Use of (-)-Epicatechin in the Treatment of Becker Muscular Dystrophy (Pilot Study)

NCT01856868

March 8, 2013

UCD0113: An open-label pilot study of purified tea-derived epicatechin to improve mitochondrial function, strength and skeletal muscle exercise reponse in Becker Muscular Dystrophy

Original: March 8, 2013

STUDY COORDINATING CENTER: Neuromuscular Medicine and Rehabilitation Research Center
Department of Physical Medicine and Rehabilitation
University of California, Davis

STUDY CHAIR: Craig M. McDonald, MD
University of California, Davis

STUDY CO-CHAIR: Erik Henricson, MPH
University of California, Davis

CO-INVESTIGATOR(S): Richard T. Abresch, MS
Lee-Way Jin, MD, PhD
Frank Sharpe, PhD
Glenn Jickling, PhD
Bjorn Oskarsson, MD
University of California, Davis

DATA MANAGER(S): Evan de Bie, BS
University of California, Davis

PROJECT COORDINATOR: TBA

UCD0113: An open-label pilot study of purified tea-derived epicatechin to improve mitochondrial function, strength and skeletal muscle exercise reponse in Becker Muscular Dystrophy

KEY CONTACTS

STUDY CHAIR
Craig M. McDonald, MD
Professor and Chair of Physical Medicine and Rehabilitation
University of California, Davis
4860 Y Street, Suite 3850
Sacramento, CA 95817
TEL: (916) 734-2923
FAX: (916) 734-7838
E-Mail: cmmcdonald@ucdavis.edu

PROJECT COORDINATOR
TBA

PROJECT CENTRAL PHARMACIST
TBA from UC Davis Investigational Drug Pharmacy

INDEPENDENT MEDICAL MONITOR
TBA (from UC Davis)

PROTOCOL SIGNATURE PAGE

UCD0113: An open-label pilot study of purified tea-derived epicatechin to improve mitochondrial function, strength and skeletal muscle exercise reponse in Becker Muscular Dystrophy

Original: March 8, 2013

Instructions to Investigators: Please sign and date this signature page, print your name, your title and the name of the facility in which the study will be conducted, and return a copy of this page to the Project Coordinator.

I confirm that I have read this protocol, I understand it, and I will work according to this protocol and to the ethical principles stated in the latest version of the Declaration of Helsinki, the applicable guidelines for good clinical practices, and the applicable laws and regulations of the country of the study site for which I am responsible, whichever provides the greater protection of the individual. I will accept the monitor's overseeing of the study.

Signature of Investigator	Date
Printed Investigator Name	Investigator Title
Name of Facility	Location of Facility (City, State, Country)
Signature of Representative of Study Sponsor	Date

TABLE OF CONTENTS

TABLES

FIGURES

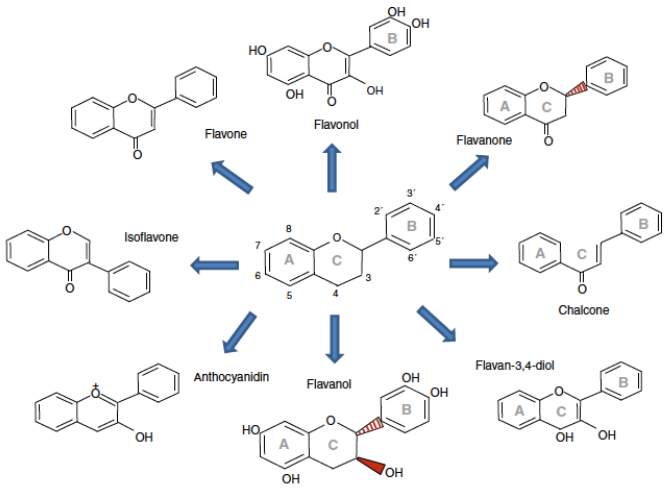
1 STUDY SUMMARY

1.1 Background

(-)-Epicatechin will be evaluated for the treatment of progressive muscle loss and impaired skeletal muscle function in Becker Muscular Dystrophy (BMD) patients. (-)-Epicatechin has been shown to improve muscle structure and function in animal and human studies by inducing muscle proteins, mitochondrial biogenesis and endogenous anti-oxidant mechanisms. (-)-Epicatechin and (-)-epicatechin-rich preparations have been well-tolerated with no significant safety issues, suggesting that (-)-epicatechin will be compatible with chronic administration. GMP grade (-)-Epicatechin will be provided by Cardero Therapeutics (CT).

(-)-Epicatechin (-)-Epicatechin is a naturally occurring compound found in many plants and fruits, including cocoa seeds, tea and grape [1, 2]. (-)-Epicatechin is a member of the flavanol subfamily of flavonoids, a chemically defined family of plant polyphenols (Figure 1) that have a basic structure of two aromatic rings (A and B) linked through three carbons that typically form an oxygenated heterocycle (C ring). The chemical characteristics of the C ring define the various subfamilies of flavonoids by providing different arrangements of hydroxy, methoxy, and glycosidic groups, and the bonding with other monomers [3].

Figure 1: Chemical structures of flavonoid families and (-)-Epicatechin



The majority of studies of (-)-epicatechin have utilized flavanol-rich cocoa preparations or concentrated tea extracts. Common commercial products that contain 60-90% cocoa can provide a total of ~12 mg of flavanols (monomers and multimers)/gram of chocolate. For example, 60% cocoa chocolate by Hershey will provide 0.84 mg/gram of (-)-epicatechin and 10.6 mg/gram of total flavanols (9.8 mg as multimers). (-)-Epicatechin, monomeric, isolated from tea, will be supplied by Cardero Therapeutics in gelatin capsules, each containing 25 mg (-)-epicatechin combined with excipients.

Therapeutic Activities Recent studies by scientists at the University of California, San Diego (UCSD) have suggested that (-)-epicatechin could reverse progressive muscle loss associated with aging and muscular dystrophy through stimulation of muscle-promoting proteins and mitochondrial biogenesis, and reduction in tissue oxidative stress, inflammation and fibrosis [4-7]. A detailed description of published and unpublished preclinical and clinical data supporting the evaluation of (-)-epicatechin in BMD is presented in Sections 5 and 6.

It must be stressed that these muscle-related therapeutic activities are unique to (-)-epicatechin and do not represent general flavonoid properties, as other flavonoids such as quercetin fail to exert similar effects. (-)-Epicatechin biology is chirally specific, as its diastereomer, catechin, is not only inactive in stimulating mitochondrial biogenesis, it antagonizes (-)-epicatechin's effects, possibly due to steric competition at a binding site. Nor is the effect due to anti-oxidation, as catechin and quercetin are anti-oxidants equal in potency to (-)-epicatechin but are inactive with respect to mitochondria.

Cocoa flavonoids have also been intensively studied as potential therapies due to their antioxidant and cardiovascular protective activities (reviewed in [8, 9]), and a recent meta-analysis of randomized controlled studies of flavonoid-rich cocoa (FRC) determined that FRC consumption significantly improves several cardiovascular risks, including blood pressure, insulin resistance, lipid profiles, and flow-mediated vascular dilation (FMD)[10]. (-)-Epicatechin was shown to be the FRC component primarily responsible for positive effects on vascular endothelium [11], mediated in part by stimulation of the nitric oxide pathway, which plays a pivotal role in maintaining vascular endothelial health (reviewed in [12-14]).

Safety and Pharmacokinetics Published reports indicate that (-)-epicatechin and flavanol-rich extracts have thus far been well tolerated in animals and humans, with no significant adverse events have been reported in acute or chronic studies (reviewed in [8, 9]). Human pharmacokinetic (PK) studies in the literature indicate that (-)-epicatechin is orally bioavailable, with well defined absorption (plasma T_{max} ~ 2 hours) and clearance (plasma elimination half-life of ~2 hours) rates for (-)-epicatechin and its major metabolites [15-22]. The majority of (-)-epicatechin and metabolites are cleared from plasma by 8 hours. Recently a human PK study was completed at the University of San Francisco (UCSF) evaluating (-)-epicatechin provided by Cardero Therapeutics; this study is described in detail in Section 6.3.

Preclinical Studies - University of California, San Diego (UCSD) Collaborators at UCSD examined the muscle-promoting effects of (-)-epicatechin in human skeletal muscle cells *in vitro* and in mice and rats. In human cells treated with (-)-epicatechin in culture, treated cells had a statistically significant increase in mitochondrial cristae membrane area relative to controls, suggesting (-)-epicatechin-treated cells have a greater capacity of ATP generation (unpublished data). In mice, significant increases in muscle performance, myocardial angiogenesis and indicators of mitochondrial structure and biogenesis were observed in (-)-epicatechin-treated animals [4]. A comparison of recognized regulators (markers) of skeletal muscle (SkM) growth (myostatin, follistatin), differentiation (myogenin, MyoD, MEF2A, Myf5) and senescence (senescence-associated β-galactosidase ,SA-β-Gal) in young and old mice revealed that aging is associated with decreases in muscle-promoting capacity (manuscript submitted for publication). Two weeks of (-)-epicatechin (1 mg/kg bid) significantly improved the profile of muscle markers in old mice. Recent (-)-epicatechin studies in the mdx and δ-sarcoglycan KO mouse models of muscular dystrophy (MD) have shown positive structural and functional effects on damaged muscle, supporting the evaluation of (-)-epicatechin as a myopathy therapy for BMD patients.

Completed Clinical Studies Three clinical studies have been completed by collaborators of Cardero Therapeutics. Two proof of concept clinical studies were conducted at UCSD to examine the effects of (-)-epicatechin on skeletal muscle (SkM) structure and function and mitochondrial biogenesis, while a pharmacokinetic (PK) study in healthy volunteers was conducted at UCSF. These studies are described in detail in Sections 6.1-6.3.

The first study was performed in five heart failure, type 2 diabetes mellitus patients using (-)-epicatechin-enriched cocoa to assess the effects on skeletal muscle mitochondria structure and indicators of mitochondrial biogenesis [7]. Apparent major losses in normal mitochondria and muscle (sarcomere) structure were observed before treatment. Epicatechin-enriched cocoa increased protein and/or activity of mediators of biogenesis and cristae abundance, and in a follow-up study, reduced markers of tissue oxidative stress. Increases in several muscle structural proteins, including dystrophin, were noted, as well as improvements in sarcomere organization.

In the second study, healthy adult subjects (average age of 41 years) were treated for 7 days with 25 mg of (-)-epicatechin in capsules BID in order to assess treatment effects on muscle strength and circulating markers of SkM growth (follistatin, myostatin). A pre-study examination of tissue bank muscle samples from young and old subjects had determined that aging is associated with a decrease in follistatin (promoter of muscle growth) and an increase in myostatin (inhibitor of muscle growth), in addition to changes in other markers of muscle growth and differentiation. (-)-Epicatechin treatment significantly improved hand-grip strength and increased the ratio of follistatin to myostatin in the plasma. Taken together, these POC clinical results suggest that (-)-epicatechin may be a useful therapy for the progressive loss of muscle function associated with Becker Muscular Dystropy.

In the PK study, (-)-epicatechin was well tolerated over the 50-200 mg dose range, with rapid absorption and first pass metabolism. (-)-Epicatechin and its metabolites were rapidly cleared from the body with a plasma elimination half-life of approximately 2.5 hrs for the 100 and 200 mg doses. Plasma concentration of (-)-epicatechin was generally proportional to the administered dose.

1.2 Study Purpose

This is a proof-of-concept phase 1/2a pilot and endpoint development study that is designed to provide initial evidence of biological activity of (-)-epicatechin. Primary endpoints include initial assessment of tissue-specific evidence of efficacy from muscle biopsy samples. Secondary endpoints include measures of strength and physical function, and safety and adverse event data. Pilot endpoints include assessment of mRNA and miRNA peripheral blood profiles and validation of non-invasive near-infrared spectroscopy (NIRS) muscle perfusion studies during exercise and a recumbent cycle exercise test that may be employed as endpoints in future clinical trials.

1.3 Study Design

This single center open-label pilot study will enroll 10 adults with genetically-confirmed Becker muscular dystrophy, who will receive the purified nutritional extract (-)-epicatechin 100mg/day orally for 8 weeks. After screening visits, participants will be enrolled in the study if they meet all inclusion criteria. They will be evaluated at baseline and at screening, day 1, and weeks 1, 2, 4 and 8. The main criterion for success of the study will be presence of one or more biologic or strength and performance outcome measures (Aims 1 and 2 below) that yield a response magnitude that allows for sufficient power in a Phase II B study with a sample size of 30 individuals.

Evaluations of efficacy will include:

- Assessment of peripheral blood creatine phosphokinase, follistatin, (-)-epicatechin pharmacokinetics, blood lactate during exercise, and baseline and post-treatment mRNA and miRNA biomarker profiles.
- Assessment of baseline and post-treatment muscle biopsy by histology, Western blot, immunostain and electron microscopy.
- Assessment of strength by isokinetic dynamometry and quantitative grip testing, exercise performance during a standardized recumbent cycle test, muscle perfusion of the quadriceps by NIRS during the exercise test, a 6-minute walk test, and body composition by dual-energy x-ray absorptiometry (DEXA).

Evaluations of safety will include comprehensive review of

- vital signs, height and weight
- medical history
- physical and neurological exam
- previous and concomitant medication history
- electrocardiogram
- laboratory safety panels for hematology and coagulation, standard blood chemistries (lipid, hepatic and metabolic profiles)

This is a greater than minimal risk study with prospect for direct benefit. A medical monitor will review safety-related evaluations throughout the duration of the study.

1.4 Specific Aims

Aim 1. (Efficacy Primary Endpoint): Evaluation of (-)-epicatechin on blood and muscle tissue markers of mitochondrial biogenesis and muscle regeneration in adults with Becker muscular dystrophy. Treatment with 8 weeks of (-)- epicatechin 100mg daily will induce mitochondrial biogenesis, muscle regeneration, and improved histological appearance in sarcomere morphology.

Hypothesis: (-)- Epicatechin will show evidence of mitochondrial biogenesis by Western blot assays of biceps brachii muscle biopsies through increases in ETC1, ETC5 and SOD2 proteins.

Hypothesis: (-)- Epicatechin will show evidence of induction of muscle regeneration through increases in PGC1a, decreases in acetylated (inactive) PGC1a, and and increases in follistatin,

myogenin, and MyoD compared to pre-treatment baseline as determined by Western blot assays of biceps brachii muscle biopsies.

Hypothesis: Administration of (-)- epicatechin will be associated with increases in plasma concentrations of follistatin, a known hormonal regulator of muscle regeneration and a potential pharmacodynamic biomarker.

Hypothesis: Administration of (-)- epicatechin will be associated with electron microscopy evidence of increases in mitochondrial number and cristae density within the mitochondria compared to pre-treatment baseline.

Hypothesis: Administration of (-)- epicatechin will be associated with increased nNOS expression in skeletal muscle compared to pre-treatment baseline and this increased expression will be associated with improved muscle perfusion during exercise.

Aim 2 (Efficacy Secondary Endpoints): Evaluation of the effects of (-)-epicatechin on exercise capacity in adults with Becker muscular dystrophy. Individuals with Becker muscular dystrophy who receive 100mg/day of (-)-epicatechin will demonstrate improved exercise performance and strength that is associated with increases in mitochondrial biogenesis and muscle regeneration.

Hypothesis: Using the 6 minute walk test, and a standardized recumbent cycle exercise test with measurement of lactic acid levels, VO_2 max, Work max, anaerobic threshold, and NIRS-defined skeletal muscle perfusion (measures of oxyhemoglobin, deoxyhemoglobin, and tissue saturation index) we will detect pre-/post-treatment differences in exercise capacity and endurance that will be clinically significant (≥ 5-10%) and reflect increases in mitochondrial biogenesis.

Hypothesis: Changes in grip strength (≥ 5%), elbow flexion/extension isometric strength (≥ 5%), knee extension isometric muscle strength (≥ 1 pound = 4.448 Newtons), pulmonary function derived maximal static airway pressures (MIP and MEP ≥ 10 cm H_2O), and regional lean tissue mass by DEXA (≥ 2%) will be clinically significant and correlate with biomarker evidence for muscle regeneration and histological improvement in sarcomere morphology.

Hypothesis: Pilot data from exercise performance and strength evaluations in adults with Becker muscular dystrophy will determine the magnitude of response over time that will enable accurate power calculations for future clinical trials.

Aim 3 (Safety Secondary Endpoints): Evaluation of safety and pharmacokinetic profiles of (-)-epicatechin in adults with Becker muscular dystrophy. Assessments of safety will include a standard clinical safety panel including hematologic, hepatologic, renal and metabolic profiles. Pharmacokinetic studies will include repeat assessments of trough, 2 hour-post (peak) and 4-hour post dose (-)-epicatechin levels.

Hypothesis (safety): (-)-epicatechin given at 100mg/d orally will display an acceptable safety profile in BMD patients.

Hypothesis (pharmacokinetics): (-)-epicatechin given at 100mg/d orally in BMD patients will produce pharmacokinetic profiles similar to those seen in previously described studies in the literature.

Aim 4 (Pilot Biomarker Endpoints): Pilot evaluation of disease- and epicatechin-specific circulating mRNA and miRNA blood profiles as pilot biomarkers for monitoring treatment efficacy. Epicatechin stimulates follistatin expression in tissue and blood. In addition to its effects on muscle regeneration, follistatin is known to exert anti-inflammatory and anti-fibrotic effects via antagonism of activin and myostatin.

Hypothesis: Using gene array assays directed toward fibrosis- and inflammatory-oriented pathways, we expect treatment with (-)-epicatechin to induce detectable pre-/post-treatment differences in mRNA and miRNA expression that may be utilized as biomarkers of treatment effect for future clinical trials.

2 BACKGROUND AND SIGNIFICANCE

2.1 Becker Muscular Dystrophy

Muscular dystrophies are a group of diverse genetic diseases featuring progressive muscle weakness, degradation of muscle fibers, and loss of function [23]. BMD is an inherited disease primarily seen in males, with an incidence of 3-6 per 100,000 live births [24]. Onset of symptoms can occur over a wide age range, with most patients diagnosed between 5 and 15 years of age [25]. BMD is characterized by progressive muscle loss (sarcopenia), with loss of strength, muscle injury, degeneration, atrophy, and eventually fibrosis [25]. Most patients can no longer walk by age 25-30, and lung (e.g., breathing problems, infections) and heart (e.g., cardiomyopathy) complications can significantly reduce lifespan [25].

Many muscular dystrophies, including BMD and the more common Duchenne Muscular Dystrophy (DMD), result from mutations in the gene encoding dystrophin, a key subsarcolemmal protein in the dystrophin-associated protein complex (DAPC) at the muscle cell membrane [26, 27]. The DAPC links the muscle cytoskeleton with the extracellular matrix and appears to play a role in muscle stabilization during contraction as well as nNOS signaling [28, 29]. Loss of dystrophin destabilizes the DAPC and results in abnormal muscle membrane permeability, detectable as large elevations in plasma creatine kinase at birth and before the appearance of physical symptoms [30]. BMD is attributed to quantitative or qualitative decrements in dystrophin expression rather than complete or near-complete loss of dystrophin as in DMD.

While a dystrophin mutation is the primary defect, the progressive loss of muscle function in BMD is due to a secondary cascade of chronic destructive events, including calcium influx and overload, mitochondrial swelling and apoptosis, disruption of nNOS/NO leading to muscle ischemia during exercise, inflammation, increased levels of ROS (oxidative stress) and protease activation, that eventually exhaust muscle repair mechanisms and result in muscle degeneration [26-31]. Mitochondrial damage appears to play a central role in this muscle damage cascade as evidenced by studies in sarcopenia. SkM from older subjects demonstrate increases in mitochondrial ETC abnormalities (accumulation of cytochrome c oxidase negative and succinate dehydrogenase hyper-positive fibers), increases in multiple markers of oxidative stress, accumulation of mtDNA mutations, and transcriptome changes indicative of mitochondrial dysfunction [32-34]. Studies in mitochondrial DNA mutator mice (mtDNA polymerase proof-reading mutation) demonstrated that mutations in mtDNA leads to skeletal muscle apoptosis and sarcopenia [35]. Myocyte apoptosis, the end result of severe mitochondrial dysfunction, is mediated in part by the opening of the mitochondrial permeability transition pore (mPTP), the rupture of the outer mitochondrial membrane and release of mitochondrial apoptotic signals [36]. In the absence of genetic repair, there is a great unmet clinical need for palliative therapies that can slow the progressive loss of muscle function due to this secondary damage cascade and/or improve skeletal muscle function.

Published studies in mouse models of MD support targeting the secondary damage cascade to preserve mitochondria and improve muscle function. Muscle cell calcium overload can lead to calcium-induced mitochondrial permeability transition (MPT), resulting in swelling, loss of function, rupture and necrotic cell death [37, 38]. Genetic or pharmacologic inhibition of cyclophilin D, the enzyme that regulates calcium-dependent MPT, in mouse models of MD (mdx, scgd$^{-/-}$) significantly reduced mitochondrial swelling, improved myofiber organization and reduced fibrosis and necrosis [39]. In a separate study, stimulating mitochondrial metabolism by the provision of an endurance mimetic compound AICAR, which activates AMP-activated protein kinase (AMPK) activity and promotes mitochondrial biogenesis and metabolism, led to markedly improved muscle function and overall activity in mdx mice and restored structural integrity of muscle fibers [40].

2.2 Justification of Current Approach

(-)-Epicatechin has been shown to improve muscle structure and function by positively influencing several aspects of the secondary damage cascade leading to progressive muscle loss in MD. These animal and human data are outlined in Table 1. (-)-Epicatechin is an attractive therapeutic candidate due to combination of its muscle-restoring and promoting activities and excellent safety profile.

Table 1: Effects of (-)-epicatechin on progressive muscle loss parameters.

Progression of MD due to:	(-)-Epicatechin Effects
Loss of Dystroglycan Proteins	Stimulated the expression of multiple components of the dystroglycan protein assembly in mice and humans, including compensatory expression in a MD mouse model
Mitochondrial Depletion	Increased expression of transcription factor PGC1α and stimulated mitochondrial biogenesis, increased expression of all electron transport complex proteins and increased mitochondrial density and mitochondria cristae density in mouse and human muscle
nNOS Deficiency	In a pilot clinical trial in patients with diabetes and heart failure, (-)-epicatechin-treated patients showed a statistically significant increase in quadriceps muscle nNOS expression
Oxidative injury	Increased the expression of endogenous anti-oxidant enzymes superoxide dismutase and catalase and increased total thiols in skeletal muscle of aged patients with diabetes and heart failure
Muscle Degeneration	Reduced creatine kinase activity (plasma marker for muscle membrane injury); improved histological appearance in sarcomere morphology in aged patients with diabetes and heart failure
Impaired Muscle Regeneration	Increased markers of muscle regeneration in skeletal muscle of mdx mice and aged patients with diabetes and heart failure
Muscle Weakness	Improved muscle strength in mdx mouse model of MD and physical activity in the delta Sarcoglycan KO mouse model of limb girdle dystrophy; increased grip strength in middle-aged human volunteers
Inflammation And Fibrosis	Prevented the myocardial fibrosis associated with the δ-sarcoglycan KO mouse model of MD

3 EPICATECHIN OVERVIEW

3.1 Pharmacokinetics / Pharmacodynamics

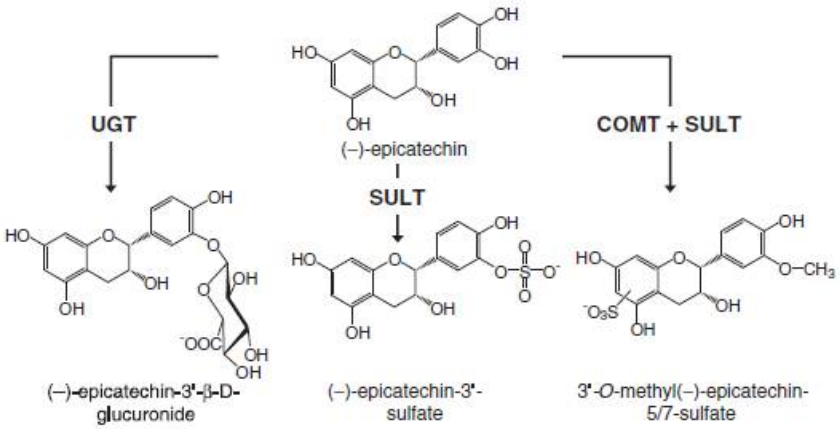
(-)-Epicatechin is orally bioavailable, as the consumption of (-)-epicatechin or cocoa products can result in pharmacologically relevant levels of (-)-epicatechin and its metabolites in blood. In addition to the literature reviewed below, the results of a recent human pharmacokinetic study are presented in Section 6.3.
The absorption of flavonoids occurs mainly in the small intestine and takes place within minutes. In the small intestine, flavanols are extensively glucuronidated and partially methylated [41, 42], allowing negligible amounts of native (-)-epicatechin in the mesenteric circulation.

In the liver, further glucuronidation, methylation, and sulfation can take place [41, 43, 44]. Several studies have determined the presence of these conjugates in the plasma and urine of rodents and humans [15, 19, 44-47], as well as in rat bile [43] and brain [48]. In plasma, (-)-epicatechin is present almost exclusively as conjugated metabolites [19, 47] .

Total plasma concentrations of (-)-epicatechin plus its metabolites are found in the low-micromolar range as soon as 1 h after the consumption of a flavanol-rich food (cocoa), with T_{max} ~2 hours [11, 18-21, 45, 47-50]. The most abundant (-)-epicatechin metabolites detected in plasma after dark chocolate ingestion were (-)-epicatechin-3'-β-D-glucur- onide,(-)-epicatechin3'-sulfate,and 3'-O-methyl-(-)-epicatechin-5-sulfate [47]. A schematic representation of these metabolites is shown in Figure 2. In addition, significant levels of 3'-O-methyl-(-)- epicatechin sulfates substituted in the 4' and 7 positions were identified [47].

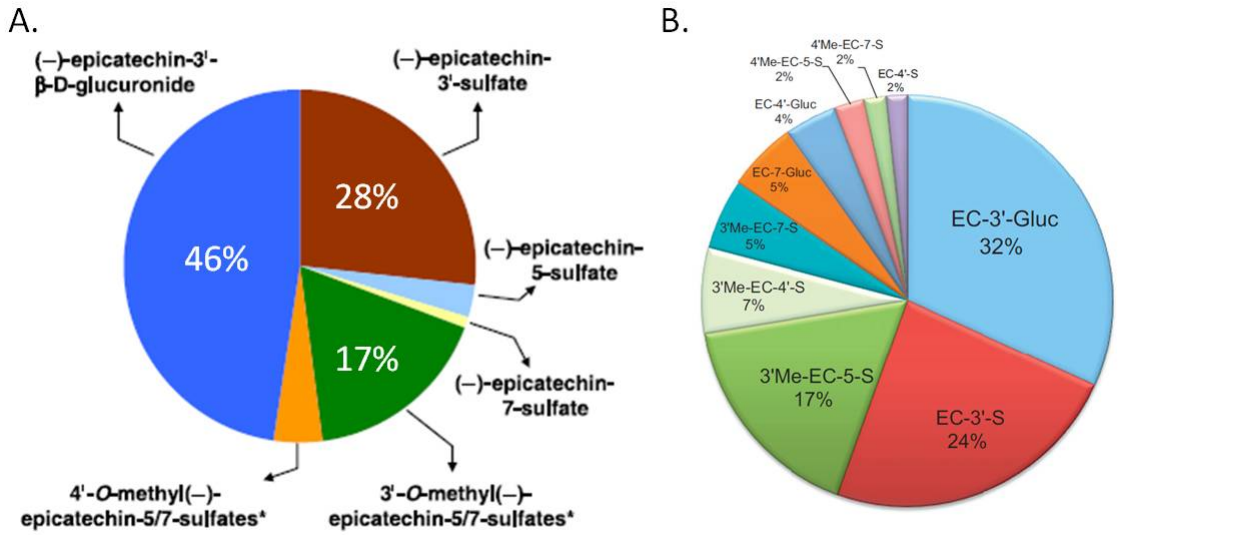
Relative amounts of (-)-epicatechin metabolites identified in plasma in two separate studies are shown in Figure 3.

Figure 2: Major (-)-epicatechin metabolites in plasma.



Schematic representation of the primary structurally-related (-)-epicatechin metabolites found in plasma after injestion of (-)-epicatechin-containing test beverage [19]. UGT, UDP-glucuronosyltransferase; SULT, sulfotransferase; COMT, catechol O-methyltransferase.

Figure 3: (-)-Epicatechin metabolite profile in plasma



A. *(-)-Epicatechin metabolite profile in plasma 2 hours after ingestion of 1.8 mg/kg body weight (-)-epicatechin [19]. Unmodified (-)-epicatechin represented <0.5%.* ***B.*** *Profile of (-)-epicatechin metabolites in plasma after ingestion of 100g of dark chocolate ((79 mg (-)-epicatechin)) [47]. Data are expressed as the percentage of total plasma AUC0–24 h. Unmodified (-)-epicatechin not detected. EC-3'-Gluc, (-)-epicatechin-3'-β-D-glucuronide; EC-4'-Gluc, (-)-epicatechin-4'- β-D-glucuronide; EC-7-Gluc,(-)-epicatechin-7-β-D-glucuronide; EC-3'-S, (-)-epicatechin-3'-sulfate; 3'Me-EC-5-S, 3'-O-methyl-(-)-epicatechin 5-sulfate; 3'Me-EC-4'-S, 3'-O-methyl-(-)-epicatechin 4'-sulfate; 3'Me-EC-7-S, 3--O-methyl- (-)-epicatechin 7-sulfate; and 4'Me-EC-5-S, 4'-O-methyl-(-)-epicatechin 5-sulfate.*

The T_{max} (time to maximum plasma concentration) of (-)-epicatechin and most of its metabolites is 1-2 hours, elimination half-time ($t_{1/2}$) from plasma is ~2-2.5 hours, and over 90% of the urinary excretion of these compounds was complete by 8 hours [17, 19, 21].
Oral bioavailability (peak plasma concentration/mg ingested) for (-)-epicatechin in cocoa/chocolate is ~10-20 nM/mg injested (Table 2). Assuming ~3L plasma in a 75 kg man [51], oral bioavailability of (-)-epicatechin (% ingested dose in plasma at Cmax, total epicatechin and metabolites), can be estimated as 1-2%. A value of 1.1% was reported for a 45 mg (-)-epicatechin dose in a green tea catechin mixture [17].

Table 2: Selected human oral bioavailability studies of (-)-epicatechin

Reference	Test Article	(-)-Epicatechin (mg)	Peak (-)-Epicatechin Species in Plasma (nM)	Peak (-)-Epicatechin Exposure (nM/mg)
Loke, W.M., et al., 2008 [52]	(-)-Epicatechin	200	3570	17.85
Ottaviani, J.I., et al., 2011 [18]	(-)-epicatechin added to cocoa vehicle	125 (mean)	~900	~7.2
Ottaviani, J.I., et al., 2012 [19]	**Cocoa drink**	**135 (mean)**	1245	9.3
Actis-Goretta, L., et al., 2012 [47]	**Dark Chocolate**	**79**	873	11.1
Donovan, J.L., et al., 2006 [53]	**Cocoa drink**	**55**	630	11.5
Schroeter, H., et al., 2006 [11]	**Cocoa flavanols:** 917 mg	174	~1950	~11.2
Baba, S., et al., 2000 [15]	**Dark chocolate**	**220**	4770	21.7

Oral doses of (-)-epicatechin between 25 and 200 mg have been shown to be pharmacologically relevant. A 200 mg oral dose increased measures of plasma NO and decreased the vasoconstrictor endothelin-1 [52], while administration of an ~75 mg dose (1 mg/kg body weight) resulted in significant improvement in vascular function (FMD) [11]. A recent meta-analysis of clinical data reported that an intake of 25 mg (-)-epicatechin per day was associated with significant reductions in SBP and DBP [54].

4 PRELIMINARY STUDIES

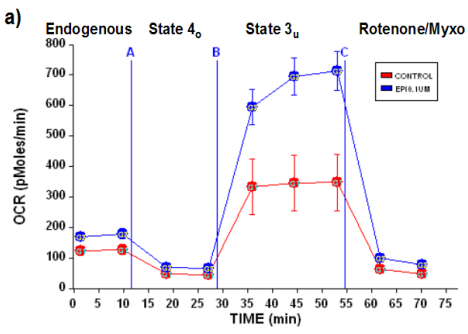
4.1 Preclinical Data

Collaborators of Cardero Therapeutics at the University of California, San Diego (UCSD) have conducted cell culture and animal studies examining the ability of (-)-epicatechin to induce mitochondrial biogenesis and SkM protein synthesis, resulting in improved muscle structure and function.

4.1.1 (-)-Epicatechin Increases Mitchondrial Respiration and Biogenesis in Cultured Human Skeletal Muscle Cells (unpublished data)

The effect of (-)-epicatechin on mitochondrial respiration, biogenesis and structure in human skeletal muscle cells *in vitro* was examined. Human skeletal muscle cells from a healthy subject were treated with 0.1 µM of (-)-epicatechin (in cell culture) for 48 hours. The treated muscle cells manifested a markedly enhanced capacity for oxidative phosphorylation (Figure 4). The ability of epicatechin to stimulate the capacity for oxidative phosphorylation in human muscle fibers treated with epicatechin allows the muscle cell to substantially increase its rate of oxidative phosphorylation in response to the metabolic demand placed on the cells by FCCP, which decreases the mitochondrial membrane potential. Importantly for safety reasons, neither endogenous nor resting oxidative phosphorylation in muscle cells is affected.

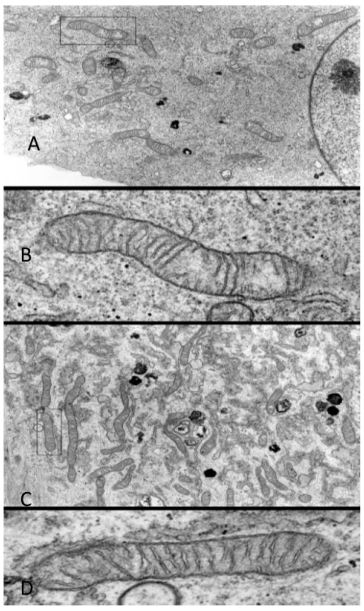
Figure 4: (-)-Epicatechin enhances mitochondrial respiration



. Treatment of Primary Cultures of Human Skeletal Muscle Myocytes with Epicatechin Enhances Maximal Rates of Mitochondrial Respiration. Effects of treatment of cultured myocytes for 48 hours with epicatechin on mitochondrial respiratory rates was monitored using the Seahorse XF24. Cells were plated at 30,000/well and treated with the indicated concentration of epicatechin in normal culture medium. Respiration (O_2 consumption rate, OCR) of the intact cells was measured in unbuffered DMEM containing 10 mM glucose, 10 mM pyruvate, and 2 mM glutamine. In **a)** endogenous respiration was measured on cells from subject 1, followed by state 4_o (resting) respiration with the addition of 1 uM oligomycin (after 'A'), and then maximal rates (state 3_u) were measured after the addition of 300 nM FCCP, a chemical uncoupler (after 'B'). Rotenone plus myxothiazol (100 nM each) was then added to assess non-mitochondrial O_2 consumption (after 'C'). In b) a dose response to epicatechin for 48 hours was performed with cells from subject 2, and maximal rates of respiration were measured with addition of 300 nM FCCP.

Treated cells were then examined using electron microscopy (Fig 5). The electron micrographs illustrate two effects. One is the marked increase in mitochondrial number. Even more unusual is the increased number of cristae per mitochondrion, suggestive of the potential for increased ATP synthesis per mitochondrion. To our knowledge, cristae density within a mitochondrion has not previously been demonstrated to be acutely modulatable before. Mitochondrial biogenesis can also be blunted by the use of eNOS inhibitors, supporting a role for the NO system in the mechanism of action for (-)-epicatechin's effects.

Figure 5: (-)-Epicatechin increases mitochondrial number and cristae density in human skeletal muscle cells (unpublished data)

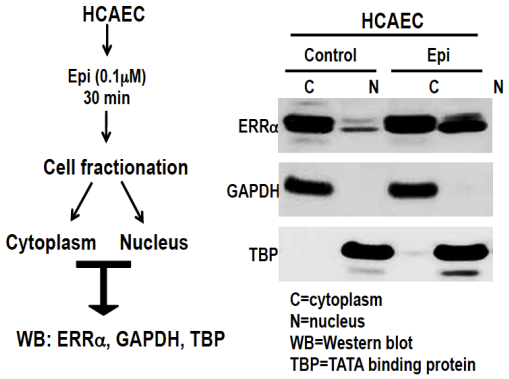


Electron microscopy of human skeletal muscle cells from healthy human subjects treated with 0.1 uM of (-)-epicatechin (in cell culture) for 48 hours are shown in panels C and D; cells from the same subject without treatment are shown in A and B. Based on blinded independent analysis of cristae/mitochondrial membrane ratio the (-)-epicatechin treated cells (1.33 ±.0 34 vs. control 0.88 ± 0.49) had statistically significant (p=0.03) more cristae membrane where the oxphos complexes are located, suggesting (-)-epicatechin-treated cells have a greater capability for ATP generation. In addition to increases in cristae membrane, treated cells also had an increased number of mitochondria.

4.1.2 (-)-Epicatechin Induces Mitochondrial Biogenesis Pathway in Human Coronary Artery Endothelial Cells (unpublished data)

Recent unpublished experiments established that (-)-epicatechin initiates mitochondrial replication via the activation of two co-factors that participate in the classical transcription pathway for mitochondrial biogenesis: The Estrogen Related Receptor (ERR) pathway and the activation of PGC1α. The ERR pathway comprises an orphan nuclear receptor complex consisting of 3 subunits, α, β and γ. When exposed to the unknown ligand, they trimerize in the cytoplasm and form a complex with PGC1α, and subsequently localize to the nucleus, where they initiate the transcription pathway for mitochondrial biogenesis [55]. Nuclear localization of the α subunit is accepted as an indicator of ERR activation. Human coronary artery endothelial cells (HCAEC) exposed to (-)-epicatechin demonstrated localization of ERRα to the nucleus within 30 minutes (Fig 6).

Figure 6: (-)-Epicatechin induces estrogen-related receptor alpha translocation (activation) into the nucleus of HCAEC



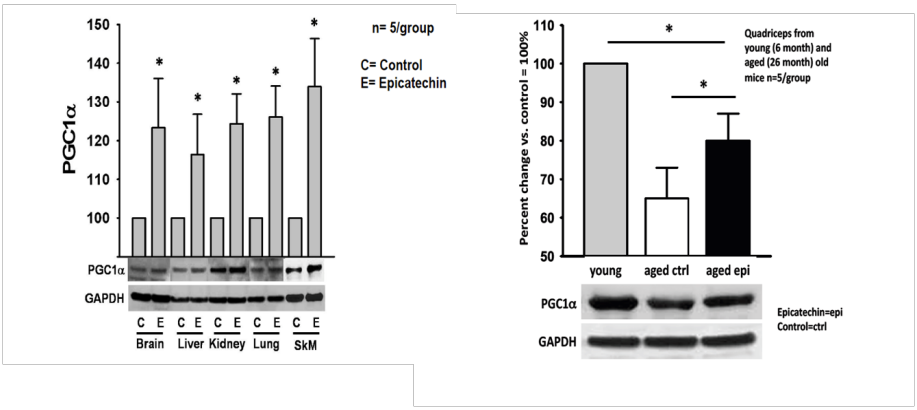
4.1.3 (-)-Epicatechin Induces Expression of Mitochondrial Biogenesis Factor PGC-1α in Aged Mice (unpublished data)

The peroxisome proliferator-activated receptor gamma (PPARγ) coactivator-1α (PGC-1α) is a transcriptional coactivator of nuclear receptors and other transcriptional factors that can enhance multiple aspects of cellular energy metabolism, including mitochondrial biogenesis and angiogenesis [56, 57]. Expression of PGC-1α in cultured mammalian cells or specific tissues of transgenic mice increases number and mass of mitochondria together with a strong enhancement of cellular respiratory capacity [58], suggesting that PGC-1α would be expected to enhance mitochondrial biogenesis and promote muscle repair and regeneration. The effects of (-)-epicatechin on PGC-1α levels were examined in young (6 mo), and old (26 mo) mice. Senile mice treated with epicatechin for two weeks exhibited increased expression of PGC-1α across all tissues evaluated (Fig 7A). These animals also demonstrated correlative mitochondrial biogenesis (not shown). When PGC-1α level of expression was compared in the senile mice to young mice, they were markedly diminished, and stimulated by epicatechin treatment in as little as two weeks (Fig 7B).

Figure 7: (-)-Epicatechin increases PGC1-alpha in aged mice.

A. Effects of epicatechin on PGC-1α in 26 month old mice tissues

B. PGC-1α in young and old (+/- Epi) mice quadriceps tissues



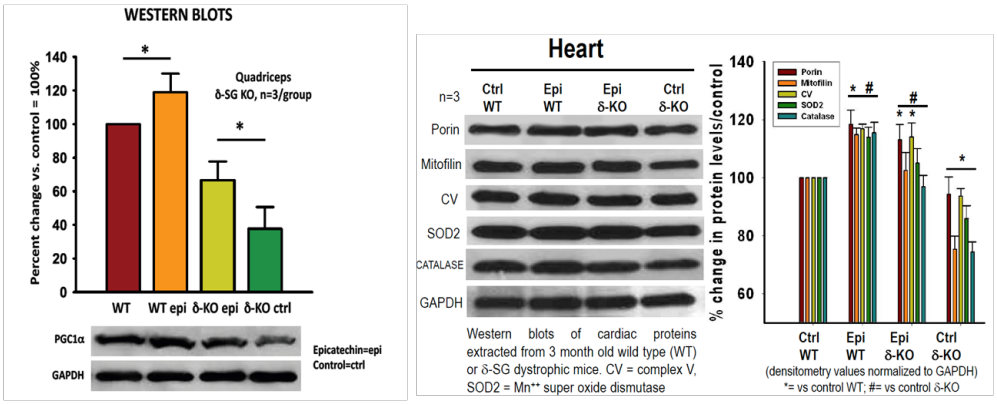
4.1.4 (-)-Epicatechin Induces Expression of Mitochondrial Biogenesis Factor PGC-1α and Mitochondrial Proteins in δ-sarcoglycan KO Mice (unpublished data)

The effects of (-)-epicatechin on PGC-1α levels were examined in the δ-sarcoglycan KO mouse model of MD. 3 month old WT and δ-sarcoglycan mice were treated with (-)-epicatechin or water for 1 month. In the δ-sarcoglycan KO mice, the loss of PGC1α is striking, as is its stimulation with 1 month of epicatechin treatment (Fig 8A). The change in PGC1α correlated with loss and regain of mitochondrial density (Fig 8B). The KO mice treated with water exhibited a significant depletion of mitochondrial proteins – enzymes and structural proteins, during a phase where they developed fibrotic cardiomyopathy. Epicatechin restored mitochondrial protein levels to normal levels, suggesting that (-)-epicatechin induced PGC-1a-mediated mitochondrial biogenesis.

Figure 8: (-)-Epicatechin increases PGC1-alpha and mitochondrial proteins in gamma-sarcoglycan knock out mice.

A. Effects of epicatechin on PGC-1α in WT and δ-sarcoglycan KO mice

B. Mitochondrial proteins in WT and δ-sarcoglycan KO mice

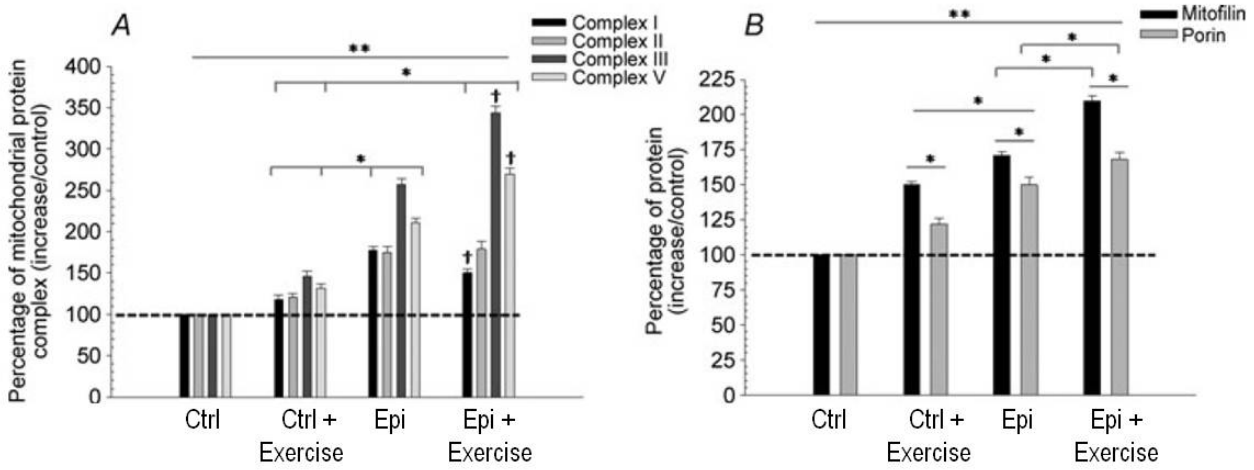


4.1.5 (-)-Epicatechin Increases I*n Vivo* Mitchondrial Biogenesis and Muscle Function in Aged 1 Year Old Mice

To test for the *in vivo* effects of (-)-epicatechin on muscle performance and indicators of mitochondrial structure (porin, mitofilin) and biogenesis (Tfam), studies were conducted in mice to compare the effects of (-)-epicatechin +/- daily exercise with vehicle +/- daily exercise [4]. Aged one year old male mice were subjected to two weeks of (-)-epicatechin treatment (1 mg/kg BID, dissolved in water) by oral gavage. Significant increases in treadmill performance (≈50%) and enhanced in situ muscle fatigue resistance

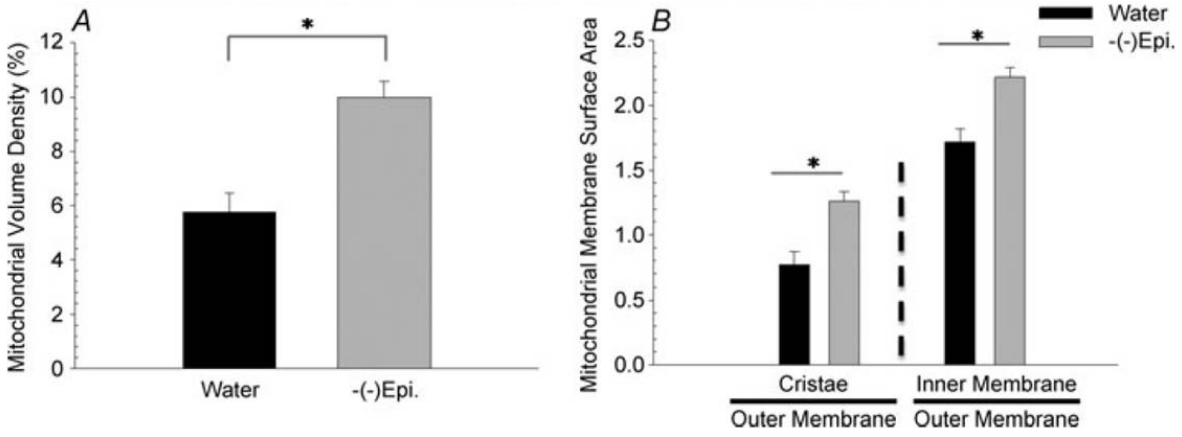
(≈30%) were observed with (-)-epicatechin. Components of oxidative phosphorylation complexes, mitofilin, porin, nNOS, and Tfam as well as mitochondrial volume and cristae abundance were significantly higher with (-)-epicatechin treatment for hindlimb and cardiac muscles than exercise alone (Figures 9 and 10). In addition, there were significant increases in skeletal muscle capillarity. The combination of (-)-epicatechin and exercise resulted in further increases in oxidative phosphorylation complexes proteins, mitofilin, porin, and capillarity than (-)-epicatechin alone. These findings indicate that (-)-epicatechin alone or in combination with exercise induces an integrated response that includes structural and metabolic changes in skeletal and cardiac muscles resulting in greater endurance capacity.

Figure 9: (-)-Epicatechin increases mitochondrial proteins in 1-year old mice.



Effect of (-)-epicatechin and exercise on A) mitochondrial oxidative phosphorylation complexes and B) mitochondrial membrane proteins. Ctrl = water only, Epi=1 mg/kg BID. Exercise = 30 minutes of treadmill exercise 5 times per week.

Figure 10: (-)-Epicatechin increases mitochondrial volume density and mitochondrial membrane surface area in 1-year old mice.



Electron microscopic examination of plantaris muscle to calculate A) mitochondrial volume density (% of cytoplasm occupied by mitochondria) and B) mitochondrial membrane surface area for cristae and inner membrane, normalized to outer membrane area.

These results are consistent with a recent study by Hutteman et al which described the ability of (-)-epicatechin to maintain exercise-induced improved capillarity and mitochondrial capacity in mice after discontinuation of exercise training, in part by induction of mitochondrial complex proteins [59].
In a follow up study the capacity of (-)-epicatechin treatment to stimulate myocardial angiogenesis was examined in the same animals. Results indicate that exercise training or (-)-epicatechin significantly stimulated myocardial angiogenesis by 30-35% above control levels (as judged by biochemical and histological measures) whereas the use of both lead to further significant increases (to ~50%). Exercise training effects were associated with significant increases in protein levels and/or activation (i.e. phosphorylation) of canonical angiogenesis pathway associated events [vascular endothelial growth factor

(VEGF), eNOS, NO and cGMP]. In most cases, (-)-epicatechin generated comparable degrees of stimulation of these pathways. The use of combined treatment led from incremental to additive outcomes in these signaling pathway endpoints.

4.1.6 (-)-Epicatechin Increases Mitchondrial Biogenesis and Improves Myocardial Function in Rats

Results from a series of studies in a rat myocardial infarction model suggests that (-)-epicatechin may facilitate preservation of myocardial function via preservation of mitochondrial structure and function. In the first study [60] (-)-epicatechin (1 mg/kg/day) or water (control) pretreatment was administered daily via oral gavage to male rats for 2 or 10 days. Ischemia was induced via a 45-min coronary occlusion. Reperfusion was allowed until 48 h or 3 wk while treatment continued. With 2 days of treatment, no reductions in myocardial infarct (MI) size occurred. After 10 days, a significant ~50% reduction in MI size occurred in the (-)-epicatechin-treated animals. (-)-Epicatechin rats demonstrated no significant changes in hemodynamics. Tissue oxidative stress was reduced significantly with (-)-epicatechin treatment. Matrix metalloproteinase-9 activity demonstrated limited increases in the infarct region with (-)-epicatechin. By 3 wk, a significant 32% reduction in MI size was observed with treatment, accompanied with sustained hemodynamics and preserved chamber morphometry.

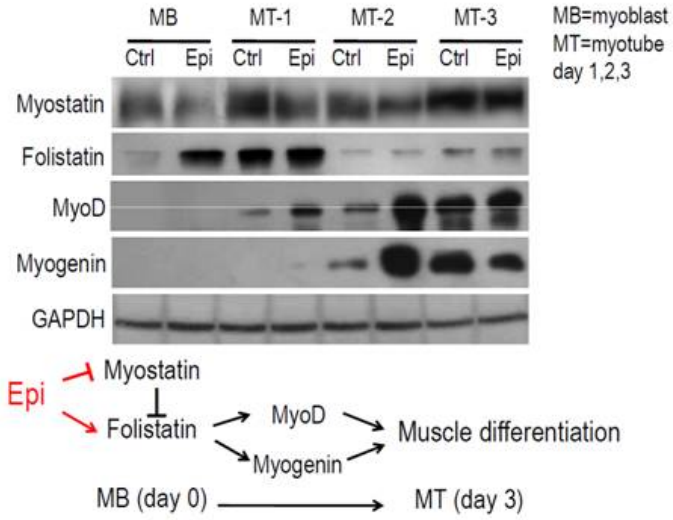
A subsequent study mirroring the one noted above was performed but using a more severe modality of myocardial injury which over time can trigger the development of heart failure (permanent coronary occlusion) [61]. Results from this study also indicated significant reductions in MI size by (-)-epicatechin (1 mg/kg/day) early after occlusion (48 h) that were sustained over time (3 weeks). Treatment did not alter hemodynamics. These effects were accompanied by the significant long-term (3 week) preservation of myocardial structure and function.

An additional study was implemented to test the potential of (-)-epicatechin to exert cardioprotection during I/R via modulation of mitochondrial function [60]. Ischemia was induced in rats via a 45 min occlusion, followed by reperfusion for 1 h, 48 h, or 3 weeks (wk). (-)-Epicatechin (10 mg/kg) was administered IV 15 min prior to reperfusion for the single dose group and again 12 h later for the double dose group. Controls received water. A single dose of (-)-epicatechin significantly reduced infarct size by 27% and 28% at 48h and 3 wk, respectively, compared to controls. Double dosing further decreased infarct size at 48 h by 80%, which was sustained at 3 wk (52% reduction). In order to assess if (-)-epicatechin-induced cardioprotection was mediated by protection of mitochondrial function, mitochondria were isolated from the left ventricle of sham, I/R, and I/R + (-)-epicatechin animals 1 h after ischemia. I/R animals had a significant decrease in mitochondrial O2 consumption, significant increase in mitochondrial Ca2+ levels, and decreased ATP and NADH pools. (-)-Epicatechin protected against these changes and had levels similar to sham animals. Taken together, results suggest that (-)-epicatechin preserves myocardial bioenergetics which likely underlies the cardioprotection observed.

4.1.7 (-)-Epicatechin Modulates the Synthesis of Muscle Growth and Differentiation Proteins in Cultured Cells and Mice (Aged and MD)(unpublished data)

The ability of (-)-epicatechin to effect markers of muscle growth and differentiation were assessed in cultured C2C12 myoblasts, 6 and 26 month old mice and mdx mice (MD model).
Muscle Regulatory Protein Expression in C2C12 Cells C2C12 cells were cultured +/- (-)-epicatechin in the presence of 1% horse serum, which induces differentiation (myoblast to myotube transition). Figure 11 depicts the three day time course of differentiation (by Western blots). (-)-Epicatechin treatment accelerated and increased protein levels of the muscle growth factor follistatin and those of differentiation (myogenin, MyoD), while decreasing levels of the inhibitor myostatin, suggesting that (-)-epicatechin upregulates muscle differentiation.

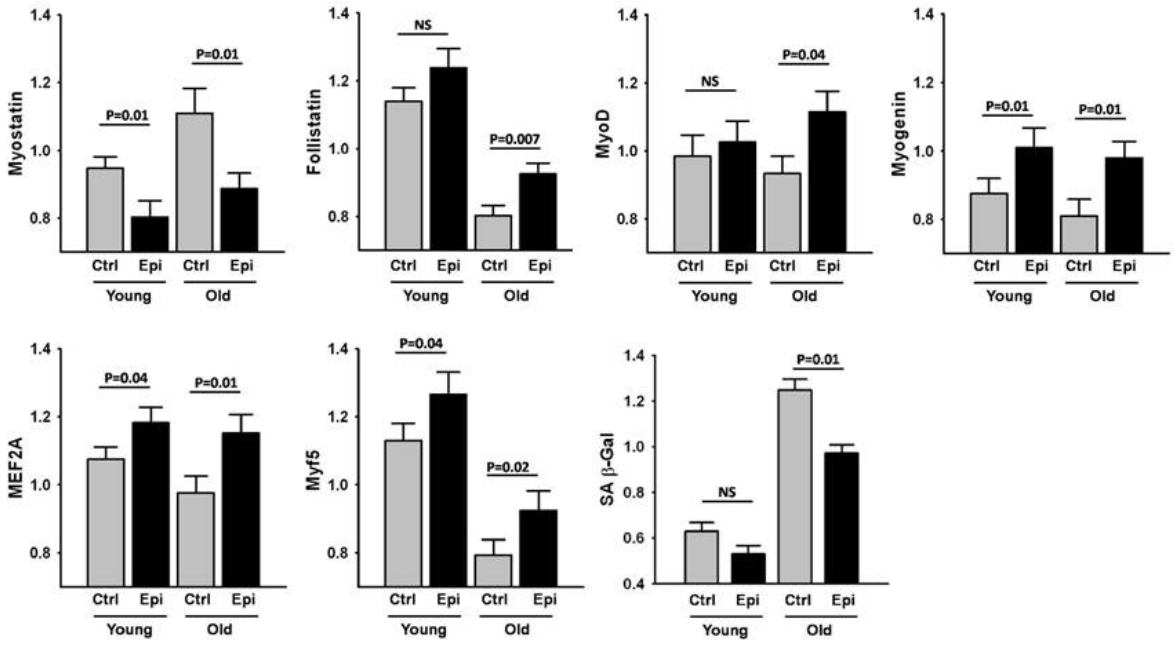
Figure 11: Muscle differentiation in time course in C2C12 cells +/- (-)-epicatechin.



4.1.1.1 Muscle Regulatory Protein Expression in 6 and 26 month old Mice

The expression of muscle growth, differentiation and senescence proteins in quadriceps SkM biopsies from 6 and 26 month old mice, either untreated (control) or treated with 1 mg/kg (-)-epicatechin for 15 days, was quantified by Westerns (Figure 12). There were clear age-related changes in protein expression, with notable increases in SkM myostatin and decreases in follistatin protein levels. Modest decreases were observed in MEF2, MyoD and myogenin with larger differences noted for Myf5. As expected, large increases were observed in senescence-associated β-galactosidase: (SA-β-Gal), a cell senescence marker, in older animals. As in the C2C12 cells, (-)-epicatechin treatment decreased myostatin while increasing the levels of differentiation-promoting factors, with three of the factors (MyoD, MEF2 and myogenin) reaching levels in the old mice that were similar or greater than those seen in young control mice.

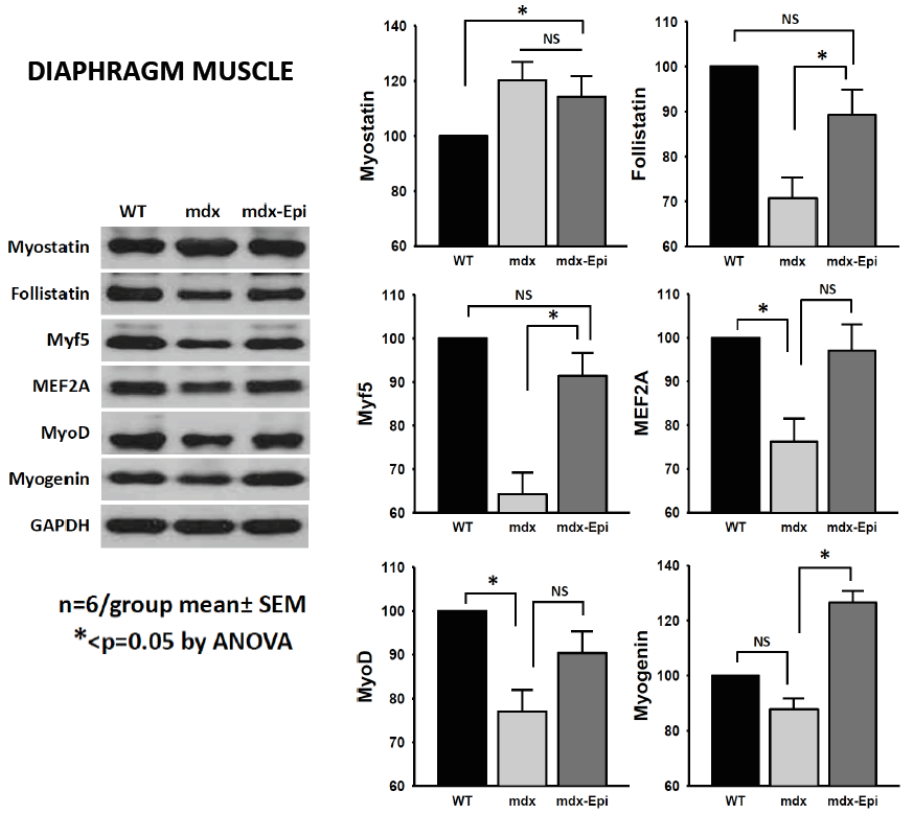
Figure 12: Muscle regulatory protein expression in 6 and 26 month old mice +/- (-)-Epicatechin



4.1.1.2 Muscle Regulatory Protein Expression in mdx Mouse Model of MD

Figure 13 depicts representative images obtained from Western blots performed on diaphragm muscle samples from mdx mice treated for month with (-)-epicatechin starting at 12-16 weeks of age. Muscle samples were probed to evaluate changes in protein levels for the muscle growth modulators, myostatin and follistatin. As can be observed in the upper panels, in mdx mice there is a significant upregulation of myostatin that did not improve with treatment. In contrast, follistatin levels in the diaphragm were significantly reduced in water treated mdx mice and recovered with (-)-epicatechin. Follistatin is a trophic muscle hormone well known to promote muscle regeneration and well as inhibit fibrosis and inflammation. Changes in recognized regulators of muscle differentiation were evaluated (Myf5, MEF2A, MyoD and myogenin). As can be observed, with the exception of MyoD where (-)-epicatechin treatment did not fully recover protein levels, all other modulators were restored to wild type (WT) levels with treatment. Similar results were obtained in an analysis of the mdx gastrocnemius muscle (data not shown).

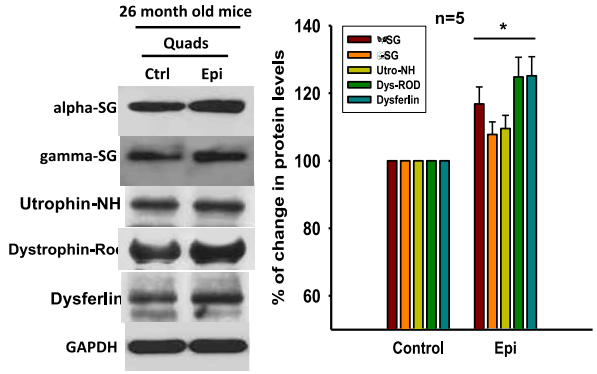
Figure 13: (-)-Epicatechin modulates muscle regulatory proteins in diaphragm muscle in *MDX* mice.



4.1.8 (-)-Epicatechin Stimulates the Expression of MD-Relevant Muscle Structural Proteins in Aged and MD Mice (unpublished data)

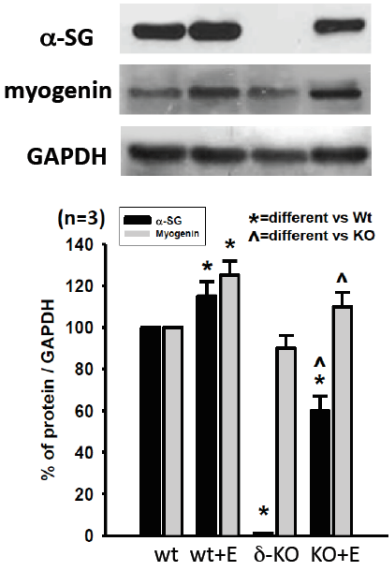
The effects of (-)-epicatechin in animals experiencing progressive muscle loss was examined in aged normal mice (model of sarcopenia) and δ-sarcoglycan KO mice (model of limb-girdle muscular dystrophy). In examining muscle loss associated with age, 26 month old mice, within 6 months of the end of their natural lifespan, were treated with (-)-epicatechin (1mg/kg) for just two weeks. All mice demonstrated increases in dystrophin, the sarcoglycans, and desferlin when compared by Western blot to age-matched controls (Fig 14).

Figure 14: (-)-Epicatechin increases DAPC proteins in middle aged mice.



The effect of epicatechin's stimulation of the dystrophin protein complex was also examined in the δ-sarcoglycan KO mouse. As shown in Fig.15, such mice also manifest a marked loss of α sarcoglycan protein expression, a loss which was quickly reversed by two weeks of epicatechin treatment, 1 mg/kg/twice a day, potentially representing compensatory sarcoglycan expression.

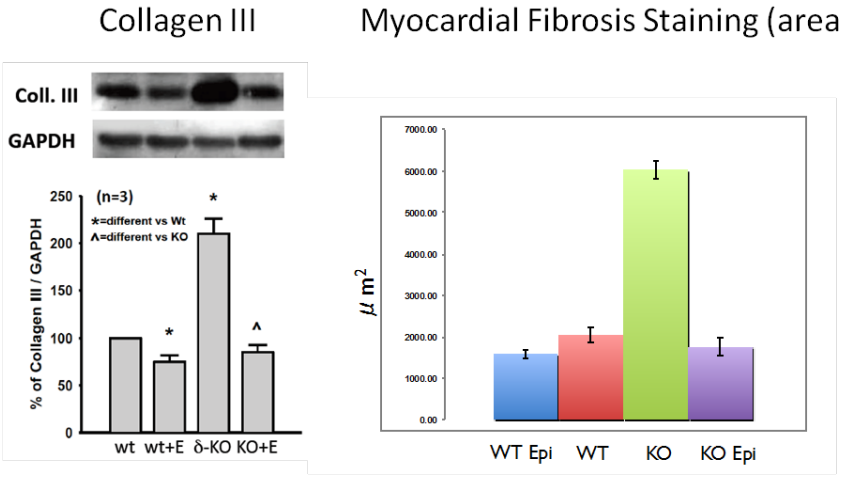
Figure 15: (-)-Epicatechin increases alpha-sarcoglycan protein expression in gamma-sarcoglycan knock out mouse muscle.



4.1.9 (-)-Epicatechin Reduces Fibrosis in δ-Sarcoglycan KO Mice (unpublished data)

Treated animals from the experiment described above also showed a dramatic decrease in expression of collagen III, a marker of fibrosis (Fig 16, left panel). The reduction in collagen III translated into reduction of myocardial fibrosis as judged by quantitative histology of heart valve tissue sections (Fig 5.13, right panel). These data suggest the potential of (-)-epicatechin to reduce cardiomyopathy often associated with MD.

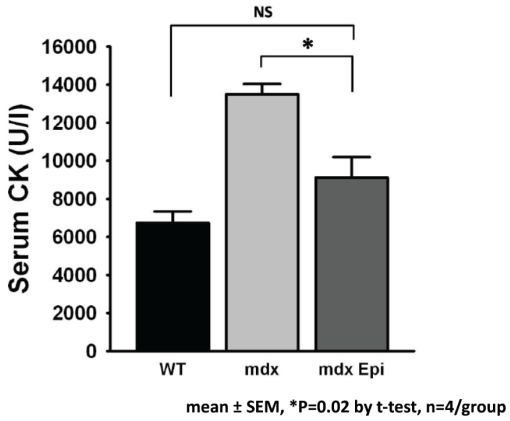
Figure 16: (-)-Epicatechin reduces fibrosis in the gamma-sarcolgycan knock out mouse.



4.1.10 (-)-Epicatechin Reduces Plasma Marker of Muscle Damage and Improves Muscle Strength in MDX Mouse Model of MD (unpublished data)

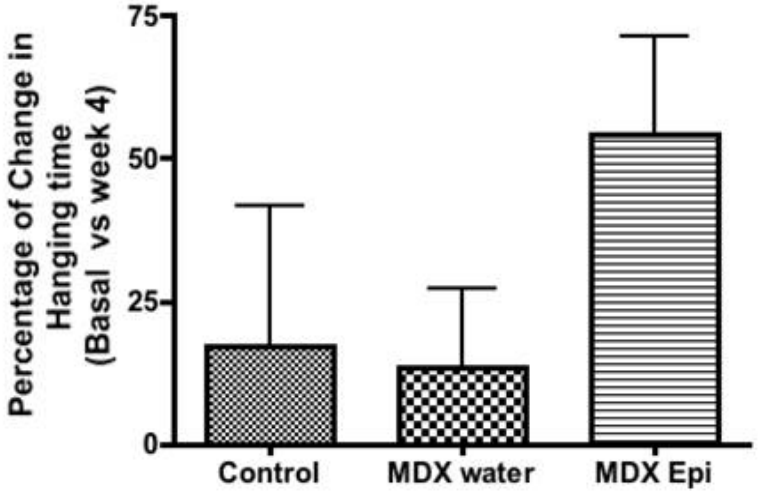
In order to confirm ongoing muscle injury in the mdx mouse model and assess the effect of (-)-epicatechin on plasma creatine kinase (CK), mice were treated with water or (-)-epicatechin (1mg/kg, bid) by oral gavage for 4 weeks; normal mice (WT) were treated with water. Excess activity of CK in plasma is recognized as a marker of skeletal and/or cardiac muscle damage. As shown in Fig 17, the levels of CK activity rise in water treated mdx mice and they are significantly reduced with epicatechin treatment to levels similar of those obtained in wild type animals.

Figure 17: (-)-Epicatechin reduces plasma CK activity (muscle injury biomarker) in *MDX* mouse model of muscular dystrophy.



In a separate study to evaluate the effect of (-)-epicatechin on muscle strength, mdx mice were treated with (-)-epicatechin (1mg/kg, qd) by oral gavage for 4 weeks; normal mice (control) were treated with water. Muscle strength testing was performed at baseline and at 4 weeks, consisting of measuring hang time of each mouse from a wire grid (30), with percent change from baseline calculated for each mouse. Figure 18 depicts (mean±SD) percent change in hang time for control and mdx mice. Control and mdx mice receiving water showed modest 18% and 14% increases, respectively, while (-)-epicatechin treatment resulted in a 55% increase in hang time. Although this was a small pilot study, the results are consistent with the ability of (-)-epicatechin to improve muscle function in animals lacking dystrophin, providing initial proof-of-concept MD efficacy data.

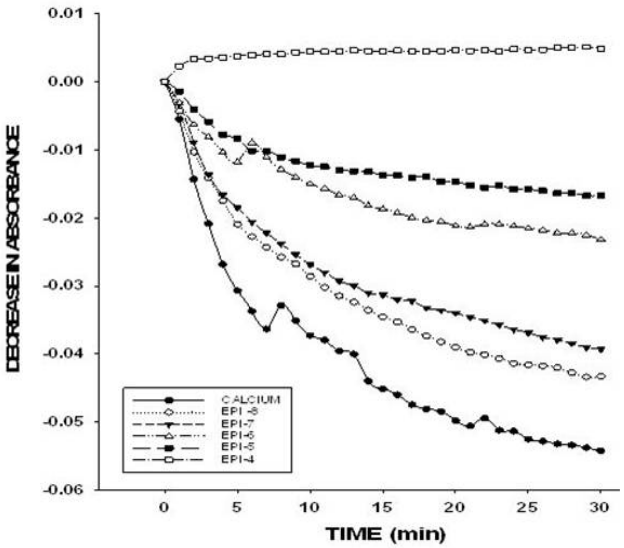
Figure 18: (-)-Epicatechin improves skeletal muscle strength in the *MDX* mouse model of muscular dystrophy.



4.1.11 (-)-Epicatechin Blocks Calcium-Induced Pore Formation in Cardiac Mitochondria (unpublished data)

Cardiac myocyte mitochondria were isolated from rat hearts and used to assess (-)-epicatechin's ability to block calcium influx and prevent damage. Mitochondrial swelling (measured by changes in light transmission) was induced by exposure to 33 μM calcium chloride alone or calcium chloride plus increasing doses of (-)-epicatechin (10^{-8}-10^{-4}M)(Figure 19). (-)-Epicatechin blocked the opening of the mitochondrial PTP in a dose-dependent manner, suggesting that (-)-epicatechin could be effective at preserving mitochondrial function in calcium-overloaded MD muscle cells and thus reducing mitochondrial-related muscle necrosis in MD patients.

Figure 19: (-)-Epicatechin blocks calcium-induced pore formation in cardiac mitochondria in a dose-dependent manner.



Epi = (-)-epicatechin. Epi-4 – Epi-8 = (-)-epicatechin concentrations of 10^{-4} – 10^{-8}M.

5 HUMAN STUDIES

5.1 POC Clinical Study #1: Mitochondrial Biogenesis and Muscle Improvement in Patients with Heart Failure and Type 2 Diabetes Patients(UCSD)

A proof of concept (POC) study was performed at UCSD in five heart failure, type 2 diabetes mellitus patients using (-)-epicatechin rich cocoa (mixture of a beverage and chocolate) in order to assess the effects on skeletal muscle mitochondria structure, indicators of mitochondrial biogenesis and SkM proteins and structure [7]. Published reports indicate that in patients suffering from heart failure or diabetes there is a significant compromise in mitochondrial structure and muscle bioenergetics [62-64]. Thus, altered mitochondrial bioenergetics may explain (at least in part) the impaired exercise capacity that is present in these patient populations. Changes were assessed in protein and/or activity levels of oxidative phosphorylation proteins, porin, mitofilin, nNOS, nitric oxide, cGMP, SIRT1, PGC-1α, Tfam, and mitochondria volume and cristae abundance by electron microscopy in skeletal muscle biopsies. Apparent major losses in normal mitochondria structure were observed before treatment.

5.1.1 Markers of Mitochondrial Biogenesis, Structure and Function

Fig 20 demonstrates that (-)-epicatechin-rich cocoa increased active PGC-1α with correlative decreases in acetylated (inactive) PGC1α, consistent with induction of mitochondrial biogenesis. This upregulation in mitochondrial biogenesis correlated with increased mitochondrial protein expression and cristae abundance (Figure 21).

Figure 20: (-)-Epicatechin-rich cocoa increases active PGC-1a and decreases acetylated (inactive) PGC1a in HF/DM2 patients.

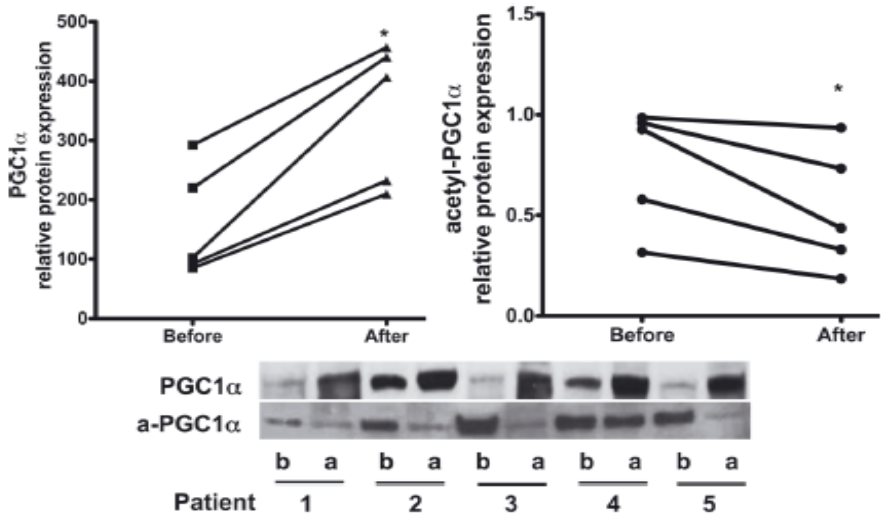
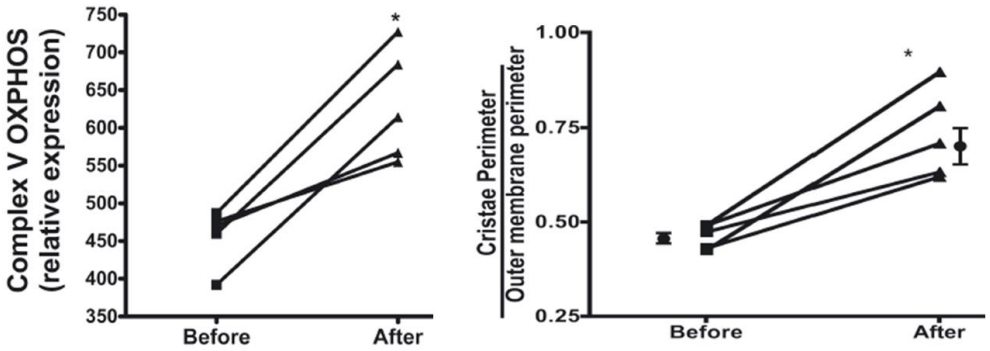
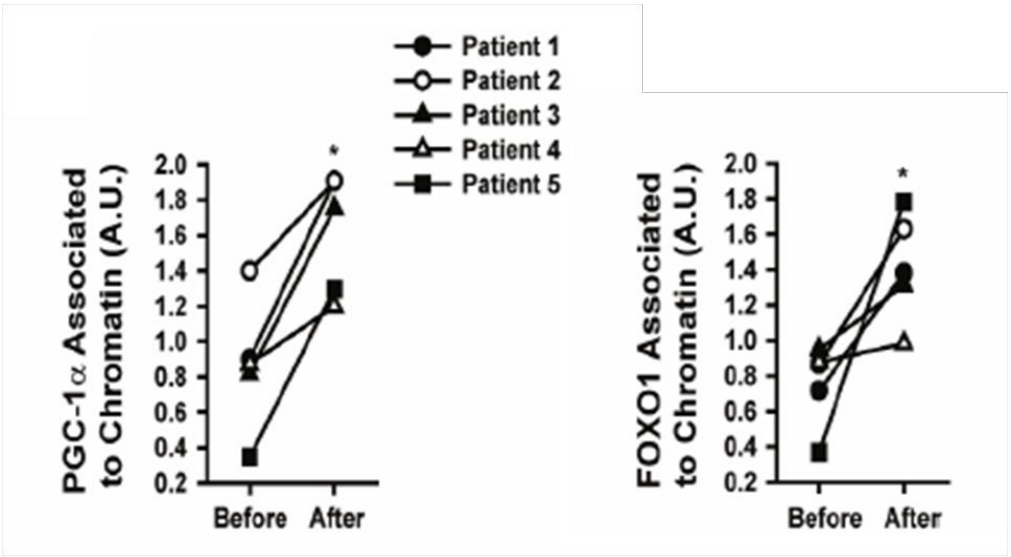


Figure 21: (-)-Epicatechin-rich cocoa increases mitochondrial complex V protein and cristae abundence in HF/DM2 patients.



The same patients demonstrated a marked increase in PGC-1α localized to the nuclear chromatin (22). There was a concurrent co-localization of Fox01, a cofactor with PGC-1α for inducing transcription of endogenous anti-oxidant pathways (and an important regulator of muscle energy metabolism) [65]. One of the characteristics of muscles in sarcopenia is evidence of marked oxidative injury. These anti-oxidant enzymes (e.g., superoxide dismutase, catalase, see Fig 23) would be predicted to have a beneficial effect on the injured muscle of muscular dystrophy.

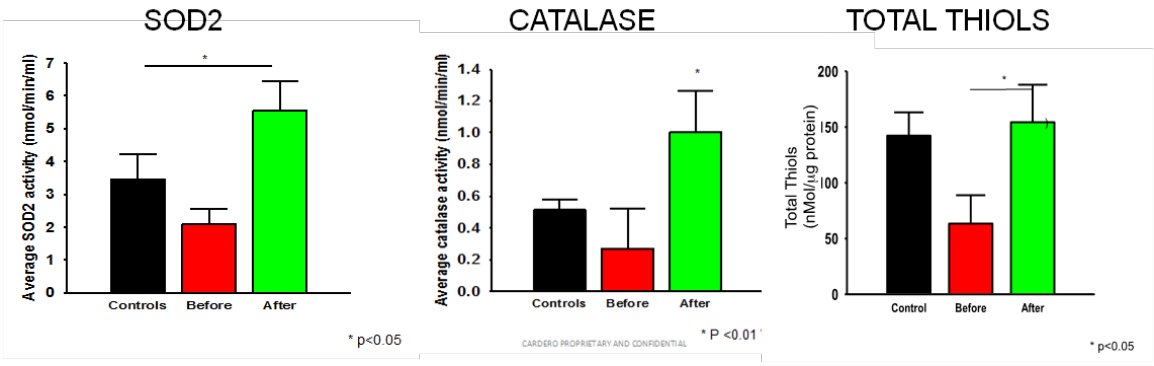
Figure 22: (-)-Epicatechin-rich cocoa increases PGC1a and FOX01 association to chromatin from quadriceps of HF/DM2 patients.



5.1.2 Markers of Tissue Oxidative Stress

Using the same group of patients but incorporating into the study a group of control subjects of similar ages (n=4) markers of tissue oxidative stress were compared before and after treatment with (-)-epicatechin-rich chocolate (Fig 23).

Figure 23: (-)-Epicatechin-rich cocoa increases antioxidant proteins and total tissue thiols (reduced glutathione) in HF/DM2 patients.

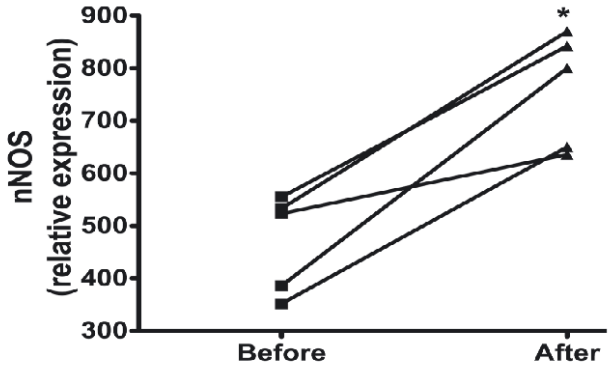


SOD2 and catalase expression were restored to levels exceeding age-matched controls and approaching levels normally associated with the young. A significant ~60% decrease in SkM tissue thiol levels were observed before treatment and these were fully restored to levels similar to controls by (-)-epicatechin-rich chocolate. Protein nitrosylation levels (determined using a slot blot) can be used as a general indicator of tissue oxidative stress levels. SkM samples of control and HF/DM2 patients were examined (data not shown). Prior to treatment, patient protein nitrosylation levels were significantly higher by ~2.5 fold than controls (=100%) and were restored to ~1.5 fold by treatment with (-)-epicatechin-rich chocolate. Altogether these results indicate that the use of (-)-epicatechin-rich chocolate has a potential to restore tissue mitochondria structure and oxidative stress levels back towards control.

5.1.1.1 nNOS Expression

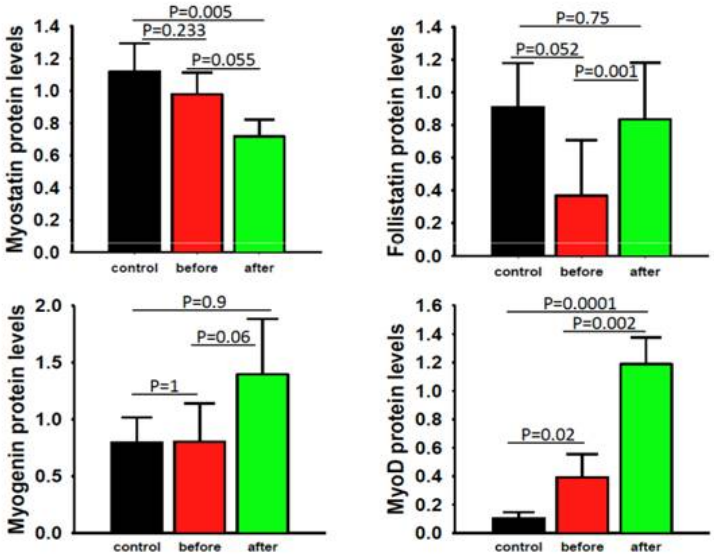
Patient muscle biopsies were assessed for nNOS expression, as myopathy, particularly BMD, is often associated with both depletion of neuronal NO synthase (nNOS) and its dislocalization from the sarcolemma. Loss of nNOS, in turn, is linked with impaired mitochondrial biogenesis, increased muscle atrophy and autophagy. Depletion and dislocalization of nNOS has a negative impact on muscle function in muscular dystrophy, causing increased muscle fatigability and functional ischemia during exercise [28, 66]. In this clinical trial, HF/DM2 patients treated with (-)-epicatechin-rich chocolate showed a statistically significant increase in quadriceps muscle nNOS expression compared to pre-treatment baseline (Fig 24).

Figure 24: (-)-Epicatechin-rich cocoa increases quadriceps muscle nNOS expression in HF/DM2 patients.



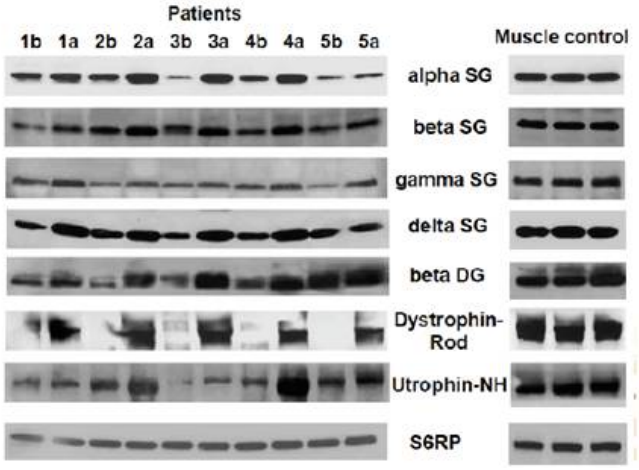
Muscle Protein Expression and Sarcomere Organization Patient SkM biopsies were evaluated for levels of muscle differentiation and structural proteins, and for sarcomere organization (unpublished data). Similar to the results of studies in C2C12 cells and mice, treatment with (-)-epicatechin increased the expression of proteins involved in muscle differentiation (follistatin, myogenin, MyoD), while levels of the inhibitor myostatin decreased (Figure 25).

Figure 25: Muscle growth/differentiation protein expression +/- (-)-epicatechin-rich cocoa in HF/DM2 patients.



Biopsies from the HF/DM2 patients were analyzed by Western blot to assess the effect (-)-epicatechin-rich cocoa on the dystrophin-associated protein complex (DAPC), a key structural component in muscle cells. As shown in Fig 26, prior to treatment there was a striking deficiency of dystrophin, which recovered to control levels with treatment. Significant increases with treatment were also noted in all the sarcoglycans, beta-dystroglycan and utrophin.

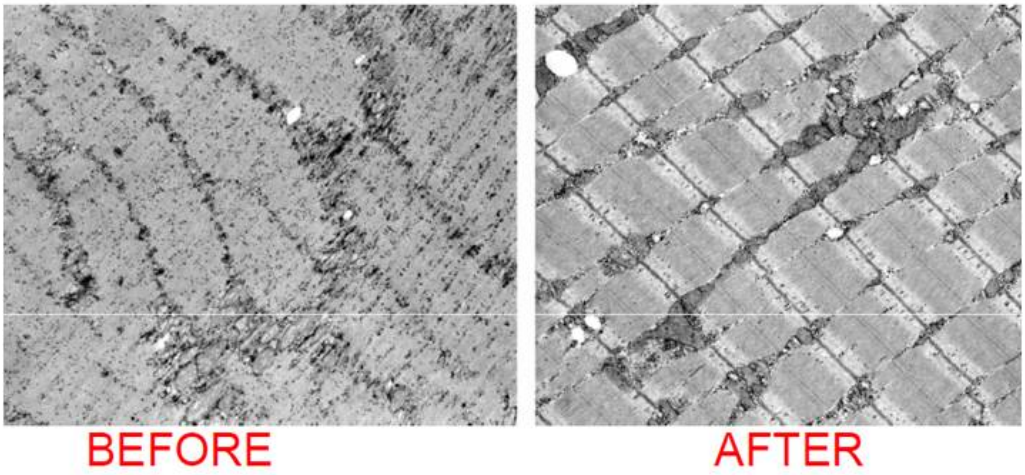
Figure 26: Muscle structural proteins induced by (-)-epicatechin-rich cocoa in HF/DM2 patients.



HF/DM2 patients before (b) and after (a) 30 day treatment with epicatechin-rich cocoa. Muscle control = healthy volunteers. SG: sarcoglycans, DG: dystroglycan

As the perturbation of dystrophin levels can greatly compromise sarcomere organization and thus, contractile function, SkM samples were evaluated using electron microscopy. Figure 27 shows a representative sample from one patient obtained before *vs* after treatment. As observed, at baseline sarcomere “organization” was very poor (no clear definition of Z and other bands). With treatment, sarcomere organization markedly recovered. Results were “quantified” using blinded, naïve graders and a highly significant improvement ($P<0.0001$) was observed in sarcomere organization (1.7±0.5 before vs. 3.1±0.5 after out of a maximum scale of 4) after scoring 100 EM images (10 before, 10 after/patient).

Figure 27: Changes in sarcomere ultrastructure in representative HF/DM2 patient before and after treatment with (-)-epicatechin-rich cocoa.



5.2 POC Clinical Study #2: Age-Related Changes in Muscle Regulatory Proteins and Effect of (-)-Epicatechin on Muscle Function and Proteins in Healthy Volunteers (UCSD)

A two part study was conducted to evaluate age-related muscle changes and the effects of (-)-epicatechin on those changes (manuscript submitted for publication, 2012). In the first part, tissue bank SkM samples were obtained from young (28.5±7 years, n=6) and old (62±2 years, n=6) subjects and analyzed for the amounts of muscle regulatory proteins. Levels of SkM growth (myostatin, follistatin), differentiation (myogenin, MyoD, MEF2A, Myf5) and senescence (senescence-associated β-galactosidase: SA-β-Gal) proteins in each group are shown in Figure 28. Muscle levels of myostatin (differentiation inhibitor) and SA-β-Gal (senescence) were increased in the older group, while those of follistatin, MyoD and myogenin decreased, suggesting an age-related loss of muscle growth and differentiation potential. These data defined a profile of age-related changes in muscle regulatory proteins and informed the selection of muscle protein outcome measures to be used in the second part of the study: (-)-epicatechin treatment of middle aged subjects.

In part 2, a small pilot treatment study was performed in human subjects (n=6, average age 41±5 years) to assess the effects of (-)-epicatechin on muscle strength and blood levels of myostatin and follistatin. Subjects were treated for 7 days with 25 mg of (-)-epicatechin in capsules BID. Muscle strength was assessed by hand grip dynamometry (thrice with each hand, alternating hands between trials and resting for 10 seconds in order to prevent fatigue, maximum strength attained was used for analysis). Myostatin and follistatin was measured in blood samples taken before and after the 7 days of treatment. Treatment resulted in a bilateral increase in hand strength of ~7% which was accompanied by a significant increase in the ratio follistatin/myostatin (Figure 29). This coupling of functional and molecular changes suggests that (-)-epicatechin has potential as a therapy to reduce progressive muscle loss.

Figure 28: Age-related muscle protein changes in human skeletal muscle samples.

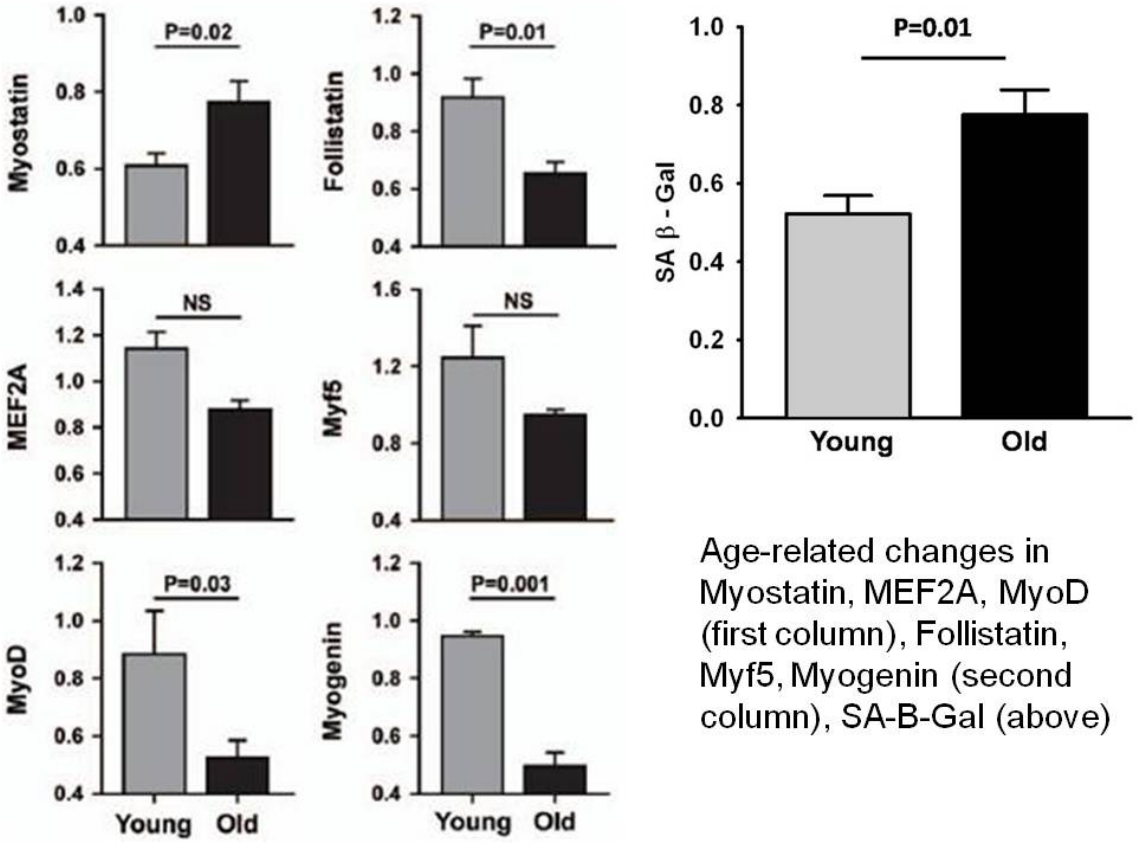
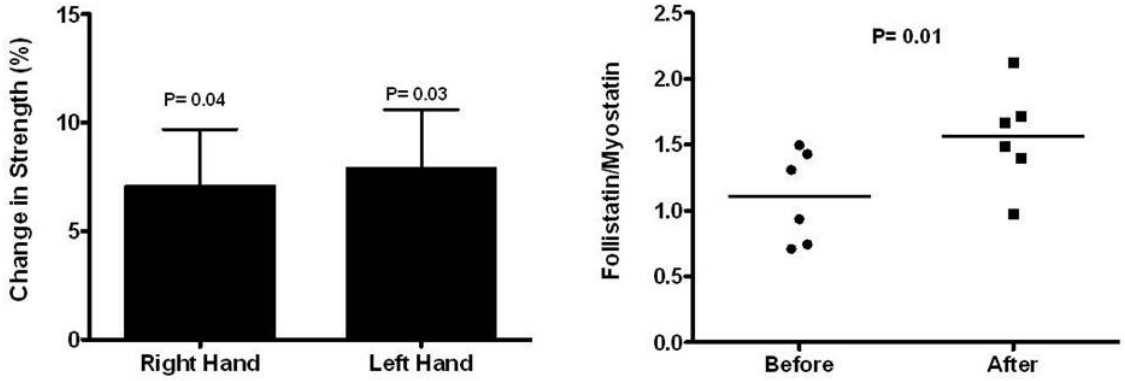


Figure 29: Changes in muscle strength and follistatin/myostatin ratio following 7 day of (-)-epicatechin treatment.



5.3 Clinical Study #3: Pharmacokinetics of (-)-Epicatechin in Healthy Volunteers (UCSF)

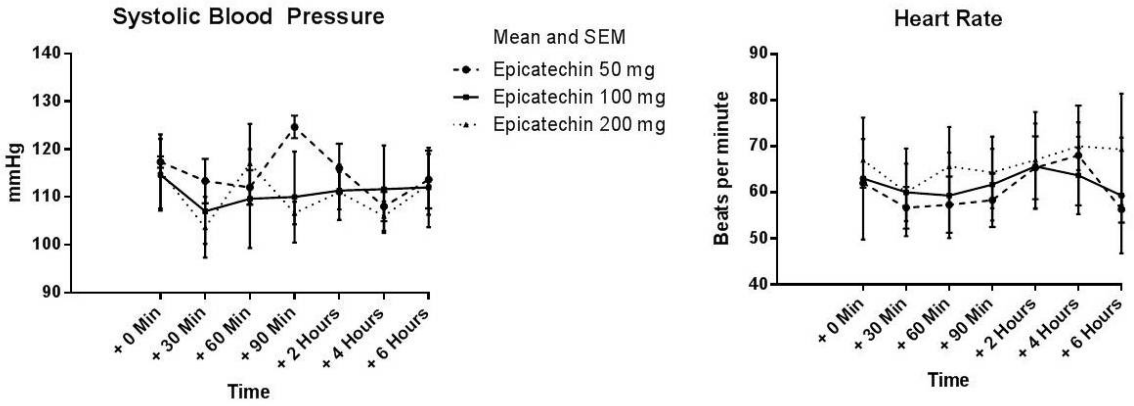
An open label study in was carried out by Dr. Christopher Barnett at the University of California, San Francisco (UCSF) to assess the safety and pharmacokinetics of a single dose of purified (-)-epicatechin in healthy volunteer subjects (n=9). (-)-Epicatechin doses of 50, 100 or 200 mg were administered in 40 ml of water.

5.3.1 Safety and Tolerability

No adverse effects were reported or observed in any of the 9 subjects. Subjects specifically denied symptoms of light headedness, dizziness, fatigue, chest discomfort or changes in breathing during the observation period. Data from vital sign monitoring is shown in Figure 30.

At time +0.5 hours there was a trend towards reduced mean systolic blood pressure with a concomitant decrease in heart rate that is consistent with subjects being relaxed, and not with a blood pressure lowering from (-)-epicatechin. In one subject blood pressure at time +0.5 hours decreased markedly, but the value was obtained immediately upon waking, the subject denied symptoms and the heart rate was also reduced from baseline, findings that are consistent with relaxation and not (-)-epicatechin induced vasodilatation.

Figure 30: Vital signs following (-)-epicatechin administration in healthy subjects.



5.3.2 Pharmacokinetics

Pharmacokinetic parameters for each dose administered are summarized presented in detail in the investigator's brochure. The (-)-epicatechin concentration and metabolite concentration rose rapidly following oral administration consistent with rapid absorption and first pass metabolism as has been previously described [11, 18-21, 45, 47-50]. Complete plasma concentration profiles of (-)-epicatechin and its metabolites after oral administration can be found in the complete PK study report in the investigator's brochure. The shape of the natural log transformed plasma concentration curves was consistent with first order pharmacokinetics.

The ratio of the AUC for (-)-epicatechin and each metabolite to administered dose of (-)- epicatechin is given in Table 6.4. The AUC/dose ratio remains nearly constant for (-)-epicatechin but increases with increasing dose of each of the metabolites suggesting either dose dependent excretion of these metabolites, or perhaps saturation of some competing pathway for epicatechin metabolism. Investigator brochure Table 6.5 shows that $AUC_{metabolite}/AUC_{epicatechin}$ is high for each of the metabolites suggesting that one or more of the metabolites may be an important mediator of biological effects.

5.3.3 Conclusions

(-)-Epicatechin was well tolerated over the 50-200 mg dose range, with rapid absorption and first pass metabolism. Non-metabolized (-)-epicatechin represented <5% of the total (-)-epicatechin compounds by 30 minutes. (-)-Epicatechin and its metabolites were rapidly cleared from the body with a plasma elimination half-life of approximately 2.5 hrs for the 100 and 200 mg doses. Plasma concentration of (-)-epicatechin was generally proportional to the administered dose. The 3 dominant groups of metabolites in our study were glucuronidated, sulfated and methyl sulfated, consistent with a recently published evaluation of (-)-epicatechin pharmacokinetics and metabolism [47]. A novel finding in this study was that the $AUC_{Metabolite}/AUC_{epicatechin}$ ratio increased with increasing doses suggesting dose dependency of (-)-epicatechin metabolism.

6 RESEARCH DESIGN AND METHODS

6.1 Selection and Withdrawal of Participants

6.1.1 Criteria for Enrollment

Ten participants will be studied in this initial pilot project.

Participant Inclusion Criteria

- Male
- Age 18 years to 60 years
- Average to low daily physical activity
- Ability to ambulate for 75 meters without assistive devices
- Diagnosis of BMD confirmed by at least one the following:
    - Dystrophin immunofluorescence and/or immunoblot showing partial dystrophin deficiency, and clinical picture consistent with typical BMD, or
    - Gene deletions test positive (missing one or more exons) of the dystrophin gene, where reading frame can be predicted as 'in-frame', and clinical picture consistent with typical BMD, or
    - Complete dystrophin gene sequencing showing an alteration (point mutation, duplication, or other mutation resulting in a stop codon mutation) that can be definitely associated with BMD, with a typical clinical picture of BMD, or
    - Positive family history of BMD confirmed by one of the criteria listed above in a sibling or maternal uncle, and clinical picture typical of BMD.
- Nutritional, herbal and antioxidant supplements taken with the intent of maintaining or improving skeletal muscle strength or functional mobility have been discontinued at least 2 weeks prior to screening (daily multivitamin use is acceptable).
- Hematology profile within normal range
- Baseline laboratory safety chemistry profile within normal range
- No plan to change exercise regimen during study participation

Participant Exclusion Criteria

- Currently enrolled in another treatment clinical trial.
- History of significant concomitant illness or significant impairment of renal or hepatic function.
- Use of regular daily aspirin or other medication with antiplatelet effects within 3 weeks of first dose of study medication.
- Regular participation in vigorous exercise.
- Symptomatic heart failure with cardiac ejection fraction <25%

6.1.2 Participant Screening Schedule

Potential participants for the study will be evaluated using a two visit screening method that will allow investigators to evaluate the participant's ability to provide a reproducible (15%) (*primary endpoint*) test scores to be enrolled. The entry procedures will be conducted according to the schedule in **Table 3**. After potential participants have completed the screening visits and the site has verified they meet all study inclusion criteria the participant will be randomized into the study.

Table 3: Study procedures

Event	Screening[1] (Week -2 to Day 1)	Baseline (Day 1)	Week 1	Week 2	Week 4	Week 8	Early with-drawal
Informed consent	X						
Medical history	X	X		X	X	X	X
Vital signs	X	X		X	X	X	X
Physical examination	X	X		X	X	X	X
Blood & urine collection (safety, efficacy, PK)	X	X		X	X	X	X
ECG	X	X		X	X	X	
Other medications and dietary supplements	X	X			X	X	X
Adverse events		X		X	X	X	X
Functional assessments strength, 6MWT, recumbent graded exercise test, NIRS, PFTs		X			X	X	X
Blood tests: baseline and efficacy		X			X	X	X
Muscle Biopsy Tests: baseline and efficacy		X				X	
Dispense Epicatechin		X		X			

6.1.3 Randomization

This is an open-label pilot study, and will not utilize randomization in the study design.

6.2 Withdrawal of Participants

Participants are free to withdraw from the study at any time. Study investigators may elect to withdraw a participant from the study for reasons including an adverse event and inability to comply with study procedures. Reasons for withdrawal of all participants will be recorded for review by the Study Medical Monitor.

6.2.1 Follow-Up of Withdrawn Participants

Those participants who are withdrawn from study medication, but are still willing to finish study participation per protocol, will be followed with (*method*) testing for the remainder of the 8-week study period (8 months from participant's baseline visit). If participant agrees to follow-up, adverse event and medical event follow-up, along with basic safety data, will also be collected at scheduled study visits or via phone every *2 weeks* for the remainder of the study period (through week 8).

6.3 Treatment of Participants

6.3.1 Epicatechin Administration

Participants will receive epicatechin by mouth 50mg twice per day (100mg per day total dose). Study medication will be supplied as a clear 25mg (#3) gelatin capsule without any inert fillers. Participants will take two capsules in the morning at approximately 7:30AM at least 15 minutes before the morning meal and two with the evening at approximately 7:30PM at least 1 hour after the evening meal as absorption is limited by some milk products.

For details on participant enrollment and randomization, please refer to the study procedures chart (Table 3) and pharmacy sections. When medications have been received, they should be dispensed to participants from their assigned investigational drug supply along with the medication instructions handout for parents and physicians.

6.3.2 Criteria for Dose Reductions

Dose reductions will occur due to adverse drug events and participants will not have their doses re-escalated during the course of the study. Criteria for dose reduction will include:

- Peak (-)-epicatechin PK level >4umol/ml
- Recurrent non-manageable headache
- Increase in baseline safety evaluations >1.5 ULN or 100% change from baseline, excepting CK, AST, ALT
- Abnormal coagulation studies (PT/PTT)
- Other Grade 4 adverse event

If participants require a dose reduction based on the criteria listed above follow the guidelines in Table 4 when reducing a participant's study medication dose.

Table 4: Dose reduction schedule.

Dose Reduction 1	Reduce participant's dose by 50mg/day (25mg AM, 25mg PM dose).
Dose Reduction 2	Withdraw participant from study medication

The Common Terminology Criteria for Adverse Events (CTCAE) published by the Cancer Therapy Evaluation Program will be used to grade adverse events for this trial (See Appendix F).

6.3.3 Study Drug Holiday as Result of Surgical Procedure

If a participant experiences an SAE that necessitates an unanticipated surgical procedure, the study drug should be withdrawn and a "drug holiday" begins. The day the study drug is withdrawn (first day not taking study drug) is Day One of the drug holiday. A drug holiday can last up to 2 weeks (14 calendar days).

Study drug should be withdrawn 2-5 days prior to the scheduled surgery date. The study drug should be restarted 2 days post surgery unless PI has medical rational for other regimen for restarting study drug.

6.3.4 Concomitant Therapy

No new medications may be taken, except over the counter cold remedies, daily multivitamin, and Zantac, during the study period without the agreement of the Study Chair. The exception is any case where such medications are used to prevent injury or disability due to unforeseen adverse events.

6.3.5 Patient Care Outside UC Davis

Participants participating in UC Davis studies may not receive protocol therapy at non-UC Davis institutions. However, the following are acceptable:

- For interim blood work done at a non-UC Davis site, a copy of the laboratory slip or other documentation MUST be forwarded to UC Davis for inclusion in the medical record and case report forms.

These guidelines do NOT override any federal, international or sponsoring agency requirements.

Records from any non-UC Davis institutions must be available for audit.

7 PHARMACY

7.1 Dosing Information and Safety Studies

The safety of this supplement has not been proven in the BMD population. We propose to use a dose at approximately 1.1-1.4 mg/kg/day spit between the morning and evening doses. The maximum dose in our population will not exceed 100 mg/day.

7.1.1 Preclinical Rodent Safety Studies

Studies in rodents indicate that (-)-epicatechin can be delivered safely at doses far in excess of the anticipated 1mg/kg dose in human studies. In a study with green tea extracts containing (-)-epicatechin given orally to rats daily for 6 months, the no-observable-adverse-effect level (NOAEL) corresponded to 85 mg (-)-epicatechin/kg [67]. In a developmental toxicity study in pregnant rats using a different tea extract,

the no-observable-adverse-effect level (NOAEL) corresponded to 100 mg (-)-epicatechin/kg (the highest dose tested)[68]. The intraperitoneal (-)-epicatechin median lethal dose (LD_{50}) reported for mice is 1000 mg/kg as stated in the MSDS provided by suppliers such as Sigma-Aldrich. Clarke and Clarke proposed that any substance with an intraperitoneal LD_{50} of above 1000 mg/kg may be regarded as safe [69].

7.1.2 Human Safety Studies

Clinical studies with purified (-)-epicatechin (1-2 mg/kg) in the dose range of Cardero's proposed studies did not reported any adverse events [11, 52]. An extensive literature of human studies using high-flavanol cocoa or chocolate human studies exists, indicating that flavanols could be administered safely at doses up to 1008 mg flavanols per day for 15 days and 444 mg flavanols per day for 6 weeks (reviewed in [8, 9]). A safety study of a green tea extract containing ~110 mg (-)-epicatechin per dose showed that once a day dosing for 4 weeks yielded the same safety profile as placebo, with no significant differences in hemotologic or clinical chemistry [16].

In addition to formal clinical studies, cocoa, chocolate and tea have been used globally on a daily basis for centuries, suggesting that these compounds pose no significant safety risk. Tea is considered a Generally Regarded As Safe (GRAS) compound by the FDA (21 CFR 182.20).

7.2 Study Drug Formulation and Procurement

Epicatechin for this study will be obtained from Cardero Therapeutics, Inc. and will be supplied as 25mg clinical trial grade (#3) clear gelcaps in bulk containers. Distribution of medication will be supervised by the UC Davis Investigational Drug Service Pharmacy.

7.2.1 (-)-Epicatechin as a Natural Test Substance (Cardero Therapeutics)

(-)-Epicatechin is a naturally-occurring product found in chocolate and tea. It is a member of the flavonoid family. The (-)-epicatechin is purchased commercially and is obtained from tea extracts:

Manufacturer	Sigma-Aldrich
Product #	E1753
Country of Origin:	India

It has >90% purity by HPLC, and has been purified in a GMP facility as follows: it is dissolved in ethanol, treated with charcoal, filtered to remove insolubles, the solvent is exchanged to purified water, and then the solvent is removed by lyophilization.

The final test substance is tested in a GMP analytical lab using appropriate qualified HPLC methods for impurities, including chirality testing. Release specifications require > 90% purity, <5% of the enantiomer, and 5% of catechin. It is also tested for other characteristics typical in GMP materials (identity by 1H NMR, IR; water content by Karl Fischer titration; ethanol content by GC; and the general USP tests of residue on ignition and heavy metals). Based on test results, a Certificate of Analysis is generated (see Appendix) and a %-content by weight is calculated.

Chemical name:	(-)-Epicatechin or (2R, 3R)-2-(3,4-dihydroxyphenyl)chroman-3,5,7-triol
Molecular formula:	$C_{15}H_{14}O_6$
CAS #:	490-46-0
Molecular weight:	290.27 grams/mole
Appearance:	off-white to light brown solid
Solubility:	>5 mg/ml in water at 20^{o}C
pKa:	neutral
Taste:	moderately bitter, resembling aspirin in degree of bitterness

(-)-Epicatechin is a single enantiomer containing two defined chiral centers. The structure of (-)-epicatechin is depicted in Figure 1. The absolute stereochemistry of (-)-epicatechin is as shown because it is either isolated from natural sources and further purified, or it is synthesized and compared to the natural isomer.

Stability (-)-Epicatechin has been demonstrated to be stable in the lyophilized state for at least six months under refrigeration. A stability study is ongoing and retest dates will be generated based on results.

7.2.2 (-)-Epicatechin as supplies as a natural test product

The intended route of administration is oral. (-)-Epicatechin isolated from tea will be supplied by Cardero Therapeutics in gelatin capsules, each containing 25 mg (-)-epicatechin combined with excipients. The test product is used as is, and swallowed with some water. Table 5 describes the (-)-epicatechin capsule formulation.

Table 5: Contents of the 25mg (-)-epicatechin capsule.

Component	Role	Amount
Epicatechin	Natural Ingredient	25.00mg
Microcrystalline cellulose@ (Avicel PH 102)	Diluent	127.00mg
Crospovidone@ (Kollidon CL)	Disintegrant	4.80mg
Citric acid monohydrate@	Acidifying agent	1.60mg
Colloidal Silicon dioxide@	Glidant	0.80mg
Magnesium Stearate@	Lubricant	0.80mg
Total		**160.00mg**

Gelatin Capsule The gelatin has been formulated into hard gelatin capsule shells for use in human pharmaceuticals. The gelatin itself is limed bone gelatin, and has been obtained from Rousselot SAS (production site: Chemin Moulins Premiers, France – 84800 Isle-Sur-La-Sorgue). The applicant has obtained Certificate of Suitability No. R1-CEP 2000-029-Rev 03, dated 22 July 2011, from Rousselot. According to this certificate, the substance GELATIN meets the criteria described in the current version of the monograph *Products with risk of transmitting agents of animal spongiform encephalopathies* (no. 1483 of the European Pharmacopoiea).

Sterility and pyrogen testing Sterility and pyrogen testing has not been conducted, as test product is supplied as lyophilized powder for oral use only. All products to be included in (-)-epicatechin formulation (capsules) will be USP grade (vitamin C, EDTA, cellulose, lactose). Sterile water will be used to prepare liquid doses for immediate and single use.
Stability A stability study of the test product in capsules is ongoing. Retained samples of clinical trial supplies will be tested to confirm potency.

7.3 Treatment Cycles Drug Dispensation

Medications will be dispensed for the entire period of time between study visits, and will be clearly marked as investigational drugs per UC Davis Investigational Drug Service Pharmacy procedures. The site will dispense study medication/placebo to the participant and will keep a medication log to document all study medication dispensed to participants. The participant will be instructed to take the study medication per protocol dosing schedule, every day during the eight-week treatment period.

7.4 Investigational Product Accounting Procedures

At each study visit, participants will be asked to return used medication containers from the previous month(s), as well as any unused medications. The participating centers will measure any unused medication and the site coordinator will keep records of the participant's medication use.

7.5 Maintenance of Randomization Codes and Emergency Unblinding

This study is open-label and no emergency unblinding procedures are necessary.

8 ASSESSMENT OF EFFICACY

8.1 Laboratory Efficacy Parameters

- Peripheral Blood Tests

  Peripheral venous blood will be collected at points specified in the study visit chart to evaluate:
  - Creatine phosphokinase (CPK)

- Follistatin
- mRNA and miRNA biomarker profiles
- Pharmacokinetics: epicatechin metabolites [serum tested at trough, 2 hours (Tmax), 4 hours after dose at baseline, week 2, week 4 and week 8].
- Blood lactate testing during exercise: Blood lactate levels will be collected at baseline and 2-minute intervals by finger-stick or ear-stick during 6-minute cycle testing.

- Muscle Biopsy (Biceps Brachii)

  Open biopsies of the mid-biceps will be conducted at baseline and after 8 weeks of study medication dosing. The muscle biopsy samples will be collected by open biopsy or according to standard hospital procedures. The minimum amount of muscle tissue required is a small piece of muscle of at least 0.5 x 1.0 x 1.0 cm. If possible, the tissue collected will be of sufficient size to provide 4 (four) pieces of tissue (each 0.5x0.5x0.5cm). Muscle for histological evaluation will be sent fresh to hospital pathology. Muscle tissue for evaluation at outside labs will be immediately frozen in liquid nitrogen-cooled 2-methylbutane and stored at -80°C or -70°C till shipment. Biopsy at baseline will be collected from the lateral aspect of the midpoint of the biceps muscle without the use of depolarizing agents or Bovie catheterization prior to tissue extration. Biopsy at 8 weeks will be collected from medial aspect of the midpoint of the biceps muscle of the same arm, and must be separated by a margin of at least 1cm to avoid scar tissue from the earlier biopsy.

  Histology samples will be evaluated (Lee-Way Jin lab, UC Davis) to examine:
  - Fiber size variation
  - Tissue necrosis
  - Degree of central nucleation
  - Presence of basophilic/regenerating fibers
  - Fiber type (myosin IHC stains)
  - NADH-TR and succinic dehydrogenase (mitochondria)

  Muscle biopsy samples will be examined by Western blot (Robert Henry lab, UC San Diego) to evaluate:

  Initial Phase (done immediately upon biopsy)
  - Muscle regeneration: follistatin, MyoD, myogenin, dystrophin, sarcoglycans, dysferlin
  - Oxidation/oxidative stress: SOD1, SOD2, catalase, GSK, nNOS, eNOS
  - Mitochondrial function: ETC-I, ETC-V, mitofilin, porin
  - Transcriptional regulation: PGC1-alpha
  - Collagen deposition: Collagen I, collagen III, fibronectin

  Delayed Phase (completed on stored material at a later date if warranted by initial data)
  - Glucose transport: Glut1, Glut 4 (and ratio)
  - Apoptosis: cytochrome-c, caspase
  - Dystrophin-associated complex (DAP) (Steve Moore lab, University of Iowa): sarcoglycans (alpha, beta, gamma, delta, epsilon), laminin-2 (alpha, beta, gamma), alpha dystrobrevin-2, syntrophin
  - Matrix metalloproteinase-9

  Muscle biopsy samples will be examined by electron microscopy (Robert Henry lab, UC San Diego) to evaluate:

  Initial Phase (done immediately upon biopsy)
  - Sarcomeric structure
  - Mitochondrial number and localization

  Delayed Phase (completed on stored material at a later date if warranted by initial data)
  - Myofiber volume
  - Mitochondrial volume per myofiber volume

- Mitochondrial christae density

8.2 Functional Efficacy Parameters

- Six-Minute Walk Test

Participants will complete the Duchenne muscular dystrophy six-minute walk test (6MWT) as described by McDonald et al [70]. Methods will be adapted for the adult population by removing the element of constant verbal encouragement. Measurements recorded will include 25-meter split times and total distance traveled. During the 6MWT, participants will undergo respiratory gas exchange testing using a Cosmed "backpack" portable metabolic system and muscle perfusion testing using a non-invasive portable near-infrared spectroscopy (NIRS) system.

- Graded exercise test using a recumbent cycle ergometer

Each participant will perform a graded exercise test on an electronically braked recumbent cycle ergometer as previously described [71, 72]. Modifications for Becker muscular dystrophy related proximal weakness will be made as described by others [72, 73]. The exercise test will began by participants pedaling at a rate of 60 revolutions/min with no load for 1 min (warm up). After the warm-up period, the work rate (WR) on the cycle ergometer will start at 10 W and increase either 10 W/min or every other minute for Becker muscular dystrophy participants until volitional exhaustion. Level of exertion will be monitored by heart rate and the Borg visual analogue scale [74]. Increments will be adjusted so that the duration of the test will be kept between 12 and 15 min. Twelve-lead ECG, blood pressure, O2 consumption ($V\dot{}O2$), CO2 production ($V\dot{}CO2$), and ventilation (VMax, SensorMedics/VIASYS Healthcare) will be continuously measured. After volitional exhaustion, participants will be immediately placed in the supine position, where a postexercise ECG will be performed within 15–30 s. Exercise tests will be considered to be maximal if the peak heart rate (HR) is 85% of that predicted for age (220 - age) and/or the peak respiratory exchange ratio (RER; $V\dot{}CO2/V\dot{}O2$) is ≥ 1.15. Muscle perfusion testing of a quadriceps muscle will be performed during cycle exercise using a non-invasive portable near-infrared spectroscopy (NIRS) system as described by Allart et al. [75]. We will use a non-invasive three channel, portable continuous-wave NIRS device (PORTAMON, Artinis Medical Systems, Zetten, The Netherlands). Participants will undergo blood lactate testing at baseline and at 2-minute intervals as specified in the "Peripheral Blood Tests" section.

- Quantitative Muscle Testing

Isometric and isokinetic QMT of elbow and knee flexors and extensors will be conducted using the BIODEX ergometer. The highest value of three consecutive maximal strength testing efforts will be recorded. Specific methods for testing can be found in the BIODEX manual of operations. Quantitative hand grip strength will be assessed using the CiTEC handheld ergometer, again recording the highest value of three consecutive tests for each hand. Methods for testing can be found in the CiTEC manual of operations.

- Range of Motion Testing

Passive ranges of motion for knee extension, ankle dorsiflexion, elbow extension and wrist extension will be measured to the nearest 5 degrees using goniometry techniques as described by Pandya et al [76] and Fowler [77]. Knee and elbow extensions ranges from 20 to -150 degrees. Ankle dorsiflexion range is from 20 to -80 degrees, with 0 degrees considered full passive range of motion. Wrist extension with fingers extended range is from 100 to -90 degrees, with 90 degrees considered full range of motion.

- Pulmonary Function Tests

Standard PFTs will be done using the Renaissance II spirometer. Spirometry has been used extensively as a measure of respiratory function. PFTs include maximal inspiratory pressure (MIP), maximal expiratory pressure (MEP), forced vital capacity (FVC), forced expiratory volume 1 (FEV1), peak expiratory flow (PEF), maximum ventilatory volume (MVV) and peak cough expiratory flow (PCEF).

- Timed Function Testing

Timed Function Testing has frequently been used to assess functional abilities in three important areas: rising from the floor, climbing four standard stairs and walking/running 10 meters. Each of these skills is timed to provide a quantitative measure of function. Specific methods are outlined in Florence et al [78].

- Functional Evaluation

The functional classification used by CINRG utilizes a scale modified from the upper extremity scale reported by Brooke et al [79] and the lower extremity scales used by Vignos et al [80]. The functional grades consist of six levels of function for the upper extremities and eleven levels for the lower extremities. Specific methods are outlined in the CQMS User's Manual.

- Anthropometric Measurements

Standing height will be measured to the nearest 0.1cm in all participants. Weight will be measured to the nearest 0.1kg. Waist and hip circumference will be measured to the nearest 0.1cm and waist-hip ratio will be calculated.

- 'Body Composition Assessment by Dual energy X-ray absorptiometry (DEXA) (15 minutes):

DEXA scans are primarily used to evaluate bone mineral density. It can also be used to measure total body composition and fat content with a high degree of accuracy. For this study it will be used to measure total and regional body composition. DEXA uses X-rays to assess bone mineral density or measure total body composition. However, the radiation dose is approximately 1/10th that of a standard chest X-ray. Bone density will be evaluated based on whole-body and subcranial total bone mass and areal bone mass (adjusted mass for bone size).

- Multifrequency Bioimpedence Assessment (MFBIA)

Participants will undergo multifrequency bioimpedence assessment (MFBIA) testing using a Xitron model 4000 MFBIA system to painlessly and non-invasively assess the amount of total body water, intracellular water, extracellular water and fat-free mass. One electrode is placed to bisect the ulnar head, and the other electrode is placed just behind the middle finger. On the ankle, one electrode is placed to bisect the medial malleolus, and the other electrode is placed just behind the middle toe. Impedence is measured between upper and lower extremity electrodes.

- Upper Extremity Range of Motion Testing (20 minutes)

Upper Extremity Range of Motion Testing (20 minutes). The subject will be asked to perform the following range of motion activities while wearing the Cal-FIT physical activity motion analysis system and recorded by the stereo-camera optical measurement system:

1. move their elbow through a complete range of motion from maximally flexed to maximal extension
2. move the forearm through a complete range of motion from maximal pronation to maximal supination
3. move the wrist through a complete range of motion from maximal extension (dorsiflexion) to maximal flexion (palmar flexion) and from radial deviation through ulnar deviation
4. move the shoulder through a complete range of motion from maximal extension to maximal flexion, maximal abduction to maximal adduction, and from maximal internal rotation to maximal external rotation.

While being monitored by a motion sensing system (KINECT):

1. perform a series of standardized range of motion and functional upper extremity tasks
2. perform a series of standardized range of motion and functional upper extremity tasks while holding a weight

9 ASSESSMENT OF SAFETY

9.1 Safety Parameters

Safety parameters for this study will include:

- Review of Systems
- Review of Adverse Events

- Laboratory Assessments: Concurrent with peripheral blood draws listed in the efficacy section and according to the study visit procedure chart, participants will provide blood samples for standard clinical safety testing. Safety panels will include:
  - CBC/Diff
  - PT/PTT
  - CPK
  - Total cholesterol
  - Albumin
  - Total Bilirubin
  - Conjugated Bilirubin
  - GGT
  - ALT/AST
  - Alkaline Phosphatase
  - Sodium
  - Potassium
  - CO2
  - Chloride
  - Calcium
  - BUN
  - Serum Creatinine
  - Serum Glucose
  - Serum Total Protein
  - Fasting lipid profile
  - BNP
- EKG
- Review of Medical History
- Review of Medical and Surgical Events
- Physical and Neurological Exam
- Review of Medication History
- Review of Current Medications and Therapies
- Review of any Adverse Events
- Collection of Vital Signs
- Collection of Height & Weight

9.2 Procedures for Reporting Adverse Events

Adverse events must be recorded in the study source documentation and eCRFs. All events occurring after the informed consent is signed must be reported regardless of whether or not they are believed to be related to the study drugs or procedures. During study participation, adverse events should be followed by the study site principal investigator until resolved. All adverse events are graded according to the CTCAE.

9.3 Data and Safety Monitoring Board / Medical Monitor

This study will not employ a Data and Safety Monitoring Board (DSMB) but adverse events will be reviewed by an appointed Medical Monitor who is otherwise not affiliated with the study.

In the event of a SAE, the Medical Monitor will be notified within 24 hours of notice of the event to the Coordinating Center, and details of the event will be provided for review at that time. Additional follow-up information will be provided to the Medical Monitor within 15 days of the initial event. Summaries of all other reported adverse events will be reviewed by the Medical Monitor on a quarterly basis.

9.4 Duration of Participation / Follow-Up

Participants will participate in the study for a period of approximately eight to ten weeks from the date of enrollment (eight weeks from start of study drug administration).

9.5 Trial Stopping Rules or Discontinuation Criteria

Participants may withdraw from the study at any time without prejudice. Study investigators may withdraw participants at any time for reasons such as:

- Inability to comply with study protocol
- SAEs
- Non-tolerable or non-manageable adverse events (AE)
- Severe or sustained unexplained laboratory abnormalities
- Recommendation of suspension of the study by the Medical Monitor

10 HUMAN PARTICIPANTS

10.1 Characteristics of the Study Population

Ambulatory male adults with a confirmed diagnosis of BMD, 18 years to 60 years, will be recruited.

Local Participant Population Available

The incidence of BMD is equal across racial and ethnic groups. It is assumed that the participant population of the study site should closely mirror the racial and ethnic distribution of the US population.

Projected Number of Participants Needed for this Study

	American Indian or Alaskan Native	Asian or Pacific Islander	Black, not of Hispanic Origin	Hispanic	White, not of Hispanic Origin	Other	Total
MALE	0	1	1	2	6	0	10

10.2 Participation of Children, Women and Minority Populations

BMD is an X-linked recessive disease affecting only males. However, female carriers of the disease can be symptomatic due to skewed X-inactivation. We have opted to study the most commonly affected population, males, to ensure patient homogeneity.

10.3 Sources of Research Material from Living Participants

Study data, blood and tissue samples collected will only be for research use or monitoring of study safety.

10.4 Recruitment of Participants

Participants will be recruited through the clinics of participating investigators, advertising and medical record screening in participating clinics. This trial will also be listed and updated on www.clinicaltrials.gov.

10.4.1 Advertising

Any local advertising through newsletters or muscular dystrophy-associated organizations' mailings will be submitted to the local institutional review board (IRB) that provides overview of this project. An advertisement is included in Appendix C.

10.4.2 Screening

Patients for this study will be identified through advertising, self-referral, referrals from other physicians and review of patients' clinic medical records currently under the care of the study site's principal investigator or co-investigators. Personnel who review existing patient medical records must be designated by the study site's principal investigator and must be an employee of that institution. A list of those personnel must be supplied to the institution's IRB. A telephone script (See Appendix B) will be used for the initial contact of potential study participants. If the potential participant is to be contacted by mail, the mailing letter template (Appendix B) should be used.

10.5 Informed Consent/Assent and Ethical Considerations

Informed consent/assent must be documented for each participant. The date and time of the consent/assent must be prior to the initiation of any study-related tests or procedures, including diagnostics that might be required to confirm a participant's study eligibility. The consent/assent form should supplement, not replace dialogue between the study principal investigator and the patient.

10.6 Retention of Participants

Care of each participant will be supervised by the participating site's principal investigator and study coordinator, who will schedule all visits and assessments.

If a participant is non-compliant, efforts will be made by site staff to ensure participant compliance, including participant/family teaching and increased frequency of phone contact to reiterate necessary information. If compliance does not improve, the investigator may decide to withdraw the participant from the trial. Any withdrawn participant will complete a follow-up visit along with an overview of systems and any adverse events that were noted during their study participation. Any ongoing adverse events will be monitored for 30 days after the participant's follow-up/withdrawal visit.

10.7 Potential Risks

10.7.1 Risks of Epicatechin

Animal and human safety studies suggest that (-)-epicatechin at doses of 1-2 mg/kg should be safe and well tolerated. In a study with green tea extracts containing (-)-epicatechin given orally to rats daily for 6 months, the no-observable-adverse-effect level (NOAEL) corresponded to 85 mg (-)-epicatechin/kg [67]. For the human dosing plan of 1 mg/kg, this provides a safety margin relative to the rat NOAEL of at least 85X. The dosing plan is also supported by published human studies, in which (-)-epicatechin doses of 1 and 2 mg/kg were administered with no adverse events were reported [11]. Potential risks based on the biological activities of (-)-epicatechin include:

Hypotension Given the reported effects of (-)-epicatechin on blood vessels, it is reasonable to assume that a potential risk may be associated with vasodilation. Of note is the fact that so far, no blood pressure reducing effects by (-)-epicatechin have been reported in normal subjects [81]. With cocoa based studies, blood pressure reducing effects are only reported in humans that have high blood pressure [81]. There is the possibility that patients undergoing pharmacologic treatment for high blood pressure if given (-)-epicatechin may develop hypotension through additive or synergistic effects.

Migraines The facilitation of migraines has been associated with the action of vasoactive substances [82]. The consumption of cocoa products has been reported to be associated with increased likelihood of migraine development [83, 84]. Thus, it is reasonable to surmise that (-)-epicatechin may increase the chances for migraine development in susceptible individuals.

Bleeding Anti-clotting like effects have been described for multiple members of the flavonoid family including (-)-epicatechin [85]. There is no report as to the effects that (-)-epicatechin per se may have on phenomena such as platelet aggregation or clotting times. There is a limited number of reports on the effects of cocoa on these endpoints. In general, they report effects on platelet aggregate
As with any compound, hypersensitivity reactions may occur, although no such reactions have been seen to date.

Contraindications and Warnings No contraindications are known. However, due to the possibility that (-)-epicatechin may interact with drugs with known antiplatelet effects to potentiate their activity, participants who are currently on long-term therapy with any such agents will be excluded from the study.

Overdose Neither the effects of overdose of (-)-epicatechin nor an antidote to overdose are known. The intraperitoneal (-)-epicatechin median lethal dose (LD_{50}) reported for mice is 1000 mg/kg as stated in the MSDS provided by suppliers such as Sigma-Aldrich.

Pregnancy and Lactation In a developmental toxicity study in pregnant rats using a green tea extract, the no-observable-adverse-effect level (NOAEL) corresponded to 100 mg (-)-epicatechin/kg (the highest dose tested)[68]. No information is available on levels of (-)-epicatechin in breast milk.Risks of

10.7.2 Risks of Blood Tests

The risks of blood drawing include soreness or bruising at the site of the needle. A local numbing cream (EMLA) will be applied to the area. There are no side effects associated with the use of this cream. Rarely, a more serious injury, such as hematoma (bleeding under skin) or infection may develop.

10.7.3 Risks of Muscle Biopsy

After the muscle biopsy patient may feel pain at the place of the biopsy. Patients usually find the pain easy to tolerate and that they rarely need to take a painkiller although Tylenol or ibuprofen is appropriate as needed for the next 24 hours. The muscle biopsy may leave a small scar and it is possible that the strength of that muscle might be slightly reduced in the short term.

10.7.4 Risks of QMT

QMT will be done at each visit to measure strength in the participant's muscles. At this time, there are no known risks associated with functional evaluation or muscle strength testing methods used in this protocol. However, the participant may experience mild muscle soreness the day after muscle testing.

10.7.5 Risks of PFT

These tests may cause dizziness and lightheadedness during and shortly after the test.

10.7.6 Risks of EKG

The EKG has no known risks.

10.7.7 Risks of Functional Evaluation and Muscle Strength Testing

At this time, there are no known risks associated with functional evaluation or muscle strength testing methods used in this protocol. However, the participant may experience mild muscle soreness the day after muscle testing.

10.7.8 Risks of DEXA

DEXA scans involve exposure to a very small amount of radiation (<1.0mrem). DEXA is contraindicated in pregnancy (not applicable in this project).

10.7.9 Risks of Multi-Frequency Bioimpedence Assessment (MFBIA)

There are no known risks of MFBIA testing.

10.7.10 Risks of Upper Extremity Range of Motion Evaluation

There are no known risks of upper extremity range of motion evaluation.

10.8 Procedures for Minimizing Risks

Confidentiality of medical information will be maintained throughout the study. Participants will be assigned identification numbers that will be used on all case report forms. No personally identifiable information will be released beyond the Study Coordinating Center without the participant's prior written consent. Data entered into electronic case report forms will be handled by the RedCap online CRF system and managed in compliance with FDA privacy and data retention standards for electronic clinical research data collection.

Safety data recorded during the conduct of this study will be transmitted directly to the Medical Monitor through the RedCap system. Data will be collected at each study visit and over the phone (family reported AEs) between study visits. Both AEs and SAEs will be reviewed by the Medical Monitor. AEs will be reviewed on a quarterly basis by the Medical Monitor.

10.9 Justification of Risks to Participants

Due to the low toxicity profiles of (-)-epicatechin, risks to subjects associated with participation in this study are less than or similar to standard clinical interventions in patients with BMD.

10.10 Benefits

The intended benefits outweigh the risks of using the study medication and study procedures in BMD patients. The participants may experience an increase in muscle strength or a delay in strength decline. The participants will receive additional care during study participation including muscle strength training, contracture measurements and spirometry during each study visit. Medical and adverse event history will be closely monitored. The additional medical monitoring allows for increased interaction with medical staff above expected routine clinical care. The increased monitoring of safety labs will also be performed.

It is possible the participants will not experience any direct benefit as a result of their study participation. However, the data collected during this trial may provide information that will benefit the scientific community as well as other individuals with BMD

10.11 Financial Considerations

No financial compensation will be given to participants or their families for participation in this trial other than minor assistance with transportation expenses (parking, etc.).

11 STATISTICS

Presented herein is the overall analysis plan. The main criterion for success of the study will be presence of one or more biologic or strength and performance outcome measures (Aims 1 and 2) that yield a response magnitude that allows for sufficient power in a Phase II B study with a sample size of 30 individuals. Analysis, employing both statistical and graphical presentations of data, will generally proceed from descriptions and simple comparisons to multiple variable models. This will help to ensure proper understanding of the data at each level before proceeding to the next. Descriptive analysis of means and proportions will characterize study participants overall as well as evaluate assumptions of normality and homoscedasticity. Any significant departures from these assumptions especially for the measurement variables will lead to normalizing or variance stabilizing transformations or, in the unlikely event these are not successful, to conversion to ranks. Baseline and screening measures will be used to calculate test-retest reliability using the intraclass correlation coefficient. Level of statistical significance is set at <0.05. Simple comparative analyses involving chi squares ttests and correlations will be used to assess and understand simple relationships, including defining first order interactions. Where possible we will perform longitudinal analyses using mixed models (using xtreg or xtmixed in STATA 12) to evaluate raw scale and percent change over time in study outcomes measures. These procedures will allow us to account for fixed and random effects as well as correlation between observations when we introduce repeated measures on the same individuals. If feasible with the small number of participants in the study and because there is little available data for individuals with Becker muscular dystrophy, this pilot data will be used to determine estimates of minimally important clinical difference (MCID) for each measure (defined as 1/3 S.D. for the population). Safety data will be compiled as tables of frequency and severity of adverse events by body system.

12 DATA COLLECTION

12.1 Data Management System

A web-based clinical data entry system, RedCap is being used for electronic case report form (eCRF) data collection. The data management system complies with all federal regulations pursuant to 12 CFR Part 11.

12.2 Data Quality Control and Quality Assurance

The RedCap System will provide validity checks on participant and visit identifier fields, as they are entered, to ensure that the visit is unique and appropriately timed according to protocol criteria. A secure web-based ‘smart’ eCRF system will detect some inaccuracies in data entry immediately to alert site study staff prior to data submission.

12.3 Security and Backups

To ensure patient confidentiality, no patient identifiers are entered into this system. Sequential study numbers are assigned to all participants. All computers are password protected and accessible only to study personnel.

All data entered into the RedCap System are copied by the UC Davis CTSC Data Coordinating Center to a secure back-up server at another site several times per day.

12.4 Data Monitoring

Over the course of the study, the Study Chair, Project Manager, biostatisticians and Medical Monitor will require access to the entire study dataset. This may be for the purposes of monitoring a specific site's data, performing quality control, performing periodic data analysis or for safety or efficacy monitoring. The above named individuals will have read-only access to the study data via the RedCap web interface.

13 REFERENCES

1. Crozier, A., I.B. Jaganath, and M.N. Clifford, *Dietary phenolics: chemistry, bioavailability and effects on health.* Nat Prod Rep, 2009. **26**(8): p. 1001-43.
2. Landolino, A.B. and D.R. Cook, *Phenylpropanoid metabolism in plants: biochem- istry, functional biology, and metabolic engineering.* In: Fraga, C.G. (Ed.), Plant Phenolics and Human Health: Biochemistry, Nutrition, and Pharmacology. Wiley, Hoboken, 2010: p. 489-563.
3. Steinberg, F.M., M.M. Bearden, and C.L. Keen, *Cocoa and chocolate flavonoids: implications for cardiovascular health.* J Am Diet Assoc, 2003. **103**(2): p. 215-23.
4. Nogueira, L., et al., *(-)-Epicatechin enhances fatigue resistance and oxidative capacity in mouse muscle.* J Physiol, 2011. **589**(Pt 18): p. 4615-31.
5. Ramirez-Sanchez, I., et al., *(-)-epicatechin activation of endothelial cell endothelial nitric oxide synthase, nitric oxide, and related signaling pathways.* Hypertension, 2010. **55**(6): p. 1398-405.
6. Ramirez-Sanchez, I., et al., *(-)-Epicatechin induces calcium and translocation independent eNOS activation in arterial endothelial cells.* Am J Physiol Cell Physiol, 2011. **300**(4): p. C880-7.
7. Taub, P.R., et al., *Alterations in skeletal muscle indicators of mitochondrial structure and biogenesis in patients with type 2 diabetes and heart failure: effects of epicatechin rich cocoa.* Clin Transl Sci, 2012. **5**(1): p. 43-7.
8. Katz, D.L., K. Doughty, and A. Ali, *Cocoa and chocolate in human health and disease.* Antioxid Redox Signal, 2011. **15**(10): p. 2779-811.
9. Corti, R., et al., *Cocoa and cardiovascular health.* Circulation, 2009. **119**(10): p. 1433-41.
10. Shrime, M.G., et al., *Flavonoid-rich cocoa consumption affects multiple cardiovascular risk factors in a meta-analysis of short-term studies.* J Nutr, 2011. **141**(11): p. 1982-8.
11. Schroeter, H., et al., *(-)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans.* Proc Natl Acad Sci U S A, 2006. **103**(4): p. 1024-9.
12. Kolluru, G.K., J.H. Siamwala, and S. Chatterjee, *eNOS phosphorylation in health and disease.* Biochimie, 2010. **92**(9): p. 1186-98.
13. Moncada, S. and E.A. Higgs, *The discovery of nitric oxide and its role in vascular biology.* Br J Pharmacol, 2006. **147 Suppl 1**: p. S193-201.
14. Sessa, W.C., *Regulation of endothelial derived nitric oxide in health and disease.* Mem Inst Oswaldo Cruz, 2005. **100 Suppl 1**: p. 15-8.
15. Baba, S., et al., *Bioavailability of (-)-epicatechin upon intake of chocolate and cocoa in human volunteers.* Free Radical Research, 2000. **33**(5): p. 635-641.
16. Chow, H.H., et al., *Pharmacokinetics and safety of green tea polyphenols after multiple-dose administration of epigallocatechin gallate and polyphenon E in healthy individuals.* Clin Cancer Res, 2003. **9**(9): p. 3312-9.
17. Lee, M.J., et al., *Pharmacokinetics of tea catechins after ingestion of green tea and (-)-epigallocatechin-3-gallate by humans: formation of different metabolites and individual variability.* Cancer Epidemiol Biomarkers Prev, 2002. **11**(10 Pt 1): p. 1025-32.
18. Ottaviani, J.I., et al., *The stereochemical configuration of flavanols influences the level and metabolism of flavanols in humans and their biological activity in vivo.* Free Radic Biol Med, 2011. **50**(2): p. 237-44.
19. Ottaviani, J.I., et al., *Structurally related (-)-epicatechin metabolites in humans: assessment using de novo chemically synthesized authentic standards.* Free Radic Biol Med, 2012. **52**(8): p. 1403-12.
20. Rein, D., et al., *Epicatechin in human plasma: in vivo determination and effect of chocolate consumption on plasma oxidation status.* J Nutr, 2000. **130**(8S Suppl): p. 2109S-14S.

21. Richelle, M., et al., *Plasma kinetics in man of epicatechin from black chocolate.* Eur J Clin Nutr, 1999. **53**(1): p. 22-6.
22. Wang JF, S.D., Holt RR, Ensunsa JL, Fraga CG, Schmitz HH, Keen CL., *A dose-response effect from chocolate consumption on plasma epicatechin and oxidative damage.* J. Nutr. , 2000. **130**: p. 2115S-2119S.
23. Emery, A.E., *The muscular dystrophies.* BMJ, 1998. **317**(7164): p. 991-5.
24. Emery, A.E., *Population frequencies of inherited neuromuscular diseases--a world survey.* Neuromuscul Disord, 1991. **1**(1): p. 19-29.
25. Kliegman RM, B.R., Jenson HB, Stanton BF, *Muscular Dystrophies*, in *Nelson Textbook of Pediatrics*, B.R. Kliegman RM, Jenson HB, Stanton BF, Editor. 2007, Saunders Elsevier: Philadelphia, Pa.
26. Blain, A.M. and V.W. Straub, *delta-Sarcoglycan-deficient muscular dystrophy: from discovery to therapeutic approaches.* Skelet Muscle, 2011. **1**(1): p. 13.
27. Gumerson, J.D. and D.E. Michele, *The dystrophin-glycoprotein complex in the prevention of muscle damage.* J Biomed Biotechnol, 2011. **2011**: p. 210797.
28. Rando, T.A., *Role of nitric oxide in the pathogenesis of muscular dystrophies: a "two hit" hypothesis of the cause of muscle necrosis.* Microsc Res Tech, 2001. **55**(4): p. 223-35.
29. Tidball, J.G. and M. Wehling-Henricks, *The role of free radicals in the pathophysiology of muscular dystrophy.* J Appl Physiol, 2007. **102**(4): p. 1677-86.
30. Allen, D.G. and N.P. Whitehead, *Duchenne muscular dystrophy--what causes the increased membrane permeability in skeletal muscle?* Int J Biochem Cell Biol, 2011. **43**(3): p. 290-4.
31. Bodensteiner, J.B. and A.G. Engel, *Intracellular calcium accumulation in Duchenne dystrophy and other myopathies: a study of 567,000 muscle fibers in 114 biopsies.* Neurology, 1978. **28**(5): p. 439-46.
32. Bua, E., et al., *Mitochondrial DNA-deletion mutations accumulate intracellularly to detrimental levels in aged human skeletal muscle fibers.* Am J Hum Genet, 2006. **79**(3): p. 469-80.
33. Melov, S., et al., *Resistance exercise reverses aging in human skeletal muscle.* PLoS One, 2007. **2**(5): p. e465.
34. Parise, G., et al., *Antioxidant enzyme activity is up-regulated after unilateral resistance exercise training in older adults.* Free Radic Biol Med, 2005. **39**(2): p. 289-95.
35. Hiona, A., et al., *Mitochondrial DNA mutations induce mitochondrial dysfunction, apoptosis and sarcopenia in skeletal muscle of mitochondrial DNA mutator mice.* PLoS One, 2010. **5**(7): p. e11468.
36. Marzetti, E., et al., *Multiple pathways to the same end: mechanisms of myonuclear apoptosis in sarcopenia of aging.* ScientificWorldJournal, 2010. **10**: p. 340-9.
37. Orrenius, S., B. Zhivotovsky, and P. Nicotera, *Regulation of cell death: the calcium-apoptosis link.* Nat Rev Mol Cell Biol, 2003. **4**(7): p. 552-65.
38. Zamzami, N. and G. Kroemer, *The mitochondrion in apoptosis: how Pandora's box opens.* Nat Rev Mol Cell Biol, 2001. **2**(1): p. 67-71.
39. Millay, D.P., et al., *Genetic and pharmacologic inhibition of mitochondrial-dependent necrosis attenuates muscular dystrophy.* Nat Med, 2008. **14**(4): p. 442-7.
40. Jahnke, V.E., et al., *Metabolic remodeling agents show beneficial effects in the dystrophin-deficient mdx mouse model.* Skelet Muscle, 2012. **2**(1): p. 16.
41. Piskula, M.K. and J. Terao, *Accumulation of (-)-epicatechin metabolites in rat plasma after oral administration and distribution of conjugation enzymes in rat tissues.* J Nutr, 1998. **128**(7): p. 1172-8.
42. Kuhnle, G., et al., *Epicatechin and Catechin are O-Methylated and Glucuronidated in the Small Intestine.* Biochemical and Biophysical Research Communications, 2000. **277**(2): p. 507-512.
43. Donovan, J.L., et al., *Catechin Is Metabolized by Both the Small Intestine and Liver of Rats.* The Journal of Nutrition, 2001. **131**(6): p. 1753-1757.
44. Spencer, J.P.E., *Metabolism of Tea Flavonoids in the Gastrointestinal Tract.* The Journal of Nutrition, 2003. **133**(10): p. 3255S-3261S.
45. Baba, S., et al., *In vivo comparison of the bioavailability of (+)-catechin, (-)-epicatechin and their mixture in orally administered rats.* J Nutr, 2001. **131**(11): p. 2885-91.
46. Donovan, J.L., et al., *Catechin Is Present as Metabolites in Human Plasma after Consumption of Red Wine.* The Journal of Nutrition, 1999. **129**(9): p. 1662-1668.

47. Actis-Goretta, L., et al., *Elucidation of (-)-epicatechin metabolites after ingestion of chocolate by healthy humans.* Free Radic Biol Med, 2012. **53**(4): p. 787-95.
48. Abd El Mohsen, M.M., et al., *Uptake and metabolism of epicatechin and its access to the brain after oral ingestion.* Free Radical Biology and Medicine, 2002. **33**(12): p. 1693-1702.
49. Holt, R.R., et al., *Procyanidin dimer B2 [epicatechin-(4ß-8)-epicatechin] in human plasma after the consumption of a flavanol-rich cocoa.* The American Journal of Clinical Nutrition, 2002. **76**(4): p. 798-804.
50. Feng, W.Y., *Metabolism of green tea catechins: an overview.* Curr Drug Metab, 2006. **7**(7): p. 755-809.
51. Frank, H., Gray, Seymour *The determination of plasma volume in man with radioactive chromic chloride.* J Clin Invest, 1953. **32**(10): p. 991-999.
52. Loke, W.M., et al., *Pure dietary flavonoids quercetin and (-)-epicatechin augment nitric oxide products and reduce endothelin-1 acutely in healthy men.* Am J Clin Nutr, 2008. **88**(4): p. 1018-25.
53. Donovan, J.L., et al., *(+)-Catechin is more bioavailable than (−)-catechin: Relevance to the bioavailability of catechin from cocoa.* Free Radical Research, 2006. **40**(10): p. 1029-1034.
54. Ellinger, S., et al., *Epicatechin ingested via cocoa products reduces blood pressure in humans: a nonlinear regression model with a Bayesian approach.* Am J Clin Nutr, 2012. **95**(6): p. 1365-77.
55. Ranhotra, H.S., *The estrogen-related receptors: orphans orchestrating myriad functions.* J Recept Signal Transduct Res, 2012. **32**(2): p. 47-56.
56. Handschin, C., *Regulation of skeletal muscle cell plasticity by the peroxisome proliferator-activated receptor gamma coactivator 1alpha.* J Recept Signal Transduct Res, 2010. **30**(6): p. 376-84.
57. Handschin, C., et al., *PGC-1alpha regulates the neuromuscular junction program and ameliorates Duchenne muscular dystrophy.* Genes Dev, 2007. **21**(7): p. 770-83.
58. Lin, J., C. Handschin, and B.M. Spiegelman, *Metabolic control through the PGC-1 family of transcription coactivators.* Cell Metab, 2005. **1**(6): p. 361-70.
59. Huttemann, M., I. Lee, and M.H. Malek, *(-)-Epicatechin maintains endurance training adaptation in mice after 14 days of detraining.* FASEB J, 2012. **26**(4): p. 1413-22.
60. Yamazaki, K.G., et al., *Short- and long-term effects of (-)-epicatechin on myocardial ischemia-reperfusion injury.* Am J Physiol Heart Circ Physiol, 2008. **295**(2): p. H761-7.
61. Yamazaki, K.G., et al., *Effects of (-)-epicatechin on myocardial infarct size and left ventricular remodeling after permanent coronary occlusion.* J Am Coll Cardiol, 2010. **55**(25): p. 2869-76.
62. Drexler, H., et al., *Alterations of skeletal muscle in chronic heart failure.* Circulation, 1992. **85**(5): p. 1751-9.
63. Nisoli, E., et al., *Defective mitochondrial biogenesis: a hallmark of the high cardiovascular risk in the metabolic syndrome?* Circ Res, 2007. **100**(6): p. 795-806.
64. Patti, M.E., et al., *Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1.* Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8466-71.
65. Kousteni, S., *FoxO1: a molecule for all seasons.* J Bone Miner Res, 2011. **26**(5): p. 912-7.
66. Finanger Hedderick, E.L., et al., *Loss of sarcolemmal nNOS is common in acquired and inherited neuromuscular disorders.* Neurology, 2011. **76**(11): p. 960-7.
67. Matsumoto, N., et al. , *Repeated dose toxicity study of green tea extract in rat. .* Jpn.Pharmacol.Ther, 1999. **27**(9): p. 1701-7. [Results presented in GRAS Notice 000225: Catechins from green tea extract. U.S. FDA website: www.accessdata.fda.gov/scripts/fcn/gras_notices/grn000225.pdf].
68. Faqui, A., et al. , *Developmental toxicity of Polyphenon E in rats. .* Toxicologist, 2001. **60**(1): p. 220. . [Results presented in GRAS Notice 000225: Catechins from green tea extract. U.S. FDA website: www.accessdata.fda.gov/scripts/fcn/gras_notices/grn000225.pdf].
69. Clarke EG, C.M., *Vetinary Toxicology*. 3rd ed. 1975, New York: Bailliere Tindal.
70. McDonald, C.M., et al., *The 6-minute walk test as a new outcome measure in Duchenne muscular dystrophy.* Muscle Nerve, 2010. **41**(4): p. 500-10.
71. Spencer, C.T., et al., *Impaired cardiac reserve and severely diminished skeletal muscle O(2) utilization mediate exercise intolerance in Barth syndrome.* Am J Physiol Heart Circ Physiol, 2011. **301**(5): p. H2122-9.
72. Sveen, M.L., et al., *Endurance training improves fitness and strength in patients with Becker muscular dystrophy.* Brain, 2008. **131**(Pt 11): p. 2824-31.

73. Jansen, M., et al., *The assisted 6-minute cycling test to assess endurance in children with a neuromuscular disorder.* Muscle Nerve, 2012. **46**(4): p. 520-30.
74. Neely, G., et al., *Comparison between the Visual Analogue Scale (VAS) and the Category Ratio Scale (CR-10) for the evaluation of leg exertion.* Int J Sports Med, 1992. **13**(2): p. 133-6.
75. Allart, E., et al., *Evaluation of muscle oxygenation by near-infrared spectroscopy in patients with Becker muscular dystrophy.* Neuromuscul Disord, 2012. **22**(8): p. 720-7.
76. Pandya, S., et al., *Reliability of goniometric measurements in patients with Duchenne muscular dystrophy.* Phys Ther, 1985. **65**(9): p. 1339-42.
77. Fowler, W.M., Jr., et al., *Profiles of neuromuscular diseases. Design of the protocol.* Am J Phys Med Rehabil, 1995. **74**(5 Suppl): p. S62-9.
78. Florence, J.M., et al., *Clinical trials in Duchenne dystrophy. Standardization and reliability of evaluation procedures.* Phys Ther, 1984. **64**(1): p. 41-5.
79. Brooke, M.H., et al., *Clinical trial in Duchenne dystrophy. I. The design of the protocol.* Muscle Nerve, 1981. **4**(3): p. 186-97.
80. Vignos PJ Jr., S.G.J., Archibald KC, *Management of progressive muscular dystrophy of childhood.* JAMA, 1963. **184**: p. 89-96.
81. Ried, K., et al., *Does chocolate reduce blood pressure? A meta-analysis.* BMC Med, 2010. **8**: p. 39.
82. Appenzeller, O., *Pathogenesis of migraine.* Med Clin North Am, 1991. **75**(3): p. 763-89.
83. Fukui, P.T., et al., *Trigger factors in migraine patients.* Arq Neuropsiquiatr, 2008. **66**(3A): p. 494-9.
84. Gibb, C.M., et al., *Chocolate is a migraine-provoking agent.* Cephalalgia, 1991. **11**(2): p. 93-5.
85. Rein, D., et al., *Cocoa inhibits platelet activation and function.* Am J Clin Nutr, 2000. **72**(1): p. 30-5.