

**THE EFFECTS OF BETA-1 AND -2 ADRENERGIC RECEPTOR GENOTYPES ON  
CARDIOPULMONARY OUTCOMES IN DUCHENNE MUSCULAR DYSTROPHY**

A DISSERTATION  
SUBMITTED TO THE FACULTY OF  
UNIVERSITY OF MINNESOTA  
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS  
FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY

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May 2019

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## **ACKNOWLEDGMENTS**

We are sincerely grateful to the subjects and physicians who were willing to donate their time and efforts to support Duchenne muscular dystrophy research. We would also like to thank the CINRG-DNHS and all associated entities for their work in collecting and making these data available. The following dissertation would not have been possible without the generous help and guidance of Dr. Eric Snyder and the help of Dr. Troy Cross.

## ABSTRACT

**Introduction:**

The main contributor of mortality in Duchenne muscular dystrophy (DMD) patients is cardiorespiratory failure. The beta-1 adrenergic receptor (ADRB1) has been shown to play a functional role in cardiomyocyte function with ADRB1 stimulation increasing cardiac rate, contractility, and work. Multiple polymorphisms of the ADRB1 have been identified such as the Gly49 polymorphism that includes at least one glycine (Gly) for serine (Ser) substitution at amino acid 49 resulting in either homozygous for Gly (Gly49Gly) or heterozygous (Gly49Ser) polymorphisms and a Gly for Arg substitution at amino acid 389 resulting in either homozygous for Gly (Gly389) or heterozygous (Arg389). Heart failure patients with these polymorphisms (Gly49 and Arg389) have been shown to have improved cardiac function and decreased mortality risk. Furthermore, the beta-2 adrenergic receptor (ADRB2) has been shown to influence respiratory muscle strength and function. Multiple polymorphisms of the ADRB2 have been identified as including a glycine (Gly) for arginine (Arg) substitution at amino acid 16. The Gly16 polymorphism has been shown to have higher receptor density on lymphocytes, be more resistant to receptor downregulation, and functionally demonstrate improved respiratory function when compared with the Arg16 genotype in humans.

**Purpose:**

The purpose of this dissertation was to assess the functional consequences of ADRB1 genotypes on cardiac function and ADRB2 genotypes on pulmonary function in patients with DMD. We hypothesize DMD patients with the Gly389 polymorphism would have a lower incidence of cardiac events compared with those expressing Arg389 polymorphism and that DMD patients with the Gly16 polymorphism would have a reduced risk of nocturnal ventilation (NV) use at any given age compared with those patients expressing the Arg16 polymorphism.

**Methods:**

For study 1, we performed genotyping of the ADRB1 (amino acid 49) and high-intensity, steady-state exercise on 71 healthy subjects (Ser49Ser = 52, Gly49Ser = 19). For study 2, we performed genotyping of the ADRB2 (amino acid 16) and high-intensity, steady-state exercise on 77 healthy subjects (AA = 18, AG = 25, GG = 34). Data from CINRG-DNHS including 175 DMD patients (ages 3-25 yrs) with up to 9.7 years follow-up were analyzed focusing on ADRB1 and ADRB2 functional variants for studies 3 and 4. We performed Cox proportional hazard and Kaplan-Meier time to event analyses for the age of NV use and the age of cardiac outcomes and interventions.

**Results:**

There were no differences between ADRB1 genotype groups in age, height, weight, BMI, or watts achieved in the healthy patients. Additionally, there were no differences between genotype groups for cardiac output (CO), systolic blood pressure ( $BP_{sys}$ ), or diastolic blood pressure ( $BP_{dias}$ ) at rest, maximal exercise, or in change from rest to maximal exercise. There were, however, differences between genotype groups for resting CI and SVR and for HR at peak exercise ( $HR_{max}$ ) with the Gly49Ser genotype presenting improved CI and a lower SVR at rest, and a higher HR at peak exercise. There was a

trend towards significance ( $p = 0.058$ ) for the change in stroke volume from rest to peak exercise ( $\Delta SV$ ) with the Ser49Ser genotype demonstrating a larger change in SV. There were no differences between ADRB2 genotype groups in age, height, weight, or BMI in the healthy patients. The genotype groups differed significantly in watts, and watts/ $VO_2$  with heavy exercise with the Gly16 genotype achieving higher workloads. There was a trend towards significance ( $p=0.058$ ) for watts/kg. There were no differences between ADRB1 genotype groups in age, height, weight, number of ambulatory patients, or age of loss of ambulation in our DMD cohort. The Arg389 polymorphism demonstrated a higher mean corticosteroid use compared with the Gly389 polymorphism. The genotype groups differed significantly ( $P<0.05$ ) in the risk of diuretics use with the Gly389 polymorphism demonstrating a 5.01-fold increased risk of diuretics use at any age compared with the Arg389 polymorphism. There were no differences between ADRB2 genotype groups in age, height, weight, corticosteroid use, number of ambulatory patients, or age of loss of ambulation in our DMD cohort. The Gly16 polymorphism demonstrated a higher probability ( $P<0.05$ ) for the use of NV assistance at any given age compared with the Arg16 polymorphism. The genotype groups differed significantly in the risk of NV use with the Gly16 polymorphism demonstrating a 2.77-fold increased risk of using NV at any given age compared with the Arg16 polymorphism.

**Conclusion:**

These data suggest genetic variation in the ADRB1 gene may influence the age of diuretics use in DMD patients with DMD patients expressing the Gly389 polymorphism being more likely to use diuretics compared with patients expressing the Arg389 polymorphism. Additionally, these data suggest genetic variation in the ADRB2 gene may also influence the age of NV use in DMD patients. Specifically, DMD patients expressing the Gly16 polymorphism were more likely to use NV at any given age compared with patients expressing the Arg16 polymorphism.

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## LIST OF ABBREVIATIONS

AC.....	Adenylyl cyclase
ACE.....	Angiotensin-converting enzyme
ADRB1.....	$\beta_1$ adrenergic receptor
ADRB2.....	$\beta_2$ adrenergic receptor
Akt.....	Protein kinase B
AMI.....	Acute myocardial infarction
ARG.....	Arginine
BB.....	Beta-blocker
BP.....	Blood pressure
$\beta$ -AR.....	$\beta$ -adrenergic receptor
CaMK.....	$\text{Ca}^{2+}$ /calmodulin dependent protein kinase
cAMP.....	Adenosine 3'-5' monophosphate
CDK2.....	Cyclin dependent kinase 2
CDK4.....	Cyclin dependent kinase 4
CFTR.....	Cystic fibrosis transmembrane conductance regulator
CREB.....	cAMP response element binding protein
CSQ.....	Calsequestrin
DAGC.....	Dystrophin-associated glycoprotein complex
DMD.....	Duchenne muscular dystrophy
ECHO.....	Echocardiogram
ECG.....	Electrocardiogram
ENaC.....	Epithelial $\text{Na}^+$ channels
ESC.....	European society of cardiology
FEF.....	Forced expiratory flow
FEF <sub>25-75</sub> .....	Forced expiratory flow from 25-75% of forced vital capacity
FEF <sub>50</sub> .....	Forced expiratory flow at 50% of forced vital capacity
FEF <sub>max</sub> .....	Maximal forced expiratory flow
FEV <sub>1</sub> .....	Forced expiratory flow at one second
FEV <sub>1</sub> /FVC.....	Forced expiratory volume in 1 second over forced vital capacity

FOXO.....	Forkhead box O transcription factors
FVC.....	Forced vital capacity
GLY.....	Glycine
GTP.....	Guanine triphosphate
HF.....	Heart failure
IC.....	Inspiratory capacity
IGF.....	Insulin-like growth factor
IL-6.....	Interleukin-6
IL-1 $\beta$ .....	Interleukin-1 $\beta$
IP <sub>3</sub> .....	Inositol 1,4,5-trisphosphate
MAP.....	Mean arterial blood pressure
MAPK.....	Mitogen activated protein kinase
MEF2.....	Myocyte enhancer factor-2
MEP.....	Maximal expiratory pressure
MIP.....	Maximal inspiratory pressure
MLC.....	Myosin light chain
MLCK.....	Myosin light chain kinase
NF $\kappa$ B.....	Nuclear factor- $\kappa$ B
NOR-1.....	Neuron-derived orphan receptor-1
NOS.....	Nitric oxide synthase
NV.....	Nocturnal ventilation
PCF.....	Peak cough flow
Pdi.....	Esophageal balloon transdiaphragmatic
PGC-1 $\alpha$ 4.....	Peroxisome-activated receptor $\gamma$ coactivator-1 $\alpha$ 4
PI3K-Akt.....	Phosphoinositide 3-kinase-protein kinase-B
PKA.....	Protein kinase A
PLB.....	Phospholamban
PLC.....	Phospholipase C
RPE.....	Rating of perceived exertion
SAR.....	Sarcalumenin

SDB.....	Sleep disordered breathing
SERCA1.....	Sarcoplasmic/endoplasmic-reticulum $\text{Ca}^{2+}$ -ATPase 1
SIK1.....	Ser/Thr kinase salt induced kinase-1
sPLA2.....	Phospholipase A2
SV.....	Stroke volume
TGF- $\beta$ .....	Transforming growth factor
TNF- $\alpha$ .....	Tumor necrosis factor- $\alpha$
TV.....	Tidal volume
VC.....	Vital capacity

## **CHAPTER 1: INTRODUCTION**

Duchenne muscular dystrophy (DMD) is the most commonly inherited X-linked disease with a 1 in 3500 birthrate incidence in males [1]. DMD is characterized by progressive muscle weakness as a result of degeneration of skeletal, cardiac, and respiratory muscles, and infiltration of fibrofatty deposits. These mechanisms all contribute to a functional decrease in the contractile ability of muscles [2]. Progressive muscle weakness and degeneration typically confines DMD patients to wheelchairs by age twelve [1]. In patients with DMD, death resulting from cardiopulmonary failure associated with dilated cardiomyopathy and restrictive pulmonary disease typically occurs in one's mid to late-twenties [1].

Major clinical milestones in DMD patients include adverse cardiac events and the use of cardiac interventions. Additional important clinical milestones include the loss of sufficient respiratory pressure generation leading to the emergence of sleep disordered breathing (SDB) and nocturnal ventilation (NV) use [3, 4]. With respiratory treatment for DMD improving and patients living longer, the implications of cardiomyopathies in DMD is becoming more clinically relevant [5]. In fact, an estimated 20% of DMD patients will die of congestive heart failure (HF) as a result of left ventricular failure [6]. In fact, left ventricular dysfunction is a strong predictor of mortality in patients with DMD [7]. Subclinical cardiomyopathies associated with DMD, typically left ventricular failure, are first evident at ten years of age and are present in almost all DMD patients over the age of 18 [5, 8]. However, approximately 70% of patients with DMD remain asymptomatic at cardiomyopathy diagnosis [9]. Given the current clinical guidelines to delay cardiomyopathy treatment until the emergence of symptoms, cardiomyopathy in

DMD patients remains less often treated at diagnosis than other dilated cardiomyopathies, allowing for further cardiac progression [9].. Of particular clinical importance is the concomitant decline in respiratory and cardiac function in DMD patients — this linear decline may arise from interdependent or independent pathologies [4, 10, 11]. The relationship between reduced cardiac function and reduced respiratory function and all-cause mortality suggests the incidence of adverse cardiac events and age of cardiomyopathy diagnosis are important clinical milestones for DMD patients.

The mechanism of cardiac involvement in DMD is similar to that of skeletal muscle in the disease. This includes a loss of muscular integrity, fiber necrosis, and replacement of contractile tissue with fibrotic tissue or fat [12]. The fibrotic region in cardiomyocytes will gradually stretch, become thinner and lose contractility, resulting in cardiac dilation [13]. Clinically, this cardiac dilation presents as increased ventricular volume, decreased ejection fraction, and decreased systolic function [12, 13]. 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 [13]. The systolic dysfunction associated with DMD can be attributed to two main mechanisms: increased inflammatory response and altered  $\text{Ca}^{2+}$  handling [12-14].

The inflammatory response, initiated by cardiomyocyte cell death in patients with DMD, results in significant fibrofatty depositions in cardiac muscles [12]. These fibrofatty depositions appear initially in the left ventricular wall behind the posterior mitral valve leaflet and progressively spreads to affect the entire ventricle [13]. As noted

above, the fibrotic region will gradually stretch, become thinner and lose contractility, resulting in dilation, reduced contractility, and disruption of conduction pathways secondary to fibrosis, are the primary contributors to cardiomyopathy in patients with DMD [13].

Furthermore, in patients with DMD, dystrophin-deficiency disrupts the function of membrane ion channels, and as a result of impaired sarcolemmal integrity, sarcolemmal stretch-activated channels are particularly affected [14]. When dystrophin-deficient cardiomyocytes stretch during ventricular filling, stretch-activated channels do not open appropriately, resulting in increased  $\text{Ca}^{2+}$  flux [13]. These resulting  $\text{Ca}^{2+}$  influxes will activate calpains,  $\text{Ca}^{2+}$ -dependent, non-lysosomal proteases, a significant component of the dystrophic pathway, which further compromises cardiomyocyte contractile ability [13]. Calpain-mediated damage of membrane proteins allows additional  $\text{Ca}^{2+}$  loading; this chronic  $\text{Ca}^{2+}$  loading leads to further cardiomyocyte damage [13]. This  $\text{Ca}^{2+}$  leak into the cardiomyocyte can also modulate L-type  $\text{Ca}^{2+}$  channels, causing unnecessary contractions and further  $\text{Ca}^{2+}$  loading [14]. In addition, dystrophin-deficient cardiomyocytes exhibit a reduction in the  $\text{Ca}^{2+}$  binding proteins [15]. This suggests that in addition to increased  $\text{Ca}^{2+}$  loading in cardiomyocytes, the cardiomyocyte's ability to handle  $\text{Ca}^{2+}$  is also dysfunctional, further disrupting  $\text{Ca}^{2+}$  homeostasis. The altered  $\text{Ca}^{2+}$  homeostasis in DMD affected cardiomyocytes contributes significantly to the cardiomyopathy in DMD patients.

One pathway that has been identified as capable of preserving cardiac function in patients with DMD is the  $\beta_1$ -adrenergic receptor (ADRB1) coupled pathway [16-20]. The

ADRB1 subtype is found primarily in the heart, comprising about 80% of total beta adrenergic receptors in the heart and playing a role in cardiac function [16, 21-23]. Furthermore, ADRB1 activity has been shown to influence cardiac: (i) inotropy, lusitropy, and chronotropy [24, 25]; (ii) nuclear and perinuclear  $\text{Ca}^{2+}$  handling [26]; (iii) reuptake of cytosolic  $\text{Ca}^{2+}$  [27]; and (iv) cardiomyocyte relaxation [22, 28]. Given the above mentioned mechanisms, the ADRB1 may play a role in preserving cardiac function in patients with DMD.

A polymorphism of the ADRB1 that affects functionality has been identified as a glycine (Gly) for arginine (Arg) substitution at amino acid 389 [16, 17, 22, 23]. Specifically, the Gly389 polymorphism demonstrates: decreased receptor density, cAMP accumulation, and a dampening response to norepinephrine infusion [16, 22, 23]. Functionally, HF patients with the Gly389 polymorphism have significantly lower diastolic, systolic, and mean arterial blood pressure — contributing to improved cardiac function and decreased mortality risk [17-19, 29]. Furthermore, literature demonstrates autoantibodies against ADRB1 are associated with more favorable myocardial recovery in patients with recent-onset cardiomyopathy [20]. The association between the Gly389 polymorphism and more favorable cardiac measures and outcomes in HF patients suggest a therapeutic target for preserving cardiac function and delaying cardiomyopathy in patients with DMD. However, to date, there is no literature investigating the relationship between ADRB1 genotype and cardiac events in DMD patients.

As mentioned previously, another major clinical milestone in DMD is the use of ventilatory assistance, particularly NV [3]. Sleep-disordered breathing (SBD) tends to

precede daytime hypoventilation in DMD and has been associated with significant increases in cardiac morbidity and neurocognitive deficits [3, 30]. Research has shown a correlation between lung function and SDB. DMD patients with nighttime hypoventilation present significantly worse lung function than normocapnic patients [31]. The negative consequences of nighttime hypoventilation support the need for early and effective NV intervention. As the DMD pathology progresses, respiratory muscles will weaken to the point of constant alveolar hypoventilation in DMD and the need for either part- or full-time daytime invasive or noninvasive ventilation is indicated [32]. The need for ventilatory assistance, whether that is NV, part, or full-time daytime ventilation, is considered an important respiratory event in the clinical analysis of disease severity in DMD.

Ventilatory assistance in DMD is indicated by progressive respiratory muscle weakness. Clinically, this respiratory muscle weakness in DMD is characterized by a progressive loss of inspiratory pressures, slow and forced vital capacities, forced expiratory volume at one second ( $FEV_1$ ), forced expiratory flow (FEF), and peak cough flow (PCF) [33, 34]. Decreases in pressure and flow generation in DMD can be attributed to diaphragm remodeling [33]. This remodeling in DMD is characterized by an increased resting diaphragm thickness (pseudo-hypertrophy) and decreased contractility [33]. An increase in resting diaphragm thickness decreases mechanical contractility in two ways. First, increased thickness due to non-contractile properties alters the length tension relationship; second, this increase in thickness may also decrease the mechanical advantage of the diaphragm. [35]. Further, DMD patients have shown an increase in

diaphragmatic tension-time index (a measure of the diaphragm's load capacity) after age 14, suggesting a higher likelihood of diaphragm fatigue [36]. This is due in part to the diaphragmatic remodeling present in this population. These data suggest diaphragmatic remodeling and the resultant weakness is an important contributor to respiratory muscle weakness in DMD.

Diaphragmatic fiber necrosis has been shown to be a major contributor to the DMD respiratory pathology [33, 35, 36]. Diaphragmatic cell death results in the formation of scar tissue and fibrosis in the diaphragm. Fibrotic tissue formation is coupled with the infiltration of connective tissues as well as fat deposits and results in diaphragm remodeling [33]. Fiber necrosis and diaphragmatic remodeling in DMD can be attributed to three primary mechanisms: decreased muciliary clearance, increased inflammatory response, and increased proteolytic pathway activation.

Respiratory muscle weakness in DMD has been associated with impaired mucociliary clearance and an increase in inflammatory response [34]. The ability for mucociliary clearance is important in the DMD pathophysiology due to their impaired respiratory secretion clearance and increased susceptibility to pulmonary infections [34]. Impaired mucociliary clearance is associated with an increased inflammatory response, fibrosis, and hospitalization due to pulmonary complications [37]. The impaired ability to clear inhaled particulates in DMD is primarily due to decreased PCF and results in particulate settling in the small airways [38]. This particulate settling stimulates an inflammatory cascade and is associated with increased levels of circulating interleukin-6

(IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) [39]. Furthermore, ineffective clearance has been shown to hasten the onset of respiratory failure in neuromuscular disorders [37].

The upregulation of proteolytic pathways also contribute significantly to respiratory muscle weakness in DMD, particularly Ca<sup>2+</sup>-modulated pathways. Decreased sarcolemmal integrity, mechanical cell membrane tears, and Ca<sup>2+</sup> channel dysregulation associated with DMD have been shown to result in increased Ca<sup>2+</sup> flux and calpain concentration and activity [40]. Calpains initiate myofibrillar protein degradation which decreases myofibrillar protein integrity [40]. The increased activation of these pathways is considered a primary contributor to muscle degradation and fiber necrosis in DMD [41]. Muscle fiber necrosis, as a result of impaired mucociliary clearance, increased inflammatory responses, and increased proteolytic activity, is a major contributor to the diaphragmatic remodeling present in DMD.

Due to the progressive nature of respiratory muscle weakness in DMD, it is imperative that target pathways are identified to slow respiratory muscle degradation. One novel pathway that has been identified as capable of combatting muscle degradation is the  $\beta_2$ -adrenergic receptor (ADRB2) coupled pathway.  $\beta_2$ -adrenergic receptors play a functional role in muscle size, strength and muscle regeneration [42-44]. Additionally, ADRB2 knockout mice had an increased inflammatory response to damage, particularly macrophage migration, a significant component of the dystrophic pathway in skeletal, cardiac, and respiratory muscles [43]. Furthermore, ADRB2 stimulation has also been shown to: (i) increase diaphragmatic cross-sectional area, strength, and contractility [43, 45, 46], (ii) improve mucociliary clearance [47-52], (iii) inhibit inflammatory pathways

[53-61], and (iv) to directly and indirectly inhibit calpain activity and concentration [62-69]. These data suggest the ADRB2 may play a significant role in the protection of respiratory muscles from the dystrophic pathways and provide a novel therapeutic target in DMD.

Given that both cardiac and respiratory function are significant contributors to mortality in DMD patients, we sought to investigate the influence of ADRB1 and ADRB2 genotype on clinically derived cardiorespiratory outcomes, specifically the risk of adverse cardiac events, cardiac medication intervention, and NV use in these patients. Considering the reduction in cardiac function over time in patients with DMD, and that the ADRB1 Gly389 polymorphism is associated with improved cardiac function and favorable outcomes in HF patients, we hypothesized DMD patients with the Gly389 polymorphism would have a lower risk of cardiac events when compared with DMD patients expressing the Arg389 polymorphism. Furthermore, given that ADRB2 stimulation has been shown to: increase diaphragmatic contractility [43, 45, 46], improve mucociliary clearance [47-52], inhibit inflammatory pathways [53-61], and inhibit calpain activity [62-69], we hypothesized DMD patients with the more functional (Gly16) polymorphism would have an increased time to NV compared with DMD patients with the less functional (Arg16) polymorphism.

### ***1.1 Aims and Hypotheses***

*Study 1. Influence of Beta-1 Adrenergic Receptor Genotype on Cardiovascular Response to Exercise in Healthy Subjects*

**Aim 1:** To examine the influence of ADRB1 genotype on cardiac response to exercise in healthy patients.

**Related Hypotheses:** Given the positive influence of the Gly49 polymorphism of the ADRB1, we hypothesize that healthy patients expressing the Gly49 polymorphism would have decreased cardiac output, stroke volume, and blood pressure at maximal exercise compared with patients with the Ser49 polymorphism. Furthermore, we hypothesize that healthy patients expressing the Gly49 polymorphism would have decreased cardiac index, heart rate, and systemic vascular resistance at maximal exercise compared with patients with the Ser49 polymorphism.

*Study 2. Beta-2 Adrenergic Receptor Genotype Influences Power Output in Healthy Subjects*

Running Title: ADRB2 Variants Influence Power Output in Healthy Subjects.

**Aim 1:** To examine the influence of ADRB2 genotype on power output in healthy subjects.

**Related Hypotheses:** Given the positive influence of the Gly16 polymorphism on skeletal muscle strength and function, we hypothesize that healthy patients expressing the Gly16 polymorphism would have increased absolute and relative power output compared with patients expressing the Arg16 polymorphism. Further, healthy patients expressing the Gly16 polymorphism would report decreased indices of relative and perceived intensity at a given workload compared with patients expressing the Arg16 polymorphism.

*Study 3.* Influence of Beta-1 Adrenergic Receptor Genotype on Incidence of Cardiac Events in Patients with Duchenne Muscular Dystrophy.

**Aim 1:** To examine the influence of ADRB1 genotype on the risk of adverse cardiac events in DMD patients.

**Aim 2:** To examine the influence of ADRB1 genotype on the risk of cardiac medication intervention in DMD patients.

**Related Hypotheses:** Given the positive influence of the Gly389 polymorphism of the ADRB1 on cardiac outcomes in HF patients, we hypothesize DMD patients expressing the Gly389 polymorphism would have a lower risk of cardiac events when compared with patients expressing the Arg389 polymorphism. Additionally, we hypothesize those DMD patients expressing the Gly389 polymorphism would have a lower risk of cardiac medication intervention compared with patients expressing the Arg389 polymorphism.

*Study 4.* Influence of  $\beta_2$  Adrenergic Receptor Genotype on Risk of Nocturnal Ventilation in Patients with Duchenne Muscular Dystrophy.

**Aim 1:** To examine the influence of ADRB2 genotype on the risk of NV use at any given age in patients with DMD.

**Related Hypothesis:** Given the positive influence of the Gly16 polymorphism of the ADRB2 on respiratory strength and function, we hypothesize that DMD patients expressing the Gly16 polymorphism would have a lower risk of NV use at any given age when compared with patients expressing the Arg16 polymorphism.

## **CHAPTER 2: LITERATURE REVIEW**

## 2.1 Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is an X-linked, inheritable disease with a 1 in 3500 male-birthrate, making it the most common lethal X-linked disease [1, 70]. DMD is characterized by progressive muscle weakness and degeneration of skeletal, cardiac and respiratory muscles. Muscle degeneration is accompanied by infiltration of fatty acid and bone mineral deposits, rendering the affected muscle functionally compromised [2]. 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, progresses proximally to distally, and typically confines patients to wheelchairs by age twelve [70]. Death results from cardiopulmonary failure associated with dilated cardiomyopathy and restrictive pulmonary disease typically occurring in the late teens to mid-twenties [70].

DMD is caused by a deletion of the dystrophin gene that encodes for the dystrophin protein. Dystrophin acts as a membrane stabilizing protein by linking cytoskeletal proteins to sarcolemmal glycoproteins to form the dystrophin-associated glycoprotein complex (DAGC) [2]. This functional link between muscle fiber cytoskeletal proteins, most notably actin, to the sarcolemmal glycoproteins, allows for the distribution of forces associated with myofibril contraction across the sarcolemma, limiting mechanical stress [2]. Normal contractions in healthy muscle cause myofibrillar micro tears and an influx of  $\text{Ca}^{2+}$  that activates degenerative pathways via calpains. Calpains, comprised of two main isoforms m-calpain and  $\mu$ -calpain, tend to be

concentrated on the Z band of the sarcomere where protein disassembly begins [71]. 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 [40].

Due to the lack of dystrophin in DMD, the  $\text{Ca}^{2+}$  influx to the intracellular space is augmented by the lack of DAGC and a resulting loss of selective permeability to  $\text{Ca}^{2+}$  in the cellular membrane. Additionally, repeated injurious events eventually exhaust the regenerative capacity of dystrophic muscles. Infiltration of adipose and connective tissue ensues leading to progressive functional impairments in affected patients [2]. Effectively, DMD renders muscle fibers fragile and susceptible to damage during contractions.

## 2.2 DMD and Skeletal Muscle

In DMD, not only is the functional link between the DAGC and actin compromised due to dystrophin deficiency, the proteins that comprise the DAGC are also greatly affected [72]. This reduction in DAGC proteins augments the distribution of contractile forces across the sarcolemma, rendering the sarcolemma more prone to mechanical tears. These tears result in increased membrane permeability and  $\text{Ca}^{2+}$  influx due to a loss of myofibril integrity during contraction and relaxation [73]. The DAGC also plays a role in  $\text{Ca}^{2+}$  channel stabilization, whereby the deformation of the DAGC due to dystrophin deficiency results in  $\text{Ca}^{2+}$  channel dysfunction [74]. In addition to mechanical tears,  $\text{Ca}^{2+}$  channel dysfunction is also believed to be a source of  $\text{Ca}^{2+}$  homeostasis dysregulation [74]. This increased  $\text{Ca}^{2+}$  influx results in the activation of

calpains, a  $\text{Ca}^{2+}$  dependent ubiquitous-proteasome, which degrades muscle protein. In addition to the activation of calpains,  $\text{Ca}^{2+}$  flux into the mitochondria can also lead to swelling and bursting of the mitochondria; mitochondrial death is followed by cell death and necrosis [1]. Following cell death and necrosis, muscle cells are infiltrated by macrophages and, due to decreased membrane stabilization, fibrofatty tissue and  $\text{Ca}^{2+}$  deposits [1]. This macrophage and fibrofatty infiltration results in muscle weakness, loss of muscular control, and loss of ambulatory abilities. This process of protein degradation and loss of functional strength seems to preferentially affect fast twitch muscle fibers [75].

In addition to  $\text{Ca}^{2+}$  channel dysfunction, research suggests there is an altered protein expression that may further increase cytosolic  $\text{Ca}^{2+}$  concentrations. Research has demonstrated the concentration and activity of key  $\text{Ca}^{2+}$ -binding and shuttling proteins play a critical role in  $\text{Ca}^{2+}$  homeostasis dysfunction in the dystrophic pathway [76]. In mdx mice (a mouse model presenting with the DMD pathology) luminal sarcalumenin (SAR), the key  $\text{Ca}^{2+}$ -shuttling protein of the sarcoplasmic reticulum, has been shown to be decreased by as much as 70% compared to healthy controls [76]. Research has also demonstrated a decreased concentration of calsequestrin (CSQ), the main  $\text{Ca}^{2+}$ -binding protein in the sarcoplasmic reticulum, in mdx mice compared to healthy controls [76]. This research also shows a mechanical linkage between SAR and the sarcoplasmic/endoplasmic-reticulum  $\text{Ca}^{2+}$ -ATPase 1 (SERCA1). However, dystrophic membrane preparations have not shown a relative decrease in SERCA1 [76]. Additionally, impaired  $\text{Ca}^{2+}$ -shuttling between SERCA units and CSQ clusters via SAR

and decreased luminal  $\text{Ca}^{2+}$ -binding capacity may amplify cytosolic  $\text{Ca}^{2+}$  levels. 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  $\text{Ca}^{2+}$  flux, it may also render the muscles ischemic during exercise [77]. Neuronal nitric oxide synthase (NOS) is localized to the sarcolemma as part of the DAGC and the loss of sarcolemmal integrity results in a loss of NOS [72, 78]. Specifically, without dystrophin to anchor the DAGC, NOS disassociates from the sarcolemma and is no longer functional. During exercise, nitric oxide has been shown to play a role in blunting exercise-induced vasoconstriction due to  $\alpha$ -adrenergic receptor activation [79]. This protective mechanism is defective in DMD resulting in unopposed vascular vasoconstriction during exercise further contributing to the pathogenesis of DMD [79, 80].

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. In DMD, the ability to regenerate skeletal muscle is surpassed by the muscle cell degradation pathways in DMD. 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 [79, 80]. 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 [2].

### 2.3 DMD and the Heart

With recent advances in DMD treatment, patients are living longer, resulting in cardiac involvement becoming more prevalent [5]. It is believed the mechanism of cardiac involvement in DMD is similar to that of skeletal muscle — loss of integrity, fiber necrosis, and replacement of contractile tissue with connective tissue or fat [12]. Similar to skeletal muscle, loss of DAGC stability results in a loss of membrane stability and  $\text{Ca}^{2+}$  channel dysfunction [81]. 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 [14]. When cardiomyocytes with augmented dystrophin levels stretch during ventricular filling, the stretch-activated channels do not open appropriately, leading to an increased influx of  $\text{Ca}^{2+}$  [14]. These resulting  $\text{Ca}^{2+}$  influxes will activate calpains which degrades troponin I and compromises cardiomyocyte contractile ability. Further, this calpain-mediated damage of membrane proteins allows additional  $\text{Ca}^{2+}$  influx; this chronic  $\text{Ca}^{2+}$  loading leads to cardiomyocyte necrosis [13]. Additionally,  $\text{Ca}^{2+}$  leak into the cardiomyocyte can modulate L-type  $\text{Ca}^{2+}$  channels, causing unnecessary contractions and further  $\text{Ca}^{2+}$  loading which contributes to cell death [14]. Similarly to skeletal muscles, DMD affected cardiomyocytes exhibit a reduction in the  $\text{Ca}^{2+}$ -binding proteins, namely CSQ [15]. This suggests that in addition to increased  $\text{Ca}^{2+}$  loading in cardiomyocytes, their ability to handle  $\text{Ca}^{2+}$  is also dysfunctional, further disrupting  $\text{Ca}^{2+}$  homeostasis.

Cardiomyocyte cell death initiates an inflammatory response wherein macrophages migrate to the damaged area to clear the cell of debris. This process is

followed by fibroblast invasion of the damaged area and the formation of scar tissue and fibrosis in the heart [12]. 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 [13]. Cardiac dilation 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 [13].

Subclinical cardiomyopathies associated with DMD are first evident at ten years of age and are present in all DMD patients over the age of 18 [5]. Cardiomyopathy, most typically left ventricular failure, is very prevalent in DMD patients and is the cause of mortality in an estimated 20% of this population [5, 6]. 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 [7]. After a 76 month follow-up period, they concluded that 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.

## 2.4 Cardiac Diagnosis in DMD

Assessment of cardiac function should be initiated at six years of age and occur at least once every two years until the age of 10. Annual complete assessments should begin at the age of 10 or at the onset of cardiac signs and symptoms if they occur earlier [82]. Clinically, these assessments include electrocardiograms (ECG) and echocardiograms (ECHO). Continual ECG screenings may help to identify and diagnose cardiomyopathies that may need further screening or treatment [82]. The prevalence of ECG abnormalities is common. Early DMD can be characterized by multiple ECG changes including tachycardia. This tachycardia presents itself secondarily to a reduced ejection fraction caused by fibrosis and a dilated cardiomyopathy to maintain cardiac output [83]. Another early ECG change is a large R/S ratio in V1 and large Q waves in leads I, II and V5 which is likely caused by the dilated cardiomyopathy and the loss of conductive cardiac cells due to fibrosis. These ECG abnormalities are present with a decreased excursion of the left ventricular posterior wall and interventricular septum as well as a decreased rate of ventricular relaxation [83].

As DMD progresses, ECG abnormalities become more prevalent. One common ECG change is a short PR interval, affecting about 50% of patients [1, 70]. This may be due to Lown-Ganong-Levine syndrome caused by a pre-excitation of the ventricles from augmented L-type  $\text{Ca}^{2+}$  channels or an accessory pathway. Conversely, research has suggested this shortened PR interval may be due to a compensatory autonomic influence rather than pre-excitation [84]. While typically this is not a dangerous abnormality, it may degrade into ventricular fibrillation, leading to sudden cardiac death. Another

common ECG change is a prolonged QTc interval ( $\geq 450$  ms). As DMD in the cardiomyocytes is associated with fibrosis-induced conduction problems, as well as cardiomyocyte dilation, especially in the ventricles, the QTc interval is commonly lengthened suggesting a decreased relaxation time of the ventricles. Additionally, it is not uncommon to see prominent Q waves in leads I, II, III, aVL, aVF, V5 or V6 [84]. This is mainly due to the dilation of the left and right ventricles caused by pulmonary hypertension and increased preload and afterload, resulting in a stretching of the fibrotic cardiomyocyte. This may also be due to irregular conduction patterns caused by fibrotic regions [1, 70]. Further, ST segment depressions, defined as  $>0.5$ mm, are common ECG changes associated with DMD. The cause of this is similar to myocardial infarction, where there is a partial or full loss of conductive cardiac tissue associated with an increase in the fibrotic region [85]. Interestingly, Thrush et al (2009) did not find ECG changes to be determinant of dilated cardiomyopathy; ECG changes were similar in DMD patients with or without dilated cardiomyopathy [85].

With advanced DMD fibrosis, serious arrhythmias, can be detected by an ECG. These include atrial fibrillation, atrioventricular block, ventricular tachycardia and ventricular fibrillation [13]. These are believed to be caused by advanced dilated cardiomyopathy and the associated decreased ejection fraction as well as irregular conductions due to fibrosis. Additionally, postmortem studies determined selective scarring at the posterobasal region of the heart resulting in a loss of posteriorly directed forces and creating electrically silent regions [86]. Such rhythms can be lethal and are a major cause of cardiac arrest and death in DMD.

These ECG changes can help professionals to not only diagnose cardiomyopathies, but to also determine their severity. Fayssoil, Nardi, and Orlikowski (2009) determined mean QRS duration was  $117 \pm 29$  ms in DMD patients with ejection fraction greater than 35% whereas mean QRS duration was  $98 \pm 17$  ms in DMD patients with ejection fraction less than 35% [87]. However, once a cardiomyopathy is determined via an ECG, further screening is needed to determine the full extent of the damage and the effect on cardiac hemodynamics.

If an ECG abnormality suggesting a cardiomyopathy is determined in screening, it is recommended that the patient has an ECHO done to further determine the damage and the resulting cardiac decompensation [86]. One major concern with DMD is left ventricular dysfunction resulting in cardiac failure. This dysfunction manifests itself as a diminished contractile excursion of the left ventricular posterior wall and interventricular septum accompanied by a decreased rate of relaxation of the left ventricular posterior wall [86]. This presents itself mechanically as a diminished change in left ventricular diameter from diastole to systole and a reduced ejection fraction. Additionally, an ECHO may determine a decreased rate of circumferential fiber shortening and reduced maximal systolic velocity [83]. Again, this ventricular dysfunction is due to myocardial dilation and fibrosis resulting in diminished contractile ability. The resultant loss in ejection fraction is especially dangerous for DMD as left ventricular dysfunction is a major predictor of mortality.

Further, Fayssoil et al (2009) sought to quantify ECHO changes associated with DMD [87]. They determined mean indexed left ventricular end diastolic diameter was 30

mm/m<sup>2</sup>, which is far below normal, suggesting severe left ventricular dysfunction. This augmented diameter was accompanied by a severely attenuated ejection fraction ranging from 10 to 62% with almost a quarter of their patients have an ejection fraction of less than 35%. Additionally, Fayssoil et al (2009) found interventricular asynchrony in 12% of their patients with an ejection fraction of less than 35% and only 2.6% of patients with an ejection fraction more than 35% [87]. This suggests that, as the disease progresses and the left ventricle becomes more dilated and fibrotic, patients not only lose the ability to eject blood, but their ventricles fall out of synchrony. This may further attenuate left ventricular systolic function.

## **2.5 Cardiac Management in DMD**

As improvements have been made in the respiratory management in DMD, cardiomyopathy has become an increasing contributor to mortality. Progressive left ventricular dysfunction is inevitable in patients with DMD. However, the onset of cardiomyopathy is highly variable, with the Pediatric Cardiomyopathy Registry Study Group reporting ages ranging from seven to 27 years of age at onset with a mean age of  $14.8 \pm 4.6$  years [12]. Possible factors influencing the age of cardiomyopathy onset may include modifier genes, age and duration of corticosteroid treatment, and use of nocturnal ventilatory support [12]. While corticosteroid use has been demonstrated effective in preserving skeletal muscle strength and function in DMD, it remains unclear what effect corticosteroids truly have on the prevention or progression of cardiomyopathy in DMD patients. Research has demonstrated corticosteroid use to be cardioprotective, with one

study reporting Kaplan–Meier freedom from ventricular dysfunction was 93% for steroid-treated cases versus 53% for untreated cases at 1500 days of treatment [88]. However, multiple studies have contradicted this, reporting corticosteroid use to negatively affect the progression of cardiomyopathy in patients with DMD [89, 90].

While there are contradicting views on the effect of corticosteroid use on cardiomyopathy in DMD, multiple studies have demonstrated the efficacy of angiotensin-converting enzyme (ACE) inhibitors. According to the European Society of Cardiology (ESC) guidelines, angiotensin-converting enzyme inhibitors are recommended as a first-line treatment for DMD patients with reduced left ventricular systolic function [91]. The decreased afterload associated with ACE inhibitors is believed to be the primary contributor to this beneficial effect. Duboc and colleagues (2007) randomly assigned 29 DMD patients to a perindopril treated group and 28 patients to a placebo group for 3 years, followed by open label treatment for up to 10 years [92]. At the 10 year follow up, survival for treated patients was 92.9% and for untreated was 65.5%. Angiotensin-converting enzyme inhibitors have also been demonstrated to be an effective treatment for normalizing shortening fraction [93]. According to the ESC, beta-blockers (BB) are indicated for patients with symptomatic, stable systolic function [91]. The addition of BB therapy to ACE inhibitors has been shown to be beneficial in DMD patients' long term survival (assessed at 5 to 7 year follow-up) when they present with left ventricular dysfunction [94]. Additionally, DMD patients treated with an ACE inhibitor with or without the addition of a BB were shown to have improved cardiomyopathy at a six to 12

months follow up, compared to the pretherapy group [95]. These data suggest an ACE inhibitor and BB can delay cardiomyopathy in DMD patients.

Furthermore, the American College of Cardiology and American Heart Association have long recommended the use of diuretics in the treatment of HF with reduced left ventricular function [96]. Consequently, the American Academy of Pediatrics recommends considerations to be given to the use of diuretics in the treatment of cardiomyopathy in DMD patients [97]. Current literature suggests the use of diuretics to treat tachycardia and lipothymia in later stages of cardiac involvement in DMD patients [98]. Despite the efficacy of diuretic therapy in the treatment of HF and recommendations for diuretic considerations in DMD patients, clinical adoption is slow [99].

Unfortunately, cardiac management in DMD patients is reactive rather than proactive. This treatment paradigm must change as the deleterious effects of cardiac decompensation are present before symptoms arise [84]. Additionally, cardiac management strategies for patients with DMD are highly variable and remain underutilized in this population [9, 100]. Currently, there is no clinical consensus regarding the proper timing and type of pharmacological therapy intervention in DMD patients [101]. Further complicating this is the fact that symptoms of cardiomyopathy in DMD are often masked by respiratory dysfunction [101]. As cardiomyopathies become increasingly contributory to DMD mortality, additional prospective studies are warranted to evaluate the benefit of ACE inhibitors, BBs, aldosterone antagonists, or other transforming growth factor- $\beta$  inhibitors in DMD. Furthermore, additional investigations

looking for other early indicators of cardiomyopathies can help guide therapy before the development of overt ventricular dysfunction should be conducted.

## 2.6 DMD and the Lungs

Respiratory muscle weakness tends to exhibit itself in the second decade of life and is the major contributor to mortality in DMD [35]. DMD associated respiratory impairment is the result of deterioration and necrosis of the diaphragm, which has similar contractile properties of skeletal muscle and also involves DAGC dysregulation [102, 103]. Similar to skeletal muscle, the loss of dystrophin to stabilize the DAGC results in reduced membrane integrity,  $\text{Ca}^{2+}$  channel dysfunction, and reduced expression of  $\text{Ca}^{2+}$ -handling proteins [102]. The implications of diaphragmatic deterioration are important in DMD because this is the primary muscle responsible for respiration. Similar to skeletal muscle deterioration, it is believed the resultant  $\text{Ca}^{2+}$  flux from either  $\text{Ca}^{2+}$  channel dysregulation or mechanical tears in the sarcolemma are the main contributors to diaphragm muscle cell death [104]. Unlike skeletal muscle, mdx diaphragm strips have revealed regucalcin as the main  $\text{Ca}^{2+}$ -dependent protein involved in diaphragmatic  $\text{Ca}^{2+}$  dysregulation. Regucalcin, a cytosolic  $\text{Ca}^{2+}$ -binding protein involved in intracellular signaling is the main  $\text{Ca}^{2+}$ -binding protein in the liver. However, research has identified regucalcin of 33.9 kDa and pI5.2 also exists in the diaphragm. Similar to the reduction of SAR and CSQ in affected skeletal muscle, there is a 2-fold reduction of regucalcin in mdx diaphragm [105]. This reduction results in disturbed intracellular signaling due to abnormal handling of cytosolic  $\text{Ca}^{2+}$ , an insufficient maintenance of  $\text{Ca}^{2+}$  homeostasis,

and abnormal regulation of  $\text{Ca}^{2+}$ -dependent enzymes [105]. This suggests that  $\text{Ca}^{2+}$  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 in patients with DMD [33]. DMD patients have also been shown to have compromised lung function and volumes, including reduced forced expiratory volume at one second ( $\text{FEV}_1$ ), forced expiratory flow (FEF), and forced vital capacity (FVC) when compared to healthy, non-DMD patients [33]. Decreases in pressure and flow generation in DMD can be attributed to diaphragm remodeling [33]. This remodeling in DMD is characterized by an increased resting diaphragm thickness (pseudo-hypertrophy) and decreased contractility resulting from two primary mechanisms. First, diaphragm cell death results in an inflammatory response wherein macrophages migrate to the damaged area to clear the cell of debris. Macrophage migration is followed by fibroblast invasion of the damaged area and the formation of scar tissue and fibrosis in the diaphragm. Second, infiltration of connective tissues as well as fat deposits has been shown to increase resting diaphragm thickness and decrease its contractility [33].

An increase in resting diaphragm thickness decreases mechanical contractility in patients with DMD in two ways. First, increased thickness due to non-contractile properties alters the length tension relationship; second, this increase in thickness may also decrease the mechanical advantage of the diaphragm [35]. Accordingly, research suggests *mdx* mice present a significant increase in the extracellular matrix protein

collagen in the diaphragm [106]. Furthermore, DMD patients have shown an increase in diaphragmatic tension-time index after age 14, suggesting a higher likelihood of fatigue [35]. 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. The diaphragm remodeling present in patients with DMD is also associated with the loss of sarcomeres in series and an almost 900-fold increase in stiffness [107]. In addition to increased fatigue, DMD patients have demonstrated a decrease in gastric pressure with cough, suggesting the onset and advancement of respiratory muscle weakness [36].

## **2.7 Respiratory Diagnosis in DMD**

The American Thoracic Society recommends that clinics treating DMD patients be capable of performing pulmonary function tests (PFT), capnography, peak cough flow, maximal inspiratory and expiratory pressures, pulse oximetry, and arterial blood gas sampling and analysis for diagnostic testing [82, 108-110]. The most common clinical means of evaluating and diagnosing respiratory muscle weakness in DMD are PFTs. These tests consist of the patient breathing into a mouthpiece in a sitting position to prevent the risk of fall; patients are advised to not smoke for at least one hour before testing, not eat a large meal two hours before testing, and not wear tight fitting clothing as these may adversely affect testing [111]. PFTs consist of three types of measurements. The first measurements

are flows, maximal forced expiratory flow ( $FEF_{max}$ ), forced expiratory flow from 25-75% of forced vital capacity ( $FEF_{25-75}$ ), and forced expiratory flow at 50% of forced vital capacity ( $FEF_{50}$ ), which may be useful in diagnosing and monitoring obstructive airway disease but have shown to not significantly contribute to clinical decision making [112, 113]. The second types of measurements consist of assessment of lung volumes, vital capacity (VC) and tidal volume (TV), and can be useful in the diagnosing and monitoring of restrictive lung disease. The third set of measurements are volume against time measurements, forced expiratory flow in 1 second ( $FEV_1$ ), FVC, and forced expiratory volume in 1 second over forced vital capacity ( $FEV_1/FVC$ ); these tests can be useful in the diagnosing and monitoring of both obstructive and restrictive lung diseases [113]. These measurements can be useful in the monitoring of respiratory decline in DMD as these patients have been shown to have compromised  $FEV_1$ ,  $FEF$ , and FVC [33].

Capnographs are used to measure partial pressures of  $CO_2$  in respiratory gases. They work on the principle that  $CO_2$  absorbs infrared radiation and that an infrared light passing through  $CO_2$  results in a reduction in the amount of light falling on a sensor [114]. It is recommended that capnography and pulse oximetry are performed during the course of a neuromuscular disease to detect respiratory insufficiency and can be a sensitive measurement of respiratory impairment even when artificial ventilation is being used [115]. This is important in DMD patients because as respiratory muscles weaken, they become more susceptible to nocturnal hypercapnia and the proper use of inspiratory and expiratory aids can prolong survival and decrease pulmonary morbidity [37, 116].

In addition to monitoring pulmonary gas tensions, the evaluation of peak cough flow (PCF) is extremely important in DMD. This is done using a peak flow meter and pneumotachograph to measure peak flow during a maximal expiration against a closed glottis [117]. As the respiratory muscles weaken, DMD patients lose the ability to adequately produce pressures for airway clearance and it is suggested that PCF should be measured when FVC < 2.1 L or FEV<sub>1</sub> < 2.1 L/sec so that techniques to assist in mucociliary clearance can be effectively used [118]. Furthermore, PCF has been correlated to maximal inspiratory pressure (MIP) and maximal expiratory pressure (MEP) in DMD patients and could be a useful measure to evaluate respiratory muscle weakness [119].

Arterial blood gas sampling is often used in conjunction with capnography to determine CO<sub>2</sub> clearance and blood gas tension which may be predictors of sleep hypoventilation in patients with DMD. This may be useful in determining nocturnal hypercapnia [120]. Additionally, arterial blood gas samples can be used to determine the effectiveness of nocturnal ventilatory assistance, which can improve treatment and prolong life [121].

One effective measurement of respiratory muscle strength, of the diaphragm in particular, is esophageal balloon transdiaphragmatic (Pdi) measures; however, it is not often used clinically due to its invasive nature. This test is done via catheter placement through either the nose or mouth to measure esophageal and gastric pressures and it is believed esophageal balloon measures of diaphragmatic strength can provide a better understanding of respiratory function than some traditional measurements [122]. Additionally, research suggests sniff Pdi pressure measures are more reproducible than

MIP and MEP, providing more accurate monitoring of maximal pressure tests [123]. Pdi can also be conducted during hypoxia, hypercapnia, chemical airway stimulation, spontaneously occurring breaths, sustained airway or tracheal occlusion, and maximal efforts produced by phrenic nerve stimulation, demonstrating its functionality as a measure [124]. Further, Pdi measurements make it feasible to individualize the level of muscle effort during mechanical ventilation [125]. With so many respiratory function tests available, it is important to note that individual tests of inspiratory, expiratory, and diaphragm muscle function tend to over diagnose respiratory muscle weakness. It is recommended to use a combination of tests to improve diagnostic precision to determine disease progression and treatment [126].

## **2.8 Respiratory Management in DMD**

A majority of DMD patients die due to respiratory failure and that respiratory muscle weakness tends to exhibit itself in the second decade of life [35]. DMD associated respiratory impairment is a result of deterioration and necrosis of the diaphragm [102, 103]. The implications of diaphragmatic deterioration are of importance in DMD because this is the primary muscular organ responsible for respiration and production of cough pressures.

The management of respiratory muscle weakness in patients with DMD is dependent on disease progression. One of the first steps of respiratory management in DMD patients is glucocorticoid treatment while ambulatory which is often continued after loss of ambulation [127]. The goal of glucocorticoid treatment is the preservation of

skeletal muscle strength and delaying the decline in respiratory and cardiac function [128, 129]. Further, steroid-treated DMD patients have exhibited an improved PCF and MEP compared to non-steroid treated DMD patients, with one study citing PCF values to be 27 L/min higher in steroid-treated patients with DMD [109, 130]. Prednisone and deflazacort treatment is believed to work similarly in reducing respiratory function decline in patients with DMD [131, 132].

As the pathology of DMD progresses, patients lose diaphragm contractility. This is important in DMD because the pulmonary system is the body's first line of defense against infection; it is the only place where the inside of the body interfaces with microbes and particulates from the environment. Inhaled particulates tend to settle in the small airways as air velocity slows [38]; this settling stimulates an inflammatory cascade and is associated with increased levels of circulating interleukin-6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) [39]. Further, an increase in circulating IL-6 has been correlated to pulmonary hypertension [133, 134]. In addition to IL-6, TNF- $\alpha$  is another important pulmonary cytokine associated with inflammation and has been shown to modulate cardiac remodeling [135, 136].

Ineffective clearance has been shown to hasten the onset of respiratory failure; however, early detection and intervention can prevent hospitalization due to pneumonia [37, 116]. As mentioned previously, in children with DMD PCF should be measured when FVC < 2.1 L or FEV<sub>1</sub> < 2.1 L/sec [118]. The assessment of cough effectiveness can be done using MIP, MEP, PCF, inspiratory capacity, or vital capacity. However, PCF is preferred as it has been shown to correlate directly to the effectiveness to clear airways

[137]. While PCF values below 160 L/min have been shown ineffective for mucociliary clearance, values above 160 L/min may not be adequate either as respiratory function deteriorates during infection [37, 116, 138]. Therefore, a PCF of 270 L/min has been recommended to identify DMD patients who could benefit from assisted cough techniques [116]. The ability to generate adequate flow for clearance has been correlated to MEPs of 60 cm H<sub>2</sub>O and is absent below 45 cm H<sub>2</sub>O [139]. Pulse oximetry can also be used to determine changes in gas diffusion associated with lower airway complications and as a screening tool to increase cough therapy [116]. This demonstrates the importance of using multiple respiratory function tests to evaluate clearance effectiveness.

When respiratory pressures deteriorate to the point that cough assistance is indicated, several cough assisting methods have been proposed for patients with respiratory muscle weakness including: manual cough assistance, mechanical cough assistance, functional electrical stimulation of respiratory muscles, and respiratory muscle training [140-142]. Manual assisted coughing involves inspiratory assistance followed by augmentation of the expiratory effort. This can be done with air stacking, increasing inspiratory capacity (IC) with glossopharyngeal breathing, positive pressure breathing with a bag and mask, and an intermittent positive pressure breathing device [110]. Mechanical assisted cough techniques involve simulating a cough by applying a positive pressure breath followed by a negative pressure exsufflation. This technique has been shown effective in clearing airways in DMD patients with inadequate cough pressures [110].

Another method of respiratory management for DMD patients is respiratory muscle training. Maximum insufflation capacity, the maximal air volume that can be held against a closed glottis, has been shown to improve in response to air stacking training in patients with DMD. This can be employed to improve the range of motion of the lung and chest wall and may help in assisted cough by increasing lung volume [141, 143]. The aim of respiratory muscle training is to improve respiratory muscle strength, thereby preserving function. This could be beneficial in DMD patients as MEP and MIP have both been correlated to cough effectiveness and preserving these functions could prove useful in prolonging life [143]. However, the effectiveness of this method in doing so is not clear [144, 145]. 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 [72, 78]. During exercise, nitric oxide has been shown to play a role in blunting exercise-induced vasoconstriction due to  $\alpha$ -adrenergic receptor activation; this protective mechanism is defective in DMD resulting in unopposed vascular vasoconstriction during exercise which further contributes to the pathogenesis of DMD [79, 80]. This defective mechanism may contraindicate respiratory muscle training in DMD.

As respiratory muscle weakness progresses, DMD patients may present with SDB [146]. SDB has also been associated with significant increases in cardiac morbidity and neurocognitive deficits [30]. In healthy individuals during sleep, there is a dampening in the slope of the ventilatory response to both hypercapnia and hypoxia, as well as reduction in tonic activity of the intercostal muscles [147, 148]. However, due to

functional declines in diaphragm strength, the intercostal muscles must share in the generation of inspiratory pressures in DMD patients and in the face of reduced tonic activity of the intercostal muscles during sleep, the diaphragm may not be able to generate sufficient pressures for adequate ventilation. Research has shown a correlation between lung function and SDB with night hypercapnic DMD patients presenting significantly worse lung function than normocapnic patients [31]. Further, it has been demonstrated that arterial blood gases should be measured when FEV<sub>1</sub> falls below 40% and that screening for SDB should be considered when Pa<sub>CO<sub>2</sub></sub> is greater than 45 mm Hg [120]. Once SDB is identified, the best treatment option is a mechanical ventilator which has been shown effective in the treatment of SDB in DMD patients [149]. Treatment with non-invasive NV has been shown to improve sleep, improve day-time gas exchange, and slow the decline of respiratory function [150]. It is also important to note that NV and assisted cough techniques have been shown to prolong life in DMD [151-153].

As the disease progresses, respiratory muscles will weaken to the point of constant hypoventilation in DMD patients and the need for either daytime invasive or noninvasive ventilation is indicated. The most common type of ventilation is an intermittent positive pressure mouth piece which has been used successfully in patients with an FVC of 0.6 L for up to eight years [32]. Another method used is glossopharyngeal breathing, using oral muscles to inhale a small bolus of air until the patient reaches TV. This can be a useful technique to learn in the event of ventilator failure [154]. If patients show an aversion to the mouth piece ventilator, a pneumo-belt can be used. This involves a belt placed around the abdomen to create negative pressures

and drive inspiration [110]. In patients without the oromotor or neck control to use a mouthpiece, a tracheostomy is recommended. The benefits of this method are that it can provide higher ventilatory pressures and airway suctioning during an infection [138]. However, a tracheostomy tube may increase pulmonary secretions, make swallowing more difficult, increase risk of aspiration, and increase bypassing of airway defenses which increases risk of infection [155].

## **2.9 Beta-Adrenergic Receptor Structure and Function**

The  $\beta$ -adrenergic receptors ( $\beta$ -AR) are part of a family of membrane proteins known as G-protein coupled receptors which, upon binding of a catecholamine to the receptor, stimulate a conformational change in the  $\beta$ -AR that causes coupling with G-proteins. G-proteins consist of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits;  $\beta$ -AR coupling leads to the dissociation of the G-protein into active  $G\alpha$  and  $G\beta$  subunits to mediate downstream signaling [156]. Moreover,  $\beta$ -AR agonist binding stimulates the dissociation of the  $G_{\alpha s}$  protein [157, 158].  $G_{\alpha s}$ -bound guanine triphosphate (GTP) then phosphorylates the enzyme adenylyl cyclase (AC). Nine isoforms of AC are known to exist [159]. AC catalyzes the conversion of ATP to adenosine 3'-5' monophosphate (cAMP) which phosphorylates protein kinase A (PKA) into its active form [160]. Both AC and cAMP have the ability to regulate downstream mechanisms via multiple internal cell signaling pathways [161-165]. Conversely,  $\beta$ -AR antagonist binding causes a competitive inhibition against catecholamine and sympathetic nervous stimulation, thereby inhibiting the dissociation of  $G_s$  proteins [166].

### 2.9.1 Beta-Adrenergic Receptor Cardiac Signaling

The ADRB1 subtype is found primarily in the heart and comprises 75-80% of  $\beta$ -AR found in the heart with the ADRB2 subtype comprising the remaining 20-25% [21]. The G-protein signaling pathway associated with the  $\beta$ -AR is important in the modulation of several key target proteins. When activated, cardiomyocyte ADRB1 preferentially binds to the  $G_{as}$  protein which phosphorylates AC, generating the secondary messenger cAMP. Increased cAMP accumulation activates PKA [25]. Activated PKA then phosphorylates troponin I, the L-type  $\text{Ca}^{2+}$  channel, and phospholamban (PLB), increasing cardiac inotropy, chronotropy, and lusitropy [24].

Research has also shown  $G_s$  activation can increase L-type  $\text{Ca}^{2+}$  current directly [166]. L-type  $\text{Ca}^{2+}$  channels play an integral role in cardiomyocyte excitability and contractility [156]. Phosphorylation of cardiac L-type  $\text{Ca}^{2+}$  channels by PKA results in an influx of  $\text{Ca}^{2+}$  into cardiomyocytes. The  $\text{Ca}^{2+}$  then binds to the sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA) triggering further sarcoplasmic  $\text{Ca}^{2+}$  loading resulting in the removal of troponin and tropomyosin inhibition of myosin binding sites [105, 167]. Additionally, research suggests the phosphorylation of phospholamban (PLB) via PKA, a downstream protein from  $\beta$ -AR stimulation, results in the removal of inhibition of SERCA. This increases the quantity and rate of reuptake of cytosolic  $\text{Ca}^{2+}$  in the sarcoplasmic reticulum [27]. 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  $\text{Ca}^{2+}$  handling [26]. Troponin I is a regulatory

protein of cardiac myofibrils and its phosphorylation by PKA inhibits actomyosin ATPase activity resulting in relaxation of cardiomyocytes in response to catecholamines [22, 28]. Research has demonstrated genetic variation of the ADRB1 modulate the cardiac response to catecholamine binding regulating the aforesaid mechanisms of action.

Further, ADRB2 stimulation has been shown to activate the G<sub>i</sub> pathway in cardiomyocytes, depressing cAMP accumulation modulating downstream mechanisms [168, 169]. Gα<sub>i</sub>-linked Gβγ subunits activate the phosphoinositide 3-kinase-protein kinase-B (PI3K-Akt) signaling pathway [170]. The PI3K-Akt signaling pathway has been shown to regulate protein synthesis, gene transcription, cell proliferation, and cell survival [171, 172]. It has been demonstrated that Akt1 inhibits the forkhead box O transcription factors (FOXO) [173]. The activation of this pathway has been shown to have cardioprotective effects in HF [174].

ADRB1 receptors have a high affinity for both epinephrine and norepinephrine while ADRB2 receptors have a high affinity for epinephrine and a low affinity for norepinephrine [175]. In cardiomyocytes, as compared to pulmonary tissue, ADRB1 is distributed much more densely than ADRB2. However, unlike pulmonary tissue, this does not necessarily indicate a large ADRB1 receptor reserve. In fact, research suggests there is no ADRB2 receptor reserve and a possible small ADRB1 receptor reserve for positive ionotropic effects in the heart, suggesting the primary influence of B-AR agonist treatment on cardiac function arises from ADRB1 activity [176-178].

### 2.9.2 Beta-Adrenergic Receptor Pulmonary Signaling

In human lungs, the ratio of ADRB2 to ADRB1 has been shown to be approximately 3:1 [179].  $\beta$ -AR were shown to be widely distributed with a higher density over airway epithelium, alveolar walls, and submucosal glands and a lower density over airway and vascular smooth muscle. Similarly, the  $\beta$ -AR of both large and small airway smooth muscle was comprised entirely of the ADRB2 subtype [179, 180]. Conversely, both  $\beta$ -AR subtypes appear to coexist in bronchial submucosal glands and alveolar walls with ADRB1 accounting for 10% and 30% of  $\beta$ -AR in the submucosal glands and alveoli respectively with ADRB2 accounting for the remainder [179, 181].

One of the most important aspects of  $\beta$ -AR, ADRB2 in particular, in the pulmonary tissue is the regulation of alveolar fluid. The activation of  $\beta$ -AR results in the dissociation of GTP which phosphorylates AC, catalyzing the conversion of ATP to cAMP and the phosphorylation of PKA into its active form [160].  $\beta$ -AR stimulation and the concomitant increase in cAMP is important in lung fluid balance [182]. The ADRB2 are important in lung fluid regulation by the activation of epithelial  $\text{Na}^+$  channels (ENaC) located on the apical membrane of alveolar cells. The stimulation of ENaC has been shown to result in an increase in the number and open probability of ENaC as well as an increase in the likelihood of an interaction with the cystic fibrosis transmembrane conductance regulator (CFTR) [183]. This ENaC-CFTR interaction causes an augmentation in the function of the  $\text{Cl}^-$  channel on the apical membrane of alveolar cells, resulting in the transcellular apical to basolateral movement of salt and an associated osmotic movement of fluid [184]. Further, research suggests ADRB2 stimulation may

play a role in regulating  $\text{Na}^+\text{K}^+$ -ATPase resulting in smooth muscle relaxation of the pulmonary lymphatics, which can augment lung fluid clearance [185].

Additionally,  $\beta$ -AR stimulation is important in the regulation of respiratory smooth muscle reactivity. AC is believed to be the principal effector of  $G_s$ -receptor transmembrane signaling.  $G_{as}$  activation of AC catalyzes ATP to cAMP, which in turn dissociates and activates PKA. PKA then phosphorylates and regulates the activity of numerous proteins, most notably the transcription factor cAMP response element binding protein (CREB) [186]. Research suggests PKA activity is responsible for the majority of intracellular actions including relaxation, inhibition of cell growth, reduced airflow resistance, and ion channel gating [187-189]. Additionally, PKA can phosphorylate certain  $G_q$ -coupled receptors as well as phospholipase C (PLC), thereby inhibiting G protein-coupled receptor-PLC-mediated phosphoinositide (PI) generation, thus reducing  $\text{Ca}^{2+}$  flux. Further, PKA can also phosphorylate the inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) receptor to reduce its affinity for  $\text{IP}_3$  which further limits  $\text{Ca}^{2+}$  flux. Additionally, PKA phosphorylates myosin light chain kinase (MLCK) and decreases its affinity to  $\text{Ca}^{2+}$  calmodulin, hence reducing activity and myosin light chain (MLC) phosphorylation [190, 191]. This downstream regulation via PKA results in smooth airway relaxation and dilation. These mechanisms of action may be of clinical importance in DMD patients by decreasing respiratory load for sufficient alveolar ventilation and by improving mucociliary clearance.

### 2.9.3 Beta-Adrenergic Receptor Vascular Signaling

Research suggests differential distribution of  $\beta$ -AR receptors in smooth muscle with ADRB2 functioning as the primary vasodilator of smooth muscle with ADRB1 predominating and mediating vasodilation only in a few major blood vessels [192].  $\beta$ -AR modulation of smooth muscle is achieved via receptor catecholamine binding and dissociation of the  $G_{\alpha s}$  protein.  $G_{\alpha s}$ -bound GTP then phosphorylates AC, catalyzing the conversion of ATP to cAMP which phosphorylates PKA into its active form [160, 193]. PKA has been shown to phosphorylate and inactivate myosin light chain kinase (MLCK), an enzyme involved in muscle contraction, resulting in smooth muscle relaxation [194]. Research suggests the phosphorylation of PLB via PKA, a downstream protein from  $\beta$ -AR stimulation, results in the removal of inhibition of SERCA1. This increases the quantity and rate of reuptake of cytosolic  $\text{Ca}^{2+}$  in the sarcoplasmic reticulum, further contributing to smooth muscle relaxation [195, 196].

ADRB2 represents a higher receptor density than does ADRB1 and is the predominant  $\beta$ -AR vasodilator in vascular smooth muscle. Research has shown the ADRB2 to have a high receptor reserve in vascular smooth muscle and a high binding affinity to epinephrine [175, 197, 198]. However, due to its low receptor density, ADRB1 likely has little or no receptor reserve in vascular smooth muscle [192]. These mechanisms suggest  $\beta$ -AR receptors may play a functional role in mitigating the aforementioned  $\alpha$ -adrenergic vasoconstriction in DMD patients, thereby improving blood flow to the skeletal, cardiac, and respiratory muscles. An improved blood flow in patients with DMD may reduce the inflammatory response associated with the DMD pathology.

## 2.10 Genetic Variations of the Beta-Adrenergic Receptor

Multiple polymorphisms of the ADRB1 have been identified, including a glycine (Gly) for serine (Ser) substitution at amino acid 49 and a Gly for arginine (Arg) substitution at amino acid 389. Both the Gly49 and Gly389 polymorphisms have been shown to have decreased receptor density, decreased cAMP accumulation, and an improved agonist-promoted downregulation [22, 23]. Heart failure patients with these polymorphisms (Gly49 and Gly389) have also been shown to have improved cardiac function and decreased mortality risk [17, 199, 200]. This suggests the Gly49 and Gly389 polymorphisms may have cardioprotective effects.

Further, 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, 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 healthy and HF patients than Arg16 [187, 201, 202]. These studies suggest a protective effect of the Gly16 polymorphism on cardiopulmonary function.

### 2.10.1 Beta-1 Adrenergic Receptors and Heart Failure

Research has suggested that ADRB1 and ADRB2 play important roles in mediating the progression of cardiomyopathies. Both ADRB1 and ADRB2 tend to be sequestered in cardiomyocyte microdomains close to their downstream targets mediated

by scaffolding of A-kinase anchoring domains [203]. Research has also demonstrated ADRB1 induced cAMP activation occurs throughout the cell, activating both PKA and PLB. Conversely, ADRB2 induced cAMP activation is highly compartmentalized, wherein it activates localized pools of AC and L-type  $\text{Ca}^{2+}$  channels [157]. This suggests that despite receptor concentration differences inherent in cardiomyocytes; these receptors have domain-specific differences in functionality. Furthermore, this suggests the ADRB1 activation has a global effect on cardiac function and is believed to be largely responsible for the cardiotoxic effects of sympathetic stimulation. These cardiotoxic effects are mediated through PKA and  $\text{Ca}^{2+}$ /calmodulin dependent protein kinase (CaMK), increased intracellular  $\text{Ca}^{2+}$  and its downstream proteolytic enzymes, inhibition of the anti-apoptotic effects of protein kinase B (Akt), and phosphorylation of the ryanodine receptor which increases diastolic  $\text{Ca}^{2+}$  leak and arrhythmogenesis [157, 204, 205]. This has been demonstrated in vitro with B1-AR antagonist treatment shown to block catecholamine-induced cardiac apoptosis [206, 207].

The cardiomyopathies associated with DMD are similar to that of HF with the formation and growth of fibrotic tissue in the heart that gradually stretches, become thinner and loses contractility, resulting in an increased ventricular volume, decreased ejection fraction, and decreased systolic function. 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 [13]. Additionally, left ventricular function as determined by an ECHO has been identified as a strong predictor of mortality [7].

As the predominant  $\beta$ -AR in cardiomyocytes, ADRB1 plays a functional role in cardiac function. The Gly49 and Gly389 polymorphisms of the ADRB1 have been associated with negative ionotropy, improved cardiac function, and decreased mortality risk associated with HF [16, 17, 22, 23, 199, 208]. This is likely due to the decrease in cAMP accumulation associated with these polymorphisms. In fact, ADRB1 overexpression in rat HF models has shown even more rapid cardiac deterioration than untreated rats [209, 210]. The influence of the Gly49 and Gly389 polymorphisms has been further supported by a decreased mortality risk and an improvement in left ventricular mass and shape with  $\beta$ 1-AR antagonist treatment [18, 19, 29].

The Gly49 and Gly389 polymorphisms have been shown to have decreased receptor density, decreased cAMP accumulation, and a dampening response to the cardio-stimulant effect of norepinephrine infusion [16, 22, 23]. Additionally, patients with the Gly389 polymorphism have significantly lower diastolic and systolic blood pressure (BP) and mean arterial blood pressure (MAP) [17]. HF patients with these polymorphisms (Gly49 and Gly389) have also been shown to have improved cardiac function and decreased mortality risk [18, 19, 29]. Furthermore, studies have demonstrated autoantibodies against ADRB1 are associated with more favorable myocardial recovery in patients with recent-onset cardiomyopathy [20]. This suggests the Gly49 and Gly389 polymorphisms may have inherent cardioprotective effects similar to that of a BB.

ADRB1 signaling has been shown to play an important role in HF with the degree of sympathetic activity being inversely correlated with survival [211]. Deleterious effects

of ADRB1 signaling include apoptosis, myocyte growth, fibroblast hyperplasia, myopathy, fetal gene induction, and proarrythmia [206, 212]. Interestingly, the Arg389 polymorphism has been shown to preferentially upregulate inflammatory and apoptotic signaling pathways [213]. As an adaptive mechanism in HF, cardiac ADRB1s become less responsive, either downregulating or uncoupling from the G<sub>s</sub> pathway [214]. This suggests the less functional variants of the ADRB1 to be clinically important in HF.

The influence of ADRB1 signaling in HF has been supported by association studies as well, demonstrating a statistically significant association between the Arg389 polymorphism and acute myocardial infarction (AMI), suggesting ADRB1 genotype plays a role in the development of AMI [200]. Specifically, experimental models of HF suggest the Arg389 polymorphism is associated with a greater susceptibility to HF with an approximate 10.11 fold increase in risk [215].

#### 2.10.2 Beta Blockers in DMD

The efficacy of BB therapy has been demonstrated in non-DMD HF patients with long exposure to BB therapy decreasing mortality risk in congestive HF; BB therapy was also independently associated with decreased mortality and rates of rehospitalization in patients with decreased ejection fraction [216, 217]. These findings have also been extended to the DMD patient population. Retrospective analyses have demonstrated improved survival and cardiac parameters in DMD patients with BB use [94, 218]. Specifically, BB treatment resulted in significantly improved left ventricular shortening fraction with no increases in left ventricular end-diastolic dimensions [218]. Additionally,

BB use in DMD patients has been shown to decrease mortality and hospitalizations due to congestive HF [6]. Intervention studies with BB and ACE inhibitor combination therapy was demonstrated to relieve symptoms of cardiomyopathy in DMD patients and resulted in decreased levels of atrial natriuretic protein, brain natriuretic protein, norepinephrine, and left ventricular end-diastolic diameter [219]. Furthermore, this combination therapy increased left ventricular ejection fraction, suggesting the capability to reverse signs and symptoms of congestive HF in DMD patients [219]. Cumulatively, these data suggest early intervention with BB is effective in combatting cardiomyopathy and may delay the progression of cardiomyopathy in DMD [95].

The Gly49 and Gly389 polymorphisms have been shown to have decreased receptor density, decreased cAMP accumulation, and a dampening response to the cardio-stimulant effect of norepinephrine infusion [22, 23]. HF patients with the Gly49 polymorphism have also been shown to have improved cardiac function and decreased mortality risk [18, 19, 29]. Furthermore, studies have demonstrated autoantibodies against ADRB1 are associated with more favorable myocardial recovery in patients with recent-onset cardiomyopathy [20]. This suggests the “less” functional ADRB1 polymorphisms (Gly49 and Gly389) may have inherent cardioprotective effects similar to that of a BB and these polymorphisms may preserve cardiac function in DMD patients.

#### 2.10.3 Beta-2 Adrenergic Receptors and Heart Failure

While B2-AR agonists are typically contraindicated in HF, recent research suggests the ADRB2 may have a cardioprotective effect in HF. Interestingly, research has

demonstrated ADRB2 activation can switch between cardiotoxic and cardioprotective, the latter through Gi and PI3K-Akt pathways [158, 169, 206, 220]. However, recent research suggests the predominating effects of ADRB2 activation are cardioprotective. This has been shown in ADRB2 knockout mice demonstrating enhanced injury when treated with doxorubicin and an increased susceptibility to isoproterenol cardiotoxicity, further supporting the cardioprotective mechanisms of ADRB2 stimulation [221, 222]. Additionally, ADRB2 knockout mice have also demonstrated increased p38 subunits of the mitogen-activated protein kinase (MAPK), resulting in apoptosis, while ADRB2 stimulation inhibits the apoptotic effects of Akt [157, 221]. Furthermore, ADRB2 overexpression has been shown to improve cardiac function in young mice; however, as the mice aged, overexpression resulted in dilated cardiomyopathy [223, 224].

While this may suggest long term exposure to a B2-AR agonist to be detrimental in cardiomyopathies, combination with an ultra-long acting B2-AR agonist in conjunction with a B1-AR 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 HF [225-227]. This type of combination therapy of super-long acting B2-AR agonists and B1-AR -selective antagonists has been shown to reduce ischemic injury after cardiac artery ligation. These studies also suggest the efficacy of this combination therapy in HF surpasses that of the traditional BB and ACE inhibitor therapy [226, 228]. This may be attributed to the ADRB2s capacity to activate the G<sub>i</sub> pathway, which depresses cAMP activity through activation of the G<sub>i</sub>/PI3K/Akt signaling pathway. ADRB2 coupling to G<sub>i</sub> proteins is upregulated in HF with the decrease in ADRB1 receptor density [168, 169].

Further, research suggests the phosphorylation of 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  $\text{Ca}^{2+}$  in the sarcoplasmic reticulum [27]. 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  $\text{Ca}^{2+}$  handling [26]. This suggests a genetic interaction between ADRB1 and ADRB2 for cardioprotective capacity, especially in regards to  $\text{Ca}^{2+}$  handling, an integral part of the DMD pathology.

#### 2.10.4 Beta-2 Adrenergic Receptors and Pulmonary Function

$\beta$ -2 adrenergic receptors have been shown to: (i) play a functional role in diaphragmatic cross-sectional area, strength, and contractility [43, 45, 46]; (ii) improve mucociliary clearance [47-52]; (iii) inhibit inflammatory pathways [53-61]; and (iv) inhibit calpain activity and concentration [62-69].  $\beta$ -2 adrenergic receptors are G-protein coupled receptors whose primary products from catecholamine binding are PKA and cAMP [160]. Both PKA and cAMP have the ability to regulate downstream mechanisms via multiple internal cell signaling pathways [165]. Functionally, the concentrations of these products reflect the density and activity of the ADRB2 suggesting the more functional polymorphism (Gly16) would result in increased PKA and cAMP concentration [43].

One of the main respiratory concerns in DMD is the loss of diaphragmatic contractility due to increased diaphragm fibrosis and pseudo-hypertrophy [33]. The

increase in diaphragm fibrosis in patients with DMD is a function of three main mechanisms: a decrease in mucociliary clearance, an increased inflammatory response, and the upregulation of  $\text{Ca}^{2+}$ -modulated proteolytic pathways [34, 36, 58, 59, 229]. Impaired mucociliary clearance is also associated with increased fibrosis and hospitalization due to pulmonary complications in DMD patients [37]. Ineffective clearance has been shown to upregulate inflammatory pathways and hasten the onset of respiratory failure in neuromuscular disorders [37]. Dystrophin-deficient muscle cells have been shown to have increased  $\text{IL-1}\beta$  levels compared to healthy controls and research suggests significant levels of  $\text{TNF-}\alpha$  were detected in 62% of human DMD tissue biopsies [53, 54, 58, 59]. Furthermore, decreased sarcolemmal integrity, mechanical cell membrane tears, and  $\text{Ca}^{2+}$  channel dysregulation associated with DMD have been shown to result in increased  $\text{Ca}^{2+}$  flux and increased calpain concentration and activity [40]. Calpains –  $\text{Ca}^{2+}$ -dependent, non-lysosomal proteases – initiate myofibrillar protein degradation which decreases myofibrillar protein integrity [40]. The increased activation of these pathways is considered a primary contributor to muscle degradation and fiber necrosis in DMD [41].

B2-AR agonist treatment has been shown to improve airway clearance by 3-4 times in humans and to have anti-inflammatory properties by inhibiting cytokine production including:  $\text{TNF-}\alpha$ ,  $\text{IL-6}$ , and  $\text{IL-1}\beta$  [49, 50, 53, 54]. It has also been demonstrated that B2-AR agonist treatment can increase calpastatin, a calpain-specific inhibitor, and decrease calpain concentration and activity [64, 67-69]. B2-AR agonist supplementation has also been shown to increase diaphragm protein concentration,

maximal tetanic force production, and transdiaphragmatic pressure in the mouse model [45, 46, 229]. Functionally, the increased receptor density and resistance to downregulation associated with the Gly16 polymorphism would result in increased cAMP accumulation, further upregulating these mechanisms [43]. Cumulatively, these data suggest the Gly16 polymorphism may result in: improved mucociliary clearance, decreased inflammatory pathways, inhibition of  $\text{Ca}^{2+}$ -dependent proteolysis, and improved diaphragmatic function in DMD.

#### 2.10.4.1 Beta-2 Adrenergic Receptors and Mucociliary Clearance

The ability for mucociliary clearance is of particular importance in the pathophysiology associated with DMD due to patients' impaired respiratory secretion clearance and susceptibility to pulmonary infections [34, 230]. For an effective cough, one must inspire a large amount of air and apply expiratory force against a closed glottis, generating high thoraco-abdominal pressures. As a result, when the glottis opens, there is a strong expiratory flow [132]. However, in DMD patients this ability is compromised due to the mechanisms described above.

Clinically, mucoactive agents are used to improve airway clearance in DMD patients. These mucoactive agents aim to improve clearance via: (i) reducing the viscosity and elasticity of airway secretions [231]; (ii) increasing cilia beat frequency [232]; and (iii) reducing the adhesivity of airway secretions through surfactant production [232]. However, most of these medications are ineffective at increasing airway water and those that are often also mucus secretagogues, increasing both the volume of mucus

and water in the airways [233]. This could be deleterious as over-hydration has been shown to decrease mucociliary clearance [234].

B2-AR agonist treatment has been shown to act similarly to mucoactive agents by improving airway clearance [50]. Research has shown an up to 5-fold increase in ciliary beat frequency with B2-AR agonist treatment [47, 51]. This increase cilia beat frequency results in an increase in the number of metachronal waves moving across the epithelial and could aid in mucociliary clearance [235]. B2-AR agonist treatment has also been shown to enhance mucus secretion and these secretions were shown to have higher viscosity and lower elasticity, properties that allowed for improved ability for clearance with cough [52, 236]. This suggests that even with impaired respiratory strength, patients with DMD could be more efficient in airway clearance with B2-AR agonist treatment. Additionally, treatment with an B2-AR agonist has been shown to increase the production of surfactant by increasing the incorporation of choline and is effective in restoring phosphatidylcholine to normal levels in bronchoalveolar lavage fluid and lung tissue in septic lungs [237]. Research has also shown ADRB2 stimulation enhances the rate of release of newly synthesized surfactant into the alveoli [238]. This may be of clinical significance in DMD as surfactant improves airway patency, aiding in clearance [48]. Furthermore, the ability of the ADRB2 to regulate lung water via the mechanisms discussed above has been shown to increase mucociliary clearance by 3-4 times after agonist treatment [49]. These data suggest B2-AR agonists may work similarly to mucoactive agents and could be beneficial in improving airway clearance in DMD patients.

The influence of ADRB2 stimulation on improving mucus and lung fluid clearance has also been demonstrated in various disease states. Improved mucus clearance following B2-AR agonist treatment has been demonstrated in asthma and chronic bronchitis [239-241]. Further, ADRB2 genotype has been associated with lung fluid accumulation. Research suggests subjects with the Arg16 polymorphism have a greater susceptibility for lung fluid accumulation than those with the Gly16 polymorphism. The ability of ADRB2 to regulate lung fluid clearance has been demonstrated in disease states as well. B2-AR agonist treatment has been shown effective in lung fluid clearance in HF with pulmonary edema, acute lung injury, and acute respiratory distress syndrome [183, 242, 243].

#### 2.10.4.2 Beta-2 Adrenergic Receptors and Inflammation

ADRB2s have also been shown to play a functional role in inflammatory responses. B2-AR agonists have anti-inflammatory properties by inhibiting cytokine production, TNF- $\alpha$ , IL-6, and interleukin-1 $\beta$  (IL-1 $\beta$ ), and by inhibiting the nuclear factor- $\kappa$ B (NF $\kappa$ B) pathway through the production of cAMP and PKA [53, 54]. This may be of clinical importance in DMD as dystrophin-deficient muscle cells have been shown to produce significant levels of IL-1 $\beta$  and research suggests significant levels of TNF- $\alpha$  were detected in 62% of DMD tissue biopsies [58, 59]. Further, a positive correlation between cytokine levels and fibrosis was observed in the dystrophin-deficient diaphragm suggesting TNF- $\alpha$  may serve as a marker of dystrophy in the diaphragm [60]. This is consistent with mdx mouse studies demonstrating that inhibiting TNF- $\alpha$  production

delayed the appearance of muscle pathology [61]. This relationship has been demonstrated functionally as well. Research has shown with the treatment of an B2-AR agonist, there is a reduction in plasma leakage and reduction in the number of neutrophils and eosinophils that adhere to sites of inflammation on the endothelium [55]. Additionally, cells treated with cAMP, a downstream product of ADRB2 stimulation, have decreased IL-1 $\beta$  and TNF- $\alpha$  levels [56, 57]. These data suggest the ADRB2 may play a significant role in reducing the inflammatory response associated with DMD and thereby slow the emergence of dystrophinopathies in DMD patients.

#### 2.10.4.3 Beta-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<sub>as</sub> protein from the tightly associated  $\beta$  and  $\gamma$  subunits. G<sub>as</sub>-bound GTP then phosphorylates AC. Adenylyl cyclase produces cAMP which phosphorylates PKA into its active form [160]. Both cAMP and phosphorylated PKA can either directly or indirectly inhibit calpain activity. Research has shown a non-hydrolysable cAMP analog along with the activation of ADRB2 inhibits Ca<sup>2+</sup>-dependent protein degradation in both rats and chicks, suggesting cAMP may directly phosphorylate calpains to inhibit activity [63, 196]. 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 [244]. This suggests a direct pathway whereby cAMP signaling can lead to increased calpastatin gene transcription (a calpain

inhibitor) thus reducing calpain-mediated protein degradation. Further, multiple phosphorylation sites have been identified directly on calpastatin, particularly those found in the L and XL domain coded by exon 6, providing additional evidence for the direct phosphorylation of calpastatin via cAMP [245]. Research also suggests PKA has 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 [65, 246]. In addition to its ability to phosphorylate calpains, research suggests the C-domain of PKA can also directly phosphorylate calpastatin [66]. PKA and cAMP phosphorylation and increased 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 [67, 68].

B2-AR agonist supplementation has also been shown to reduce calpain activity. Research has indicated a decreased rate of protein degradation following epinephrine supplementation that was prevented by propranolol, a non-selective beta-antagonist and by M ICI 118.551, a B2-AR antagonist in rat skeletal muscle models. Additionally, dibutyryl cAMP and isobutylmethylxanthine reduced proteolysis in both soleus and extensor digitalis longus muscles [62]. Similar results have been shown in bovine species as well. B2AR agonist treatment of 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 [247]. In fact, following clenbuterol administration in the rat model, there was an approximate 50% decrease in the muscle  $\text{Ca}^{2+}$ -dependent proteolytic pathway with no resultant change in the activations of the lysosomal or ubiquitin-proteasome pathways, further supporting that the activity and gene expression of calpastatin are increased after B2-AR agonist supplementation [64, 69, 247]. Additionally, evidence supports the role of adrenergic tonus on the inhibition of proteolysis. After chemical and surgical sympathectomy, rat models demonstrated an increase in  $\text{Ca}^{2+}$ -dependent proteolytic pathways, suggesting adrenergic tonus exerts its effect via  $\text{Ca}^{2+}$ -dependent pathway inhibition [195, 196].

#### 2.10.5 Beta-2 Adrenergic Receptors and Other Myogenic Effects

Research has demonstrated exogenous ADRB2 stimulation may also demonstrate other myogenic actions. ADRB2 stimulation has been found to stimulate the production of growth factors such as insulin-like growth factors (IGF) and transforming growth factor  $\beta$  (TGF- $\beta$ ) which play an integral role in muscle development, growth and regeneration [248, 249]. Additionally, catecholamine administration has demonstrated a significant increase in cell proliferation associated with a mitogen activated protein kinase (MAPK)-dependent increase in cell cycle proteins cyclin E and D1 and cyclin-dependent kinase 2 and 4 (CDK2 and CDK4) [250]. Further, previous work suggests the  $\text{G}\alpha_i$ -linked  $\text{G}\beta\gamma$  subunits activate the phosphoinositide 3-kinase-protein kinase-B (PI3K-AKT) signaling pathway [170]. An isoform of this pathway (AKT1) has been shown to inhibit the forkhead box O transcription factor (FOXO), which has been implicated in

muscle atrophy [170, 173]. These data suggest the PI3K-AKT signaling pathway may regulate protein synthesis, gene transcription, cell proliferation, and cell survival. Additional myogenic effects of ADRB2 stimulation include the regulation of CREB and its downstream products via phosphorylation by the free C-subunits of PKA [162]. Increased CREB activity has been associated with increased activity of the transcription factor myocyte enhancer factor-2 (MEF2) and increased expression of neuron-derived orphan receptor-1 (NOR-1), both of which play roles in cell proliferation, differentiation, adaptation, and survival [161, 251]. In addition to these actions, NOR-1 expression was associated with a significant increase in the levels of myostatin expression. Myostatin is a member of the transforming growth factor- $\beta$  superfamily and the primary negative regulator of muscle mass [251]. These data suggest the ADRB2 may directly modulate muscle size through various mechanisms independent of the DMD pathology, providing evidence of the therapeutic benefits of B2-AR agonism and by extension, the Gly16 polymorphism.

#### 2.10.6 Beta-2 Adrenergic Receptors and Vascular Function

The dystrophin associated glycoprotein complex (DAGC) dysfunction associated with DMD may render the muscles ischemic during exercise [77]. 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 [72, 78]. Nitric oxide during exercise has been shown to play a role in blunting exercise-induced vasoconstriction due to  $\alpha$ -adrenergic receptor activation. This protective mechanism is defective in DMD

resulting in unopposed vascular vasoconstriction during exercise further contributing to the pathogenesis of DMD [79, 80]. The Gly16 polymorphism of the ADRB2 has shown improved vasodilation. Research using a non-selective  $\beta$ -AR antagonist has shown an increase in renin production thus increasing angiotensin II formation and upregulating its vasoconstrictive activity [252, 253]. This suggests the ADRB2 plays a functional role in not only promoting vasodilation but also in opposing vasoconstriction. Additionally, ADRA1 and ADRA2 have similar binding affinities for epinephrine and norepinephrine but still have a lower affinity for epinephrine than does the ADRB2. This suggests the physiologic plasma levels of epinephrine associated with the Gly16 polymorphism may be beneficial in opposing  $\alpha$ -adrenergic vascular vasoconstriction in DMD patients.

### **CHAPTER 3: RESEARCH PROJECTS**

3.1 Influence of Beta-1 Adrenergic Receptor Genotype on Cardiovascular Response to Exercise in Healthy Subjects

3.2 Beta-2 Adrenergic Receptor Genotype Influences Power Output in Healthy Subjects

3.3 Influence of Beta-1 Adrenergic Receptor Genotype on Time to Cardiovascular Intervention in Duchenne Muscular Dystrophy

3.4 Influence of Beta-2 Adrenergic Receptor Genotype on Time to Nocturnal Ventilation in Duchenne Muscular Dystrophy

### **3.1 Influence of Beta-1 Adrenergic Receptor Genotype on Cardiovascular Response to Exercise in Healthy Subjects**

#### **Short title: ADRB1 Influences Cardiovascular Response**

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**Background & Aims:** The beta-1 adrenergic receptor (ADRB1) has been shown to play a functional role in cardiomyocyte function and accounts for up to 80% of the cardiac tissue adrenergic receptors with ADRB1 stimulation increasing cardiac rate, contractility, and work. Multiple polymorphisms of the ADRB1 have been identified such as the Gly49 polymorphism that includes at least one glycine (Gly) for serine (Ser) at amino acid 49 resulting in either homozygous for Gly (Gly49Gly) or heterozygous for Gly (Gly49Ser) polymorphisms. Heart failure patients with this polymorphism (Gly49) have been shown to have improved cardiac function and decreased mortality risk, but if there is an effect in healthy subjects is less clear.

**Purpose:** The purpose of this study was to determine the effects of the Gly/Ser polymorphism at position 49 of the ADRB1 on the cardiovascular response to exercise in healthy subjects. **Methods:** We performed genotyping of the ADRB1 (amino acid 49) and high-intensity, steady-state exercise on 71 healthy subjects (Ser49Ser = 52, Gly49Ser = 19).

**Results:** There were no differences between genotype groups in age, height, weight, BMI, or watts achieved (age =  $28.9 \pm 5.6$  yrs.,  $30.6 \pm 6.4$  yrs., height =  $173.6 \pm 9.9$  cm,  $174 \pm 7.5$  cm, weight =  $74.4 \pm 13.3$  kg,  $71.9 \pm 13.5$  kg, BMI =  $24.6 \pm 3.5$ ,  $23.6 \pm 3.3$ , and watts =  $223.8 \pm 76.8$ ,  $205 \pm 49.4$ , for Ser49Ser and Gly49Ser respectively). Additionally, there were no differences for genotype groups for cardiac output (CO), systolic blood pressure (BP<sub>sys</sub>), or diastolic blood pressure (BP<sub>dias</sub>) at rest, maximal exercise, or in change from rest to maximal exercise. The genotype groups differed significantly in heart rate (HR<sub>max</sub>) at maximal exercise and cardiac index at rest (CI) (HR<sub>max</sub> =  $184.2 \pm 9.5$  bpm,  $190.7 \pm 10.6$  bpm, CI =  $0.063 \pm 0.014$ ,  $0.071 \pm 0.013$ , for Ser49Ser and Gly49Ser respectively). There was a trend towards significance ( $p = 0.058$ ) for the change in stroke volume from rest to peak exercise ( $\Delta$ SV) ( $0.016 \pm 0.018$  L,  $0.0076 \pm 0.012$  L, for Ser49Ser and Gly49Ser respectively).

**Conclusion:** These data suggest genetic variations of the ADRB1 may influence cardiovascular responses to exercise in healthy subjects.

**Keywords:** Exercise; ADRB1 polymorphism; Cardiovascular; Beta-1 genotype; Healthy

## INTRODUCTION

The  $\beta$ -adrenergic receptors ( $\beta$ -AR) are part of a family of membrane proteins known as G-protein coupled receptors where, upon binding of a catecholamine to the receptor, stimulates a conformational change in the  $\beta$ -AR that causes coupling with G-proteins. G-proteins consist of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits and  $\beta$ -AR coupling leads to the dissociation of the G-protein into active  $G\alpha$  and  $G\beta$  subunits to mediate downstream signaling [156]. Moreover,  $\beta$ -AR agonist binding results in a dissociation of the stimulatory  $G\alpha$  ( $G_{as}$ ) protein [193].  $G_{as}$ -bound guanine triphosphate (GTP) then phosphorylates the enzyme adenylyl cyclase (AC). Both AC and cAMP have the ability to regulate downstream mechanisms via multiple internal cell signaling pathways [161-165]. Conversely,  $\beta$ -AR antagonist binding causes a competitive inhibition against catecholamine and sympathetic nervous stimulation, thereby inhibiting the dissociation of  $G_s$  proteins [166].

One subtype of the  $\beta$ -ARs is the beta-1 adrenergic receptor (ADRB1). The ADRB1 subtype has been shown to play a functional role in cardiomyocyte function and accounts for approximately 70% of the cardiac tissue adrenergic receptors in the atria, 80% in the ventricles, and 95% in the sinoatrial (SA) node [176]. Research has demonstrated that ADRB1 stimulation increases cardiac rate, contractility, and work [199, 208]. Multiple polymorphisms of the ADRB1 have been identified as including a glycine (Gly) for serine (Ser) at amino acid 49 resulting in either homozygous for Gly (Gly49Gly) or heterozygous for Gly (Gly49Ser) polymorphisms. The Gly49 polymorphism has been shown to have decreased receptor density, decreased cAMP

accumulation, and a dampening response to the cardio-stimulant effect of norepinephrine infusion [22, 23]. Heart failure (HF) patients with this polymorphism (Gly49) have also been shown to have improved cardiac function and decreased mortality risk [18, 19, 29]. Furthermore, studies have demonstrated autoantibodies against ADRB1 are associated with more favorable myocardial recovery in patients with recent-onset cardiomyopathy [20]. This suggests the Gly49 polymorphism may have inherent cardioprotective effects similar to that of a beta-blocker.

While many studies have demonstrated the cardioprotective effect of the Gly49 polymorphism in HF, little is known of the effect of this polymorphism in healthy subjects or on the function in response to endogenous agonist (catecholamine) changes. Additionally, there is a lack of research exploring the effect of the Gly49 polymorphism on cardiovascular response to exercise in healthy subjects. This study aimed to identify the effect of ADRB1 polymorphisms on resting cardiovascular function and on cardiovascular response to exercise in healthy subjects.

## METHODS

### Subjects

Data analyzed for this article were part of a larger study on adrenergic receptor genotypes and cardiopulmonary function at rest and during exercise but the data have not been assessed as presented in this study [44, 201]. This study received approval by the appropriate ethics committee and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Appropriate consent has been obtained pursuant to law, and the subjects were informed of the benefits and risks of the investigation before signing an institutionally-approved informed consent prior to participation. Seventy-one untrained subjects, ages 20–40, agreed to participate and were genotyped for Gly49Ser polymorphisms of the ADRB1. All subjects were healthy nonsmokers and not on medication.

### Procedures

Subjects underwent baseline screening tests including pulmonary function testing, an incremental cycle ergometry test to exhaustion on a cycle ergometer, a blood draw for a complete blood count (to rule out anemia) and in women, a pregnancy test. The baseline exercise study, served as an initial familiarization session, was used to determine work intensities for subsequent sessions, and acted as a screening study to rule out myocardial ischemia and abnormal arrhythmias. After these initial tests, subjects met with the Clinical Research Center (CRC) nutritionist and were put on a controlled sodium diet ( $3,450 \text{ mg}\cdot\text{d}^{-1}$ ) for three days with a 24-hour urine collection to confirm sodium intake. Subjects subsequently returned to the CRC on two occasions for exercise testing while maintaining a salt neutral diet.

The next session consisted of a cycle ergometry test similar to the first visit but with the additional measurement of Q using a previously validated open-circuit acetylene uptake method [254]. This session served as a further familiarization with the measurements to be made on the final study day and also allowed for confirmation of workloads for the final visit.

On the last study visit, resting measurements of Q, heart rate (HR), stroke volume (SV), and arterial blood pressure (BP) were made. Cardiac output was measured with the open-circuit acetylene method, BP was measured via an arterial catheter, HR was measured via a 3-lead EKG, and SV was calculated by dividing Q by HR, and CI was calculated by dividing Q by weight. Subjects then exercised for 9 minutes at ~40% and 9 minutes at ~75% of their peak workload achieved during the initial exercise studies while measurements were repeated every 2–3 minutes. Nine minutes of exercise was performed because pilot data suggested that this was an adequate time frame to obtain three sets of measures and brought the subjects close to exhaustion with the higher workload with minimal physiologic drift in VO<sub>2</sub> and cardiovascular function. All visits were conducted in the morning to account for testing variability.

### **ADRB1 genotyping**

Buffy coat, obtained from whole blood collected on EDTA, was used for genomic DNA extraction using the Gentra Puregene DNA Isolation Kit (Gentra Systems Inc., Minneapolis, MN, USA), and DNA samples were sent to the University of Arizona Genomics Core for genotype analysis. A polymerase chain reaction (PCR) was conducted according to standard methods, using the following primer sequences (e.g., for Ser49Gly): (forward) 5'-CCG GGC TTC TGG GGT GTT CC-3' and (reverse) 5'-GGC GAG GTG TGG CGA GGT AGC-3', resulting in a PCR product 564 bps in length. For genotype analysis, a plate of sample DNA was normalized to 1ng/uL. 10uL of this stock DNA was plated into an AB1400/W reaction plate (Thermo Fisher) and was vacuum desiccated. A reaction mixture of Taqman Fast Advance Mastermix (Life Technologies),

reaction-grade water (Thermo Fisher), and pre-designed Taqman SNP genotyping assays (Life Technologies) was created and pipetted into the wells containing vacuum dried DNA. The plate was sealed with AB1170 Absolute qPCR Plate Seals (Thermo Fisher), centrifuged, and pre-read in an ABI 7300 qPCR instrument. The plates were relocated to a BioRad Tetrad Thermocycler system (BioRad) and reacted. The thermocycling protocol was as follows: 1 cycle of 15m at 45°C, 3m at 95°C, followed by 40 cycles of 15s at 95°C and 60s at 58°C. The reacted PCR plates were cooled to 4°C, centrifuged, and post-read on the ABI 7300 instrument. The data was analyzed using the SDS 1.4.1 software. The Ser49Ser homozygous genotype is represented by a single 564 bp band and the Ser49Gly by 2 products of 219 and 343 bp.

### **Statistical analyses**

All statistical comparisons were made using a statistical software package (SPSS; SPSS Inc, Chicago, IL, USA, version 19). Group demographics were compared with a 1-way ANOVA using an  $\alpha$  level of 0.05 to determine statistical significance. Genotype differences in performance were compared with an ANOVA to detect differences among the specific genotype groups. An  $\alpha$  level of 0.05 was used for the ANOVA and post hoc analyses. An  $\alpha$  level of 0.05 was used for the ANOVA and post hoc analyses.

## **RESULTS**

### **Subject characteristics**

Seventy-one subjects were enrolled in this study (36 male and 35 female). Genotyping for ADRB1 was completed for amino acid position 49 with individuals who

were homozygous for serine (Ser49Ser,  $n = 52$ ) or heterozygous (Gly49Ser,  $n = 19$ ) at codon 49. There was no statistically significant difference between genotype groups for age, height, weight, BMI, or peak watts achieved, during the maximal exercise test (Table 1).

### **Cardiac output, stroke volume, and blood pressure**

There were no statistically significant differences between genotype groups for cardiac output (Q) or systolic blood pressure ( $\text{BP}_{\text{sys}}$ ) at rest, peak exercise, or in change from rest to peak exercise (Figure 1). There were no significant differences between genotype groups for diastolic blood pressure ( $\text{BP}_{\text{dias}}$ ) or stroke volume (SV) at rest or peak exercise (Fig 1). There was a trend towards significance between genotype groups for change in SV ( $p = 0.058$ ) from rest to peak exercise. Furthermore, while not statistically significant ( $p = 0.08$ ), there was a clinically significant difference between genotype groups for change in  $\text{BP}_{\text{dias}}$  from rest to peak exercise (- $5.5 \pm 15.4$  and  $1.3 \pm 11.5$  mmHg for Ser49Ser and Gly49Ser respectively) (Figure 1). We have defined this as clinically significant as small changes in BP (~5 mmHg) can drastically influence survival in HF patients [255].

### **Cardiac index, heart rate, and systemic vascular resistance**

There were no statistically significant differences between genotype groups for cardiac index (CI) at peak exercise or for change in CI from rest to peak exercise. Nor were there any differences between genotype groups for resting heart rate (HR) or for change in HR from rest to peak exercise. Further, there were no statistically significant

differences between genotype groups in systemic vascular resistance (SVR) at peak exercise or for change in SVR from rest to peak exercise. There were, however, differences between genotype groups for resting CI ( $p = 0.037$ ) and SVR ( $p = 0.046$ ) and for HR at peak exercise (HR<sub>max</sub>) ( $p = 0.016$ ), with the Gly49Ser genotype presenting improved CI and a lower SVR at rest, and a higher HR at peak exercise (Figure 2).

## DISCUSSION

In the present study, we demonstrate that genetic variation of the ADRB1 is associated with differences in some components of the cardiovascular responses to peak exercise in healthy subjects. Individuals with one glycine allele (Gly49Ser) at amino acid 49 demonstrate a trend towards significance for change in SV from rest to peak exercise. The Gly49Ser polymorphism demonstrated a blunted response in SV in response to peak exercise and a significant change in BP<sub>dias</sub> from rest to peak exercise and the Gly49Ser polymorphism demonstrated a greater increase in BP<sub>dias</sub> and the Ser49Ser polymorphism demonstrating a decrease in BP<sub>dias</sub>. Further, the Gly49Ser polymorphism demonstrated an improved CI at rest, an increased HR<sub>max</sub> at peak exercise, and a lower SVR at rest compared to the Ser49Ser polymorphism.

The ADRB1 subtype is found primarily in the heart and comprises 75-80% of total  $\beta$ -AR found in the heart and approximately 95% of  $\beta$ -AR in the SA node [21]. The G protein signaling pathway associated with the  $\beta$ -AR is important in the modulation of several key target proteins. When activated, cardiomyocyte ADRB1 preferentially binds to the G<sub>as</sub> protein which phosphorylates AC, generating the secondary messenger cAMP.

Increased cAMP accumulation activates PKA [25]. Activated PKA then phosphorylates troponin I, the L-type  $\text{Ca}^{2+}$  channel and phospholamban (PLB), increasing cardiac inotropy, chronotropy, and lusitropy [24].

Research has also shown  $G_s$  activation can increase L-type  $\text{Ca}^{2+}$  current directly [166]. L-type  $\text{Ca}^{2+}$  channels play an integral role in cardiomyocyte excitability and contractility [156]. Phosphorylation of cardiac L-type  $\text{Ca}^{2+}$  channels by PKA results in an influx of  $\text{Ca}^{2+}$  into cardiomyocytes. The  $\text{Ca}^{2+}$  then binds to the sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA) triggering further sarcoplasmic  $\text{Ca}^{2+}$  loading resulting in the removal of troponin and tropomyosin inhibition of myosin binding sites [105, 167]. Additionally, the phosphorylation of phospholamban (PLB) via PKA, a downstream protein from  $\beta$ -AR stimulation, has been shown to result in the removal of inhibition of SERCA. This increases the quantity and rate of reuptake of cytosolic  $\text{Ca}^{2+}$  in the sarcoplasmic reticulum [27]. 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  $\text{Ca}^{2+}$  handling [26]. Troponin I is a regulatory protein of cardiac myofibrils and its phosphorylation by PKA inhibits actomyosin ATPase activity resulting in relaxation of cardiomyocytes in response to catecholamines [22, 28].

Genetic variants of the ADRB1 have been shown to modulate the cardiac responses to catecholamine binding; the Gly49 polymorphism has been shown to produce a dampening effect to these responses. These data suggest genetic variations of the ADRB1 may influence cardiovascular responses to exercise in healthy subjects. In the

present study, subjects with the Gly49Ser polymorphism present with a statistically significantly improved cardiac index as compared to the Ser49Ser polymorphism at rest; this difference is abolished at peak exercise. This suggests improved cardiac function at rest with no deleterious effect on cardiac function at peak exercise. Additionally, there is a statistically significant difference between the two polymorphisms for HR<sub>max</sub> and no difference at rest, with the Gly49Ser polymorphism demonstrating a higher HR<sub>max</sub>. This suggests the Gly49Ser polymorphism has an improved HR reserve. Further, this polymorphism (Gly49Ser) also demonstrated a lower SVR at rest, suggesting the Gly49Ser polymorphism has decreased cardiac work at rest. This coupled with the abolishment of this difference at peak exercise suggests subjects with the Gly49Ser polymorphism have an improved cardiac work reserve. Furthermore, the trend towards significance in change in stroke volume from rest to peak exercise, with the Gly49Ser polymorphism presenting a damped increase, in addition to there being no difference in HR at rest and improved CI at rest, suggests an improved left ventricular contractility in the Gly49Ser polymorphism. It is interesting to note the clinically significant difference between genotype groups in change in BP<sub>dias</sub> rest to peak exercise may suggest a systemic influence of the ADRB1 genotype, particularly in the typically observed arterial vasodilation observed in response to aerobic exercise. These data suggest the Gly49Ser polymorphism to have improved cardiovascular function at rest and peak exercise.

ADRB1 signalling has been shown to play an important role in HF with the degree of sympathetic activity being inversely correlated with survival [211]. deleterious effects of ADRB1 signalling include apoptosis, myocyte growth, fibroblast hyperplasia,

myopathy, fetal gene induction, and proarrythmia [206, 212]. As an adaptive mechanism in HF, cardiac ADRB1s become less responsive, either downregulating or uncoupling from the G<sub>s</sub> pathway [214]. This suggests the less functional variant of the ADRB1 to be clinically important in HF and our data confirms that further study is certainly warranted.

The present study did not demonstrate as large of an effect of ADRB1 polymorphisms on cardiovascular parameters in healthy subjects as the current research has demonstrated in HF. We postulate this may be due to the change in β-AR ratios in HF and epinephrine/norepinephrine binding affinities for ADRB1 and ADRB2. Previous work has demonstrated a decrease in cardiomyocyte ADRB1 concentration by as much as 61% in HF with no or little corresponding decrease in ADRB2 concentrations [214, 256-258]. Furthermore, ADRB1 receptors have a high binding affinity for both epinephrine and norepinephrine while ADRB2 receptors have a high binding affinity for epinephrine and a low binding affinity for norepinephrine [175]. Additionally, there is little to no ADRB1 receptor reserve for positive cardiovascular ionotropic effects [176, 177, 259]. This decrease in β-AR concentration, particularly ADRB1 concentration, and successive decreased catecholamine sensitivity coupled with a low ADRB1 reserve, suggests this decrease in ADRB1 receptor concentrations is a protective mechanism in HF and modulates the effect of ADRB1 polymorphisms on cardiovascular parameters.

Current research suggests a cardioprotective effect of the Gly49 polymorphism in HF and other cardiac pathologies. This present study supports this notion, suggesting improved cardiovascular function at rest and response to peak exercise in healthy subjects and supporting the use of beta-blockers to improve exercise tolerance in HF patients.

These findings may be clinically important as exercise is an essential aspect of cardiac rehabilitation following cardiac exacerbations. The ability to improve exercise tolerance in this population may prove integral in improving the efficacy and adherence to exercise as a part of cardiac rehabilitation.

## LIMITATIONS

There are inherent limitations regarding genetics studies including sample size and genotype distribution. Limited statistical power because of the modest sample size and different genotype distribution in the present study ( $N = 71$ ) may have played a role in limiting the significance of some of the statistical comparisons conducted. A post hoc power analysis revealed the power to detect statistically significant differences between groups for CI at rest and  $HR_{max}$  to be .625 and .613 respectively at  $\alpha = 0.05$ , suggesting sufficient sample size for the present study. Furthermore, our study population was void of the homozygous Gly49Gly genotype. This can be explained by the genotype frequency of the three genotypes present at amino acid 49 (0.69, 0.29, and 0.04 for Ser49Ser, Gly49Ser, and Gly49Gly respectively) but may vary among different racial/ethnic groups [260]. However, present research has demonstrated the Gly49Ser and Gly49Gly polymorphisms function similarly, suggesting there is no additive effect of an additional glycine present at amino acid 49 and that the Gly49Ser polymorphism may be representative of the Gly49 polymorphism as a whole for ADRB1 [176, 208].

## CONLCUSIONS

The present study examined the influence of ADRB1 genotype on cardiovascular response to exercise in healthy subjects. Our data demonstrated the Gly49Ser genotype has a blunted SV response to peak exercise and a significant change in BP<sub>dias</sub> from rest to peak exercise compared to the Ser49Ser genotype. Additionally, the Gly49Ser demonstrated improved CI at rest, increased HR<sub>max</sub> at peak exercise, and a lower SVR at rest compared to the Ser49Ser genotype. The current study suggests improved cardiovascular function at rest and response to peak exercise in healthy subjects and provides further evidence to the cardioprotective effect of the Gly49 polymorphism in a healthy population.

### **COMPETING INTERESTS**

All authors declare they have no competing interests.

### **GRANT SUPPORT**

This study was funded by National Institute of Health (NIH) grants: HL108962-06.

### **FINANCIAL DISCLOSURE**

All authors declare they have no conflict of interest.

**TABLES****Table 1.** Subject characteristics (mean  $\pm$  standard deviation, N or p-value)**Study Demographics**

	N	Mean $\pm$ SD	p-value
<b>Sex (Male/Female)</b>			
Ser49Ser	26/26	-	-
Gly49Ser	10/9	-	-
Total	36/35	-	-
<b>Age (yrs)</b>			
Ser49Ser	52	28.9 $\pm$ 5.6	0.25
Gly49Ser	19	30.6 $\pm$ 6.4	
Total	71	29.3 $\pm$ 5.8	
<b>Height (cm)</b>			
Ser49Ser	52	173.6 $\pm$ 9.9	0.87
Gly49Ser	19	174 $\pm$ 7.4	
Total	71	173.7 $\pm$ 9.3	
<b>Weight (kg)</b>			
Ser49Ser	52	74.4 $\pm$ 13.3	0.5
Gly49Ser	19	71.9 $\pm$ 13.5	
Total	71	73.8 $\pm$ 13.3	
<b>BMI</b>			
Ser49Ser	52	24.6 $\pm$ 3.5	0.33
Gly49Ser	19	23.6 $\pm$ 3.3	
Total	71	24.3 $\pm$ 3.4	
<b>Watts</b>			
Ser49Ser	52	223.8 $\pm$ 76.8	0.33
Gly49Ser	19	205 $\pm$ 49.4	
Total	71	218.7 $\pm$ 70.6	

Subject characteristics (N, mean  $\pm$  standard deviation, and p-value)

Ser49Ser = genotype (homozygous for ADRB1 resulting in serine at amino acid 49), Gly49Ser = genotype (heterozygous for ADRB1 resulting in one glycine and one serine at amino acid 49). BMI – body mass index. There were no statistically significant differences in demographic data

## FIGURE LEGENDS

**Table 1.** Subject characteristics (mean  $\pm$  standard deviation, N or p-value)

Subject characteristics (N, mean  $\pm$  standard deviation, and p-value)

Ser49Ser = genotype (homozygous for ADRB1 resulting in serine at amino acid 49), Gly49Ser = genotype (heterozygous for ADRB1 resulting in one glycine and one serine at amino acid 49). BMI – body mass index. There were no statistically significant differences in demographic data

### Figure 1

Panels depict the change from rest to peak exercise in (A) Cardiac Output (Q), (B) Systolic Blood Pressure ( $BP_{sys}$ ), (C) Diastolic Blood Pressure ( $BP_{dias}$ ), and (D) Stroke Volume (SV). The error bars represent the SE of the mean.

† Trend towards significance for change in SV ( $p = 0.058$ ) from rest to peak between genotype groups.

‡ Clinically significant difference between genotype groups for change in  $BP_{dias}$  from rest to peak exercise ( $-5.5 \pm 15.4$  and  $1.3 \pm 11.5$  for Ser49Ser and Gly49Ser respectively).

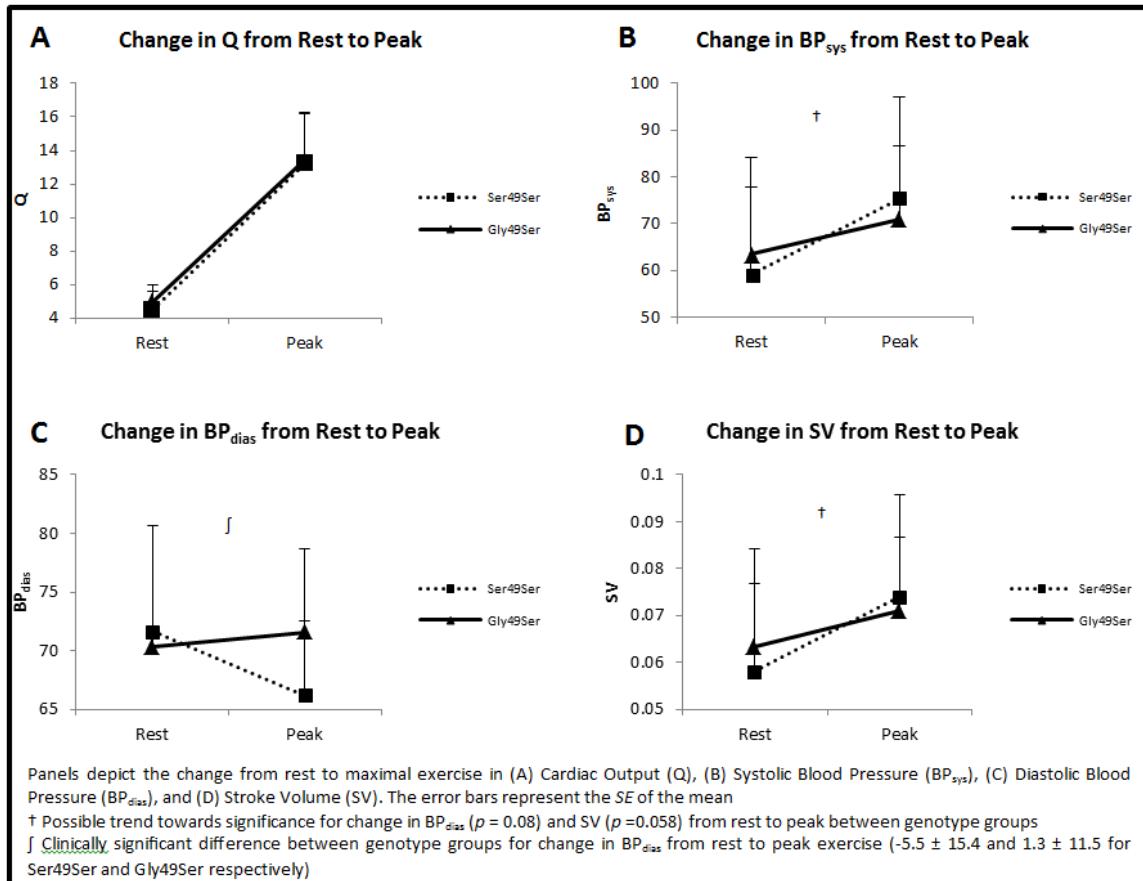
### Figure 2

Panels depict the change from rest to peak exercise in (A) Cardiac Index (CI), (B) Heart Rate (HR), and (C) Systemic Vascular Resistance (SVR). The error bars represent the SE of the means.

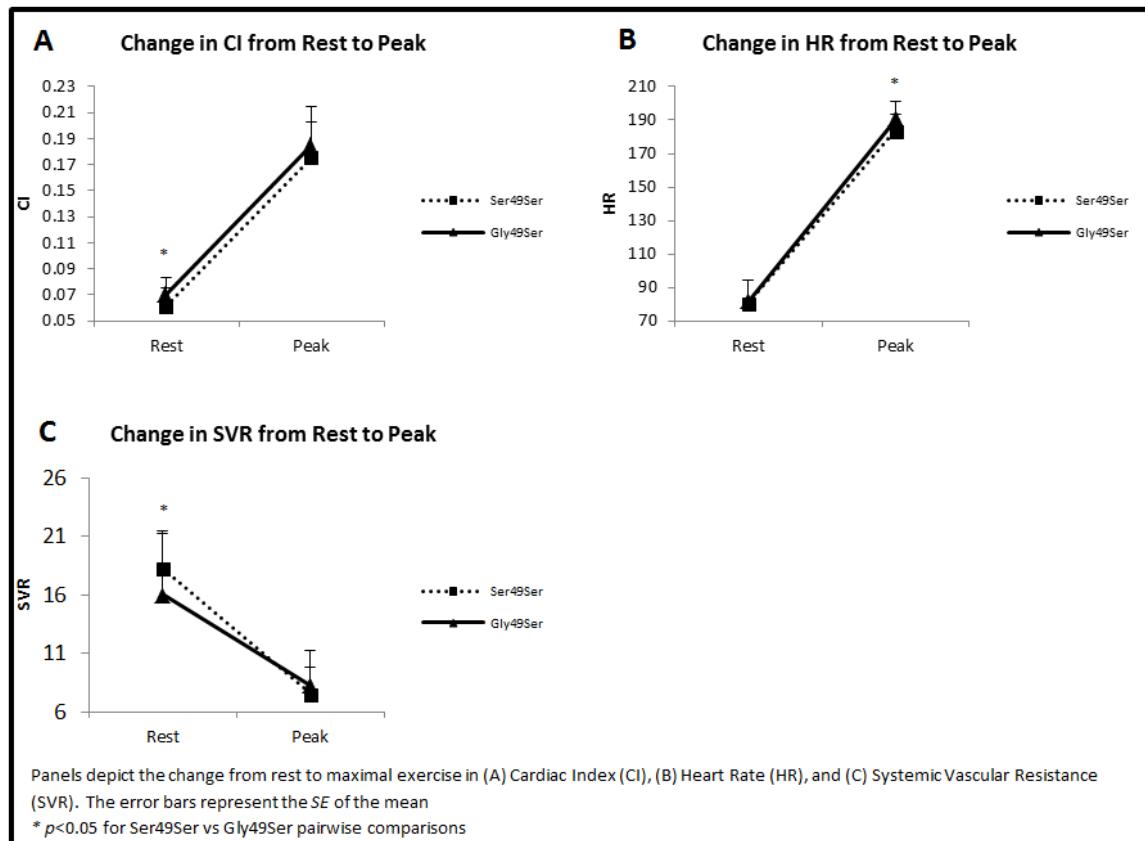
\* $p < 0.05$  for Ser49Ser vs Gly49Ser pairwise comparisons.

## FIGURES

**Figure 1.** Change from rest to peak exercise in Cardiac Output (Q), Systolic Blood Pressure ( $BP_{sys}$ ), Diastolic Blood Pressure ( $BP_{dias}$ ), and Stroke Volume (SV).



**Figure 2.** Change from rest to peak exercise in Cardiac Index (CI), Heart Rate (HR), and Systemic Vascular Resistance (SVR).



### **3.2 Beta-2 Adrenergic Receptor Genotype Influences Power Output in Healthy Subjects**

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#### **ABSTRACT**

The purpose of this study was to determine the effects of ADRB2 genotypes on muscle function (absolute power and relative power) in healthy subjects. We performed genotyping of the ADRB2 (amino acid 16) and high-intensity, steady-state exercise on 77 healthy subjects (AA = 18, AG = 25, GG = 34). There were no differences between genotype groups in age, height, weight, or BMI (age =  $28.9 \pm 5.7$  yrs,  $27.9 \pm 5.7$  yrs,  $29.2 \pm 5.9$  yrs, height =  $170.7 \pm 8.6$  cm,  $174.9 \pm 8.7$  cm,  $173.4 \pm 9.6$  cm, weight =  $68.5 \pm 13.0$  kg,  $75.0 \pm 12.9$  kg,  $74.4 \pm 12.9$  kg, and BMI =  $23.4 \pm 3.9$ ,  $24.4 \pm 2.9$ ,  $24.7 \pm 3.4$ , for AA, AG, and GG, respectively). The genotype groups differed significantly in watts, and watts/VO<sub>2</sub> with heavy exercise (watts =  $186.3 \pm 54.6$ ,  $237.8 \pm 54.4$ ,  $219.4 \pm 79.5$ , watts/VO<sub>2</sub> =  $0.08 \pm 0.006$ ,  $0.09 \pm 0.005$ ,  $0.08 \pm 0.006$ ). There was a trend towards significance ( $p=0.058$ ) for watts/kg ( $2.7 \pm 0.4$ ,  $3.2 \pm 0.5$ ,  $2.9 \pm 0.8$ , for AA, AG, and GG, respectively). These data suggest that genetic variation of the ADRB2 may influence relative strength in healthy subjects and may become an important genetic determinant of muscular strength and functional capacity in patients with diseases that result in a loss of muscle strength.

**Keywords:** exercise; ADRB2 polymorphism; strength; beta-2 genotype; beta-2 receptor

## INTRODUCTION

Muscular strength and power are important aspects to athletic performance.  $\beta_2$  agonist supplementation has been shown to increase power and increases the rates of glycolysis and glycogenolysis during sprinting in men (16). Further, Kalsen et al (2015) also demonstrated increased mean and peak power during the sprint with increased anaerobic adenosine-triphosphate (ATP) utilization following  $\beta$ -agonist administration. In this same study,  $\beta$ -agonist administration preserved whole muscle ATP concentrations with no difference in phosphocreatine breakdown. These findings suggest the  $\beta_2$  adrenergic receptors (ADRB2) influence anaerobic power and capacity, possibly improving anaerobic performance in power athletes.

The  $\beta_2$  adrenergic receptor plays a functional role in muscle size, strength and muscle regeneration (7, 30). Church et al, (2014) demonstrated reduced peak twitch force, rate of contraction, maximal force along with significantly reduced rates of regeneration in ADRB2 receptor knockout mice compared to controls. Further, supplementation with  $\beta_2$  agonists has demonstrated enhanced sarcoplasmic reticulum  $\text{Ca}^{2+}$  release rates, maximal voluntary contraction strength and peak Wingate power in trained human and rat models (5, 14, 25). Hodge et al, (2002), also demonstrated that denervation-induced atrophy was attenuated through  $\beta_2$  agonist treatment in rats. These data suggest the ADRB2 regulates muscular size, strength, contractility and protects against denervation-induced atrophy, suggesting ADRB2 may play a functional role in reducing disuse atrophy associated with injury.

The mechanisms by which ADRB2 stimulation may increase muscle size, strength, and contractility are associated with its role in internal cell signalling. 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 (11, 30). Skeletal muscle cAMP signaling is shown to regulate contractility, sarcoplasmic  $\text{Ca}^{2+}$  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 (2). This previous work suggests individuals 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. Multiple polymorphisms of the ADRB2 have been identified as including a glycine (Gly) for arginine (Arg) substitution at amino acid 16. The Gly16 polymorphism has been shown to have higher receptor density on lymphocytes, be more resistant to receptor down regulation, and functionally demonstrate higher cardiac output, stroke volume, left ventricular function, ejection fraction and vascular function when compare to the Arg16 genotype in humans (9, 13, 32, 34, 35).

Although not specifically studied previously, demographic data from previous studies demonstrate that subjects with the Gly16 polymorphism tend to have a greater

body mass index, despite higher 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 through other down stream mechanisms.

Although the clinical implications of ADRB2 genotypes on muscular development and strength are important, the influence of ADRB2 genotype on performance may identify novel supplementation for improvement of sports performance. Currently, little research has investigated the direct effect of ADRB2 genotype on skeletal muscle function. Therefore, the purpose of this study was to determine the effects of ADRB2 genotypes on muscle function (absolute power and relative power) in healthy subjects.

## METHODS

### Experimental Approach to the Problem

This study used a one way ANOVA to compare genotype groups for indices of intensity, power (watts, watts/kg, and watts/kg), and perceived intensity (watts/hr) at high intensity cycling for healthy, untrained subjects. Peak measures were compared for genotype groups for dependent variable measures. Subjects attended three sessions of testing with 72 hours between sessions one and two on a controlled low-sodium diet and at least 24 hours between sessions two and three.

### Subjects

Data analyzed for this manuscript were part of a larger study on ADRB2 genotypes and cardiopulmonary function at rest and during exercise but the data have not been assessed as presented in the present study (32, 33). This study received Institutional Board approval for the research and appropriate consent has been obtained pursuant to law and the subjects were informed of the benefits and risks of the investigation prior to signing an institutionally approved informed consent document to participate. Seventy-seven untrained subjects, ages 20 to 40, agreed to participate and were genotyped for Arg16Gly polymorphisms of the ADRB2. Individuals who were homozygous for arginine (ArgArg,  $n = 18$ ), glycine (GlyGly,  $n = 34$ ) or heterozygous (ArgGly,  $n = 25$ ) at codon 16 agreed to participate in the study. All subjects were healthy non-smokers and not on medication.

### Procedures

Subjects underwent baseline screening tests including pulmonary function testing, an incremental cycle ergometry test to exhaustion on a lode cycle ergometer, a blood draw for a complete blood count (to rule out anemia) and, in women, a pregnancy test. The baseline exercise study served as an initial familiarization session, was used to determine work intensities for subsequent sessions, and acted as a screening study to rule out myocardial ischemia and abnormal arrhythmias. Following these initial tests, subjects met with the Clinical Research Center (CRC) nutritionist and were put on a controlled sodium diet (3450 mg day<sup>-1</sup>) for 3 days with a 24-h urine collection to confirm sodium intake. This controlled sodium diet was used because previous studies have suggested that the ADRB2 may be sensitive to changes in dietary sodium (31, 18). Subjects

subsequently returned to the CRC on two occasions for exercise testing while maintaining a salt neutral diet.

The next session consisted of a cycle ergometry test similar to the first visit but with the additional measurement of Q using a previously validated open-circuit acetylene uptake method (15). This session served as a further familiarization with the measurements to be made on the final study day and also allowed for confirmation of workloads for the final visit.

On the last study visit, resting measurements of Q, HR, SV and arterial BP were made. Subjects then exercised for 9 min at ~40% and 9 min at ~75% of their peak workload achieved during the initial exercise studies while measurements were repeated every 2–3 min. Nine minutes of exercise was performed because pilot data suggested that this was an adequate time frame to obtain three sets of measures and brought the subjects close to exhaustion with the higher workload. All visits were conducted in the morning to account for testing variability.

#### ADRB2 Genotyping

$\beta_2$  adrenergic receptor 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  $\mu$ l 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 Analyses

All statistical comparisons were made using a statistical software package (SPSS; SPSS Inc; Chicago, IL, version 19). Group demographics were compared with a one-way analysis of variance (ANOVA) using an  $\alpha$  level of 0.05 to determine significance. Genotype differences in performance were compared with an ANOVA using a Tukey post hoc test to detect differences among the specific genotype groups. An  $\alpha$  level of 0.05 was used for the ANOVA and post hoc analyses.

## **RESULTS**

#### Subject Characteristics

There was no difference between genotype groups in age, weight, height, body mass index (BMI), or body surface area (BSA) (Table 1).

(Table 1 here)

### Power Measures

There were no differences in any of the power parameters (watts, watts/VO<sub>2</sub>, watts/kg) with light exercise. With heavy exercise, there were significant effects of genotype on power parameters ( $p\text{ANOVA}<0.05$ ) (Figure 1). Specifically, following post-hoc analysis, it was determined that the Arg/Gly group achieved significantly higher watts ( $p=0.04$ ) than the Arg/Arg group ( $237.8 \pm 54.4$ ,  $186.3 \pm 54.6$ , for AG and AA respectively, SE=20.67, 95% CI (2.00, 100.82)). Additionally, the Arg/Gly group had significantly greater relative power as measured by watts/kg ( $p=0.046$ ) than the Arg/Arg group ( $3.2 \pm 0.5$ ,  $2.7 \pm 0.4$ , for AG and AA respectively, SE=0.202, 95% CI (0.007, 0.97)). Further, the Arg/Gly group demonstrated significantly greater watts/VO<sub>2</sub> ( $p=0.034$ ) than the Gly/Gly group ( $0.09 \pm 0.005$ ,  $0.08 \pm 0.006$ , for AG and GG respectively, SE=0.002, 95% CI (0.0003, 0.008)). There was no difference in watts/VO<sub>2</sub> between the Arg/Gly and Arg/Arg groups, nor were there any differences in VO<sub>2</sub> between genotype groups.

(Figure 1 here)

### Intensity Measures

Similar to power indices, there were significant genotype differences in indices of relative and perceived intensity. The Arg/Gly group demonstrated significantly higher watts/hr ( $p=0.019$ ) than the Arg/Arg group ( $1.3 \pm 0.3$ ,  $0.99 \pm 0.3$ , for AG and AA respectively, SE=0.119, 95% CI (0.046, 0.62)) (Table 2). This is likely due to the ability of the Arg/Gly group to exercise at a lower relative intensity (higher peak watts while maintaining lower heart rates) ( $p=0.038$ ;  $181 \pm 9.7$ ,  $189 \pm 9.9$ , for AG and AA respectively, SE=2.97, 95% CI (0.35, 14.57)). Further, the Arg/Gly group reported lower RPEs ( $p=0.032$ ) than the Arg/Arg group ( $18.6 \pm 0.51$ ,  $18.9 \pm 0.24$ , for AG and AA respectively, SE=0.15, 95% CI (0.03, 0.74)) despite producing higher watts (Figure 2).

(Table 2 here)

(Figure 2 here)

## DISCUSSION

In the present study we demonstrate that genetic variations of the ADRB2 are associated with differences in muscular power, efficiency and intensity. Individuals with one arginine and one glycine allele (Arg/Gly) demonstrated significantly higher peak power (watts), relative power (watts/kg), muscular efficiency (watts/VO<sub>2</sub>) and exercise intensity (watts/hr) during heavy, steady-state exercise. Interestingly, despite producing higher peak watts, the Arg/Gly group also reported significantly lower rating of perceived exertion (RPE). Similar to previous observations, although not statistically significant, individuals with at least one glycine allele (Arg/Gly and Gly/Gly) were heavier than those homozygous for arginine ( $68.5 \pm 13.1$ ,  $75.1 \pm 12.9$ ,  $74.4 \pm 12.9$ , for AA, AG and

GG respectively). These findings may be due to the regulation of several downstream mechanisms by the ADRB2.

There are several pathways by where activation of the ADRB2 may regulate skeletal muscle size and strength. One such pathway is through phosphorylation by catecholamines. ADRB2s are g-coupled protein receptors where upon binding of a catecholamine to the receptor stimulates a dissociation of the guanine protein which phosphorylates adenylyl cyclase (AC). Adenylyl cyclase produces cAMP which phosphorylates protein kinase A (PKA) into its active form (2).

The first process whereby ADRB2 activation may increase muscular size and strength is binding of a catecholamine to the ADRB2 resulting in dissociation of the guanine-linked subunits. Further, previous work suggests the G $\alpha_i$ -linked G $\beta\gamma$  subunits activate the phosphoinositide 3-kinase-protein kinase-B (PI3K-AKT) signaling pathway (28). The PI3K-AKT signaling pathway has been shown to regulate protein synthesis, gene transcription, cell proliferation, and cell survival (3, 12). 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) (36). This is significant because FOXO has been implicated in muscle atrophy (17). 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 (17). This suggests that the ADRB2 plays a functional role in attenuating skeletal muscle atrophy in addition to promoting skeletal muscle growth. Further study of the genotypes in disease models is certainly warranted.

Another process by which ADRB2 stimulation may increase muscular size and strength is by phosphorylation of PKA via cAMP. PKA phosphorylation into its active state results in dissociation of the PKA subunits. It has been demonstrated that the 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) (6). CREB is a nuclear transcription factor that is universally expressed and has many processes, including cell proliferation, differentiation, adaptation, and survival (21). 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 (1). ADRB2 activation is also associated with an increased expression of neuron-derived orphan receptor-1 (NOR-1) (24). 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. Myostatin is a member of the transforming growth factor- $\beta$  superfamily and the primary negative regulator of muscle mass (24). 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. Collectively, these data

suggest the ADRB2 plays multiple roles in regulating skeletal muscle growth through downstream signaling.

In addition to the mediation of internal cell signaling, stimulation of the ADRB2 can also regulate  $\text{Ca}^{2+}$ -mediated proteolysis. Both cAMP and phosphorylated PKA can either directly or indirectly inhibit calpain activity. Calpains are  $\text{Ca}^{2+}$ -mediated proteases degrade myofibrils and by inhibiting calpain activity, myofibril size and integrity are preserved. This would decrease muscle damage and loss due to increased  $\text{Ca}^{2+}$  flux from exercise. 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 (22, 23). Research also suggests PKA demonstrates the ability to phosphorylate calpains, which is important in the context of this study because increased ADRB2 activation attributed to the Gly16 genotype would result in increased PKA concentrations and decreased calpain activity and myofibril degradation. 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 (27, 29). The ability of cAMP and PKA to modulate calpain concentrations and activity suggests a mechanism whereby the Gly16 genotype may influence muscular size and strength.

Furthermore, ADRB2 stimulation may also regulate calpastatin activity, a calpain-specific inhibitor, thereby decreasing calpain concentrations and activity. Recent research has demonstrated that the calpastatin promoter sequence between nt -1653 and +130 contains a single cAMP binding site located at nt -76 (8). This suggests a direct pathway whereby cAMP signaling can lead to increased calpastatin gene transcription 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 (37). In addition to its ability to phosphorylate calpains, research suggests the C-domain of PKA can also directly phosphorylate calpastatin (25). These data suggest another pathway whereby ADRB2 stimulation may inhibit calpain activity and regulate muscle size and strength.

Hence, there are multiple pathways whereby ADRB2 activation may increase muscle size, strength, and contractility as well as protect against disuse atrophy. These implications suggest ADRB2 stimulation as a viable supplementation site, thus improving sport performance and attenuating muscular loss resulting from injury. In the current study, the Arg/Gly polymorphism demonstrated significantly higher power measures, muscular efficiency, and exercise intensity. This suggests the more functional polymorphism of the ADRB2 may have an effect on these measures and that ADRB2 stimulation may improve muscular size, strength, and contractility.

## PRACTICAL APPLICATIONS

There are many pathways by which the ADRB2 can influence muscular development and strength. This study suggests that genetic variations of the ADRB2 are associated with muscular power and efficiency. We hypothesize the improved muscular function in the Arg/Gly group could be due to increased lymphocyte density and resistance to downregulation associated with the Gly16 polymorphism resulting in increased accumulation of downstream products which have been implicated in the regulation of muscle size and strength. These findings may imply a novel, safe approach to the attenuation of muscle degradation associated with disuse atrophy resulting from injury and to the improvement of muscular power and efficiency in athletes.

## LIMITATIONS

There are inherent limitations regarding a genetics study including sample size and genotype distribution. Limited statistical power because of the modest sample size and different genotype distribution in the present study ( $N = 77$ ) may have played a role in limiting the significance of some of the statistical comparisons conducted. A post hoc power analysis revealed the power to detect statistically significant differences between groups for watts, watts/kg, and watts/VO<sub>2</sub> to be .84, .95, and 1.00 respectively at  $\alpha = 0.05$ , suggesting sufficient sample size for the present study. Additionally, time of day and time of year for testing as well as training background were not controlled for in the present study, which may affect test-retest reliability. An intraclass correlation coefficient analysis revealed the test-retest reliability for watts, watts/kg, and watts/VO<sub>2</sub> to be 0.52,

0.49, and 0.59 respectively, suggesting fair test-retest reliability. Therefore, we cannot rule out the influence of uncontrolled for variables on these measures.

## **ACKNOWLEDGMENTS**

We are sincerely grateful to the subjects who donated both their time and effort to be a part of this study. The results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation. The results of the present study do not constitute endorsement by NSCA. This study was funded by NIH grants: HL108962-06. The authors have no conflict of interest to disclose.

**TABLES****Table 1.** Subject characteristics (mean  $\pm$  standard deviation, N or p-value)**Study Demographics**

	<b>N</b>	<b>Mean</b>	<b>SD</b>	<b>p-value</b>
<b>Age (years)</b>				
Arg/Arg	18	28.9	5.67	0.708
Arg/Gly	24	27.9	5.74	
Gly/Gly	34	29.2	5.94	
Total	76	28.7	5.76	
<b>Height (cm)</b>				
Arg/Arg	18	170.8	8.61	0.341
Arg/Gly	24	175	8.79	
Gly/Gly	34	173.5	9.67	
Total	76	173.3	9.17	
<b>Weight (kg)</b>				
Arg/Arg	18	68.5	13.06	0.214
Arg/Gly	24	75.1	12.96	
Gly/Gly	34	74.4	12.93	
Total	76	73.2	13.07	
<b>BMI (<math>\text{kg}/\text{m}^2</math>)</b>				
Arg/Arg	18	23.4	3.85	0.448
Arg/Gly	24	24.4	2.91	
Gly/Gly	34	24.7	3.39	
Total	76	24.3	3.35	
<b>BSA (<math>\text{m}^2</math>)</b>				
Arg/Arg	18	1.79	0.19	0.189
Arg/Gly	24	1.90	0.20	
Gly/Gly	34	1.88	0.19	
Total	76	1.87	0.20	
<b>VO2 (mL/min)</b>				
Arg/Arg	18	2257.83	761.19	0.152
Arg/Gly	24	2712.12	629.37	
Gly/Gly	34	2613.41	872.19	
Total	76	2562.34	784.55	
<b>VO2/KG</b>				
Arg/Arg	18	32.35	5.98	0.200
Arg/Gly	24	36.29	5.61	
Gly/Gly	34	35.11	8.50	
Total	76	34.85	7.18	

Arg/Arg = genotype (homozygous for ADRB2 resulting in arginine at amino acid 16), Arg/Gly = genotype (heterozygous for ADRB2 resulting in one arginine and one glycine at amino acid 16), and Gly/Gly = genotype (homozygous for ADRB2 resulting in glycine at amino acid 16). BMI = body mass index; BSA = body surface area; VO<sub>2</sub> = maximal oxygen consumption; VO<sub>2</sub>/KG = maximal oxygen consumption corrected for kilogram. There were no statistically significant differences in demographic data.

**Table 2.** Indices of intensity (mean  $\pm$  standard deviation, N or p-value) during peak exercise

### Indices of Intensity

	N	Mean	SD	p-value
<b>HR (beats/min)</b>				
Arg/Arg	18	188.5	9.89	0.024*
Arg/Gly	24	181.0	9.68	
Gly/Gly	34	186.9	9.43	
Total	76	185.4	9.98	
<b>RPE</b>				
Arg/Arg	18	18.94	0.24	0.039*
Arg/Gly	24	18.56	0.51	
Gly/Gly	34	18.76	0.55	
Total	76	18.74	0.49	
<b>RER</b>				
Arg/Arg	18	1.16	0.015	0.836
Arg/Gly	24	1.15	0.010	
Gly/Gly	34	1.15	0.010	
Total	76	1.15	0.006	
<b>RR (breaths/min)</b>				
	18	43.17	2.10	0.295
Arg/Arg	24	40.84	1.38	
Arg/Gly	34	39.74	1.21	
Gly/Gly	76	40.90	0.86	
Total				

HR = heart rate; RPE = rating of perceived exertion; RER = respiratory exchange ratio;

RR = respiratory rate.

\*p<0.05

## **FIGURE LEGENDS**

### **Figure 1.**

Panels depict the maximum values during peak exercise for A) Watts, B) Relative power (Watts/KG), and C) Muscular efficiency (Watts/VO2). The error bars represent the SE of the mean.

\* $p<0.05$  for Arg/Arg vs Arg/Gly pairwise comparison.

\*\* $p<0.05$  for Arg/Gly vs Gly/Gly pairwise comparison.

### **Figure 2.**

Depicts maximal watts corrected for heart rate at peak exercise. The error bars represent the SE of the mean.

\* $p<0.05$  for Arg/Arg vs Arg/Gly pairwise comparison.

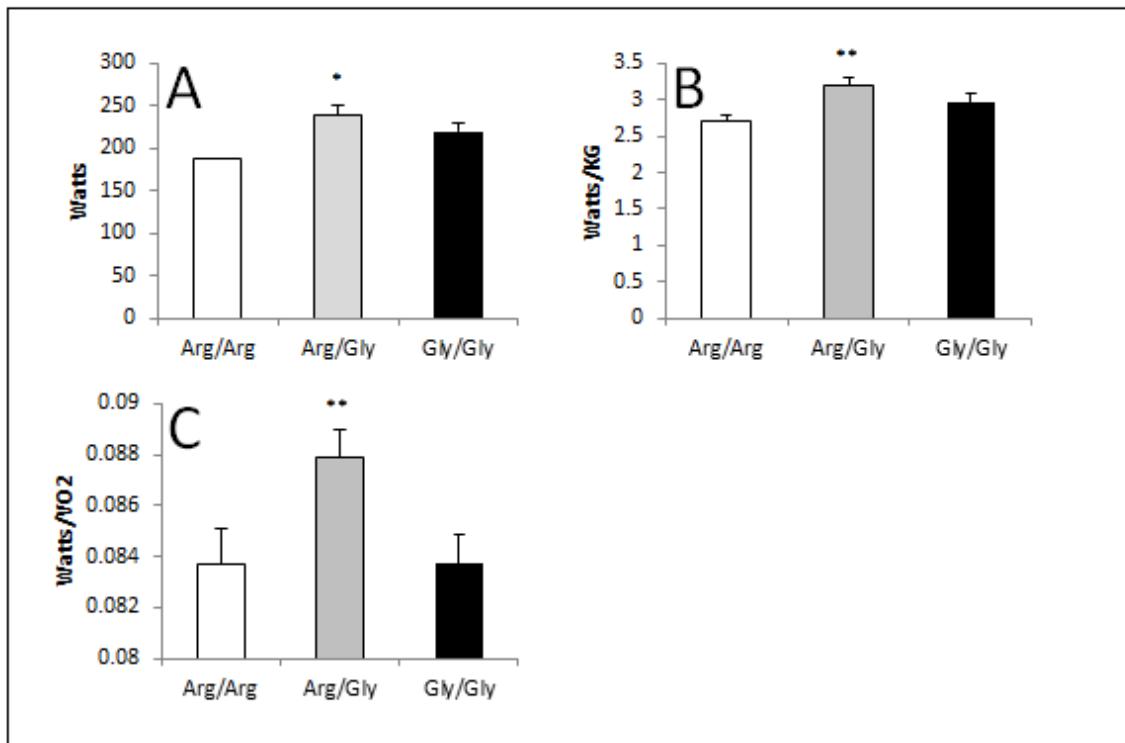
### **Table 1.**

Arg/Arg = genotype (homozygous for ADRB2 resulting in arginine at amino acid 16), Arg/Gly = genotype (heterozygous for ADRB2 resulting in one arginine and one glycine at amino acid 16), and Gly/Gly = genotype (homozygous for ADRB2 resulting in glycine at amino acid 16). BMI = body mass index; BSA = body surface area; VO2 = maximal oxygen consumption; VO2/KG = maximal oxygen consumption corrected for kilogram. There were no statistically significant differences in demographic data.

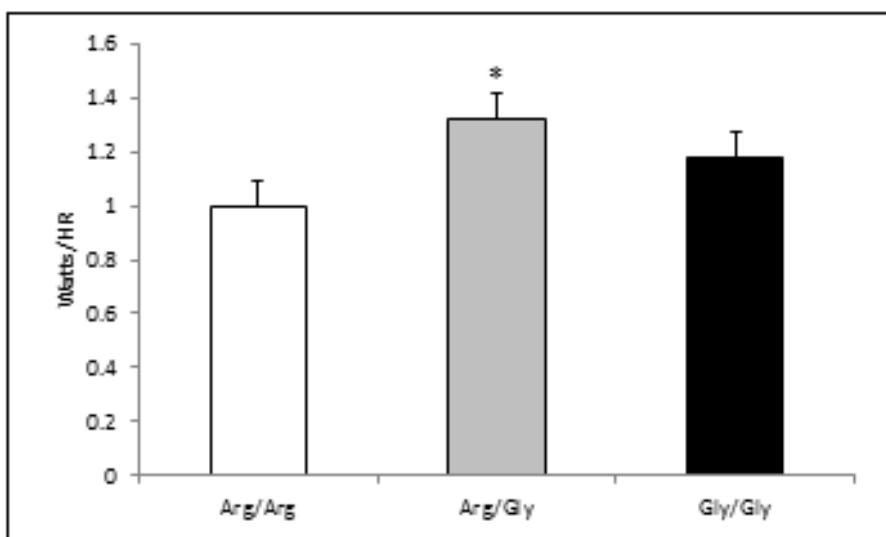
### **Table 2.**

HR = heart rate; RPE = rating of perceived exertion; RER = respiratory exchange ratio; RR = respiratory rate.

\* $p<0.05$

**FIGURES****Figure 1.** Watts, watts/kg and watts/VO<sub>2</sub>: Arg/Arg vs Arg/Gly vs Gly/Gly during peak exercise

**Figure 2.** Watts/hr: Arg/Arg vs Arg/Gly vs Gly/Gly during peak exercise



### 3.3 Influence of Beta-1 Adrenergic Receptor Genotype on Risk of Cardiac Events in Patients with Duchenne Muscular Dystrophy.

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#### ABSTRACT

**Background:** As respiratory care in Duchenne muscular dystrophy (DMD) patients improve, cardiomyopathies are emerging as significant contributors to mortality. The Gly389 polymorphism of the  $\beta_1$ -adrenergic receptor (ADRB1) has been associated with favorable cardiac outcomes and decreased mortality risk in heart failure patients.

**Purpose:** The aim of this study was to determine the influence of ADRB1 genotype on risk of cardiac events in DMD patients.

**Methods:** Data from CINRG-DNHS including 175 DMD patients (ages 3-25 yrs) with up to 9.7 years follow-up were analyzed focusing on ADRB2 functional variants (Arg389,  $n = 80$ ; Gly389,  $n = 95$ ). We performed Cox proportional hazard and Kaplan-Meier time to event analyses for the risk of cardiac events in DMD patients.

**Results:** There were no differences between ADRB1 genotype groups in age, height, weight, number of ambulatory patients, or age of loss of ambulation in our DMD cohort. The Arg389 polymorphism demonstrated a higher mean corticosteroid use compared with the Gly389 polymorphism ( $3.89 \pm 0.50$  yrs vs  $3.03 \pm 0.38$  yrs, respectively,  $P=0.05$ ). There were differences between ADRB1 genotype groups in risk of congestive heart failure or prescription of cardiac medication including inotropic agents, beta-blockers (BB), and idebenone for cardiac issues at any given age. The genotype groups differed significantly in the incidence of diuretics use with the Gly389 polymorphism demonstrating a 5.01-fold increased risk of diuretics use at any age compared with the Arg389 polymorphism.

**Conclusion:** These data suggest ADRB1 genotype may influence the incidence of diuretics use in DMD patients. However, the variability of cardiac management in patients with DMD may confound these findings and explain the lack of findings for other cardiac events included in this analysis.

## 1. INTRODUCTION

As respiratory care for Duchenne muscular dystrophy (DMD) patients improves, an emerging source of morbidity and mortality is cardiomyopathy leading to heart failure (HF) and dangerous arrhythmias [9]. In fact, an estimated 20% of DMD patients will die of congestive HF [6]. Subclinical cardiomyopathies, most typically left ventricular failure, associated with DMD are first evident at ten years of age and are present in 90% of DMD patients over the age of 18 [8]. However, approximately 70% of patients with DMD remain asymptomatic at cardiomyopathy diagnosis [9]. While cardiomyopathy in DMD patients is less often treated at diagnosis than other cardiomyopathies, treatment rates have increased [9]. Unfortunately, it has been demonstrated that the DMD cardiomyopathy has higher mortality rates than other dilated cardiomyopathies [9]. Additionally, there is a concomitant decline in respiratory and cardiac function in DMD patients, either from dependent or independent pathologies [4, 10, 11]. Further, left ventricular dysfunction secondary to cardiomyopathy is a strong predictor of mortality in patients with DMD [7]. Therefore, understanding the factors that affect the incidence of cardiac events in patients with DMD may provide therapeutic targets to delay the development of cardiomyopathy and decrease the risk of mortality in this population.

The cardiac-specific mechanisms of dystrophin deficiency result in disruption of membrane ion channel function, particularly the sarcolemmal stretch-activated channels — further increasing  $\text{Ca}^{2+}$  flux, activating calpains, and increasing cardiomyocyte necrosis [13, 14]. This cardiomyocyte necrosis initiates an inflammatory response and the replacement of cardiomyocyte tissue with fibrofatty tissue [12]. The myocardial fibrosis

associated with DMD, initially appearing in the left ventricular and gradually spreading to encompass the entire ventricle, gradually stretches, becomes thinner, and loses contractility [13]. 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 [13]. Furthermore, previous work has estimated that 71% of 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 [7]. The systolic dysfunction associated with DMD cardiomyopathy can be attributed to two main mechanisms: increased cardiac work and altered  $\text{Ca}^{2+}$  handling [12-14]. Due to the progressive nature of the cardiac involvement in DMD, it is imperative that target pathways are identified to slow cardiomyocyte degradation and decrease the risk of adverse cardiac events.

One pathway that has been identified as capable of preserving cardiac function in cardiomyopathies is the  $\beta_1$ -adrenergic receptor (ADRB1) coupled pathway [16-20]. The ADRB1 subtype is found primarily in the heart, comprising about 80% of total beta adrenergic receptors found in the heart and playing a role in cardiac function [16, 21-23]. Furthermore, ADRB1 activity has been shown to influence cardiac: (i) inotropy, lusitropy, and chronotropy [24, 25]; (ii) nuclear and perinuclear  $\text{Ca}^{2+}$  handling [26]; (iii) reuptake of cytosolic  $\text{Ca}^{2+}$  [27]; and (iv) cardiomyocyte relaxation [22, 28]. Given the above mentioned mechanisms, the ADRB1 may play a role in preserving cardiac function in patients with DMD.

A polymorphism of the ADRB1 that affects functionality includes a glycine (Gly) for arginine (Arg) substitution at amino acid 389 [16, 17, 22, 23]. Specifically, the Gly389 polymorphism demonstrates decreased receptor density and cAMP accumulation and a dampening response to norepinephrine infusion [16, 24, 261]. Functionally, HF patients with the Gly389 polymorphism have significantly lower diastolic, systolic, and mean arterial blood pressure — contributing to improved cardiac function and decreased mortality risk [17-19, 29]. Furthermore, literature demonstrates autoantibodies against ADRB1 are associated with more favorable myocardial recovery in patients with recent-onset cardiomyopathy [20]. The association between the Gly389 polymorphism and more favorable cardiac measures and outcomes in HF patients suggest a therapeutic target for preserving cardiac function and delaying cardiomyopathy in patients with DMD. However, to date, there is no literature investigating the relationship between ADRB1 genotype and cardiac events in DMD patients.

The aim of this study was to investigate the influence of ADRB1 genotype on the incidence of cardiac events in patients with DMD at any given age. Given the ADRB1 Gly389 polymorphism is associated with improved cardiac function and favorable outcomes in HF patients, we hypothesize DMD patients with the Gly389 polymorphism would have a lower incidence of cardiac events when compared with DMD patients with the Arg389 polymorphism.

## 2. METHODS

### 2.1 Participants

Data analyzed for this study were a part of a larger dataset from the Cooperative International Neuromuscular Research Group Duchenne Natural History Study (CINRG-DNHS). All participants included in this study and/or their legal guardians consented/assented specifically to genotyping of genetic variants for research purposes, and the study was approved by local institutional or ethics review boards at each participating institution. One-hundred seventy-five patients with a clinical diagnosis of DMD (ages 3-25 years at entry into the study) were identified and included in the dataset used in this study. Patients were followed for a maximum of 9.7 years. This study focused on a functional ADRB1 protein altering variant at codon 389 employing an Exome Chip (a technique for sequencing all of the protein-coding genes in a genome). Exome Chip genotyping and data cleaning methods in the CINRG-DNHS cohorts have been previously described [3]. Patients were homozygous for arginine (AA,  $n = 11$ ), glycine (GG,  $n = 95$ ), or heterozygous (AG,  $n = 69$ ) at codon 389.

## *2.2 Data Analyses*

Patient age, height, weight, corticosteroid use, and ambulatory status at entry into the study were obtained from the CINRG-DNHS database. All reported patient heights were calculated from ulnar length, as described by others [262]. Corticosteroid use was derived from clinically reported start and stop dates. Cardiac events in the analyses included age of: congestive HF hospitalization, other cardiovascular issues (tachycardia, arrhythmia, and elective admission for cardiac prophylactic), and prescription of cardiovascular medication (i.e. angiotensin converting enzyme inhibitor (ACEI) or angiotensin-receptor

blocker (ARB), diuretics, cardiac inotropic agents, beta-blockers (BBs), idebenone for cardiac issues, and other cardiovascular medications).<sup>9</sup>

### *2.3 Statistical Analyses*

Group demographics were compared using a one-way analysis of variance (ANOVA). Genotype differences in the incidence of cardiac events at any given age were estimated by a Kaplan-Meier analysis and the log-rank test. A Cox proportional hazard (PH) model was used to examine the effects of genotype variant on the incidence of cardiac events in DMD patients, after adjusting for patient height, weight, ambulatory status, and corticosteroid use. An additional Cox PH analysis with the inclusion of clinic site (20 clinic sites) as a covariate was used to examine the variability of medication prescription between clinic sites. Time dependence of the covariates was ruled out after inspection of the Schoenfeld residuals scores [263]. We chose to group genotypes as Gly389 and Arg389 because preliminary analyses of the data demonstrated no difference between polymorphisms containing at least one arginine. This genotype grouping is consistent with previous literature [16-19, 22, 23, 29]. When we collapsed the genotype groups into functionally similar groups, patients were homozygous for glycine (Gly389,  $n = 95$ ) or homozygous for arginine or heterozygous (Arg389,  $n = 80$ ). This genotype grouping improves ease of interpretation. For all covariates entered into the Kaplan-Meier and Cox PH analyses, outliers were identified as observations above or below the 1.5 times interquartile range (IQR). All statistical comparisons were made using a statistical software packages (RStudio; RStudio Inc., Boston, MA, USA, version 1.1.456). Statistical analyses were considered significant if  $P < 0.05$ .

### 3. RESULTS

#### 3.1 Subject Characteristics

There was no difference in age, height, weight, number of patients on corticosteroids, number of ambulatory patients, or age of loss of ambulation between genotype groups (Table 1). There was no significant difference between genotype groups for corticosteroid use in years ( $3.03 \pm 0.38$  vs.  $3.85 \pm 0.50$  for Arg389 and Gly389 respectively).

#### 3.2 Kaplan-Meier Analysis

The Kaplan-Meier analysis did not identify a difference in probability of cardiac events in DMD patients for age of congestive HF and other cardiovascular issues or prescription of cardiovascular medication including: ACEI, ARB, cardiac inotropic agents, BBs, idebenone for cardiac issues, and other cardiovascular medications. For the age of diuretics use, three observations were identified as outliers in a preliminary analysis of the Kaplan-Meier analysis and omitted from further analyses (172 observations remaining). The results of the Kaplan-Meier analysis for age of diuretics use are illustrated in Figure 1. Kaplan-Meier mean and median ages of first use of diuretics are presented in Table 2. The Gly389 polymorphism demonstrated a higher probability ( $P<0.05$ ) for the use of diuretics at any given age compared with the Arg389 polymorphism ( $22.06 \pm 3.12$  yrs. vs.  $19.03 \pm 1.73$  yrs respectively).

#### 3.3 Cox Proportional Hazard

The Cox PH analysis did not identify a difference in the incidence of congestive heart failure or prescription of cardiac medication including inotropic agents, BBs, and idebenone for cardiac issues at any given age. The Cox PH analysis determined a significant difference in the incidence for other cardiac issues and use of ACEI, ARB, and other cardiovascular medications at any given age.

### *3.3.1 Diuretics*

Eleven observations were identified as outliers in a preliminary analysis of the PH analysis and omitted from further analyses (164 observations remaining). The results of the Cox PH are presented in Table 3. Height and weight were strong, negative predictors of the incidence of diuretics use at any given age in patients with DMD ( $P<0.05$ ). ADRB1 genotype was identified as a strong, positive predictor of diuretic use ( $P<0.05$ ). Specifically, DMD patients with the Gly389 polymorphism were 5.01 times more likely to be given diuretics at any given age compared with patients with the Arg389 polymorphism.

### *3.3.2 Other Cardiac Issues*

Twelve observations were identified as outliers in a preliminary analysis of the PH analysis and omitted from further analyses (163 observations remaining). Corticosteroid use was a strong, negative predictor of the incidence of other cardiac issues at any given age in patients with DMD ( $P<0.05$ ).

### *3.3.3 Angiotensin Converting Enzyme Inhibitor and Angiotensin-Receptor Blocker*

Ten observations were identified as outliers in a preliminary analysis of the PH analysis and omitted from further analyses (165 observations remaining). Corticosteroid use and height were strong, negative predictors of the incidence of ACEI and ARB use at any given age in patients with DMD ( $P<0.05$ ).

### *3.3.4 Other Cardiovascular Medications*

Ten observations were identified as outliers in a preliminary analysis of the PH analysis and omitted from further analyses (163 observations remaining). Height was a strong, negative predictors of the incidence of the use of other cardiovascular medications at any given age in patients with DMD ( $P<0.05$ ).

## **4. DISCUSSION**

Our original hypothesis was that DMD patients with the Gly389 polymorphism would have a lower incidence of cardiac events compared with those expressing Arg389 polymorphism. The principle findings of this study were that DMD patients with the Gly389 polymorphism of the ADRB1 had a higher incidence of diuretics use compared with those patients expressing the Arg389 polymorphism. Specifically, patients with the Gly389 polymorphism were 5.01 times more likely to be on diuretics compared with patients with the Arg389 polymorphism at any given age. Furthermore, ADRB1 genotype had no influence on the incidence of other cardiac events included in this study. However, corticosteroid use was a negative predictor of the incidence of other cardiac issues and ACEI and ARB use and height was a negative predictor of the incidence of ACEI, ARB, and other cardiovascular medications use. The present study refutes our

hypothesis and demonstrates DMD patients with the Gly389 polymorphism may have an increased incidence of diuretics use compared with patients with the Arg389 polymorphism and that ADRB1 genotype has no influence on other cardiac events in these patients.

As respiratory management in DMD patients improves, the development of cardiomyopathies and the accompanying left ventricular dysfunction is emerging as a significant contributor to mortality in this population [6-8]. While cardiomyopathies are present in 90% of DMD patients over the age of 18, only 30% of patients present with symptoms at the time of diagnosis [8, 9]. Furthermore, a majority of DMD patients with normal left ventricular function exhibited dysfunctional myocardial strain at the posterolateral wall (the initial site of fibrofatty deposition in myocardium), suggesting abnormal myocardial contraction before left ventricular decompensation [13, 264]. The cardiomyopathy associated with DMD has a higher mortality than other dilated cardiomyopathies but often remain less treated at diagnosis [9]. Further, left ventricular dysfunction secondary to cardiomyopathy is a strong predictor of mortality in patients with DMD [7]. Therefore, understanding the factors that affect the incidence of cardiac events in patients with DMD may provide therapeutic targets to delay the development of cardiomyopathy and decrease the incidence of mortality in this population.

The multifactorial nature of the cardiac involvement in DMD necessitates a battery of therapy options to treat cardiomyopathy symptoms and limit the side-effect profile [82]. Both the DMD Care Considerations Working Group and the European Society of Cardiology guidelines recommend ACEI as first-line therapy for dilated cardiomyopathy

and/or congestive heart failure in DMD patients [82, 91, 101]. However, the side-effect profile of ACEI, such as cough, renal dysfunction, hyperkalemia, and angioedema, led to the testing of ARBs as a replacement therapy for cardiac dysfunction [265-267]. These findings have led to ACEIs and ARBs becoming more mainstream therapy in patients with DMD [9]. Additionally, the American Academy of Pediatrics recommends considerations to be given to the use of diuretics in the treatment of cardiomyopathy in DMD patients [97]. Secondary to cardiomyopathy, DMD patients also present with tachycardia and other arrhythmias [98]. These additional complications present further clinical care considerations and signal the emergence of extensive fibrofatty deposition and conduction disorders [268]. Additional therapies used to address these clinical considerations in our cohort included  $\text{Ca}^{2+}$  channel blockers, blood thinners, and cholesterol lowering drugs.

Current research and clinical findings in the literature has led investigators to suggest the use of diuretics to treat tachycardia and lipothymia in later stages of cardiac involvement in DMD patients [98]. Despite the efficacy of diuretic therapy in the treatment of HF and recommendations for diuretic considerations in DMD patients, clinical adoption is slow [99]. In our study population, only 18 patients were prescribed a diuretic at any point (14 and four for Gly389 and Arg389 respectively). However, the Cox PH analysis determined genotype, height, and weight to be strong predictors of diuretic use in DMD patients. Specifically, DMD patients with the Gly389 polymorphism were approximately 5 times more likely to be on diuretics compared with patients with the Arg389 polymorphism at any given age.

In light of the burden of literature demonstrating the beneficial influence of the Gly389 polymorphism in cardiac outcomes and mortality in HF patients, the contradictory findings of this study may be difficult to explain. One explanation is the lack of widespread clinical adoption of specific guidelines for treatment of cardiomyopathy in patients with DMD. Despite a greater appreciation for the clinical importance of DMD cardiomyopathy, cardiac management strategies are highly variable and remain underutilized in this population [9, 100]. Currently, there is no consensus on the proper pharmacological therapy class and timing for treatment of cardiomyopathy in DMD patients [101]. This is demonstrated by the Cox PH model including clinic site as an additional covariate with 5 clinic sites differing significantly in the prevalence of diuretics prescription at any given age. Given this, the variability of cardiac management for DMD patients in different clinics may explain these contradictory findings.

#### *Other factors influencing incidence of cardiac events in DMD patients*

The Cox regression analysis also demonstrated that corticosteroid use and height were significant predictors of diuretics use in DMD patients. Firstly, it is not surprising that corticosteroid use decreases the incidence of diuretics use in our cohort, given that corticosteroid use has been demonstrated to delay the emergence of cardiomyopathy and decrease cardiac-associated mortality [10]. That height was a negative predictor of diuretics use is harder to explain; height has been positively correlated with cardiac function, but this relationship has not been demonstrated in DMD patients [269]. Furthermore, the Cox regression analysis demonstrated that corticosteroid use was a significant predictor of ACEI and ARB use, and was a significant predictor of the

incidence of presenting with other cardiac issues (i.e. tachycardia, arrhythmia, and elective admission for cardiac prophylactic). Height was also identified as a strong predictor of ACEI, ARB, and other cardiac medications use in our cohort.

### *Limitations*

Our cohort included DMD patients who were homozygous for arginine (AA,  $n = 11$ ), glycine (GG,  $n = 95$ ), or heterozygous (AG,  $n = 69$ ) at codon 389 and were collapsed into two study groups (Gly389,  $n = 95$  and Arg389,  $n = 90$ ). This even genotype distribution is surprising given the frequency in the healthy population (0.70 and 0.30 for Arg389 and Gly389 respectively) [270]. However, there is no data available regarding ADRB1 389 genotype frequency in a DMD population. Furthermore, the DNHS protocol did not specify when clinicians should prescribe diuretics to a patient. The variability of diuretic prescription between clinicians is demonstrated by the influence of clinic site on the prevalence of diuretics prescription. A second consideration is the number of events in our data. It is generally recommended that for each covariate, a minimum of 10 events are needed to ensure statistical power. In our analysis including 5 variables, 50 events are needed. Our cohort only had 18 DMD patients who were prescribed diuretics, violating this rule. However, literature has demonstrated a range of circumstances in which coverage and bias standards were adequate with fewer than 10 events per variable, suggesting the rule of 10 events per variable may be relaxed [271]. Another consideration is the longitudinal nature of the study. As such, the data acquisition utilized a rolling accrual, resulting in left-truncated data, which may influence the accuracy of the Cox model to identify the age of first diuretics use and therefore the incidence of diuretics use.

However, the aim of this study was to determine the influence of ADRB1 genotype on the incidence of diuretics use and there is no reason to believe genotype as a covariate would influence the model's accuracy to identify the timing of first use of diuretics in our cohort.

## **5. CONCLUSION**

The findings of this study suggest that DMD patients with the Gly389 polymorphism have a significantly higher incidence of diuretics use compared with patients expressing the Arg389 polymorphism. Specifically, the Gly389 polymorphism is 5 times more likely to use diuretics compared with the Arg389 polymorphism. However, there remains high variability in the type and timing of implementation of cardiac management therapies for DMD patients. This variability may present complications in studies of this nature in the future. With cardiomyopathies continuing to emerge as a significant contributor to mortality and the related decline in cardiac and respiratory function, it is important to identify the factors that contribute to cardiac dysfunction in DMD patients. Therefore, it is important that clinical guidelines are established and clinically adopted regarding cardiac management in the DMD population.

## **6. ACKNOWLEDGMENTS**

The authors are sincerely grateful for the patients that dedicated their time and agreed to participate in the collection of data used in this study. We would also like to thank all CINRG-DNHS personnel and the participating clinics for the collection and distribution of this data. This study could not have been done without their participation.

**FIGURE CAPTIONS**

**Figure 1. Cumulative risk of diuretics use in Duchenne muscular dystrophy (DMD) patients stratified by  $\beta_1$ -adrenergic receptor genotype (Arg389 and Gly389).** The risk incidence of diuretics use in DMD patients was calculated from the survival curve produced by the Kaplan-Meier analysis.

**Figure 2. The cumulative risk of diuretics use in Duchenne muscular dystrophy (DMD) patients stratified by  $\beta_1$ -adrenergic receptor genotype (Arg389 and Gly389).** The cumulative risk function was obtained from Cox regression modeling of the risk of diuretics use, where genotype group, ambulatory status, corticosteroid-use, and height were entered into the model as covariates. The risk curve was produced for each genotype variant by holding all other covariates in the Cox model constant at their respective means (Height = 140.87cm, weight = 42.99kg, corticosteroid use = 3.4yrs, ambulatory status = 0.55).

**TABLES****Table 1. Subject characteristics, corticosteroid use status, corticosteroid use in years, ambulatory status, and age of loss of ambulation.**

		N (%)	Mean	SE	<i>p-value</i>
<b>Age (yrs)</b>					
	Arg389	—	12.72	0.69	0.55
	Gly389	—	12.19	0.55	
	Total	—	12.43	0.44	
<b>Height (cm)</b>					
	Arg389	—	140.19	2.45	0.70
	Gly389	—	141.44	2.29	
	Total	—	140.86	1.67	
<b>Weight (kg)</b>					
	Arg389	—	42.78	2.57	0.91
	Gly389	—	43.18	2.31	
	Total	—	42.99	1.71	
<b>Corticosteroid Use Status and Corticosteroid Use (yrs)</b>					
	Arg389	57 (71%)	3.85	0.50	0.05
	Gly389	65 (68%)	3.03	0.38	
	Total	122 (70%)	3.40	0.31	
<b>Ambulatory Status and Age of Loss of Ambulation (yrs)</b>					
	Arg389	46 (58%)	9.97	0.57	0.98
	Gly389	51 (54%)	9.99	0.49	
	Total	97 (55%)	9.98	0.37	

SE: standard error; Arg389: patients who were homozygous for the  $\beta_1$ -adrenergic receptor (ADRB2) resulting in an arginine substitution at amino acid 389 ( $n = 90$ ), Gly389: patients who were homozygous or heterozygous for the  $\beta_1$ -adrenergic receptor (ADRB1) resulting in at least one glycine substitution at amino acid 389 ( $n = 95$ ); N are reported as number of non-ambulatory patients and patients on corticosteroids and percent of the total study population.

**Table 2. Kaplan-Meier mean and median ages at first use of diuretics in patients with Duchenne muscular dystrophy (DMD).**

	Mean Age			Median Age		
	Estimate (yrs)	SE	95% CI	Estimate (yrs)	SE	95% CI
<b>Arg389</b>	22.06*	3.12	15.94 – 28.18	22.99*	2.07	18.94 – 27.03
<b>Gly389</b>	19.03	1.73	16.09 – 21.97	16.86	0.78	15.33 – 18.38
	19.70	1.49	16.76 – 22.64	18.47	1.01	16.49 – 20.44
<b>Overall</b>						

SE: standard error; CI: confidence interval; Arg389: patients who were homozygous for the  $\beta_1$ -adrenergic receptor (ADRB1) resulting in an arginine substitution at amino acid 389 ( $n = 90$ ), Gly389: patients who were homozygous or heterozygous for the  $\beta_1$ -adrenergic receptor (ADRB1) resulting in at least one glycine substitution at amino acid 389 ( $n = 95$ ); \* $P < 0.05$

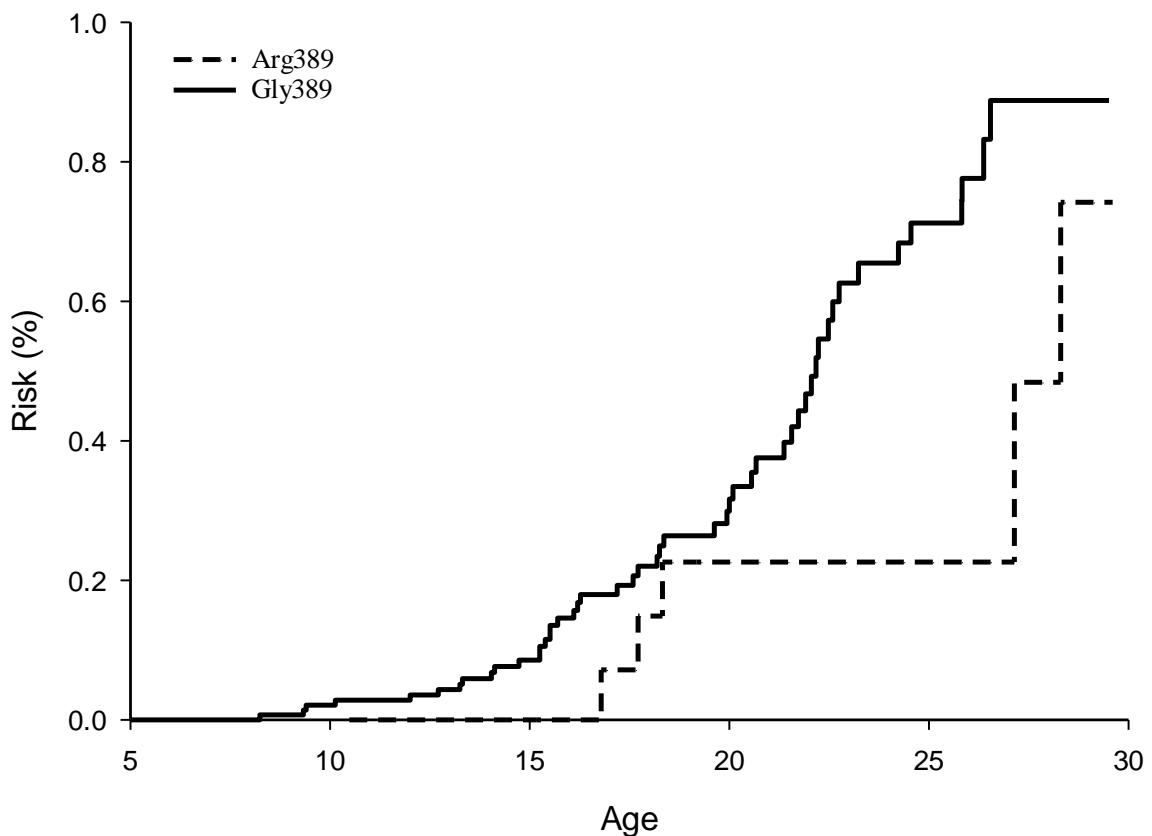
**Table 3.** Cox regression analysis for risk of diuretics use at any given age in patients with Duchenne muscular dystrophy (DMD).

	<b>β</b>	<b>SE</b>	<b>Wald</b>	<b>p-value</b>	<b>HR</b>	<b>95% CI</b>
Genotype	1.61	0.78	2.06	<0.05	5.01	1.08 – 23.2
Ambulatory Status	0.23	0.85	0.27	0.79	1.26	0.24 – 6.65
Corticosteroid Use (yrs)	–	0.09	-1.91	0.06	0.84	0.70 – 1.00
Height (cm)	–	0.04	-3.54	<0.05	0.88	0.83 – 0.95
Weight (kg)	0.07	0.02	2.83	<0.05	1.07	1.02 – 1.13

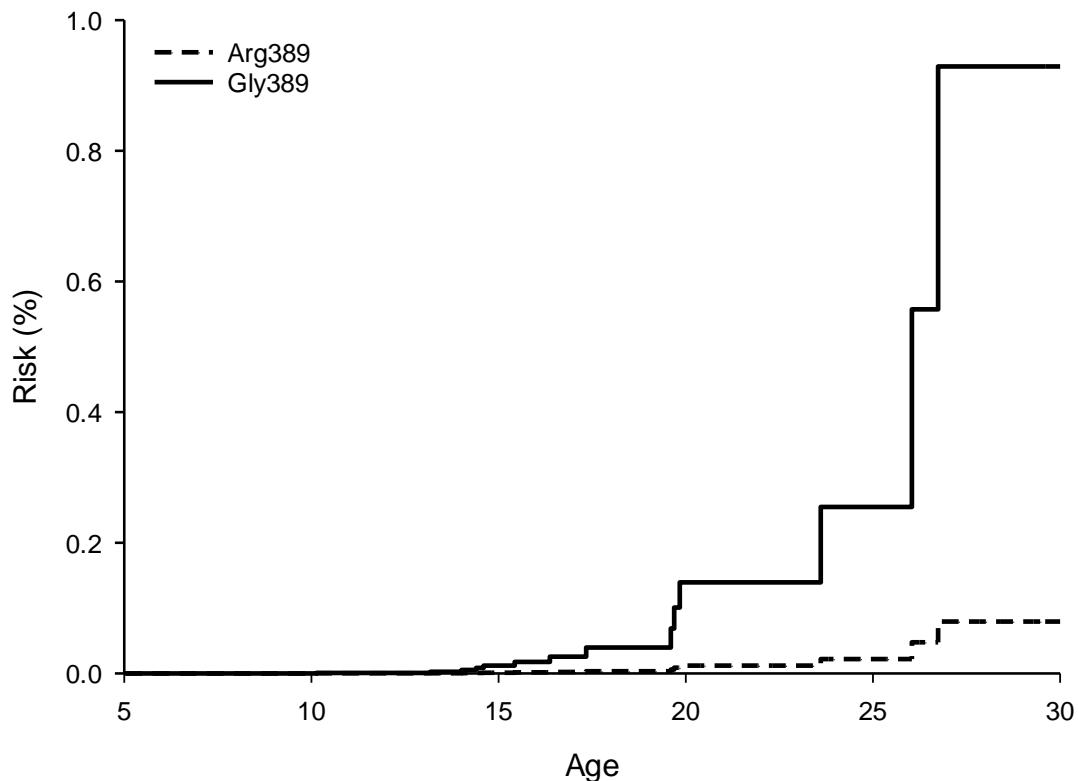
SE: standard error; HR: hazard ratio; CI: confidence interval; genotype was coded as: 0 = Arg389: patients who were homozygous for the  $\beta_1$ -adrenergic receptor (ADRB2) resulting in an arginine substitution at amino acid 389 ( $n = 90$ ), 1 = Gly389: patients who were homozygous or heterozygous for the  $\beta_1$ -adrenergic receptor (ADRB1) resulting in at least one glycine substitution at amino acid 389 ( $n = 95$ ); ambulatory status was coded as: 0 = non-ambulatory, 1 = ambulatory; there was a significant influence of genotype, height, and weight on risk of diuretics use.

**FIGURES**

**Figure 1. Cumulative risk of diuretics use in Duchenne muscular dystrophy (DMD) patients stratified by  $\beta_1$ -adrenergic receptor genotype (Arg389 and Gly389).**



**Figure 2. The cumulative risk of diuretics use in Duchenne muscular dystrophy (DMD) patients stratified by  $\beta_1$ -adrenergic receptor genotype (Arg389 and Gly389).**



### 3.4 Influence of Beta-2 Adrenergic Receptor Genotype on Time to Ventilatory Assistance in Duchenne Muscular Dystrophy

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#### ABSTRACT

The purpose of this study was to identify the influence of beta-2 adrenergic receptor (ADRB2) genotype on the risk of nocturnal ventilatory use in Duchenne muscular dystrophy (DMD). Data from CINRG-DNHS including 175 DMD patients (3-25 yrs) with up to 9.7 years follow-up were analyzed focusing on ADRB2 genotype variants. Time-to-event analyses were used to examine differences in the age at “first use” of nocturnal ventilation (NV) differed between genotypes. There were no differences between genotype groups in age, height, weight, corticosteroid use, or number of ambulatory patients, or age of loss of ambulation. DMD patients expressing the Gly16 polymorphism had a significantly ( $P<0.05$ ) lower mean age at “first use” of NV compared with those patients expressing the Arg16 polymorphism ( $21.80\pm0.59$  yrs vs  $25.91\pm1.31$  yrs, respectively). In addition, a covariate-adjusted Cox model revealed that the Gly16 variant group possessed a 2.77-fold higher risk of using NV at any given age compared with the Arg16 polymorphism group. These data suggest that genetic variations in the ADRB2 gene may influence the age at which DMD patients are first prescribed NV , whereby patients with the Gly16 polymorphism are more likely to require NV assistance at an earlier age than their Arg16 counterparts.

## 1. INTRODUCTION

It is estimated that 32% of Duchenne muscular dystrophy (DMD) patients suffer from nighttime alveolar hypoventilation as a result of sleep-disordered breathing [3]. Sleep-disordered breathing in this population is a result of respiratory weakness, and is associated with cardiac morbidity, neurocognitive deficits, and impaired lung function [3, 30]. As disease status worsens, the progressive weakening of respiratory muscles results in a loss of sufficient respiratory pressure generation to maintain adequate alveolar ventilation, leading to constant alveolar hypoventilation [31]. Sleep-disordered breathing tends to precede daytime hypoventilation in patients with DMD, the latter of which is a significant contributor to hospitalization and mortality rates in this population [116]. The age at first use of NV is therefore considered an important clinical milestone in these patients, and indicates significant respiratory derangement [3, 4]. It is therefore important to understand the factors which affect the risk of NV use, such that we may identify novel therapeutic interventions to delay its prescription, and offset the progression to day-time ventilator assistance, ultimately decreasing hospitalization and mortality rates, and improving quality-of-life.

Respiratory muscle weakness in DMD is characterized by a progressive loss in the ability to generate respiratory pressures, resulting in severe ventilatory derangements [33, 34]. Decreases in respiratory pressure and airflow generation can be attributed to a primary weakness of the diaphragm secondary to intramuscular remodeling in DMD [33]. This remodeling is characterized by an increased resting diaphragm thickness due to an

infiltration and deposition of non-contractile elements (pseudo-hypertrophy), concomitant with the loss of sarcomeres in series [35, 36]. Diaphragm pseudo-hypertrophy, and the loss of sarcomeres in series, together adversely affect the length-tension relationship and contractility of the diaphragm [35]. Due to the progressive nature of respiratory weakness in DMD, it is imperative that therapeutic targets are identified to slow respiratory muscle degradation, and thus decrease the risk of NV prescription.

One novel pathway that has been identified as capable of attenuating skeletal muscle degradation in DMD is the  $\beta_2$ -adrenergic receptor (ADRB2) coupled pathway [42, 43, 45, 46].  $\beta_2$ -adrenergic receptors influence bulk muscle size, strength and regeneration [12, 13]. ADRB2 stimulation has also been shown to: (i) increase diaphragmatic cross-sectional area, strength, and contractility [43, 45, 46]; (ii) improve mucociliary clearance [47, 50, 51]; (iii) inhibit inflammatory pathways [53, 54, 56, 58, 60]; and to (iv) inhibit calpain activity and decrease concentration [62, 63, 65, 66, 69]. In light of the above, the functionality of ADRB2 may play a significant role in the protection of respiratory muscles from dystrophic pathways, and provide a novel therapeutic target in DMD to delay the use of NV.

A polymorphism of the ADRB2 that improves receptor functionality includes the substitution of glycine (Gly) for arginine (Arg) at amino acid 16. The Gly16 polymorphism is globally expressed, has a higher receptor density, and is more resistant to receptor down-regulation than its Arg16 counterpart [202, 272]. The Gly16 polymorphism is associated with sustained bronchodilation at rest and following intense exercise, and improved lung function in healthy pediatric patients and patients with heart

failure [29, 273, 274]. The association between the Gly16 polymorphism and improved lung function in healthy and diseased populations suggests a possible therapeutic target for preserving respiratory function and the delaying of NV usage in DMD patients. However, to date, no studies have investigated the influence of ADRB2 genotype and the risk of NV use in this clinical population.

The aim of this study was to examine the influence of ADRB2 genotype on the risk of NV prescription in patients with DMD. Given that ADRB2 stimulation may provide several benefits to respiratory function (e.g., increased diaphragmatic contractility, improved mucociliary clearance, inhibited inflammatory pathways and calpain activity), we hypothesized that DMD patients with the more “functional” ADRB2 polymorphism (i.e., Gly16) would demonstrate a lower risk of NV use compared with DMD patients expressing the less functional polymorphism (i.e., Arg16).

## 2. METHODS

### 2.1 Participants

Data analyzed for this study were a part of a larger dataset from the Cooperative International Neuromuscular Research Group Duchenne Natural History Study (CINRG-DNHS). All participants included in this study and/or their legal guardians consented specifically to genotyping of genetic variants for research purposes, and the study was approved by local institutional or ethics review boards at each participating institution. One-hundred seventy-five patients with a clinical diagnosis of DMD (ages 3-25 years at entry into the study) were identified and included in the dataset used in this study.

Patients were followed for a maximum of 9.7 years. This study focused on a functional ADRB2 protein altering variant at codon 16 employing an Exome Chip (a technique for sequencing all of the protein-coding genes in a genome). Exome Chip genotyping and data cleaning methods in the CINRG-DNHS cohorts have been previously described [275]. Patients were homozygous for arginine (AA,  $n = 26$ ), glycine (GG,  $n = 59$ ), or heterozygous (AG,  $n = 90$ ) at codon 16.

## *2.2 Data Analyses*

Patient age, height, weight, corticosteroid use, and ambulatory status at entry into the study were obtained from the CINRG-DNHS database. All reported patient heights were calculated from ulnar length, as described by others [262]. Corticosteroid use was derived from clinically reported start and stop dates. Nocturnal ventilation status was determined by the clinic visit at which full-time NV (e.g., Bi-PAP mask,  $n = 40$ ; Bi-PAP nasal pillows,  $n = 9$ ; C-PAP,  $n = 3$ ; and mouthpiece,  $n = 3$ ) was first prescribed to the patient.

## *2.3 Statistical Analyses*

Group demographics were compared using a one-way analysis of variance (ANOVA). To investigate differences among the specific genotype groups, a Tukey honest significant difference (HSD) post-hoc comparison was used. Genotype differences in the risk of NV use at any given age were estimated by a Kaplan-Meier analysis and the log-rank test. A Cox proportional hazard (PH) model was used to examine the effects of genotype variant

on the risk of NV in DMD patients, after adjusting for patient height, weight, ambulatory status, and corticosteroid use. Time dependence of the covariates was ruled out after inspection of the Schoenfeld residuals scores [263]. We chose to group ADRB2 genotypes as homozygous for arginine at amino acid spot 16 (Arg16,  $n = 26$ ) and homozygous or heterozygous for ADRB2 resulting in at least one glycine at amino acid 16 (Gly16,  $n = 149$ ) because preliminary analyses of the data demonstrated no difference between polymorphisms containing at least one glycine. This genotype grouping improves ease of interpretation and is consistent with previous literature [273, 274, 276]. For all covariates entered into the Kaplan-Meier and Cox PH analyses, outliers were identified as observations above or below the 1.5 times interquartile range (IQR). All statistical comparisons were made using a statistical software packages (RStudio; RStudio Inc., Boston, MA, USA, version 1.1.456). Statistical analyses were considered significant if  $P < 0.05$ .

### 3. RESULTS

#### 3.1 Subject Characteristics

There were no differences in age, height, weight, corticosteroid use, number of ambulatory patients, or age of loss of ambulation between genotype groups (Table 1). Further, there was no difference in age at loss of ambulation between Gly16 and Arg16 genotype groups ( $10.96 \pm 0.23$  yrs vs  $11.93 \pm 0.75$  yrs, respectively). A total of 78 patients were non-ambulatory at entry into the study, consisting of 65 patients in the Gly16 group,

and 13 patients in the Arg16 group (72% and 50% of the genotype sample populations, respectively).

### *3.2 Kaplan-Meier Analysis*

Two observations were identified as outliers in a preliminary analysis of the Kaplan-Meier analysis, and were omitted from further analyses (173 observations remaining). The results of the Kaplan-Meier analysis are illustrated in Figure 1. The mean and median age at first use of NV are presented in Table 2. DMD patients with the Gly16 polymorphism demonstrated a higher risk ( $P<0.05$ ) of use of NV at any given age compared with those patients expressing the Arg16 polymorphism ( $21.80\pm0.59$  yrs vs  $25.91\pm1.31$  yrs, respectively).

### *3.3 Cox Proportional Hazard*

Eleven observations were identified as outliers in a preliminary analysis of the Cox PH model. These outliers were omitted from further analyses (164 observations remaining). The results of the Cox PH analysis are presented in Table 3. Ambulatory status, corticosteroid use and patient height were strong, negative predictors of the risk of NV assistance in patients with DMD at any given age ( $P<0.05$ ). In contrast, ADRB2 genotype was identified as a strong, *positive* predictor of NV use in DMD patients ( $P<0.05$ ). Specifically, those DMD patients with the Gly16 genotype variant were approximately 2.77 times more likely to be given NV assistance at any given age than those patients with the Arg16 polymorphism.

#### 4. DISCUSSION

The original hypothesis of this study was that DMD patients with the Gly16 polymorphism would have a reduced risk of using NV at any given age compared with those patients expressing the Arg16 polymorphism. Our rationale for this hypothesis was constructed on the basis that ADRB2 stimulation may: increase diaphragmatic cross-sectional area, strength, and contractility [43, 45, 46]; improve mucociliary clearance [47, 50, 51]; inhibit inflammatory pathways [53, 54, 56, 58, 60]; and inhibit calpain activity and decrease concentration [62, 63, 65, 66, 69]. As such, it was expected that possessing the “functional” ADRB2 genotype (i.e., Gly16) would confer a positive effect on retarding the progressive loss of respiratory function and, by extension, delaying the age at first use of NV in DMD patients. Our results did not support this hypothesis. Instead, the present study demonstrated DMD patients with the Gly16 polymorphism display an almost 3-fold *increase* in the risk of NV than those with the Arg16 variant. Certainly, our finding that the Gly16 polymorphism confers a higher (not lower) risk of NV use is difficult to explain at first. To this end, we propose two potential explanations for these unexpected findings; namely that the Gly16 polymorphism may (i) increase the rate of contraction-induced injuries and (ii) may increase intracellular  $[Ca^{2+}]$ .

ADRB2 stimulation increases myofibril expression of type IIa myosin heavy chain isoform in animal models, promoting a fiber-type shift from slow-oxidative to more fast-glycolytic muscle fibers [277, 278]. Type II fibers have an increased peak contractile strength compared with type I fibers [279]. Indeed, healthy adults expressing the Gly16 polymorphism often display increased muscular strength and power relative to their

Arg16 counterparts [276]. While it may seem at first that increased strength is beneficial for respiratory muscle fatigue and weakness, the augmentation of contractile force likely increases the number, and rate of contraction-induced injurious events in DMD patients. Dystrophin-deficient muscle fibers lack adequate membrane stability to efficiently distribute forces associated with myofibril contraction across the sarcolemma [2]. As a result, dystrophin-deficient muscle fibers are highly susceptible to contraction-induced injuries — a significant component of muscle fiber degradation in DMD [280]. Further, the type I to type II fiber type shift renders the muscle fiber more susceptible to eccentric contraction-induced damage, and increased fatigability [281, 282]. In light of the above, it is at least conceivable that the Gly16 polymorphism, by increasing the rate of contraction-induced injuries, hastens the progression of respiratory muscle weakness in DMD patients, leading to a higher risk of NV use than those patients expressing the Arg16 polymorphism.

A secondary explanation for our current findings is that DMD patients with the Gly16 polymorphism, by virtue of increased ADRB2 activity [202, 272], may have incurred additional skeletal muscle damage due to relatively higher intracellular concentrations of  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]$ ). Certainly, ADRB2 stimulation enhances  $\text{Ca}^{2+}$  efflux from the sarcoplasmic reticulum via a G-protein linked pathway [250], promoting a rise in intracellular  $[\text{Ca}^{2+}]$ . This facilitatory effect of ADRB2 stimulation on rising intracellular  $[\text{Ca}^{2+}]$  is amplified by the abnormally low gene expression of regucalcin in DMD patients, i.e., the principal  $\text{Ca}^{2+}$ -binding protein in the diaphragm [105]. Further to the above, the potentially higher rate of contraction-induced injuries in DMD patients with the Gly16 polymorphism (see

explanation above) may promote further  $\text{Ca}^{2+}$  influx into the cytosol from the extracellular space [40]. It is emphasized that increased intracellular  $[\text{Ca}^{2+}]$  promotes calpain activity, upregulating proteolytic cellular damage and myofibril degradation [105, 250]. As such, we speculate that the Gly16 polymorphism is associated with poorer intracellular  $[\text{Ca}^{2+}]$  homeostasis and greater calpain activity, the result of which may hasten the onset of respiratory muscle weakness, leading to a higher risk of NV use than those patients expressing the Arg16 polymorphism.

#### *Implications of our findings*

Pilot studies have shown an improvement in isometric knee-extensor strength and manual muscle test scores in DMD patients following 12 weeks of ADRB2 agonist treatment [283]. A larger follow-up study also demonstrated that ADRB2 agonist treatment improves lean body mass and time to walk/run 30 ft. in patients with DMD [284]. Importantly, however, both study populations consisted of young DMD patients whose ages ranged between 5 to 11 years. We emphasize here that although our findings demonstrate that the Gly16 polymorphism confers a higher risk of NV use at any given age, the absolute difference in risk between Gly16 and Arg16 groups was marginal for patients aged between 5 to 11 years (Figure 2B). Indeed, the absolute risk difference between genotype groups was less than 1.4% up to 15 years of age in our cohort of DMD patients. The difference in risk of NV between groups widened thereafter to reach >40% at patient ages of 25 years and older. One may infer from these observations that the increase in risk of NV use in the Gly16 compared with the Arg16 polymorphism is relatively trivial below the adolescent years, and becomes more important when DMD

patients enter the second decade of life. Further studies are needed to examine whether the effects of the Gly16 polymorphism on respiratory outcomes in DMD are indeed dependent on patient age.

#### *Other factors influencing risk of nocturnal ventilation in DMD*

Our Cox regression analysis demonstrated that ambulatory status, corticosteroid-use, and height were also significant predictors of the risk of NV in DMD patients. Firstly, it is not surprising that ambulatory status decreased the risk of NV in our cohort, seeing that DMD patients who are ambulatory typically display a better clinical prognosis [285]. Secondly, it is expected that height should confer a protective effect on the risk of NV use in DMD, given that height is strongly correlated with vital capacity in these patients [33, 286]. However, that a greater corticosteroid-use also reduced the risk of NV is harder to explain. It is possible that a greater corticosteroid usage delayed the loss of ambulation and emergence of cardiomyopathy, reduced the risk of scoliosis, together preserving respiratory function for a longer period of time in our cohort of patients [285].

#### *Methodological considerations*

Our cohort included DMD patients who were homozygous for arginine (AA,  $n = 26$ ), glycine (GG,  $n = 59$ ), or heterozygous (AG,  $n = 90$ ) at codon 16 and were collapsed into two study groups (Arg16,  $n = 26$  and Gly16,  $n = 149$ ). While it is certain that this distribution is not representative of a typical *healthy* population (0.40 and 0.60 for Arg16 and Gly16 respectively), no data is available regarding ADRB2 16 genotype distribution in the DMD population [287]. Thus, we do not currently know whether the genotype

frequency observed in our cohort is representative of the wider DMD patient population. A second consideration is the retrospective nature of the data and the rolling accrual of patients used for data collection. As a result, our cohort did include left-censored data which may influence the accuracy of the Cox model to identify the age of first NV use and therefore the risk of NV use. However, the aim of this study was to determine the influence of ADRB2 genotype on the risk of NV use. At present, we do not believe that genotype status, *per se*, would have influenced the model's accuracy to identify the timing of first NV use.

## 5. CONCLUSIONS

The findings of the present study indicate that DMD patients with the Gly16 ADRB2 polymorphism are almost 3 times more likely to require NV assistance at a given age compared with those patients expressing the Arg16 polymorphism. It is emphasized, however, that the absolute difference in risk of NV between genotypes is relatively trivial (<2%) below the age of 15 years. Despite prior speculation that ADRB2 stimulation may be a useful therapeutic approach, our data suggest that ADRB2 agonist treatment may be inappropriate for post-adolescent DMD patients.

## 6. ACKNOWLEDGEMENTS

The authors are sincerely grateful for the patients that dedicated their time and agreed to participate in the collection of data used in this study. We would also like to thank all CINRG-DNHS personnel and the participating clinics for the collection and distribution

**FIGURE CAPTIONS**

**Figure 1. The cumulative risk of nocturnal ventilation in Duchenne muscular dystrophy (DMD) patients stratified by  $\beta_2$ -adrenergic receptor genotype (Arg16 and Gly16).** The cumulative risk of nocturnal ventilation in DMD patients was calculated from the survival curve produced by the Kaplan-Meier analysis.

**Figure 2. The cumulative risk (A) and absolute difference in risk (B) of nocturnal ventilation in Duchenne muscular dystrophy (DMD) patients stratified by  $\beta_2$ -adrenergic receptor genotype (Arg16 and Gly16).** The cumulative risk function was obtained from Cox regression modeling of the risk of nocturnal ventilation, where genotype group, ambulatory status, corticosteroid-use, and height were entered into the model as covariates. The risk curves in panels A and B were produced for each genotype variant by holding all other covariates in the Cox model constant at their respective means (Height = 141 cm; Mass = 43 kg; Corticosteroid-use = 3.4 yrs; Ambulatory status = 0.55). The reference lines in Panel B denote the absolute difference in risk between genotype variants at the ages of 15, 20 and 25 years of age. Note that the difference in risk of NV between Arg16 and Gly16 genotype groups is marginal (<1%) at ages below 15, yet widens greatly to over 40% by the age of 25 years.

**Table 1.** Subject characteristics.

		Mean	SE	<i>p-value</i>
<b>Age (yrs)</b>				
	Arg16	12.97	1.23	0.61
	Gly16	12.34	0.47	
	Total	12.43	0.44	
<b>Height (cm)</b>				
	Arg16	138.85	4.27	0.62
	Gly16	141.22	1.82	
	Total	140.87	1.67	
<b>Weight (kg)</b>				
	Arg16	42.91	4.12	0.98
	Gly16	43.01	1.89	
	Total	42.99	1.71	
<b>Corticosteroid Use (yrs)</b>				
	Arg16	3.47	0.72	0.93
	Gly16	3.39	0.34	
	Total	3.40	0.31	

SE: standard error; Arg16: patients who were homozygous or heterozygous for the  $\beta_2$ -adrenergic receptor (ADRB2) resulting in at least one arginine substitution at amino acid 16 ( $n = 26$ ); Gly16: patients who were homozygous for ADRB2 resulting in a glycine substitution at amino acid 16 ( $n = 149$ ).

**Table 2.** Kaplan-Meier mean and median ages at first use of nocturnal ventilation (NV) in patients with Duchenne muscular dystrophy (DMD).

	Mean Age			Median Age		
	Estimate (yrs)	SE	95% CI	Estimate (yrs)	SE	95% CI
<b>Arg16</b>	25.91*	1.31	23.35 – 28.49	28.30*	4.14	20.19 – 36.41
<b>Gly16</b>	21.80	0.59	20.86 – 23.42	22.17	0.40	21.38 – 22.96
<b>Overall</b>	22.71	0.62	21.49 – 23.93	22.48	0.39	21.72 – 23.24

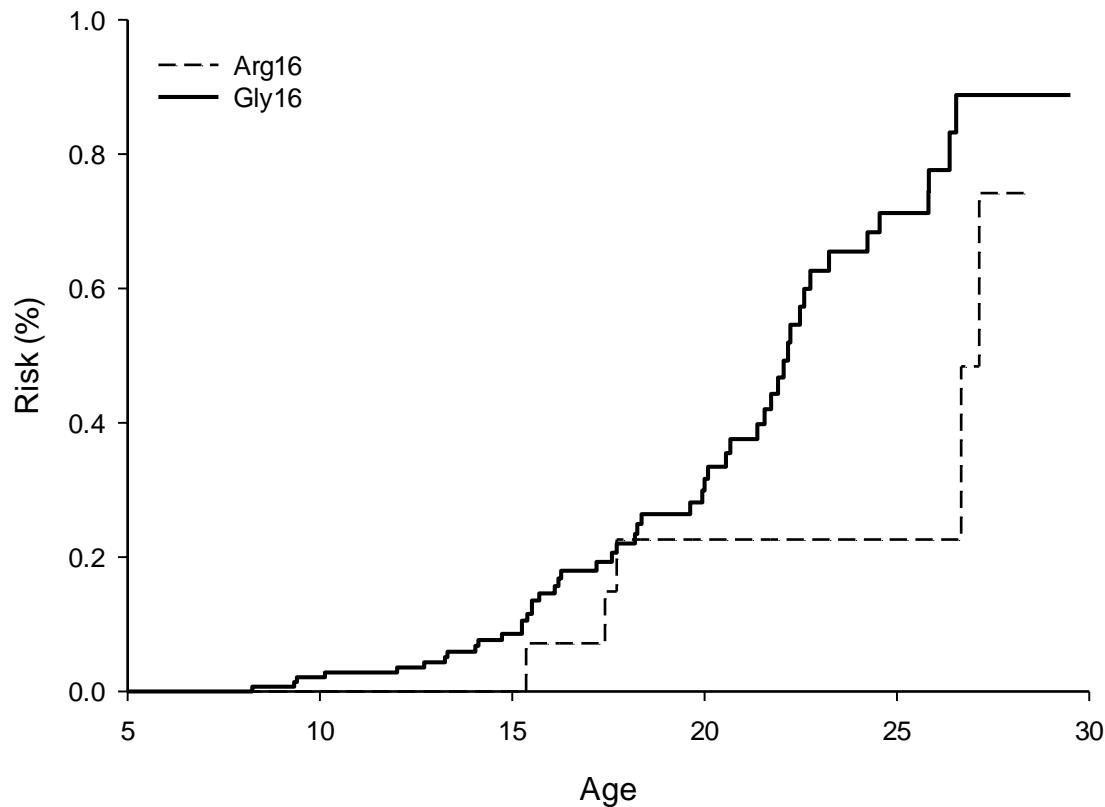
SE: standard error; CI: confidence interval; Arg16: patients who were homozygous or heterozygous for the  $\beta_2$ -adrenergic receptor (ADRB2) resulting in at least one arginine substitution at amino acid 16 ( $n = 26$ ); Gly16: patients who were homozygous for ADRB2 resulting in a glycine substitution at amino acid 16 ( $n = 147$ ). \*Significant difference in mean or median age at first use of NV between genotype groups,  $P < 0.05$ .

**Table 3.** Cox regression analysis of the risk of using nocturnal ventilation (NV) at any given age in patients with Duchenne muscular dystrophy (DMD).

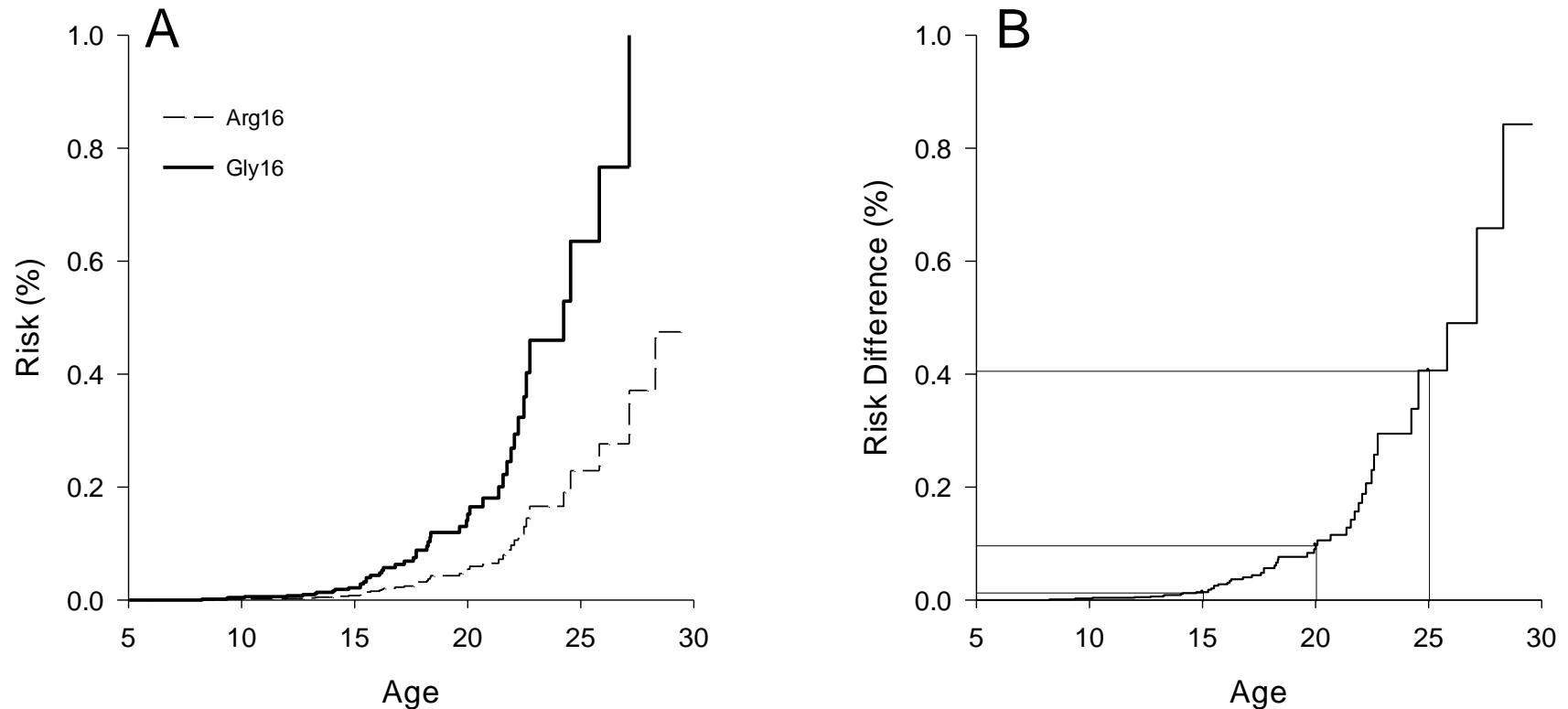
	<b>β</b>	<b>SE</b>	<b>Wald</b>	<b>p-value</b>	<b>HR</b>	<b>95% CI</b>
Genotype	1.02	0.50	2.04	<0.05	2.77	1.04 – 7.39
Ambulatory Status	-1.45	0.57	-2.56	<0.05	0.23	0.07 – 0.71
Corticosteroid-use (yrs)	-0.21	0.05	-4.11	<0.05	0.81	0.73 – 0.89
Height (cm)	-0.04	0.02	-2.41	<0.05	0.96	0.93 – 0.99
Weight (kg)	0.004	0.01	-0.34	0.73	1.01	0.98 – 1.03

SE: standard error; HR: hazard ratio; CI: confidence interval; Arg16: patients who were homozygous or heterozygous for the  $\beta_2$ -adrenergic receptor (ADRB2) resulting in at least one arginine substitution at amino acid 16 ( $n = 26$ ); Gly16: patients who were homozygous for ADRB2 resulting in a glycine substitution at amino acid 16 ( $n = 147$ ); genotype was coded as: 0 = Arg16, 1 = Gly16; ambulatory status was coded as: 0 = non-ambulatory, 1 = ambulatory; there was a significant influence of genotype, ambulatory status, corticosteroid use, and height on risk of NV use.

**Figure 1.** The cumulative risk of nocturnal ventilation in Duchenne muscular dystrophy (DMD) patients stratified by  $\beta_2$ -adrenergic receptor genotype (Arg16 and Gly16).



**Figure 2.** The cumulative risk (A) and absolute difference in risk (B) of nocturnal ventilation in Duchenne muscular dystrophy (DMD) patient stratified by  $\beta_2$ -adrenergic receptor genotype (Arg16 and Gly16).



**CHAPTER 4: DISCUSSION**

DMD is a debilitating disorder caused by a deletion of the dystrophin gene. Dystrophin deficiency renders muscle fibers fragile and susceptible to contraction-mediated injuries which affect skeletal, diaphragmatic, and cardiac muscles. The result is a majority of DMD patients dying from dilated cardiomyopathy and restrictive pulmonary pathologies. Therefore, it is important to further understand the factors that affect respiratory and cardiac health in patients with DMD. Understanding these factors may aid in identifying therapeutic interventions to delay the loss of respiratory and cardiac function and decrease hospitalization rates and mortality in this population.

Two primary pathways have been identified as possible therapeutic targets to preserve respiratory and cardiac function in DMD patients. These pathways are the ADRB1 and ADRB2 coupled pathways. Polymorphisms of these receptors that influence functionality include the Ser49Gly and Arg389Gly of the ADRB1 and Arg16Gly of the ADRB2. These ADRB1 polymorphisms (Ser49Gly and Arg389Gly) have been demonstrated to influence cardiac measures and response to beta-blockers. The ADRB2 polymorphism (Arg16Gly) has been demonstrated to influence skeletal muscle size, strength, and respiratory function.

#### 4.1 Beta-1 Adrenergic Receptor Genotype in Healthy Patients

The ADRB1 subtype is found primarily in the heart and comprises 75-80% of total  $\beta$ -AR found in the heart and approximately 95% of  $\beta$ -AR in the SA node [21]. The G-protein signaling pathway associated with the  $\beta$ -AR is important in the modulation of several key target proteins. When activated, cardiomyocyte ADRB1 preferentially binds

to the G<sub>as</sub> protein which phosphorylates AC, generating the secondary messenger cAMP. Increased cAMP accumulation activates PKA [25]. Activated PKA then phosphorylates troponin I, the L-type Ca<sup>2+</sup> channel and phospholamban (PLB), increasing cardiac inotropy, chronotropy, and lusitropy [24].

Research has also shown G<sub>s</sub> activation can increase L-type Ca<sup>2+</sup> current directly [166]. These L-type Ca<sup>2+</sup> channels play an integral role in cardiomyocyte excitability and contractility [156]. Phosphorylation of cardiac L-type Ca<sup>2+</sup> channels by PKA results in an influx of Ca<sup>2+</sup> into cardiomyocytes. The Ca<sup>2+</sup> then binds to the sarco/endoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA) triggering further sarcoplasmic Ca<sup>2+</sup> loading which results in the removal of troponin and tropomyosin inhibition of myosin binding sites [105, 167]. Additionally, research suggests the phosphorylation of phospholamban (PLB) via PKA, a downstream protein from β-AR stimulation, results in the removal of inhibition of SERCA. This increases the quantity and rate of reuptake of cytosolic Ca<sup>2+</sup> in the sarcoplasmic reticulum [27]. Recent research also suggests PLB pools exist in the nuclear envelope which allows them to regulate perinuclear/nuclear Ca<sup>2+</sup> handling [26]. Troponin I is a regulatory protein of cardiac myofibrils and its phosphorylation by PKA inhibits actomyosin ATPase activity resulting in relaxation of cardiomyocytes in response to catecholamines [22, 28]. Research has demonstrated that genetic variants of the ADRB1 modulate the cardiac responses to catecholamine binding and the aforementioned mechanisms; the Gly49 polymorphism has been shown to produce a dampening effect to these responses. These data suggest genetic variation of the ADRB1 may influence cardiovascular responses to exercise in healthy subjects.

Our original hypothesis was that healthy patients expressing the Gly49 polymorphism would have decreased cardiac output, stroke volume, and blood pressure at maximal exercise compared with patients with the Ser49 polymorphism. Furthermore, we hypothesized that healthy patients expressing the Gly49 polymorphism would have decreased cardiac index, heart rate, and systemic vascular resistance at maximal exercise compared with patients with the Ser49 polymorphism. The principle findings of project 1 were that patients with the Gly49Ser demonstrated an improved cardiac response to exercise. Specifically, patients with the Gly49Ser polymorphism had a significantly improved cardiac index as compared to the Ser49Ser polymorphism at rest; this difference is abolished at peak exercise. This suggests improved cardiac function at rest with no deleterious effect on cardiac function at peak exercise. Additionally, there is a significant difference between the two polymorphisms for  $HR_{max}$  and no difference in HR at rest, with the Gly49Ser polymorphism demonstrating a higher  $HR_{max}$ . This suggests the Gly49Ser polymorphism has an improved HR reserve, suggesting a higher capacity for cardiac work. This polymorphism (Gly49Ser) also demonstrated a lower SVR at rest, suggesting the Gly49Ser polymorphism has decreased cardiac work at rest. This coupled with the abolishment of this difference at peak exercise suggests subjects with the Gly49Ser polymorphism have an improved cardiac work reserve. Furthermore, the trend towards significance in change in SV from rest to peak exercise – with the Gly49Ser polymorphism presenting a dampened increase – in addition to there being no difference in HR at rest and improved CI at rest, suggest an improved left ventricular contractility in the Gly49Ser polymorphism. The clinically significant difference between genotype

groups in change in BP<sub>dias</sub> rest to peak exercise may suggest a systemic influence of the ADRB1 genotype, particularly in the arterial vasodilation observed in response to aerobic exercise. These data support our original hypothesis and suggest the Gly49Ser polymorphism to have improved cardiovascular function at rest and peak exercise.

#### *4.2 Beta-1 Adrenergic Receptor Genotype in Duchenne Patients*

ADRB1 signaling has been shown to play an important role in HF with the degree of sympathetic activity being inversely correlated with survival [211]. Deleterious effects of ADRB1 signaling include apoptosis, myocyte growth, fibroblast hyperplasia, myopathy, fetal gene induction, and proarrythmia [206, 212]. As an adaptive mechanism in HF, cardiac ADRB1s become less responsive, either downregulating or uncoupling from the G<sub>s</sub> pathway [214]. This suggests the less functional variants of the ADRB1 to be clinically important in HF and other HF-like cardiomyopathies.

Literature has identified the ADRB1 coupled pathway as capable of preserving cardiac function in cardiomyopathies [16-20]. The ADRB1 subtype is found primarily in the heart, comprising about 80% of total beta adrenergic receptors found in the heart and playing a role in cardiac function [16, 21-23]. Research has demonstrated ADRB1 activity influences cardiac: (i) inotropy, lusitropy, and chronotropy [24, 25]; (ii) nuclear and perinuclear Ca<sup>2+</sup> handling [26]; (iii) reuptake of cytosolic Ca<sup>2+</sup> [27]; and (iv) cardiomyocyte relaxation [22, 28]. Given the above mentioned mechanisms, the ADRB1 may play a role in preserving cardiac function in patients with DMD.

A polymorphism of the ADRB1 that affects functionality includes a glycine (Gly) for arginine (Arg) substitution at amino acid 389 [16, 17, 22, 23]. Specifically, the Gly389 polymorphism demonstrates: decreased receptor density and cAMP accumulation as well as a dampening response to norepinephrine infusion [16, 22, 23]. Functionally, HF patients with the Gly389 polymorphism have significantly lower diastolic, systolic, and mean arterial blood pressure — contributing to improved cardiac function and decreased mortality risk [17-19, 29]. Furthermore, literature demonstrates autoantibodies against ADRB1 are associated with more favorable myocardial recovery in patients with recent-onset cardiomyopathy [20]. The association between the Gly389 polymorphism and more favorable cardiac measures and outcomes in HF patients suggest a therapeutic target for preserving cardiac function and delaying cardiomyopathy in patients with DMD. To date, however, there is no literature investigating the relationship between ADRB1 genotype and cardiac events in DMD patients.

Our original hypothesis was that DMD patients with the Gly389 polymorphism would have a lower risk of cardiac events compared with those expressing Arg389 polymorphism. The principal findings of project 3 were that DMD patients with the Gly389 polymorphism of the ADRB1 had a higher incidence of diuretics use compared with those patients expressing the Arg389 polymorphism. Specifically, patients with the Gly389 polymorphism were 5.01 times more likely to be on diuretics compared with patients with the Arg389 polymorphism at any given age. Furthermore, ADRB1 genotype had no influence on the risk of other cardiac events included in project 3 in patients with DMD. However, corticosteroid use was a negative predictor of the risk of other cardiac

issues and ACEI and ARB use and height was a negative predictor of the incidence of ACEI, ARB, and other cardiovascular medications use. The present study refutes our hypothesis and demonstrates DMD patients with the Gly389 polymorphism may have an increased incidence of diuretics use compared with patients with the Arg389 polymorphism and that ADRB1 genotype has no influence on other cardiac events in these patients.

As respiratory management in DMD patients improves, the development of cardiomyopathies and the accompanying left ventricular dysfunction are emerging as significant contributors to mortality in this population [6-8]. While cardiomyopathies are present in 90% of DMD patients over the age of 18, only 30% of patients present with symptoms at the time of diagnosis [8, 9]. Additionally, a majority of DMD patients with normal left ventricular function exhibited dysfunctional myocardial strain at the posterolateral wall (the initial site of fibrofatty deposition in myocardium), suggesting abnormal myocardial contraction before left ventricular decompensation [13, 264]. The cardiomyopathy associated with DMD has a higher mortality than other dilated cardiomyopathies but often remains less treated at diagnosis [9]. Further, left ventricular dysfunction secondary to cardiomyopathy is a strong predictor of mortality in patients with DMD [7]. Therefore, understanding the factors that affect the risk of cardiac events in patients with DMD may provide therapeutic targets to delay the development of cardiomyopathy and decrease the risk of mortality in this population.

Both the DMD Care Considerations Working Group and the European Society of Cardiology guidelines recommend ACEI as first-line therapy for dilated cardiomyopathy

and/or congestive heart failure in DMD patients [82, 91, 101]. However, the side-effect profile of ACEI, such as cough, renal dysfunction, hyperkalemia, and angioedema, led to the testing of ARBs as a replacement therapy for cardiac dysfunction [265]. The Evaluation of Losartan in the Elderly (ELITE) 1 and 2 trials determined ARB therapy to be as effective as ACEI in the treatment of HF [266, 267]. These findings have led to ACEIs and ARBs becoming more mainstream therapy in patients with DMD [9]. Although the age of initiation of these therapies remains an important clinical question, the use of ACEIs and ARBs is an important milestone in cardiac therapy in DMD patients [9]. The Cox PH analysis identified corticosteroid use and height as strong, negative predictors of the incidence of ACEI and ARB use in patients with DMD. That a greater corticosteroid use decreased incidence of ACEI and ARB use in DMD patients is not surprising as corticosteroid use delays the emergence of cardiomyopathy [10].

The American College of Cardiology and American Heart Association have long recommended the use of diuretics in the treatment of HF with reduced left ventricular function [96]. Consequently, the American Academy of Pediatrics recommends considerations to be given to the use of diuretics in the treatment of cardiomyopathy in DMD patients [97]. Current literature has led investigators to suggest the use of diuretics to treat tachycardia and lipothyrmia in later stages of cardiac involvement in DMD patients [98]. Despite the efficacy of diuretic therapy in the treatment of HF and recommendations for diuretic considerations in DMD patients, clinical adoption is slow [99]. In our study population, only 18 patients were prescribed a diuretic at any point (14 and four for Gly389 and Arg389 respectively). However, the Cox PH analysis determined

genotype, height, and weight to be strong predictors of diuretic use in DMD patients. Specifically, DMD patients with the Gly389 polymorphism were approximately 5 times more likely to be on diuretics compared with patients with the Arg389 polymorphism at any given age. Further, height and weight were identified as strong, negative predictors of diuretics use in our population of DMD patients.

The multifactorial nature of the cardiac involvement in DMD necessitates a battery of therapy options to treat cardiomyopathy symptoms and limit the side-effect profile [82]. Literature supports the use of ACEIs and ARBs as first-line therapy and considerations for BBs and diuretics in the treatment of cardiomyopathy in DMD patients and recommends the adoption of clinical guidelines for HF management [82, 96, 97]. Additional therapies used in our clinical population included  $\text{Ca}^{2+}$  channel blockers, blood thinners, and cholesterol lowering drugs. In our population, height was a negative predictor for the incidence of use of the above mentioned therapies.

Secondary to cardiomyopathy, DMD patients also present with tachycardia and other arrhythmias [98]. These additional complications present further clinical care considerations and signal the emergence of extensive fibrofatty deposition and conduction disorders [268]. Further, sinus tachycardia is correlated with cardiac dysfunction in DMD patients and other conduction abnormalities present more commonly in DMD patients with left ventricular ejection fraction <35% [288, 289]. These findings demonstrate the clinical importance of the emergence of arrhythmias in DMD patients. These conditions may point to advanced myocardial fibrosis and decompensated left ventricular ejection fraction and thus provide important clinical

milestones in cardiac management in this population. Corticosteroid use was identified as a negative predictor of a DMD patient's risk of cardiac issues including tachycardia and other arrhythmias. As mentioned previously, corticosteroid use has been shown to delay cardiomyopathy in DMD patients [10].

In light of the burden of literature demonstrating the beneficial influence of the Gly389 polymorphism in cardiac outcomes and mortality in HF patients, the contradictory findings of this study may be difficult to explain. One explanation is the lack of widespread clinical adoption of specific guidelines for treatment of cardiomyopathy in patients with DMD. Despite a greater appreciation for the clinical importance of DMD cardiomyopathy, cardiac management strategies are highly variable and remain underutilized in this population [9, 100]. Currently, there is no consensus on the proper pharmacological therapy class and timing for treatment of cardiomyopathy in DMD patients [101]. In fact, when clinic site was entered into the Cox PH analysis as an additional covariate, 5 clinic sites differed significantly from the other sites in the prevalence of diuretics prescription at any given age. Given this, the variability of cardiac management for DMD patients in different clinics may explain these contradictory findings.

#### 4.3 Beta-2 Adrenergic Receptor Genotype in Healthy Patients

Research suggests B2-AR agonist supplementation may have an efficacious effect on respiratory function, primarily as a mechanism of improved diaphragmatic strength and contractility. This improved diaphragmatic function is demonstrated by increased

diaphragmatic myofibril protein concentration and maximal tetanic force production following B2-AR agonist [45]. Furthermore, ADRB2 treatment has been shown to increase diaphragm cross-sectional area and myosin heavy chain concentration with an increased transdiaphragmatic pressure [46, 290]. These beneficial effects have been demonstrated in other clinical populations, such as chronic obstructive pulmonary disease, with improved respiratory strength and endurance following B2-AR agonist treatment [291]. These data suggest the ADRB2 may play a role in the regulation and preservation of diaphragm strength and respiratory function.

The ADRB2 may also play an important role in skeletal muscle size and strength through multiple internal mechanisms. Free C-subunits of PKA, a downstream product of ADRB2 stimulation, are capable of phosphorylating multiple regulator genes of CREB [162]. CREB is ubiquitously expressed and influences cell proliferation, differentiation, adaptation, and survival [164]. Further, CREB plays an integral role in mediating MEF2, SIK1, and myostatin activity, pathways that regulate skeletal muscle turnover [161, 292]. An additional pathway influenced by ADRB2 stimulation is the PI3K-Akt signaling pathway which regulates protein synthesis, gene transcription, cell proliferation, and cell survival [170-172]. The primary mechanism of skeletal muscle regulation through the PI3K-Akt pathway is via FOXO inhibition, thereby decreasing FOXO-mediated cellular apoptosis [293]. ADRB2 activation is also associated with an increased expression of NOR-1 and peroxisome-activated receptor  $\gamma$  coactivator-1 $\alpha$ 4 (PGC-1 $\alpha$ 4), both negative regulators of myostatin expression [163, 165, 251]. Collectively, these data suggest the

ADRB2 plays multiple roles in regulating skeletal muscle growth through downstream regulation.

In addition to the mediation of internal cell signaling, stimulation of the ADRB2 can also regulate  $\text{Ca}^{2+}$ -mediated proteolysis. Both cAMP and phosphorylated PKA have been shown to directly and indirectly inhibit calpain activity [40]. Specifically, research suggests cAMP and PKA may directly phosphorylate calpains to an inactive state to inhibit activity [63, 65, 196, 246]. Additionally, research has identified calpastatin (a calpain-specific inhibitor) binding sites for both cAMP and PKA, suggesting the capacity for direct phosphorylation and increased expression of calpastatin [66, 244, 245]. The multiple pathways whereby ADRB2 stimulation may improve skeletal and diaphragmatic muscle function and protect against calpain-mediated atrophy suggest an effective therapeutic target for preservation of respiratory function in DMD patients. Project 2 demonstrates that genetic variation of the ADRB2 is associated with differences in muscular power, efficiency and intensity. Our original hypothesis was that healthy patients expressing the Gly16 polymorphism would have increased absolute and relative power output compared with patients expressing the Arg16 polymorphism. We further hypothesized those healthy patients expressing the Gly16 polymorphism would report decreased indices of relative and perceived intensity at a given workload compared with patients expressing the Arg16 polymorphism.

The principal findings of study 2 support our original hypothesis. Specifically, patients with the Arg16Gly polymorphism demonstrated significantly higher peak power, relative power, muscular efficiency, and exercise intensity during heavy, steady-state

exercise. Additionally, subjects with this polymorphism (Arg16Gly) reported significantly lower RPE despite producing higher peak watts, suggesting improved muscular efficiency. While not statistically significant, Gly16 subjects were heavier than Arg16 subjects. Project 2 suggests the Arg16Gly polymorphism may improve power measures, muscular efficiency, and exercise intensity in healthy subjects compared with the Arg16Arg polymorphism. Considering the beneficial influence of the Gly16 polymorphism on skeletal muscle, it is conceivable to posit there are also positive influences of this polymorphism on the diaphragm and accessory respiratory muscles.

#### *4.4 Beta-2 Adrenergic Receptor Genotype in Duchenne Patients*

The results of Project 2 suggest ADRB2 genotype, specifically the Gly16 polymorphism, may play a functional role in improving skeletal muscle strength and function. Additionally, a pilot study has shown an improvement in isometric knee extensor strength and manual muscle test scores in DMD patients following 12 weeks of B2-AR agonist treatment [283]. A larger, follow-up study further demonstrated B2-AR agonist treatment improves lean body mass and time to walk/run 30 feet in patients with DMD [284]. There were no improvements in isometric knee moments or manual muscle tests following B2-AR agonist treatment in this study population. Collectively, these data suggest ADRB2 genotype may influence skeletal muscle function in patients with DMD. However, to date there has been no investigation into the influence of ADRB2 genotype on respiratory function and the age of important clinical respiratory milestones, particularly NV.

An estimated 32% of DMD patients present with nighttime alveolar hypoventilation [3]. The negative effects of SBD include increased cardiac morbidity, neurocognitive deficits, decreased lung function, and increased hospitalization rates and mortality [3, 30, 31, 294]. Once patients with DMD present with SBD and abnormal overnight oximetry, patients are prescribed NV — the age at which this occurs is an important clinical milestone in this population [3, 151]. Clinically, NV in DMD patients is indicated by progressive respiratory muscle weakness, the primary contributor of which is a loss of diaphragmatic contractility due to diaphragm remodeling [33, 35]. Diaphragmatic remodeling in DMD can be attributed to two main mechanisms: increased inflammatory response and an increased calpain activity [34, 36, 58, 59, 229]. The deleterious effect of these mechanisms on diaphragm function suggests the abatement of these mechanisms would prove clinically important in DMD patients. Given ADRB2 stimulation has been shown to: increase diaphragmatic cross-sectional area, strength, and contractility [43, 45, 46]; improve mucociliary clearance [47-52]; inhibit inflammatory pathways [53-61]; and to inhibit calpain activity and decrease concentration [62-69], we reasoned DMD patients with the Gly16 polymorphism would have a reduced risk of NV use.

One of the main respiratory concerns in DMD is the loss of diaphragmatic contractility due to increased diaphragm fibrosis and pseudo-hypertrophy [33]. The increase in diaphragm fibrosis in patients with DMD is a function of two main mechanisms: a decrease in mucociliary clearance and the resultant increased inflammatory response and the upregulation of  $\text{Ca}^{2+}$ -modulated proteolytic pathways [34,

36, 58, 59, 229]. Impaired mucociliary clearance is also associated with increased fibrosis and hospitalization due to pulmonary complications in DMD patients [37]. Ineffective clearance has been shown to upregulate inflammatory pathways and hasten the onset of respiratory failure in neuromuscular disorders [37]. Dystrophin-deficient muscle cells have been shown to have increased IL-1 $\beta$  levels and research suggests significant levels of TNF- $\alpha$  were detected in 62% of human DMD tissue biopsies [53, 54, 58, 59]. Furthermore, decreased sarcolemmal integrity, mechanical cell membrane tears, and Ca<sup>2+</sup> channel dysregulation associated with DMD have been shown to result in increased Ca<sup>2+</sup> flux and increased calpain concentration and activity [40]. Calpains, Ca<sup>2+</sup>-dependent, non-lysosomal proteases, initiate myofibrillar protein degradation thereby decreasing myofibrillar protein integrity [40]. The increased activation of these pathways is considered a primary contributor to muscle degradation and fiber necrosis in DMD [41].

B2-AR agonist treatment has been shown to improve airway clearance by 3-4 times in humans and have anti-inflammatory properties by inhibiting cytokine production including: TNF- $\alpha$ , IL-6, and interleukin-1 $\beta$  (IL-1 $\beta$ ) [49, 50, 53, 54]. It has also been demonstrated that B2-AR agonist treatment can increase calpastatin, a calpain-specific inhibitor, and decrease calpain concentration and activity [64, 67-69]. B2-AR agonist supplementation has also been shown to increase diaphragm protein concentration, maximal tetanic force production, and transdiaphragmatic pressure in the mouse model [45, 46, 229]. Functionally, the increased receptor density and resistance to downregulation associated with the Gly16 polymorphism results in increased cAMP accumulation, further upregulating these mechanisms [43]. Cumulatively, these data

suggest the Gly16 polymorphism may result in: improved mucociliary clearance, decreased inflammatory pathways, inhibition of  $\text{Ca}^{2+}$ -dependent proteolysis, and improved diaphragmatic function in DMD.

Our original hypothesis was that DMD patients expressing the Gly16 polymorphism would have a lower risk of NV use at any given age when compared with patients expressing the Arg16 polymorphism. However, the principal findings of project 4 directly contradict this hypothesis, with the Gly16 polymorphism demonstrating an almost 3-fold increase in the risk of NV use at any given age. The finding that the Gly16 polymorphism confers a higher (not lower) risk of NV use is difficult to explain at first. When considering the aforementioned benefits of ADRB2 stimulation on respiratory and skeletal muscle strength, literature has suggested B2-AR agonist therapy may be efficacious in stymying muscle degradation in DMD patients and preserving respiratory function [295, 296]. The findings of study 4 contradicting these speculations can be explained by two main mechanisms: increased contraction-induced injuries and  $\text{Ca}^{2+}$  flux.

ADRB2 stimulation results in a phenotypic shift from type I oxidative to type II glycolytic muscle fibers, resulting in increased conduction velocity and contraction strength [277-279]. Secondary to increased type II fiber concentrations, B2-AR agonists have been shown to have inotropic effects in animal diaphragms resulting in increased speed and force of contraction [297]. Furthermore, the Gly16 polymorphism is associated with increased muscular strength and power [276]. While increased strength and contractility may seem beneficial for respiratory muscle fatigue and weakness, this likely

increases the number and likelihood of contraction-mediated injurious events in DMD patients — a significant component of muscle fiber degradation in DMD [280]. Additionally, type II fast oxidative-glycolytic fibers are more susceptible to eccentric contraction-induced damage [281]. In addition to increased contraction-induced injuries, the inotropic effect of ADRB2 stimulation may also increase intracellular  $\text{Ca}^{2+}$  concentrations, further increasing calpain activity and muscle fiber degradation [250]. These data present mechanisms whereby the Gly16 polymorphism may have deleterious effects on respiratory function and risk of NV use in DMD patients.

While the Gly16 polymorphism may have a positive influence on skeletal muscle function (as demonstrated in study 2) and respiratory function in various populations, study 4 suggests this polymorphism may have a negative effect on respiratory function and risk for NV compared with the Arg16 polymorphism in patients with DMD. Specifically, DMD patients with Gly16 polymorphism present an almost 3-fold increase in risk of NV compared to patients with the Arg16 polymorphism.

**CHAPTER 5: LIMITATIONS**

There are inherent limitations regarding genetics studies including sample size and genotype distribution. Limited statistical power because of the modest sample size and different genotype distribution in project 1 and 2 ( $N = 71$  and 77 respectively) may have played a role in limiting the significance of some of the statistical comparisons conducted. Post hoc power analyses for project 1 revealed the power to detect statistically significant differences between groups for CI at rest and  $HR_{max}$  to be .625 and .613 respectively at  $\alpha = 0.05$ . For project 2, post hoc power analyses revealed the power to detect statistically significant differences between groups for watts, watts/kg, and watts/VO<sub>2</sub> to be .84, .95, and 1.00 respectively at  $\alpha = 0.05$ . These post hoc analyses suggest a sufficient sample size for both project 1 and 2.

Furthermore, the sample population for project 1 was void of the Gly49Gly genotype. This can be explained by the genotype frequency of the three genotypes present at amino acid 49 (0.69, 0.29, and 0.04 for Ser49Ser, Gly49Ser, and Gly49Gly respectively) but may vary among different racial/ethnic groups [260]. However, literature has demonstrated the Gly49Ser and Gly49Gly polymorphisms function similarly and that the Gly49Ser polymorphism may be representative of the Gly49 polymorphism as a whole for ADRB1 [176, 208]. Additionally, time of day and time of year for testing as well as training background were not controlled for in project 2, which may affect test-retest reliability. An intraclass correlation coefficient analysis revealed the test-retest reliability for watts, watts/kg, and watts/VO<sub>2</sub> to be 0.52, 0.49, and 0.59 respectively, suggesting fair test-retest reliability.

Project 3 included DMD patients who were homozygous for arginine (AA,  $n = 11$ ), glycine (GG,  $n = 95$ ), or heterozygous (AG,  $n = 69$ ) at codon 389 and were collapsed into two study groups (Gly389,  $n = 95$  and Arg389,  $n = 80$ ). This even genotype distribution is surprising given the frequency in the healthy population (0.70 and 0.30 for Arg389 and Gly389 respectively) [270]. However, there is no data available regarding ADRB1 389 genotype frequency in a DMD population. A second consideration is the number of events in our data. It is generally recommended that for each covariate, a minimum of 10 events are needed to ensure statistical power. In our analysis including 5 variables, 50 events are needed. Our cohort only had 18 DMD patients who were prescribed diuretics, violating this rule. However, literature has demonstrated a range of circumstances in which coverage and bias standards were adequate with fewer than 10 events per variable, suggesting the rule of 10 events per variable may be relaxed [271]. A second consideration is the retrospective nature of the data and the rolling accrual of patients used for data collection. As a result, our cohort did include left-censored data which may influence the accuracy of the Cox model to identify the age of first diuretics use and therefore the incidence of diuretics use. However, the aim of this study was to determine the influence of ADRB1 genotype on the incidence of diuretics use. At present, we do not believe that genotype status, *per se*, would have influenced the model's accuracy to identify the timing of first diuretics use. Further, we used a Kaplan Meier analysis, which is more effective when dealing with left-censored data given a <50% left-truncated distribution [298], and also identified a difference between genotypes in survival without NV use.

Project 4 included DMD patients who were homozygous for arginine (AA,  $n = 26$ ), glycine (GG,  $n = 59$ ), or heterozygous (AG,  $n = 90$ ) at codon 16 and were collapsed into two study groups (Arg16,  $n = 26$  and Gly16,  $n = 149$ ). While this distribution is not representative of a healthy population (0.40 and 0.60 for Arg16 and Gly16 respectively), no data is available regarding ADRB2 16 genotype distribution in a DMD population [287]. A second consideration is the retrospective nature of the data and the rolling accrual of patients used for data collection. The rolling accrual resulted in our cohort data having a left-truncation which may influence the accuracy of the Cox model to identify the age of first NV use and the risk of NV use [see above]. However, the aim of this study was to determine the influence of ADRB2 genotype on the risk of NV use. At present, we do not believe that genotype status would have influenced the model's accuracy to identify the timing of first NV use. Again, we used a Kaplan Meier analysis, which is more suited for data with left truncation, and also identified a difference between genotypes groups for age of NV use.

**CHAPTER 6: FUTURE DIRECTIONS AND CONCLUDING REMARKS**

The findings of project 3 suggest that DMD patients with the Gly389 polymorphism have a significantly higher incidence of diuretics use compared to patients with the Arg389 polymorphism. Specifically, the Gly389 polymorphism is 5 times more likely to use diuretics compared to the Arg389 polymorphism. However, there remains high variability in the type and timing of implementation of cardiac management therapies for DMD patients, as demonstrated by different incident rates of diuretic prescription between the clinics included in our analysis. The current standard of care is to treat cardiomyopathies when the patient becomes symptomatic, which poses complications as a majority of DMD patients are asymptomatic at cardiomyopathy diagnosis [9]. Fortunately, an emerging school of thought recommends treating cardiomyopathies before fibrofatty depositions emerge [9]. This variability may present complications in studies of this nature in the future. With cardiomyopathies continuing to emerge as a significant contributor to mortality and the related decline in cardiac and respiratory function, it is important to identify the factors that contribute to cardiac dysfunction in DMD patients. Therefore, it is important that clinical guidelines are established and adopted regarding cardiac management in the DMD population. Given the highly variable nature of cardiomyopathy management, future research aimed at investigating the influence of ADRB1 genotype on cardiac measures in DMD patients may be best served by focusing on annual and bi-annual ECHO measures. Using clinically derived ECHO measures would mitigate the effect of the variability of cardiac treatment in the clinic. Therefore, further research is necessary to identify if there is a

positive influence of the Arg389 polymorphism on clinically derived ECHO measures in DMD patients.

Preliminary research by our group has demonstrated DMD patients with the Gly16 polymorphism have improved FVC, FEV<sub>1</sub>, and FEF<sub>max</sub> as percent predicted compared with those expressing the Arg16 polymorphism earlier in life [299]. However, this improvement in respiratory function was almost completely abolished as the patient ages. Furthermore, a pilot study demonstrated an improvement in isometric knee extensor strength and manual muscle test scores in DMD patients following 12 weeks of B2-AR agonist treatment [283]. A larger, follow-up study by this same group supported their previous findings with B2-AR agonist treatment improving lean body mass and time to walk/run 30 feet in patients with DMD — there were no improvements in isometric knee moments or manual muscle tests [284]. However, both study populations consisted of young DMD patients whose ages ranged between 5 to 11 years. It is important to emphasize that our findings demonstrate the Gly16 polymorphism confers a significantly higher risk of NV use; however, the absolute difference in risk between the Gly16 and Arg16 is marginal for ages between 5 and 11 years (project 4; Figure 2B). Indeed, the absolute difference in risk between genotype groups is less than 1.4% up to 15 years of age in our cohort of DMD patients. The difference in risk of NV between groups widens thereafter to reach >40% at patient ages of 25 years and older. One may infer from these observations that the additional risk of NV use associated with the Gly16 is relatively trivial below the adolescent years, and becomes more important when DMD patients enter the second decade of life. Further studies utilizing longitudinal clinically-derived

respiratory measures are needed to examine whether the effects of the Gly16 polymorphism on respiratory outcomes in DMD are indeed dependent on patient age.

Current literature clearly demonstrates the need to further both cardiac and respiratory management in DMD patients. The interconnectedness of cardiac and respiratory function demonstrates a need for a treatment protocol to preserve the function of both in tandem. Our original hypotheses were that the Gly389 polymorphism would have a reduced probability and risk of cardiac events and interventions and that the Gly16 polymorphism would have a reduced probability and risk of NV use in DMD patients. The current studies have demonstrated a deleterious influence of the Gly389 polymorphism on the incidence of diuretics use and the Gly16 polymorphism on risk of NV use in patients with DMD. However, the burden of the literature suggests a protective effect of these polymorphisms (Gly389 and Gly16) on cardiac and respiratory function respectively. The principal findings of our studies refuting our original hypotheses may be explained by the aforementioned reasoning. These studies were the first of their kind in a DMD population and identified multiple pitfalls and limitations of these types of studies in this population. A longitudinal approach implementing more objective cardiopulmonary measure (i.e. ECHO and PFT measures) may further elucidate the complex relationship between the ADRB1 389 and ADRB2 16 genotypes and cardiopulmonary health in DMD patients.

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