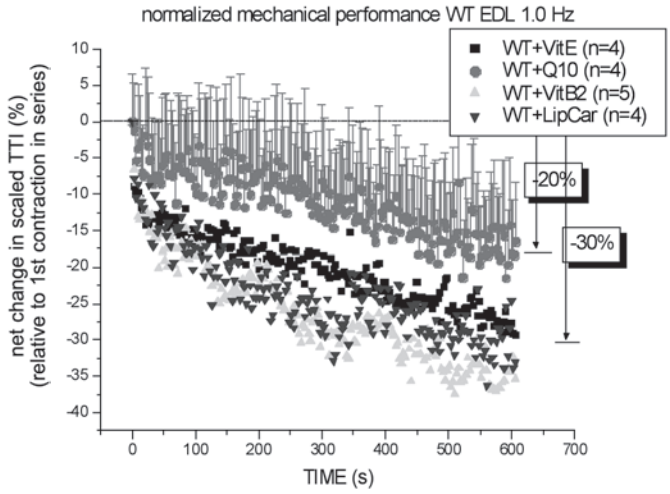


Figure 5b. The time-course of the net change in scaled tension-time integral (TTI) of twitch contractions of transgenic CF EDL muscle stimulated at 1.0 Hz after the direct supplementation of the different micronutrients for each of group. Scaled mechanical performance (TTI) for CF after various additions at 1.0 Hz

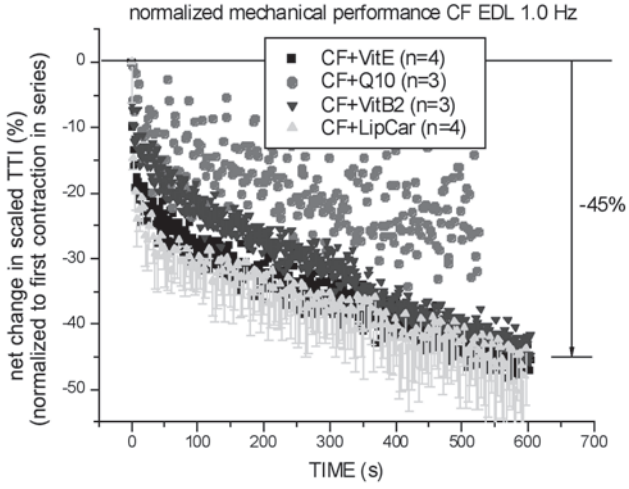


Figure 5c. The time-course of the net change in scaled tension-time integral (TTI) of twitch contractions of wild type and transgenic CF EDL muscle stimulated at 1.0 Hz after the direct supplementation of vitamin E.

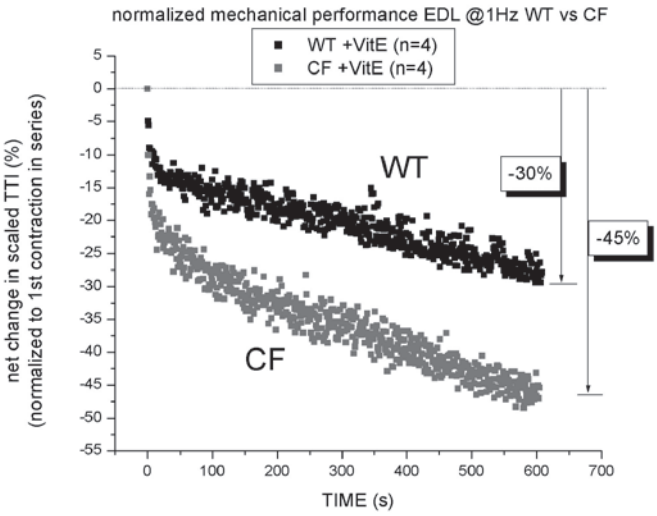
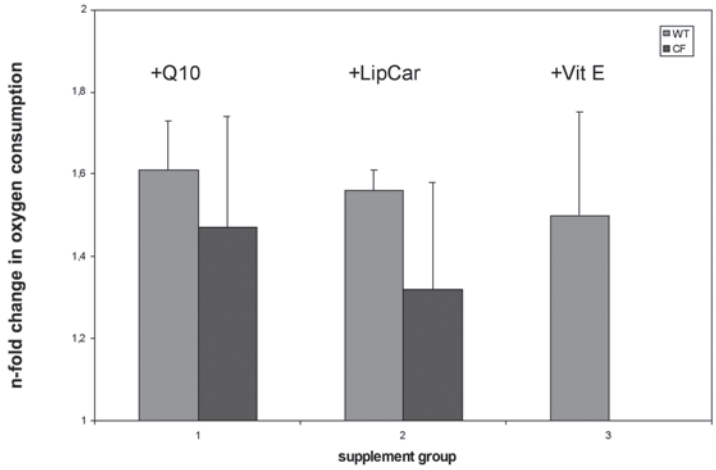


Figure 6. The net change in oxygen consumption after doubling of the serial contraction frequency from 0.5 to 1 Hz after the direct supplementation of coenzyme Q10, a-lipoic acid/ acetylcarnitine, and vitamin E for each group of WT and CF.



CoQ10 supplementation groups: WT n=2, CF n=2
Lipcar supplementation groups: WT n=3, CF n=3
Vitamin E supplementation group: WT n=2

Effects of direct micronutrient supplementation on mechanical performance: oxygen consumption

The oxygen consumption rate during serial contraction at 0.5 Hz at steady-state prior to any supplementation of micronutrient was the same in WT and CF (2.27 ± 0.54 versus 2.17 ± 0.51 nmol/s/g above basal rate (mean ± SD; n=10), respectively). Figure 6 shows the net change in oxygen consumption after doubling of the contraction frequency from 0.5 to 1 Hz for the various micronutrient supplementation groups for WT and CF. In the WT groups, oxygen consumption increased 1.6-fold after doubling of the contraction frequency (Figure 6). This result was the same as we previously found (Oudshoorn, this thesis (chapter 4.1)). For the CF groups, oxygen consumption data at 0.5 and 1 Hz were only available for two micronutrient supplementation groups (i.e., CoQ10 and lipcar). The difference observed as compared to WT was NS (Figure 6).

Discussion

We found that direct supplementation of CoQ10, but not vitamins B2 or E, nor a mixture of α-lipoic acid and acetylcarnitine, improves mechanical performance of serially stimulated superfused intact fast-twitch muscle isolated from both wild-type FVB mice and a transgenic strain with the DeltaF508 CF mutation. This result together with its mechanistic basis and possible implications for clinical management of human CF patients presenting with reduced exercise performance are discussed.

Mouse model and experimental design of the study

The present study was done in a different mouse strain than our previous study (FVB versus C57BL/6, respectively; Oudshoorn, this thesis, Chapter 4.1). Although we found the same abnormalities in the FVB CF phenotype with respect to body weight (Table 1) and EDL muscle weight and specific force (Table 2) that we first reported for the C57BL/6 CF phenotype (Oudshoorn, this thesis, Chapter 4.1), there were a number of phenotypic strain differences for the WT FVB and C57BL/6 EDL muscles (Table 3) indicating that EDL muscle in FVB mice is faster than in the C57BL/6 strain. Specifically, the rate of EDL relaxation following isometric twitch contraction (and, as such, half-width duration of a twitch contraction) for the FVB strain was 1.3-fold faster than for C57BL/6; rise time was unaltered (Table 3). Surprisingly, specific force of FVB EDL muscle was 1.3-fold lower than for C57BL/6. These findings suggested that in FVB EDL muscle either total calcium released upon a single stimulation is reduced or calcium buffering and/or resequestration is increased compared to C57BL/6. These apparent phenotypic strain differences influenced the present study as FVB EDL muscles were found to have a significantly more limited operational range of

mechanical performance compared to C57BL/6. Whereas WT EDL muscles of the latter strain show significant fatigue only at stimulation frequencies of 2 Hz and higher (Ter Veld et al., Pflugers Archives, in press), WT FVB EDL muscle already showed significant fatigue at 1 Hz (Figure 5c). This was even more evident in the CF group. As such, the experimental design used in our previous study, measuring EDL mechanical and concomitant mitochondrial performance during two consecutive doublings of stimulation frequencies between 0.5 and 2 Hz (Oudshoorn, this thesis, chapter 4.1) could not be used here. At 1 Hz, the high rate of fatigue (and resulting drop in ATP utilization rate) in the CF group subdued any ancillary activation of mitochondrial respiration in the vitamin B2 and E groups, if present at all (Figure 6).
The design of the present study – i.e. to test the effects of direct supplementation of micronutrients to isolated EDL muscles, rather than dietary administration in intact animals, similar to the approach taken in human patients (Oudshoorn, this thesis), was chosen for the following reason: we have previously found significant, acute effects of supplementation of various oxidative substrates (i.e., glucose, lactate and pyruvate) on mitochondrial respiration and cytosolic ATP free energy potential in unstimulated mouse EDL and SOL muscles using this exact same experimental set-up and design of compound delivery (Wiseman RW, Jeneson JAL and Kushmerick MJ, unpublished results). The significant, direct effects of CoQ10 supplementation on TTI and HW in both the WT and CF groups found in the present study confirmed that these small molecules are indeed rapidly taken up by the muscle-cells, reach the mitochondrial compartment and exercise their molecular function. Therefore, the lack of significant effects of the other compounds tested should be more likely attributed to lack of any significant metabolic effect than to a failure for these molecules to reach the mitochondria. As we now have determined the direct effects, future studies should test if the observed effects for CoQ10 can also be found after dietary administration.

Direct effects of micronutrient supplementation on EDL mechanical performance during serial stimulation

The main finding of the present study was that direct supplementation of CoQ10, but not vitamins B2 or E, nor a mixture of α-lipoic acid and acetylcarnitine, improved mechanical performance of serially stimulated superfused intact fast-twitch muscle isolated from both wild-type FVB mice and a transgenic strain with the DeltaF508 CF mutation. This conclusion was based on the observation that the net drop in TTI of isometric twitch contractions during a series of subsequent contractions relative to TTI of the first contraction in the series was 25% less after supplementation of CoQ10 (Figures 1d and 2b). This effect was absent for each of the other tested compounds (Figures 1a-c and Figure 2a).
Further analysis of individual twitch contractions with respect to amplitude and rise- and relaxation kinetics revealed the major cause of this improved maintenance of TTI. It was found that after CoQ10 supplementation, the typical biphasic, dramatic acceleration of muscle relaxation during the first two minutes of serial stimulation reflected in a biphasic

drop of isometric twitch duration from 80 to 45 ms (e.g., Figures 3 and 4, pre-supplementation groups) was considerably blunted. Specifically, the initial twitch HW post-CoQ10 supplementation was on average 30 ms shorter than prior to supplementation in both the WT and CF groups (Figures 3d and 4b). Furthermore, curve-fit analysis of the HW time-course with respect to the time constants of the bi-exponential decay showed that a steady-state with respect to this contraction parameter post-CoQ10 supplementation was more swiftly attained than pre. This was more pronounced in the WT group than in CF (Figures 3d and 4b).

We have only once before made a similar observation. In a previous study of intact EDL muscles isolated from transgenic creatine kinase knock-out (CK k.o.) mice in a C57BL/6 strain, it was likewise found that the net drop in TTI after the first contraction in a series was heavily blunted compared to WT (Ter Veld et al., Pflugers Archives, in press). We analyzed the time course of twitch HW during serial stimulation at 0.5 Hz for these muscles and found that initial HW in the CK k.o. group was 50 ms and remained constant throughout the entire series of stimulations, whereas in WT initial HW was 65 ms and typically dropped to 45 ms (data not shown). This suggests that identification of a common factor in the remodeled CK k.o. EDL muscle and the CoQ10-supplemented EDL muscle could lead us to the mechanistic basis for the faster EDL relaxation observed in the latter group.

It has been well documented that fast-twitch skeletal muscle of CK k.o. mice has an almost twofold higher mitochondrial density than WT and, as such, a twofold higher Vmax of respiration (29). In addition, it has been reported that chronic CoQ10 supplementation to human patients diagnosed with muscle disease caused by mitochondrial dysfunction likewise increased Vmax of respiration and ATP synthesis (30). Taken together, an explanation of the observed effects of CoQ10 supplementation on EDL relaxation after contraction in the present study could be the following: CoQ10 increases Vmax of respiration, resulting in a higher 'gain' of mitochondrial ATP utilization sensing (through detection of alterations in cytosolic ADP concentration)(31). During a twitch, one and the same amount of ADP produced by ATP hydrolysis by the actomyosin ATPase and sarcoplasmic reticular calcium pumps (SERCa) therefore results in a higher ATP synthesis rate supporting faster SERCA calcium recovery and thereby faster relaxation of the muscle. A consequence of this mechanism is that CoQ10 would need to function as an electron donor rather than free radical electron scavenger. CoQ10 is found in the inner membrane of the mitochondria first tightly bound to NADH (complex I), where four protons are released in a reductive cycle, before electrons are transferred to a second loosely bound CoQ10 to form CoQ10red. This can travel through the lipid in the membrane to complex III where the CoQ10red is oxidized again via an oxidation-reduction cycle, allowing four protons to cross the membrane for each CoQ10red oxidation cycle (19).

There is an important implication with respect to striated muscle physiology of this hypothesis for the mechanistic basis of the measured acute CoQ10 effect. In smooth muscle, it has been argued that cation pumps such as the sarcolemnal Na-K-ATPase and SERCa are

preferentially driven by ATP derived non-oxidatively from glycogenolysis (32). It has been further argued that such compartmentation should also be physiologically relevant in striated muscle. However, the proposed mechanistic explanation for the faster muscle relaxation following CoQ10 supplementation entails that SERCa pumps are driven by mitochondrial ATP. If proven correct, this would refute any preferential non-oxidative ATP supply of the SERCa pumps. Testing of this proposed mechanism for faster calcium recovery following CoQ10 supplementation can be performed by determining whether the beneficial effect of CoQ10 can be reversed by inhibition of mitochondrial respiration at complex III or IV using standard inhibitors such as antimycin and cyanide, respectively. The oxygen consumption measurements in the present study unfortunately did not provide data of sufficient quality to test this hypothesis by means of comparing oxygen consumption rate pre and post-CoQ10 supplementation. The ratio of the steady-state rates measured during serial stimulation at 0.5 and 1 Hz (Figure 6) likewise did not provide any information on this question. Although the ratio of 1.6 for the WT oxygen consumption rates at 1 versus 0.5 Hz was identical to our previous study (Oudshoorn, this thesis, chapter 4.1), as a result of the high fatigue rate of FVB CF EDL muscles stimulated at 1 Hz (see above) there was insufficient oxygen consumption data to draw any conclusion regarding the effects of micronutrient supplementation on mitochondrial function.

In summary, we have found a direct effect of CoQ10 supplementation on mechanical performance of intact superfused fast-twitch mouse muscle. We propose that this beneficial effect is the result of directly boosting mitochondrial capacity for ATP synthesis through its role as enzyme cofactor in the respiratory chain. Future studies are needed to test this hypothesis as well as to investigate whether these beneficial effects can also be obtained through dietary administration.

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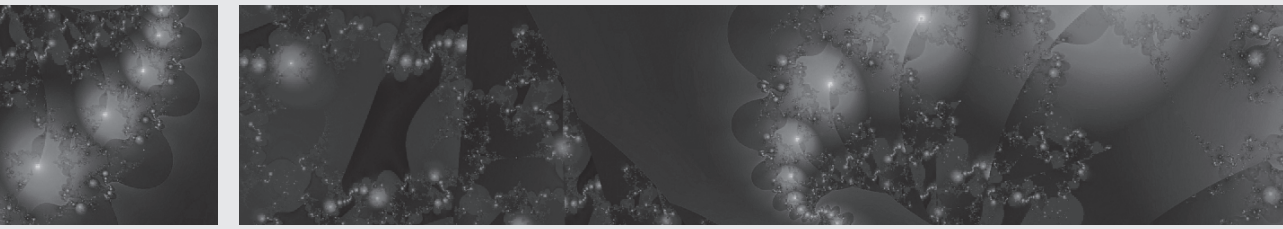

GENERAL DISCUSSION

The increase in median survival of patients with cystic fibrosis (CF) during the past 20 years is the result of optimal nutrition, pro-active use of antibiotics, meticulous physiotherapy and exercise programs, and a better understanding of the disease. The research described in this thesis aimed to contribute to our knowledge of the effect of exercise and macro- and micronutrients on the physical condition of pediatric patients with cystic fibrosis.

Exercise in cystic fibrosis: anaerobic exercise

Effects of anaerobic training in children with cystic fibrosis

Several studies investigating aerobic exercise programs have shown a positive effect on pulmonary function (1,2,3), but others have failed to detect this improvement (4,5,6,7). Apart from these inconsistent effects, compliance to aerobic training regimes is suboptimal. As anaerobic training might be a good alternative to preserve physical fitness and pulmonary function, we investigated whether a high-intensity anaerobic training program could improve overall muscle performance, lung function, body composition, peripheral muscle strength, and health-related quality of life (HRQOL) in children and adolescents with CF (chapter 1). No positive or adverse effects on pulmonary function, fat free mass or peripheral muscle strength were found. Nevertheless, we could describe that children with mild-to-moderate CF, like healthy children (8,9,10), can enhance their anaerobic and aerobic performance and HRQOL through participation in a structured anaerobic exercise training program, which mimicked their natural activity pattern and also contained the necessary variation. In contrast to the poor adherence to aerobic exercise, which generally involves several hours per week sub-maximal intensity training and is often perceived monotonous (4), the anaerobic exercise program we offered showed excellent compliance. So the ideal training program for CF patients might be an exercise program, that is individually tailored to his/her preference, and consists of aerobic, anaerobic, and strength training activities with wide variation.

Macronutrients in cystic fibrosis

Short-term protein intake and stimulation of protein synthesis in stunted children with cystic fibrosis

At present, nutritional strategies in CF focus primarily on high-energy and high-fat intake (11-16). Surprisingly, little effort has been made to determine the optimal intake of dietary protein in pediatric CF patients, especially as adequate protein is necessary for normal linear growth and efficacious immune defense mechanisms (17,18). Current recommendations are 1.0-1.5 g protein $\cdot$ $kg^{-1} \cdot d^{-1}$, which is the same for healthy children (19). These recommendations might be suboptimal, since a portion of the CF patients are stunted, and recurrent

infections induce protein catabolism. Posthoc analysis of the data of 123 CF patients from our CF Centre at the Wilhelmina Children's Hospital indeed showed a mean height of -0.6 ± 0.9 SD, while 8 patients were genuinely stunted (height < -2 SD). This group of 123 children (age range between 6 and 16 year; mean age 11.0 ± 2.9 yrs) had a protein intake of 2.4 ± 0.6 g · kg^{-1} · d^{-1} (data not shown). The most recent North American CF Foundation Annual Report documented that 16.3 % of CF children were below the 5^{th} percentile for height and 15.7 % for weight (20). Furthermore, in pediatric CF patients during acute pulmonary exacerbation, protein synthesis rates of 50% of normal were reported by Holt et al. (21). Protein synthesis was also reduced as compared to CF patients with chronic but stable pulmonary disease. Conversely, in the same study, whole-body protein breakdown in stable CF patients was higher than in the exacerbation group or control subjects. The resulting net protein deposition was 0.14 ± 0.11 g · kg^{-1} · 10 h in CF patients with pulmonary exacerbation versus 0.02 ± 0.15 g · kg^{-1} · 10 h in stable CF patients versus 0.65 ± 0.19 in controls (10 h overnight [^{15}N] glycine isotope study) (21).

The marginal protein synthesis rate in CF patients might be boosted by enhancing protein intake (22). However, the few studies that have addressed the effect of different amounts of protein intake on rates of protein synthesis and breakdown in patients with CF, varied the protein intake simultaneously with total energy intake (23,24). So it remains unclear whether the positive results obtained could be explained solely by the different amounts of dietary protein used. In Chapter 2, we therefore investigated the influence of three isoenergetic diets containing normal (1.5 g protein · kg^{-1} · d^{-1}), intermediate (3 g protein · kg^{-1} · d^{-1}), and high (5 g protein · kg^{-1} · d^{-1}) levels of dietary protein, in a group of pediatric CF patients with chronic but stable pulmonary disease (mean height -1.1 SD). Whole-body protein synthesis in the high protein group was 130%, respectively 113%, higher as compared to the normal and intermediate protein group. Since this study was a short term study we could not determine the effect of high dietary protein on linear growth. Two other studies have addressed this question, but in both energy and protein intake were optimized simultaneously. The first study showed a non significant increase in height velocity ($P < 0.1$), from –1.03 ± 0.54 (mean SD ± SEM) in the first year (intake of 2.9 g whole protein · kg^{-1} · d^{-1}; energy intake of 65 ± 2 kcal · kg^{-1} · d^{-1}) to +0.26 ± 0.70 in the second year (3.8 g whole protein · kg^{-1} · d^{-1}; energy intake of 82 ± 1 kcal · kg^{-1} · d^{-1}) (21). Another long term study consisting of one year observation period on conventional treatment, followed by one year of energy and protein supplementation (≥120% of RDA) and subsequently 1 to 2-y follow up, showed a catch-up weight gain and sustained improvement in linear growth, with fewer pulmonary infections per year than during the initial observation period in 10 stunted pediatric CF patients (25). Better weight gain and linear growth, as well as a significant reversal of the trend for deteriorating lung function were observed in comparison with matched controls, who received conventional therapy. Given these and our results, a long term study needs to establish whether at optimal energy intake a higher dietary protein can still increase linear growth, improve body composition, and decrease (pulmonary) infections.

Micronutrients in cystic fibrosis: human studies

Cystic fibrosis (CF) is predominantly characterized by chronic pulmonary inflammation and infection in combination with maldigestion and malabsorption due to pancreatic insufficiency (26). The airway inflammation is dominated by activated neutrophils, which antimicrobial efficiency depends on the generation of reactive oxygen species (ROS), resulting in oxidative stress (27). The surplus of ROS, combined with the impaired absorption of fat soluble anti-oxidant nutrients, results in an oxidant-antioxidant imbalance (28,29,30).

Decreased Coenzyme Q10 concentration in plasma of children with cystic fibrosis

Patients with CF generally have malabsorption of the lipid soluble vitamins A, D, E and K (31-33). This same mechanism seems to apply to CoQ10, which is also lipid soluble, as we found significantly diminished plasma concentrations of CoQ10. Contrary to expectations, a normal ratio between oxidized and reduced CoQ10 was found, so it seems that in these patients regeneration of reduced CoQ10 by NADH/NADPH was adequate (34-38). This could have been caused by the relative good clinical condition of our patient group, who had stable pulmonary disease without any signs of pulmonary infection. In addition, since plasma levels of CoQ10 are not a good reflection of tissue levels (39), the normal redox status in plasma we found here might not represent the actual CoQ10red/ox balance in the different tissues. Further investigations of CoQ10 tissue levels (lymphocytes, platelets, or buccal mucosal cells, all containing mitochondria) and including CF patients with advanced pulmonary disease are warranted to address these questions.

If indeed a normal redox status is present, would CoQ10 supplementation be beneficial to CF patients? CoQ10 supplementation in "healthy" volunteers resulted in a higher plasma level of reduced CoQ10 and lower thiobarbituric acid reactive substances (TBARS; marker of lipid peroxidation) concentrations (40). This suggests that in CF patients the same effect might be attained even in the presence of a normal redox status. Dietary supplementation with CoQ10 was able to elevate both plasma and tissue levels of coenzyme Q in young mice (41), while in rats a similar enhancement resulted also in a selective decrease in protein oxidative damage, and an increase in antioxidative potential (39). In healthy adults, receiving 3 mg/kg/day, CoQ10 levels could also be boosted both in plasma and platelets (42).

Dietary supplementation with multiple micronutrients mixture: no beneficial effects in pediatric cystic fibrosis

Distinct antioxidant vitamin deficits (α-tocopherol, ascorbic acid, and carotenoids) and elevated indicators of oxidative stress (malondialdehyde and protein carbonyls) in plasma, buccal mucosal cells and breath condensate were found to correlate with progression of clinical disease in adult CF patients (43). Signs of oxidative stress are present as early as two months of age, as significantly elevated myeloperoxidase (measure of neutrophils inflammation) and

3-chlorotyrosine (biomarker of the potent oxidant hypochlorous acid) in bronchoalveolar lavage fluid of young children were reported (44). Since oxidative stress is already present at a very early age and the oxidant-antioxidant imbalance worsens in later life, the hypothesis was generated that a change in dietary habits and innovative supplementation strategies might optimize the antioxidant status of patients with CF. Therefore, several studies have investigated the effect of supplementing a single or a few micronutrients, especially β-carotene and α-tocopherol, and some also investigated their influence on the oxidant-antioxidant imbalance and/or the pulmonary function in CF patients (45-48). Renner et al performed a high dose (1 mg/kg/d) and subsequently low dose (10 mg/d) β-carotene supplementation study, and showed a higher β-carotene concentration on high dose, a decrease of MDA levels, and significant fewer days on antibiotics during pulmonary exacerbation, but no effect on lung function (47).

Since CF is characterized by both oxidative stress and poor exercise tolerance we investigated the effect of a mixture of multiple micronutrients (ML1) with either antioxidant and/or muscle fortifying action in chapter 3.2. It showed no beneficial effects on either pulmonary function or anaerobic and aerobic muscle performance in pediatric cystic fibrosis patients compared to placebo. For several parameters a trend towards a negative effect for the micronutrient mixture was found, e.g. FEV_1 and FVC. A similar trend in favor of placebo was found by Wood et al (48), when supplying CF patients with a micronutrient mixture containing several antioxidants (500 μg vitamin A, 25 mg β-carotene, 300 mg vitamin C, 200 mg vitamin E, and 90 μg selenium). However, Wood et al. found no differences in the oxidant-antioxidant balance when measuring plasma 8-iso-prostaglandin $F_{2\alpha}$, while we found a weak trend to a pro-oxidative state (change of plasma MDA concentrations of -0.263 μmol/l ± 0.66 μmol/l during placebo and of 0.023 μmol/l ± 0.61 μmol/l during ML1; *P*=0.15).

High-dose carotenoid supplementation might indeed induce a pro-oxidative state (49) and in a few clinical studies harmful effects of β-carotene and other carotenoids have been observed, e.g. a higher incidence of lung cancer after treatment of smokers with 30 mg β-carotene (49). Also, doubts have been raised whether high vitamin E supplementation or plasma levels are always beneficial. A review of nine vitamin E supplementation studies in healthy individuals performing aerobic or resistance exercise revealed no decrease in exercise-induced lipid peroxidation (50). Although α-tocopherol therapy, especially at high doses (600-1200 IU=mg), has been shown to decrease the release of pro-inflammatory cytokines (such as IL-1β, IL-6, and TNF-α) (51), a meta-analysis reported that high-dosage (≥400 IU/d) vitamin E supplements may increase all-cause mortality and should be avoided (52). Nevertheless this has been challenged since the reviewed high-dosage (≥400 IU/d) trials were often small and were performed in patients with chronic diseases (52). Therefore, Hathcock et al concluded that vitamin E supplements in amounts ≤ 1600 IU/d (1073 mg RRR-α-tocopherol) appear safe for most adults (53).

Creatine and carnitine supplementation have been reported to enhance muscular performance, but are most effective in combination with high-intensity physical training (54,55).

Healthy, essentially sedentary persons and untrained individuals consuming adequate diets, generally do not improve performance after vitamin and mineral supplements (51,52). However, a pilot study in 18 adolescent CF patients showed a significant increase in maximal isometric muscle strength, but no change in lung function after creatine supplementation (56). In that study higher doses than in our study were used (initial loading dosage 12 g/d first 7 d, maintenance dose 6 g/d for 11 weeks as compared to 1.2 g/d in our study). This large difference in dosage might explain why we did not find a positive effect. Most studies in healthy subjects taking carnitine have failed to demonstrate an objective performance improvement (57). Despite isolated reports of positive carnitine effects in athletes, who usually undergo aggressive training regimens and nutritional interventions, the totality of the data do not support the conclusion that carnitine supplementation improves exercise performance. Also, no primary abnormality of carnitine metabolism seems to exist in CF, and only transient decreased levels of carnitine have been described (58,59). This could explain why carnitine did not enhance skeletal muscle function or exercise performance in our CF patients.

Since we, and also others, found some evidence of untoward effects of micronutrient mixtures, we believe that no further studies with combination supplements are warranted. Nevertheless, careful designed interventions with antioxidants that normalize parameters of oxidative stress, such as β-carotene or α-tocopherol, or muscle performance enhancers, like creatine, might have a place.

Muscle performance and micronutrients in cystic fibrosis: animal studies

CF patients often demonstrate altered skeletal muscle performance and exercise intolerance (60). At first this has been attributed to diminished nutritional status and decreased oxygen delivery due to restricted pulmonary function (61,62,63). However, evidence for a defect in skeletal muscle itself has been found (60), and to investigate this we used a mouse model of CF.

Abnormal mechanical and energetic properties of skeletal muscle in a mouse model of cystic fibrosis

In chapter 4.1 we investigated whether an intrinsic abnormality exists in fast-twitch extensor digitorum longus (EDL) and slow-twitch soleus (SOL) muscles in a transgenic mouse, with a C57BL/6 background, and the most common mutation in man, wich is the delta F508 mutation. We found significantly lower *absolute*, but significantly higher *specific* isometric force (force per gram muscle; 1.3- and 1.4-fold, respectively) of twitch contraction of EDL and SOL muscles isolated from CF mice as compared to wild type (WT). Since the specific force of isometric twitch contraction is determined by two factors, i.e. the myosin isoform composition of the muscle,

and the gain of excitation-contraction (E-C) coupling, we tried to find an explanation for this phenomenon of higher specific force. There is an interesting analogy with thyroid hormone receptor-deficient (TR$\alpha^{-/-}\beta^{-/-}$) mice. Isometric force of EDL and SOL isolated from these mice showed similar force per cross-sectional area as WT mice, although the muscle weights were lower (64). SOL muscles from TR$\alpha^{-/-}\beta^{-/-}$ mice showed increased contraction and relaxation times, and were more fatigue resistance than WT controls. Protein analysis of TR$\alpha^{-/-}\beta^{-/-}$ SOL muscles showed a marked increase in expression of the slow isoform of the sarcoplasmatic reticulum Ca^{2+} pump (SERCa2), while expression of the fast SERCa1 was decreased. EDL muscles from both mice showed no significant difference in contraction and relaxation times, fatigue resistance and protein expression. Furthermore, expression of myosin heavy chain (MHC) isoforms in SOL muscles are significantly altered in TR$\alpha^{-/-}\beta^{-/-}$ SOL muscles, and showed a 100% increase in twitch contraction and relaxation times compared to WT. No difference in the expression of the mitochondrial enzyme citrate synthase was seen between TR$\alpha^{-/-}\beta^{-/-}$ and WT muscles, indicating no major changes in mitochondrial density in TR$\alpha^{-/-}\beta^{-/-}$ muscles, and therefore no increased capacity for oxidative ATP production (64). A generalized shift in myosin composition of hindlimb muscles towards faster myosin isoforms in transgenic CF mice would explain our results (65), but this would cause faster rise time, and decreased energetic efficiency of twitch contractions, and we did not find evidence for either.
The alternative explanation would be an increased gain of E-C coupling in CF skeletal muscle, which may result from increased calcium release from the sarcoplasmic reticulum upon excitation or increased calcium sensitivity of the myofilaments. Further investigations into determining the MHC and SERCa isoforms in the EDL and SOL muscles of CF mice are warranted to clarify the differences in CF and WT muscles.

Influence of micronutrients on the contractile properties of skeletal muscle in a mouse model of cystic fibrosis

Diminished skeletal muscle performance and exercise intolerance in mitochondrial myopathies can be enhanced by administration of antioxidants (vitamin E, lipoic acid), electron donors and acceptors (coenzyme Q10, vitamin B2), alternative energy sources (creatine monohydrate), lactate reduction strategies (dichloroacetate) and exercise training (66). In chapter 4.2 we investigated whether we could boost muscle performance in EDL muscle of transgenic CFTR-deltaF508 (CF) mice by direct administration of either vitamin E, vitamin B2, coenzyme Q10, or α-lipoic acid and carnitine. We found that direct supplementation of CoQ10, but not vitamins B2 or E, nor a mixture of α-lipoic acid and acetylcarnitine, improves mechanical performance of serially stimulated superfused intact fast-twitch muscle isolated from both wild type FVB mice and a transgenic strain with the DeltaF508 mutation. The significant, direct effects of CoQ10 supplementation on tension time integral and half width in both the WT and CF groups confirmed that these small molecules are indeed rapidly taken up by the muscle-cells, reach the mitochondrial compartment and exercise their molecular function.

Therefore, we suggest that the observed lack of significant effects of the other compounds tested is more likely attributed to lack of any significant metabolic effect than to a failure for these molecules to reach the mitochondria. Having established these direct effects of CoQ10 supplementation, future follow-up studies in this mouse model should test if the same effect can be found after dietary administration.

If indeed mechanical performance in transgenic mice can be improved by dietary supplementation with CoQ10, further investigation on the effect of CoQ10 in CF patients is warranted. This is based on the fact that CF patients exhibit diminished exercise performance due to an intrinsic defect in the muscle itself (60,67), which might be influenced by boosting CoQ10 levels thus enhancing mitochondrial ATP production (68).

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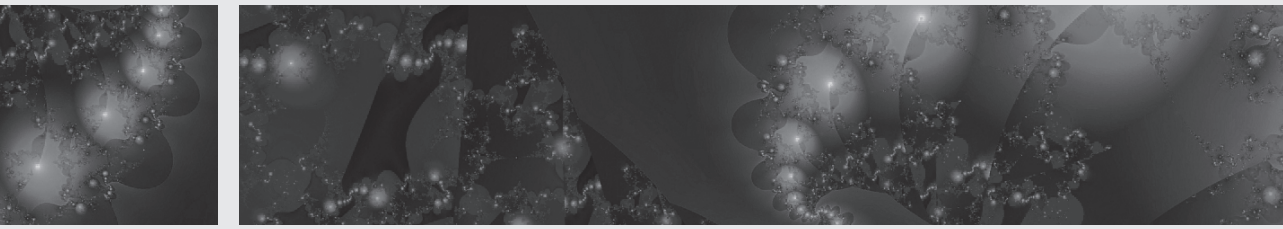


SAMENVATTING IN HET NEDERLANDS

Cystic Fibrosis (CF), ook wel taaislijmziekte genaamd, is een erfelijke ziekte waarbij de verschillende door het lichaam uitgescheiden vloeistoffen anders van samenstelling zijn en daardoor stroperig worden. Hierdoor kunnen diversen organen en systemen niet meer goed functioneren of worden kwetsbaar voor infecties. De werking van de longen gaat langzaam achteruit als gevolg van chronische ontsteking en herhaaldelijke infecties. De alvleesklier kan geen of onvoldoende verteringssappen meer maken, waardoor de voeding slecht verteerd wordt. Ook kunnen er lever en galproblemen ontstaan en is er een toegenomen zoutverlies door een verhoogd zoutgehalte in het zweet.
Het is de meest voorkomende levensbedreigende erfelijke aandoening onder de blanke bevolking. De ziekte komt voor bij 1 op de 2000-3000 blanken en 1 op de 22-28 is drager. Het gen dat CF veroorzaakt heet CFTR en is in 1989 ontdekt. Het draagt zorg voor de aanmaak van een eiwit dat de samenstelling van vloeistoffen reguleert. Er zijn op dit moment meer dan 1000 mutaties, 'fouten in het CFTR-gen', beschreven, maar de meest voorkomende is de delta F508 mutatie. Deze mutatie komt wereldwijd bij 66 % van de CF chromosomen voor. Er bestaan grote verschillen in verschijnselen tussen de individuele CF patiënten, en zelfs tussen broers en zussen onderling met dezelfde mutatie.
De afgelopen 10-15 jaar is de levensverwachting beduidend toegenomen van gemiddeld 20 naar 35 jaar. Dit komt door een beter inzicht in de onderliggende oorzaak en mechanismen van CF waardoor een betere behandeling mogelijk is. Deze bestaat uit laagdrempelige, agressieve behandeling van luchtweginfecties met antibiotica met intensieve fysiotherapie gecombineerd met fitness programma's. Een optimaal dieet met 120-150% van de aanbevolen dagelijkse hoeveelheid aan calorieën en een hogere dan normale vetinname gecombineerd met alvleesklier-vervangende medicijnen, wordt hierbij nagestreefd.
Dit proefschrift beschrijft de resultaten van onderzoeken naar zowel het effect van fysieke training als voeding op de lichamelijke conditie van kinderen met CF.

Deel 1: Lichaamsbeweging in cystic fibrosis: anaerobe training

Hoofdstuk 1: Effecten van anaërobe training in kinderen met cystic fibrosis
Verscheidene studies betreffende aërobe (zuurstof afhankelijke) training hebben een wisselend effect op de longfunctie laten zien. Aangezien de patiënten deze aërobe duurtraining moeilijk vol bleken te kunnen houden, hebben we bekeken of anaërobe (zuurstof onafhankelijke) training een alternatief is om een goede lichamelijke conditie en longfunctie te behouden. Deze anaërobe training bootst het natuurlijke, dagelijkse activiteitenpatroon van een kind na. Dit bestaat uit korte, intensieve, lichamelijke activiteiten en krachtsinspanningen. Wij vonden geen verbetering of verslechtering van de longfunctie, spierkracht of spier-vet verhouding. Wel was er een verbetering van het anaërobe en aërobe prestatievermogen in de patiënten met milde-tot-matige CF, zoals ook beschreven is bij gezonde

kinderen. Wel was het gevarieerde trainingsprogramma goed vol te houden. Het meest ideale trainingsprogramma lijkt te bestaan uit een op het individu toegesneden programma met een variatie aan aërobe, anaërobe en krachttrainingactiviteiten.

Deel 2: Macronutrienten in cystic fibrosis

Hoofdstuk 2: Invloed van eiwitinname en stimulatie van eiwitaanmaak op de korte termijn in groeivertraagde kinderen met cystic fibrosis

Op dit moment richten de voedingsstrategieën zich op een hoog energetische voeding met een hoge vetinname. Verrassend genoeg is er weinig aandacht besteed aan het bepalen van de optimale eiwitbehoefte bij kinderen met CF, zeker gezien het feit dat een adequate hoeveelheid eiwit nodig is voor een goede lengtegroei en een doeltreffend afweersysteem. De huidige aanbevelingen voor kinderen met CF zijn hetzelfde als voor gezonde kinderen, maar zijn mogelijk te laag, aangezien een deel van de kinderen groeivertraging vertoont. Een analyse van de gegevens van kinderen onder behandeling in ons CF-centrum toonde een gemiddelde lengte onder het gemiddelde van de Nederlandse jeugd, ondanks het feit dat ze een gemiddeld hogere eiwitinname hadden. De Noord-Amerikaanse CF vereniging rapporteerde in 2004, dat 16.3 % van de CF kinderen te klein en 15.7 % te licht is. CF kinderen met stabiele CF en tijdens een acute longontsteking hebben een verminderde eiwitaanmaak en een hogere eiwitafbraak t.o.v. gezonde kinderen. De matige eiwitaanmaak zou gestimuleerd kunnen worden door het verhogen van de eiwitinname. Tot op heden is er nog geen studie gedaan, die naar het effect van een hoger eiwit aanbod zonder verhoging van het energie aanbod heeft gekeken. Wij hebben in een groep CF patiënten met chronische maar stabiele longziekte, de invloed van drie verschillende diëten met hetzelfde aantal kilocalorieën en verschillende hoeveelheden eiwit, onderzocht. De eiwitaanmaak was 130% t.o.v. normaal (1.5 g eiwit/kg/dag) in de hoge eiwitinname groep (5 g eiwit/kg/dag) en 113% t.o.v. normaal in de tussengroep (3 g eiwit/kg/dag). Aangezien onze studie slechts van korte duur was, konden we geen gunstig effect van hogere eiwitinname op de lengtegroei vaststellen. Onze resultaten zijn een goed uitgangspunt voor een lange termijn onderzoek studie.

Deel 3: Micronutrienten in cystic fibrosis: studie in mensen

Een van de kenmerken van CF is chronische ontsteking en herhaaldelijke infecties van de longen. Daarnaast is er een slechte vertering en slechte opname van voedingsbestanddelen in de darmen als gevolg van een slechte alvleesklierfunctie. De veelvuldig optredende ontstekingsreacties in de luchtwegen worden gedomineerd door geactiveerde witte bloedcellen, die zuurstofradicalen (= oxidant = geactiveerde zuurstof moleculen) maken om daarmee de

aanwezige bacteriën en virussen te bestrijden. Dit vrijwel chronische teveel aan zuurstofradicalen lijdt tot schade aan gezond longweefsel. Normaal gesproken wordt gezond weefsel beschermd door antioxidanten die via de voeding worden opgenomen. Echter door de verminderde opname van vetoplosbare antioxidanten resulteert dit in een oxidant-antioxidant dysbalans, ook wel 'oxidatieve stress' genaamd.

Hoofdstuk 3.1: Verlaagde Coenzyme Q10 concentratie in het bloed van kinderen met cystic fibrosis

Patiënten met CF vertonen meestal een slechtere opname van de vetoplosbare vitaminen A, D, E and K. In hoofdstuk 3.1 beschrijven we bij CF patiënten significant verlaagde bloedspiegels van het vetoplosbare Coenzyme Q10 (CoQ10), waarvan de gereduceerde vorm als antioxidant werkt, dus lijkt hiervoor hetzelfde te gelden als voor de vetoplosbare vitaminen. Tot onze verassing vonden we een normale verhouding tussen de geoxideerde en gereduceerde vorm van CoQ10 (redox status genaamd), dus waarschijnlijk is het recycling proces van gereduceerde CoQ10 adequaat. De normale verhouding kan het gevolg zijn van de relatief goede klinische conditie van onze patiënten groep, met stabiele longziekte zonder tekenen van infectie waardoor er minder verbruik is van antioxidanten. Daarbij komt dat bloedspiegels van CoQ10 geen goede weerspiegeling zijn van de weefselspiegels en weefsel spiegels belangrijker zijn voor de beschermende werking.

De vraag rijst of bij een normale redox status het geven van CoQ10 aan CF patiënten nuttig is? Er is aangetoond, dat door toediening van CoQ10 het mogelijk is om hogere bloedspiegels van gereduceerd CoQ10 en minder tekenen van oxidatieve stress te bereiken in proefdieren en gezonde vrijwilligers, evenals hogere weefsel spiegels van CoQ10 in weefsels bij proefdieren.

Hoofdstuk 3.2: Geen voordelige effecten van een mengsel van voedingssupplementen bij kinderen met cystic fibrosis

De mate van specifieke tekorten aan vitaminen met antioxidant werking (nl. vitamine A, C en E) en tekenen van verhoogde oxidatieve stress (afbraakproducten van vetten en eiwitten) bij volwassen CF patiënten kwamen overeen met het stadium van de longziekte. Bij jonge CF patiënten zijn er op de leeftijd van twee maanden al tekenen van oxidatieve stress aangetoond in spoelvloeistof van de longen. Gezien het bovenstaande wordt er gedacht dat een verandering in dieetgewoonten en het geven van voedingssupplementen de antioxidant status zou kunnen verbeteren. Onderzoek naar het effect van één of een paar micronutriënten, voornamelijk vitamine A en E, hebben wisselende resultaten op de oxidant-antioxidant dysbalans en de longfunctie opgeleverd. Aangezien CF wordt gekenmerkt door zowel oxidatieve stress als een slecht uithoudingsvermogen, hebben we het effect van een mengsel van meerdere (totaal 32) micronutriënten met antioxidant en/of spierversterkende werking beschreven. Het mengsel had in vergelijking met een nepmiddel (placebo) noch een voordelig effect op de longfunctie, noch op het anaërobe en aërobe prestatievermogen.

In een aantal andere studies zijn ook afwezige en zelfs negatieve effecten beschreven van antioxidante micronutriënten (vooral vitamine E en A, maar ook multivitamine preparaten). Beschreven is dat extra creatine en carnitine een prestatieverhogend effect op de spieren hebben, doch toediening lijkt het meest effectief in combinatie met het volgen van een intensief trainingsprogramma. Echter, een kleine studie in 18 adolescenten met CF, die creatine kregen, toonde een significante toename van de spierkracht, maar geen verandering van de longfunctie. In die, en ook andere studies, zijn hogere doses gebruikt (beginfase 12 g/dag eerste 7 dagen, onderhoud 6 g/dag gedurende 11 weken) in vergelijking met onze studie (1.2 g/dag). Aangezien wij, en ook anderen, geen of zelfs negatieve effecten hebben gevonden van mengsels van multipele micronutriënten, hebben we het idee dat vervolgstudies met combinatiepreparaten niet gerechtvaardigd zijn.

Deel 4: Prestatievermogen en micronutrienten in cystic fibrosis: proefdier studies

CF patiënten vertonen vaak zowel een veranderd prestatie- als uithoudingsvermogen van de skeletspieren. Eerst veronderstelde men dat het kwam door de slechtere voedingstoestand en een verminderde zuurstoftoevoer naar de spieren door een slechtere longfunctie. Het bleek dat een defect in de skeletspier zelf waarschijnlijk hiervoor verantwoordelijk is. Om dit te onderzoeken hebben we een muizenmodel van CF gebruikt.

Hoofdstuk 4.1: Abnormale mechanische en energetische eigenschappen van skeletspier in een muizenmodel van cystic fibrosis

In hoofdstuk 4.1 bespreken we de resultaten van een onderzoek naar een afwijking in de snel samentrekkende extensor digitorum longus (EDL) skeletspier of de langzaam samentrekkende soleus (SOL) spier van een genetisch gemanipuleerde muis, met de meest voorkomende delta F508 mutatie, aanwezig is. We vonden lagere absolute, maar hogere specifieke spierkracht (kracht per gram spier; 1.3- en 1.4-maal, respectievelijk) in de EDL en SOL spieren geïsoleerd uit de CF muizen in vergelijking tot normale, wildtype (WT) muizen. De specifieke kracht wordt bepaald door de samenstelling van de spier en de snelheid van zenuwprikkeling tot samentrekking van de spier.

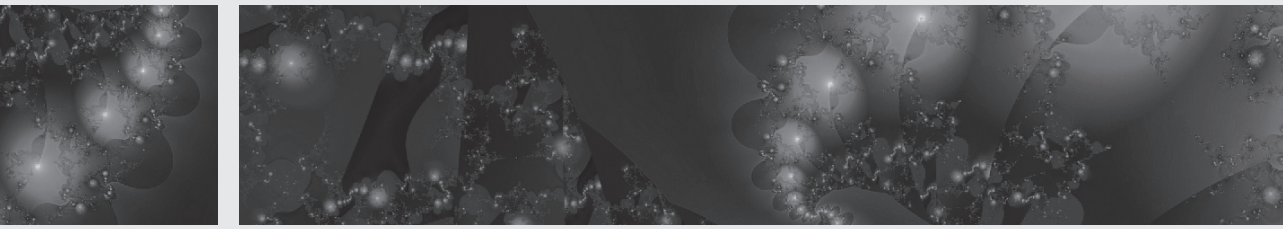
We hebben geprobeerd een verklaring te vinden voor de hogere specifieke kracht. Het is mogelijk dat de calciumpomp in de spiercelmembraan een rol speelt. De stroom van calcium de spiercel in is de voornaamste verantwoordelijke voor de samentrekkingskracht. De hoeveelheid aanwezige mitochondriën zou ook een rol kunnen spelen. Mitochondriën zijn grotendeels verantwoordelijk voor de energie voorziening van de cel. Wij konden geen verschil vaststellen tussen de CF en de wildtype spieren. Een verschuiving in de CF spiersamenstelling zou onze bevindingen kunnen verklaren.

Nader onderzoek moet plaatsvinden om dit verder op te helderen. Vervolgens zouden meer ingrijpende methoden, zoals een spierbiopsie, nodig kunnen zijn om bij de CF patiënten zelf te kijken naar de veranderingen in de spieren.

Hoofdstuk 4.2: De invloed van micronutriënten op de samentrekkende eigenschappen van skeletspier in een muizenmodel van cystic fibrosis

Het verminderde prestatievermogen van de skeletspier en het uithoudingsvermogen in mitochondriële spieraandoeningen kunnen verbeterd worden door de toediening van antioxidanten (vitamine E, lipoic acid), electrondonoren en -ontvangers (coenzyme Q10, vitamine B2), alternatieve energiebronnen (creatine), melkzuurverlagende maatregelen (dichloroacetate) en spiertraining. In hoofdstuk 4.2 beschrijven we het effect van directe toediening van vitamine E, vitamine B2, coenzyme Q10, of α-lipoic acid en carnitine aan de EDL spier van CF en wildtype muizen op de afwijkende spierprestatie. We vonden, dat directe toevoeging van CoQ10, maar niet van de vitaminen B2 of E, noch een mengsel van α-lipoic acid en acetylcarnitine, de spierprestatie van continu gestimuleerde EDL spier, van zowel de wild type als de CF muis, positief beïnvloed kon worden.

Aangezien we nu het effect van directe toediening van CoQ10 hebben onderzocht, zouden toekomstige studies moeten onderzoeken of eenzelfde effect bereikt kan worden met het geven van CoQ10 in het dieet.



DANKWOORD

De volgende opsomming is in willekeurige volgorde van belangrijkheid. Dit proefschrift zou nooit zonder een van de onderstaande personen in deze vorm tot stand zijn gekomen, jullie vormden samen een warm bad waarin ik mijn eureka kon uitroepen.

Allereerst alle CF kinderen en hun ouders. Jullie inzet, enthousiasme en doorzettingsvermogen is een voorbeeld voor een ieder en zorgt ervoor dat je weet waarvoor je onderzoek doet. En natuurlijk ook de controle kinderen bedankt voor hun donatie van bloed.

Roderick, jou ideeën over hoe een artikel in elkaar moet zitten kwamen niet altijd overeen met de mijne, en de artikelen zijn dan wel geen spannend boek geworden maar hebben wel geleid tot veel spanning. Dank voor je inzet, je heldere kijk en de tijd die in de vele correcties is gaan zitten. Misschien moet je toch maar geloven aan het gebruik van automatische spellingscontrole, in plaats van het woordenboek.

Jeroen, jou geloof in mij was een enorme stimulans. Je liet me inzien dat het doen van proefdieronderzoek ook zelfs door iemand zonder proefdierervaring met precisie en toewijding gedaan kan worden en de mooie resultaten zeggen de rest. Jouw immense energie en enthousiasme waren aanstekelijk en maakte het doen van proefdieronderzoek niet meer eng, maar leuk, interessant en inspirerend.

Peter, onze nauwe samenwerking in mijn eerste onderzoeksproject is de basis geweest voor veel van mijn verdere onderzoeken. Je enthousiasme, je energie om de kinderen tot hun beste prestaties op te zwepen, je SPSS kennis en natuurlijk je gezelligheid, was een uitstekende stimulans. Jouw promotie was mijn goede voorbeeld waarvan de vrucht nu voor je ligt.

Vincent, we hebben tussen de bloedafnamen door heel wat uren met de CF kinderen video's gekeken en spelletjes gespeeld. En met het rondbrengen van de studievoedingen hebben we aardig wat kilometers West-Nederland doorgereden. De uurtjes samen achter de computer, met een mooi uitzicht op de aanvliegroute van Schiphol, waren soms zwoegen, maar het resultaat was er ook naar. Veel succes met het afronden van jou proefschrift.
Hans, bedankt voor je scherpe kijk op het gehele proces van opzet van de studie tot het afronden van het artikel.

Alle medewerkers van het CF-centrum, Tineke, Sylvia, Mirjam, Ingeborg en Joyce. Bedankt voor jullie inzet, secretariële ondersteuning, en natuurlijk de gezelligheid.

Het CF-team: de pulmo-dokters Kors, Han, Bert, en Walter. Han en Kors, bedankt voor jullie nuttige en opbouwende adviezen met betrekking tot het onderzoek en de artikelen.

En natuurlijk ook Jan, Cora, Willy, Peter en Gerben. Ik bewonder jullie toewijding in de zorg voor de CF patiënten.

Ruud en Wietse, bedankt voor onze voortgangsgesprekken en jullie commentaar op mijn stukken.

Judith en Jan, jullie collegialiteit zorgde ervoor dat ik de gelegenheid kreeg mijn proefschrift binnen niet al te lange tijd af te ronden.

Arend, je hebt uitstekend voor mijn muizen gezorgd en ik was blij dat jij het euthanaseren het grootste deel van de tijd tot je rekening nam, aangezien ik de keren dat ik het zelf moest doen, het ervoer alsof ik het zelf moest ondergaan. Arie, je apparatuurtechnische en computertechnische ondersteuning was onmisbaar.

Ron, bedankt voor je laboratoriumtechnische bijdrage.

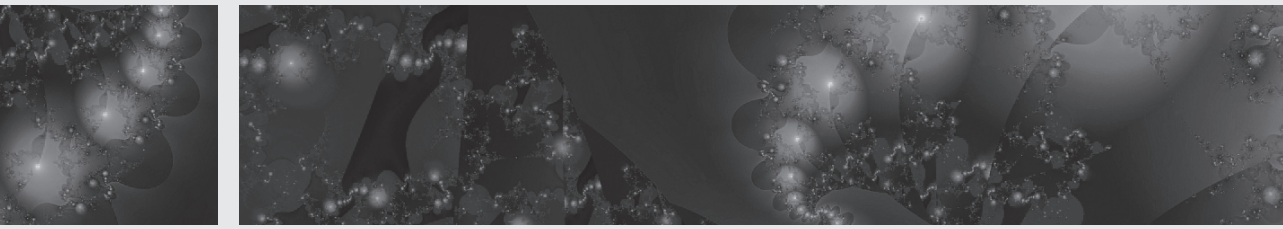
Wouter en Robin, bedankt voor de laboratoriumtechnische en inhoudelijke ondersteuning en de prettige samenwerking.

Fenneke, Anke en Michiel, jullie hebben met je onderzoeksstage voor een mooie bijdrage gezorgd. En Fenneke, ons gezamenlijk werk moet nog bekroond worden met een mooi artikel.

Henk en Cuno, jullie bijdrage in de statistiek was erg belangrijk voor deze dame voor wie statistiek in het begin nog het gevoel gaf van het vinden van een schat op de bodem van een troebel meer.

Dank aan mijn trouwe auto, die mij, en vaak ook man en kinderen, overal heen bracht om de verse studie voedingen bij de CF kinderen af te leveren, om daarna toch nog iets leuks te kunnen gaan doen.

Tot slot, mijn lieve Bert. Jij bent altijd mijn rots in de branding geweest. Door je heldere kijk op dingen zag ik weer alles in perspectief als het tegenzat. Je wist altijd weer het begrip en geduld op te brengen als dat hard nodig was, vooral het laatste jaar. Ook dank aan mijn kinderen. Mama hoeft nu niet meer steevast achter de computer en heeft weer uitgebreid de tijd om meer dan één verhaaltje voor te lezen en leuke dingen met jullie te doen in het weekend.



CURRICULUM VITAE

Johanna Hermiena (Annemarie) Oudshoorn werd geboren op 27 juli 1966 in Rotterdam. Ze volgde het middelbaar onderwijs aan de S.G. "De Krimpenerwaard" en S.G. "Van Oldenbarneveldt" en deed eindexamen VWO-B in 1984. Via het uitwisselingsprogramma van de Educational Foundation for Foreign Study (EFFS) verbleef ze een jaar in Erskine, Minnesota, USA en volgde daar de 12th grade op de high-school en was actief in vele buitenschoolse activiteiten (muziek, toneel, sport). In 1985 startte ze haar studie Geneeskunde aan de Erasmus Universiteit te Rotterdam. Tijdens de studie heeft ze gewerkt als student/verpleegkundige in het Medisch Studententeam van het Sophia Kinderziekenhuis, alwaar haar interesse voor de ziekte Cystic Fibrosis al werd gewekt. Na het behalen van het artsexamen in 1993, was zij een jaar arts-assistent kindergeneeskunde in het Academisch Medisch Centrum van Amsterdam op de afdeling Kinderchirurgie, gevolgd door een jaar in het Reinier de Graaf Gasthuis te Delft onder leiding van Dr. P.J.C. van der Straaten.

Daar bracht zij later ook haar perifere stage kindergeneeskunde door. In december 1995 startte ze met haar opleiding kindergeneeskunde in het Leids Universitair Medisch Centrum onder leiding van Prof. J.M. Wit. Na het behalen van de kinderartsentitel in augustus 2000 is ze aansluitend gestart als fellow-kindergastroenterologie in het Wilhelmina Kinderziekenhuis, UMC Utrecht, hetgeen ze in december 2004 voltooide. In september 2000 is ze gestart met onderzoek binnen het Cystic Fibrosis Centrum Utrecht/afdeling kindergastroenterologie, wat geleid heeft tot dit proefschrift. Annemarie is gehuwd met Bert Pos en heeft tijdens haar loopbaan van arts-assistent tot kindergastroenteroloog drie kinderen gekregen, Ruben, Lyanne en Laurens.