THE EFFECTS OF BETA2-ADRENERGIC RECEPTOR GENOTYPE ON CARDIOPULMONARY AND BODY COMPOSITION MEASURES IN DUCHENNE MUSCULAR DYSRTOPHY

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ABSTRACT

Objective: Duchenne muscular dystrophy (DMD) is an inherited x-link disease caused by the deletion of the dystrophin gene. DMD is characterized by progressive muscle weakness and degeneration of skeletal, cardiac and respiratory muscles resulting in severe functional impairment. The beta-2 adrenergic receptor (ADRB2) plays a functional role in muscle size, strength and regeneration through regulation of multiple downstream mechanisms. Multiple polymorphisms of the ADRB2 have been identified as including a glycine (Gly) for arginine (Arg) substitution at amino acid 16. This polymorphism (Gly16) has been shown to have higher receptor density on lymphocytes, be more resistant to receptor down regulation, and have improved lung function. The objective of this study was to determine the influence, if any, of ADRB2 genotype on body composition and cardiopulmonary health outcomes in DMD.

Methods: Nineteen patients were recruited through the University of Minnesota Fairview Clinic. DNA was collected via buccal swabs and analyzed for ADRB2 genotype. Medical records were then accessed to obtain retrospective data on age of loss of ambulation and start of corticosteroid treatment, DEXA, and cardiopulmonary measures when available.

Results: There were no differences between genotype groups in age of loss of ambulation (AMB) or start of corticosteroid treatment (STER) (AMB = 11.3 ± 1.09, 12 ± 0.71, STER = 8.67 ± 3.68, 7.22 ± 2.23 for Arg and Gly respectively). Additionally, there were no differences between genotype groups in age, height, weight, BMI, or BSA (age = 11.9 ± 4.1, 11.3 ± 3.5, height = 140.8 ± 18.7, 143.1 ± 19.7, weight = 47.7 ± 22.8, 45.7 ± 19.3, BMI = 23.6 ± 18.6, 21.5 ± 5.5, and BSA = 1.3 ± 0.39, 1.3 ± 0.33 for Arg and Gly respectively). Further, there were no differences between genotype groups in L1-L4, TBD, LBM, or LBM/BSA (L1-L4 = 0.66 ± 0.13, 0.69 ± 0.09, TBD = 0.69 ± 0.12, 0.64 ± 0.01, LBM = 62.6 ± 16.1, 65.5 ± 17.1, and LBM/BSA = 56.1 ± 29.6, 65.5 ± 17.1 for Arg and Gly respectively). There was a statistically significant difference between genotype groups for FEFmax at the patients' youngest age (FEFmax = 77.1 ± 14.1, 97.4 ± 16.9 for Arg and Gly respectively). There may also have been a clinically significant difference between genotype groups for FVC and FEV_1 at the patients' youngest age (FVC = 85.1 ± 18.5, 94.5 ± 15.7, and FEV_1 = 84.7 ± 16.1, 99.1 ± 18.9 for Arg and Gly respectively). However, there were no differences between genotype groups for FEFmax, FVC, and FEV_1 at the patients' mid-point and oldest age nor were there differences for MEP and MIP at any age. Furthermore, there were no differences between genotype groups for any of the cardiac measures at any age.

Discussion: These data suggest the ADRB2 may play a role in an improved functional pulmonary capacity but does not influence body composition or cardiac measures. This suggests early intervention with ADRB2 treatment may serve to preserve functional pulmonary capacity and health outcomes in DMD.

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LIST OF ABBREVIATIONS

DMD……Duchenne muscular dystrophy

DAGC……Dystrophin-associated glycoprotein complex

SAR……Sarcalumenin

CSQ……..Calsequestrin

SERCA1……..Sarcoplasmic/endoplasmic-reticulum calcium-ATPase 1

FEV_1……..Forced expiratory flow at one second

FEF……...Forced expiratory flow

FVC…….Forced vital capacity

ADRB2…….β_2 adrenergic receptors

GLY……..Glycine

ARG…….Arginine

GTP……....Guanine Triphosphate

cAMP……Adenosine 3'-5' monophosphate

DEXA…….Dual-energy x-ray absorptiometry

AC……..Adenylyl cyclase

PKA…….Protein kinase A

BMI……...….Body mass index

BSA……...Body surface area

L1-L4……..Lower lumbar density

TBD……..Total body density

LBM……..…Lean body mass

LBM/BSA…….Lean body mass corrected for body surface area

MEP…….Maximal expiratory pressure

MIP…….Maximal inspiratory pressure

CREB……....cAMP response element binding protein

MEF2……..Myocyte enhancer factor-2

SIK1…….Ser/Thr kinase salt induced kinase-1

NOR-1…….Neuron-derived orphan receptor-1

PI3K-AKT……Phosphoinositide 3-kinase-protein kinase-B

FOXO……..Forkhead box O transcription factors

ADRB1……..... β_1 adrenergic receptors

PLB…….…..Phospholamban

CHAPTER 1: INTRODUCTION

Duchenne muscular dystrophy (DMD) is an inherited X-linked disease with a 1/3500 birth rate incidence in males (Emery & Mutoni, 2003). Females can be carriers of DMD but do not display the rapid muscle wasting indicative of the disease (Emery & Mutoni, 2003). DMD is characterized by progressive muscle weakness and degeneration of skeletal, cardiac and respiratory muscles (Prior, 2015). Patients with DMD are diagnosed at an early age when their physical ability deviates significantly from their age-matched peers. Progressive muscle weakness and degeneration is symmetrical and affects the lower limbs before the upper limbs and occurs proximally to distally. Progressive muscle wasting and fibrosis typical of DMD typically confines patients to a wheelchair by age twelve with death due to cardiac or respiratory failure by age twenty (Emery, 2002).

The β_2 adrenergic receptors (ADRB2) play a functional role in muscle size, strength and muscle regeneration (Church et al., 2014; Silva, Wensing, Brum, Camara, & Miyabara, 2014). Church et al (2014) demonstrated reduced peak twitch force and rate of contraction, maximal force and significantly reduced rates of regeneration in ADRB2 receptor knockout mice compared to controls. Additionally, these knockout mice had an increased inflammatory response to damage, particularly macrophage migration, which is a significant component of the dystrophic pathway in skeletal, cardiac and respiratory muscles. These data suggest the ADRB2 may play a significant role in the protection of muscles from the dystrophic pathways.

Multiple polymorphisms of the ADRB2 have been identified as including a glycine (Gly) for arginine (Arg) substitution at amino acid 16. This polymorphism

(Gly16) has been shown to have higher receptor density on lymphocytes, be more resistant to receptor down regulation, and functionally demonstrate higher cardiac output and stroke volume, improved left ventricular function and ejection fraction, sustained bronchodilation following intense exercise, and better lung function in patients with heart failure than Arg16 in humans (Green, Turki, Innis, & Liggett, 1994; Snyder, Hulsebus, Turner, Joyner, & Johnson, 2006; Tang et al., 2013). Collectively, previous work suggests a protective effect of the Gly16 polymorphism on cardiac function; this implication is of particular importance to DMD as those patients are at a high risk of cardiomyopathies and left ventricular dysfunction (Nigro, Comi, Politano, & Bain, 1989).

Although not specifically studied previously, human subjects with the Gly16 polymorphism tend to have a greater body mass index, despite greater fitness levels, possibly suggesting greater muscle mass. Therefore, the Gly16 polymorphism may not only have a protective effect on the cardiac muscles, but may also attenuate skeletal muscle degeneration throught other down stream mechanisms. The activation of the ADRB2 results in the formation of adenosine 3'-5' monophosphate (cAMP), a secondary messenger that plays many roles in the body. An increase in ADRB2 density has been correlated with increased cAMP accumulation in animal models (George, Berrios, Hadcock, Wang, & Malbon, 1988; Silva, Wensing, Brum, Câmara, & Miyabara, 2014). Skeletal muscle cAMP signaling is shown to regulate contractility, sarcoplasmic calcium dynamics, and recovery from sustained contractile activity. The net result of cAMP activation is characterized by increased contractile force and rapid recovery of ion balance. Further, in rodent DMD models, cAMP production was shown to slow

degeneration as well as promote regeneration of skeletal muscles (Berdeaux & Stewart, 2012). This previous work suggests DMD patients with the more functional variant of the ADRB2 may have increased cAMP accumulation, resulting in improved skeletal muscle contractile activity and ability to recover as well as an attenuated degeneration of skeletal muscle.

This study will be novel because it will be the first study, to our knowledge, to analyze ADRB2 genotype and its interaction with cardiac and pulmonary function, strength and body composition in DMD. The more functional variants of the ADRB2 may have a protective effect on both cardiopulmonary function as well as skeletal muscle degradation by reducing receptor down regulation and increasing density, thus improving receptor activity and cAMP accumulation.

CHAPTER 2: REVIEW OF LTERATURE

Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is an inherited X-linked disease with a 1/3500 birthrate incidence in males and is the most common lethal X-linked disease (Emery & Muntoni, 2003). DMD is characterized by progressive muscle weakness and degeneration of skeletal, cardiac and respiratory muscles and infiltration of fatty acid and bone mineral deposits, rendering the affected muscle compromised (Prior, 2015). Patients are diagnosed at an early age when their physical ability deviates significantly from their age-matched peers. Progressive muscle weakness and degeneration is symmetrical and affects the lower limbs before the upper limbs, proximally to distally and typically confines patients to wheelchairs by age twelve with death resulting from cardiopulmonary failure associated with dilated cardiomyopathy and restrictive pulmonary disease typically occurring in the late teens to early twenties (Emery & Muntoni, 2003).

DMD is caused by a mutation of the dystrophin gene that encodes for the dystrophin protein which acts as a membrane stabilizing protein. Dystrophin normally links cytoskeletal proteins to sarcolemmal glycoproteins to form the dystrophin-associated glycoprotein complex (DAGC) (Prior, 2015). This functional link between muscle fiber cytoskeletal proteins, most notably actin, to the sarcolemmal glycoproteins, allows for distribution of forces across the sarcolemma associated with myofibril contraction limiting mechanical stress. Normal muscle contractions in healthy muscle cause tears in muscle fibers and an influx of calcium that activates degenerative pathways via calpains. Calpains, comprised of two main isoforms m-calpain and μ-calpain, tend to

be concentrated on the Z band of the sarcomere where protein disassembly begins (Kumamoto et al., 1992). Calpains do not degrade proteins to amino acids or even small peptide, but rather initiate myofibrillar protein degradation by disassembling the outer layer of proteins, thus releasing them as myofilaments, further decreasing myofibrillar protein integrity (Navegantes, Baviera, & Kettelhut, 2009).

Due to the lack of dystrophin associated with DMD, this calcium influx is augmented by the lack of DAGC and a resulting loss of selective permeability in the cellular membrane. Additionally, repeated injurious events eventually exhaust the regenerative capacity of dystrophic muscles and infiltration of adipose and connective tissue ensues leading to progressive functional impairments in affected patients (Prior, 2015). Effectively, DMD renders muscle fibers fragile and susceptible to damage during contractions.

Duchenne Muscular Dystrophy and Skeletal Muscle

In DMD, not only are the DAGC compromised due to dystrophin deficiency, the other proteins of the DAGC are also greatly reduced (Ervasti & Campbell, 1991). This reduction in DAGC proteins leaves the membrane more prone to mechanical tears resulting in increased membrane permeability and calcium flux due to a loss of stabilization of myofibril integrity during contraction and relaxation (Talsness, Belanto, & Ervasti, 2015). The DAGC also plays a role in calcium channel stabilization, whereby the deformation of the DAGC due to dystrophin deficiency results in calcium channel dysfunction. Further, in addition to mechanical tears, calcium channel dysfunction is also believed to be a source of calcium homeostasis dysregulation (Spencer, Croall, & Tidball,

1995). This increased calcium flux results in the activation of calpains, a calcium dependent ubiquitous-proteasome, which degrades muscle protein. In addition to the activation of calpains, calcium flux into the mitochondria can also lead to swelling and bursting of the mitochondria; mitochondrial death is followed by cell death and necrosis. Following cell death and necrosis, muscle cells are infiltrated by macrophages and, due to decreased membrane stabilization, fatty tissue deposits and calcium deposits (Emery & Muntoni, 2003). This macrophage infiltration results in muscle weakness, loss of muscular control and ambulatory abilities. Additionally, the process of protein degradation and loss of functional strength seems to preferentially affect fast twitch muscle fibers (Webster, Silberstein, Hays, & Blau, 1988).

In addition to calcium channel dysfunction, research suggests there is an altered protein expression that may further increase cytosolic calcium concentrations. Research has demonstrated the role of calcium homeostasis dysfunction in the dystrophic pathway in mdx mice, homologous to the DMD pathology, by showing luminal sarcalumenin (SAR), the key calcium-shuttling protein of the sarcoplasmic reticulum, to be decreased by as much as 70% in affected mdx skeletal muscle fibers compared to healthy controls and a decreased concentration of calsequestrin (CSQ), the main calcium-binding protein in the sarcoplasmic reticulum (Doran, Dowling, Lohan, McDonnell, Poetsch, & Ohlendieck, 2004). This research also shows a mechanical linkage between SAR and the sarcoplasmic/endoplasmic-reticulum calcium-ATPase 1 (SERCA1). However, dystrophic membrane preparations have not shown a relative decrease in SERCA1 (Doran, Dowling, Lohan, McDonnell, Poetsch, & Ohlendieck, 2004). Further, impaired

calcium-shuttling between SERCA units and calsequestrin clusters via SAR and decreased luminal calcium-binding capacity may amplify cytosolic calcium levels due to calcium channel dysfunction. This suggests that the reduction in SAR and CSQ plays a significant role in the skeletal muscle dystrophic pathway.

Furthermore, DAGC dysfunction not only results in decreased membrane stabilization and increased calcium flux, it may also render the muscles ischemic during exercise (Kobayashi et al., 2008). Because neuronal nitric oxide synthase is localized to the sarcolemma as part of the DAGC, loss of sarcolemmal integrity results in loss of nitric oxide synthase (Brenman, Chao, Xia, Aldape, & Bredt, 1995; Ervasti & Campbell, 1991). Nitric oxide during exercise has been shown to play a role in blunting exercise-induced vasoconstriction due to α-adrenergic receptor activation and this protective mechanism is defective in DMD resulting in unopposed vascular vasoconstriction during exercise further contributing to the pathogenesis of DMD (Sander et al., 2000; Thomas, Sander, Lau, Huang, Stull, & Victor, 1998).

Functional skeletal muscles have the ability of regeneration but due to the upregulation of contraction-induced injuries and cell necrosis in DMD, this ability is overwhelmed and the ability to regenerate skeletal muscle is surpassed by the muscle cell degradation pathways. Not only do DMD patients experience progressive muscle weakness due to cell death and necrosis, but functional capacity is limited as a result of ischemia. This rampant cell death and necrosis results in DMD patients being wheel chair bound by their early teens and experiencing a severe decline in quality of life (Prior, 2015).

Duchenne Muscular Dystrophy and the Lungs

A majority of DMD patients die due to respiratory failure and that respiratory weakness tends to exhibit itself in the second decade of life (Back et al., 2006). DMD associated respiratory impairment is a result of deterioration and necrosis of the diaphragm, which has similar contractile properties of skeletal muscle and also involves DAGC (Ishizake et al., 2008; Lewis, Carberry, & Ohlendiek, 2009). Similarly to skeletal muscle, the loss of dystrophin to stabilize the DAGC results in reduced membrane integrity, calcium channel dysfunction and reduced expression of calcium-handling proteins (Lewis, Carberry, & Ohlendiek, 2009). The implications of diaphragmatic deterioration are of importance in DMD because this is the primary muscular organ responsible for respiration. Similar to skeletal muscle deterioration, it is believed the resultant calcium flux from either calcium channel dysregulation or mechanical tears in the sarcolemma are the main contributor to diaphragm muscle cell death (Petrof et al., 1993). Unlike skeletal muscle, mdx diaphragm strips have revealed regucalcin as the main calcium-dependent protein involved in diaphragmatic calcium dysregulation. Regucalcin, a cytosolic calcium-binding protein involved in intracellular signaling, is the main calcium-binding protein in the liver. However, research has identified regucalcin of 33.9 kDa and pl5.2 also exists in the diaphragm muscle. Similar to the reduction of SAR and CSQ in affected skeletal muscle, there is a 2-fold reduction of regucalcin in mdx diaphragm (Doran, Dowling, Donoghue, Buffini, & Ohlendieck, 2006). This reduction results in disturbed intracellular signaling due to abnormal handling of cytosolic calcium and an insufficient maintenance of calcium homeostasis and abnormal regulation of

calcium-dependent enzymes (Doran, Dowling, Donoghue, Buffini, & Ohlendieck, 2006). This suggests that calcium homeostasis plays a significant role in the dystrophin-associated pathology of the DMD diaphragm.

Clinically, muscle fiber necrosis of the diaphragm presents itself in progressive loss of inspiratory pressures and vital capacity (De Bruin et al., 1997). DMD patients have also been shown to have compromised forced expiratory volume at one second (FEV_1), forced expiratory flow (FEF), and forced vital capacity (FVC) (De Bruin et al., 1997). Further, diaphragmatic cell death results in an inflammatory response wherein macrophages migrate to the damaged area to clear the cell and debris which is followed by fibroblast invasion of the damaged area and the formation of scar tissue and fibrosis in the diaphragm. Additionally, infiltration of connective tissues as well as fat deposits has been shown to increase resting diaphragm thickness (pseudo-hypertrophy) and decrease its contractility (De Bruin et al., 1997). Not only may this increase in resting diaphragm thickness be problematic for mechanical contractility, but the resulting effect on the diaphragm curvature may also affect contractility due to the altered length-tension relationship (Back et al., 2006). Accordingly, research suggests mdx mice present a significant increase in the extracellular matrix protein collagen in the diaphragm (Carberry, Zweyer, Swandulla, & Ohlendieck, 2012). Furthermore, DMD patients have shown an increase in diaphragmatic tension-time index after age 14, suggesting this population's diaphragm fatigues easier. This is due in part to diaphragmatic remodeling present in this population. In the canine model of DMD, diaphragmatic remodeling was demonstrated such that the primary role of the diaphragm becomes the passive elastic

storage of energy transferred from the abdominal walls. This results in the expiratory muscles sharing in the generation of inspiratory pressure and flow. This remodeling was also associated with the loss of sarcomeres in series and an almost 900 fold increase in stiffness (Mead et al., 2014). In addition to increased fatigue, DMD patients have demonstrated a decrease in gastric pressure with cough, suggesting the onset and advancement of muscle weakness in respiratory muscles (Khirani et al., 2014).

Duchenne Muscular Dystrophy and the Heart

With recent advances in DMD treatment, patients are living longer, resulting in cardiac involvement being of more importance. It is believed the mechanism of cardiac involvement in DMD is similar to skeletal muscle; loss of integrity, fiber necrosis and replacement of tissue with connective tissue or fat (Beynon & Ray, 2008). Similar to skeletal and diaphragmatic muscle, loss of DAGC stability results in a loss of membrane stability and calcium channel dysfunction (Parvatiyar et al., 2015). However, there are cardiac-specific mechanisms affected by DMD; the absence or mutation of dystrophin disrupts the function of membrane ion channels, particularly the sarcolemmal stretch-activated channels (Aguettaz et al., 2016). When cardiomyocytes with augmented dystrophin levels stretch during ventricular filling, the stretch-activated channels do not open appropriately, leading to an increased influx of calcium. These resulting calcium influxes will activate calpains, a calcium-induced protease, which degrades troponin I and compromises cardiomyocyte contractile ability. Further, this calpain-mediated damage of membrane proteins allows additional calcium influx; this chronic calcium loading leads to cardiomyocyte necrosis (Kaspar, Allen, & Montanaro, 2009).

Additionally, this calcium leak into the cardiomyocyte can modulate L-type calcium channels, causing unnecessary contractions and further calcium loading, contributing to cell death. Similarly to skeletal and diaphragmatic muscles, DMD affected cardiomyocytes exhibit a reduction in the calcium-binding proteins, namely CSQ (Beard, Laver, & Dulhunty, 2004). This suggests that in addition to increased calcium loading in cardiomyocytes, its ability to handle calcium is also dysfunctional, further disrupting calcium homeostasis.

Cardiomyocyte cell death initiates an inflammatory response wherein macrophages migrate to the damaged area to clear the cell and debris which is followed by fibroblast invasion of the damaged area and the formation of scar tissue and fibrosis in the heart. Myocardial fibrosis associated with DMD initially appears in the left ventricular wall behind the posterior mitral valve leaflet and progressively spreads inferiorly towards the apex and will gradually affect the entire ventricle. The fibrotic region will gradually stretch, become thinner and lose contractility. This results in an increased ventricular volume, decreased ejection fraction, and decreased systolic function. Mitral valve regurgitation is also a common pathology as fibrosis spreads to encompass this area. The result of this systolic dysfunction is decreased cardiac output and hemodynamic decompensation and is the major contributor to the left ventricular dysfunction accompanying DMD (Kaspar et al., 2009).

Subclinical cardiomyopathies associated with DMD are first evident at ten years of age and is present in all DMD patients over the age of 18. Cardiomyopathy, most typically left ventricular failure, is very prevalent in DMD patients and will be the cause

of mortality in an estimated 10-15% of this population (Nigro, Comi, Politano, & Bain, 1989). Additionally, Corrado et al (2001) determined that 71% of their DMD patients had evidence of electocardiographic abnormalities with 32% of those having frequent premature ventricular complexes, 28% with ventricular late polarizations and 35% having left ventricular systolic dysfunction. After a 76 month follow-up period, they determine left ventricular dysfunction as determined by an echocardiograph is a strong predictor of mortality. This suggests that if left ventricular systolic dysfunction can be identified and treated, it could improve DMD life span.

β_2 Adrenergic Receptors and Duchenne Muscular Dystrophy

The β_2 adrenergic receptors (ADRB2) play a functional role in muscle size, strength and muscle regeneration (Church et al., 2014; Silva, Wensing, Brum, Camara, & Miyabara, 2014). Church etal (2014) demonstrated reduced peak twitch force and rate of contraction, maximal force and significantly reduced rates of regeneration in β_2 receptor knockout mice compared to controls. Additionally, these ADRB2 knockout mice had an increased inflammatory response to damage, particularly macrophage migration; this is a significant component of the dystrophic pathway in skeletal, cardiac and respiratory muscles. These data suggest the ADRB2 may play a significant role in the protection of muscles from the dystrophic pathways.

Functional Single Nucleotide Polymorphism

Multiple polymorphisms of the ADRB2 have been identified as including a glycine (Gly) for arginine (Arg) substitution at amino acid 16. This polymorphism (Gly16) has been shown to have higher receptor density on lymphocytes, be more

resistant to receptor down regulation, and functionally demonstrate higher cardiac output and stroke volume, improved left ventricular function and ejection fraction, sustained bronchodilation following intense exercise, and better lung function in patients with heart failure than Arg16 in humans (Green, Turki, Innis, & Liggett, 1994; Snyder, Hulsebus, Turner, Joyner, & Johnson, 2006; Tang et al., 2013). These studies suggest a protective effect of the Gly16 polymorphism on cardiac function; this implication is of particular importance to DMD as those patients are at a high risk of cardiomyopathies and left ventricular dysfunction (Nigro, Comi, Politano, & Bain, 1989).

Gly16 Functional Polymorphism and Duchenne Muscular Dystrophy

Although not specifically studied previously, human subjects with the Gly16 polymorphism tend to have a greater body mass index, despite greater fitness levels, possibly suggesting greater muscle mass (Snyder et al., 2006). Therefore, the Gly16 polymorphism may not only have a protective effect on the cardiac muscles, but may also attenuate skeletal muscle degeneration through other down stream mechanisms. The activation of the ADRB2 results in the formation of adenosine 3'-5' monophosphate (cAMP), a secondary messenger that plays many roles in the body. Additionally, an increase in ADRB2 density has been correlated with increased cAMP accumulation in animal models (George, Berrios, Hadcock, Wang, & Malbon, 1988; Silva et al., 2014). Skeletal muscle cAMP signaling is shown to regulate contractility, sarcoplasmic calcium dynamics, and recovery from sustained contractile activity. The net result of cAMP activation is characterized by increased contractile force and rapid recovery of ion balance. Further, in rodent DMD models, cAMP production was shown to slow

degeneration as well as promote regeneration of skeletal muscles (Berdeaux & Stewart, 2012). Therefore we hypothesize that DMD patients with the more functional variant of the ADRB2 may have increased cAMP accumulation, resulting in improved skeletal muscle contractile activity and ability to recover as well as an attenuated degeneration of skeletal muscle.

β_2 Adrenergic Receptors and Calpain Inhibition

The mechanism whereby ADRB2 activation may inhibit proteolysis is via calpain inhibition. ADRB2s are g-protein coupled receptors where upon binding of a catecholamine to the receptor stimulates a dissociation of the $G_{\alpha s}$ protein from the tightly associated β and γ subunits. $G_{\alpha s}$-bound guanine triphosphate (GTP) then phosphorylates adenylyl cyclase (AC). AC produces cAMP which phosphorylates protein kinase A (PKA) into its active form (Berdeaux & Stewart, 2012). Both cAMP and phosphorylate PKA can either directly or indirectly inhibit calpain activity. Research has shown using a nonhydrolyzable cAMP analog and activation of ADRB2 to inhibit protein degradation in both rats and chicks, suggesting cAMP may directly phosphorylate calpains to inhibit activity (Navegantes, Resano, Migliorini, & Kettelhut, 2001; Navegantes, Machado, Resano, Migliorini, & Kettelhut, 2003). Further, it has been demonstrated that the calpastatin promoter sequence between nt -1653 and +130 contains a single cAMP binding site located at nt -76 (Cong, Goll & Antin, 1998). This suggests a direct pathway whereby cAMP signaling can lead to increased calpastatin gene transcription, a calpain inhibitor, reducing calpain-mediated protein degradation. Further, multiple phosphorylation sites have been identified on calpastatin, particularly those found in the

L and XL domain coded by exon 6, suggesting cAMP to have the ability to directly phosphorylate calpastatin (Tullio et al., 2009). Research also suggests PKA as having the ability to phosphorylate calpains. Studies in rat models have demonstrated a phosphorylation site at serine 369 which would restrict domain movement and keep m-calpain in an inactive state, suggesting direct phosphorylation of calpain by PKA to have a negative-control effect on calpain activation (Salamino et al., 1994; Shiraha, Glading, Chou, Jia, & Wells, 2002). In addition to its ability to phosphorylate calpains, research suggests the C-domain of PKA can also directly phosphorylate calpastatin (Reiken et al., 2003). PKA and cAMP phosphorylation and increase in gene transcription of calpastatin are of importance to DMD as calpastatin overexpression has been shown to result in skeletal muscle hypertrophy and to protect against atrophy in rats, suggesting calpastatin is also important in the regulation of skeletal muscle protein turnover (Otani et al., 2001; Tidball & Spencer, 2002).

ADBR2 agonist supplementation has also been shown to reduce calpain activity. Research has indicated a decreased rate of protein degradation following epinephrine supplementation which was prevented by propranolol, a non-selective beta-antagonist and by M ICI 118.551, a β_2-antagonist in rat skeletal muscle models. Additionally, dibutyryl cAMP and isobutylmethylxanthine reduced proteolysis in both soleus and extensor digitalis longus muscles (Navegantes, Resano, Migliorini, & Kettelhut, 2000). Similar results have been shown in bovine species as well. β_2-agonist in Friesian steer was shown to induce hypertrophy as well as increase calpastatin-specific activity by 76% with an overall 96% increase in calpastatin mRNA levels (Parr, Bardsley, Gilmour, &

Buttery, 1992). In fact, following clenbuterol administration in the rat model, there was an approximate 50% decrease in the muscle calcium-dependent proteolytic pathway with no resultant change in the activations of the lysosomal or ubiquitin-proteasome pathways, further support that the activity and gene expression of calpastatin are increased after beta-agonist supplementation (Parr, Sensky, Arnold, Bardsley, & Buttery, 2000; Salem, Levesque, Moon, Rexroad, & Yao, 2006). Further evidence supports the role of adrenergic tonus on the inhibition of proteolysis. After chemical and surgical sympathectomy, rat models demonstrated an increase in calcium-dependent proteolytic pathways, suggesting adrenergic tonus exerts its effect via calcium-dependent pathway inhibition (Navegantes, Resano, Migliorini, & Kettelhut, 1999; 2001).

Purpose

DMD is an x-linked disease with a 1/3500 male birthrate and is characterized by progressive muscle weakness caused by a deletion of the gene encoding for dystrophin. This resultant absence of dystrophin renders the muscle cell membrane fragile and susceptible to mechanical tears and calcium channel dysregulation, leading to an increased intracellular calcium flux. High intracellular calcium concentrations activate multiple degradation pathways, most notably calpains. These mechanical tears not only modulate membrane permeability to calcium, but to fatty acids as well, resulting in both calcified and fatty acid deposits in the muscle cell. Once muscle degeneration exceeds its regenerative capacity, fibrotic tissue formation occurs. These processes diminish the muscles functional capacity and contractile ability. The ADRB2 plays a functional role in muscle size, strength and regeneration through regulation of multiple downstream

mechanisms. Multiple polymorphisms of the ADRB2 have been identified as including a glycine (Gly) for arginine (Arg) substitution at amino acid 16. This polymorphism (Gly16) has been shown to have higher receptor density on lymphocytes, be more resistant to receptor down regulation, and have improved lung function. Although not specifically studied previously, human subjects with the Gly16 polymorphism tend to have a greater body mass index, despite greater fitness levels, possibly suggesting greater muscle mass. This suggests the Gly16 polymorphism may attenuate skeletal, cardiac, and pulmonary muscle degeneration. Therefore, for this recent study, we sought to investigate the influence of ADRB2 genotype on cardiopulmonary and body composition measures on subjects with DMD.

CHAPTER 3: METHODOLOGY

Subjects/Participants

Subjects ages 6 to 18 (n=19) with a clinical diagnosis of DMD were recruited at the Riverside Fairview Clinic, Minneapolis MN. Inclusion criteria included a clinical diagnosis of DMD. Exclusion criteria included the inability to provide assent. The study was approved by the University of Minnesota Institutional Review Board, each subject provided written informed assent prior to the study, each subject's legal guardian also provided written informed consent prior to the study, and all aspects of the study were performed according to the Declaration of Helsinki.

Data Collection

DNA was collected via buccal swabs and analyzed for ADRB2 genotype. Medical records were then accessed to obtain retrospective data on pulmonary function tests, echocardiograms, and DEXA body composition measures.

Data Analysis

ADRB2 genotyping was PCR-based according to methods of Bray et al (2000). Buffy coat, obtained from whole blood collected on EDTA, was extracted using the Gentra Puregene DNA Isolation Kit (Gentra Systems Inc., Minneapolis, MN, USA). The PCR reaction was conducted according to standard methods, using the following primer sequences (e.g. for Arg16Gly): (forward) 5′-AGC CAG TGC GCT TAC CTG CCA GAC-3′ (at −32) and (reverse) 3′-CA TGG GTA CGC GGC CTG GTG CTG CAG TGC-5′, resulting in a PCR product 107 base pairs in length. The reaction included 30 ng of DNA, 1.5 mM magnesium chloride, 0.5 U taq polymerase (Invitrogen, Carlsbad, CA, USA), 8.5% DMSO and standard concentrations of nucleotides and buffer in a 20 µl

reaction volume. After initial denaturation at 94°C for 4 min, the fragments were amplified by 35 cycles of 1 min at 94°C, 1 min at 61°C, 1 min at 72°C, followed by 5 min at 72°C and 5 min at 98°C. The amplicons were then digested by exposure to 5 U of the restriction enzyme *Kpn*I, followed by electrophoretic separation on 3% aragose gels, staining with ethidium bromide and visualization using UV light. The ArgArg homozygous genotype is represented by a single 107 bp band, the ArgGly group is represented by 25, 82 and 107 bp bands, and the GlyGly homozygous group by 82 and 25 bp bands.

Statistical Analysis

All statistical comparisons were made using a statistical software package (SPSS; SPSS Inc; Chicago, IL, version 19). Group demographics and genotype differences in body composition were compared with a one-way analysis of variance (ANOVA) using an α level of 0.05 to determine significance. Genotype differences in pulmonary and cardiac measures were compared with two-way repeated measures ANOVA for three time points, the subjects' youngest age, mid-point age, and oldest age. An α level of 0.05 was used for the two-way repeated measures ANOVA analysis.

CHAPTER 4: RESULTS

Subject Characteristics

There were no differences between genotype groups in age, height, weight, body mass index (BMI), or body surface area (BSA) (Table 1). Additionally, there were no differences between genotype groups in lower lumbar density (L1-L4), total body density (TBD), lean body mass (LBM), or lean body mass corrected for body surface area (LBM/BSA) (Table 2).

Ambulatory and Corticosteroid Status

There were no differences between genotype groups in age of loss of ambulation (eight of the 19 patients were non-ambulatory and confined full-time to a wheelchair) (Figure 1.A). Further, there were no differences between genotypes in the age of start of corticosteroid treatment (17 of the 19 patients were on a corticosteroid use regimen) (Figure 1.B).

Pulmonary Measures

Forced Vital Capacity

There were no statistically significant differences between genotype groups in FVC measured as percent of predicted at any of the three time points (young, mid-point, and old). However, there may be a clinically significant difference between genotype groups at the youngest age (85.1 ± 18.5 vs. 94.5 ± 15.7, for Arg carriers and Gly homozygous respectively) (Figure 2.A).

Forced Expiratory Volume

Similar to FVC measures, there were no statistically significant differences between genotype groups in FEV_1 measured as percent of predicted, but there may be

clinically significant differences in FEV_1 at the youngest age (84.7 ± 16.1 vs. 99.1 ± 18.9, for Arg carriers and Gly homozygous respectively) (Figure 2.B).

Forced Expiratory Flow

There were no statistically significant differences between genotype groups at the mid-point and oldest age for FEF. There was a statistically significant difference between groups at the youngest age (*p-value* = 0.02) (Figure 2.C).

Maximal Pressures

There were no statistically significant differences between genotype groups in maximal expiratory pressure (MEP) (Figure 2.D). Further, there were no significant differences between groups for maximal inspiratory pressure (MIP) (Figure 2.E).

Cardiac Measures

There were no statistically significant differences between genotype groups in left ventricular ejection fraction (LVEF) (Figure 3.A), left ventricular end diastolic volume (LVED) (Figure 3.B), left ventricular end systolic volume (LVES) (Figure 3.C), or left ventricular shortening fraction (LVSF) (Figure 3.D).

CHAPTER 5: DISCUSSION

In this present study, we demonstrated that ADRB2 genotype may play a role in pulmonary mechanics in DMD. We demonstrated a statistically significant difference between genotype groups in FEFmax at the patients youngest age (*p-value* = 0.02) and a possible clinically significant difference in FVC and FEV_1 at the patients youngest age. However, we did not demonstrate any significant differences between genotype groups in any of the cardiac measures or the body composition measures. This suggests ADRB2 genotype may not have an effect on these outcome measures in DMD

Research suggests ADRB2 agonist supplementation may have an efficacious effect on pulmonary mechanics. Research has shown that ADRB2 agonist supplementation results in increased diaphragmatic myofibril protein concentration in the mouse model. Clinically, this manifested itself in increased diaphragmatic maximal tetanic force production (Smith et al., 2002). ADRB2 treatment has also been shown to increase diaphragm cross-sectional area and myosin heavy chain concentration with a concomitant increase in transdiaphragmatic pressure (Pellegrino et al., 2003; Numata, Suzuki, Miyashita, Suzuki, & Okubo, 1993). These effects have also been shown in other clinical populations with an increase in respiratory strength and endurance in chronic obstructive pulmonary disease following ADRB2 treatment (Nava, Crotti, Gurrieri, Fracchia, & Rampulla, 1992). These data suggest the ADRB2 plays an important role in the regulation and preservation of diaphragm strength and pulmonary capacity. In this study, the only statistically significant interaction between ADRB2 genotype and age was for FEF at the patients' youngest age. Although not statistically significant, there does seem to be a clinically significant difference between genotype

groups at the subjects' youngest age for FVC (9.4 mean difference in percent predicted) and FEV_1 (14.4 mean difference in percent predicted). Therefore, our data suggest patients with the Gly16 polymorphism may have an improved functional pulmonary capacity and that early intervention with an ADRB2 agonist may preserve this capacity. Further research is needed with a larger sample population to increase statistical power to investigate these relationships.

Because chronic ADRB2 stimulation has been shown to alter cardiac electrical stability and increase the propensity for the formation of harmful arrhythmias, ADRB2 agonist treatment is contraindicated in patients suffering from left heart failure (Matera, Martuscelli, & Cazzola, 2010). However, recent research suggests the ADRB2 may have a cardioprotective effect in heart failure. Treatment with ultra-long acting ADRB2 agonist in conjunction with a β_1 adrenergic receptor (ADRB1) antagonist has been shown to decrease infarct size, reduce blood pressure and heart rate, and reverse the decrease in ejection fraction in rat models for heart failure (Rinaldi et al., 2015; Ahmet et al. 2008; 2004). Further, research suggests the phosphorylation of phospholamban (PLB) via PKA, a downstream protein from ADRB2 stimulation, results in the removal of inhibition of SERCA. This increases the quantity and rate of reuptake of cytosolic calcium in the sarcoplasmic reticulum (Haghighi, Gregory, & Kranias, 2004). Recent research also suggests PLB is not sequestered only to the sarcoplasmic reticulum but rather, PLB pools exist in the nuclear envelope which allows them to regulate perinuclear/nuclear calcium handling (Wu et al., 2016). These data suggest the ADRB2 may have a cardioprotective effect, especially in regards to calcium handling, an integral part of the DMD pathology.

However, our data does not suggest this. We were unable to identify a statistically significant difference between genotype groups on any of the cardiac measures at any age, suggesting ADRB2 genotype does not have an influence on the degradation of cardiac measure in DMD.

It has been demonstrated that free C-subunits of PKA diffuse passively into the nucleus, where they have the capability for direct phosphorylation of multiple regulator genes of the cAMP response element binding protein (CREB) (Carlezon, Duman, & Nestler, 2005). CREB is a nuclear transcription factor that is universally expressed and has many processes, including cell proliferation, differentiation, adaptation, and survival (Mayr & Montminy, 2001). Current research suggests CREB plays a role in mediating the activity of the transcription factor myocyte enhancer factor-2 (MEF2), a family of transcription factors that play a key role in the differentiation of muscle cells by triggering the expression of the Ser/Thr kinase salt induced kinase-1 (SIK1), thereby enhancing the promotion of MEF2 target genes (Berdeaux et al., 2007). It has also been demonstrated that CREB interacts with MyoD on the follostatin promotor site, promoting follostatin gene expression, inducing skeletal muscle hypertrophy through myostatin suppression (Gilson etal, 2009). Myostatin is a member of the transforming growth factor-β superfamily and the primary negative regulator of muscle mass (Pearen et al., 2006). ADRB2 activation is also associated with an increased expression of neuron-derived orphan receptor-1 (NOR-1) (Pearen et al., 2006). Pearen et al. (2006) also demonstrated siRNA-mediated inhibition of NOR-1 expression was associated with a significant increase in the levels of myostatin mRNA. This suggest the ADRB2 plays a

functional role in the regulation of muscle mass through increased NOR-1 expression resulting in decreased myostatin levels, promoting skeletal muscle growth. In addition to NOR-1 modulation, ADRB2 activation has been shown to increase expression of peroxisome-activated receptor γ coactivator-1α4 (PGC-1α4), which has been shown to repress the expression of myostatin and the myostatin high-affinity receptor, activin type IIB (Ruas et al., 2012; Lee & McPherron, 2001). Collectively, these data suggest the ADRB2 plays multiple roles in regulating skeletal muscle growth through downstream regulation.

Further, research suggests the $G\alpha_i$-linked Gβγ subunits activate the phosphoinositide 3-kinase-protein kinase-B (PI3K-AKT) signaling pathway (Schmidt, Holsboer, & Spendler, 2001). The PI3K-AKT signaling pathway has been shown to regulate protein synthesis, gene transcription, cell proliferation, and cell survival (Bodine et al., 2001; Glass, 2005). Although there are three distinct isoforms of AKT, the predominant skeletal muscle isoform is AKT1. It has been demonstrated that AKT1 inhibits the forkhead box O transcription factors (FOXO) (Tran, Brunet, Griffith, & Greenberg, 2003). This is significant because FOXO has been implicated in muscle atrophy (Kline, Panaro, Yang, & Bodine, 2007). Thus, by phosphorylating and inactivating FOXO, AKT1 blocks the induction of FOXO-mediated atrophy signaling. Additionally, ADRB2 activation has been found to reduce the expression of FOXO-mediated atrophy signaling in skeletal muscle from denervated and hindlimb-suspended rats (Kline, Panaro, Yang, & Bodine, 2007). This suggests that the ADRB2 plays a

functional role in attenuating skeletal muscle atrophy in addition to promoting skeletal muscle growth.

CHAPTER 6: CONCLUSIONS

Our data suggest patients with the Gly16 polymorphism of the ADRB2 may have improved functional pulmonary capacity. While only FEFmax at the subjects' youngest age demonstrated a statistically significant difference between genotype groups, there was a clear trend for the subjects with the Gly16 polymorphism for an improved FVC and FEV_1 at their youngest age. This difference may be clinically significant for FVC and FEV_1 measures but any difference was lost at the subjects' mid-point age. This suggests early intervention with an ADRB2 agonist treatment may help to preserve this capacity. However, our data does not suggest any influence of ADRB2 genotype on any of the cardiac measures. Considering this lack of statistical evidence and the contraindication of ADRB2 agonist treatment in heart failure, treatment with an ADRB2-specific agonist cannot be advised for DMD. We believe further research is needed with a larger sample population which would provide more statistical power, allowing us to further investigate the influence of ADRB2 genotype on cardiopulmonary measures.

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APPENDIX

Table 1. Subject characteristics (mean ± standard deviation, N or p-value)

Study Demographics

		N	Mean	SD	*p-value*
Age (years)					
	Arg	13	11.9	4.1	0.497
	Gly	6	11.3	3.5	
	Total	19	11.7	3.6	
Height (cm)					
	Arg	13	140.8	18.7	0.844
	Gly	6	143.1	19.7	
	Total	19	141.5	16.4	
Weight (kg)					
	Arg	13	47.7	22.8	0.775
	Gly	6	45.7	19.3	
	Total	19	47.1	12.5	
BMI (kg/m^2)					
	Arg	13	23.6	8.6	0.158
	Gly	6	21.5	5.5	
	Total	19	22.9	7.1	
BSA (m^2)					
	Arg	10	1.3	0.39	0.921
	Gly	6	1.3	0.33	
	Total	16	1.3	0.34	

Arg = genotype (homozygous or heterozygous for ADRB2 resulting in arginine or one arginine and one glycine at amino acid 16), and Gly = genotype (homozygous for

ADRB2 resulting in glycine at amino acid 16). BMI = body mass index; BSA = body surface area. There were no statistically significant differences in demographic data.

Table 2. Body composition measures (mean ± standard deviation, N or p-value)

Body Composition

		N	Mean	SD	*p-value*
L1-L4 (g/cm^2)					
	Arg	10	0.66	0.13	0.692
	Gly	5	0.69	0.09	
	Total	15	0.67	0.12	
TBD (g/cm^2)					
	Arg	10	0.69	0.12	0.331
	Gly	6	0.64	0.01	
	Total	16	0.67	0.09	
LBM (%)					
	Arg	13	62.6	16.1	0.755
	Gly	6	65.5	17.1	
	Total	19	63.7	16.6	
LBM/BSA ($\%/m^2$)					
	Arg	13	56.1	29.6	0.919
	Gly	6	57.8	29.7	
	Total	19	56.8	29.6	

L1-L4 = lower lumbar density; TBD = total body density; LBM = lean body mass; LBM/BSA = lean body mass corrected for body surface area. There were no statistically significant differences in body composition data.

Figure Captions

Figure 1. Comparison of age of loss of ambulation **(A)** [Arg = 4, Gly – 4], and age of start of corticosteroid treatment **(B)** [Arg = 12, Gly = 5].

Figure 2. Comparison of variables of pulmonary function of FVC **(A)** [FVC_{young} = 7 Arg, 4 Gly; FVC_{mid} = 7 Arg, 4 Gly; FVC_{old} = 7 Arg, 4 Gly], FEV_1 **(B)** [FEV_{1young} = 7 Arg, 4 Gly; FEV_{1mid} = 7 Arg, 4 Gly; FEV_{1old} = 7 Arg, 4 Gly], FEF **(C)** [FEF_{young} = 7 Arg, 4 Gly; FEF_{mid} = 7 Arg, 4 Gly; FEF_{old} = 7 Arg, 4 Gly], MIP **(D)** [MIP_{young} = 6 Arg, 2 Gly; MIP_{mid} = 6 Arg, 2 Gly; MIP_{old} = 6 Arg, 2 Gly], and MEP **(E)** [MEP_{young} = 6 Arg, 2 Gly; MEP_{mid} = 6 Arg, 2 Gly; MEP_{old} = 6 Arg, 2 Gly]. * = $p < 0.05$ Arg vs Gly.

Figure 3. Comparison of variables of cardiac function of LVEF **(A)** [$LVEF_{young}$ = 9 Arg, 3 Gly; $LVEF_{mid}$ = 9 Arg, 3 Gly; $LVEF_{old}$ = 9 Arg, 3 Gly], LVED **(B)** [$LVED_{young}$ = 8 Arg, 2 Gly; $LVED_{mid}$ = 8 Arg, 2 Gly; $LVED_{old}$ = 8 Arg, 2 Gly], LVES **(C)** [$LVES_{young}$ = 8 Arg, 2 Gly; $LVES_{mid}$ = 8 Arg, 2 Gly; $LVES_{old}$ = 8 Arg, 2 Gly], LVSF **(D)** [$LVSF_{young}$ = 7 Arg, 2 Gly; $LVSF_{mid}$ = 7 Arg, 2 Gly; $LVSF_{old}$ = 7 Arg, 2 Gly].

Figure 1. Genotype differences in the age of loss of ambulation and start of corticosteroid treatment

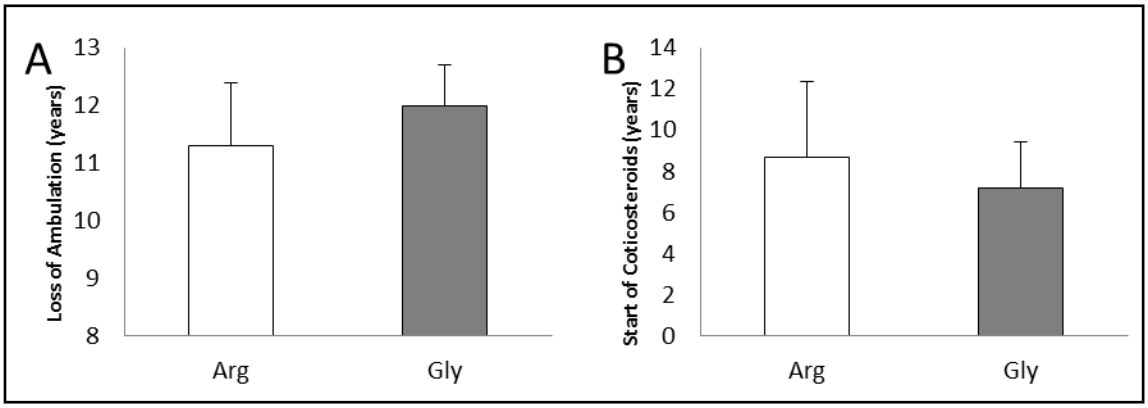
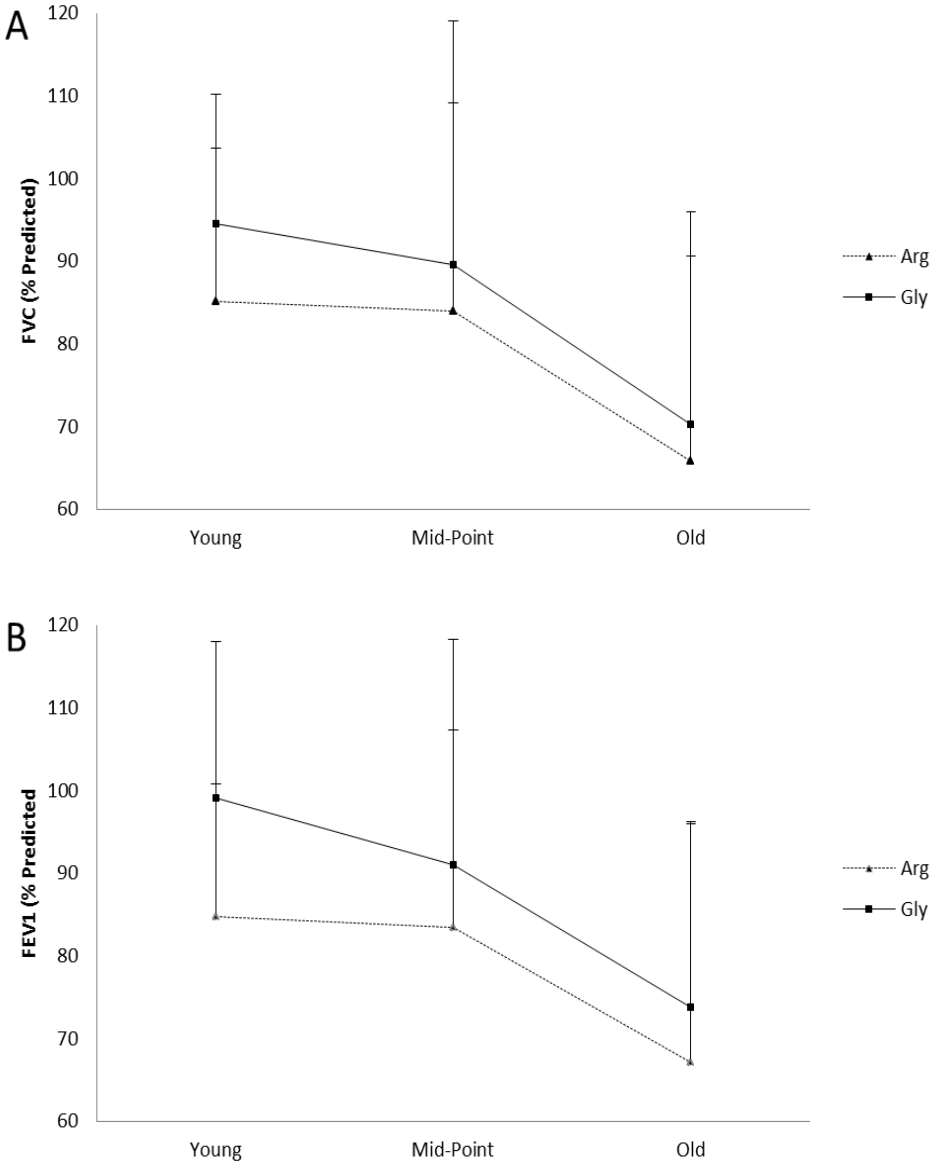
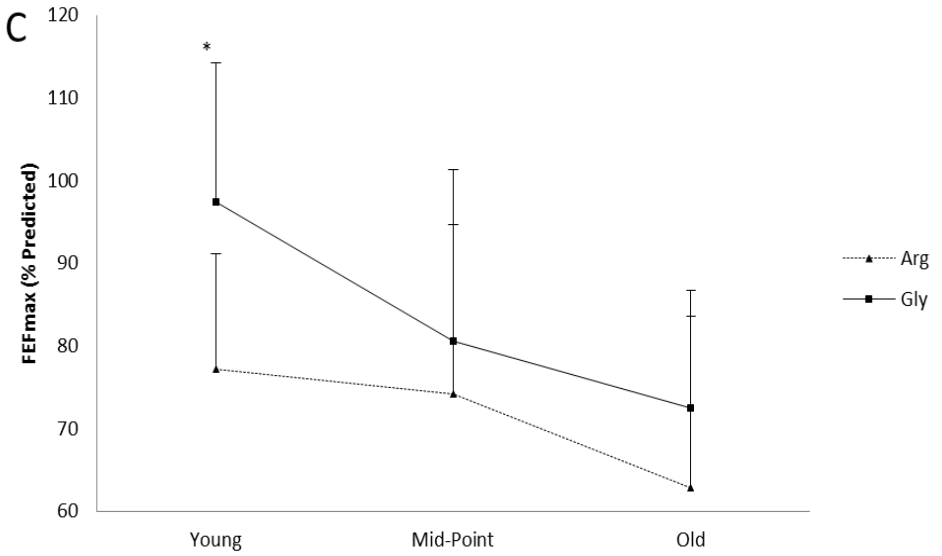
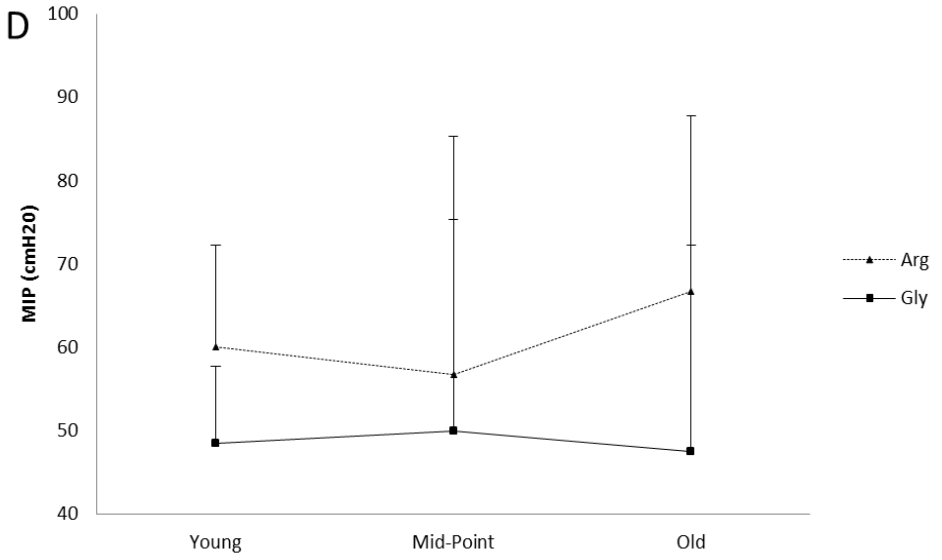


Figure 2. Genotype Comparisons of Pulmonary Function




C
120
110
100
90
80
70
60
FEFmax (% Predicted)
*
Young
Mid-Point
Old
Arg
Gly


D
100
90
80
70
60
50
40
MIP (cmH20)
Young
Mid-Point
Old
Arg
Gly

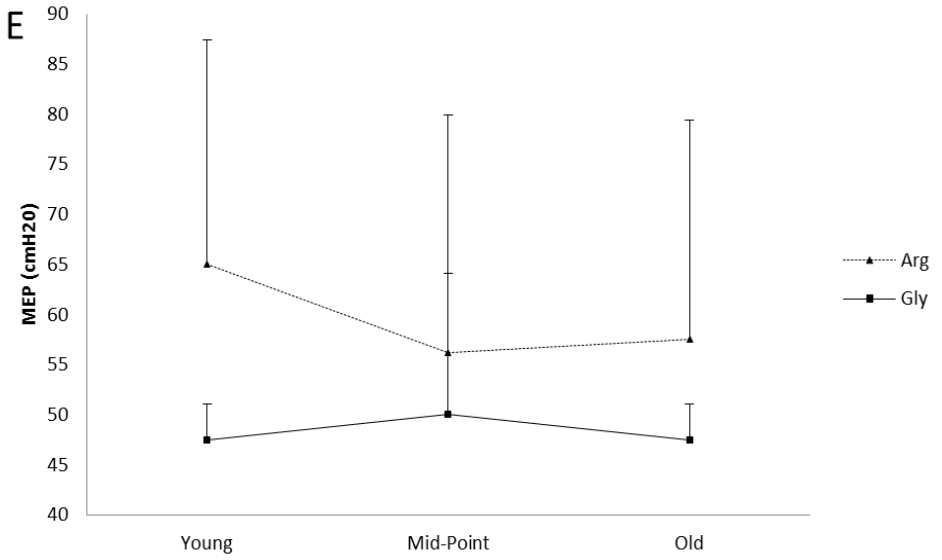
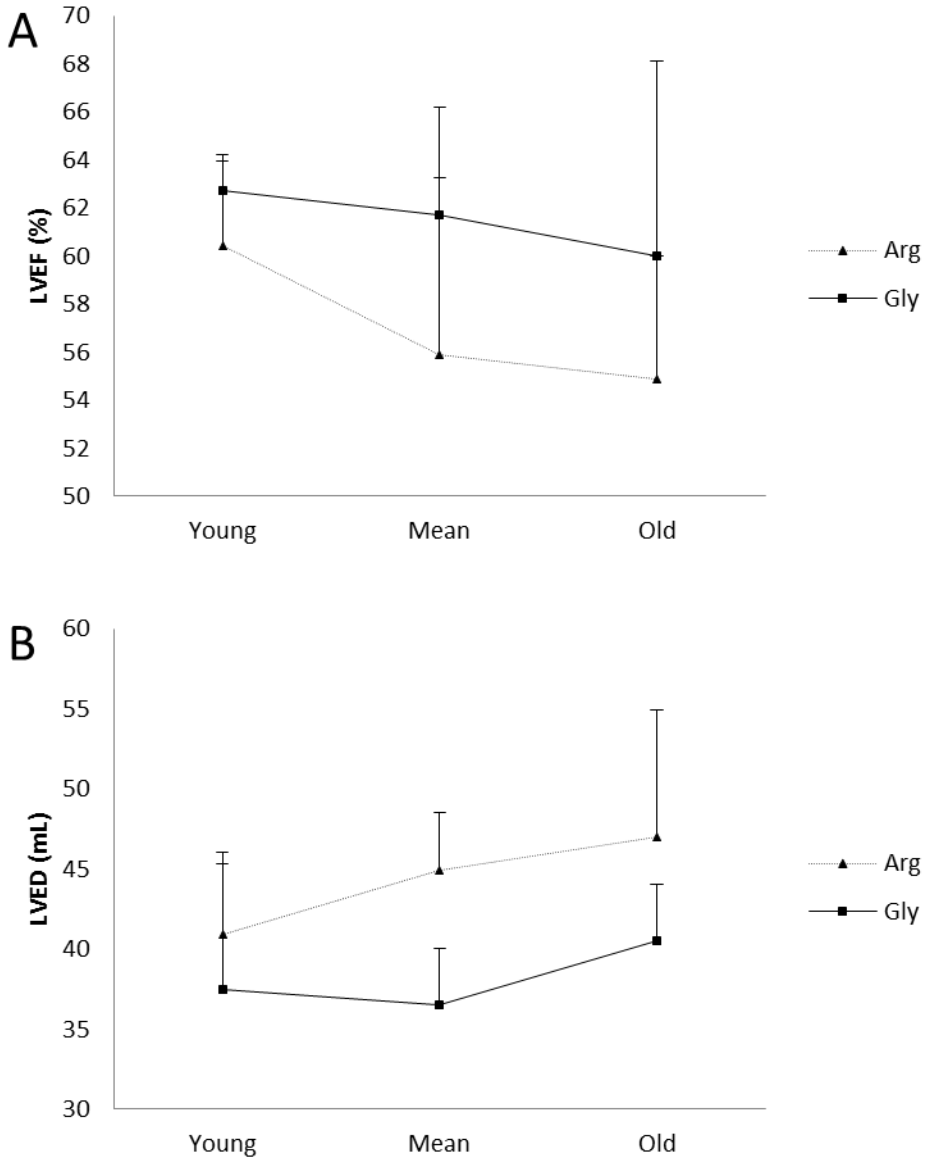
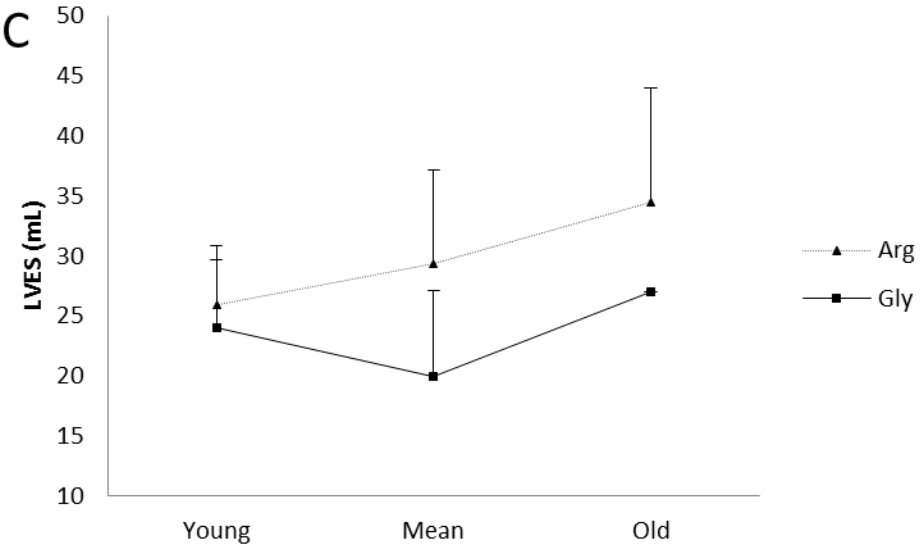

E
90
85
80
75
70
65
60
55
50
45
40
MEP (cmH20)
Young
Mid-Point
Old
Arg
Gly

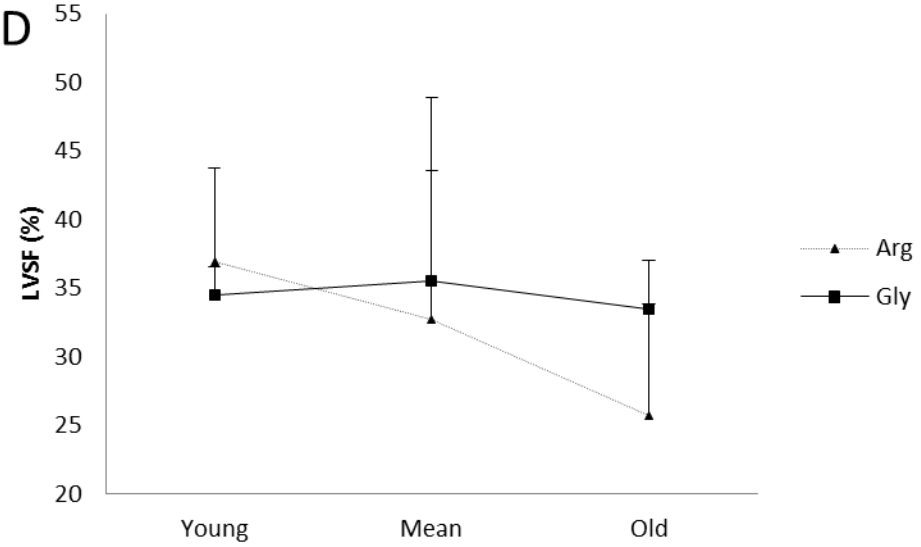
Figure 3. Genotype Comparisons of Cardiac Function



C


50
45
40
35
30
25
20
15
10
LVES (mL)
Young
Mean
Old
Arg
Gly

D


55
50
45
40
35
30
25
20
LVSF (%)
Young
Mean
Old
Arg
Gly