Plus Epicatechin Duchenne Muscular Dystrophy in Non-ambulatory Adolescents

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UCD0316: A single center dose ranging pilot study of (+)-epicatechin (Cardero Therapeutics, Inc) in non-ambulatory adolescents with Duchenne muscular dystrophy and pre-symptomatic cardiac dysfunction

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PROTOCOL SIGNATURE PAGE

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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

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APCI	atmospheric pressure chemical ionization	MPT	mitochondrial permeability transition
AMPK	adenosine monophosphate protein kinase	mPTP	mitochondrial permeability transition pore
BID	twice a day	NOAEL	no-observable-adverse-effect level
BP	blood pressure	NO	nitric oxide
C_{max}	maximum concentration	NOS	nitric oxide synthase
cGMP	cyclic guanosine monophosphate	OXPHOS	oxidative phosphorylation
CHD	coronary heart disease	PAT	peripheral arterial tonometry
CT	Cardero Therapeutics	PD	Pharmacodynamics
CVD	cardiovascular disease	PGC1✔	peroxisome proliferator-activated receptor gamma, coactivator-1✔
DBP	diastolic blood pressure	PK	Pharmacokinetic
eNOS	endothelial nitric oxide synthase	PO	Oral
FMD	flow mediated vascular dilation	POC	proof of concept
ETC	electron transport chain	ROS	reactive oxygen species
ERC	(-)-epicatechin-rich cocoa	RXNO	total plasma NO species
GRAS	Generally Regarded As Safe	SBP	systolic blood pressure
HCAEC	human coronary artery endothelial cells	SkM	skeletal muscle
HPLC	high-performance liquid chromatography	$T_{1/2}$	half-life
IP	intraperitoneal, intraperitoneally	T_{max}	time to maximum concentration
I/R	ischemia/reperfusion	UCD	University of California, Davis
IV	intravenous, intravenously		
L-NMMA	L-N^6-mono-methyl-arginine		
LC-MS	liquid chromatography mass spectrometry		
LD_{50}	median lethal dose		

Table 1: List of Terms and Abbreviations

1. STUDY SUMMARY

1.1 Summary

Duchenne muscular dystrophy (DMD) is a disabling and life-threatening X-linked genetic disorder caused by defects in the gene for dystrophin, a protein that stabilizes muscle cell membranes [1]. It is the most common neuromuscular disease of childhood affecting 1 in 3,500-5,500 males with an estimated prevalence in the U.S. of 15,000 [2, 3]. Complete loss of dystrophin causes increased muscle fragility, and males with dystrophinopathy develop progressive loss of muscle fibers with replacement by fat and connective tissue, severe disabling weakness, contractures and scoliosis, loss of ambulation and self-care skills, respiratory and cardiac failure, and premature death [4, 5]. Becker muscular dystrophy (BMD), is a phenotypically milder form of dystrophinopathy due to in-frame mutations and partial loss of dystrophin and these patients exhibit later manifestation of symptoms [6]. DMD creates tremendous psychological and emotional burden as well as financial burden on patients, parents and caregivers, siblings, and other family and friends [7-12]. Patients with DMD also utilize considerable health, education, and community resources. DMD has been estimated to have the highest per capita costs for medical rehabilitation services of any childhood disability [13].

The active compound (-) epicatechin is found in cocoa powder, green tea and other plants (Shay et al, 2015). Recent research has indicated that its enantiomer, (+)-epicatechin is more potent and more efficacious in vitro and in vivo compared to (-)-epicatechin with respect to stimulating new mitochondrial formation. (+)-Epicatechin is also a natural product, found in the guarana berry that serves as the source of widely consumed beverages in South America. Guarana extracts are found in over 100 sports and energy drinks marketed in the U.S., Europe, and South America. Its principal components are caffeine, theobromine, and theophylline. Components of guarana seed powder were identified as 8.8% humidity, 1.51% ash, 2.1% caffeine and 16% tannins. Chemical analysis indicates that in guarana extracts, (-)-epicatechin and (+)-epicatechin can be found in a ratio of about 10:1 respectively, that is, ~10% of total tannins correspond to (+)-epicatechin (i.e. 1-2% of total extract weight)[14, 15].

A recent study has shown that self-reported habitual consumption of guarana is associated with a lower prevalence of hypertension, obesity, and metabolic syndrome [16]. Guarana is Generally Recognized as Safe and a permitted food additive by FDA [17].

Many of today's medications are derived from plant extracts, including such commonly used medications as digoxin (foxglove), atropine (belladonna), codeine (poppy), irinotecan (dogwood) and taxol (pacific yew). Recent studies in animal models and humans demonstrate that epicatechin, the primary flavanol present in cacao, enhances cardiac and skeletal muscle structure and function to levels comparable to those triggered by exercise. Epicatechin works as an "exercise mimetic" in humans mimicking as a chemical homolog to an endogenous hydroxysterol released in humans as a normal response to exercise training. This recently discovered gene regulation pathway induced by exercise leads to: 1) improved muscle bioenergetics (via mitochondrial biogenesis whereby mitochondria replicate and/or undergo hypertrophy); 2) muscle structural growth and regeneration (via increasing follistatin and decreasing myostatin levels); 3) improved fat metabolism (increasing lipolysis, and decreasing lipogenesis and triglyceride levels); and 4) improved glucose metabolism and insulin sensitivity. As an exercise hormone mimetic, epicatechin bypasses the generation of oxygen radicals associated with exercise while mediating the benefits of exercise. It additionally reverses oxidative damage to heart and muscle by increasing the expression of superoxide dismutase, catalase, thioredoxin, and glutathione peroxidase.

The relative mitochondrial biogenesis effects of a recently discovered natural endogenous exercise hormone 11-β-OHP and it's structural homologs – both (-)-epicatechin and (+)-epicatechin have been explored extensively in animal models. (+)-epicatechin is a closer structural homolog to 11-β OHP and this small molecule has been shown by Cardero Therapeutics to be 400X more potent than (-)-epicatechin in inducing mitochondrial complex IV protein in tissue culture. The effects of 15 day treatment in 10 month old mice using 1 mg/kg/day of (-)-epicatechin vs. 0.1, 0.3 and 1 mg/kg/day of (+)-epicatechin were studied by Cardero Therapeutics and (+)-epicatechin was more potent at equivalent doses in inducing PGC1α production. There are many commercial sources of tea extracts containing (-)-epicatechin contaminated with numerous other flavanols, some of which are known inhibitors of the epicatechin effect on mitochondria. In contrast, the (+) isoform is very rare and not available commercially in either purified or in extract form. Therefore there is much less potential for unscrupulous marketing of ineffective preparations to families via marketing channels outside the purview of the FDA. Cardero

Therapeutics has developed the only known manufacturing process for pharmaceutical grade (+)-epicatechin, providing a means for effective commercialization of a validated, tested, effective drug product. Therefore (+)-epicatechin makes sense for a Duchenne muscular dystrophy (DMD) trial in terms of both potency and potential for future commercialization. Given encouraging results from an 8-week pilot trial of the weaker (-)-isoform of epicatechin in humans with Becker muscular dystrophy (BMD)(tissue and plasma biomarker responses, BNP reduction, and improved exercise capacity on graded exercise testing), animal literature demonstrating improved skeletal muscle and cardiac fibrosis, evidence of mitochondrial biogenesis from tissue proteomics in humans with Becker muscular dystrophy, there is a clear indication of the need for studies of (+)-epicatechin in dystrophinopathy patients with DMD. This proposed trial would also comprise the introduction of a novel therapeutic strategy- inducing mitochondrial biogenesis- as a therapeutic strategy in the treatment of Duchenne muscular dystrophy

In all disease contexts, loss of mitochondria is associated with accelerated muscle atrophy. The purpose of this pilot trial is to determine whether the addition of inducers of mitochondrial biogenesis (formation of new mitochondria), a new class of therapeutics, administered in conjunction with standard of care cardiac therapy, may ameliorate or reverse the loss of UE function (using state of the art measures of upper limb function in DMD), and improve the early signs of cardiac dysfunction seen in a spectrum of early non-ambulatory patients with DMD.

1.2 Study Purpose

This single center open-label pilot study will enroll 15 non-ambulatory children with Duchenne muscular dystrophy at least 8 years of age and who demonstrate pre-clinical cardiomyopathy (defined as a cardiac ejection fraction ≥40% with abnormal LV strain by cardiac MRI). They will receive (+)-epicatechin at one of three doses during an 8-week dose-ranging study with assessments at baseline, 2 Weeks, 4weeks, and 8 weeks. The study will determine optimal dosing for future cardiac efficacy studies based on serum / plasma biomarker response using follistatin: myostatin ratio, nitrite/nitrate ratio, cardiac troponins and cardiac BNP. Secondary endpoints will include additional biomarker assessments by SOMAscanTM, cardiac functional evaluations by cardiac MRI (LV strain), and echocardiogram (LV strain by speckle tracking) and measures of strength, range of motion and mobility, and clinical safety assessments. Results of secondary endpoint analysis will be used to refine design of subsequent clinical trials powered to detect changes in clinical outcomes.

1.3 Specific Aims

Aim 1: Establish the optimal dose of (+)-epicatechin over an 8 week exposure as determined by plasma follistatin: myostatin ratio (primary outcome), and nitrite/nitrate ratio, cardiac troponins, and cardiac BNP. We will test three unit doses: 25 mg PO BID (50mg daily), 25 mg PO TID (75mg daily), and 75 mg PO BID (150mg daily) (n=5 per group, stratified based on 6MWD) over 8 weeks.

Aim 2: (Biomarker Efficacy Primary Endpoints): Demonstrate Evaluation of the effect of (+)-epicatechin on circulating blood biomarkers of mitochondrial biogenesis and muscle regeneration in children with Duchenne muscular dystrophy Individuals who receive 8 weeks of epicatechin daily will show evidence of improved mitochondrial biogenesis. This will be reflected by plasma biomarkers associated with muscle growth and repair (follistatin:myostain ratio), vascular response to exercise (nitrite/nitrate ratio), inflammation and membrane integrity (MMP-9, plasma TNF-alpha, TGF-Beta, and CK), and selected SOMAscan™ validated measures associated with age-related DMD disease severity (Troponin I, fast skeletal muscle, Carbonic anhydrase 3, Troponin I, cardiac muscle, creatine kinase M-type, Mitogen-activated protein kinase 12 [MAPK12, proto-oncogene tyrosine-protein kinase receptor Ret, GDF 11, Cadherin-5).

Aim 3 (Clinical Efficacy Secondary Endpoints): Evaluation of the effect of (+)-epicatechin on cardiac and exercise capacity in children with Duchenne muscular dystrophy. We will explore responsiveness of clinical endpoints to (+) epicatechin. Individuals who receive epicatechin will be evaluated for evidence of short-term improvements in a) cardiac function by cardiac MRI, and b) exercise performance (HR, and VO2/Kg at defined workloads, 6MWT, 6-minute cycle test, Kinect reachable workspace fatigue). Short-term clinical benefits may be seen early at 8 weeks in a similar fashion to exercise “training-like” responses attributed to increases in mitochondrial biogenesis, muscle regeneration, and improvements in skeletal muscle perfusion. In addition traditional clinical efficacy endpoints will be explored.

Aim 4: (Safety and Pharmacokinetics Primary Endpoints): Evaluation of the safety and pharmacokinetic profiles of epicatechin in children with Duchenne muscular dystrophy. Assessments of safety will include a

standard safety panel including hematologic, hepatologic, renal and metabolic profiles. Phamacokinetic studies will include repeat assessments of trough and 2-hour post (peak) epicatechin plasma levels.

2. Background and Significance

2.1 The dystrophinopathies: Duchenne and Becker Muscular Dystrophy (DBMD)

Muscular dystrophies are a group of diverse genetic diseases featuring progressive muscle weakness, degradation of muscle fibers, and loss of function [3]. DBMD is an inherited spectrum disorder primarily seen in males, with an incidence of 1:3500-5000 live births [3]. Onset of symptoms can occur over a wide age range, with most patients diagnosed between 3 and 15 years of age [4, 5]. DBMD is characterized by progressive muscle loss (sarcopenia), with loss of strength, muscle injury, degeneration, atrophy, and eventually fibrosis and fatty replacement [18]. Patients with the more severe Duchenne (DMD) phenotype typically begin to lose ambulatory ability by 9-11 years of age and most are wheelchair dependent by 12-14 years. Patients with DBMD experience secondary complications of weakness including lung (e.g., breathing problems, infections) and heart (e.g., cardiomyopathy) complications that significantly impair mobility and participation and reduce lifespan [18, 19].

DBMD results from mutations in the gene encoding dystrophin, a key subsarcolemmal protein in the dystrophin-associated protein complex (DAPC) at the muscle cell membrane [1]. 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 [20, 21]. 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 [4].

2.2 Epicatechin directly targets multiple points in DBMD pathophysiology

The process of progressive strength loss in individuals with dystrophinopathies is due to a complex pathophysiologic cascade that occurs as a secondary effect of the loss of the dystrophin protein. Progressive damage to muscle tissue occurs due to changes in the structure of the contractile apparatus due to partial or complete loss of the dystrophin associated protein complex (DAPC), and results in secondary damage to and depletion of mitochondria. The mitochondrial injury itself is attributed to oxidation injury, calcium accumulation and excessive metabolic demands of regenerating muscle, where cellular energy demands are not sufficient to compensate. Alteration of expression profiles associated with improved oxidative phosphorylation in normal exercise response is decreased in DMD and reduces effectiveness of tissue remodeling [22, 23]. Activity or exercise-induced mitochondrial upregulation is also associated with improvements in insulin resistance and reduced incidence of diabetes, and decreases in mitochondrial capacity seen in dystrophinopathy patients are also seen in diabetic patients, suggesting that reduced activity or capacity in these pathways more accurately reflects physical activity status rather than any specific disease process. Thus, it is expected that drugs targeting mitochondrial biogenesis will have wide-ranging applications outside of the dystrophinopathies, and may also include groups such as patients with diabetes and obesity, age-related muscle wasting (sarcopenia), and sarcopenias that occur secondary to other conditions such as spinal cord injury and stroke

Epicatechins represent a novel small molecule approach to intervention directly upregulating this mitochondrial pathway. Treatment with epicatechins increases mitochondrial synthesis of ATP in response to metabolic demand within 48 hours, and concurrently activates the transcription pathway for muscle growth and regeneration by upregulating the protein PGC1a, which in turn upregulates follistatin, which is a key regulator of the growth antagonist myostatin. This has been demonstrated in neonatal mice, where PGC1a over expression resulted in increased expression of key muscle proteins including utrophin and type-1 myosin heavy chain as well as key mitochondrial proteins [24]. Overexpression of PGC1a also drives oxidative gene expression, aiding damaged mitochondria and supporting ATP production [25-30]. Selsby et al demonstrated that the increase in production of muscle proteins secondary to PGC1a overexpression increased Type 1 muscle fiber count, improved resistance to eccentric contraction-induced injury and fatigue and reduced evidence of muscle tissue necrosis in the *mdx* model of dystrophinopathies, and provides evidence that the approach improves dystrophic muscle resistance to structural damage secondary to activity and exercise [24].

Further evidence suggests that NF-kB, a protein which contributes to healthy tissue remodeling and adaptation in response to exercise, paradoxically reduces activation of PGC1a pathways in diseased conditions such as

dystrophinopathies, diabetes and cachexia, contributing to increased metabolic and oxidative stress to muscle tissue and subsequent loss of mitochondria, increased inflammation, and tissue degeneration [31].

In addition to decreased resistance to mechanical stressors, patients with dystrophinopathies also under express nitric oxide (NO) and show evidence of a subsequent lack of normal vascular response to exercise [32]. This reduces oxygen transport capacity to muscles during exercise, creating an environment where the tissue is chronically oxygen-starved during activity. Oxygen depravation leads to increased oxidative stress as the remaining mitochondria are forced to utilize relatively greater proportions of anaerobic mechanisms to create energy for the muscle tissue. The by products of this anaerobic process include damaging pro-inflammatory reactive oxygen species (ROS) that have been shown to trigger chronic activation of the NF-kB pathway discussed above.

The combination of reduced resistance to mechanical stress and reduced oxygen supply and resulting chronic inflammation creates a "double hit" that promotes a vicious cycle of tissue damage, inflammation and impaired remodeling that ultimately results in gradual and eventually almost complete replacement of muscle with non-functional fibrotic and fatty tissue.

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 secondary damage and/or improve skeletal muscle function. The epicatechin signaling pathway represents a *direct* intervention that addresses many of the defects that occur secondary to the loss of dystrophin that are currently under investigation. Treatment with the drug directly increases expression of follistatin resulting in reduction in production of myostatin and improved muscle growth and regeneration. Indirect immune-mediated reductions in myostatin using targeted antibodies have become a key therapeutic approach in dystrophinopathies [33]. Epicatechin use also results in upregulation of PGC1a which increases the number and quality of mitochondria in muscle tissue that leads to reduced oxidative stress, improved muscle function and improved insulin resistance. Indirect approaches to reduce oxidative stress using CoenzymeQ10 and idebenone are currently in development in DMD and have shown positive results [34, 35]. Indirect approaches to reducing inflammation and tissue remodeling that occur secondary to oxidative stress include ongoing study of anti-fibrotic drugs such as pentoxifylline and halofuginon [36-38]. Treatment with epicatechin directly results in increased NO production, improves vascular response to exercise and reduces tissue hypoxia and oxidative stress. Studies of the ability of PDE5 inhibitors (sildenafil, tadalafil) to block downstream degradation of of NO-derived vasodilators is currently being explored in DMD patients to improve oxygen supply to muscle tissue [39]. A summary of published and unpublished preclinical and clinical data supporting the evaluation of epicatechin in DBMD is presented in Table 2.

Table 2: 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 PGC1a 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 delta-sarcoglycan KO mouse model of MD

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.

2.3 Congestive Heart Failure

Congestive heart failure (CHF) represents a state of impaired cardiac pump function such that perfusion of the peripheral organs is inadequate. It is a syndrome associated with conditions representing a variety of injurious factors, such as diabetes, ischemic heart disease, and hypertension. Impaired cardiomyocyte contraction is associated with apoptosis of cardiac myocytes, prolonged increased levels of cytosolic calcium, and diminished capacity to generate the bioenergetics required for contraction. It has become increasingly clear that altered mitochondrial function sits at the confluence of toxic factors inhibiting contractile function of the heart in heart failure. Mitochondrial content is diminished in both animal and human models of the failing heart. The cardiac expression and activation of the mitochondrial transcription factor, PGC1 ✔, is inhibited in CHF; and down regulation of the mitochondrial transcription factor, Tfam, results in cardiomyopathy. Both sets of data suggest that cardiac mitochondrial biogenesis is impaired in CHF, resulting in an ability to maintain mitochondrial production of energy in response to cardiac demands. Elevated cytosolic calcium is toxic to mitochondria by opening up mitochondrial permeability transition pores (mPTP), which are non-selective pores that allow electrolyte flux into mitochondria, eventuating in swelling, decreased oxidative phosphorylation, and eventual fragmentation of mitochondria. This is turn is associated with initiation of myocyte apoptosis and consequent death of the host cell. Loss of mitochondria would additionally decrease the supply of bioenergetics required for cardiac cell contraction, and loss of the host cardiac cells themselves due to internal ischemia would result in impaired pump function, just as occurs with vascular ischemia-associated loss of cardiac myocytes. Myocyte apoptosis, ischemic myocyte necrosis, and opening of mitochondrial mPTP have all been documented in animal models of heart failure, suggesting they are an important contributing factor in the progressive dysfunction of the failing heart (reviewed in [40, 41]).

2.4 Cardiovascular Studies of (-)-Epicatechin

Epidemiological studies have correlated flavanol-rich diets with improved cardiovascular prognosis, and numerous human trials of flavonol-rich cocoa and chocolate preparations have shown these compounds to improve vascular function, including vasodilation, increased cerebral blood flow, blood pressure reduction in hypertensive individuals, insulin sensitivity and lipid reduction (reviewed in [42-47]). A recent meta analysis of interventional and observational studies encompassing ~114,000 subjects reports that the highest levels of chocolate consumption were associated with a 37% reduction in cardiovascular disease (relative risk 0.63 (95% confidence interval 0.44 to 0.90) and a 29% reduction in stroke compared with the lowest levels [48]. Cocoa flavanols produced positive effects on cerebral blood flow, including an increase in blood flow in the middle cerebral artery in elderly patients, as well as improvements in angiogenesis and cognitive function, suggesting that (-)-epicatechin may have benefit in dementia and stroke [49-53].

Positive vascular effects were confirmed in studies evaluating (-)-epicatechin in humans [54]. These studies used normal subjects and single dose treatments. In a randomized cross-over study, healthy individuals received water or (-)-epicatechin dissolved in water. The (-)-epicatechin doses were 1 (n=6) or 2 mg/kg (n=3). Flow mediated dilation (FMD) was measured before and 1, 2, 3, and 4 h (1 mg/kg) or 2 h (2 mg/kg) after treatment. Results demonstrate that 2 h after the ingestion of (-)-epicatechin all subjects had significant increases in flow mediated dilation and peripheral arterial tonometry that were similar between the two doses [54]. In a separate study by the same research group using a high-flavanol preparation, it was determined by multivariate analysis that the flavanol-dependent increase in vasodilation could be ascribed only to (-)-epicatechin and its primary metabolite [54].

2.5 Cardiovascular Mechanism of Action

The vascular effects of (-)-epicatechin appear to result from increased plasma nitric oxide (NO) and activation of endothelial nitric oxide synthase (eNOS) [54, 55]. While (-)-Epicatechin has well-characterized anti-oxidant properties [56-60], its ability to activate and induce NO in vascular endothelium and other tissues distinguishes it from other anti-oxidants and appears to be the therapeutically significant Mechanism of Action [61]. Endothelium-derived NO is essential for vascular health, as reduced eNOS and/or NO is associated with human athero-

sclerotic disease [62]. The stimulation of eNOS (and NO release) has been documented to trigger "healthy" outcomes under a variety of scenarios. A physiological means by which sustained increases in endothelial cell eNOS protein levels can occur is through exercise as shear stress is a well known stimulator of such effects [63]. It is thought that this is a prominent mechanism by which aerobic physical activity favors a healthier profile in particular, as it relates to blood pressure control. The upregulation of eNOS has also been associated with improved outcomes under pathological scenarios such as with myocardial infarction [64, 65].

When (-)-epicatechin is administered at increasing doses (0.1-1 μM) to cultured human endothelial cells, a progressive release of nitric oxide (NO) occurs secondary to the activation of the endothelial nitric oxide synthase (eNOS) that peaks by 10 min and plateaus at 1 μM. This effect is associated with biochemical (i.e. signaling) changes that indicate the presence of cell membrane like mediated responses. When (-)-epicatechin is given repeatedly over a periods of up to several days an upregulation in eNOS protein levels occurs, indicating enhanced capacity to produce NO for a given stimulus [61]. Flavanols and their metabolites induce vasodilation in isolated rabbit aortic rings *ex vivo*; this effect is blocked by the eNOS inhibitor L-N^6-mono-methyl-arginine (L-NMMA)[54]. In human subjects the vasodilatory effects of cocoa flavanols can also be blocked *in vivo* by infusion of L-NMMA [54]. Administration of purified (-)-epicatechin to healthy male volunteers correlated with increased NO bioavailability and reduced the plasma levels of endothelian-1, a potent vasoconstrictor [51].

2.6 Sympathetic / Parasympathetic Disorder, Cardiomyopathy and Congestive Heart Failure in Duchenne Muscular Dystrophy

Individuals with DMD exhibit early sinus tachycardia and reduced heart rate variability prior to onset of cardiac fibrosis, suggesting parasympathetic signaling defects precede and may contribute to myocardial remodeling. While reductions in CNS parasympathetic pathway alpha3beta2/beta4-AChRs [66] in *mdx* mice secondary to dystrophin loss has not been demonstrated in humans, there is consistent evidence of reduced parasympathetic vagal tone and increased heart rate in clinical populations. Six-minute walk test studies by McDonald et al demonstrated mild resting sinus tachycardia in 4-12 year old ambulatory boys with DMD, with a subsequently reduced cardiac response to exercise compared to healthy age-matched controls [67, 68]. Increased heart rate variability (HRV) due to autonomic dysfunction can precede onset of symptomatic cardiomyopathy, and HRV has been used as a predictive biomarker of morbidity and mortality in mutiple cardiac conditions. Inoue et al demonstrated that significant proportions of DMD patients without echocardiographic or serum BNP evidence of cardiomyopathy displayed measurable abnormalities in sympathetic tone as measured by R-R intervals >50ms, ratio of low-to-high frequency intervals (LF/HF ratio), and standard deviation for normal RR intervals in 24H (SDNN), with abnormal levels in 89%, 59%, 97% of patients, respectively [69]. Yotsukura et al showed that abnormalities in RR intervals and LF/HF ratio are progressive, but are present even in very young children with DMD, indicating that baseline disruptions are compounded by the development of cardiorespiratory insufficiency later in the disease [70, 71]. A recent study by Thomas et al [72] in 74 individuals with DMD and similar aged controls demonstrated that DMD-associated tachycardia and increased R-R intervals (decreased HRV associated with a predominance of sympathetic activity and decreased parasympathetic activity) were positively associated with late gadolinium enhancement of fibrosis, that differences in beta blockade and ACEi use were not associated with HRV, and that HRV was abnormal prior to the onset of clinical heart failure.

Patients with DMD ultimately suffer from severe cardiomyopathy, which manifests around ten years of age and is universally present in DMD patients by age 20. The initial stage of DMD cardiomyopathy consists of left ventricle dilatation which progresses to a fibrotic stage of dilated cardiomyopathy. It has been estimated that up to 60% of the premature mortality experienced by DMD patients is attributed to direct complications of progressive cardiomyopathy [41]. Most DMD patients with established heart failure are not symptomatically responsive to currently approved heart failure therapeutics, although initiating therapy with inhibitors of the angiotensin pathway in the earliest stages of cardiac dysfunction has been shown to delay the onset of clinically significant CHF by 1-3 years [73]. In DMD, both skeletal muscle and cardiac muscle are affected by progressive mitochondrial injury secondary to oxidative stress and cellular calcium overload, with a consequent excessive opening of mPTP resulting in a loss of mitochondrial density and an increase in myocyte death. Progressive functional weakness and fibrosis of both skeletal muscle and cardiac muscle ensues [74].

2.7 Proposed Therapeutic Effect of Epicatechin in CHF

Epicatechin has been shown to reduce skeletal muscle abnormalities that are shared with cardiac muscle in both animal models and human examples of muscular dystrophy. In cardiac myocytes, epicatechin stimulates mitochondrial biogenesis, reverses oxidative damage to muscle by increasing the expression of endogenous,

mitochondrial anti-oxidant enzymes, inhibits fibrosis through inhibition of endogenous myostatin, and prevents the calcium-induced opening of mPTP in mitochondria. As shown in mitochondria, isolated mitochondria placed in 1 ¥ M of calcium rapidly swell, due to opening of the mPTP, causing a loss of refraction by the mitochondria (the lowest declining line in Fig X), which has been shown to be associated with loss of mitochondrial function. Adding increasing amounts of epicatechin at the same time as the calcium demonstrates an increasing, dose-dependent protection against calcium-induced mPTP opening by (-)-epicatechin (data courtesy of Dr. Francisco Villarreal, UC San Diego)

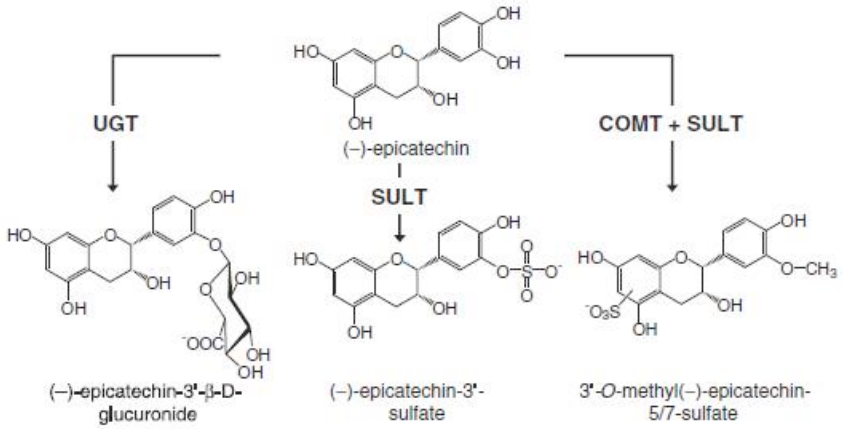
3. Epicatechin overview

3.1 Pharmacokinetics / Pharmacodynamics

3.1.1 (-)-Epicatechin

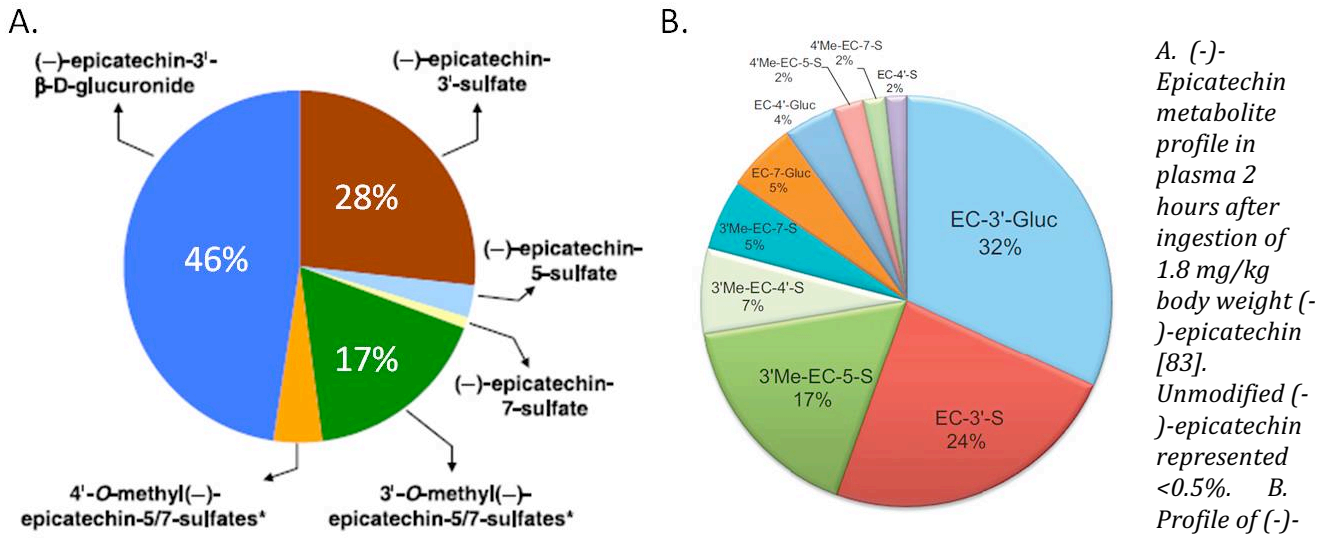
(-)-Epicatechin is orally bioavailable, as the consumption of (-)-epicatechin or cocoa products can result in pharmacologically relevant levels of (-)-epicatechin and its metabolites in blood. The absorption of flavonoids occurs mainly in the small intestine and takes place within minutes. In the small intestine, flavanols are extensively glucuronidated and partially methylated [75, 76], allowing negligible amounts of native (-)-epicatechin in the mesenteric circulation. In the liver, further glucuronidation, methylation, and sulfation can take place [75, 77, 78]. Several studies have determined the presence of these conjugates in the plasma and urine of rodents and humans [78-83], as well as in rat bile [77] and brain [84]. In plasma, (-)-epicatechin is present almost exclusively as conjugated metabolites [82, 83]. 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 [54, 79, 82-90]. 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[82]. A schematic representation of these metabolites is shown in Figure 1. In addition, significant levels of 3'-O-methyl-(-) - epicatechin sulfates substituted in the 4' and 7 positions were identified [89]. Relative amounts of (-)-epicatechin metabolites identified in plasma in two separate studies are shown in Figure 2.

Figure 1: Major (-)-epicatechin metabolites in plasma.



Schematic representation of the primary structurally-related (-)-epicatechin metabolites found in plasma after injestion of (-)-epicatechin-containing test beverage [83]. UGT, UDP-glucuronosyltransferase; SULT, sulfotransferase; COMT, catechol O-methyltransferase.

Figure 2: (-)-Epicatechin metabolite profile in plasma



A. (-)-Epicatechin metabolite profile in plasma 2 hours after ingestion of 1.8 mg/kg body weight (-)-epicatechin [83]. Unmodified (-)-epicatechin represented <0.5%. B. Profile of (-)-epicatechin metabolites in plasma after ingestion of 100g of dark chocolate ((79 mg (-)-epicatechin)) [82]. 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[83, 90, 91]. Oral bioavailability (peak plasma concentration/mg ingested) for (-)-epicatechin in cocoa/chocolate is ~10-20 nM/mg injested (Table 4). Assuming ~3L plasma in a 75 kg man, 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 [92].

Table 3: 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 [51]	(-)-Epicatechin	200	3570	17.85
Ottaviani, J.I., et al., 2011 [87]	(-)-epicatechin added to cocoa vehicle	125 (mean)	~900	~7.2
Ottaviani, J.I., et al., 2012 [83]	Cocoa drink	135 (mean)	1245	9.3
Actis-Goretta, L., et al., 2012 [82]	Dark Chocolate	79	873	11.1
Donovan, J.L., et al., 2006 [93]	Cocoa drink	55	630	11.5
Schroeter, H., et al., 2006 [54]	Cocoa flavanols: 917 mg	174	~1950	~11.2
Baba, S., et al., 2000 [80]	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 [51], while administration of an ~75 mg dose (1 mg/kg body weight) resulted in significant improvement in vascular function (FMD) [54]. 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 [44].

3.1.2 (+)-Epicatechin

(+)-Epicatechin Pharmacokinetics

Single-dose pharmacokinetics: (+)-Epicatechin was administered orally to female SD rats once a day (QD) at 3 different doses of 1, 3 and 10 mg/kg. Animals were also dosed 1mpk IV. Blood was collected for pharmacokinetics study. Pharmacokinetics (PK) data indicated reasonable dose linearity (Table 4, Table 5) and (+)-Epicatechin was found to have higher than 40% bioavailability. A glucoronide metabolite was detected only at the 10 mg/kg oral dose.

Table 4: PK parameters for oral (PO) dosing in female SD rats

Parameters	10 mg/kg (m/SD)	3 mg/kg (m/SD)	1 mg/kg (m/SD)
C_{max} (nM)	325.46 (193.16)	102.45 (27.21)	20.75 (3.12)
T_{max} (h)	0.16 (0.0)	0.44 (0.48)	0.27 (0.2)
AUC (nM.h)	245.37 (116.78)	136.97 (18.75)	22.30 (8.08)
$T_{1/2}$ (h)	2.61 (0.42)	3.83 (0.88)	2.22 (1.52)

Table 5: PK parameters for 1 mpk (IV) dosing in female SD rats

Parameters	Average	SD
C_{max} (nM)	278.08	118.94
T_{max} (h)	0.00	0.00
AUC (nM.h)	51.65	15.30
$T_{1/2}$ (h)	2.67	0.27

Summary of AUC Values	
Dose	AUC (nM.h)
1mpk IV	51.65
1mpk PO	22.30
3mpk PO	136.97
10mpk PO	245.37

Repeat-dose pharmacokinetics and tolerability: (+)-Epicatechin was administered orally 100mg/kg once per day (QD) to female SD rats for 10 days. Blood was collected for pharmacokinetics study on Days 1, 3, 5 and 10. Animals were observed to measure weight, food consumption, water intake, general activity and coat/skin changes. At sacrifice, organs (brain, liver, skeletal muscle) were collected and stored for toxicity evaluation. Pharmacokinetics data indicated a steady rise in C_{max} and AUC over the study period (Table 6). Glucoronide and dimethyl metabolites were detected in trace amounts (Figure 3, Figure 4).

Table 6: Pharmacokinetics	Day			
	1	3	5	10
C_{max} (nM)	1111.0	2484.5	2855.6	3352.2
T_{max} (h)	1	1.33	1.00	0.50
AUC (nM.h)	3106.0	5518.0	6952.7	8141.3
$T_{1/2}$ (h) elimination	3.39	1.31	3.68	3.57

Figure 3: Levels of Metabolites

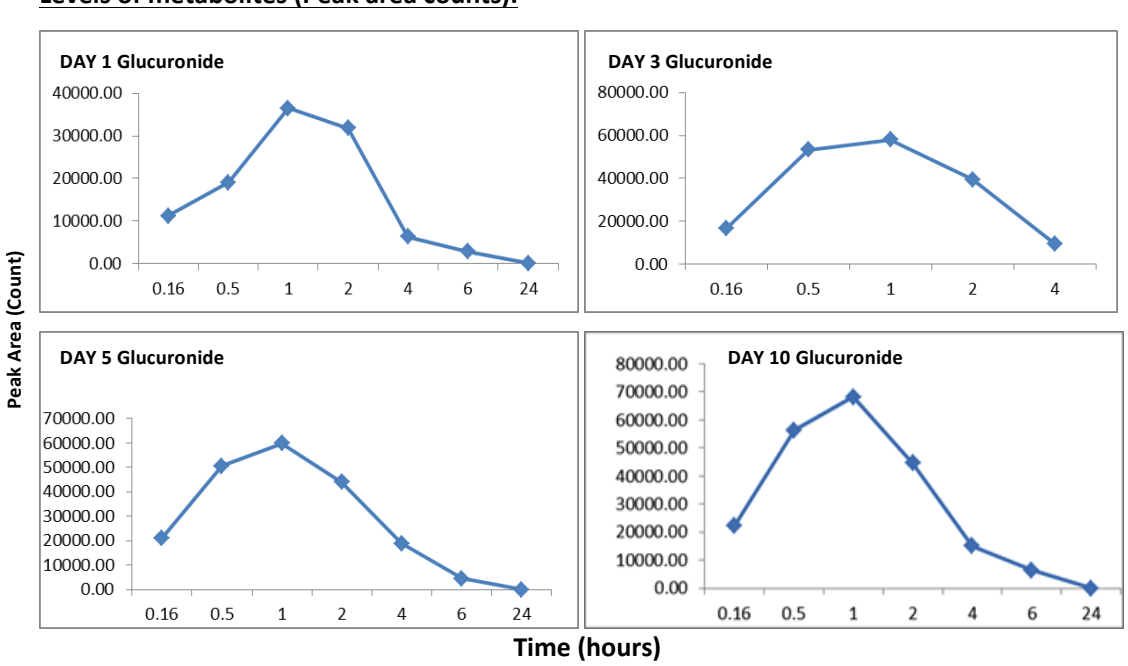
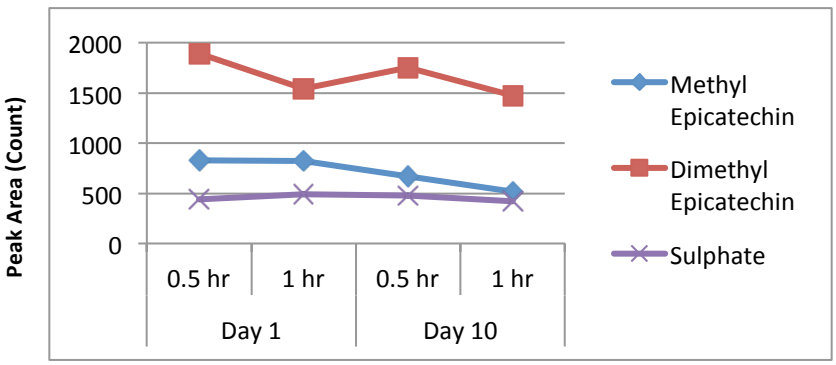


Figure 4: Other Metabolites Detected (Day 1 and Day 10, Peak Area Count)



Tolerability studies showed that the animals' average increase in body weight was comparable from the 1st to the 10th day in treated and control groups. There was a slight increase in food consumption between treated (8.91g) and control (6.07g) groups, and water intake remained the same. Treated animals were found to be active and comparable to control animals. At sacrifice, there were no significant coat/skin changes, and no organ discoloration or change in organ texture was observed.

4. Preclinical Studies

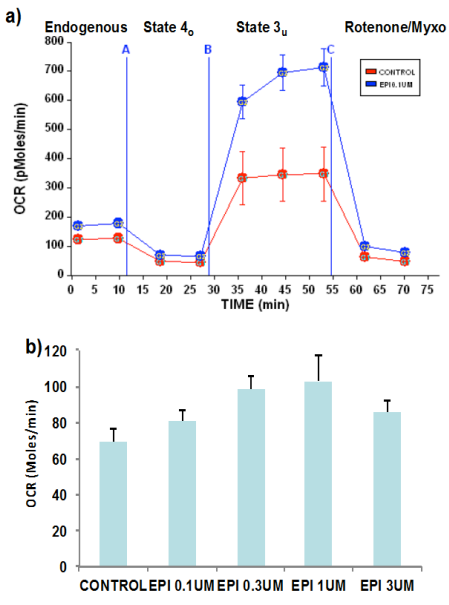
4.1 Preclinical Data

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 5). 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.

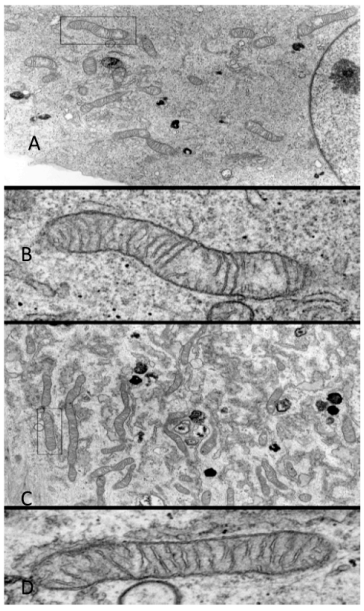
Figure 5: (-)-Epicatechin enhances mitochondrial respiration



. 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_0 (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 6). 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 6: (-)-Epicatechin increases mitochondrial number and cristae density in human skeletal muscle cells (unpublished data)

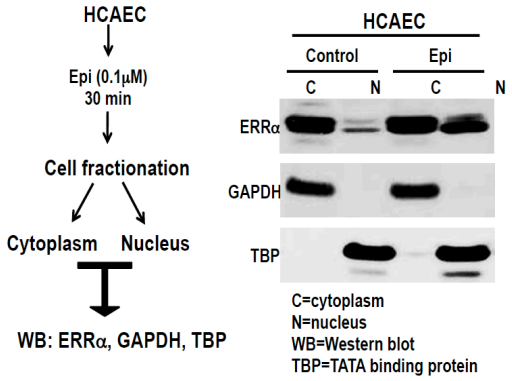


Electron microscopy of human skeletal muscle cells from healthy human subjects treated with 0.1 uM of (-)-epicatechin (in cell culture) for 48 hours are shown in panels C and D; cells from the same subject without treatment are shown in A and B. Based on blinded independent analysis of cristae/mitochondrial 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, ✔, 🚲 and ■ When exposed to the unknown ligand, they trimerize in the cytoplasm and form a complex with PGC1 ✔, and subsequently localize to the nucleus, where they initiate the transcription pathway for mitochondrial biogenesis [94]. Nuclear localization of the ✔ subunit is accepted as an indicator of ERR activation. Human coronary artery endothelial cells (HCAEC) exposed to (-)-epicatechin demonstrated localization of ERR ✔ to the nucleus within 30 minutes (Fig 7).

Figure 7: (-)-Epicatechin induces estrogen-related receptor alpha translocation (activation) into the nucleus of HCAEC



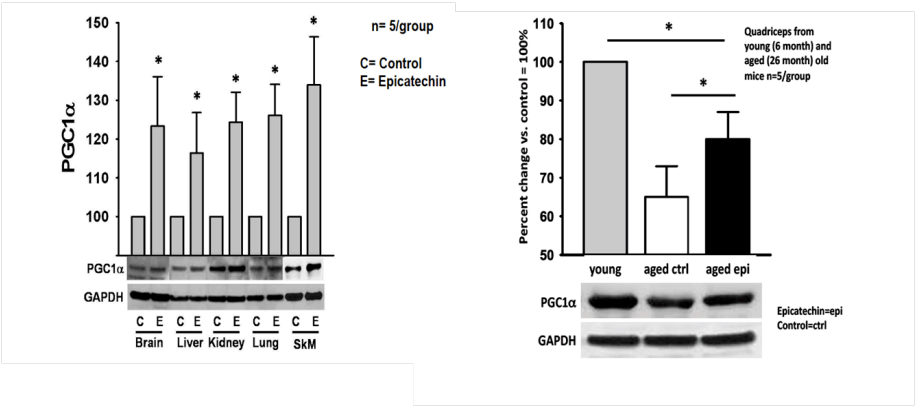
4.1.3 (-)-Epicatechin Induces Expression of Mitochondrial Biogenesis Factor PGC-1α in Aged Mice (unpublished data)

The peroxisome proliferator-activated receptor gamma (PPAR■ coactivator-1α (PGC-1✔) 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 [95, 96]. Expression of PGC-1✔ 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[27], suggesting that PGC-1✔ 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 8A). 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 8B).

Figure 8: (-)-Epicatechin increases PGC1-alpha in aged mice.

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

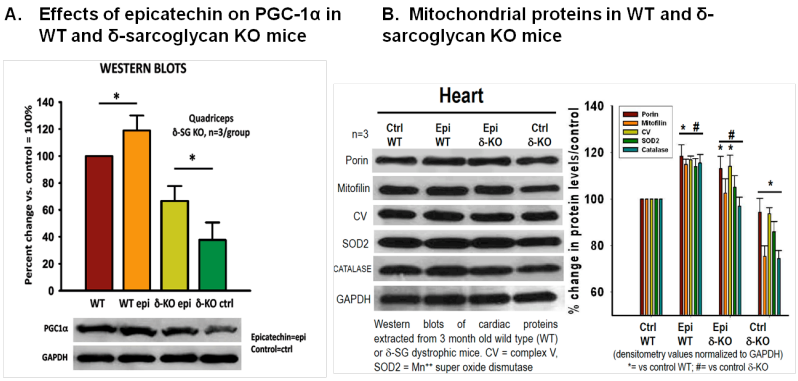
B. PGC-1α in young and old (+/- Epi) mice quadriceps tissues



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 9A). The change in PGC1α correlated with loss and regain of mitochondrial density (Fig 9B). 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.

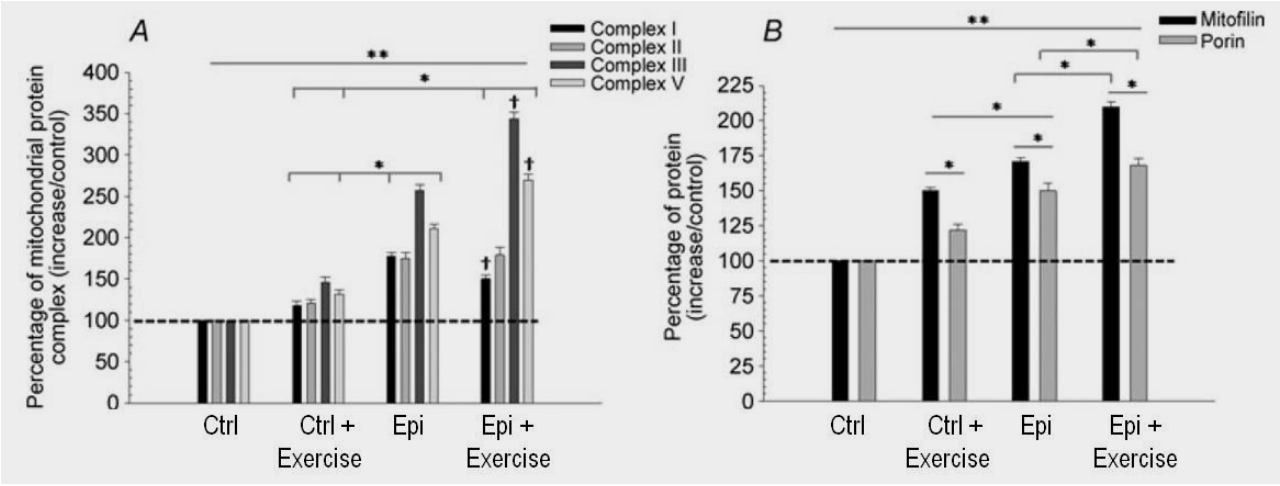
Figure 9: (-)-Epicatechin increases PGC1-alpha and mitochondrial proteins in gamma-sarcoglycan knock out mice.



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

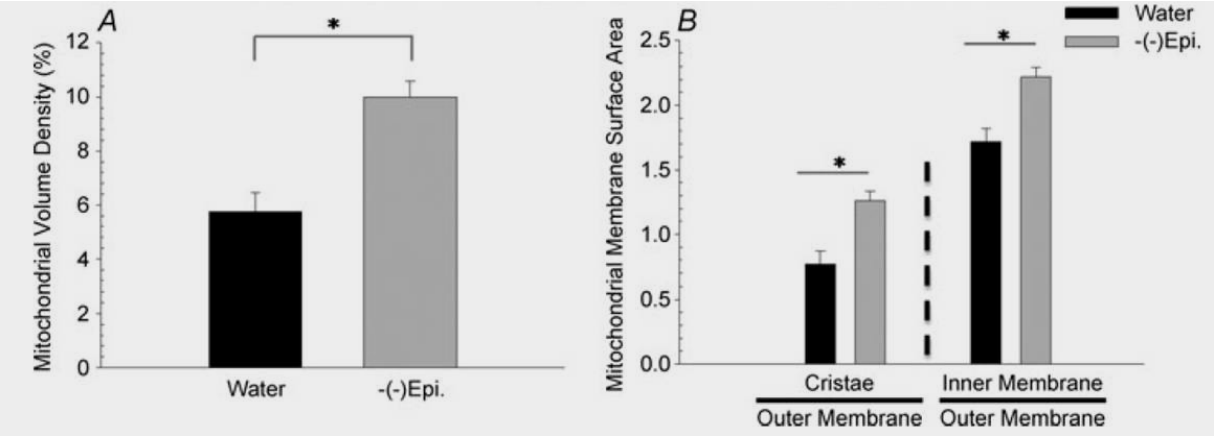
To test for the *in vivo* effects of (-)-epicatechin on muscle performance and indicators of mitochondrial structure (porin, mitofilin) and biogenesis (Tfam), studies were conducted in mice to compare the effects of (-)-epicatechin +/- daily exercise with vehicle +/- daily exercise[97]. Aged one year 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 10 and 11). 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 10: (-)-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 11: (-)-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 [98].

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 [99] (-)-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) [100]. 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 [99]. 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,

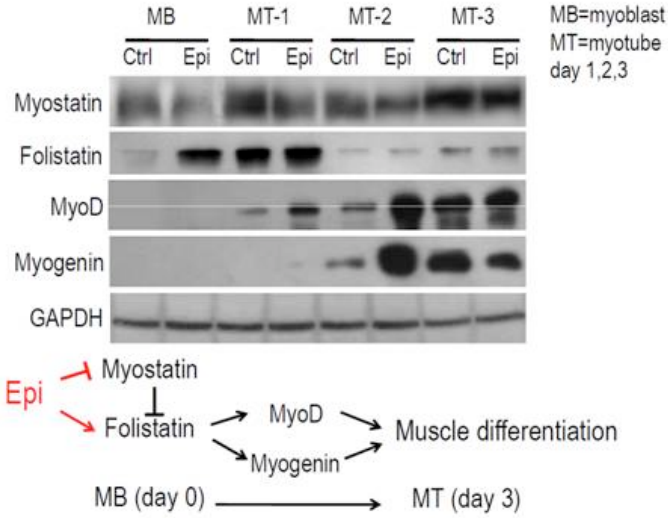
compared to controls. Double dosing further decreased infarct size at 48 h by 80%, which was sustained at 3 wk (52% reduction). In order to assess if (-)-epicatechin-induced cardioprotection was mediated by protection of mitochondrial function, mitochondria were isolated from the left ventricle of sham, 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 12 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.

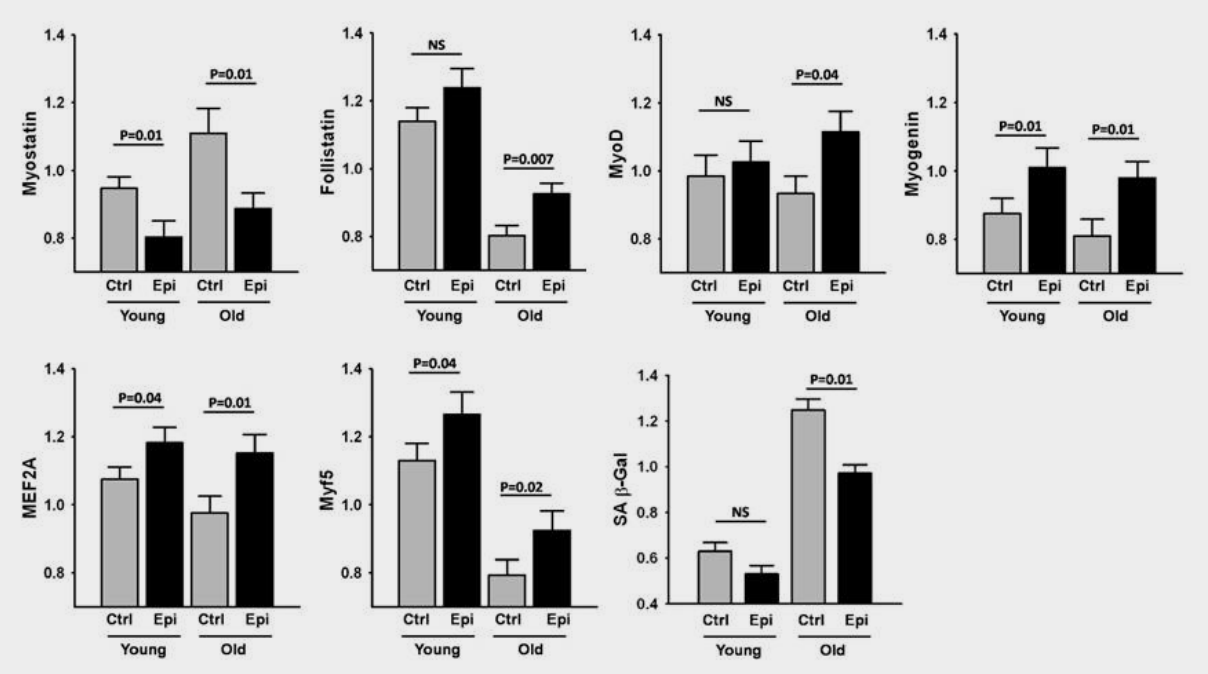
Figure 12: Muscle differentiation in time course in C2C12 cells +/- (-)-epicatechin.



4.1.8 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 13). 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.

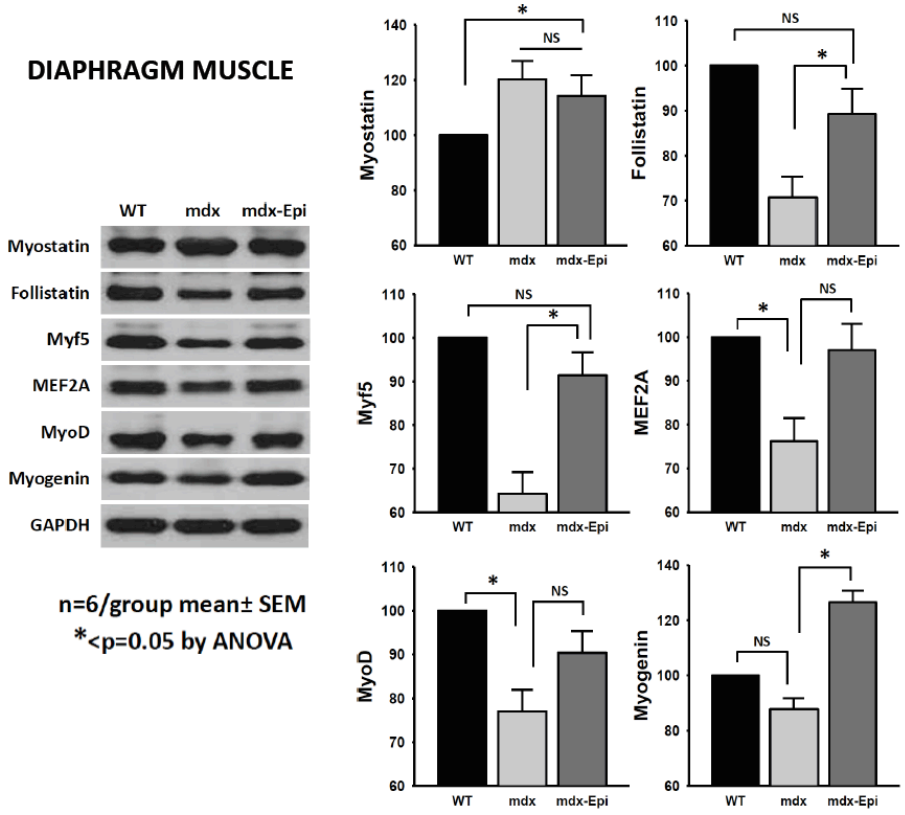
Figure 13: Muscle regulatory protein expression in 6 and 26 month old mice +/- (-)-Epicatechin



4.1.8.1 Muscle Regulatory Protein Expression in mdx Mouse Model of MD

Figure 14 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).

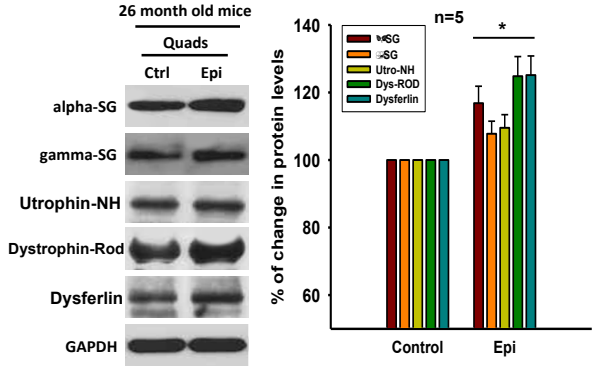
Figure 14: (-)-Epicatechin modulates muscle regulatory proteins in diaphragm muscle in *MDX* mice.



4.1.9 (-)-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 15).

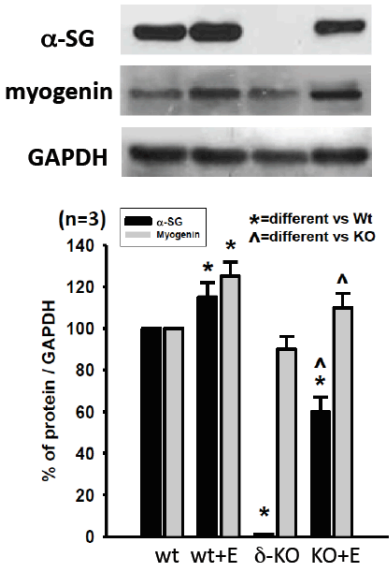
Figure 15: (-)-Epicatechin increases DAPC proteins in middle aged mice.



The effect of epicatechin's stimulation of the dystrophin protein complex was also examined in the δ-sarcoglycan KO mouse. As shown in Fig.16, 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.

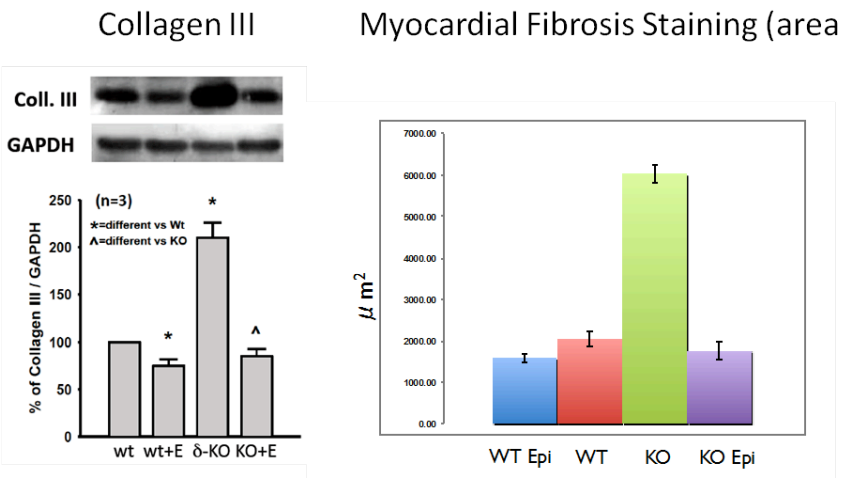
Figure 16: (-)-Epicatechin increases alpha-sarcoglycan protein expression in gamma-sarcoglycan knock out mouse muscle.



4.1.10 (-)-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 17, left panel). The reduction in collagen III translated into reduction of myocardial fibrosis as judged by quantitative histology of heart valve tissue sections (Fig 17, right panel). These data suggest the potential of (-)-epicatechin to reduce cardiomyopathy often associated with MD.

Figure 17: (-)-Epicatechin reduces fibrosis in the gamma-sarcolgycan knock out mouse.

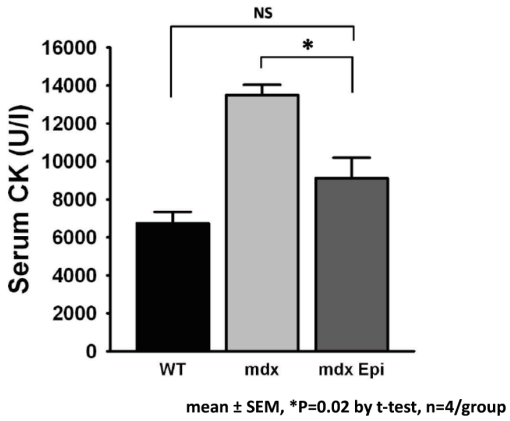


4.1.11 (-)-Epicatechin Reduces Plasma Marker of Muscle Damage and Improves Muscle Strength in MDX Mouse Model of MD (unpublished data)

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 18, the levels of CK activity rise in water treated mdx

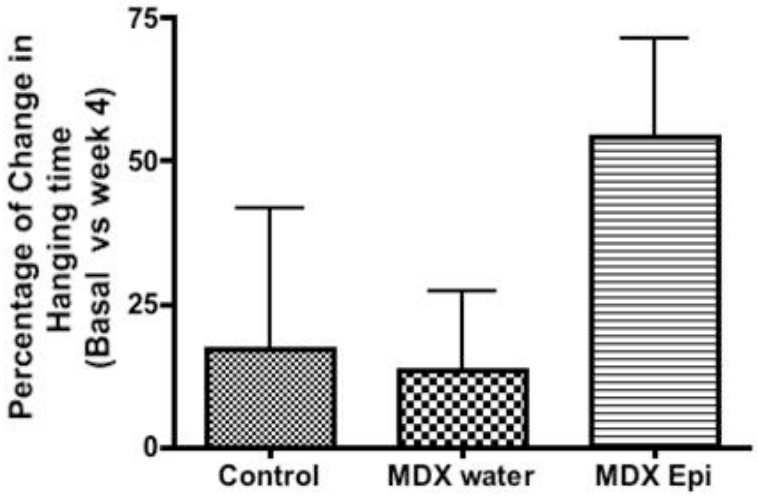
mice and they are significantly reduced with epicatechin treatment to levels similar of those obtained in wild type animals.

Figure 18: (-)-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 19 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.

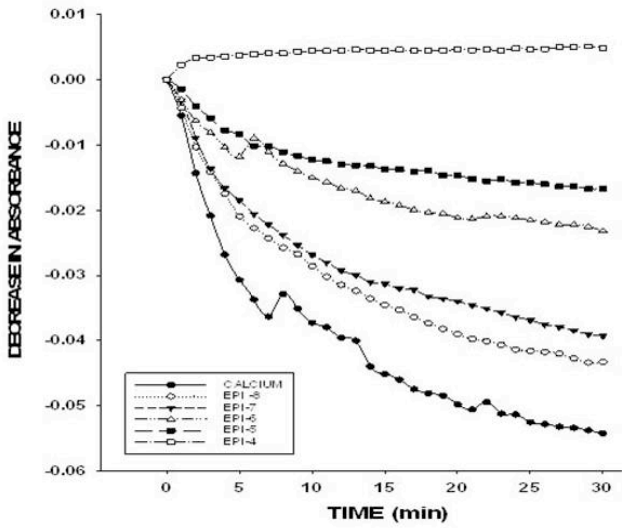
Figure 19: (-)-Epicatechin improves skeletal muscle strength in the *MDX* mouse model of muscular dystrophy.



4.1.12 (-)-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 20). (-)-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 20: (-)-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.

The biological activity of the (+)-enantiomer of epicatechin is significantly more potent than the (-) - enantiomer of epicatechin. Studies have been performed to evaluate effects on exercise capacity using wild type mice treated with either water (control), (-)-epicatechin (1 mg/kg/day) or (+)-epicatechin (0.1, 0.3 or 1 mg/kg/day). Endpoints evaluated include the mitochondrial biogenesis transcription factors AMPK and PGC1-α, Figure 21 Results indicate that following 2 weeks of treatment, (+)-epicatechin could enhance exercise capacity (as assessed by treadmill testing, Figure 22, in a magnitude similar to 1 mg/kg/day of (-)-epicatechin but at lower doses (equivalency noted at 0.3 mg/kg/day). Biochemical analyses (Western blots) of muscle samples from the above mice demonstrated a similar pattern of responses, whereby (+)-epicatechin was as effective at lower doses as those employed for (-)-epicatechin or more effective at comparable doses when probing for effects on recognized key modulators of metabolism.

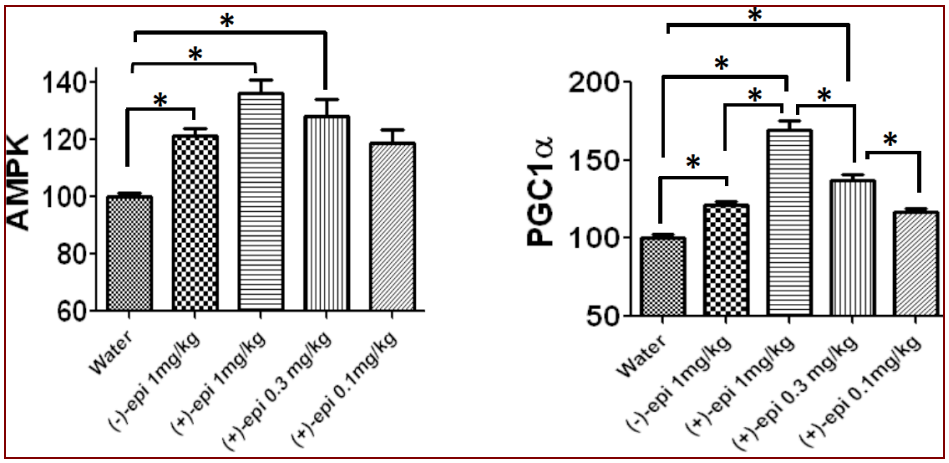


Figure 21: Results on 2 weeks of treatment

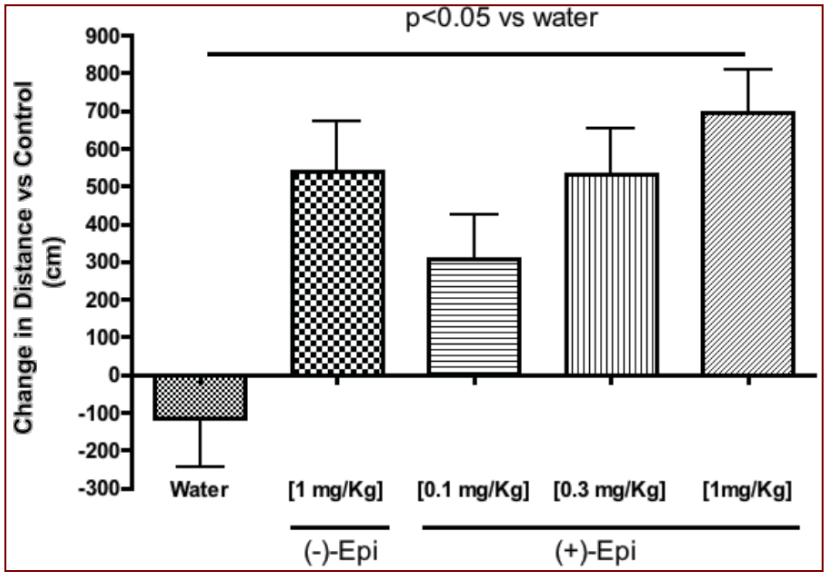
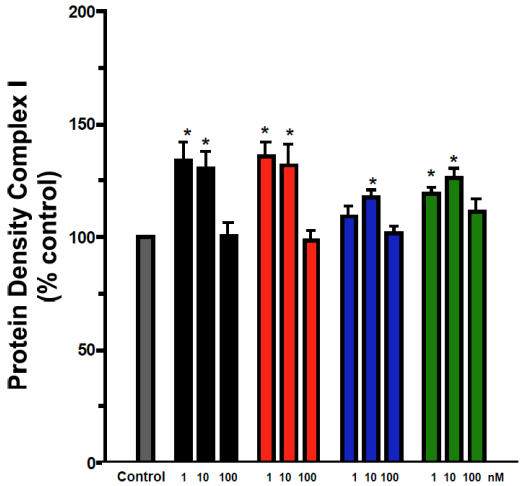


Figure 22: Change in distance vs control

4.2 Effects of Epicatechin Epimers on Cultured Cells

Experiments were performed in cultured HCAEC to ascertain the effects that (+) and (-)-epicatechin have on MiB. As the chemical synthesis of epicatechin generates a 50/50 ratio of the enantiomers, comparisons were also implemented using the racemate. Figure 23 demonstrates the effects that all three different forms of epicatechin have on oxidative phosphorylation (OXPHOS) complex I at varying doses. As can be observed, the use of either the racemic (+/-) mixture or (+)-epicatechin led to significant increases in complex I at lower comparable doses vs. (-)-epicatechin. The observation that the racemic mixture was as effective as (+)- suggests that the (+)- form of epicatechin exerts maximal stimulation with minimal interference from the (-)- form. The extent of the differences noticed also lends credence to the hypothesis that specific molecular spatial interactions occur between epicatechin and a putative "receptor" such that the enantiomeric (+)- version of the molecule enhances its end-effects. These results have now been verified in a preliminary fashion using C2C12 cells in culture.

Figure 23 Effects of Epicatechin Epimers on OXPHOS Complex I Protein Levels



Compounds tested include purified, natural (-)-epicatechin (blue) and synthetic, Cardero Therapeutics (-)- in green, (+)- red and (+/-)- black epicatechins.

4.3 Effects of (+)-Epicatechin in Normal Mice

Studies have been performed using wild type 10 month old mice treated by gavage with either water (control), (-)-epicatechin (1 mg/kg/day) or 0.1, 0.3 or 1 mg/kg/day of (+)-epicatechin. Results indicate that following 2 weeks of treatment, (+)-epicatechin can enhance exercise capacity (as assessed by treadmill testing, Figure 24) in a magnitude similar to 1 mg/kg/day of (-)-epicatechin but at lower doses (equivalency noted at 0.3 mg/kg/day). Biochemical analyses (Western blots) of muscle samples from the above mice demonstrate a similar pattern of responses whereby, (+)-epicatechin is as effective at lower doses or more effective at comparable doses when probing for effects on recognized key modulators of metabolism. Endpoints evaluated include the kinases AMPK, LKB1, PGC1-α, as well as follistatin, a stimulator of muscle growth (Figures 25 and 26).

Figure 24 Effects of (-)- and (+)-Epicatechin on Treadmill Performance

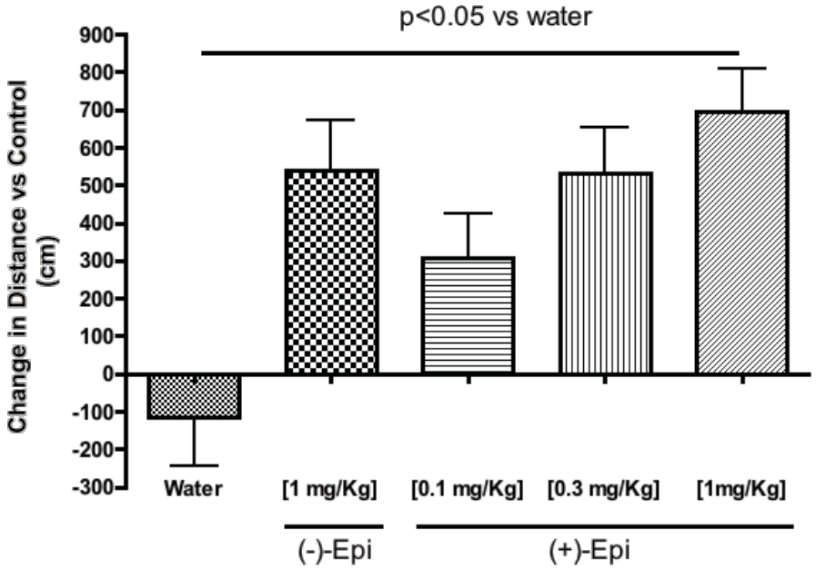


Figure 25 Effects of (-)- and (+)-Epicatechin on Follistatin and p-LKB1

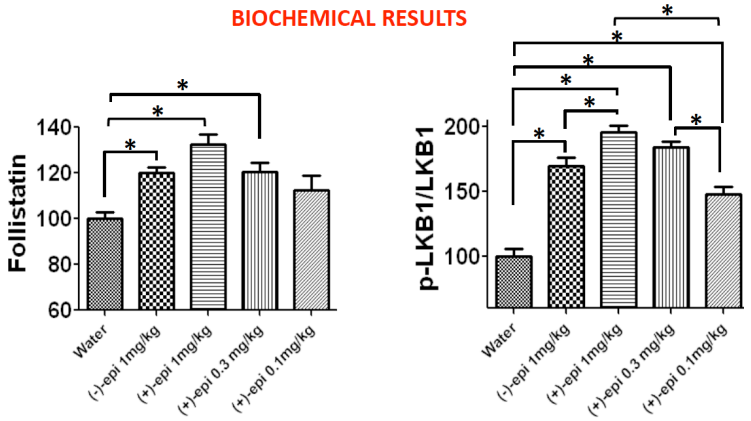
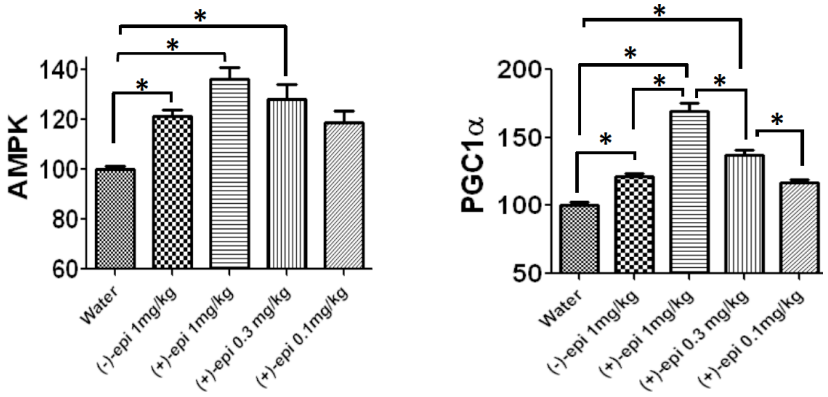


Figure 26 Effects of (-)- and (+)-Epicatechin on AMPK and PGC1α



5. Human Studies

5.1 *Clinical Studies in Diseases Other Than Duchenne/Becker Muscular Dystrophy*

Numerous clinical studies have been conducted with flavonoid extracts containing epicatechin derived from cocoa that demonstrate human safety. As reviewed recently, and confirmed in a 12 week, randomized, controlled, double-blind study in which healthy participants received either cocoa flavonoids (n=46) or control flavonoid free compounds (n=28), administration of up to 2 grams/day of cocoa flavonoids for 12 weeks demonstrated no difference from controls with respect to vital signs, platelet function, metabolism, or adverse events [87] (NCT02447783, clinical trials.gov).

With respect to patients with heart failure and sarcopenia, one small study was completed using (-)-epicatechin-enriched cocoa (ERC). Five patients with Type 2 diabetes, congestive heart failure, and sarcopenia, ranging in age from 60-75, received 100 mg of (-) epicatechin per day for 12 weeks. All patients demonstrated severe severe loss of mitochondrial density and disrupted muscle sarcomere structures in baseline biopsies of the quadriceps muscle. Over the course of 12 weeks, significant decreases in plasma BNP ($p<0.06$) were observed. In muscle biopsies conducted at the end of treatment, ERC induced significant differences in the quadriceps expression of biomarkers of mitochondrial biogenesis, including PGC1alpha, SIRT1, Tfam, mitofilin, porin, and the ETC complexes I and (V) ($p<0.05$). Ultrastructural analysis revealed increased mitochondrial density and increased number of cristae per mitochondria (indicative of enhanced capacity for oxidative phosphorylation). ERC reversed biomarkers of oxidative muscle injury, normalizing glutathione levels and reversing baseline increases in carbonylated proteins, while increasing expression of the mitochondrial anti-oxidant enzymes superoxide dismutase and catalase ($p<0.05$). ERC markedly restored the disrupted sarcomeres to near normal structures, increasing the expression of the sarcoglycans and biomarkers of skeletal muscle biogenesis, including MEF2a, My5, MyoD, and myogenin (all $p<0.04$ or less)[101-103].

Five clinical studies in the United States and abroad have been completed in indications other than dystrophinopathies using purified enantiomers [either (-) or (+)] of epicatechin provided by our supplier, Cardero Therapeutics. Briefly, the findings of those studies are:

(1) In a Phase I PK and safety study conducted at UCSF, purified, monomeric (-)-epicatechin was administered to healthy volunteers in an SAD format (50,100, 200 mg orally, n=9) and an MAD format (50 mg qd or b.i.d X 5 days, n=8). (-)-Epicatechin and its metabolites were rapidly cleared from the body with a plasma elimination half-life of approximately 2.5 h for the 100 and 200 mg doses. No adverse events were noted. On day 5, significant increases in plasma nitrite were observed (30% in the qd group and 17% in the bid group). The induction of mitochondrial biogenesis was confirmed in platelets on Day 5 in the qd group, with significant increases in ETCI and IV and increases in citrate synthase activity observed. Average day 5 levels of follistatin, an endogenous hormone trophic for muscle, were 2.5 fold higher vs. day 1 AUC levels in the b.i.d group. No difference in PK after 1 day or 5 days of exposure was observed.[104]

(2) The (+)-enantiomer of epicatechin has been studied in an Investigator-Initiated IND, Phase 1 study, by Dr Robert Henry at UCSD ("Pharmacokinetics of (+)-epicatechin, IND # 122158). Part 1 is an SAD format of 10,30,and 100 mg orally, n=4 each dose. Part 2 is an multiple dose format of 100 mg administered orally as 100 mg qd or 50 mg b.i.d (n=8) for 7 days. The clinical phase of the program has been completed. No adverse events or abnormal lab values were observed. PK is comparable to that of (-)-epicatechin

(3) In a study conducted in India, patients with hypertriglyceridemia (200-500 mg/dL) were treated for 4 weeks in a double-blind, placebo-controlled trial, with 10 patients on control and 20 patients receiving oral (-)-epicatechin, 50 mg b.i.d for 4 weeks. In all patients, (-)-epicatechin significantly decreased triglyceride levels by 26% ($p<0.05$), and CRP ($p<0.01$). In patients with a fasting hyperglycemia (> 100 mg/dL), epicatechin significantly decreased plasma triglycerides by 30% ($p<0.02$), fasting glucose by 17%, and CRP by 30%. No adverse events were noted. Lab tests showed either no change or improvement [105]

(4) In a small pilot study conducted at the Instituto Politechnico Nacional, Mexico, healthy adult subjects (average age of 41 years) were treated for 7 days with 25 mg of (-)-epicatechin in capsules, b.i.d, in order to assess treatment effects on muscle strength and circulating biomarkers of skeletal muscle modulation (follistatin as a hormone trophic for muscle, myostatin as a hormone mediating muscle atrophy). (-)-Epicatechin treatment significantly improved hand-grip strength and increased the ratio of follistatin to myostatin in plasma [106]. No adverse events were observed

(5) In the same institution, in a separate study, 20 healthy, normal and overweight adult subjects were provided a single dose of 50 mg (-)-epicatechin before a standardized metabolic meal, and metabolic endpoints measured. (-)-Epicatechin increased postprandial lipid catabolism as evidenced by a significant decrease in the respiratory quotient and kilocalories obtained from fat oxidation. The effect was associated with significantly lower postprandial plasma glucose and triglycerides concentrations. Such effects were more prominent in overweight subjects. No adverse events were observed [107].

5.2 Clinical Study in Becker Muscular Dystrophy

As a proof of concept in adults with dystrophinopathies, we conducted a single-center open-label proof-of-concept pilot study of oral epicatechin 50mg twice daily in ambulatory adults with genetically-confirmed Becker muscular dystrophy from August 2013 to June 2014.

Study participants: This was a single-center proof-of-concept pilot study and due to the limited availability of both genetically-confirmed BMD patients and a limited drug supply, we employed a convenience sample for this study. The main criterion for success of the study was presence of one or more biologic or strength and performance outcome measures that yield a response magnitude that allows for sufficient power in a Phase II B study with a sample size of 30 individuals. A maximum of ten participants were to be enrolled in our study. Participants were males between the ages of 8 and 17 years with a genetically-confirmed diagnosis of BMD, average to low daily physical activity and the ability to ambulate at least 75 meters without the use of assistive devices.

Study drug: Participants received epicatechin by mouth 50mg twice per day (100mg per day total dose) for 8 weeks.

Study procedures and evaluation: Participants had a total of 7 study visits each at baseline and at screening, day 1, and weeks 1, 2, 4 and 8. A comprehensive safety review was conducted at each visit, and a medical monitor reviewed suspected study-related adverse events throughout the duration of the study. Evaluations of efficacy are shown in the table below.

Table 7: BMD epicatechin pilot study efficacy evaluations.
1) assessment of peripheral blood biomarker profiles;
2) assessment of baseline and post-treatment muscle biopsy by histology, Western blot and electron microscopy, and;
3) assessment of strength by isokinetic dynamometry and quantitative grip testing [18, 108], 4) exercise performance and metabolic testing during a standardized graded recumbent cycle test[109, 110],
5) muscle perfusion of the vastus lateralis by NIRS during the exercise test [111], and
6) the 6-minute walk test.

5.2.1 Results

Table 8: Participant characteristics at baseline.					
Measure	N	Mean	SD	Min	Max
Age at screening (Yrs)	7	46.6	9.5	31.5	60
Height (cm)	7	179.2	4.3	172.5	185.5
Weight (kg)	7	83.2	18.3	53.3	104.5
Forced Vital Capacity (%)	7	93.4	12.5	77	116
6MWD (m)	7	372	87.2	245	502
Time to Stand (s)	5	5.5	2.3	2.9	8.1
Time to Climb 4 Stairs (s)	7	7.4	7.4	4.4	12.3
Time to Run 10m (s)	7	7.9	7.9	5.1	12.8

Recruitment and baseline data: Seven participants with sufficient genetic diagnostics passed screening. One participant who failed screening was not sufficiently ambulatory to perform the required functional evaluations. One participant withdrew consent during screening. The remaining participants all began treatment. Baseline characteristics of the final study cohort are shown in **Table 8**. One participant was unable to participate in the full set of strength testing measures at baseline due to the advanced level of his disease. Tissue for electron microscopy was limited to 3 patients due to poor tissue quality and an error in tissue fixation for one sample.

5.2.2 Biomarker outcomes at 8 weeks

All enrolled participants completed 8 weeks of follow-up on study medication. One participant sustained a fall at home (unrelated to study medications) that resulted in a minor knee injury and temporary limitation of mobility just prior to his 8-week evaluation and was unable to undergo week 8 exercise testing or biopsy.

Biomarkers of mitochondrial biogenesis from muscle biopsy tissue: Notable changes in muscle tissue as measured by Western blot included significant increases in transcription factors LKB1, PGC1a, AMPK, and mitofilin associated with mitochondrial biogenesis and function **(Figure 27).** We were able to collect bicep muscle tissue of sufficient quality to directly image mitochondria for 3 participants. Those participants showed increases in both numbers of mitochondria and cristae abundance supportive of our overall concept **(Figure 28)**. One participant demonstrated approximately double the number of muscle mitochondria per unit area after 8 weeks of treatment **(Figure 28, first panel)**.

Figure 27: Increased muscle / mitochondrial transcription factors.

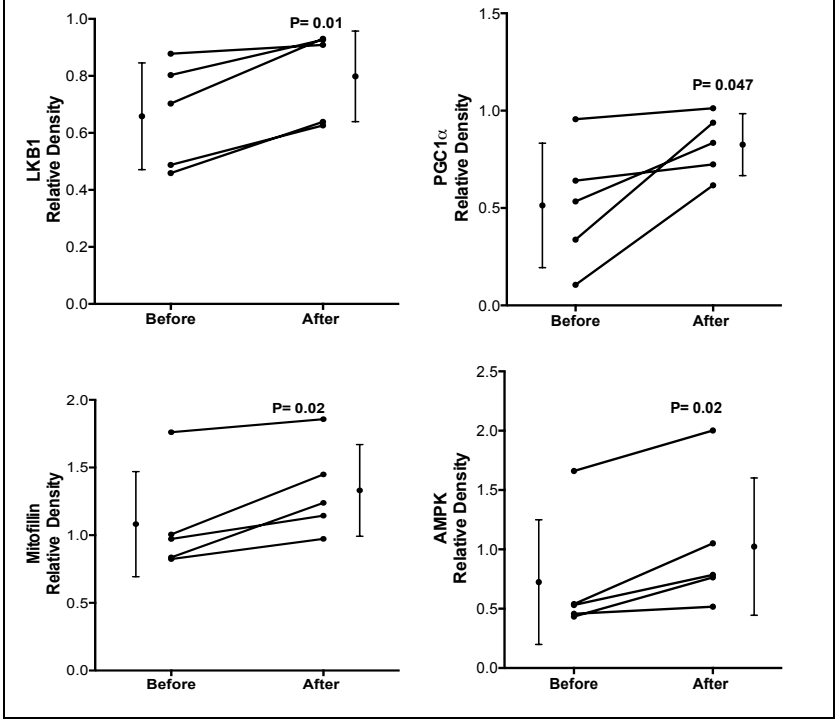
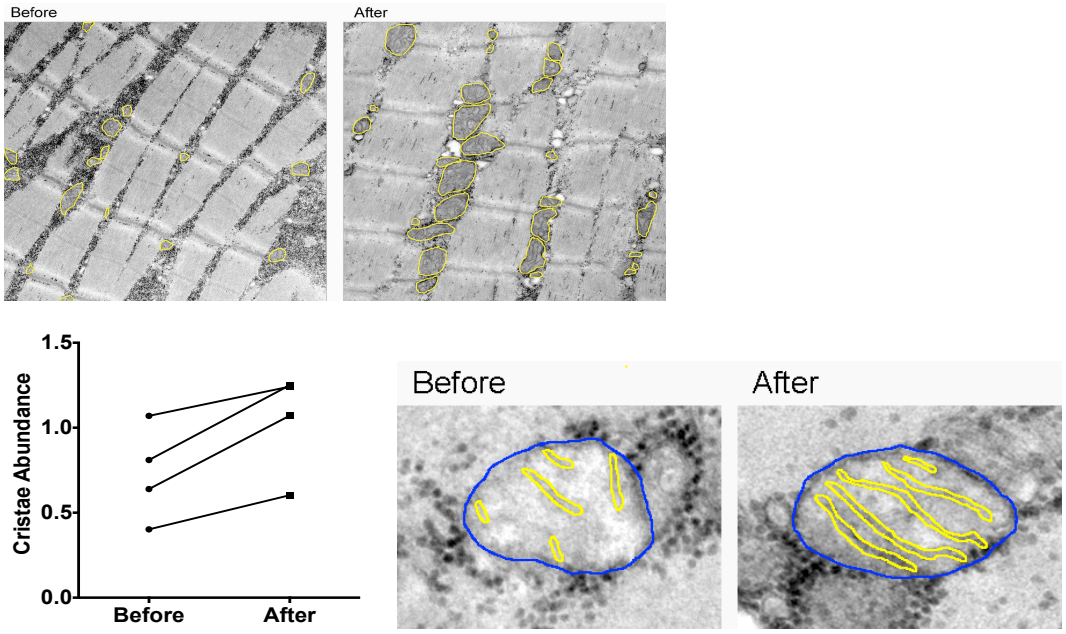
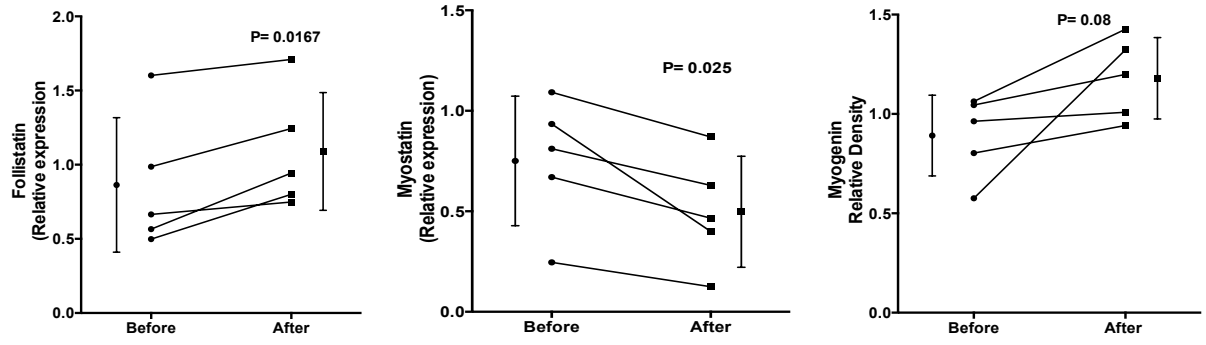


Figure 28: Increases in mitochondrial count and cristae abundance in 4 participants with viable muscle biopsies.



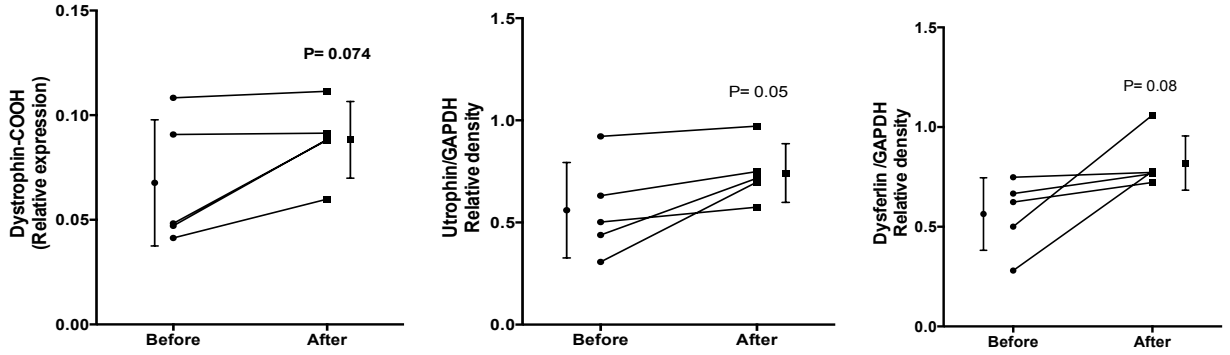
Biomarkers of regeneration from muscle biopsy tissue: Western blot results from muscle biopsy also indicated significant alterations in expression of tissue follistatin and myostatin **(Figure 29)**, which are major regulators of muscle growth and repair, suggesting a shift toward proliferation of mitochondria and muscle tissue overall.

Figure 29: Pro-repair regulation of muscle growth and regeneration-related proteins.



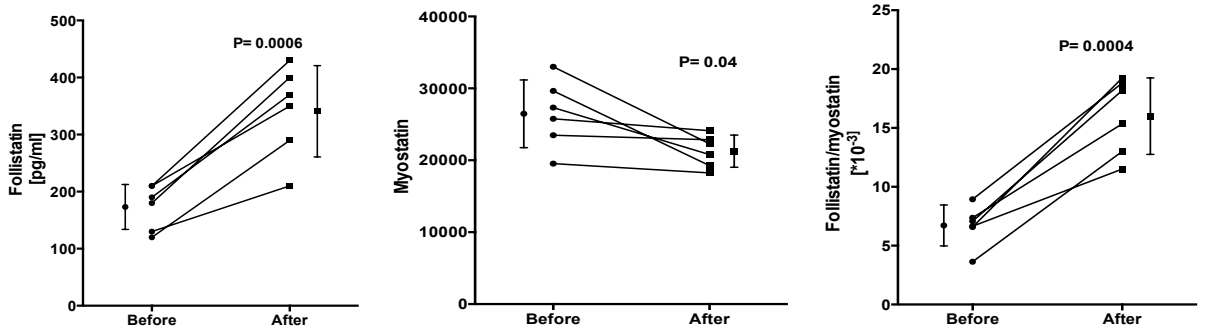
Dystrophin-related proteins from muscle biopsy tissue: Increases were seen in muscle tissue Western blot for key structural proteins dystrophin, utrophin and dysferlin (**Figure 30**), with non-significant increasing trends in other related skeletal muscle proteins suggesting a downstream pattern of growth and repair that is consistent with the previously mentioned increases in regulatory proteins.

Figure 30: Increased dystrophin-associated proteins in muscle by Western blot.



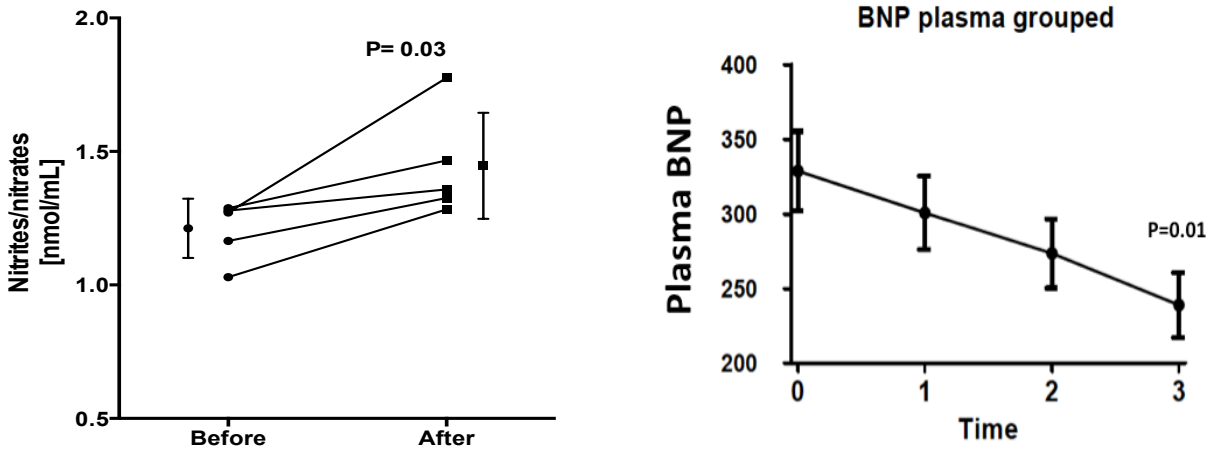
Biomarkers of muscle regeneration and mitochondrial growth from plasma: Changes in muscle tissue follistatin and myostatin were replicated in circulating plasma as well, with increased follistatin and decreased myostatin expression. The striking change in the ratio between plasma levels of growth potentiating follistatin and growth inhibiting myostatin suggests a likely biomarker of initiation of a tissue repair state **(Figure 31)** that will be useful in future clinical trials. Mass spectrometry yielded statistically significant increases ($p<0.05$) of between 8-fold and 140-fold in mitochondrial proteins acyl-coenzyme A thioesterase 2, acyl-CoA synthetase family 3, mitochondrial chaperone BCS1, paraplegin, 28-s ribosomal protein S31 (mitochondrial) and threonine synthase-like 1, all of which are indicators of upregulated mitochondrial production.

Figure 31: Follistatin and myostatin changes in circulating plasma act as a biomarker of muscle growth and repair.



Biomarkers of heart failure and NO reserve from plasma: In addition to indicators of improved energetics and muscle repair, we demonstrate significant increases in plasma nitrates, including nitric oxide (NO), a key regulator of vascular tone that promotes tissue oxygenation during exercise **(Figure 32)**. Nitric oxide synthetase (NOS), which is responsible for NO production, co-localizes with dystrophin and is known to be reduced in individuals with dystrophinopathies. We also noted significant reductions in plasma B-natriuretic peptide (BNP) which is expressed during heart failure and by skeletal muscle satellite cells in response to ischemic stress **(Figure 32)** [112]. These results again suggest an overall pattern of growth and repair and normalization of function.

Figure 32: Changes in NO and BNP expression in plasma show reduced ischemic stress.

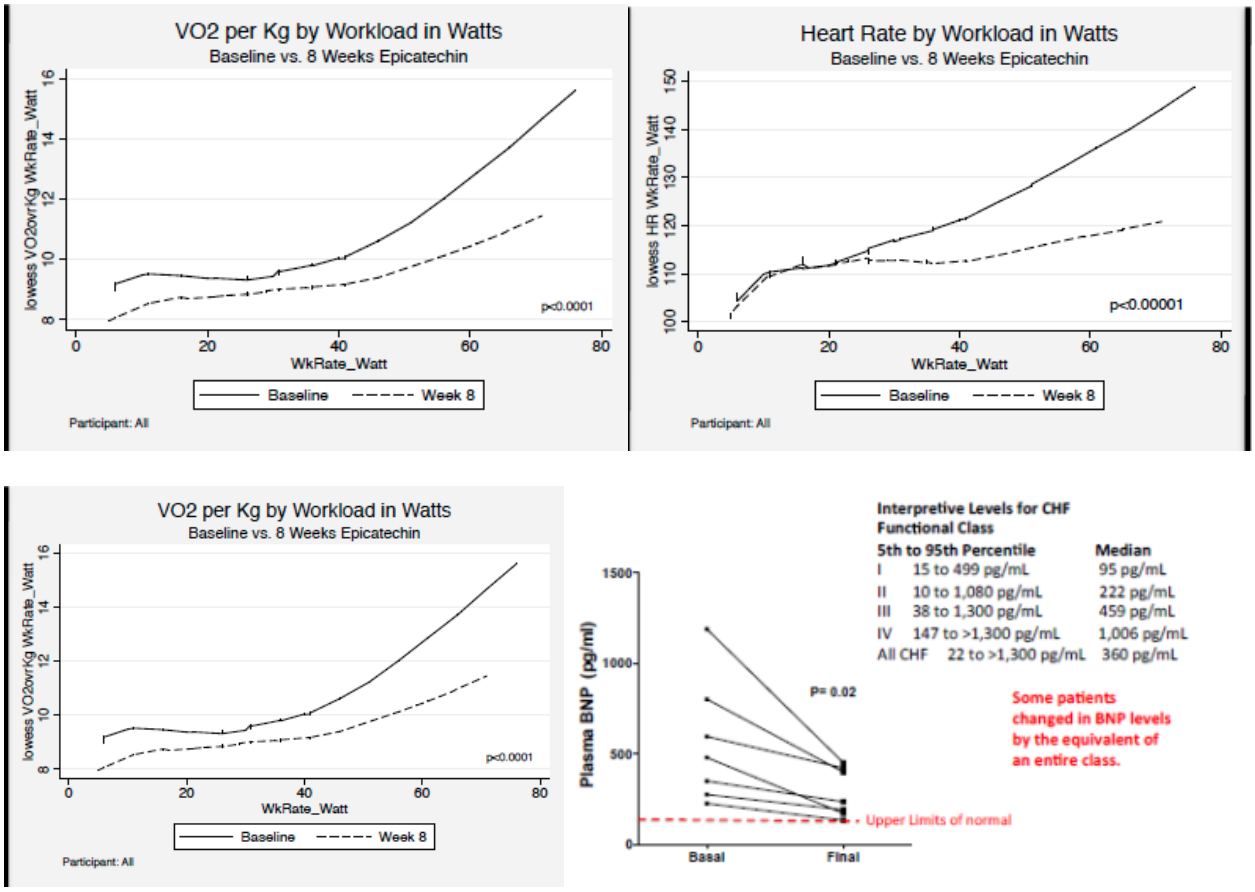


5.2.3 Strength and function and safety outcomes at 8 weeks.

Metabolic indicators during graded exercise cycle testing: Overall, duration of exercise and maximal attainable workload did not increase on the graded cycle exercise test. We did observe significant improvements in metabolic exercise performance that are consistent with and similar in magnitude to those seen in response to short-duration (8-10 weeks) exercise training regimens. During the graded cycle exercise test, participants demonstrated significant decreases from baseline at 8 weeks in VO2, blood lactate and heart rate for a given level of workload in watts (**Figure 33**) indicating a reduced energy cost of exercise, and improved muscle energy utilization and respiratory efficiency. All treated patients demonstrated decreased plasma lactate levels, increased oxygen efficiency, and decreased tachycardia. In order to maintain cardiac output, if there is a smaller increase in heart rate in the setting of graded exercise, there must be an increase in stroke volume, indicating an improvement in cardiac function. This inference was confirmed by the finding that within 8 weeks there was a very significant decrease in BNP levels per patient, indicating an improvement in the severity of underlying cardiomyopathy (**Figure 33**).

These effects are highly consistent with our biological marker, and further support our concept that supplementation with epicatechin provides an exercise training-like effect for muscle in people with dystrophinopathies in a manner consistent with moderate exercise but without the damaging mechanical stress. Participants demonstrated significant changes in tissue oxygen saturation index (TSI) by near infrared spectroscopy (NIRS) in the vastus lateralis during the six-minute walk test ($p<0.001$). These changes suggest an improved degree of vasodilation during exercise that is consistent with the overall increase in expression of plasma NO observed in the biomarker studies.

Figure 33: Metabolic exercise test parameters show efficiency gains during exercise and improvement in plasma BNP



Clinical measures of strength and function: Despite metabolic testing evidence demonstrating reduced work of exercise via V02, lactate and heart rate reductions at a given level of work, we failed to show significant group-wise improvements in commonly-used clinical measures of timed motor performance, strength and pulmonary function that we included in our evaluation set. However, our participant who demonstrated a doubling of mito-chondrial volume as noted above also increased his 6MWT distance from 350m to 494m while other participants showed mild increases or stability over 8 weeks. We feel that the lack of response in clinical measures is due mostly to the short duration of the study and the small sample size. It has been demonstrated in randomized clin-ical drug trials in patients with more rapidly progressing Duchenne muscular dystrophy diagnoses that evidence of groupwise differences between treated and non-treated participants do not become evident until between 24 and 48 weeks of treatment [113]. Given the current evidence there is a clear indication of the need for long-term stud-ies of epicatechins in dystrophinopathy patients.

5.2.4 Safety outcomes at 8 weeks

No serious adverse events occurred during the treatment phase of the study. Mild to moderate health events were consistent with community living (e.g. cold, headache) and were not present in a pattern that suggested that they were related to epicatechin therapy. One participant experienced a biopsy wound infection that resolved with oral antibiotics. Moderate short-duration bruising from muscle biopsies was present in all participants and was consistent with standard clinical care, and resolved without incident.

6. Research design and methods

6.1 Selection and Withdrawal of Participants

6.1.1 Criteria for Enrollment

Up to 15 participants will be studied in this initial pilot project.

Participant Inclusion Criteria

- Male
- Age 8 years to 17 years
- Non-Ambulatory (unable to complete 10m run/walk under 10s)
- Weight </=100Kg
- Diagnosis of DMD confirmed by at least one the following:
    - Dystrophin immunofluorescence and/or immunoblot showing complete dystrophin deficiency, and clinical picture consistent with typical DMD, or
    - Gene deletions test positive (missing one or more exons) of the dystrophin gene, where reading frame can be predicted as 'out-of-frame', and clinical picture consistent with typical DMD, 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 DMD, with a typical clinical picture of DMD, or
    - Positive family history of DMD confirmed by one of the criteria listed above in a sibling or maternal uncle, and clinical picture typical of DMD.
- Cardiac ejection fraction >40% on echocardiogram
- Use of nutritional, herbal and antioxidant supplements taken with the intent of maintaining or improving skeletal muscle strength or functional mobility has been discontinued at least 4 weeks prior to screening (daily multivitamin use is acceptable).
- Glucocorticoid therapy, if used, must have a stable weight-based dose for at least 3 months prior to enrollment
- Cardiac therapy, if used, includes prophylactic ACE inhibitors, aldosterone receptor antagonists (e.g. spironolactone, eplerenone, etc.), and/or beta-blocker therapy, and must be stable for 3 months prior to enrollment.
- Hematology profile within normal range.
- Baseline laboratory safety chemistry profile within typical range for DMD (elevated ALT / AST acceptable in the absence of elevated GGT, elevated CK acceptable).

Participant Exclusion Criteria

- Inability to complete cardiac or strength, range of motion and mobility assessments per protocol
- Current enrollment 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.
- Cardiac symptoms that, in the opinion of the investigator, may be suggestive of imminent moderate to severe cardiac events, irrespective of LVEF.

6.1.2 Participant Screening Schedule

The entry procedures will be conducted according to the Study Matrix presented below. After potential participants have completed the screening visits and the site has verified they meet all study inclusion criteria the partiipant will be entered into the treatment phase of the study.

Study Matrix: Open-label dose-ranging study of (+)-epicatechin in non-ambulatory DMD

Assessments	Primary Phase				
	Screening	Baseline	Week 2	Week 4	Week 8
	Day -28 - Day 0		Day 14*	Day 28*	Day 56*
Review of Entry Criteria	X				
Medication Administration		- Daily -			
Pharmacokinetics		X	X	X	X
Biomarker Efficacy Assessments					
Plasma Biomarkers					
Follistatin		X		X	X
Myostatin		X		X	X
Follistatin:Myostatin Ratio		X		X	X
Nitric Oxide		X		X	X
Nitrite/Nitrate Ratio		X		X	X
Creatine Kinase		X		X	X
MMP-9		X		X	X
TNF-alpha		X		X	X
TGF-beta		X		X	X
Cardiac troponins		X		X	X
BNP		X		X	X
SomaScan Biomarker Panel (Serum)		X		CK	X
Clinical Efficacy Assessments					
Cardiac Function					
Screening Echocardiogram	X				X
Cardiac MRI		X			X
Strength, Range of Motion and Mobility					
Kinect Reachable Surface Area		X		X	X
Performance of the Upper Limb (PUL)		X		X	X
6-min cycle test w/metabolic testing		X		X	X
Person-Reported Outcomes					
POSNA PODCI		X		X	X
PROM-UL		X		X	X
Body Composition / Anthropometrics					
Height, Weight	X	X		X	X
DEXA for Body Composition		X		X	X
Safety					
Medical History	X	X		X	X
Physical/Neurological Examination	X	X	X	X	X
AE/Concomitant Medications	X	X	X	X	X
12-lead Electrocardiogram	X	X	X	X	X
Laboratory Safety					
Complete Blood Count	X	X	X	X	X
Differential	X	X	X	X	X
PT/PTT	X	X	X	X	X
Comprehensive Chemistry Panel	X	X	X	X	X
Electrolytes	X	X	X	X	X
Urinalysis	X	X	X	X	X

*** Visits may occur +/- 2 days**

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 all specified testing for the remainder of the 8-week study period. If a 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

Three groups (n=5) will receive either a total daily dose of 50 mg per day (one 25mg capsule BID), 75 mg/day (25 mg TID), or 150 mg/day (75 mg BID). Participants will receive (+)-epicatechin by mouth according to the following escalating sequential dose cohort schedule:

Cohort *1:* Participants will receive epicatechin by mouth 25mg BID per day (50mg per day total dose) for 56 days

Study medication will be supplied as a clear 25mg (#3) gelatin capsule without any inert fillers. Participants will take one capsule in the morning at approximately 7:30AM at least 15 minutes before the morning meal and one in the evening before bedtime at least 1 hour after the evening meal as absorption is limited by some milk products.

Cohort *2:* Participants will receive epicatechin by mouth 25mg TID per day (75mg per day total dose) for 56 days

Study medication will be supplied as a clear 25mg (#3) gelatin capsule without any inert fillers. Participants will take one capsule in the morning at approximately 7:30AM at least 15 minutes before the morning meal, one capsule early afternoon at approximately 2:30PM and one capsule in the evening before bedtime at least 1 hour after the evening meal as absorption is limited by some milk products.

Cohort *3:* Participants will receive epicatechin by mouth 75mg BID per day (150mg per day total dose) for 56 days

Study medication will be supplied as a clear 25mg (#3) gelatin capsule without any inert fillers. Participants will take three capsules in the morning at approximately 7:30AM at least 15 minutes before the morning meal and three capsules in the the evening before bedtime at least 1 hour after the evening meal as absorption is limited by some milk products.

6.3.2 Criteria for Dose Reductions

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

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

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. If participants require a dose reduction based on the criteria listed above, follow the guidelines in the Dose Reduction Table 9 below.

Table 9. Dose Reduction Guideline

	Cohort 1 (25mg BID)	Cohort 2 (25mg TID)	Cohort 3 (75mg BID)
Dose Reduction 1	Reduce participant's dose to 25mg/day (eliminate morning dose)	Reduce participant's dose to 50mg/day (eliminate afternoon dose)	Reduce participant's dose to 125mg/day (administer 50mg of evening dose)
Dose Reduction 2	Withdraw participant from study medication.	Reduce participant's dose to 25mg/day (eliminate morning dose)	Reduce participant's dose to 100mg/day (administer 25mg of evening dose)
Dose Reduction 3		Withdraw participant from study medication.	Reduce participant's dose to 75mg/day (eliminate evening dose)
Dose Reduction 4			Reduce participant's dose to 50mg/day (administer 50mg of morning dose)
Dose Reduction 5			Reduce participant's dose to 25mg/day (administer 25mg of morning dose)
Dose Reduction 6			Withdraw participant from study medication.

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 a medical rationale for altering the date of restarting the study drug (such as evidence of impairment of coagulation, etc.).

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. All concomitant medications will be recorded for the duration of study participation.

6.3.5 Patient Care outside UC Davis

Participants participating in UC Davis studies may not undergo protocol-associated procedures at non-UC Davis institutions. However, interim safety studies may be conducted at other institutions. In such a case, a copy of the laboratory orders and other documentation MUST be forwarded to UC Davis for inclusion in the patient's medical record and study documentation.

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 Epicatechin dosing information and safety studies

Pharmacokinetics of both (+)- and (-)- isoforms of epicatechin have been described in a previous INDs filed by Drs. Craig McDonald and Erik Henricson (IND 118118), Dr. Robert Henry (IND 122158), by Barnett et al, 2015, and Ottaviani et al., 2011 [83, 114]. No Phase I hepatic metabolism, such as that mediated by cytochrome p450 oxidases, has been identified for purified or dietary polyphenol epicatechin. (+)-epicatechin has no inhibitory effect on the five most important P450 oxidases in a hepatic microsome assay. In the liver, (-)-epicatechin undergoes modification by uridine-5-diphosphate glucuronosyl-transferases, sulfo-transferases, and catechon-O-methyltransferases. The metabolic result is the presence of glucuronides, sulfates, and/or methyl conjugates in the blood stream, accounting for more than 85% of the absorbed epicatechin with Tmax ranging from 3.2 - 3.8 hours and T1/2 ranging from 1.8 - 3.8 hours. Virtually no unconjugated epicatechin is observed after 1 hour. (+)-Epicatechin is metabolized in the same way, with variations in the relative frequency of the same conjugated metabolites [83, 89] Bioavailability is approximately 30%. (+)-Epicatechin demonstrates moderately less bioavailability and less conjugation with glucuronide or sulfate compared to (-)-epicatechin. It is less effective as an inducer of NO synthesis in vitro and in vivo. The half-life is less than 90 minutes for both enantiomers [83]. In vitro, (+)-epicatechin demonstrates, no Human Ether-a-Go-go Related Gene (HERG) activity and no non-selective interactions with a diverse panel of receptors and enzymes.

The safety of (+)-epicatechin has not been proven in the DMD population. The maximum dose in our population will not exceed 150 mg/day. For this study, (+)-epicatechin will be administered orally in an open label study at a daily dose of 50 mg (25 mg BID), 75mg (25 mg TID), and 150 mg (75mg BID), n=5 each group, for 8 weeks. (+)-epicatechin plasma levels will be measured at 2 weeks and 8 weeks.

The dosage is supported by 3 factors:

- Human efficacy has been demonstrated with (-) epicatechin at 1.0-1.5 mg/kg [114, 115] given at 50 mg twice a day for up to 8 weeks. The twice a day dosing was chosen because once a day dosing did not demonstrate efficacy, presumably because of the short half-life [114].
- At 1 mg/kg in animals, (+)-epicatechin is more effective in stimulating exercise endurance and activating muscle AMPK and PGC1alpha than (-)-epicatechin.

Because epicatechin is not subject to Phase 1 hepatic metabolism, the pharmacokinetics in rats are similar to that of humans and the effective dosage in rodents predicts effective dosage in humans [102]. DMD patients will range between 25-70 kg in weight (est. for age). Therefore, we will be well within the in vivo documented range of efficacy in rodent models and within the safe administration of (+)-epicatechin in previously published pharmacokinetic studies [83].

7.1.1 Preclinical / Rodent Safety studies

Evaluation of (-)-epicatechin: 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 85mg (-)-epicatechin/kg [116]. 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)[117]. 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 [118].

Evaluation of (+)-epicatechin: Guarana effects on toxic and behavioral parameters were assessed in rats and mice subsequent to acute and chronic administrations. Experimental parameters included tests for antioxidant capacity in vitro, in vivo, toxicological screening, body weight, motor activity, death rate, and histopathological examination of viscera. Guarana demonstrated antioxidant effects even at low concentrations by inhibiting lipid peroxidation. At high doses (1-2 g/kg IP and PO) it did not induce significant alterations in toxicological parameters. Consumption of liquids containing guarana evidenced unaltered weight of the animals even after prolonged (23 months) administration. The percentage mortality was equivalent in control and guarana groups. The absence of toxicity of guarana was also demonstrated by histopathological examination, with no alterations being detected in heart, lungs, stomach, small and large intestine, liver, pancreas, kidneys, bladder and spleen [119]. A 10-day study in which rats received 100 mg/kg/day, approximately 50X our proposed dose in humans, demonstrated that (+)-epicatechin at this high dose demonstrated no adverse effects with respect to appetite, behavior, alertness, or appearance. No abnormalities of any organs were observed at necropsy.

7.1.2 Human safety studies

Evaluation of (-)-epicatechin: Clinical studies with purified (-)-epicatechin (1-2 mg/kg) in the dose range of proposed DMD studies did not generate any significant adverse events [51, 54]. 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 [42, 43]). 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 [120]. 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).

Evaluation of (+)-epicatechin: (+)-Epicatechin. Campos *et al* recently evaluated the effects of standardized Guarana encapsulated dried extract on fatigue, sleep quality, anxiety, depression symptoms, and menopause in a cohort of breast cancer-associated chemotherapy patients. Patients with progressive fatigue after their first cycle of chemotherapy were randomized to receive either guarana 50 mg orally twice daily (n=32) or placebo (n=43) for 21 days. After a 7-day washout period, patients were crossed over to the opposite experimental arm. All patients

were evaluated on days 1, 21, and 49. Guarana significantly improved fatigue measures vs. placebo on days 21 and 49. Guarana treatment did not produce any Common Terminology Criteria for Adverse Event grade 2, 3, or 4 toxicities and did not worsen sleep quality or cause anxiety or depression [121].

7.2 Study Drug Formulation and Procurement

7.2.1(+)-epicatechin as a natural test substance

(+)-Epicatechin is a naturally-occurring product found in guarana, grapes, chocolate and tea **(Table 10)**. It is a member of the flavonoid family. (+)-Epicatechin is not commercially available in current Good Manufacturing Practices (GMP) grade monomeric form, for any purpose, research or otherwise. Cardero Therapeutics has developed the first scalable manufacturing process that provides pharmaceutical grade (+)-epicatechin. This manufacturing process has already been accepted by the FDA in the context of a previously filed IND for determining the tolerability and PK profile of (+)-epicatechin in healthy human volunteers (Henry IND#122158).

Synthetic, GMP (+)-epicatechin is produced using quercertin as the starting material:

Manufacturer: Syngene

Batch #: GF15000259

Country of Origin: India

It has >98% purity by HPLC, and has been purified in a cGMP facility. 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 cGMP analytical lab using appropriate qualified HPLC methods for impurities, including chirality testing. Release specifications require >95% purity, <3% of the (-)-epicatechin enantiomer, and <3% 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. The current batch is >95% pure, with <0.1% of catechin, <1% of chiral impurities. A stability study is ongoing and retest dates are scheduled.

Table 10: Analysis of (+)-Epicatechin

Chemical name	(+)-Epicatechin or (+)-*cis*-3,3′,4′,5,7-Pentahydroxyflavane, (2S,3S)-2-(3,4-Dihydroxy-phenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol
Molecular formula	$C_{15}H_{14}O_6$
CAS #:	35323-91-2
Molecular weight	290.27 grams/mole
Appearance	brown color solid
Solubility	>5 mg/ml in water at 20^{o}C
pKa	Neutral
Taste	Moderately bitter, resembling aspirin in degree of bitterness

7.2.2 (+)-EPICATECHIN AS SUPPLIED AS A NATURAL TEST SUBSTANCE

The intended route of administration is oral. (+)-Epicatechin synthesized from quercertin 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 water. **Table 11** describes the (+)-epicatechin capsule formulation.

Table 11: Quantitative composition of the (+)-epicatechin capsule.

Sl. No.	Ingredient	Functional Category	Qty/Capsule (mg)	Qty /Capsule (%)
1	(+)-Epicatechin	Active Ingredient	25.00	15.63
2	Microcrystalline cellulose (Avicel PH 102)	Diluent	127.00	79.37
3	Crospovidone (Kollidon CL)	Disintegrant	4.80	3.00
4	Citric acid monohydrate	Acidifying agent	1.60	1.00
5	Colloidal Silicon dioxide (Aerosil 200 Pharma)	Glidant	0.80	0.50
6	Magnesium Stearate	Lubricant	0.80	0.50
7	Hard gelatin capsule shells "Size 2" (White opaque cap / White opaque body)	Encapsulant	60.00	-
Total theoretical capsule weight			160.00 mg	100.00 %

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). Cardero Therapeutics 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).

The following excipients were investigated for potential use in the formulation of (+)-Epicatechin capsules as they are typical excipients utilized in the pharmaceutical industry with established properties and characteristics. They were successfully employed in the previously described clinical studies of (-)-epicatechin.

Diluents [Microcrystalline Cellulose (Avicel PH 102), Mannitol (Pearlitol 200SD), partially pregelatinized maize starch (Lycatab C)]:

The excipients mentioned functions as a diluent in the formulation where is it used in direct filling process. In addition to its use as diluents, it also has some disintegrant properties that make it useful in formulation. This is required to make up the volume of the capsule for low dose formulation. All diluents mentioned are available in compendial grades complying USP / NF, Ph.Eur, JP. It is incorporated in the formulation at levels of between 75 – 95%. Microcrystalline Cellulose (Avicel PH 102) was considered as diluent for (+)- Epicatechin capsules.

Disintegrant [Crospovidone (Kollidon CL), Croscarmellose sodium (Ac-di-sol)]:

Although epicatechin not found to be hygroscopic, still there are chances of API forming agglomerates on storage during stability; which may affect the dissolution. Disintegrants helps in rapid swelling (4 -18 fold in 10 seconds) and promotes disintegration of the slug and thus dissolution of the active ingredient. All disintegrants mentioned are available in compendial grades complying USP / NF, Ph.Eur, JP. It is incorporated in the formulation at a level of about 3 - 5%. Crospovidone (Kollidon CL) was considered as disintegrant for (+)- Epicatechin capsules.

Lubricants (Magnesium Stearate, Talc):

It functions as a lubricant, preventing the blend from sticking to tamping pins upon tamping and allowing for ease of ejection of the slug from the bed cavity. It is shear sensitive, which allows the other ingredients to be covered with a thin layer of this material. Inter-particle friction is also reduced. All lubricants mentioned are available in compendial grades complying USP / NF, Ph.Eur, JP. It is incorporated in the formulation at typical levels of 0.2 to 1%. Magnesium Stearate was considered as lubricant for (+)- Epicatechin capsules.

Glidant [Colloidal Silicon Dioxide (Aerosil 200 pharma)]:

It functions as a glidant, its small particle size and large surface area give it desirable flow characteristics that are exploited to improve the flow properties of dry powders in a number of processes such as capsule filling. It is available in compendial grades complying USP / NF, Ph.Eur, JP. It is incorporated in the formulation at a level of 0.2 to 1%.

Acidifying agent / Antioxidants (Citric acid monohydrate, Ascorbic acid):

API is known to be unstable in basic environment and susceptible to oxidation. Hence, an acidifying agent and antioxidant would help in increasing the overall stability of the formulation. These are available in compendial grades complying USP / NF, Ph.Eur, JP It is incorporated in the formulation at a level of 0.1 to 2%. Citric acid monohydrate was considered as acidifying agent for (+) - Epicatechin capsules.

A stability study is ongoing and retest dates are scheduled. Stability has been demonstrated with a previous batch at 20^{o}C for 1 year. Retained samples of clinical trial supplies will be tested to confirm potency.

7.3 Packaging

25 mg capsules of open-label study medication will be packaged 42 capsules per bottle. The bottles will be subsequently labeled by the UC Davis Investigational Drug Pharmacy for each subject, based on a written prescription by a medically qualified Study Investigator. In addition to dosing directions, the bottles shall bear a label with the statement "Caution: New Drug--Limited by Federal (or United States) law to investigational use."

7.3.1 Receipt of Drug Supplies

The UC Davis Investigational Drug Service will maintain drug shipment and dispensing accountability records of the investigational product per institutional standard operating procedures. Expiry dating/retest dates will be provided by Cardero Therapeutics based on ongoing stability testing. Documentation of expiry/re-test dates will be retained with study records. Study drug reconciliation will be documented in study records.

7.3.2 Storage Conditions

Capsules will be stored in HDPE bottles as packaged by Syngene, and maintained at ambient room temperature within the UC Davis Investigational Drug Service Pharmacy. Subjects/LAR will be advised to keep bottles at room temperature in their homes.

7.4 Treatment Cycles Drug Dispensation

Medications will be dispensed for the entire period of time between study visits and will include a 3-day reserve supply to account for the +/- 2-day study window as well as weekends and holidays. The study drugs will be clearly marked as investigational drugs per UC Davis Investigational Drug Service Pharmacy procedures. The site will dispense study medication 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.

7.4.1 Dispensing of Study Drug

A prescription will be written, signed by a qualified physician investigator and faxed to the UC Davis Investigational Drug Service Pharmacy. Medications will be released to the study coordinator, who will provide medications to the subject with a dosing/AE diary.

7.4.2 Return or Destruction of Study Drug

Participants will be instructed to return any unused study medications at each visit. The study coordinator will inventory unused medications and review the participant medication log, and will document reasons for any missed doses. Unused medications will be returned to the UC Davis Investigational Drug Pharmacy. At the completion of the study, there will be a final reconciliation of drug amounts shipped, dispensed, returned, and remaining. This reconciliation will be included in study files. Any discrepancies noted will be documented and investigated, prior to return or destruction of unused study drug. Any investigational drug destroyed on site according to institutional waste policy will be documented by the Research Pharmacy.

7.5 Investigational Product Compliance and Accounting Procedures

Study medication will be dispensed as part of the Baseline and study visits at UC Davis, to be taken home with the subject. A dosing and adverse event diary will be provided to the subjects, who will be asked to record the dates/times of medication intake and any unusual symptoms. The subjects will be asked to bring their diary and the previously dispensed bottles (empty, partial and unused) to each visit for review by study staff. Parents or guardians of participants younger than 18 years of age will be asked to supervise and/or provide the medication to their child and ensure the diaries are completed. A count of capsules returned at each visit will be performed by a study team member/pharmacy, and the number of returned capsules and compliance will be documented. If it is determined by study staff by review of diary, returned amounts and interview that the subject did not take 50% or greater of the daily dose for 3 days in any given week, the study investigator will discuss medication compliance with the parent/guardian and patient. If a participant is determined by the above criteria to be non-compliant a second time, he will be dropped from the study.

7.6 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

Laboratory efficacy assessments will occur according to the schedule set forth in the Study Matrix, and will include:

- *Pharmacokinetics:* (+)-epicatechin serum concentrations will be evaluated. Initial sampling (3ml peripheral blood) will occur immediately pre-dose, followed by supervised self-dosing by the study participant at the prescribed morning dose level. Peak serum concentration sampling (3ml peripheral blood) will occur at T_{max}, 2 hours (+/- 5 minutes) post-dose.
- *Plasma biomarkers:* 10ml peripheral venous blood will be collected to evaluate plasma biomarekers (approx. 4ml total plasma), including follistatin, myostatin, nitrite/nitrate ratio, MMP-9, TNF-a, TGF-b, CK, cardiac troponins and BNP.
- *SomaSCAN biomarker panel:* 5ml peripheral venous blood will be collected. The assay will include selected SOMAscan validated measures associated with age-related DMD disease severity (Troponin I, fast skeletal muscle, Carbonic anhydrase 3, Troponin I, cardiac muscle, creatine kinase M-type, Mitogen-activated protein kinase 12 (MAPK12, proto-oncogene tyrosine-protein kinase receptor Ret, GDF 11, Cadherin-5). Additional biomarker response will be evaluated.

8.2 Clinical Efficacy Parameters

Clinical efficacy assessments will occur according to the schedule set forth in the Study Matrix, and will include:

- Cardiac Function
    - *Echocardiogram (1 hour)*: Participants will undergo echocardiogram with collection of left ventricular (LV) circumferential and longitudinal strain, LV shortening fraction, LV ejection fraction, end systolic volume, and end diastolic volume as described by Spurney *et al* [122].
    - *Cardiac MRI (1 hour):* Cardiac MRI (cMRI) sequences will be performed on a Siemens 3T scanner at the UC Davis Imaging Research Center. Goals of cMRI imaging will be to obtain long and short imaging planes of the left ventrical, to obtain a stack of short-axis images that allow measurements of left ventricular volume at systole and asystole, and to obtain tagged cine images for cirumfrential strain analysis. Imaging will consist of:
        - scout images to identify cardiac structures in sagittal, axial and coronal planes;
        - a stacked axial fast-spin black blood image sequence;
        - a gradient echo sequence to define the long axis of the left ventricle;
        - a stacked gradient echo sequence to define the short axis of the left ventricle;
        - creation of a 4-chamber long-axis view localizer image;
        - a series of 12-16 steady state free precision gradient echo cine images based on the 4-chamber scout view; and

- tagged cine gradient echo images for circumferential strain analysis

Scans will last approximately 45-60 minutes including subject positioning and preparation. No sedation or contrast will be utilized during collection of these images.

- Strength, Range of Motion and Mobility
    - *Kinect Reachable Surface Area (15 minutes):* The Kinect camera is a product for Xbox designed by Microsoft that captures motion (http://msdn.microsoft.com/en-us/library/jj131033.aspx). The Kinect camera will be used to measure reachable workspace and fatigue of the participant using the protocol developed and published by Kurillo, Han et al. [123-128]. While being monitored by the Kinect, the participant will perform a series of standardized range of motion and functional upper extremity tasks, and perform a series of standardized range of motion and functional upper extremity tasks while holding a weight. Testing will yield bilateral 4-quadrant standardized measures of reachable surface area, normalized to the body size of the individual.
    - *Performance of the Upper Limb (PUL) assessment (15 minutes):* Mayhew et al developed the upper limb assessment tool called the Performance of the Upper Limb module for Duchenne muscular dystrophy [129]. The device was developed using upper limb functional performance items from the Brooke upper extremity functional scale [130], the Jebsen-Taylor Hand Function Test (JTHFT) [131] and the Motor Function Measure (MFM)[132] selected on the basis of a conceptual framework that items should provide assessment of upper limb and hand mobility including impact of weakness, growth and development of joint contractures across both ambulatory and non-ambulatory phases of disease.
    - *6-minute cycle test with exercise metabolic testing (15 minutes):* The COSMED K4b2, a portable cardiopulmonary metabolic system will be used to measure O2 consumption, CO2 production, and ventilation. Participants will be equipped with a mask that is placed over the nose and mouth while wearing a backpack carrying the receiver and battery pack. With the K4b2 on, the participant will perform an assisted 6-minute cycling test to assess endurance as described by Jansen et all [133]. The exercise test will begin by positioning the participant on a motorized assisted mobility trainer (KPT Cycla, Kinetec, France) that can be used with a participant's personal (electric) wheelchair or a regular chair with a backrest. The goal of the test is to cycle as fast as possible and keeps it up for 6-minutes. The KPT Cycla is set in passive mode 1 with no-load speed of 7 RPM. Participant are seated either in their wheelchair or a chair with back support in front of the KPT Cycla, with the hips and knees are positioned at 90 degrees of flexion for one leg while the other will be submaximally extended. The arm pedal axis of the KPT cycla will be adjusted a few centimeters (max of 5 cm) below shoulder level. The distance from the chair to the bicycle is determined by allowing participants to move their legs and arms over submaximal range of motion, producing a feeling of stretch. Verbal encouragement is given every 15 seconds. Participants are also told of the time completed and time left every minute. Rest is allowed if fatigued but participants are encouraged to continue cycling as soon as possible. The outcome of the test is the number of revolutions achieved in 6-minutes. Revolutions per minute (cumulative) and rest periods are also recorded.
- Person-Reported Outcomes
    - *POSNA PODCI (15 minutes):* The POSNA PODCI instrument was developed by Daltroy and colleagues with support by the Pediatric Orthopaedic Society of North America (POSNA). The PODCI is a 108-item questionnaire that evaluates global functioning in the pediatric orthopedic population utilizing four components: upper extremity functioning, transfers and basic mobility, sports and physical functioning and a comfort/pain score. Global functioning is assessed by the average of the four previous scores. All scales are scored from zero to 100, with 100 representing the highest level of functioning and least pain. The PODCI asks questions such as "During the last week, was it easy or hard for you to … lift heavy books". Both parent proxy and adolescent self-report forms have been validated. This is a self-administered questionnaire which takes about 15 to 20 minutes to complete. In DMD the PODCI transfers/basic mobility and sports/physical function domain scores are significantly associated with age (and hence disease progression), and traditional clinical outcome measures employed in ambulatory clinical trials.

- *PROM-UL (10 minutes):* The Person-Reported Outcome Measure Upper Limb (PROM-UL) is a DMD-specific self-assessment of upper limb mobility and hand dexterity in the context of daily activities, and is currently under development by Goemans and Klingels. It is a self-report analog of the Performance of the Upper Limb (PUL) clinical assessment noted above.

- Body Composition / Anthropometrics
  - *Anthropometric measures (5 minutes):* Anthropometric measures will include standing height, weight, and ulnar length. Standing height will only be measured for individuals who are able to stand unassisted; cacluated height using ulnar length will be measured according to methods established by the Cooperative International Neuromuscular Research Group [134]. Weight will be measured to the nearest 0.1kg.
  - *DEXA for body composition (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 $1/10^{th}$ 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).

9. Assessment of safety

9.1 Safety Parameters

Safety assessments will occur according to the schedule set forth in the Study Matrix, and will include:

- Vital Signs and Electrocardiogram
- Review of Medical History, including:
  - Medication History
  - Current Medications and Therapies
  - Medical and Surgical Events
  - Review of Adverse Events
- Clinical Review of Systems
  - Physical Examination
  - Neurological Exam
- Laboratory Assessments: Concurrent with biomarker peripheral blood draws listed in the Study Matrix, participants will provide blood samples for standard clinical safety testing. Safety panels will include at least:
  - 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 insulin and glucose
- Fasting lipid profile
- Urinalysis

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. Adverse events will 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 independent 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 and details 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 continue in the study for a period of approximately 10 weeks from the date of enrollment (including screening).

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
- 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

A. Characteristics of the Study Population

Non-Ambulatory males with DMD at least 8 years of age will be recruited

B. Participation of Children, Women and Minority Populations

DMD 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.

C. Sources of Research Material from Living Participants

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

D. 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.

i. 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.

ii. 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 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 should be used.

E. 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.

F. Retention of Participants

Care of each participant will be supervised by the 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.

G. Potential Risks

i. Risks of Epicatechin

Bleeding: Anti-clotting like effects have been described for foods containing members of the flavonoid family including epicatechin, such as cocoa, tea, and guarana. There is no report suggesting that epicatechin per se may have any effect on phenomena such as platelet aggregation or clotting times [14, 42, 135]. The modest effects on platelet aggregation of food containing polyphenols has been cited as one basis for beneficial cardiovascular effects. To our knowledge, no adverse event involving platelet inhibition has been described in any study of foods or their extracts of cocoa,guarana, or tea. Nonetheless, we deemed it prudent to exclude patients with thrombocytopenia or clotting disorders.

Hypotension: Potential risk based on the biological activities of (+)-epicatechin includes hypotension through presumed mechanism of nitric oxide (NO) mediated vasodilation [61]. Of note is the fact that so far, no blood pressure reducing effects by (-)-epicatechin have been reported in normotensive subjects. With cocoa based studies, blood pressure reducing effects are only reported in humans that have high blood pressure [136]. In the study by Ottaviani et al., the vasodilating effects of (+)-epicatechin in humans were only 25% of those of (-)-epicatechin when given at the same doses (1.5 mg/kg) [87]. 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 [137]. The consumption of cocoa products has been reported to be associated with increased likelihood of migraine development [138, 139]. However, the migraine effect has not been validated in subsequent clinical trials, and, if it exists, has been attributed to the presence of phenylethylamine [42]. However, it is reasonable to surmise that (+)-epicatechin may increase the chances for migraine development in susceptible individuals.

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.

Risks of Epicatechin Overdose: Neither the effects of overdose of (+)-epicatechin nor an antidote to overdose are known. For reference, the intraperitoneal (-)-epicatechin median lethal dose (LD_{50}) reported for mice is 1000 mg/kg as stated in the Material Safety Data Sheet provided by suppliers such as Sigma-Aldrich.

Pregnancy and Lactation: There are no known adverse effects of natural products containing epicatechin in pregnant women. 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). No information is available on levels of (-)-epicatechin in breast milk.

ii. Risks of Echocardiogram

Risks of echocardiogram evaluation include mild physical discomfort from holding positions required for image acquisition, and from the cool conductive gel used to optimize image quality.

iii. Risks of Cardiac MRI

Cardiac Magnetic Resonance Imaging (cMRI): With proper safety program adherence, risks of non-contrast MRI are minimal, and there are no known negative effects of exposure to magnetic fields at the 3T level. The Imaging Research Center requires extensive training of operators and staff prior to admittance to the facility to minimize hazards due to ferromagnetic projectiles, magnet quench, and to avoid burns due to incorrectly placed radio frequency coils. Participants undergo standardized screening to rule out presence of ferromagnetic metal in or on the body, and are given ear plugs and/or ear muffs to protect against noise exposure. Participants may experience feelings of claustrophobia, and may be removed from the scanner if they become too anxious. There is an additional risk of incidental MRI findings of previously unappreciated / asymptomatic health conditions. If any such findings are identified, imaging center staff will bring them to the attention of the center Medical Director, who will discuss any recommendations for follow up with the patient.

Iv. Risks of Kinect Reachable Surface Area Testing

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

v. Risks of Performance of Upper Limb Assessment Testing

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

vi. Risks of 6-minute Cycle Test Evaluation

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

vii. Risks of Person-Reported Outcome Questionnaires

Risks of self-reported functional questionnaire completion include minor risks of negative psychological stress due to inability to perform actions described in the questionnaires.

viii. Risks of Anthrometric Evaluation

There are no known risks of anthropometric evaluation.

ix. Risks of DEXA Body Composition Analysis

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

x. Risks of Electrocardiogram

The EKG has no known risks.

xi. Risks of Blood Draws for Biomarker and Safety Analysis

The risks of blood drawing include soreness or bruising at the site of the needle. A local numbing cream (EMLA) may be applied to the area at the participant's request. 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.

H. Procedures for Minimizing Risks

All study procedures will be conducted by study personnel who are experienced and licensed (as necessary) to conduct study assessments. 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. 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.

I. 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 DMD.

J. Benefits

Any health benefits to participation in this 8-week dose-finding study are likely to be transient. However, the intended benefits of long-term therapy outweigh the risks of using the study medication and study procedures in DMD patients. For long-term therapy, the participants may experience an improvement in cardiac function, an increase in muscle strength or a delay in strength decline. The participants will receive additional medical attention from the study team during their participation. 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 clinical 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 DMD.

K.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. Analysis Plan

Determination of Sample Size: Designation of sample size for this study was based on clinical considerations. Five participants will be enrolled sequentially in each of the three ascending dose cohorts (up to a maximum of 15 participants). Safety and PD endpoints will be evaluated along with PK characterization for the data obtained.

Statistical Analysis Plan: Because of the short treatment duration, the primary intent of endpoint collection is not an evaluation of drug efficacy. Instead, descriptive statistics on PD are performed to help determine preliminary estimates of variability and to explore data handling rules that can be integrated into future studies as applicable. Within-dose group estimates of the change from baseline will be summarized along with 95% confidence intervals for all variables captured. In addition, subjects will be classified as having improved, remained the same (no change), or worsened relative to their respective baseline assessment for each applicable endpoint for the post treatment follow-up visit. Analysis, employing both statistical and graphical presentations of data, will 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 standard deviations or frequencies 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. Level of statistical significance is set at <0.05. Simple comparative analyses will be used to assess and understand simple relationships, including defining first order interactions. We will compare continuous variables across groups using the Student's t-test and analysis of variance. We will compare differences between groups using Chi Square and Fisher's exact tests. We will compare simple relationships between variable using parametric or nonparametric correlations as appropriate. Power calculations will be based on simple between-group comparisons of means for treated / non-treated individuals at specified time periods. However, we will also explore complex relationships between variables and differences between groups using a combination of linear regression and longitudinal multivariate mixed model approaches (using xtreg or xtmixed in Stata12). 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. 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 Analysis: Exposure to study drug and any reasons for discontinuation of study will be tabulated, and demographics will be presented using descriptive statistics (ie, mean, standard deviation, median, and range). Safety variables will be tabulated and presented for all subjects who receive any dose of epicatechin. Safety data will be compiled as tables of frequency and severity of adverse events (AEs) by body system. AEs will also be presented by severity (as evaluated by the Common Terminology Criteria for Adverse Events and relationship to study drug). Change from baseline in clinical laboratory parameters and vital signs will be summarized across time.

Pharmacokinetics: The PK population is defined as all subjects enrolled in the study that received at least 1 dose of epicatechin and have at least 1 post-dose PK measurement. Listing of individual subject epicatechin plasma concentrations, actual blood sampling times, and PK parameters as well as graphs of concentration versus time will be prepared by actual dose regimen. PK variables will include C_{max}, T_{max} and plasma concentration at 12 hours post-dose (C_{min}).

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.

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