EARLY DETECTION OF PHYSIOLOGIC AND FUNCTIONAL CHANGES OF COMMON STRUCTURAL PROTEIN MUTATIONS UNDERLYING HUMAN DISEASES BY MRI

by

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*We also certify that written approval has been obtained for any proprietary material contained therein.
Dedicated to Henry and Harrison – the two loves of my life
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**Discussion**

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**Abstract**

**Introduction**

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<tr>
<td>ADC</td>
<td>Apparent Diffusion Coefficient</td>
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<tr>
<td>AQP4</td>
<td>Aquaporin 4</td>
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<tr>
<td>ASL</td>
<td>Arterial Spin Labeling</td>
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<tr>
<td>BBB</td>
<td>Blood-Brain Barrier</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine Kinase</td>
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<td>CMR</td>
<td>Cardiovascular Magnetic Resonance</td>
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<tr>
<td>cMyBPC</td>
<td>Cardiac Myosin Binding Protein C</td>
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<td>CS</td>
<td>Circumferential Strain</td>
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<td>DENSE</td>
<td>Displacement Encoding with Stimulated Echoes</td>
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<tr>
<td>DGC</td>
<td>Dystrophin-Glycoprotein Complex</td>
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<td>DMD</td>
<td>Duchenne Muscular Dystrophy</td>
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<td>DWI</td>
<td>Diffusion Weighted Imaging</td>
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<td>ECG</td>
<td>Electrocardiogram</td>
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<td>ECHO</td>
<td>Echocardiography</td>
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<td>EF</td>
<td>Ejection Fraction</td>
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<td>FISP</td>
<td>Fast Imaging with Steady State Free Precession</td>
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<td>HCM</td>
<td>Hypertrophic Cardiomyopathy</td>
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<tr>
<td>ICD</td>
<td>Implantable Cardioverter-Defibrillator</td>
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<tr>
<td>LV</td>
<td>Left Ventricular</td>
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<tr>
<td>LVH</td>
<td>Left Ventricular Hypertrophy</td>
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<tr>
<td>MCA</td>
<td>Middle Cerebral Artery</td>
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<tr>
<td>MLP</td>
<td>Muscle Limb Protein</td>
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<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<td>RS</td>
<td>Radial Strain</td>
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<tr>
<td>VVI</td>
<td>Velocity Vector Imaging</td>
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<td>WT</td>
<td>Wild Type</td>
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ABSTRACT

by

CANDIDA LAURA GOODNOUGH

The most common genetic disorders of skeletal and cardiac muscle result from mutations in structural proteins essential for normal tissue function. The ability to identify tissue dysfunction prior to the onset of overt clinical symptoms will provide better insight of physiology and treatment of pathology. Hypertrophic cardiomyopathy (HCM) is the single most common genetic disorder of the heart, resulting from mutations in sarcomeric proteins, and manifesting as sudden death or progressive heart failure. Characterization of early cardiac phenotypes and improved screening measures in HCM may reduce morbidity and mortality. Likewise, Duchenne Muscular Dystrophy (DMD) is an X-linked progressive, invariably fatal disease of skeletal and cardiac muscle resulting from mutations in dystrophin, one of the largest genes in the genome. One of the largest causes of mortality in this cohort is dilated cardiomyopathy. There is also a need to identify extra-cardiac sequelae of dystrophin mutations as new therapies may dramatically improve the life expectancy of patients with DMD. Indeed, dystrophin is also necessary to maintain the blood-brain barrier and cerebrovascular disease is case-reportable in DMD patients. The goal of this work is to explore the physiologic consequences of aberrant structural proteins using in vivo magnetic resonance imaging (MRI). In particular, MRI techniques were
used to detect early pathologic changes in cardiovascular and vascular function prior to the manifestation of overt symptoms in mouse models of HCM and DMD, respectively. Here, we demonstrate that in a mouse model of HCM, displacement encoding with stimulated echoes (DENSE) MRI detects left ventricle mechanical dysfunction antecedent of pathologic hypertrophy. We also demonstrate that velocity vector imaging echocardiography is comparable to DENSE MRI in identifying mechanical dysfunction in cardiomyopathy. Further, we developed an arterial spin labeling-fast imaging with steady-state free precession (ASL-FISP) MRI method as a means to measure cerebral perfusion. Finally, in a mouse model of muscular dystrophy, ASL detected decreased cerebral perfusion in setting of increased angiogenesis. Taken together, we demonstrate that the development of new imaging technologies not only might allow earlier diagnosis and thus more timely intervention, but also may facilitate identification of subtle and clinically relevant phenotypes in important disease states.
CHAPTER 1: INTRODUCTION

SIGNIFICANCE

Hypertrophic cardiomyopathy (HCM) is a common genetic myocardial disorder that is the leading cause of sudden cardiac death in young athletes (Maron et al., 1996a). An epidemiological study demonstrated a prevalence of HCM in the general adult population (ages 23-35) of 1:500 people using an echocardiographic basis of left ventricular hypertrophy (LVH) (Maron et al., 1995a). However, a large portion of adults carrying a mutant gene for HCM (particularly cMyBPC) are undetected since they do not yet exhibit overt LVH by echocardiography (Maron et al., 2001; Niimura et al., 1998a). The most devastating consequence of undetected HCM is sudden death, particularly following vigorous exercise in young adults. Sudden death was the primary mode of death in 51% (ages 45±20 yrs) of a cohort of HCM patients, followed by progressive heart failure (36%) and HCM-related stroke associated with atrial fibrillation (13%) (Maron et al., 2000). There is therefore precedence for early detection of cardiovascular dysfunction in HCM patients prior to the onset of symptoms or LVH.

Traditionally, family members of HCM patients are screened with echocardiography and electrocardiography (ECG) on an annual basis from the age of 12 to 18, and if no LVH is detected, families are typically reassured that no further echocardiographic testing is necessary (Maron et al., 2004). Nevertheless, there are some genetic variants of HCM that demonstrate incomplete penetrance and LVH is not detected until later in adulthood (Maron et al., 2001), so there is a risk that these patients may not be appropriately detected. The introduction of commercially
available genetic tests to detect the most common mutations in HCM has improved risk stratification and identifies family members suitable for further testing (Bos et al., 2009a). However, the uncertainty between the genotype-phenotype relationship still warrants a better imaging technique to identify pre-clinical expression of the genetic substrate.

Muscular dystrophies present another common cause of cardiomyopathy in the younger patient population (Hermans et al., 2010; Towbin, 1998). In particular, dilated and hypertrophic cardiomyopathy may be detectable as early as age 10 in Duchenne muscular dystrophy (DMD) boys (Nigro et al., 1990). Congestive heart failure or sudden death account for 10-20% mortality in DMD patients (American Academy of Pediatrics Section on Cardiology and Cardiac Surgery, 2005). Early detection of cardiac dysfunction is typically limited due to the extreme physical inactivity and thus a lack of symptoms in young DMD boys. However, there has been improvement in the quality of life and prolonged survival in DMD, which highlights the need to diagnose preclinical cardiovascular dysfunction and initiate treatment earlier. Abnormal myocardial strain detectable by cardiovascular magnetic resonance imaging (cMRI) actually precedes the onset of global functional deterioration (Ashford et al., 2005; Li et al., 2009a). Subclinical cardiac dysfunction is an indication for initiation of angiotensin-converting-enzyme (ACE) inhibitors, followed by β blockers and diuretics as management of heart failure (Bushby et al., 2010a).

Although significant advancements have been made in the treatment of muscle and pulmonary function (Eagle et al., 2002a; Finder et al., 2004; Simonds et al., 1998), the progression of vascular disease needs further investigation in DMD patients. In
addition to development of cardiomyopathy and/or cardiac arrhythmias, there has also been evidence of vascular dysfunction in the skeletal muscle and brains in a mouse model of DMD (Nico et al., 2002; Straino et al., 2004). Cerebrovascular integrity is essential for cognitive function and remains poorly understood in DMD.

OUTLINE

This work is dedicated to the early detection of structural protein dysfunction underlying human disease by MRI. I begin by introducing the clinical features of Hypertrophic Cardiomyopathy and Duchenne Muscular Dystrophy. Short descriptions of the MRI techniques used in my studies will follow. Chapter 2 describes our finding of early mechanical dysfunction in HCM, which was published in Circulation Imaging in 2012 (Desjardins et al., 2012), and Chapter 3 compares two different imaging techniques to quantify this dysfunction (Azam et al., 2012). Chapter 4 introduces the MRI method (Gao et al., 2014) that is used to quantify perfusion deficits in a mouse model of DMD laid out in Chapter 5 (Goodnough et al., 2014). I conclude my dissertation with future directions in studying structural protein dysfunction underlying HCM and DMD.
HYPERTROPHIC CARDIOMYOPATHY

INTRODUCTION

Cardiac myocytes are composed of parallel arrangements of myofibrils which are further divided into sarcomeres, the basic contractile unit of the heart muscle (Hoit and Walsh, 2011). The sarcomere is a cytoskeletal structure made of thick and thin myofilaments. Two units of β- or α-myosin heavy chains and four myosin light chain molecules make up the thick filament, whereas the thin filament is composed of repeating actin molecules that associate with troponins T (cTnT), I (cTnl), and C.
(cTnC). Cardiac myosin-binding protein C (cMyBPC) plays a role in the regulation of actin-myosin interaction and cross-bridge kinetics (Figure 1.1).

**CLINICAL PICTURE**

Hypertrophic cardiomyopathy (HCM) is a disease caused by mutations in genes that encode sarcomeric proteins. Hypertrophic cardiomyopathy (HCM) is primarily an inherited disease of the myocardium affecting young adults. It is characterized by left ventricular hypertrophy and myofibril disarray in the absence of other causes, which leads to a reduced left ventricular cavity (Wigle et al., 1995). HCM is frequently featured in the news as the leading cause of sudden death in young athletes. The overall prevalence of HCM in the general population has been estimated as 1:500. HCM affects many races and ethnicities, and occurs equally in males and females (Maron et al., 1995b).

**PATHOLOGY**

The pathophysiology of HCM is diastolic dysfunction, left ventricular outflow tract obstruction, mitral regurgitation, myocardial ischemia, and arrhythmias (Ommen et al., 2011). The hypertrophy in HCM is classically asymmetric, affecting the septum more so than the free wall. The largely septal hypertrophy decreases the left ventricular cavity (Davies et al., 1974). HCM can be present with or without an obstructive gradient, which impedes outflow during systolic contraction and is intensified by increased contractility. Diastolic dysfunction arises from defects in ventricular relaxation and chamber stiffness (Wigle et al., 1995). The outflow tract obstruction, asymmetric hypertrophy, and delayed inactivation due to abnormal
intracellular calcium reuptake causes impaired ventricular relaxation. The hypertrophy of the myocardium causes increased chamber stiffness and impairs relaxation. Myocardial ischemia may also affect relaxation and chamber stiffness. As a result, there is a compensatory increase of late diastolic filling during atrial systole. Approximately one third of patients with symptomatic HCM exhibit signs of obstructive gradients (Gersh et al., 2011). Microscopically, disordered myocytes swirl and branch, interspersed with fibrosis in HCM tissue (Figure 1.2, (Maron, 1993)).

**GENETICS**

It is critical to complete a full family history in patients with suspected HCM. In addition to identifying family members with a definitive diagnosis of HCM, it is
also beneficial to evaluate if any family members died suddenly at a young age, or were involved in an otherwise unexplained car accident. Approximately one half of HCM cases are identified by the typical Mendelian autosomal dominant fashion (Stevenson and Loscalzo, 2012). Clinical genetic testing may be particularly useful for identifying family members at risk for developing HCM, especially if there are no overt signs of left ventricular hypertrophy. However, the pathogenic mutation must be known for genetic testing to be of any utility, which is the case approximately 35% of the time (Richard et al., 2003). Although genetic screening may be possible in the future, the genetic heterogeneity requiring extensive resequencing and the high percentage of unknown causal genes for HCM require further investigation.

Hypertrophic cardiomyopathy is thought to be the most common genetic myocardial disorder, affecting approximately 1 in 500 people (Maron et al., 1995b). HCM is a highly heterogeneous disorder, with several hundred genetic mutations described. The most common genes affected in HCM encode for the β-myosin heavy chain and cardiac myosin-binding protein C (cMyBPC), however, mutations in cardiac troponin T and I, actin, titin, α-tropomyosin, and myosin light chains can also lead to HCM (Richard et al., 2003). cMyBPC is a thick filament protein that modulates actin-myosin interactions and thereby the rate of muscle contraction (Barefield and Sadayappan, 2010; Carrier, 2007). Mutations in cMyBPC are among the most common genetic causes of HCM, accounting for more than 40% of the total known cases worldwide (Richard et al., 2003).

Nearly 200 known mutations of the gene encoding cMyBPC have been identified as causative for HCM (Bonne et al., 1995; Harris et al., 2011; Watkins et al.,
The mutations can be deletions, insertions, missense, or splice site. Alternatively, *de novo* mutations may cause sporadic cases. A large number of disease-causing mutations in cMyBPC in humans are predicted to produce C-terminal truncated proteins that are not incorporated into the sarcomere, resulting in cMyBPC haploinsufficiency. The majority of mutations in the gene that encode cMyBPC are heterozygous and are predicted to result in expression of truncated cMyBPC lacking the C-terminal regions of the protein that binds to myosin and titin (Carrier et al., 1997). However, analysis of myocardial biopsy samples from patients with cMyBPC mutations demonstrate a reduction in the amount of full-length cMyBPC protein (van Dijk et al., 2009; Jacques et al., 2008; Marston et al., 2009). Therefore, the allele generating mutant cMyBPC effectively functions as a null allele, causing cMyBPC haploinsufficiency. However, the mechanisms that link reduced cMyBPC levels in the heart with the development and progression of HCM have remained elusive.

**PRESENTATION**

The characteristic hemodynamic defect in HCM is diastolic dysfunction, both as a primary consequence of the disease and also secondary to hypertrophy, fibrosis, and outflow obstruction. The ejection fraction and cardiac output are typically normal in HCM, unless the patient undergoes vigorous exercise, where the ventricular filling is inadequate to match the increased demand. The physical exam of an HCM patient is consistent with left ventricular hypertrophy: bifid apical impulse and fourth heart sound (Ommen et al., 2011). A physical exam of the HCM patient would also reveal a harsh systolic crescendo-decrescendo murmur heard best at the left lower sternal border. The murmur arises from left ventricular outflow turbulence/obstruction and
mitral regurgitation. The classic sign of HCM is an increase in the intensity of the murmur with maneuvers that decrease ventricular volume, like the Valsalva maneuver, and a decrease by increasing ventricular volume by squatting. It also common to hear a fourth heart sound as a result of decreased ventricular compliance.

HCM patients typically present between the ages of 20 and 40 years with dyspnea on exertion (Stevenson and Loscalzo, 2012). Dyspnea is the most common symptom in HCM patients, occurring in up to 90% of symptomatic patients. In addition, it is common for patients to complain of chest pain with or without exertion due to myocardial ischemia from an increase in demand. Conduction abnormalities may also produce palpitations, either from atrial fibrillation or ventricular arrhythmias. The majority of HCM patients are asymptomatic when they are diagnosed, however, dyspnea, angina, and/or syncope are the most common presentations.

**DIAGNOSIS**

The first manifestation of HCM may be sudden cardiac death from ventricular tachycardia or fibrillation in young patients during a sporting event (Maron et al., 1996b). Many younger individuals who carry disease-causing mutations in the cMyBPC gene do not exhibit overt LVH, because increases in LV wall thickness are often only detectable with advanced age (Maron et al., 2004; Niimura et al., 1998a). Given that these seemingly asymptomatic carriers are at risk for the development of HCM and cardiac disease later in life, the diagnosis and treatment of these patients is a major clinical challenge. The majority of HCM patients are asymptomatic at the time of diagnosis by abnormal electrocardiogram, heart murmur, or screening
echocardiogram. An abnormal electrocardiogram is present in the majority of symptomatic HCM patients (95%), demonstrating left axis deviation, left ventricular hypertrophy, prominent septal Q waves, and/or diffuse T-wave inversions (Frank and Braunwald, 1968). A chest x-ray would demonstrate mild to moderate cardiac silhouette enlargement, which is consistent with left ventricular and atrial enlargement.

The gold standard for a diagnosis of HCM is increased left ventricular wall thickness in the absence of other pathology by 2D and Doppler echocardiography (Ommen et al., 2011). Two-dimensionally (2D)-directed echocardiography has arguably become the leading method for assessing left ventricular (LV) function because it is noninvasive, relatively cost-effective, widely available, and has short acquisition and post-processing times that allow high through-put. However, 2D-directed detection of LV dysfunction by conventional echocardiography (M-mode, 2D, Doppler) is considered a late manifestation of cardiac disease that lacks the sensitivity to identify subclinical disease (Marwick et al., 2010; Stanton and Marwick, 2010). Cardiac magnetic resonance imaging (MRI) is also useful in determining the site and extent of hypertrophy. MRI studies can be particularly helpful in patients with equivocal echocardiographic results. With its high spatial resolution and superb imaging quality, MRI can be used to better delineate subtle apical or segmental hypertrophy. Cardiac catheterization is not a common study in HCM patients, however, it may demonstrate left ventricular outflow obstruction if the echocardiographic study is inconclusive.
**TREATMENT**

The therapy goals in HCM are to manage symptoms and prevent sudden death (see Figure 1.3, (Maron and Maron, 2013)). Studies have been inconclusive regarding treatment of asymptomatic family members of HCM patients (Ommen et al., 2011). Medical therapy is the first line of treatment in symptomatic HCM patients. Calcium-channel blockers (primarily verapamil) and beta-adrenergic blockers are used to prevent exertional dyspnea and chest pain. Oral anti-coagulation is indicated to prevent of embolic events in at-risk patients with atrial fibrillation. If medical management fails, particularly in patients with severe ventricular outflow obstruction, interventional myomectomies reduce the size of the septum. Similarly, ethanol-mediated ablation via catheterization may reduce the size of the septum by infarction.

![Figure 1.3: Primary treatment strategies for presentations of hypertrophic cardiomyopathy.](image-url)

Although these procedures may improve symptoms, they have not been shown to improve survival in HCM patients.

It is estimated that the incidence of sudden death in the HCM population is around 1% (Maron et al., 1996b). Sudden cardiac death occurs due to ventricular tachyarrhythmias that are caused by disarray of myocytes with interstitial fibrosis. The risk factors for sudden death include: prior sustained ventricular tachyarrhythmias, family history of sudden death, genetic mutations associated with sudden death, septal wall thickness >30 mm, recurrent syncope, and exertional hypotension. Patients with risk factors for sudden death are encouraged to have implantable cardioverter-defibrillators, and all patients with HCM are urged to minimize competitive sports and intense training.

There have been no randomized trials to evaluate treatment options in HCM patients, however, retrospective observational studies have been used to create general guidelines. In addition to counseling on treatment, it is recommended that the family of the patient undergo genetic testing since HCM is inherited in an autosomal dominant fashion. Screening echocardiography is typically performed in young first degree relatives of the patient every 12-18 months and before competitive sport activities. Screening of non-athlete adult relatives is currently recommended every five years. If the particular mutation of the patient is known, genetic analysis is the preferred method of screening in relatives. Competitive athletics are advised against and mild aerobic exercises are permitted for a healthy lifestyle in HCM patients (Ommen et al., 2011).
There has been significant progress in describing the pathophysiology, genetics, and treatment of HCM, however there is still much debate and uncertainty regarding the diagnosis, natural history, and management of HCM (Maron and Maron, 2013). The role of genetic testing in HCM is still poorly defined. It has been shown that the occurrence of a positive genetic test for HCM is associated with greater incidence of LV dysfunction (Figure 1.4, Olivotto et al., 2008), however pathogenic mutations are only identified in fewer than 50% of clinically affected HCM patients (Bos et al., 2009b; Maron et al., 2012a; Richard et al., 2003). This is largely due to significant genetic heterogeneity, presence of mutations in non-analyzed sequences, incomplete sensitivity of the screening procedure, or involvement of currently unidentified genes (Richard et al., 2003). As the genetic analysis of patients with HCM expands, there may be potential for more targeted therapies against the progression of LVH. A proof of concept study has demonstrated that in vivo gene transfer of cMyBPC directly injected into the myocardium of cMyBPC knock-out mice improved myofilament and in vivo contractile function (Merkulov et al., 2012).

Clinical genetic testing in the 1990’s revealed a population of genotype-positive phenotype-negative individuals (Rosenzweig et al., 1991), also titled preclinical HCM. It has been challenging to define the best screening/diagnostic plan and resulting management of genotype-positive phenotype-negative individuals (Maron et al., 2011a), particularly due to the long period of follow-up necessary to make evidence-based guidelines.
Following genetic identification of HCM individuals, the phenotype is characterized by diagnostic imaging. Traditionally, 2-dimensional echocardiography is used to define the LV thickness, and thus if the patient is positive for a phenotype of LVH. There have been several studies aiming to better characterize the subclinical presentation of genotype-positive phenotype-negative individuals. Abnormalities such as blood-filled myocardial crypts (Maron et al., 2012b), biomarkers of fibrosis (Ho et al., 2010), and mitral leaf elongation (Germans et al., 2006; Maron et al., 2011b) have all been described in the LV myocardium of subclinical HCM. The definition of a positive phenotype has been expanded to include subclinical diastolic dysfunction in the absence of overt LVH measured by Doppler imaging (Ho et al., 2002). Cardiovascular MRI (CMR) is becoming the preferred imaging modality in describing preclinical HCM given its better characterization of LVH as compared to echocardiography (Germans et al., 2006; Maron et al., 2009b). In addition, contrast-
enhanced CMR has been used to characterize LV myocardial fibrosis in four unrelated genotype-positive phenotype-negative HCM patients (Rowin et al., 2012).

The clinical management of genotype-positive phenotype-negative HCM individuals remains problematic even as our understanding of the pathophysiology improves. The identification of this subset of HCM patients only emerged in the past two decades, so there is a lack of clinical data following their clinical course. In particular, complex questions regarding abstinence from competitive sports will need to be addressed, given that HCM is the most common cause of sudden death in young athletes (Maron et al., 1996b, 2009a) and that there may be a decreased risk of death from avoiding vigorous exercise (Pelliccia et al., 2005). There is also a need to evaluate primary prevention of sudden death with ICDs in genotype-positive phenotype-negative HCM patients. Although ICDs have been effective in terminating life-threatening arrhythmias in HCM patients with marked LVH (Maron et al., 2007, 2013), there is no data regarding ICD placement in preclinical subjects. Thus, there is a need to better understand and define the negative phenotype in genotype-positive HCM patients.
DUCHENNE MUSCULAR DYSTROPHY

INTRODUCTION

The dystrophin-glycoprotein complex (DGC) is thought to be responsible for maintaining the structure and morphology of myocytes. Dystrophin is a major actin-binding component of the dystrophin glycoprotein complex (DGC) and links cytoskeletal and membrane elements in the muscle (Tinsley et al., 1994). (Figure 1.5, (Liew and Dzau, 2004)) The DGC provides stability to the sarcolemma, and disruption of the DGC may cause tears in the membrane, altered cell signaling, and ultimately muscle fiber necrosis.

Figure 1.5: Structural network in the cardiomyocyte.

The dystrophin-glycoprotein complex (DGC) is believed to be involved in maintaining the structural integrity of the myocyte. The complex is comprised of dystrophin, dystrobrevin, dystroglycans, sarcoglycans, and syntrophins concentrated at costameres, which anchor and maintain spatial organization of myofibrils to the plasma membrane and are the sites of mechanical coupling between the extracellular matrix and sarcomere. Dystrophin binds to F-actin to link the sarcomere and costamere.

The DGC is also a necessary component of the neurovascular units in the brain (Figure 1.6, (Abbott and Friedman, 2012)), which are formed by neurons, glia, and microvessels implicated in regulating cerebral blood flow (Abbott et al., 2006). The blood-brain barrier (BBB), which is formed by the endothelial cells lining the cerebral microvessels, acts within the neurovascular unit to ensure the proper metabolic and physical environment for neuronal function (Mäe et al., 2011). The endothelial cells lining the vasculature of the brain are joined by tight junctions in order to prevent free diffusion of large substances from the blood into the brain parenchyma (Brightman and Reese, 1969; Reese and Karnovsky, 1967). Dystrophin is necessary for anchoring of aquaporin 4 (AQP4) and tight-junctional components, which regulate the movement of water in the brain (Nico et al., 2004) (Figure 1.7, (Wolburg et al., 2009).
Duchenne’s muscular dystrophy (DMD) is an invariably fatal X-linked recessive disorder in which the cytoskeletal protein dystrophin is defective and therefore progressively disrupts muscle fiber function. The loss of muscle strength in boys with Duchenne’s muscular dystrophy is progressive, and is more severe in the proximal limb (legs more so than arms) and neck flexor muscles. The incidence of Duchenne’s dystrophy is approximately 1 in 3000-4000 live-born males (Ropper et al., 2014).
PATHOLOGY

There are two primary theories proposed to elucidate the pathogenesis of DMD (Figure 1.8 (Deconinck and Dan, 2007). The first is that a lack of dystrophin destabilizes the sarcolemma and results in muscle fiber damage from repetitive contractions, particularly eccentric contraction (Mokri and Engel, 1998; Pestronk et al., 1982; Petrof et al., 1993). The dystrophin-associated protein complex form costameres that anchor the cytoskeleton to the extracellular matrix (Campbell, 1995; Rybakova et al., 2000), and its disruption leads to membrane fragility. Secondly,
disruption of the DGC alters calcium homeostasis in the muscle cell (Hack et al., 1999; Rafael et al., 2000). The first evidence of calcium dysregulation came from muscle biopsies of DMD patients that demonstrated accumulation of calcium and hypercontracted fibers (Bodensteiner and Engel, 1978; Cullen and Fulthorpe, 1975; Duncan, 1978). Prior studies have established that there is a larger influx of calcium into the cell, likely involving the TRPC calcium channel (De Backer et al., 2002; Franco and Lansman, 1990; Tutdibi et al., 1999; Vandebrouck et al., 2002). The sustained increase in intracellular calcium leads to activation of proteases that destroy membrane components and ultimately leads to cell death (Spencer and Tidball, 1996; Spencer et al., 1995).

**GENETICS**

Duchenne's muscular dystrophy is the result of a mutation of the gene that encodes for the cytoskeletal protein, dystrophin. The dystrophin gene is one of the largest in the human genome, larger than 2000 kb in size, and is on the short arm of the X chromosome at Xp21. The disease is most commonly caused by a deletion, and the size of the mutation does not correlate with the severity of symptoms (Amato and Brown, 2012). There are several genetic mutations that may lead to DMD. Point mutations (approximately 10% of DMD cases) may generate premature termination codons that produce a truncated protein, or the point mutation may affect a critical domain of the dystrophin protein. Deletion or insertion of a single nucleotide disrupt the open reading frame of the messenger RNA (mRNA) for dystrophin, which significantly alters the structure of the protein. In addition, exon-intron splice site mutations can also alter the open reading frame of dystrophin mRNA. Reading-frame
alterations are the most common mutations of the \textit{dmd} gene (Amato and Brown, 2012). Mutations that result in a partially functional dystrophin protein clinically lead to a milder form of the disease, known as Becker muscular dystrophy (Ropper et al., 2014). Approximately 30\% of cases do not have any family history of dystrophy, which represents the rate of spontaneous mutations. In rare cases, females may exhibit symptoms of Duchenne's muscular dystrophy. For example, a female may inherit only one X chromosome (Turner syndrome) that carries the dystrophin mutation.

\textit{PRESENTATION}

Duchenne's muscular dystrophy is present at birth, however, it is not typically diagnosed until the boy is between 3 and 5 years old (Ropper et al., 2014). Children with Duchenne's have difficulty playing, fall frequently, and will demonstrate abnormal running and jumping. A classic sign is the Gowers' maneuver, in which the boy will need to use his hands to gradually climb up the shins, knees, and torso when getting up from the floor. By the age of 6 years, boys will exhibit toe walking from gastrocnemii weakness and contractures of the heel cords and iliotibial bands. A lordotic posture results from weakness of the abdominal and paravertebral muscles. It is typical for the boys to have enlarged gastrocnemii (pseudo-hypertrophy) that have a firm and rubbery feel due to the replacement of muscle tissue with connective and adipose tissue. Patients waddle when walking and have a wide stance when standing from gluteus medius weakness and instability. By the age of 10, walking requires the use of braces, and most boys are wheelchair-bound by the age of 12. Joint contractures of hip flexion, knee, elbow, and wrist extension may become fixed and painful.
Tendon reflexes eventually diminish and are not present once the muscle has atrophied.

In addition to diaphragmatic weakness, progressive scoliosis is typical and impairs pulmonary function. Pulmonary infections may be fatal in Duchenne’s muscular dystrophy teenage boys and is a common cause of mortality. Aspiration of food and acute gastric dilation are also common causes of death. The life expectancy in DMD does not commonly extend beyond the third decade (Annexstad et al., 2014; Eagle et al., 2002b; Kieny et al., 2013).

Cardiomyopathy is present in almost all Duchenne’s muscular dystrophy boys by the age of 18 (Nigro et al., 1990). Similar to the pathogenesis in skeletal muscle, the necrosis of cardiac muscle fibers triggers fibrosis. Cardiac involvement typically includes progressive atrioventricular block, arrhythmia, dilated cardiomyopathy, and akinesis/dyskinesis of the posterobasal wall of the left ventricle. An ECG may display prominent R waves in the right precordial leads, deep Q waves in the left precordial and limb leads, and diminished P-wave amplitude.

Intellectual impairment is common, with boys testing on average one standard deviation below the mean for intelligence quotient (IQ) and commonly affecting verbal performance more than anything else (Bresolin et al., 1994a). The etiology of cognitive impairment is not well understood in DMD, however studies have suggested that hypometabolism may have a role (Al-Qudah et al., 1990; Tracey et al., 1995, 1996a). There is also evidence of cerebrovascular disease in young adult DMD patients, demonstrated by case reports of cerebral infarctions (Table 1.1, (Tsakadze et al., 2011)) (Díaz Buschmann et al., 2004; Ikeniwa et al., 2006; Matsuishi et al., 1982; Tsakadze
et al., 2011). Cerebral infarction has been reported in 0.75% of DMD patients aged 16-20 years (Hanajima and Kawai, 1996a), however this may be significantly underestimated given the difficulty in detecting symptoms in setting of severe dystrophy. It is thought that the etiology of cerebral infarction in DMD is due to cardio-embolic events as a result of cardiomyopathy.

**DIAGNOSIS**

Serum CK levels are elevated 20-100 times the normal level in Duchenne's muscular dystrophy (Amato and Brown, 2012). An elevated CK may be detected at birth, and will progressively increase until the end stages of the disease where the patient is inactive and has lost significant muscle mass. A muscle biopsy (typically from the gastrocnemius) from a Duchenne's muscular dystrophy boy demonstrates fibers of varying size, necrosis, regeneration, and connective tissue and adipose tissue that has replaced muscle. An official diagnosis of Duchenne’s muscular dystrophy is

<table>
<thead>
<tr>
<th>Publication</th>
<th>Sex/age, years</th>
<th>Vascular territory</th>
<th>CMO</th>
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<tr>
<td></td>
<td>M/26</td>
<td>Left MCA</td>
<td>Present</td>
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**Table 1.1: Case reports of cerebral infarction in DMD patients.**

made by Western blot analysis of the biopsy, which shows abnormal quantity and size of the dystrophin protein. Immunohistochemical analysis of the muscle may also

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<tr>
<th>Figure 1.9: Stages of Duchenne Muscular Dystrophy and Treatment Options.</th>
</tr>
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demonstrate dystrophin deficiency or deletion. An EMG shows fibrillations, positive waves, and low-amplitude and brief polyphasic motor unit potentials.

**TREATMENT**

The current treatment of DMD is multi-disciplinary and involves therapies directed towards alleviating symptoms, including corticosteroids, respiratory, cardiac, orthopedic, and rehabilitative interventions (Figure 1.9 (Bushby et al., 2010b). Glucocorticoids are the standard of care and have been shown to delay the progression of DMD for up to 2 years by improving muscle strength and function (Manzur et al., 2008; Moxley et al., 2010). However, many patients decline this therapy given the significant side effect profile, including weight gain and increased risk of fractures in a population that is already at danger for falls. ACE inhibitors and beta-blockers are the first-line agents for treating the progression of cardiomyopathy in DMD (Bushby et al., 2010a).

**RESEARCH ADVANCES**

There has been significant emphasis on improving strategies for DMD gene and pharmacologic therapies over the past decade. Animal models of DMD have been invaluable for evaluating the efficacy of such investigations. The dystrophin-null *mdx* mouse has been used as a mouse model of muscular dystrophy for over two decades (Sicinski et al., 1989). In addition, skeletal or cardiac specific deficiency of dystrophin has been studied in dogs. The GRMD (Golden Retriever Muscular Dystrophy) is the most accurate animal model to study given its homology to the clinical disease (Kornegay et al., 2012).
There has been significant focus on investigating the use of gene therapy to restore the defective dystrophin gene or induce dystrophin production by correcting the underlying mutation as a treatment in DMD since it has the potential to be curative (Table 1.2 (van Deutekom and van Ommen, 2003)(Foster et al., 2012; Konieczny et al., 2013; Malik et al., 2012). Genetic strategies have included viral-mediated insertion of either full-length or truncated dystrophin transgenes, antisense oligonucleotides to induce exon skipping and reestablish the dystrophin reading frame, agents that read-through stop codon mutations, and upregulating surrogate proteins such as utrophin to replace dystrophin. Stem cell therapies have focused on restoring the depleted muscle fibers and inducing a functional dystrophin gene. The observation that patients with Becker muscular dystrophy maintain ambulatory capabilities and typically do not exhibit symptoms until well into adulthood led researchers to believe that even the

<table>
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<tr>
<th>Strategy</th>
<th>Action/effect</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Prospects</th>
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<tbody>
<tr>
<td>Adenoviral vectors</td>
<td>Full-length dystrophin cDNA transfer</td>
<td>High transduction levels in regenerating muscle, expression of fully functional dystrophin</td>
<td>Viral immune responses, limited persistence of transgene expression, maturation dependent</td>
<td>++</td>
</tr>
<tr>
<td>Herpes simplex viral vectors</td>
<td>Full-length dystrophin cDNA transfer</td>
<td>High transduction levels in regenerating muscle, expression of fully functional dystrophin</td>
<td>Viral toxicity and immune response, limited persistence of transgene expression, maturation dependent</td>
<td>+</td>
</tr>
<tr>
<td>Plasmid vectors</td>
<td>Full-length dystrophin cDNA transfer</td>
<td>Synthetic, non-infectious, relatively safe, flexible, simple engineering</td>
<td>Large molecule, delivery requires efficient transfection method</td>
<td>++</td>
</tr>
<tr>
<td>Myoblast transplantation</td>
<td>Introduce dystrophin-producing cells</td>
<td>Non-infectious, relatively safe</td>
<td>Low efficiencies, immune suppression required</td>
<td>+</td>
</tr>
<tr>
<td>Stem-cell therapy</td>
<td>Introduce dystrophin-producing cells</td>
<td>Conventional treatment, relatively safe</td>
<td>Low efficiencies, immune suppression required</td>
<td>++</td>
</tr>
<tr>
<td>Chimera oligonucleotides</td>
<td>Correction of mutation at the mRNA level</td>
<td>Cumulative, permanent effect</td>
<td>Low in vivo efficiencies</td>
<td>+</td>
</tr>
<tr>
<td>Gentamicin therapy</td>
<td>Ribosomal read-through of stop codons in mRNA</td>
<td>Conventional drug</td>
<td>Low reproducibility, risk of non-specific adverse effects</td>
<td>+</td>
</tr>
<tr>
<td>RAAV vectors</td>
<td>Mini- or micro-dystrophin cDNA transfer</td>
<td>High transduction efficiencies in muscle, non-pathogenic minimal immune responses</td>
<td>Unable to deliver full-length dystrophin, laborious production systems</td>
<td>+++</td>
</tr>
<tr>
<td>Antisense oligonucleotides</td>
<td>Splicing modification of pre-mRNA</td>
<td>Synthetic, small-molecule drug, relatively safe, restores all isoforms</td>
<td>Repeated administrations and targeting; delivery agent; needed, mutation specific</td>
<td>+++</td>
</tr>
<tr>
<td>Utophin upregulation</td>
<td>Replacement of dystrophin</td>
<td>Small-molecule drug, no immune response, relatively safe</td>
<td>No effective specific compound identified as yet</td>
<td>++</td>
</tr>
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*These three relatively new strategies are most likely to lead to an effective treatment for Duchenne muscular dystrophy. The symbols in the prospects column indicate a subjective assessment of the probability of a particular strategy leading to an effective treatment, ranging from low (+) to high (+++). Also recombinant adenovirus-associated virus.

**Table 1.2: Overview of strategies for Duchenne Muscular Dystrophy gene therapy.**

partial restoration of functional dystrophin may be beneficial in DMD patients (Bushby et al., 1993; Malhotra et al., 1988). It has been shown in mice and humans that the minimum level of dystrophin necessary to prevent muscular dystrophy is 20-30% (Sharp et al., 2011).

Nonsense mutations that result in premature termination codons (PTC) may be addressed by administering molecules (gentamycin and ataluren) that allow a read-through of the PTC in mRNA. Approximately 15% of DMD patients have nonsense mutations from single nucleotide DNA polymorphisms that lead to premature in-frame stop codons and would therefore benefit from development of these drugs (Dent et al., 2005). Administration of ataluren to prevent nonsense mutations has been studied in both mdx mice and DMD patients, which was able to restore some functional dystrophin expression and slow disease progression (Barton-Davis et al., 1999; Hoffman and Connor, 2013; L et al., 2003; Peltz et al., 2013; Wagner et al., 2001; Welch et al., 2007).

Disruption of the open reading frame is the primary source of DMD cases (approximately 80%), either through deletions/insertions or splicing mutations. Exon skipping is a tactic to restore the reading frame of the dystrophin gene (van Deutekom and van Ommen, 2003; Foster et al., 2012). Specific antisense oligonucleotides (AONs) are synthetic, short nucleic acid polymers that bind to the mutation site in the pre-mRNA to skip over the offending exon and restore the reading frame (Summerton and Weller, 1997). There have been great advancements in drug therapy for exon skipping in DMD patients. Trials are currently underway studying two antisense oligomer drugs (eteplirsen and drisapersen) that target a DMD gene hot spot (exon
51), however the drisapersen trial was suspended due to concerns of efficacy (Hoffman and Connor, 2013).

Given the recent advances along multiple fronts for the treatment of DMD, it is reasonable to assume that the median life expectancy of DMD will increase. Accordingly, the prevention, diagnosis, treatment for extra-cardiac manifestations of dystrophin dysfunction will likely take on new importance. Specifically, the role of dystrophin in the pathophysiology of cerebrovascular disease is poorly defined and awaits delineation with the use of advanced in vivo imaging.
Magnetic Resonance Imaging

DISPLACEMENT ENCODING WITH STIMULATED ECHOES

Cardiovascular magnetic resonance (CMR) provides a non-invasive and accurate representation of cardiovascular function. In particular, techniques have been developed to measure the mechanics of myocardial wall motion, such as strain, twist, and torsion. The myofibers of the heart are arranged in a right-handed helix in the subendocardium and transition to a left-handed helix in the subepicardium. This results in LV wall thickening in the radial direction and shortening in the longitudinal direction during contraction (Sengupta et al., 2006). Myocardial strain can be described as the deformation of the myocardial wall, and is defined in three orthogonal planes: radial, circumferential, longitudinal (Figure 1.10, Jiang and Yu, 2014). A positive strain represents lengthening or thickening and a negative strain is representative of shortening of the myocardium. Strain rate is characterized as the rate of change of strain over the course of the cardiac cycle, and is frequently used as an index of regional LV function (Edvardsen et al., 2006). CMR is also useful in describing the wringing or twisting motion of the heart. The base rotates in a clockwise direction and the apex in a counterclockwise direction when viewed from the apex (Figure 1.10, Jiang and Yu, 2014), which is quantified as the twist angle. Torsion is defined as the difference in the twist angle from the apical and basal slices normalized by the distance between the two slices. Torsion and twist have been shown to be sensitive to subtle myocardial systolic and diastolic dysfunction since they are affected by myofiber orientation, contractility, and loading conditions (Burns et al., 2008; Dong et al., 1999; Li et al., 2009b; Stuber et al., 1999). Displacement encoding with
stimulated echoes (DENSE) is one method that was developed by Aletras et al. in 1999 to provide high spatial density of tissue displacement via stimulated echoes (Aletras et al., 1999). The myocardial displacement is encoded directly in the phase of the stimulated echo, and can therefore provide myocardial strain and twist data. A representative displacement map is shown in (Figure 1.10, Jiang and Yu, 2014).

**Figure 1.10:** *Left ventricular mechanics during contraction.* (A) Myocardial wall strains during diastole and systole in the Radial-Circumferential-Longitudinal (RLC) system: radial (E_R), circumferential (E_C), and longitudinal (E_L) strains, and the principal system: along the direction of greatest length increase in myofibers (E_1), greatest length decrease in myofibers (E_2), and orthogonal to both E_1 and E_2 (E_3). (B) LV wall torsional deformation during contraction. The apex twists counterclockwise and base twists clockwise viewing from apex to base. (C) Representative 2-D displacement map at peak systole as measured by displacement encoding with stimulated echoes (DENSE).

ARTERIAL SPIN LABELING

Arterial spin labeling (ASL) MRI is a non-contrast perfusion MRI technique that has been shown to provide quantitative assessments of tissue perfusion in several applications (De Bazelaire et al., 2005; Katada et al., 2012; Mai and Berr, 1999; Martirosian et al., 2004; Wong et al., 1997). In essence, ASL is similar to traditional contrast perfusion techniques in that it “magnetically tags” arterial blood water, leading to altered tissue longitudinal magnetization that is proportional to tissue perfusion. ASL MRI techniques generate blood flow contrast between multiple images using a wide variety of blood labeling methods (Detre and Alsop, 1999; Detre et al., 1992; Edelman et al., 1994; Kim and Tsekos, 1997; Kwong et al., 1995; Williams et al., 1992). A typical ASL MRI acquisition combines an ASL preparation phase, followed by a rapid imaging readout to capture the blood flow-weighted contrast.

One major advantage of ASL over conventional dynamic contrast-enhanced (DCE) MRI perfusion techniques is the lack of exogenous, and potentially toxic, paramagnetic contrast agents, which is especially important for the imaging of patients with chronic kidney diseases (Kuo et al., 2007). This attribute is also important for longitudinal preclinical imaging applications, as multiple tail-vein injections and/or catheterizations can cause local inflammation and necrosis, resulting in reduced access to veins.

DIFFUSION-WEIGHTED MAGNETIC RESONANCE IMAGING

Diffusion-weighted MRI (DWI) is a technique that has become invaluable in defining neurological disorders, particularly in the diagnosis and therapeutic intervention of stroke (Le Bihan et al., 1986; Sevick et al., 1990; Warach et al., 1995;
Schellinger et al., 2003; Kloska et al., 2010). The signal intensity of a DW image is representative of the Brownian motion of water molecules in the tissue, and the calculated apparent diffusion coefficient (ADC) provides a means to quantify this diffusion under physiologic and pathologic states. High ADC values are characteristic of a tissue with relatively free water diffusion as opposed to tissue water with a restricted environment (Bihan, 2007). Following cerebral ischemia, the metabolic production of ATP is halted and therefore leads to sodium and calcium retention in the cell from non-functioning ionic pumps (Ito et al., 1979). The osmotic influx of water into the cell results in cytotoxic edema (Klatzo, 1987) that decreases the available extracellular space for diffusion of water. This pathologic response to ischemia is supported by an immediate decrease in ADC which occurs before any significant changes in a T₂-weighted image (Moseley et al., 1990; Kucharczyk et al., 1991).
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CHAPTER 2: CARDIAC MYOSIN BINDING PROTEIN C INSUFFICIENCY LEADS TO EARLY ONSET OF MECHANICAL DYSFUNCTION

ABSTRACT

Background—Decreased expression of cardiac myosin binding protein C (cMyBPC) as a result of genetic mutations may contribute to the development of hypertrophic cardiomyopathy (HCM); however, the mechanisms that link cMyBPC expression and HCM development, especially contractile dysfunction, remain unclear. Methods and Results — We evaluated cardiac mechanical function in vitro and in vivo in young mice (8–10 weeks of age) carrying no functional cMyBPC alleles (cMyBPC−/−) or 1 functional cMyBPC allele (cMyBPC±). Skinned myocardium isolated from cMyBPC−/− hearts displayed significant accelerations in stretch activation cross-bridge kinetics. Cardiac MRI studies revealed severely depressed in vivo left ventricular (LV) magnitude and rates of LV wall strain and torsion compared with wild-type (WT) mice. Heterozygous cMyBPC± hearts expressed 23±5% less cMyBPC than WT hearts but did not display overt hypertrophy. Skinned myocardium isolated from cMyBPC± hearts displayed small accelerations in the rate of stretch induced cross-bridge recruitment. MRI measurements revealed reductions in LV torsion and circumferential strain, as well reduced circumferential strain rates in early systole and diastole. Conclusions—Modest decreases in cMyBPC expression in the mouse heart result in early-onset subtle changes in cross-bridge kinetics and in vivo LV mechanical function, which could contribute to the development of HCM later in life.
INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is one of the most commonly occurring genetic myocardial disorders, affecting approximately 1 in 500 people (Maron et al., 1995). Cardiac myosin binding protein C (cMyBPC) is a thick filament protein that modulates actin-myosin interactions and thereby the rate of muscle contraction (Carrier et al., 2007, Barefield et al., 2010). It is well established that the gene encoding cMyBPC is one of the most common causes of inherited HCM, with nearly 200 known mutations identified (Harris et al., 2011) since the first reported mutation in this gene (Watkins et al., 1995, Bonne et al., 1995). However, many younger individuals who carry disease-causing mutations in the cMyBPC gene do not exhibit overt LVH, because increases in LV wall thickness are often only detectable with advanced age (Maron et al., 2004, Niimura et al., 1998). Because these seemingly asymptomatic carriers are at risk for the development of HCM and cardiac disease later in life, the diagnosis and treatment of these patients is a major clinical challenge.

The majority of mutations in the gene that encode cMyBPC are heterozygous and are predicted to result in expression of truncated cMyBPC lacking the C-terminal regions of the protein that binds to myosin and titin (Carrier et al., 1997). However, analysis of myocardial biopsy samples from patients with cMyBPC mutations have not detected truncated cMyBPC, but rather a reduction in the amount of full-length cMyBPC protein has been noted (Rottbauer et al., 1997, Moolman et al., 2000, van Dijk et al., 2009, Marston et al., 2009, Jacques et al., 2008). It is therefore likely that mutant cMyBPC mRNA or proteins are rapidly degraded by nonsense mediated mRNA decay or the ubiquitin-proteosome system, thereby preventing mutant proteins
from incorporating into the sarcomere (Schlossarek et al., 2011). Therefore, the allele generating mutant cMyBPC effectively functions as a null allele, causing cMyBPC haploinsufficiency. However, the mechanisms that link reduced cMyBPC levels in the heart with the development and progression of HCM have remained elusive.

Considering the functional importance of cMyBPC in regulating myofilament contractile properties, it is reasonable to suppose that decreased cMyBPC expression could affect in vivo mechanical function. In this regard, it has been shown that homozygous cMyBPC knockout mice (cMyBPC<sup>−/−</sup>) with 2 null cMyBPC alleles (i.e., a model of pure insufficiency) display early-onset impairments in systolic and diastolic contractile function and severe LVH (Harris et al., 2002, Palmer et al., 2004, Nagayama et al., 2007). In contrast, heterozygous cMyBPC knockout mice (cMyBPC±) with 1 null cMyBPC allele develop a phenotype later in life, displaying modest hypertrophy despite preserved systolic and diastolic contractile function (Carrier et al., 2004). Interestingly, cMyBPC± hearts expressed ≈25% less total cMyBPC than aged-matched wild-type (WT) mice (Carrier et al., 2004), which is similar to the amount of full length cMyBPC in patients with heterozygous cMyBPC mutations (van Dijk et al., 2009, Marston et al., 2009). These results suggest that modest decreases in cMyBPC expression in the heart may be sufficient to produce cardiac dysfunction and/or LVH; however, it is has not been established if these changes are related to altered cardiac mechanical performance.

Therefore, in the present study we examined the effects of variable cMyBPC expression on in vitro and in vivo mechanical function in young (8–10 weeks of age) cMyBPC<sup>−/−</sup> and cMyBPC± mice to determine if cMyBPC insufficiency can cause
mechanical dysfunction early in life. We used MRI to quantify both global and regional mechanical indices such as LV twist, torsion, and principal strains, over the whole cardiac cycle to observe subtle changes in mechanical function. Our goal was to link cMyBPC expression and cross-bridge function with LV strain and torsion, which are direct measures of myocardial wall deformation, to characterize the functional consequences of cMyBPC insufficiency.

METHODS

Animal Models

Adult male cMyBPC null (cMyBPC−/−), heterozygous cMyBPC mice (cMyBPC+), and WT mice of the SV/129 strain (8–10 weeks of age) were used in this study (Harris et al., 2002). All procedures involving animal care and handling were performed according to institutional guidelines set forth by the Animal Care and Use Committee at Case Western Reserve University.

Skinned Fiber Experiments

Force-pCa relationships and stretch activation kinetics were measured in skinned ventricular myocardium isolated from WT, cMyBPC−/−, and cMyBPC+ hearts as previously described (Chen et al., 2010, Stelzer et al., 2006). Myofibrillar protein content and phosphorylation were determined in LV homogenates and skinned myocardium as previously described (Chen et al., 2010, Stelzer et al., 2007).

In Vivo MRI Measurements of Cardiac Function

Two-dimensional LV myocardial motion was quantified at the apex, mid, and basal levels using multiphase displacement encoding with stimulated-echo (DENSE) MRI in a 9.4-T Bruker Biospec (Billerica, MA) horizontal scanner (Liu et al.,
LV twist, torsion, circumferential and radial strain, as well as torsion and strain rates were quantified, using custom software as previously described (Zhong et al., 2008).

**Statistical Analysis**

Cross-sectional areas of skinned preparations were calculated by measuring the width of the mounted preparation and assuming a cylindrical cross section. Submaximal Ca\(^{2+}\)-activated force (P) was expressed as a fraction of the force (P\(_o\)) generated at pCa 4.5, that is, P/P\(_o\). Rate constants of stretch-induced force decay (k\(_{rel}\)) and development (k\(_{ad}\)) were obtained by fitting the time course trace with a single exponential. Data are reported as mean±SD or mean±SEM. Means for fiber data were generated by averaging data for each variable for each mouse and then averaging the means for all mice within a group. Approximately 3 fibers were studied from each mouse. Comparisons of force-pCa relationships and stretch activation variables between groups were done using a 1-way ANOVA and a Tukey-Kramer post hoc test. Comparisons of strain and twist angles were independently analyzed at each ventricular level by 1-way ANOVA and Tukey-Kramer test. Strain and torsion time course data were evaluated by 1-way ANOVA with a Bonferroni adjustment for multiple comparisons. Additionally, a separate analysis examining only peak rates of torsion and strain in systole and diastole was performed using 1-way ANOVA and Tukey-Kramer post hoc test. Statistical significance was established at a level of \(P<0.05\).

**RESULTS**
Myofilament Protein Expression and Phosphorylation in WT and cMyBPC Mutant Myocardium

We examined the expression and phosphorylation status of myofilament proteins in age-matched male WT, cMyBPC\(^{-/-}\), and cMyBPC\(^{+/+}\) LV tissue homogenates. As expected, cMyBPC was not detected in cMyBPC\(^{-/-}\) hearts in either Coomassie-stained gels or Western blots probed with a cMyBPC specific antibody (Santa Cruz, CA); however, cMyBPC\(^{+/+}\) hearts expressed 23±5% less cMyBPC than WT hearts (\(P<0.05\), Figure 2.1). Consistent with previous findings, (Harris et al., 2002) expression of \(\beta\)-myosin heavy chain (MHC) was slightly elevated in cMyBPC\(^{-/-}\) hearts (16±3%, \(P<0.05\)) compared with WT hearts, but there was no significant difference in MHC isoform expression between cMyBPC\(^{+/+}\) and WT hearts (Figure 2.2). No significant differences in the relative abundance or phosphorylation status of other myofilament proteins in WT, cMyBPC\(^{-/-}\), and cMyBPC\(^{+/+}\) myocardium were detected (Figure 2.3). The cMyBPC content of skinned myocardium isolated from cMyBPC\(^{+/+}\) LV used for mechanical experiments was also quantified (Table 2.1) and was nearly identical to the cMyBPC content measured in LV tissue homogenates prepared from cMyBPC\(^{+/+}\) hearts (ie, reduced 24±6% compared with WT).

![Figure 2.1. Quantification of cMyBPC expression in cMyBPC\(^{+/+}\) myocardium. A representative Western blot of whole tissue homogenates probed with a cMyBPC specific antibody. Lane 1, cMyBPC\(^{-/-}\), lane 2 cMyBPC\(^{+/+}\), and lane 3 WT. cMyBPC protein content was normalized to \(\alpha\)-actinin content. On average cMyBPC\(^{+/+}\) hearts expressed 23% less cMyBPC than WT hearts.](image)
Figure 2.2. Quantification of myosin heavy chain (MHC) isoform expression in WT and cMyBPC mutant myocardium. A representative 6% SDS-PAGE coomassie stained gel of whole tissue homogenates prepared from: Lane 1, cMyBPC<sup>−/−</sup>, lane 2 cMyBPC<sup>+/−</sup>, and lane 3 WT, myocardium. α-MHC, alpha myosin heavy chain, β-MHC, beta myosin heavy chain. The β-MHC isoform was detected only in cMyBPC<sup>−/−</sup> myocardium.

Figure 2.3. Myofibrillar protein content and phosphorylation in WT and cMyBPC mutant myocardium. (A) Representative 4-12% SDS-PAGE gel of myofibrillar proteins isolated from skinned myocardium from cMyBPC<sup>+/−</sup> (lanes 1 and 4), WT (lanes 2 and 5), and cMyBPC<sup>−/−</sup> (lanes 3 and 6) hearts and stained with coomassie (lanes 1-3) to detect total proteins and Pro-Q Diamond to detect phosphorylated proteins (lanes 4-6). cMyBPC; cardiac myosin binding protein C, TnT; troponin T, TnI; troponin I, RLC; regulatory light chain. (B-E) Slopes of phosphorylation signals from Pro-Q Diamond stained gels (n=6) determined from regression analysis of plots of area x average optical density versus protein loaded (µg) for (B) cMyBPC, (C) RLC, (D) TnT, and (E) TnI. Data are means ± SD.
Mechanical Properties of Skinned Myocardium Isolated From WT and cMyBPC Mutant Hearts

The steady-state mechanical properties of skinned myocardium isolated from WT, cMyBPC−/−, and cMyBPC± hearts are summarized in the Table 2.1. Skinned preparations from the 3 groups of mice exhibited similar force-pCa relationships. There were no differences in force generation at maximal and submaximal [Ca^{2+}] or in the steepness of the force-pCa relationship (Hill coefficient, $n_H$). Consistent with previous studies (Stelzer et al., 2007), cMyBPC−/− myocardium displayed dramatically accelerated rates of stretch-induced force decay ($k_{rel}$) and delayed force development ($k_{df}$) at submaximal Ca^{2+} activations as well as greater stretch-induced force decay (P$_2$ amplitude) and stretch activation amplitude (P$_{a0}$), compared with WT myocardium (Figure 2.4). Skinned myocardium isolated from cMyBPC± hearts displayed small but significant (21%) accelerated stretch-induced delayed force development ($k_{ud}$) at submaximal Ca^{2+} activations, but P$_2$ and P$_{a0}$ amplitude were not significantly different compared with WT (Table 2.2).
In Vivo Assessment of Cardiac Morphology and Mechanical Function

**LV Morphology and Contractile Function**

Cardiac morphology and global contractile function analysis of WT, cMyBPC⁺, and cMyBPC⁻⁻ hearts are presented in Table 2.3. The cMyBPC⁻⁻ hearts displayed significant LV hypertrophy as demonstrated by increased end-diastolic wall thickness and decreased ejection fraction compared with WT controls. In contrast,
CMyBPC⁺ hearts displayed a small increase in apical thickening at end-diastole compared with WT hearts (Table 2.3, \( P<0.05 \)), but systolic function was preserved, as indicated by near-normal ejection fraction values. Global hypertrophy was seen in CMyBPC⁻⁻ hearts, as demonstrated by greater

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Data are mean±SD. WT indicates wild-type; CMyBPC, cardiac myosin binding protein C. *Significantly different from WT.

![Figure 2.5](image)

**Figure 2.5. Cardiac hypertrophy analysis.** (A) Heart-weight to body-weight ratios (HTWT/BWT), 8 hearts from each group. Relative abundance of mRNA of molecular markers of cardiac hypertrophy (values normalized to glyceraldehyde 3-phosphate dehydrogenase, GAPDH abundance): (B) Atrial natriuretic factor (ANF), (C) brain natriuretic factor (BNP), (D) alpha-skeletal actin (α-sk actin). Values are expressed as means ± SD. *Significantly different from WT.
heart weight–to–body-weight (HTWT/BWT) ratios compared with WT hearts. There was no difference in HTWT/BWT ratio between cMyBPC± and WT hearts (Figure 2.5). Molecular markers of hypertrophy, including transcript expression of ANF, BNP, and α-skeletal actin were also significantly upregulated in cMyBPC−/− hearts but not in cMyBPC± hearts (Figure 2.5).

**Myocardial Strain**

Representative displacement maps for the in-plane motion of the WT, cMyBPC±, and cMyBPC−/− groups in the mid-ventricle at peak systole are presented in Figure 2.6. The overall reduction in displacement magnitude (Figure 2.6C) for strain vectors displayed by cMyBPC−/− hearts compared with WT hearts is consistent with significant contractile dysfunction.

![Image of displacement maps](image)

**Figure 2.6.** Representative MRI principal strain vector displacement maps. Representative DENSE displacement maps at the mid-ventricle during peak systole for A, wild-type (WT); B, cardiac myosin binding protein C (cMyBPC)+/−; and C, cMyBPC−/−. The direction of radial (solid arrow) and circumferential strain (dashed arrow) is labeled in (A).

Mean LV strain was calculated at the apex, base, and mid-ventricle for each group. There was a significant reduction in maximal LV radial and circumferential strains in the cMyBPC−/− mice for all 3 ventricular levels (Figure 2.7), and there was a significant
reduction in maximal LV circumferential strain at the mid-LV of cMyBPC± mice compared with WT mice (Figure 2.7B, \( P<0.05 \)). Measurements of average radial (Figure 2.8A through 2.8C) and circumferential strain rates (Figure 2.8D through 2.8F) were significantly reduced in cMyBPC−/− mice throughout the cardiac cycle. Reduced early systolic strain rates were observed in cMyBPC± mice at the mid-LV (Figure 2.8).

Analysis of peak strain rates revealed that cMyBPC± mice displayed lower peak radial systolic strain rates in the base and mid-LV, and lower peak circumferential diastolic strain rates in the mid-LV (Figure 2.8G through 2.8L).

**Ventricular Twist and Torsion**

Figure 2.9A illustrates the difference in twist (rotation) angles between WT, cMyBPC±, and cMyBPC−/− mice at the apical, mid-LV, and basal levels during peak systole. There was a clear decrease in overall rotation in cMyBPC−/− hearts. LV

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**Figure 2.7.** Measurements of left ventricular (LV) global principal strains. Global radial (A) and circumferential (B) strain at the base, mid, and apex in the LV of wild-type (WT), cardiac myosin binding protein C (cMyBPC) +/-; and cMyBPC−/− mice. Data are mean ± SE. *Significantly different from WT.
twisting occurred in the counterclockwise direction at the apex in the WT (10.12±1.9°) and cMyBPC± hearts (7.59±1.3°, P=NS), but minimal apical twisting was observed in cMyBPC−/− hearts (0.83±0.9°, P<0.05). Clockwise twisting was observed at the base in WT (−6.48±1.0°) and cMyBPC± hearts (−4.90±0.40°, P=NS), but minimal rotation occurred in cMyBPC−/− hearts (−1.39±0.84°, P<0.05).

Figure 2.8. Analysis of principal strain rates. Time course of radial (A through C) and circumferential (D through F) strain rates were quantified throughout the cardiac cycle, and peak radial (G through I) and circumferential (J through L) strain rates were quantified (from top to bottom) at the base, mid-ventricle, and apical segments in wild-type (WT), cardiac myosin binding protein C (cMyBPC)+/−; and cMyBPC−/− mice. Data are mean ± SE. *Significantly different from WT by 1-way ANOVA with Bonferroni (A through F) or Tukey-Kramer (G through L).

Figure 8 continued on next page.
The net twist, defined as the difference between the twist angles at the base and apex, is plotted in Figure 2.9B. There was a significant reduction in net twist in cMyBPC\(^{-/-}\) (2.23±0.61°) and cMyBPC\(^{\pm}\) (12.49±1.08°) hearts compared with WT hearts (16.60±1.46°). In particular, net twist between the mid and basal LV segments was reduced in cMyBPC\(^{\pm}\) hearts compared with WT (Figure 2.9A, 3.24±2.0° and 5.98±4.5°, respectively), although this difference fell short of statistical significance (\(P=0.08\)).

There was a significant reduction in the magnitude of peak torsion generated by cMyBPC\(^{-/-}\) and cMyBPC\(^{\pm}\) hearts compared with WT (50.8°/cm ±5.7): 8.9°/cm ±2.2 in cMyBPC\(^{-/-}\) and 39.0°/cm ±2.4 in cMyBPC\(^{\pm}\) (Figure 2.10A and 2.10C), and peak
systolic torsion occurred earlier in cMyBPC−/− hearts. Maximal systolic and diastolic torsion rates were lower in cMyBPC−/− hearts (Figure 2.10B, 2.10D, 2.10E).

**DISCUSSION**

There is growing evidence that decreased cMyBPC expression may be a common feature of HCM related to mutations in the gene encoding cMyBPC (van Dijk et al., 2009, Marston et al., 2009); however, the link between cMyBPC expression, cardiac mechanical function, and the development of HCM is not well understood. We show that decreased cMyBPC expression in young cMyBPC± mice leads to altered myofilament function and in vivo torsion and principal strains in the absence of overt hypertrophy. Our data suggest that mechanical dysfunction exists in preclinical cMyBPC related HCM.
Myofilament Function in cMyBPC Mutant Myocardium

Levels of full-length cMyBPC have been shown to be decreased (24–33%) in patients with mutations in cMyBPC (van Dijk et al., 2009, Marston et al., 2009). Similarly, levels of cMyBPC were decreased in heterozygous mouse models expressing 1 functional cMyBPC allele (the present study and Carrier et al., 2004). Mutations in cMyBPC predicted to produce C-terminal truncated proteins are not
found in patients (Rottbauer et al., 1997, Moolman et al., 2000, van Dijk et al., 2009, Marston et al., 2009, Jacques et al., 2008). The mutant mRNA and/or proteins are thought to be unstable and are degraded before incorporation in the sarcomere (Schlossarek et al., 2011), such that the mutant allele effectively acts as a null allele, leading to cMyBPC haploinsufficiency.

Consistent with previous studies, we found that skinned myocardium isolated from cMyBPC\(^{+/−}\) (Harris et al., 2002) or cMyBPC\(^{−/−}\) hearts (Stelzer et al., 2007) does not display changes in Ca\(^{2+}\)-sensitivity of force generation. This suggests that cMyBPC content in the sarcomere does not directly determine myofilament Ca\(^{2+}\) sensitivity. A recent study (van Dijk et al., 2009) showed that skinned myocytes isolated from myectomy samples obtained from patients with HCM-causing cMyBPC mutations displayed increases in the Ca\(^{2+}\) sensitivity of force generation. However, changes in force generation did not correlate with cMyBPC expression and were likely a result of the large decrease in troponin I (TnI) phosphorylation (84%) in myectomy samples compared with control donor samples.

It is well established that skinned myocardium isolated from cMyBPC\(^{−/−}\) hearts displays significantly accelerated rates of cross-bridge kinetics (Stelzer et al., 2007, Stelzer et al., 2006, Palmer et al., 2004) because myosin heads are in closer juxtaposition to actin molecules, thereby enhancing the probability of acto-myosin interactions (Colson et al., 2007). In the present study, we found that a relatively small decrease in cMyBPC expression (24%) in cMyBPC\(^{+}\) skinned myocardium produced acceleration in the rate of delayed cross-bridge recruitment \(k_{df}\) after rapid stretch at submaximal Ca\(^{2+}\) activations compared with WT skinned myocardium (Table 2.2).
Decreased cMyBPC content may result in nonuniform incorporation of cMyBPC into the sarcomere and regional inhomogeneities in rates of force development. Thus, sarcomeres with reduced levels of cMyBPC could develop force at a faster rate compared with sarcomeres with normal cMyBPC content (Theis et al., 2009). This would result in simultaneous shortening and stretch of different regions of the sarcomere, which would disrupt the timing and synchronization of fiber shortening during systole. Additionally, there may be a delay in force relaxation of regions that are being actively stretched (stretch-activated) (Stelzer et al., 2007) by regions of the sarcomere that are shortening, thereby prolonging relaxation in vivo (Harris et al., 2002, Palmer et al., 2004, Nagayama et al., 2007).

**Effects of Decreased cMyBPC Expression on In Vivo Mechanical Function**

Studies of in vivo cardiac contractile function in cMyBPC−/− mice (Harris et al., 2002, Palmer et al., 2004, Carrier et al., 2004, McConnell et al., 2001) have demonstrated global systolic and diastolic dysfunction and severe LVH. However, in vivo cardiac function and morphology of cMyBPC+ hearts has been shown to be normal at a young age (Harris et al., 2002, Carrier et al., 2004, McConnell et al., 2001), with unexplained asymmetrical hypertrophy developing at 10–11 months of age despite preserved systolic and diastolic function (Carrier et al., 2004). Cardiac MRI can detect subtle changes in LV morphology and mechanical function, so we examined LV motion indices of torsion and strain throughout the cardiac cycle in cMyBPC−/− and cMyBPC+ hearts. In this study, we used DENSE MRI to quantify myocardial mechanics in the mouse heart, a technique that has been validated against rotating phantoms or traditional MRI tagging protocols in mice and humans (Kim et
al., 2004, Gilson et al., 2004). We show that the lack of cMyBPC nearly abolished LV torsion and twist in young mice. Furthermore, the magnitude of LV torsion and strain are reduced and the pattern of LV mechanical function is altered in cMyBPC± hearts in the absence of overt hypertrophy. The latter may be the early mechanical manifestation of cMyBPC haploinsufficiency, which could contribute to the progression and development of LVH with advanced age.

The counterclockwise rotation of the apex and the clockwise rotation of the base (Streeter et al., 1969) produces the wringing motion that maximizes pumping of blood into the circulation. We observed that the magnitude of LV torsion (Figure 2.10) and net twist (Figure 2.9) were severely impaired in cMyBPC−/− hearts such that LV systolic rotation was minimal. These results are consistent with overt hypertrophy and fibrosis (Harris et al., 2002), which renders the cMyBPC−/− LV extremely stiff. Additionally, myofiber architecture abnormalities disrupt the transmural progression of the helical arrangement of subendocardial and subepicardial fibers (Wang et al., 2010). This disruption may decrease the generation of mechanical torque by subepicardial fibers, which propels the rotation of the LV during systole (Taber et al., 1996). Interestingly, despite a significantly reduced magnitude of LV torsion in cMyBPC−/− hearts, peak systolic torsion occurred earlier in systole compared with WT hearts. The resulting shortening of the ejection phase in cMyBPC−/− hearts is consistent with findings that cMyBPC is crucial for modulating the rate of pressure development during isovolumic contraction and the period of systolic ejection (Nagayama et al., 2007).
In contrast to severe mechanical dysfunction and hypertrophy in cMyBPC–/– hearts, a modest 23% decrease in cMyBPC content in cMyBPC± hearts resulted in a small but significant reduction in the magnitude of peak systolic torsion (Figure 2.10). The decrease in the magnitude and duration of systolic torsion in cMyBPC± hearts was due to a reduction in the overall net twist angle between the apex and base (Figure 2.9). Specifically, there was a more prominent reduction in twist between the mid-LV and the basal LV segments (Figure 2.9), although this difference did not reach statistical significance (P=0.08). Because systolic rotation and fiber shortening in the LV proceeds in an apex-to-base sequence (Sengupta et al., 2008), a reduction in net twist could be due to global or regional abnormalities in circumferential strain. Thus, the small reduction in circumferential strain at the mid-LV in cMyBPC± hearts could impair the progression of mechanical rotation from the apex to the base during systole resulting in diminished twist between the mid-LV and basal-LV. These data are also consistent with the idea that cMyBPC is important for prolongation of fiber shortening during late systole (Nagayama et al., 2007), perhaps through continued rotation of the later-activated LV base. Accelerated rates of cross-bridge kinetics and fiber shortening in the early isovolumic contraction phase (Nagayama et al., 2007, Stelzer et al., 2007) in cMyBPC± hearts may disrupt the timing of fiber shortening in later-activated regions such as the base, thereby reducing LV rotation in late systole.

Slower systolic and diastolic circumferential strain rates (Germans et al., 2010, Ennis et al., 2003) are a common feature in HCM carriers. In the present study, cMyBPC–/- hearts displayed significant reductions in radial and circumferential strain
rates during systole and diastole compared with WT hearts (Figure 2.8), and cMyBPC± hearts displayed lower early systolic and peak diastolic circumferential strain rates at the mid-LV, and lower peak radial systolic strain rates in the base and mid-LV (Figure 2.8). Diastolic dysfunction is thought to be an early manifestation of impaired cardiac function in preclinical HCM (Ho et al., 2009). The early phase of LV reverse rotation after systole is an important determinant of diastolic filling (Doucende et al., 2010, Carasso et al., 2010), and decreases in diastolic mid-LV circumferential strain rates in cMyBPC± hearts may result in a slowing of the rate of LV relaxation and decreased diastolic filling, as has been noted previously in cMyBPC−/− hearts (Harris et al., 2002, Palmer et al., 2004).

**Potential Functional Consequences of cMyBPC Haploinsufficiency**

Our data show that small reductions in cMyBPC content in the mouse heart, similar to decreases reported in myocardial samples obtained from patients expressing cMyBPC mutations, can significantly accelerate rates of cross-bridge kinetics and alter myocardial mechanical function in vivo. Nonuniform incorporation of cMyBPC in sarcomeres may result in regional inhomogeneities in rates of force development within sarcomeres along with uncoordinated sarcomere shortening and stretching. This can result in abnormal patterns of LV mechanical torsion in vivo that decrease the efficiency of contraction and relaxation and thereby, chamber ejection and filling. Furthermore, both increased ATP turnover due to accelerated cross-bridge cycling and reduced LV mechanical efficiency would be expected to increase energy expenditure by myocytes (Crilley et al., 2003, Timmer et al., 2010), which promotes apoptotic pathways (Eijssen et al., 2008) that lead to decreased myocyte survival and
replacement of myocytes with fibrotic tissue (Morita et al., 2010). The induction of LV fibrosis progressively stiffens the myocardium and impairs active fiber shortening and stretch. Fibrotic myocardium has a reduced capacity to generate sufficient strain and torsion to meet circulatory demands, which leads to compensatory LV hypertrophy.

**Clinical Implications**

It is possible that phenotypic unpredictability in contractile function and LVH, as well as the age at which disease symptoms are apparent in patients expressing cMyBPC mutations, is related to variability in overall levels of cMyBPC expression (Morita et al., 2010). In addition, the regional distribution of cMyBPC expression in the myocardium, such that patients with more significant cMyBPC loss present with more severe dysfunction and LVH at an earlier age. Although mutations in the gene encoding cMyBPC have typically been associated with late-onset cardiac dysfunction and LVH, there is growing evidence that clinical symptoms of the disease are apparent in a significant number of cMyBPC mutation carriers at a young age, including infancy (Kaski et al., 2009, Rodriguez-Garcia et al., 2010). It is noteworthy that decreased levels of cMyBPC have been reported in myocardial samples from young HCM patients (20–30 years of age) (van Dijk et al., 2009, Marston et al., 2009) carrying heterozygous cMyBPC mutations, which parallel our findings in young cMyBPC⁻⁺ mice. Collectively, these data imply that clinical symptoms of cardiac dysfunction related to mutations in cMyBPC, and perhaps mechanical dysfunction specifically, manifest earlier than previously thought and should be screened for in young relatives of HCM patients (Kaski et al., 2009). With the emergence of improved cardiac MRI technology (Maron, 2009), it may now be possible to identify subtle
changes in mechanical function and hypertrophy in cMyBPC carriers at an earlier age, thus significantly reducing risks for development of cardiac disease later in life.

**Limitations**

The mechanical manifestation and degree of cardiac hypertrophy in human HCM is often diverse due to the underlying cause of the disease, which often involves multiple contributing factors and may or may not involve mutations in sarcomeric genes. The mechanical data presented here were collected from a homogeneous population of mice in the absence of environmental modifiers that could impact disease progression in humans. Therefore, the animal models of cardiac disease used in this study may not fully reproduce all aspects of human disease. Furthermore, the mice used in this study were deficient in cMyBPC, and thus our data may not be representative of a general population of HCM in which cardiac dysfunction and hypertrophy is not related to mutations in cMyBPC. For example, echocardiography studies in a general cohort of HCM patients of unknown genotype revealed slightly increased systolic circumferential strain compared with controls (Carasso et al., 2008), whereas we observed lower circumferential strains in cMyBPC-deficient mice. However, consistent with our data, lower systolic circumferential strains have been shown in HCM patients with cMyBPC mutations (Germans et al., 2010). Thus, it can be speculated that divergent cardiac mechanical function in different HCM populations may reflect the underlying cause of the disease.

**Clinical Perspective**
Hypertrophic cardiomyopathy (HCM) is a disease caused by mutations in genes that encode sarcomeric proteins. Mutations in cardiac myosin binding protein C (cMyBPC) are among the most common causes genetically explained HCM, accounting for more than 40% of the total known cases worldwide. A large number of disease-causing mutations in cMyBPC in humans are predicted to produce C-terminal truncated proteins that are not incorporated into the sarcomere, resulting in cMyBPC haploinsufficiency, which reduces the total amount of cMyBPC in the sarcomere. However, the link between decreased cMyBPC expression in the sarcomere and the development of cardiac disease is unclear. Our data demonstrate that a mouse model of cMyBPC haploinsufficiency with only 1 functional cMyBPC allele (cMyBPC+/−) displays a 24% reduction in total cMyBPC expression and accelerated cross-bridge kinetics, which underlies in vivo impairments in systolic and diastolic cardiac mechanics measured by MRI. Importantly, reduced mechanical indices of torsion, twist, and strain were apparent in young cMyBPC+/− mice 8–10 weeks of age, suggesting that cardiac dysfunction in cMyBPC-related HCM is not necessarily late-onset, as is commonly accepted, but may be detected early in life using high-resolution cardiac MRI. Furthermore, in contrast to the severe mechanical dysfunction and cardiac hypertrophy displayed by cMyBPC-null mice, mechanical dysfunction in cMyBPC+/− mice was apparent before the development of overt cardiac hypertrophy, implicating altered mechanical function as the first link between reduced cMyBPC expression in the sarcomere and the development of pathological hypertrophy and cardiac disease.
CHAPTER 2 REFERENCES


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CHAPTER 3: COMPARISON OF VELOCITY VECTOR IMAGING ECHOCARDIOGRAPHY WITH MAGNETIC RESONANCE IMAGING IN MOUSE MODELS OF CARDIOMYOPATHY

ABSTRACT

Background—Myocardial strain imaging using echocardiography can be a cost-effective method to quantify ventricular wall motion objectively, but few studies have compared strain measured with echocardiography against magnetic resonance imaging (MRI) in small animals.

Methods and Results—We compared circumferential strain (CS) and radial strain (RS) measured with echocardiography (velocity vector imaging [VVI]) to displacement encoding with stimulated-echo MRI in 2 mouse models of cardiomyopathy. In 3-month-old mice with gene targeted deficiency of cardiac myosin-binding protein-C (cMyBPC\(^{-/-}\), n=6) or muscle LIM protein (MLP\(^{-/-}\), n=6), and wild-type mice (n=8), myocardial strains were measured at 3 cross-sectional levels and averaged to obtain global strains. There was modest correlation between VVI and MRI measured strains, with global CS yielding stronger correlation compared with global RS (CS \(R^2=0.4452\) versus RS \(R^2=0.2794\), both \(P<0.05\)). Overall, strain measured by VVI was more variable than MRI (\(P<0.05\)) and the limits of agreement were slightly, but not significantly (\(P=0.14\)), closer for global CS than RS. Both VVI and MRI strain measurements showed significantly lower global CS strain in the knockout groups compared with the wild type. The VVI (but not MRI) CS strain measurements were different between the 2 knockout groups (−14.5±3.8% versus −6.6±4.0%, cMyBPC\(^{-/-}\) versus MLP\(^{-/-}\) respectively, <0.05).
Conclusions—Measurements of left ventricular CS and RS are feasible in small animals using 2-dimensional echocardiography. VVI and MRI strain measurements correlated modestly and the agreement between the modalities tended to be greater for CS than RS. Although VVI and MRI strains were able to differentiate between wild-type and knockout mice, only global CS VVI differentiated between the 2 models of cardiomyopathy.

**INTRODUCTION**

Two-dimensionally (2D)-directed echocardiography has arguably become the leading method for assessing left ventricular (LV) function in small animals because it is noninvasive, relatively cost-effective, widely available, and has short acquisition and post-processing times that allow high throughput. These techniques have proved useful in determining both the interplay between altered cardiovascular gene expression and compensatory physiologic regulation, as well as the effects of genetic, surgical, and pharmacological interventions in rodent models of disease (Popovic et al., 2007, Bachner-Hinenzon et al., 2010, Hoit, 2006).

However, 2D-directed detection of LV dysfunction by conventional echocardiography (M-mode, 2D, Doppler) is considered a late manifestation of cardiac disease that lacks the sensitivity to identify subclinical disease (Marwick et al., 2010, Borg et al., 2009, Bauer et al., 2011, Stanton et al., 2010). Doppler- and 2D echocardiographic-derived myocardial strain (or deformational) imaging are recent advances in noninvasive cardiac imaging intended, in part, to overcome the limitations that confound the traditional echocardiographic assessment of LV function.
Abnormalities of myocardial deformation are seen early in the development of a variety of pathophysiologic states, and thus provide a sensitive means for detecting global and regional myocardial dysfunction (Hoit, 2011). Compared with Doppler-determined strain, 2D echocardiographic (2D speckle-tracking echocardiography [STE]) strain is independent of the angle of ultrasound propagation, measures the 3 normal Lagrangian strains (radial strain [RS], circumferential strain [CS], and longitudinal strain), and is less noisy, and more reproducible. 2D STE has been previously described in humans for use in myocardial layer-specific deformation analysis (Adamu et al., 2009), and more recently has been used for phenotyping mice (Bauer et al., 2011). This imaging technique tracks speckle patterns generated by interference between the ultrasound beam and myocardium on 2D echocardiographic images (Chetboul et al., 2010). It allows a non–Doppler-based assessment of regional myocardial motion, and provides information about segmental as well as global LV function with greater sensitivity and specificity compared with conventional echocardiography (Bauer et al., 2011, Cottrell et al., 2010, Rappaport et al., 2006). Velocity Vector Imaging (VVI; Siemens Medical Solutions, Mountain View, CA) is a novel imaging technique based on 2D STE, which incorporates speckle-tracking and endocardial contour tracking that allows angle-independent measurements of strain (Hoit, 2011, Bansal et al., 2008).

With its high spatial resolution and superb imaging quality, magnetic resonance imaging (MRI)-based strain imaging is considered the accepted standard method for measuring myocardial wall strain (Chetboul et al., 2010, Liu et al., 2006). However, it is costly, time-consuming, and not widely available, precluding it from routine use in
the small animal research arena. Although other studies have compared 2D STE strain measurements with MRI (Li et al., 2007), there are no studies to date that have directly compared VVI strain measurements with MRI-based strain in small animal models of cardiovascular disease. In this study, we report LV strain measurements in normal mice and in mouse models of hypertrophic and dilated cardiomyopathy using VVI and displacement encoding with stimulated-echo MRI, thus providing a direct comparison of the strain measurements in pathological states.

**METHODS**

**Experimental Animals**

Adult male (8–10 weeks of age) mice with deficiency of either myosin-binding protein-C, a thick filament-associated protein of the sarcomere (cMyBPC$^{-/-}$; n=6), or muscle LIM protein, a promoter of myogenic cytoskeleton differentiation (MLP$^{-/-}$; n=6), and their wild-type littermates of the SV/129 strain (wild-type [WT]; n=8) were used in this study. These groups served as models of hypertrophic and dilated cardiomyopathy, and controls, respectively (Arber et al., 1997, Harris et al., 2002).

Mice were placed on a standard mouse chow diet and water ad libitum, and housed in a temperature-controlled environment with an alternating 12-hour light/dark cycle. All procedures involving animal care and handling were performed according to institutional guidelines set forth by the Animal Care and Use Committee at Case Western Reserve University.

**Magnetic Resonance Imaging**
Animals were anesthetized with 2% isoflurane with supplemented O₂ in an isoflurane induction chamber, and then moved into the magnet and kept under inhalation anesthesia with 1.5% isoflurane. With electrocardiogram and respiratory gating, cardiac functional MRI studies were performed with a 9.4-T Bruker Biospec (Billerica, MA) horizontal scanner using a volume coil. A series of scout images were first acquired to obtain the horizontal long-axis image from which 3 LV short-axis planes at basal, mid-ventricular, and apical levels were prescribed as perpendicular to the LV long-axis with an interslice distance of 1.5 mm. Two-dimensional myocardial motion was quantified using displacement encoding with stimulated-echo MRI, the details of which has been described previously (Zhong et al., 2010). Data were captured at a rate of 13 frames/cardiac cycle, resulting in a temporal resolution of ≈9 ms. Image processing (MRI reconstruction) and data analysis were performed offline using custom-built software written in Matlab (MathWorks, Natick, MA). Data acquisition and analysis required ≈3 to 3.5 hours and 1 per animal.

The epicardial and endocardial LV borders were traced using cine images to calculate LV ejection fraction. A 2D displacement map was calculated by means of vector addition of the displacement from 2 orthogonal directions to compute Lagrangian strain tensors at the base, mid, and apex of the LV. The CS and RS measurements at the base, mid, and apex of the LV were averaged to obtain global CS and RS for each animal (Figure 3.1A).

**Echocardiography**

Echocardiographic studies were performed within 5 days of the MRI. Animals were anesthetized with 2% isoflurane supplemented with O₂ in an isoflurane induction
chamber and maintained with 1.5% isoflurane by nose cone. Echocardiography was performed as previously described (Morgan et al., 2004). Acoustic capture B-mode cine clips (120 Hz) were obtained with electrocardiographic gating using a Sequoia ACUSON System (Siemens Medical Solutions, Mountain View, CA) with a 15-MHz linear array transducer. Image processing and data analysis were performed offline using Syngo Vector Imaging technology software (Siemens Medical Solutions, Mountain View, CA).

2D-directed M-mode images from the mid-papillary short-axis were used to calculate conventional measurements of the LV, which included the LV end-diastolic diameter, end-systolic diameter, anterior and posterior wall thicknesses, and fractional shortening.
B-mode clips were selected based on adequate visualization of the endocardial border and the absence of image artifacts. The epicardial and endocardial LV borders were manually traced and accurate tracking verified >3 cycles in the parasternal short-axis view at end-systole to calculate Lagrangian strain at the base, mid, and apex of the LV. Peak CS and RS values for each segment of the LV were recorded and averaged to obtain global strains for each animal (Figure 3.1B).

Data acquisition (3 short axes) and analysis (CS and RS) required ≈15 minutes per animal.

To ensure good quality images for STE-based strain analyses, image acquisition was performed at a high frame rate by using the smallest possible depth and sector...

Figure 3.1. Displacement encoding with stimulated-echo magnetic resonance imaging (A) and velocity vector imaging echocardiographic (B) vector maps for regional strain in wild type (left) vs knockout (KO) (right). The KO group has decreased strain compared with the wild type, demonstrated by smaller size of the vectors.
size. All image acquisitions and offline measurements were conducted by a single investigator (SA) who was blinded to animal groups. Using a second investigator (BDH), interobserver differences of peak regional systolic RS and CS were determined from 20 randomly selected clips as $100 \times$ the difference between 2 observations divided by the mean of the 2 observations. Intraobserver differences were determined similarly from 20 randomly selected clips measured 10 days apart by a single investigator (AL). Intra- and interreader coefficients of variation were estimated as the root mean square of the coefficients of variations and intra- and interreader intraclass coefficients were calculated using the method of Fleiss (Fleiss et al., 1996).

**Statistical Analysis**

The statistical analysis was performed using SigmaPlot 12.0 (Systat Software, Inc., San Jose, CA) and SAS (SAS Institute, Cary, NC) version 9.2 commercial software. All values are expressed as mean±SD. Nondeformation echocardiographic and MRI variables were compared with 1-way ANOVA with post hoc Tukey tests. Strain data were analyzed using linear mixed models, fitting a separate model for the RS and CS at 3 locations (base, mid, and apex) and a model each for global RS and CS. Thus, 8 models were fit; for each, Tukey multiple comparison procedure with a family-wise error rate of 0.05 was performed for pairwise comparisons of the 6 groups defined by the 3 genotypes, and for comparisons between VVI and MRI within a genotype. Bland-Altman plots were constructed to illustrate the agreement between VVI and MRI strain measurements. The Morgan-Pitman test (Wilcox, 1990) was used to test the hypotheses that variability was greater for VVI than MRI, and variability was greater for RS than CS. A $P$ value (2-sided) <0.05 was considered as statistically significant.
RESULTS

Echocardiographic and MRI Volumetric Variables

The LV ejection fraction measured by MRI for the cMyBPC<sup>−/−</sup> and MLP<sup>−/−</sup> groups (33±8% and 29±9%, respectively) was significantly lower than the WT group (69±9%; *P* <0.05), and was similar between the 2 groups of knockout mice. Similarly, the fractional shortening measured by 2D-directed M-mode echocardiography was significantly lower for the cMyBPC<sup>−/−</sup> and MLP<sup>−/−</sup> groups (38±6% and 19±5%, respectively) compared with the WT group (61±5%), but was significantly lower in the MLP<sup>−/−</sup> than cMyBPC<sup>−/−</sup> group (Table 3.1). The LV EDD and LV ESD were greater in the MLP<sup>−/−</sup> group and wall thicknesses were greater in the cMyBPC<sup>−/−</sup>group. There were no differences in heart rate among the 3 groups of mice.

Global Strain

The Bland-Altman limits were narrower for global CS compared with global RS (Figure 3.2A and 3.2B; however, the difference was not statistically
Additionally, there was modest correlation between VVI and MRI measured strains, with global CS yielding a stronger correlation compared with global RS (CS $R^2=0.4452$ versus RS $R^2=0.2794$, both $P<0.05$). The global RS measured by MRI was greater in the wild-type (24.7±1.0%) than knockout mice (cMyBP-C$^{-/-}$: 12.7±2.0%, MLP$^{-/-}$: 14.8±3.2%). In contrast, the global RS measured by VVI in the WT mice (23.5±9.2%) was statistically similar to that in the cMyBP-C$^{-/-}$ (16.4±4.6%), but significantly greater than in the MLP$^{-/-}$ mice (6.8±4.0%). The global CS determined with either VVI or MRI was significantly lower in the knockout than WT mice. However, only VVI showed significantly lower CS strain in the
MLP\(^{-/^{-}}\) mice (−6.6±4.0\%) than the cMyBP-C\(^{-/-}\) mice (−14.5±3.8\%) (Tables 3.1 and 2).

In comparing the VVI and MRI, only global CS strains in the WT mice were statistically different, with lower strains measured with MRI compared with VVI. Variability of global CS and RS strains was greater for VVI than MRI (both \(P<0.05\)), although variability was similar for global CS and RS (\(P=0.39\)).

**Regional Strain**

Values of regional RS comparing VVI and MRI were similar in both the WT and cMyBP-C\(^{-/-}\) mice, and only at the LV base of MLP\(^{-/-}\) mice were values significantly different when comparing VVI and MRI (Figure 3.3).

Regional strains measured with MRI in both knockouts were lower than those in the WT mice, whereas, only regional RS

![Figure 3.2. Bland-Altman plots comparing the magnetic resonance imaging (MRI)- and echo-derived strain. A, Circumferential strain measurements. B, Radial strain measurements. There is closer agreement between the MRI and echo imaging modalities for circumferential strain when compared with radial strain measurements. Upper and lower dotted lines are the 95\% limits of agreement.](image-url)
measured with VVI were significantly lower in the MLP$^{-/-}$ than those in the WT mice. When comparing between the 2 knockout groups, strains measured by VVI were significantly lower in the MLP$^{-/-}$ than cMyBP-C$^{-/-}$ group at the base of the LV; regional RS measured by MRI was similar between the 2 knockout groups.

Values of regional CS comparing VVI and MRI were similar in both the WT and MLP$^{-/-}$ mice, and only at the mid LV base of cMyBP-C$^{-/-}$ mice were values significantly different when comparing VVI and MRI. Regional CS measured by MRI was lower in the cMyBP-C$^{-/-}$ knockout group compared with the WT group at the mid and apex levels, and the MLP$^{-/-}$ knockouts at the mid LV. The regional CS measured by VVI was also lower for both knockout groups compared with WT except for regional strain at the mid LV in cMyBP-C$^{-/-}$ mice (Figure 3.4). Similar to regional
RS, VVI regional CS measurements (in this instance, mid LV) was significantly lower in the MLP<sup>−/−</sup> group compared with cMyBP-C<sup>−/−</sup> group.

**Interpretative Variability**

The interobserver differences for CS and RS were −3.5±14.7 and −3.9±21.9, respectively. Intraobserver differences for CS and RS were 4.7±9.2% and 4.7±13.0%, respectively. The intra- and interreader coefficients of variations were 9.6% and 15.3% for RS and 7.2% and 10.4% for CS, respectively. The intra- and interreader intraclass coefficients were 0.986 and 0.955 for RS and 0.990 and 0.932 for CS, respectively.

**DISCUSSION**

This is the first study comparing echocardiographic strain imaging using VVI and MRI in the wild-type and genetically altered mouse. The principal results of this study are (1) correlations between MRI and VVI strains are modest and are greater (and
agreement tends to be greater) for CS than RS; (2) strains measured with VVI are more variable than those measured with MRI and the variability is similar for RS and CS; and (3) VVI-measured strains can be used to rapidly phenotype mouse models of cardiomyopathy. Although MRI is currently considered the accepted standard for noninvasive myocardial strain imaging, in part because of the ability to evaluate strain in 3 dimensions, long acquisition and postprocessing times, expense, and limited availability preclude its routine use in the small animal laboratory. Thus, echocardiographic strain is a potentially advantageous method for the objective assessment of global and regional LV function and for high through-put phenotyping of murine models (Bauer et al., 2011).

Bauer et al (Bauer et al., 2011) previously reported speckle-tracking strain measurements in WT mice, which were higher than values obtained in our study. The previous study used speckle-tracking algorithm supplied by VisualSonics (VevoStrain, VisualSonics, Toronto Canada), whereas echocardiographic strain measurements in our study were obtained by VVI algorithm supplied by Siemens Medical Solutions (Syngo Vector Imaging technology software, Siemens Medical Solutions, Mountain View CA) (Bauer et al., 2011). The proprietary speckle-tracking software from the 2 companies and the superior sampling rates in the VisualSonics software may account for the difference in the strain measurements. Importantly, in this study, the VVI strains are similar to those obtained with MRI and the latter measured in the WT mice were similar to MRI strains previously reported (Liu et al., 2006).

Bansal et al (Bansal et al., 2008) previously reported validation of VVI strain with harmonic phase MRI in humans with a very modest correlation between the 2
modalities, greater with CS than RS (CS $R^2=0.12$ versus RS $R^2=0.005$). Similarly, in our study, the correlation between VVI and MRI strain was higher with CS than RS (CS $R^2=0.4452$ versus RS $R^2=0.2794$), but both CS and RS correlations in our study were much greater than in that validation study involving human subjects and were similar to both Bansal et al (Bansal et al., 2008) (CS $R^2=0.397$ and RS $R^2=0.348$, both $P<0.05$) and Cho et al (CS $R^2=0.26$ and RS $R^2=0.36$) using a different speckle-tracking algorithm, automated functional imaging (GE Medical Systems, Milwaukee WI). Nevertheless, our correlations are disappointingly less robust than those reported in a validation study in 5 mice after myocardial infarction and 2 control mice using the VisualSonics instrument with EKV (CS $R^2=0.81$ and RS $R^2=0.72$), which acquires data from multiple cardiac cycles and constructs an image sequence composed of >100 images per cardiac cycle (Li et al., 2007).

LV contraction is a complex process involving deformation resulting in shortening in 3 normal directions; longitudinal, CS, and RS. Longitudinal strain, which is a sensitive indicator of subendocardial fiber dysfunction and an early marker of ischemia and increased wall stress, was not evaluated in this study as we were comparing directly with MRI as it is measured in small animals in our institution. In addition, advantages of strain imaging compared with conventional echocardiographic parameters could not be demonstrated in this study, which was designed to compare strain imaging with MRI in fully developed models of cardiomyopathy. In a previous study in mice, longitudinal strains were sensitive to changes early after experimental myocardial infarction and were able to predict LV remodeling; CS and RS were not as consistent. Longitudinal strain is typically obtained from an apical view (although
the parasternal long-axis view was used by Bauer et al (Bauer et al., 2011)), which is difficult to obtain reliably in small animals. CS and RS are more influenced by transmural fiber dysfunction (especially the mid-myocardium), and are generally more suited for identifying dysfunction in ventricles with reduced LV systolic function (Geyer et al., 2010).

Both MRI and VVI strain measurements were able to differentiate between the WT group and the genetic models of cardiomyopathy, reinforcing the potential usefulness of strain measurements in mouse models of LV dysfunction. However, only VVI CS strain differentiated between the 2 models of cardiomyopathy, which corresponded with differences in their echocardiographic LV fractional shortening.

One potential difficulty is that VVI had greater variability in strain measurement compared with MRI, and was particularly problematic in measurement of RS. This may be because of the need to track both epicardial and endocardial borders, the former more difficult to identify with a resultant decrease in accuracy. The greater variability has been reported in previous studies (Bansal et al., 2008). Although MRI RSs are generally more variable than CS, in this study, their variability was similar.

Limitations

Several limitations of this study merit consideration. First, for logistic reasons, echocardiography and MRI were not performed on the same day for all the animals. This may be responsible, in part, for the modest correlations between the 2 techniques. Second, the echocardiographic images were obtained using 2D rather than 3D imaging used in MRI; translation of the heart remains a problem using 2D acquisition methods.
as error is introduced to strain measurements when the heart swings out of the imaging plane. In addition, out-of-plane motion occurs because of rotation and motion of the heart; as a result, only a portion of the real motion can be detected. Third, potential sources of variation include the quality of the images obtained for strain analysis. High quality of the 2D images at high frame rates is essential for accurate VVI strain measurements. Moreover, poor quality of images may hinder the investigator’s ability to perform tracing of the endocardium and epicardium which may affect the strain values, thereby introducing another source of variability. Fourth, while temporal resolution for the echocardiograms (120 Hz) was similar (8.3 ms) to the MRI, the accuracy of tracking speckles may theoretically be jeopardized at these frame rates and may have resulted in a significant underestimation of strain values; higher frame rates were reported using different instrumentation and algorithms (Bauer et al., 2011, Li et al., 2007). Finally, changes in the imaging angle of incidence can result in capturing different fiber layers at different levels and may introduce variability, particularly because we analyzed exclusively short views which are sensitive to small differences in image angle (Bauer et al., 2011).

Conclusion

Despite these limitations, we demonstrate that measuring LV CS and RS is feasible in small animals using 2D echocardiography with VVI. VVI and MRI strain measurements correlated modestly and the correlation was greater (and agreement tended to be greater) for CS than RS. VVI had greater variability in strain measurement compared with MRI. Although global VVI and MRI strains were able to differentiate
between WT and knockout mice, only VVI strain differentiated between the 2 models of cardiomyopathy.

Clinical Perspective

Although 2-dimensional echocardiographic strain imaging cost-effectively and objectively quantifies ventricular wall motion, only 1 small study has directly compared strain measured with echocardiography against magnetic resonance imaging (MRI) in mice. Using a different speckle-tracking algorithm (velocity vector imaging [VVI]), we compared circumferential (CS) and radial strain (RS) to displacement encoding with stimulated-echo MRI in 2 genetic mouse models of cardiomyopathy. CS and RS were measured in groups of wild-type mice and mice with gene targeted deficiency of cardiac myosin-binding protein-C or muscle LIM protein. There was modest correlation between VVI and MRI measured strains, with global CS yielding a stronger correlation compared with global RS (CS R²=0.4452 versus RS R²=0.2794, both P<0.05). Overall, strain measured by VVI was more variable than MRI and the limits of agreement were slightly, but not significantly, closer for global CS than RS. Both VVI and MRI strain measurements showed significantly lower global CS strain in the knockout groups compared with the wild-type, but only the VVI CS strain measurements were different between the 2 knockout groups. These data demonstrate that measurements of LV CS and RS are feasible in mice using VVI, although correlations and agreement were modest. VVI may complement conventional methods that objectively assess global and regional LV
function and be particularly useful for high through-put phenotyping of murine models.
CHAPTER 3 REFERENCES


CHAPTER 4: ARTERIAL SPIN LABELING-FAST IMAGING WITH STEADY-STATE FREE PRECESSION (ASL-FISP): A RAPID AND QUANTITATIVE PERFUSION TECHNIQUE FOR HIGH-FIELD MRI

ABSTRACT

Arterial spin labeling (ASL) is a valuable non-contrast perfusion MRI technique with numerous clinical applications. Many previous ASL MRI studies have utilized either echo-planar imaging (EPI) or true fast imaging with steady-state free precession (true FISP) readouts, which are prone to off-resonance artifacts on high-field MRI scanners. We have developed a rapid ASL-FISP MRI acquisition for high-field preclinical MRI scanners providing perfusion-weighted images with little or no artifacts in less than 2 s. In this initial implementation, a flow-sensitive alternating inversion recovery (FAIR) ASL preparation was combined with a rapid, centrically encoded FISP readout. Validation studies on healthy C57/BL6 mice provided consistent estimation of in vivo mouse brain perfusion at 7 and 9.4 T (249 ± 38 and 241 ± 17 mL/min/100 g, respectively). The utility of this method was further demonstrated in the detection of significant perfusion deficits in a C57/BL6 mouse model of ischemic stroke. Reasonable kidney perfusion estimates were also obtained for a healthy C57/BL6 mouse exhibiting differential perfusion in the renal cortex and medulla. Overall, the ASL-FISP technique provides a rapid and quantitative in vivo assessment of tissue perfusion for high-field MRI scanners with minimal image artifacts.
INTRODUCTION

Arterial spin labeling (ASL) MRI is a non-contrast perfusion MRI technique that has been shown to provide quantitative assessments of tissue perfusion in multiple clinical imaging applications, including brain (Wong et al., 2007, Brookes et al., 2007, Boss et al., 2007), kidney (Karger et al., 2000, Martirosian et al., 2004, De Bazelaire et al., Fenchel et al., 2006), lung (Mai et al., 1999, 2001, Wang et al., 2003, Bolar et al., 2006, Martirosian et al., 2006) and liver (Katada et al., 2012). One major advantage of ASL over conventional dynamic contrast-enhanced MRI perfusion techniques is the lack of exogenous, and potentially toxic, paramagnetic contrast agents. The use of an endogenous blood signal to obtain tissue perfusion information is especially important for the imaging of patients with chronic kidney diseases, which can be a contraindication for gadolinium-based MRI perfusion methods (Kuo et al., 2007). This attribute is also important for longitudinal preclinical imaging applications, as multiple tail-vein injections and/or catheterizations can cause local inflammation and necrosis, resulting in reduced access to veins.

ASL MRI techniques generate blood flow contrast between multiple images using a wide variety of blood labeling methods (Detre et al., 1992, Williams et al., 1992, Edelman et al., 1994, Kwong et al., 1995, Kim et al., 1997, Detre et al., 1999). A typical ASL MRI acquisition combines an ASL preparation phase, followed by a rapid imaging readout to capture the blood flow-weighted contrast (Fig. 1). It is important to note that a large majority of the imaging developments have focused on the optimization of the preparation phase of the ASL acquisition. As a result, a wide variety of preclinical and clinical ASL MRI techniques have been developed. These
ASL techniques can be broadly grouped into continuous [CASL (Detre et al., 1992, Williams et al., 1992, Detre et al., 1999)] and pulsed [PASL (Edelman et al., 1994, Kwong et al., 1995, Kim et al., 1997)] categories. A hybrid of these two techniques, pseudo-continuous ASL (pCASL), has also been developed recently (Wong et al., 2007, Wu et al., 2007, Duhamel et al., 2014). Each of these specialized techniques offers advantages for specific imaging applications. At the same time, many of these studies have utilized conventional imaging readouts, including fast low-angle shot (FLASH) (Pell et al., 2004, Zuo et al., 2013), echo-planar imaging (EPI) (Wang et al., 2004, Rajendran et al., 2013) and true fast imaging with steady-state free precession (true FISP) (Martirosian et al., 2004, 2006, Esparza-Coss et al., 2010, Boss et al., 2006). Unfortunately, these imaging readouts exhibit significant limitations for high-field MRI applications. Specifically, $B_0$ inhomogeneities result in increased distortion/ghosting and banding artifacts from EPI and true FISP imaging techniques, respectively, on high-field MRI scanners. These artifacts are particularly problematic for body imaging applications, where cardiac and respiratory motion, as well as adipose tissue, can make precise shimming difficult. In addition, the increase in $T_1$ magnetic relaxation times on high-field MRI scanners can result in spoiled gradient echo images with a lower signal-to-noise ratio (SNR) relative to these other imaging techniques. A lower SNR is especially problematic for ASL MRI techniques, as the differential blood flow signal is typically less than 10% of the mean tissue signal (Petersen et al., 2006, Yongbi et al., 1999). Therefore, there is a need to develop a rapid and robust ASL MRI imaging readout that is both sensitive to blood flow labeling from
the ASL preparation and also immune to $B_0$ inhomogeneities and motion artifacts on high-field MRI scanners.

Previously, our group has reported the development of a centrically encoded FISP imaging technique which can be flexibly combined with conventional diffusion and magnetization transfer (MT)/chemical exchange saturation transfer (CEST) preparation methods to rapidly generate quantitative imaging data (Lu et al., 2012, Shah et al., 2011). Importantly, the magnetic field gradients for the FISP readout sequence are not completely refocused in either the slice select or frequency encoding directions, resulting in greatly reduced off-resonance artifacts in comparison with EPI and true FISP acquisitions. In addition, centric $k$-space encoding of the FISP readout retains the image contrast generated in the diffusion and MT/CEST preparations.

Here, we describe our initial in vivo results from an ASL-FISP acquisition on 7- and 9.4-T Bruker Biospec (Bruker Inc., Billerica, MA, USA) small-animal MRI scanners. For this initial study, our ASL-FISP implementation combines a flow-sensitive alternating inversion recovery (FAIR) preparation scheme with our rapid centrically encoded FISP imaging readout to generate ASL imaging data (Martisosian et al., 2004, Kim et al., 1997, Boss et al., 2006, Gunther et al., 2001). The reproducibility of this new ASL-FISP technique was evaluated in brains of healthy C57/BL6 mice. In addition, we demonstrate the effectiveness of this technique for multiple imaging applications, including mouse models of ischemic stroke and healthy mouse kidneys.

METHODS
In vivo comparison of image artifacts at 7 T

Axial FISP images of a healthy C57/BL6 mouse brain were acquired on a 7-T Bruker Biospec MRI scanner for comparison with conventional spin echo (TR/TE = 2000/14 ms; one average; total scan time, 4 min 16 s), true FISP (TR/TE = 4.0/2.0 ms; 20 averages; tip angle, 60°; total scan time, 11 s) and EPI (TR/TE = 2500/30.8 ms; four segments; two averages; total scan time, 20 s) imaging readout methods to assess image artifacts.

ASL-FISP pulse sequence design

The ASL-FISP acquisition was implemented on Bruker Biospec 7- and 9.4-T MRI scanners equipped with 400 mT/m gradient inserts. The ASL-FISP sequence was implemented on both MRI scanners to demonstrate the robustness of the methodology to artifacts on high-field MRI scanners. The ASL-FISP pulse sequence was developed by combining a FAIR preparation (slice-selective or non-selective inversion) with a centrically encoded FISP imaging readout to acquire all lines of \( k \) space following each ASL preparation (Fig. 4.1). It should be noted that, although a FAIR scheme was implemented herein, the ASL-FISP technique is adaptable to a variety of ASL preparations. A hermite excitation radiofrequency (RF) pulse with a duration of 0.5 ms and a tip angle of 60° were selected for this implementation. The high tip angle was selected for this FISP acquisition to provide increased SNR in comparison with low tip angles (data not shown).

Uniformity of the inversion pulse is essential for accurate and precise quantification of tissue perfusion. Therefore, a hyperbolic secant adiabatic inversion pulse with a duration of 3.0 ms was used for the FAIR inversion preparation. Magnetic field
gradient spoilers were applied after the inversion preparation to limit transverse magnetization prior to the FISP imaging readout. The FISP imaging readout (including 10 dummy scans) provided in vivo images in less than 2 s with relatively high SNR in comparison with conventional spoiled gradient echo acquisitions, and minimal off-resonance distortion and ghosting artifacts in comparison with EPI (Lu et al., 2012, Shah et al., 2011). The FISP imaging readout also prevented banding artifacts typical of balanced steady-state free precession (SSFP)/true FISP acquisitions on high-field MRI scanners. The FISP imaging readout was also designed with centric encoding to retain blood flow sensitivity, as well as the $T_1$ weighting generated from the FAIR inversion preparation.

*Initial in vivo ASL-FISP perfusion assessments in mouse brains*

All animal studies were conducted in accordance with approved Institutional Animal Care and Use Committee protocols at Case Western Reserve University. The ASL-FISP technique was initially evaluated by assessing brain perfusion in healthy wild-type C57/BL6 mice (The Jackson Laboratory, Bar Harbor, ME, USA). Each
animal was anesthetized with 1–2% isoflurane with supplementary O₂ and positioned within a mouse volume coil (inner diameter, 35 mm) in a Bruker Biospec MRI scanner. Each animal's body temperature was maintained at 35 ± 1 °C with a warmed air control system. Respiratory triggering was performed through an MR-compatible, small-animal gating and control system (SA Instruments, Stony Brook, NY, USA).

Ten male C57/BL6 mice, aged 10 weeks, were scanned with the ASL-FISP imaging protocol at 7 T. Five of these mice were scanned with the ASL-FISP imaging protocol at 9.4 T at least 1 day prior to the 7-T scan for comparison. Rapid localizer scans were first used to identify an appropriate and consistent axial mid-brain imaging slice for the ASL-FISP protocol. After slice positioning, the single-slice ASL-FISP protocol consisted of three sequential scans: (1) ASL-FISP with a slice-selective inversion; (2) ASL-FISP with non-selective inversion; and (3) FISP with no inversion preparation as a reference (M₀) scan for blood flow calculation (Kim et al., 1997). An inversion delay time (TI) of 1420 ms was used for this initial implementation to generate sufficient blood flow contrast between the slice-selective and non-selective ASL-FISP images. Other than the inversion preparation, the FISP imaging readout parameters were identical for these three acquisitions (centric encoding; TR/TE = 2.4/1.2 ms; matrix, 128 × 128; field of view, 3 cm × 3 cm; imaging slice thickness, 1.5 mm; tip angle, 60°).

These scans were repeated 60 times to determine the effects of image quality on the perfusion calculations. Although not required, for this initial implementation, an additional delay time of ~13 s was incorporated between each scan repetition to allow the magnetization to return to equilibrium (M₀) between repetitions and to limit the duty cycle on the magnetic field gradients. The total acquisition time for one ASL-
FISP repetition, including the ASL preparation (1420 ms), FISP imaging readout (331 ms) and ~13-s delay, was 15 s.

For comparison, we also implemented an ASL-GRE acquisition by combining an identical FAIR preparation with a gradient echo (GRE) imaging readout (TR/TE = 5000/2 ms; five averages; tip angle, 90°). For this simplified ASL-GRE acquisition, only one line of $k$ space was acquired for each ASL preparation. All other imaging parameters, including the field of view and resolution, were identical to the ASL-FISP acquisitions described above. This conventional ASL-GRE acquisition was evaluated on a separate single healthy C57/BL6 mouse brain at 7 T for direct comparison with the ASL-FISP results.

Following the ASL-FISP scans, a FISP-based Look–Locker acquisition was implemented to generate voxel-wise $T_1$ maps according to previously described techniques (Deichmann & Haase, 1992, Jakob et al., 2001). The Look–Locker acquisition consisted of a non-selective adiabatic inversion, followed by 10 continuous and sequential FISP image repetitions (linear encoding; tip angle, 10°; TR/TE = 4.0/2.0 ms; 512 ms/image). The Look–Locker acquisition was repeated at least 40 times to ensure accurate $T_1$ relaxation time estimation. As for the ASL-FISP acquisitions above, an ~7-s delay was introduced after each repetition to reduce the duty cycle on the imaging gradients and to allow the magnetization to return to equilibrium ($M_0$). Voxel-wise $T_1$ relaxation maps were then calculated according to previously described methods (Deichmann & Haase, 1992, Jakob et al., 2001). The geometry of the Look–Locker acquisition was identical to that of the ASL-FISP
acquisitions to ensure one-to-one correspondence between the ASL and $T_1$ relaxation data, and to enable direct calculation of the quantitative ASL maps described below. Quantitative, voxel-wise perfusion maps were generated using previously described methods for the FAIR ASL technique according to (Kwong et al., 1995, Kim et al., 1997):

$$f = \frac{\lambda}{2T_1} \frac{SS(TI) - NS(TI)}{M0} \exp\left(\frac{TI}{T_1}\right)$$

where $f$ is the tissue perfusion in mL/min/100 g of tissue, NS, SS and M0 are the signal intensities for the non-selective, slice-selective and reference (M0) ASL-FISP images, respectively, $T_1$ is the longitudinal relaxation time calculated from the Look–Locker acquisition, TI is the inversion time set at 1420 ms and the tissue–blood partition coefficient ($\lambda$) was assumed to be 0.9 mL/g (Herscovitch et al., 1985). Perfusion maps were calculated using this equation for each ASL-FISP scan.

A region of interest (ROI) analysis was then used to calculate the mean perfusion over the entire mouse brain visible within the single ASL-FISP imaging slice. It should be noted that these ROIs included both white and gray matter, whereas the ventricles were excluded from the analysis. A histogram analysis of the mouse brain ROI was also used to calculate a threshold (mean ± two standard deviations) to limit the impact of large vessels (high perfusion values) on the calculation of the mean brain perfusion value for each animal, similar to previously described methods (Boss et al., 2007, Martirosian et al., 2004). The mean brain perfusion values for each individual mouse were then used to calculate an overall group average and standard deviation at 7 and 9.4 T, respectively, for comparison. This ROI analysis was repeated for 20, 40 and 60 ASL-FISP averages at 7 and 9.4 T to perform an initial determination of the
effects of noise on the brain perfusion assessments. For all C57/BL6 mice, ASL-FISP scans were obtained with an inversion slab thickness three times that of the FISP imaging slice thickness to ensure a uniform inversion over the entire FISP imaging slice. This factor of three was previously described by our group (Lu et al., 2012) and provides an effective balance between blood flow sensitivity and inversion uniformity. One C57/BL6 mouse was scanned with inversion slab thickness/imaging slice thickness ratios of 1, 3, 6 and 10 to determine the effects of the relative inversion slab thickness on the perfusion results.

Additional in vivo ASL-FISP experiments: mouse brain ischemic stroke model and healthy mouse kidneys

The ASL-FISP acquisition and analysis protocol described above was performed on two additional C57/BL6 mice to evaluate: (1) brain perfusion in the context of known pathology (ischemic stroke); (2) kidney perfusion; and (3) muscle perfusion. Except for the number of signal averages (Durukan et al., 2009), all of the imaging parameters used for these imaging studies were identical to those of the brain ASL-FISP experiments described above.

An ASL-FISP study was performed on a mouse model of ischemic stroke to assess the method's sensitivity to a known pathophysiology. A C57/BL6 mouse (male, 8 weeks of age) was anesthetized with isoflurane. A transient stroke was initiated by inserting a 0.22-mm-diameter, silicone-coated filament (Doccol Corporation, Sharon, MA, USA) into the right carotid artery, resulting in an occlusion of the middle cerebral artery (MCAo), whilst monitoring cerebral blood flow using laser Doppler flowmetry (Schmid-Elsaesser et al., 1998, Harada et al., 2005, Durukan & Tatlisumak, 2009).
The filament was removed after 1 h of occlusion to allow reperfusion as the animal recovered. At 24-h post-ictus, the animal was re-anesthetized for ASL-FISP scanning as described above. A diffusion-weighted EPI image (TR/TE = 5000/31 ms; \( b = 500 \text{s/mm}^2 \)) was also acquired to confirm the presence of the infarct from the MCAo. A brain perfusion map and a mean perfusion value for the whole brain within the imaging slice were calculated for comparison with healthy C57/BL6 mice.

For the kidney and paraspinal muscle perfusion assessment, ASL-FISP was performed on a healthy male 10-week-old C57/BL6 mouse. Respiratory triggering was performed as described above. This study was performed as an initial demonstration of the effectiveness of ASL-FISP for high-field body imaging applications. Following the ASL-FISP acquisitions, an ROI analysis was used to calculate the mean renal perfusion values for the left and right kidneys of the mouse and the mean perfusion value of the paraspinal muscles.

**RESULTS**

Axial mouse brain images from spin echo, FISP, true FISP and EPI techniques at 7 T are shown in Fig. 4.2. Banding and ghosting/distortion artifacts are clearly visible.

**Figure 4.2.** Representative axial mouse brain images at 7T using spin echo (a), fast imaging with steady-state free precession (FISP) (b), true FISP (c) and echo-planar imaging (EPI) (d) acquisitions. Note the similar lack of artifacts in the spin echo and FISP images in comparison with the true FISP (banding) and EPI (ghosting/distortion) images.
in the true FISP and EPI images, respectively. By comparison, the spin echo and FISP images show a lack of image artifacts, with the FISP images generated in less than one-tenth of the acquisition time (22 s versus 4 min 16 s). Representative brain ASL-FISP image sets, $T_1$ relaxation time maps and calculated perfusion maps are shown for a healthy C57/BL6 mouse at 7 T ($n = 10$) in Fig. 4.3. Qualitatively similar images

**Figure 4.3.** Representative arterial spin labeling-fast imaging with steady-state free precession (ASL-FISP) images of a healthy C57/BL6 mouse brain at 7T: (a) slice-selective (bright blood) ASL-FISP image (40 averages); (b) non-selective (dark blood) ASL-FISP image (40 averages); (c) M0 image (no inversion); (d) brain $T_1$ map from Look-Locker acquisition; (e, f) perfusion map from ASL-FISP (e) and ASL-GRE (f) (in mL/min/100g of tissue). Note that the ASL-FISP and ASL-GRE perfusion maps are from different mice. GRE, gradient echo.
and maps were obtained from the mice at 9.4 T \((n = 5, \text{ data not shown})\). The means and standard deviations for the groups of healthy mice scanned at 7 and 9.4 T are shown in Fig. 4.4a as a function of the number of ASL-FISP averages used to calculate the perfusion data (20, 40 and 60 averages). A two-tailed Student's \(t\)-test showed no significant difference between the mean perfusion values at the two magnetic field strengths \((p > 0.2)\) or for 20, 40 and 60 averages \((p > 0.6)\). With 40 signal averages, the mean brain perfusion values for healthy C57/BL6 mice ranged from 210–333 mL/min/100 g of tissue at 7 T to 222–268 mL/min/100 g of tissue at 9.4 T. The means ± standard deviations of the mouse brain perfusion (excluding ventricles) at 7 and 9.4 T were 249 ± 38 and 241 ± 17 mL/min/100 g of tissue, respectively.

A secondary ROI analysis of the 7-T mouse brain perfusion data showed differential perfusion values in the cortex (211 ± 30 mL/min/100 g) and thalamus (288 ± 48 mL/min/100 g), which is consistent with previous studies (Muir et al., 2008). A perfusion map from the ASL-GRE method (mean cerebral perfusion, 285 mL/min/100 g) is shown in Fig. 4.3f for comparison. The total imaging time for five averages was approximately 4 h. In order to make a valid comparison between protocols, the time under anesthesia should be consistent to ensure similar perfusion conditions. We chose to evaluate the ASL-GRE method with two averages so that the imaging time was approximately the same as ASL-FISP. The mean cerebral perfusion value from the ASL-FISP method (40 averages) was 249 ± 38 mL/min/100 g, which is quite reasonable compared with the cerebral perfusion value of 240 mL/min/100 g from the ASL-GRE method (two averages).
The mean brain perfusion values from a single C57/BL6 mouse using inversion slab thickness/imaging slice thickness ratios of 1, 3, 6 and 10 and 5, 10, 20, 40 or 60 ASL-FISP averages are shown in Fig. 4.4b. A large decrease in perfusion was observed as the inversion slab thickness/imaging slice thickness ratio was increased from 1 (inversion slab thickness = imaging slice thickness) to 3, as a result of both increased uniformity of the inversion pulse over the entire imaging slice and reduced perfusion sensitivity. The mean brain perfusion values continued to decrease at a lower rate as the inversion slab thickness/imaging slice thickness ratio was increased from 3 to 6 and 10. In addition, only minimal variation was observed in the mean perfusion values as the number of ASL-FISP averages was reduced from 60 to 5 for each ratio.

Axial ASL images, $T_1$ maps and brain perfusion maps for the MCAo model of ischemic stroke in a mouse are shown in Fig. 4.5. The region of infarct on the right side of the brain (left side of the image) is clearly visible in the ASL-FISP images and the corresponding perfusion map. It should be noted that a smaller secondary infarct
is visible on the contralateral side, most likely resulting from the extensive cytotoxic edema caused by the induced ischemic stroke. Overall, the mean brain perfusion for

Figure 4.5. Arterial spin labeling-fast imaging with steady-state free precession (ASL-FISP) images of a middle cerebral artery occlusion (MCAo) mouse model of stroke at 7T: (a) slice-selective (bright blood) ASL-FISP image; (b) non-selective (dark blood) ASL-FISP image; (c) M0 FISP image (no inversion); (d) diffusion-weighted image \( b=500\text{s/mm}^2 \) showing right brain infarct; (e) Look-Locker T\(_1\) map; (f) perfusion map. The primary infarct is visible in the right brain in all images. A potential contralateral perfusion deficit is also observed in the perfusion map, but is less evident in T\(_1\) and diffusion-weighted images.
the MCAo mouse was measured to be 143 mL/min/100 g. Axial ASL images, $T_1$ maps and perfusion maps for the C57/BL6 mouse kidneys are shown in Fig. 4.6. As expected, the aorta and renal arteries show a very bright signal in the slice-selective inversion ASL-FISP image (Fig. 4.6a), whereas the arterial blood signal is greatly attenuated for the non-selective ASL-FISP image (Fig. 4.6b). The measured mean renal perfusion values for the left and right kidneys (cortex + medulla + pelvis) were 513 and 560 mL/min/100 g, respectively. In addition, the perfusion in the renal cortex was visibly increased relative to that in the renal medulla, as expected. Importantly, mouse kidney perfusion was approximately twice that of brain perfusion, consistent with previous reports (Duhamel et al., 2008, 2014, Rajendran et al., 2013, Kober et al., 2008). The mean perfusion value in the paraspinal muscles was 81 mL/min/100 g, which was significantly lower than that of the kidneys, as expected (Duhamel et al., 2014).
DISCUSSION

In this study, initial \textit{in vivo} ASL MRI results were obtained using a rapid and artifact-resistant ASL-FISP acquisition on 7- and 9.4-T small-animal MRI scanners. The ASL-FISP technique combines an inversion preparation (slice-selective or non-selective), followed by a centrically encoded FISP imaging readout, to provide ASL data with minimal image artifacts in comparison with conventional EPI and true FISP imaging readouts for ASL MRI acquisitions.

There are several important design features of the ASL-FISP acquisition. Most importantly, the FISP imaging readout is applied with centric-$k$-space encoding and relatively few (i.e. 10) dummy scans. As shown previously, this centric encoding approach minimizes the loss of perfusion sensitivity caused by additional RF pulses and gradient lobes encountered in linear $k$-space encoding (Shah et al., 2011). A FISP imaging readout was selected for this study instead of a balanced SSFP readout primarily to limit well-known banding artifacts that are increased significantly on high-field MRI scanners. An alternative approach using balanced SSFP would be to acquire multiple images with different RF phase variation sequences to reconstruct a banding-free image (Vasanawala et al., 2000, Cukur et al., 2008). This approach may offer increased signal-to-noise, but may require additional image processing to remove the banding artifacts. As a result, this alternative approach and direct comparison with ASL-FISP were not explored for this initial study. Further studies are also needed to directly compare the FISP and true FISP techniques, as the FISP flow sensitivity may be altered by dephasing (Haacke et al., 1990).
Overall, the key advantage of the FISP imaging readout is that it provides perfusion sensitivity with a short acquisition time (~2 s/image) with minimal artifacts in comparison with EPI readouts. The ASL-FISP and ASL-GRE acquisitions generated relatively similar perfusion maps (Fig. 4.3) and mean brain perfusion values. However, the ASL-GRE method required an acquisition time that was more than 50 times that of the ASL-FISP method. In addition, the FISP imaging readout can easily be coupled with virtually any ASL preparation in order to meet the requirements for specific imaging applications. For this initial implementation, a simple FAIR ASL preparation was implemented with either a slice-selective or non-selective inversion pulse. However, more complex ASL preparations, such as pCASL, could also be implemented in order to measure transit times and other important perfusion parameters (Duhamel et al., 2014). The FISP imaging readout is particularly relevant for FAIR acquisitions as the difference between the images with the slice-selective and non-selective inversion is small relative to the M0 image (typically <10%). As a result, FAIR ASL studies generally require numerous signal averages to obtain a reasonable estimate of tissue perfusion. Therefore, the acquisition of all $k$-space lines following a single ASL preparation (with minimal artifacts) using the FISP readout results in practical imaging times to acquire sufficient signal averages. Although only single-slice ASL-FISP results are presented in this initial study, multi-slice and/or three-dimensional ASL-FISP implementations are possible, and may have significant advantages for specific imaging applications.

Initial in vivo mouse brain perfusion studies demonstrated that the ASL-FISP technique provides reasonable perfusion assessments on high-field MRI scanners.
ASL-FISP images in the healthy mouse brain and in an induced ischemic stroke model show an expected lack of distortion and artifacts, and clearly delineated perfusion deficits in the stroke model (Figs 4.3 and 4.5, respectively). Further, the kidney ASL-FISP images and perfusion maps in Fig. 4.6 show differential perfusion between the renal cortex and medulla, as expected from previous studies (Martirosian et al., 2004, Miles et al., 1991). In addition, the renal arteries are clearly visible in both the slice-selective inversion ASL-FISP images and the perfusion maps, demonstrating the flow sensitivity of the ASL-FISP technique. Overall, the reliability of the ASL-FISP technique is exhibited by the lack of differences in the perfusion results at 7 and 9.4 T (Fig. 4.4a). Most importantly, the mouse kidney images shown in Fig. 4.6 demonstrate that the ASL-FISP technique can provide high-quality ASL data for rodent brain and body imaging applications on high-field MRI scanners with no distortion and artifacts.

The ASL-FISP technique presented herein also has several important limitations. One key observation of these initial results is that the mean perfusion values for mouse brains and kidneys shown here are dependent on the perfusion preparation scheme and inversion pulse design. It has already been shown in multiple studies that the relative thickness between the slice-selective inversion and the imaging readout is directly related to the resulting tissue perfusion estimate (Brookes et al., 2007, Karger et al., 2000, Esparza-Coss et al., 2010, Yongbi et al., 1999). For example, a smaller inversion slab thickness (e.g. one times that of the imaging slice) will result in enhanced perfusion sensitivity and erroneously high perfusion estimates. Conversely, a larger inversion slab thickness (e.g. six times that of the imaging slice) will result in reduced perfusion sensitivity. These results are reflected in Fig. 4.4b
which shows that an inversion slab thickness/imaging slice ratio $\geq 3$ is needed to maintain reasonably consistent perfusion results. The perfusion results can also be influenced directly by the shape of the inversion pulse and the excitation pulses of the FISP imaging readout. However, optimization of these pulses was beyond the scope of this initial technical development. Nevertheless, the results shown herein confirm that the ASL-FISP technique can sensitively differentiate normal tissue perfusion from pathology (e.g. ischemic stroke) and relative tissue perfusion levels (renal cortex versus renal medulla versus skeletal muscle). It is important to note that the ASL-FISP technique may also be sensitive to the effects of pulsatility, which may be an underlying cause of the bright cerebrospinal fluid signal in the mouse brains.

Another observation of these initial ASL-FISP results is the trend towards lower perfusion values at 9.4 T. Although not statistically significant, this trend may be caused, in part, by the reduced $T_2^*$ relaxation times at 9.4 T. For mouse brain imaging, this reduction in $T_2^*$ can reduce the SNR of the SS, NS and M0 images, especially in regions near the ear canals. Fortunately, this potential limitation can be partially mitigated using shorter TEs which would reduce the deleterious $T_2^*$ effects at all field strengths. For the FISP acquisition, the reduction in TE would provide an additional increase in SNR, as TR would also be reduced by the same percentage, providing an increase in the coherent steady-state magnetization.

**Conclusions**

This study reports a rapid and quantitative ASL-FISP MRI technique for high-field MRI scanners. For this initial study, the ASL-FISP technique combines a FAIR ASL preparation with a rapid, centrically encoded FISP imaging readout to provide
perfusion-weighted images in less than 2 s with minimal image distortion, ghosting and banding artifacts in comparison with EPI and balanced SSFP readouts. Initial \textit{in vivo} ASL-FISP perfusion results were obtained for healthy and ischemic stroke C57/BL6 mouse brains at 7 T and healthy mouse brains at 9.4 T. As a demonstration of the invulnerability of the ASL-FISP technique to off-resonance artifacts, initial \textit{in vivo} kidney ASL results were also obtained for a C57/BL6 mouse at 7 T. This new technique provides an alternative method for many perfusion imaging applications on high-field MRI scanners where off-resonance artifacts can severely limit the use of EPI and balanced SSFP acquisitions.
CHAPTER 4 REFERENCES


CHAPTER 5: LACK OF DYSTROPHIN RESULTS IN ABNORMAL CEREBRAL DIFFUSION AND PERFUSION IN VIVO

ABSTRACT

Dystrophin, the main component of the dystrophin-glycoprotein complex, plays an important role in maintaining the structural integrity of cells. It is also involved in the formation of the blood-brain barrier (BBB). To elucidate the impact of dystrophin disruption in vivo, we characterized changes in cerebral perfusion and diffusion in dystrophin-deficient mice (mdx) by magnetic resonance imaging (MRI). Arterial spin labeling (ASL) and diffusion-weighted MRI (DWI) studies were performed on 2-month and 10-month mdx mice and their age-matched wildtype controls (WT). The imaging results were correlated with Evan's blue extravasation and vascular density studies. The results show that dystrophin disruption significantly decreased the mean cerebral diffusivity in both 2-month ($7.38 \pm 0.30 \times 10^{-4}$ mm$^2$/s) and 10-month ($6.93 \pm 0.53 \times 10^{-4}$ mm$^2$/s) mdx mice as compared to WT ($8.49 \pm 0.24 \times 10^{-4}$, $8.24 \pm 0.25 \times 10^{-4}$ mm$^2$/s, respectively). There was also an 18% decrease in cerebral perfusion in 10-month mdx mice as compared to WT, which was associated with enhanced arteriogenesis. The reduction in water diffusivity in mdx mice is likely due to an increase in cerebral edema or the existence of large molecules in the extracellular space from a leaky BBB. The observation of decreased perfusion in the setting of enhanced arteriogenesis may be caused by an increase of intracranial pressure from cerebral edema. This study demonstrates the defects in water handling at the BBB and consequently, abnormal perfusion associated with the absence of dystrophin.
INTRODUCTION

The blood-brain barrier (BBB) ensures the proper physical and metabolic environment for brain function. Endothelial cells lining the vasculature of the brain are joined by tight junctions in order to prevent free diffusion of large and hydrophilic molecules, including water, from the blood into the brain parenchyma (Brightman and Reese, 1969 and Reese and Karnovsky, 1967). A major role of the tight junctions is to maintain the physiologic and biochemical environment necessary for neuronal firing. The cytoskeleton is crucial in forming, anchoring, and maintaining the BBB (Zlokovic, 2008). Disruption of the BBB leads to increased permeability of large molecules, such as albumin, into the CNS, causing neuronal dysfunction, edema, and a lower seizure threshold (Abbott et al., 2006, Hawkins and Davis, 2005, Tomkins et al., 2008 and Van Vliet et al., 2007).

Dystrophin, a major actin-binding component of the dystrophin glycoprotein complex (DGC), links cytoskeletal and membrane elements in the muscle (Tinsley et al., 1994) and brain (Lidov et al., 1990 and Lidov et al., 1993). Inherited deficiency of functional dystrophin leads to Duchenne muscular dystrophy (DMD), an X-linked recessive disease leading to progressive muscle degeneration. In addition to voluntary muscle weakness, DMD patients suffer from ventilatory and cardiovascular failure at the end stages of the disease. Functional dystrophin is also not expressed in the brains of DMD patients (Hoffman et al., 1987). However, aside from the observation that pediatric DMD patients have lower intelligence quotient (IQ) assessments than that of healthy control children (Cotton et al., 2001 and Karagan, 1979), few studies have
characterized the structural and functional impact of dystrophin disruption in the brain.

The dystrophin-null *mdx* mouse has been used as a mouse model of muscular dystrophy for over two decades (Sicinski et al., 1989). Aside from the muscular phenotype, cognitive defects have also been observed in *mdx* mice (Vaillend et al., 1995). The tight junction proteins of the BBB were shown to be reduced and dysfunctional in the *mdx* mouse brain, leading to a leaky BBB (Nico et al., 2003). Moreover, enhanced expression of matrix-metalloproteinase (MMP)-2 and -9 in *mdx* mouse brains was associated with increased expression of vascular endothelial growth factor (VEGF, Nico et al., 2006). These in vitro observations provide the evidence of altered brain structure associated with dystrophin deletion. A full understanding of the role of dystrophin in maintaining the BBB and vascularization has yet to be studied in vivo with modern imaging technologies which will likely support future clinical investigations.

Arterial spin labeling (ASL) is a non-contrast MRI method that has made significant contributions towards assessing tissue perfusion in vivo (Detre et al., 1992, Williams et al., 1992, Edelman et al., 1994, Kwong et al., 1995, Kim and Tsekos, 1997, Wong et al., 1997, Pell et al., 1999 and Thomas, 2005). In ASL MRI, water molecules are “magnetically tagged” in the blood, leading to altered tissue longitudinal magnetization that is proportional to tissue perfusion. This method does not require exogenous paramagnetic contrast agents as in conventional Dynamic Contrast Enhanced (DCE) MRI perfusion techniques. Hence, ASL may eventually become the preferred method for longitudinal imaging studies. In spite of its utility, ASL has yet
to be fully applied to understanding the pathophysiologic consequence of dystrophin disruption with regard to water movement and perfusion in the brain.

Diffusion-weighted MRI (DWI) has been invaluable in defining neurological disorders, particularly in the diagnosis of stroke and the assessment of therapeutic interventions (Le Bihan et al., 1986, Kloska et al., 2010, Schellinger et al., 2003, Sevick et al., 1990 and Warach et al., 1995). The signal intensity of a DW image reflects the restrictions on Brownian motion of water molecules in the tissue, and the calculated apparent diffusion coefficient (ADC) provides a means to quantify this diffusion under physiologic and pathologic states. High ADC values are characteristic of tissue with relatively free water diffusion, e.g., in extracellular space, as opposed to tissue water with a restricted environment, e.g., in intracellular space (Le Bihan, 2007). Therefore, the diffusion of water molecules as detected by DWI can be used to delineate the neural structure, anatomy, and pathophysiology in the absence of dystrophin.

The goal of this study was to characterize the impact of dystrophin deletion on physiological and structural changes in the brain using both in vivo and in vitro methods. Cerebral perfusion and brain structure were evaluated by ASL and DWI, respectively. These in vivo imaging results were compared with in vitro and ex vivo studies of vascular density. The present study demonstrates the defects in perfusion and diffusion associated with dystrophin disruption in mdx mice that can be observed in vivo with MRI and the association of these in vivo imaging assessments with histopathologic measures.
METHODS

Animal Models

Studies were performed on young (2-month, n = 10) and adult (10-month, n = 10) male dystrophin-null (mdx) and wild-type (WT) mice of the C57/BL6 strain. All mice were obtained from Jackson Laboratories (Bar Harbor, ME). All procedures involving animal care and handling were performed according to institutional guidelines set forth by the Animal Care and Use Committee at Case Western Reserve University.

Perfusion and Diffusion MRI

Imaging studies were performed on a 7 T Bruker Biospec (Billerica, MA) horizontal bore MRI scanner. Anesthesia was induced with 2% isoflurane with supplemented O₂ in an isoflurane induction chamber and maintained via nosecone with 1.5% isoflurane once the animal was put in the magnet. The body temperature was monitored and maintained at approximately 36 °C by blowing hot air into the magnet through a feedback control system. Respiratory gating and monitoring was performed through an MR-compatible small animal gating and monitoring system (SA Instruments, Stony Brook, NY) to reduce motion artifacts during image acquisition. Single-slice axial ASL brain images were acquired with a Flow-Sensitive Alternating Inversion Recovery (FAIR) preparation sequence followed by a centrically-encoded Fast Imaging in Steady Precession (FISP) imaging readout (Gao, et al., 2014). Specifically, arterial spin labeling was accomplished by respiratory-triggered, slice-selective (4.5 mm thickness) and non-selective (global) inversion of magnetization,
respectively. The inversion pulse used a hyperbolic secant adiabatic inversion pulse with a duration of 3 ms, and the inversion thickness was set to three times that of the excitation pulse to ensure a uniform inversion over the entire imaging slice. An inversion efficiency value of 1 was assumed. The FISP acquisition (including 10 dummy scans) was implemented at 1420 ms following the inversion pulse. This delay time allowed data acquisition to occur during the mid- to late-stage of the subsequent breathing cycle. It is a balance between maximizing blood flow sensitivity and minimizing respiratory motion artifacts. Imaging parameters are: flip angle, 60°; TR, 2.4 ms; TE, 1.2 ms; number of averages, 40; matrix size, 128x128; FOV, 30x30 mm²; slice thickness, 1.5 mm. A proton density image (M0) with no inversion pulse was also acquired using the same imaging parameters. A voxel-wise T₁ map with the same spatial resolution was also acquired with a FISP-based Look-Locker acquisition consisting of a non-selective adiabatic inversion pulse followed by 10 continuous FISP acquisitions with the following parameters: flip angle, 10°; TR, 4.0 ms; TE, 2.0 ms; number of averages, 40. Image reconstruction and analysis were performed offline using custom-built software written in Matlab (MathWorks, Natick, MA). Perfusion maps were generated from the magnetization difference of slice-selective and non-selective inversion images (ΔM), proton density image (M0), and the T₁ maps (Gao, et al., 2014). A blood/tissue partition coefficient (λ) of 0.9 ml/g was used in this study (Herscovitch and Raichle, 1985).

Single-slice axial Diffusion-Weighted Images (DWI) were acquired with a diffusion-weighted Echo-Planar Imaging (DW-EPI) acquisition. Diffusion weighting was accomplished with two 4 ms (δ) diffusion-encoding gradients separated by 15 ms
(Δ), yielding a b value of 500 s/mm² applied in the readout direction. The DW-EPI images were acquired with the following parameters: TR, 5000; number of averages, 5; matrix, 128x128; FOV, 30 x 30 mm²; slice thickness, 1 mm. Images were reconstructed and analyzed using custom-built software written in Matlab (MathWorks, Natick, MA). Voxel-wise apparent diffusion coefficient (ADC) maps were calculated by the established equation: $\text{ADC} = \ln(I_0/I)/b$, where $I_0$ and $I$ are the signal intensity of the $b = 0$ s/mm² and the $b = 500$ s/mm² images, respectively.

**Analysis of Vascular Density by Immunocytochemistry**

WT (2 mo, n = 4; 10 mo, n = 5) and mdx (2 mo, n = 4; 10 mo, n = 5) mice were deeply anesthetized with isoflurane after imaging. The brains were excised and fixed in 4% paraformaldehyde for 24 h at 4 °C. The tissue was transferred to 5% sucrose in PBS for 1 h followed by 20% sucrose overnight at 4 °C. Subsequently, the brains were rapidly frozen in OCT media (Electron Microscopy Sciences, Hatfield, PA) with liquid nitrogen. Tissues were sectioned at 10 μm slice thickness at the location corresponding to the MR imaging slice. A total of 7 sections were obtained from each brain. Indirect immunofluorescence against CD31 (PECAM-1, 1:100, Sigma Aldrich, St. Louis, MO) was performed to assess cerebral vascular density. DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride, Sigma Aldrich, St. Louis, MO) staining was used for nuclear identification. Stained tissue sections were photographed for the DAPI (blue) and PECAM-1 (red) channels. The number of PECAM-1 positive cells per field was quantified. All images were photographed at the same exposure times and magnification. The obtained values from all animals were
averaged, and the results were expressed as the mean number of PECAM-1 positive cells per field ± SD.

**Analysis of BBB Leakage using Evans Blue Staining**

WT (2 mo, n = 2; 10 mo, n = 2) and mdx (2 mo, n = 2; 10 mo, n = 2) mice were injected intraperitoneally with a bolus of 0.1 mL/10 g Evans Blue (10 mg/ml, Sigma Aldrich, St. Louis, MO). After 24 hours, the mice were deeply anesthetized with isoflurane and the brains were excised and rapidly frozen in OCT media (Electron Microscopy Sciences, Hatfield, PA). Tissues were sectioned at 10 μm slice thickness at the location corresponding to the MR imaging slice. Evans Blue extravasation was evaluated by fluorescence in WT versus mdx.

**Analysis of Vascular Density by Cryo-Imaging**

The remaining WT (2-month, n = 2; 10-month, n = 2) and mdx (2-month, n = 2, 10-month, n = 2) mice were anesthetized with isoflurane and Fluorescein isothiocyanate-dextran (FITC-dextran, 10 mg/ml, Sigma Aldrich, St. Louis, MO) was injected intravenously at a dose of 200 mg/kg. After 10 minutes, the brains were excised and rapidly frozen with liquid nitrogen in OCT media (Electron Microscopy Sciences, Hatfield, PA) and mounted to the cryo-imaging system. The tissue was sectioned in 25 μm slices and the bright-field and fluorescent images from the cryo-imaging system were processed and analyzed with custom-built Matlab (Mathworks Inc., Natick, MA) and AMIRA (Mercury Computer Systems, San Diego, CA) software (Roy et al., 2009). The brain contour was traced manually from the bright-field images, and a mask was generated. The mask was applied to fluorescent images,
and the 3D cerebral vasculature was reconstructed using thresholding and volume rendering methods (Qutaish et al., 2012). The resolution for the reconstructed 3-D images were 10.5x10.5x25 μm³.

**Statistical Analysis**

All data are reported as mean ± SD. A two-tailed, unpaired Student’s t-test was performed to compare mdx and WT mice. Statistical significance was established at a level of p < 0.05.

**RESULTS**

**Decreased cerebral diffusivity with dystrophin disruption**

Representative brain apparent diffusion coefficient (ADC) maps of 2- and 10-month old mdx and WT mice are shown in Figs. 5.1a-d. The mean and standard

![Figure 5.1. a-d. Representative diffusion images of 2-month-old WT (a), 2-month-old mdx (b), 10-month-old WT (c), 10-month-old mdx (d). e. Mean cerebral (excluding ventricles) diffusion in C57/BL6 WT (black) and mdx (white), respectively. There was a significant decrease in mean diffusion as compared to WT observed in the mdx brains in both the 2- and 10-month old mice (*p<0.0005). There was also a significant age-dependent decrease in diffusion in young versus aged WT and mdx mice, respectively (#p<0.05). Data are represented as mean ± SD. Color bar is uniform in each panel.](image-url)
deviations for each group of mice are shown in Fig. 5.1e. Mean diffusivity in 2-month mdx mice was $7.38 \pm 0.30 \times 10^{-4} \text{mm}^2/\text{s}$, which was significantly lower than that in age-matched WT mice ($8.49 \pm 0.24 \times 10^{-4} \text{mm}^2/\text{s}$, Fig. 5.1e, $p < 0.0005$). Further, 10-month mdx mice also exhibited a decrease in cerebral mean diffusivity as compared to the age-matched WT mice ($6.93 \pm 0.53 \times 10^{-4}$ versus $8.24 \pm 0.25 \times 10^{-4} \text{mm}^2/\text{s}$, $p < 0.0005$). In addition, a 2-tailed Student’s t-test showed a significant age-related decrease in the mean ADC values of both the WT and mdx mice ($p < 0.05$).

**Figure 5.2.** a-d. Representative perfusion images of 2-month-old WT (a), 2-month-old mdx (b), 10-month-old WT (c), 10-month-old mdx (d). e-h. Representative T1 maps of 2-month-old WT (e), 2-month-old mdx (f), 10-month-old WT (g), 10-month-old mdx (h). i. Mean cerebral (excluding ventricles) perfusion from the ASL-FISP method in C57/BL6 WT (black) and mdx (white), respectively. There was a significant decrease in mean perfusion as compared to aged WT observed in the aged mdx mice (*$p<0.005$). There was no significant change in perfusion in young versus aged WT ($p>0.3$), respectively. Data are represented as mean ± SD. Color bar is uniform in each panel.
Abnormalities in Cerebral Perfusion in Aged mdx Mice

Representative cerebral perfusion maps for the 2 and 10 month old mdx and WT mice are shown in Figs. 5.2a-d. The mean and standard deviations for each group are shown in Fig. 5.2e. There was a 15% decrease in cerebral perfusion in 10 month mdx mice as compared to WT (Fig. 5.2e, $p = 0.001$), without any significant difference in mean cerebral T₁ or M₀ values ($p = N.S.$). The mean brain perfusion values for the 2-month old mdx mice were slightly lower than for 2-month old WT mice. However, no significant difference in cerebral perfusion was observed.

Disruption of Blood Brain Barrier Integrity in mdx Mice

To investigate the integrity of the BBB in the absence of dystrophin, we examined the extravasation of Evans blue from blood vessels histologically. There was a significant leakage of Evans blue into the cerebrum of both 2- and 10-month mdx mice as demonstrated in Figs. 5.3a,c. There were few vessels demonstrating Evans blue extravasation in the mdx mice.
extravasation in both young (2-month old) and aged (10-month old) WT mouse brains (Figs. 5.3b,d).

**Enhanced Cerebral Arteriogenesis in Aged mdx Mice**

Representative PECAM-1 stained immunofluorescent images for the 2- and 10-month mdx and WT mice are shown in Figs. 5.4a-d. There was no difference in the amount of cerebral vasculature (number of PECAM-1 positive cells) between the young mdx and WT mice (Fig. 5.4e). Interestingly, there was enhanced arteriogenesis in the aged (10 month) mdx mice as compared to the controls, as demonstrated by a 13% increase in PECAM-1 positive cells (Fig. 5.4e, p < 0.05). Representative 3D vessel reconstructions from the FITC-dextran cryoimaging are shown in Fig. 5.5. Qualitatively, there were more vessels visible in the 2- and 10-month mdx mouse brains as compared to WT.

![Figure 5.4. a-d. Representative images of indirect immunofluorescence against CD31 (platelet endothelial cell adhesion molecule 1, PECAM-1) on axial sections of paraformaldehyde-fixed frozen 2-month-old WT (a), 2-month-old mdx (b), 10-month-old WT (c), 10-month-old mdx (d). e. Quantification of PECAM-1 positive cells in 2- and 10-month-old mdx and WT mice. Data are represented as mean ± SD. *p<0.05 versus WT.](image)
DISCUSSION

In this study, we report the first *in vivo* evaluation of age-dependent alterations in cerebral perfusion and diffusion in mdx mice. In addition to BBB disruption, we have observed increased arteriogenesis in the cerebrum as a result of dystrophin deletion. Traditional Evans Blue extravasation confirmed an interruption at the BBB in both the 2- and 10-month mdx mice. In addition, *in vivo* DWI experiments established an alteration of the normal cerebral microstructural and physiologic environment in both 2- and 10-month mdx mice. Interestingly, decreased in vivo perfusion was also present in the 10-month mdx mice despite increased arteriogenesis observed in both cryoimaging and immunocytochemistry.

*Figure 5.5.* a-l. Representative dorsal (top row), lateral (middle row), and rostral (bottom row) views of the 3D vessel reconstructions from 2-month-old WT (a-c), 2-month-old mdx (d-f), 10-month-old WT (g-i), and 10-month-old mdx (j-l).
Consistent with the findings by immunocytochemistry, cryoimaging demonstrates the extensive arteriogenesis in mdx throughout the entire brain as compared to WT. Previously, enhanced arteriogenesis was observed in the hindlimbs of mdx mice (Straino et al., 2004). It was also shown that deletion of dystrophin results in enhanced expression of matrix-metalloproteinase (MMP)-2 and -9, which ultimately leads to an increase of VEGF and VEGFR2 expression (Nico et al., 2006). In addition to angiogenesis, increased VEGF activity may also contribute towards an opening of tight junctions at the BBB and enhanced vascular permeability (Schoch et al., 2002 and Zhang et al., 2002). The observed increase in arteriogenesis by both cryoimaging and immunocytochemistry in the current study is consistent with the observations of enhanced VEGF activity in vitro (Nico et al., 2002). However, unlike the qualitative results from cryoimaging, we did not see a difference in PECAM-1 expression in mdx versus WT mice at 2-months of age. The high resolution, but limited sampling of the immunofluorescence experiments may better reflect the molecular content of the tissue, whereas the 3D cryoimaging data are more representative of the global macrovascular structure. As such, the global nature of cryoimaging as described herein provides unique and complementary information for these types of investigation.

The decreased cerebral ADC observed in the current study likely represents cellular edema as a result of BBB disruption. BBB disruption in mdx mice due to reduced expression of tight junction proteins has been reported previously (Nico et al., 2003). Consequently, the osmotic influx of water into the cell causes cellular edema and a decrease in extracellular space, leading to a decrease in ADC (Kucharczyk et
al., 1991 and Moseley et al., 1990). While it has been shown that aqp4 facilitates the clearance of vasogenic edema as a result of BBB disruption (Papadopoulos et al., 2004), the lack of properly functioning aqp4 channels at the BBB in mdx mice (Adams et al., 2008 and Frigeri et al., 2001) may prevent the removal of water from the cerebrum and therefore worsen cellular edema. Alternatively, a leaky BBB may also lead to an increase of large molecules in the extracellular space in mdx mice, which may also limit water diffusivity in the extracellular space. Therefore, the observed ADC decrease in mdx brains may be the result of cellular edema, increased extracellular protein content, enhanced vascular permeability, or a combination of these possible mechanisms. The age-dependent reduction of ADC in the 10-month WT as compared to 2-month is supported by prior studies, which describe decreasing ADC with age likely due to cellular atrophy and an increase in cerebrospinal fluid (CSF) compartments (Heiland et al., 2002).

To the best of our knowledge, this is the first study that demonstrated decreased perfusion in the setting of increased angiogenesis and a chronically leaky BBB in the mdx brain in vivo. This decrease in cerebral perfusion is likely the result of cerebral edema, as observed by DWI. It has been established that the presence of cerebral edema and/or angiogenesis in the fixed volume of the skull leads to an increase in intracranial pressure (ICP, Adams and Ropper, 1997 and Mokri, 2001). Increased ICP in this way could decrease cerebral perfusion pressure (CPP), which is the difference between mean arterial pressure and ICP. The control of CPP ensures proper brain function as decrease in CPP may lead to ischemia and an increase may contribute towards raising the ICP (Powers, 1991 and Schumann et al., 1998). As a result of
increased ICP, the consequent decrease of CPP ultimately leads to an autoregulatory
decrease in cerebral blood flow. This decrease in perfusion in mdx mice may
compromise their ability to react to periods of hypoxia.

There are several limitations in this initial study aimed at investigating whether
there were overall changes in diffusion and perfusion in the brain of mdx mice that can
be detected by MRI. First, fast imaging sequences were used for both DWI and ASL
acquisitions. The voxel size and slice thicknesses used gave rise to partial volume
averaging that obscured the small white matter tracts in a mouse brain in vivo. Future
studies aimed at white/gray matter differentiation/evaluation will require
optimization of the imaging parameters or potentially using a different imaging
readout approach. Second, instead of performing a comprehensive diffusion tensor
imaging study, we performed DWI in order not to overtly extend the acquisition time.
While DWI is sensitive to structural changes at the cellular level (e.g., edema), a future
diffusion tensor study will be helpful to validate and refine our current findings.

Third, measurement of cerebral perfusion used a single inversion time (TI) and
the classic model that assumes equal $T_1$ for blood and tissue (Detre et al., 1992, Kwong
et al., 1995 and Kim and Tsekos, 1997). Further, transit time is not considered in the
calculation. A more recent model by Pell et al incorporated both the transit time ($\delta$)
and the $T_1$ of the blood ($T_{1a}$) and the tissue ($T_{1app}$) separately (Pell et al., 1999). While
the method requires the quantification of both $T_{1app}$ and $T_{1a}$ by using multiple inversion
times, it allows more comprehensive assessment of several physiological parameters
that can impact blood flow. In calculating the cerebral blood flow (CBF), the classic
model and the Pell model used the following equations respectively:
where $F_C$ and $F_P$ are the two terms that the classic model and the Pell model differ. These two terms are defined as:

$$F_C(T_1, T_1) = \frac{e^{\gamma T_1}}{T_1}$$

$$F_P(\delta, T_{1a}, T_{1app}, T_1) = e^{\delta/T_{1a}} \cdot \frac{1/T_{1a} - 1/T_{1app}}{e^{-(T_{1a} - \delta)/T_{1app}} - e^{-(T_{1a} - \delta)/T_{1a}}}$$

And $T_1$ and $T_{1app}$ are related by:

$$\frac{1}{T_{1app}} = \frac{1}{T_1} + \frac{\text{CBF}}{\text{T}}$$

Hence, for a measured $\frac{\Delta M(T_1)}{M_0}$, the ratio of measured blood flow by the two methods is:

$$\frac{C_{BF_C}}{C_{BF_P}} = \frac{F_C(T_1, T_1)}{F_P(\delta, T_{1a}, T_{1app}, T_1)}$$

Using a transit time of 50 ms (Thomas, 2005), a blood $T_1$ of 2.2 and 2.4 s at 7 T and 9.4 T, respectively (ex vivo measurement) (Dobre et al., 2007), and a tissue $T_1$ of 1.4, 1.6, and 1.8 s respectively, the simulated differences between the two models with CBF ranging from 100 to 400 ml/min/100 g are shown in Fig. 5.6. These results suggest that at a field strength of 7 T, the difference between the classic model and the Pell
model was < 3\% when tissue T_1 was 1.8 s. However, the classic model overestimated CBF by 12\% to 16\% when tissue T_1 was 1.4 s (Figs. 5.6.a&b). In our current study, measured tissue T_1 was \sim 1.7 s. Hence the calculated CBF difference should be within 8\% compared to the Pell model. At 9.4 T, there could be an up to 12\% over-estimation by classic model (Figs. 5.6.c&d).

**Figure 5.6.** Comparison of the classic model and the Pell model. (a) and (b). Simulated \( F_c \) and \( F_p \), and \( CBF_c/CBF_p \) at 7T, respectively. (c) and (d). Simulated \( F_c \) and \( F_p \), and \( CBF_c/CBF_p \) at 9.4T, respectively. Solid lines in (a) and (c) represent the classic model, while dotted lines represent the Pell model. Black, red, and blue represent tissue T_1 of 1.4, 1.6, and 1.8 s, respectively.

In summary, we have observed abnormal cerebral diffusivity, enhanced angiogenesis, and decreased cerebral perfusion in the mdx mouse. Our study is the
first to assess the consequence of dystrophin deletion on cerebral blood flow in the mdx mouse model. *In vivo* mouse studies are essential to further the translational value of these results. Although this work is a step towards clinical translation, better mouse models of DMD need to be studied in further detail since the mdx mouse has a mild phenotype in comparison to the clinical presentation of DMD. The leaky BBB and decreased cerebral perfusion observed in mdx mice may contribute towards the disruption of proper brain function observed in DMD patients. As the life expectancy of DMD patients increases with improved therapies, developing an understanding of the neurologic manifestations of the dystrophin-null phenotype will lead to better patient management when symptoms emerge. In particular, DMD patients suffering from cardiac arrhythmias will be at an increased risk of stroke, and thus understanding dystrophin’s role at the BBB and response to hypoxia is critical.
CHAPTER 5 REFERENCES


CHAPTER 6: FUTURE DIRECTIONS

1) Mechanical dysfunction in other models of HCM

RATIONALE

This work identified the potential of MRI in detecting early phenotypic changes of one mouse model of HCM. If future studies demonstrate the utility of MRI in sensitively predicting early phenotypes of other HCM genotypes in mice, then MRI may represent a broadly applicable early detection tool in identification of HCM in humans. The most predominant HCM-causing mutant genes are β-myosin heavy chain, myosin-binding protein C, and cardiac troponin T (Table 6.1, (Houston and Stevens, 2014)). In addition, cardiac troponin I, regulatory and essential myosin light chains, titin, α-tropomyosin, α-actin, and α-myosin heavy chain gene mutations have all been identified as causes of HCM (Houston and Stevens, 2014). In fact, more than 1400 mutations have been identified, which are largely unique to individual families (Landstrom et al., 2010a). There is considerable clinical heterogeneity of HCM given the diversity of molecular defects, including age of onset, degree of

<table>
<thead>
<tr>
<th>Gene</th>
<th>Symbol</th>
<th>Locus</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin heavy chain</td>
<td>MYH7</td>
<td>1q12</td>
<td>&gt;30%</td>
</tr>
<tr>
<td>Myosin-binding protein C</td>
<td>MYBPC3</td>
<td>11p11.2</td>
<td>&gt;20%</td>
</tr>
<tr>
<td>Cardiac troponin T</td>
<td>TNN12</td>
<td>1q32</td>
<td>&gt;20%</td>
</tr>
<tr>
<td>Troponin</td>
<td>TPM1</td>
<td>15q22.1</td>
<td>&gt;5%</td>
</tr>
<tr>
<td>Cardiac troponin I</td>
<td>TNN1</td>
<td>19p13.2</td>
<td>&gt;5%</td>
</tr>
<tr>
<td>Myosin light chain, essential</td>
<td>MYL3</td>
<td>3p21.3-p21.2</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>Myosin light chain, regulatory</td>
<td>MYL2</td>
<td>12q23-q24.3</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>Cardiac alpha-actin</td>
<td>ACTC</td>
<td>1q</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>Cardiac troponin C</td>
<td>TNN1C</td>
<td>3p21.3</td>
<td>Rare</td>
</tr>
<tr>
<td>Alpha-Myosin heavy chain</td>
<td>MYH6</td>
<td>14q</td>
<td>Rare</td>
</tr>
<tr>
<td>Protein kinase A gamma-subunit of AMP activated protein kinase</td>
<td>PRKAG2</td>
<td>7q22-q31.1</td>
<td>unknown</td>
</tr>
</tbody>
</table>
LVH, and clinical course (Maron, 2002). In order for DENSE MRI to be of clinical value in detecting preclinical HCM, the pattern of LV mechanical dysfunction must be described in each of the sarcomeric protein mutations.

**EXPERIMENTAL STRATEGY**

Adult mouse models for each of the sarcomeric protein mutations causing HCM will be obtained (see Table 6.2) with appropriate age-matched controls. The MRI DENSE protocol will be repeated as previously described in Chapter 2 for each of the mouse models of HCM at several time points to better characterize the progression of phenotypic expression (ages 2 weeks, 8 weeks, 6 months, and 10 months). LV twist, torsion, circumferential and radial train, as well as torsion and strain rates will be quantified as previously described (Zhong et al., 2008). Comparisons of strain and twist angles will be independently analyzed at each ventricular level by 1-way ANOVA and Tukey-Kramer test. Strain and torsion time course data will be evaluated by 1-way ANOVA with a Bonferroni adjustment for multiple comparisons. Statistical significance will be established at a level of $P<0.05$.

**INTERPRETATION**

I anticipate that the mechanical indices of cardiac function will be affected in other HCM-causing mutations as what was observed in cMyBPC (Desjardins et al., 2012). Given that there is significant heterogeneity in the phenotypic expression and clinical course of HCM, I suspect that there will be differences in the timing and/or severity of strain and twist alterations. For example, mutations in troponin T have been shown
to have very little LVH but high rates of sudden death (Moolman et al., 1997),
cMyBPC mutations have been associated with late-onset of HCM (Niimura et al.,
1998b), and there was a family with mutations in β-myosin heavy chain with severe
HCM and early onset of LVH. Therefore, troponin T mutated mice might
demonstrate decreased mechanical function even earlier than seen in cMyBPC
mutated mice, and β-myosin heavy chain mutated mice would show the most severe

| Table 6.2: Mouse and human genes identified in hypertrophic cardiomyopathy |
|-------------------|-------------------|-------------------|
| HUMAN GENE | MOUSE GENE | REFERENCE |
| CSRP3: cysteine and glycine-rich protein 3 (cardiac LIM protein) | Csrp3 | (Knöll et al., 2010) |
| MYBPC3: myosin-binding protein C, cardiac | Mybpc3 | (Harris et al., 2002) |
| MYH6: myosin, heavy chain 6, cardiac muscle, alpha | Myh6 | (Palmer et al., 2004) |
| ACTC1: actin, alpha, cardiac muscle 1 | Actc1 | (Song et al., 2011) |
| CALR3: calreticulin 3 | Calr3 | (Ikawa et al., 2011) |
| CAV3: calveolin 3 | Cav3 | (Galbiati et al., 2001) |
| JPH2: junctophilin 2 | Jph2 | (Takeshima et al., 2000) |
| MYH7: myosin, heavy chain 7, cardiac muscle, beta | Myh7 | (Pandya et al., 2006) |
| MYL2: myosin, light chain 2, regulatory, cardiac, slow | Myl2 | (Kazmierczak et al., 2012) |
| MYLK2: myosin light chain kinase 2 | Mylk2 | (Zhi et al., 2005) |
| MYOZ2: myozenin 2 | Myoz2 | (Ruggiero et al., 2013) |
| PRKAG2: protein kinase, AMP-activated, gamma 2 non-catalytic subunit | Prkag2 | (Arad et al., 2003) |
| TCAP: titin-cap | Tcap | (Markert et al., 2010) |
| TNNI3: troponin I type 3 (cardiac) | Tnni3 | (Wang et al., 2012) |
| TNNT2: troponin T type 2 (cardiac) | Tnnt2 | (Tardiff et al., 1998) |
| TTN: titin | Ttn | (Gramlich et al., 2009) |
| VCL: vinculin | Vcl | (Zemljic-Harpf et al., 2007) |

The bolded genes are the most common mutations causing HCM.
deficit in strain and twist seen at an early age. In addition, there has been a gene dose-dependent effect seen in humans (Olivotto et al., 2008; Richard et al., 2000) and mice (Desjardins et al., 2012), suggesting that a similar effect will be seen in the proposed study.

**POTENTIAL CONCERNS**

There is some evidence to suggest that stratifying the genetic etiology of HCM is not clinically beneficial (Landstrom et al., 2010b). It was shown that myofilament-positive HCM patients had a greater probability of LV dysfunction than those with myofilament-negative HCM, however there was no difference in dysfunction between MyBPC3 thick or thin filament affected individuals (Olivotto et al., 2008). Although this study did not detect differences in function between two myofilament mutations, I believe that a more comprehensive evaluation of several myofilament proteins will generate differences in outcome and provide data on phenotype-genotype relationships.

2) **Predictive value of mechanical dysfunction in preclinical HCM**

**RATIONALE**

The introduction of commercial genetic testing has expanded the recognition of patients with HCM. However, it can be difficult to manage the gene-positive and phenotype-negative patients. It is unclear if these patients would benefit from treatment or even close monitoring. The data is not clear if gene-positive phenotype-negative individuals are at an increased risk for sudden death or disease progression.
There is a need for better diagnostic technology and long term follow-up in this patient population.

The next step of this investigation is to translate the findings from our small animal studies to a clinical trial. We will conduct a small prospective cohort study in HCM families to test if MRI-detected mechanical dysfunction is superior to echocardiogram-identified cardiac hypertrophy in pinpointing early and clinically significant pathology. We first established that there are reduced mechanical indices of torsion, twist, and strain apparent in a mouse model of HCM (Desjardins et al., 2012). Furthermore, the mechanical dysfunction was apparent before the development of overt cardiac hypertrophy, implicating altered mechanical function as the first link of the development of pathological hypertrophy and cardiac disease.

**EXPERIMENTAL STRATEGY**

The future directions of this aim will require several phases. I would begin by obtaining Institutional Review Board (IRB) approval at my institution for a prospective cohort clinical study. I would enroll the children (ages 12-18) of families identified with HCM that are already being evaluated with annual echocardiograms, but do not yet exhibit overt LVH (i.e. genotype-positive phenotype-negative). Most phenotypes of HCM begin to emerge in young adults during accelerated adolescent growth, which is typically complete by age 18 (Maron et al., 1986). My exclusion criteria would include pregnancy, prior myocardial infarction, prior septal myectomy or alcohol septal ablation, incessant ventricular arrhythmias, known cardiovascular disease, hypertension, substance abuse, standard MRI exclusion criteria including;
claustrophobia, ferromagnetic material in the body (foreign body or implanted), and inability to lie still for one hour or more. Once enrolled, the subjects would undergo DENSE MRI on the same timing schedule as the standard of care echocardiography study. After imaging is completed, subjects will be followed for cardiovascular outcomes.

In addition to the conventional markers of cardiac function, the LV strain, twist, and torsion would be determined for each subject at every time point. The primary outcome to be studied in this clinical trial would be whether DENSE MRI can predict defects in cardiovascular function prior to standard echocardiography. This information would be correlated with the incidence of cardiac death, aborted sudden cardiac death, heart transplantation, left ventricular assist device placement, all-cause mortality, ventricular tachyarrhythmias, ventricular fibrillation or sustained ventricular tachycardia, hospitalization for heart failure, atrial fibrillation, and stroke. In particular, I would focus on twist/torsion as markers of dysfunction. In our study of cMyBPC⁺/⁻ mice, we detected a 15% reduction in net twist, and a 13% decrease in maximal torsion (Desjardins et al., 2012). Prior work has established that LV torsion is similar between mice and humans, and thus represents a uniform measure of LV function across species (Henson et al., 2000). In order to detect a 5% decrease in net twist angle with a power of 90% and type I error rate less than 5%, the necessary sample size must be n=71.

**INTERPRETATION**

I anticipate that DENSE MRI would be more sensitive than standard echocardiography in detecting early myocardial dysfunction in young adults with
HCM. Since LV torsion is a consistent marker of cardiovascular function between mice and men (Henson et al., 2000), I expect that our findings of decreased LV torsion in a mouse model of HCM would translate clinically.

**POTENTIAL CONCERNS**

The proposed study requires a long period of follow-up with subjects and may require several years to enroll a sufficient subject population. There may be difficulty ensuring that subjects continue to follow with MRI for several years. In addition, the prevalence of disease progression has not yet been established in the genotype-positive phenotype-negative population with standard echocardiography. We may need to adjust the sample size of the study if data emerges regarding the incidence of disease progression in phenotype-negative individuals. There are several clinical trials currently underway investigating the diagnosis (markers of fibrosis, echocardiography vs CMR) and treatment (diltiazem) of preclinical HCM.

Table 6.3: Hypertrophic cardiomyopathy vs physiologic athletic heart hypertrophy.

Lastly, a potential cofounder of the study is if there is also physiologic hypertrophy from athletic training (Table 6.3,(Maron and Maron, 2013) ). We have previously shown in a mouse model of physiologic hypertrophy that there are alterations from the normal LV strain pattern (Montano et al., 2013). There are clinical trials dedicated to studying the cardiovascular response to athletic training in patients with known HCM mutations, particularly because HCM is the most common cause of sudden death in trained competitive athletes (Maron et al., 1996b). The target population in the proposed study is 12-18 years old, so there may not be many subjects with intense athletic training at this point in their lives. Although the physiologic hypertrophy may add complexity to data interpretation, it will be important to characterize myocardial function in this population due to the concern for sudden death.

3) Functional consequence of cerebral ischemia in mdx mice

**RATIONALE**

The blood-brain barrier (BBB) ensures the proper physical and metabolic environment for brain function. Endothelial cells lining the vasculature of the brain are joined by tight junctions in order to prevent free diffusion of large substances from the blood into the brain parenchyma (Brightman and Reese, 1969; Reese and Karnovsky, 1967). Although the water content of the brain has been studied for decades (BERING, 1954; Bradbury et al., 1972; EDSTROM et al., 1961), the exact mechanism by which water passes through the BBB remained elusive. Discovery of the water channel, aquaporin 4 (AQP4), has contributed greatly to the understanding of water movement in the brain (Francesca and Rezzani, 2010; Nicchia et al., 2004;
Nico et al., 2001; Zador et al., 2009). In mice, AQP4 plays a prominent role in both the formation and removal of edema (cytotoxic and vasogenic respectively) following brain injury, such as in stroke or tumor formation (Hirt et al., 2009; Kaur et al., 2006; Meng et al., 2004; Promeneur et al., 2013; Saadoun et al., 2002; Tait et al., 2010; Tang et al., 2010; Tourdias et al., 2011; Vajda et al., 2002a; Verkman et al., 2006; Vizuete et al., 1999; Yang et al., 2008; Zeynalov et al., 2008). The anchoring of AQP4 by the dystrophin-glycoprotein complex (DGC) is vital for its proper function in the brain (Adams et al., 2008; Amiry-Moghaddam et al., 2004; Bragg et al., 2006; Nico et al., 2005; Perronnet and Vaillend, 2010; Vajda et al., 2002b; Waite et al., 2012). Thus, dystrophin may be indispensable in physiologic blood brain barrier maintenance, and perturbation of dystrophin could be predicted to result in pathologic dysregulation of the blood brain barrier.

The role of dystrophin in maintaining the BBB in vivo has not yet been described, despite the clinical benefit in understanding the pathophysiology of stroke in dystrophinopathy. First, dystrophinopathies are associated with cardiomyopathy and arrhythmias and therefore represent an increased risk of ischemic stroke in these patients (Atsumi et al., 2004; Finsterer, 2012; Finsterer and Stöllberger, 2010; Finsterer et al., 2012; Hanajima and Kawai, 1996b; Tsakadze et al., 2011). Secondly, as the life expectancy of DMD patients increases with improved therapies, developing an understanding of the neurologic manifestations of the dystrophin-null phenotype will lead to better patient management when symptoms emerge. A comprehensive assessment of edema development as a result of acute stroke in mice lacking
dystrophin using powerful MR techniques may represent a first step in targeting therapies towards preventing or treating stroke in dystrophinopathy patients.

Future work of this project would test the function of dystrophin in preventing vasogenic edema following ischemic insult. Ischemic stroke induced by middle cerebral artery occlusion (MCAO) demonstrates two distinct phases of edema with unique pathologies (Simard et al., 2007). Ischemic stroke is first characterized by cytotoxic edema, which is characterized as the swelling of cells as a result of movement of osmotically active molecules to the intracellular space. The lack of blood flow during ischemia leads to decline in ATP synthesis, which impairs Na⁺/K⁺ ATPase function. As a result, cellular accumulation of Na⁺, Ca²⁺, and lactate drives water into the cell and produces significant cell swelling. Vasogenic edema causes further cell damage at later time points due to the breakdown of the BBB (Simard et al., 2007). Several mechanisms have been described to alter the integrity of the BBB, including reverse pinocytosis (Castejón, 1984) and disruption of Ca²⁺ signaling (Brown and Davis, 2002). Although dystrophin is an integral part of the cytoskeletal network that supports regulation of water transport at the blood brain barrier, the function of dystrophin in these edematous conditions has not yet been explored. My hypothesis is that a lack of dystrophin will be detrimental during ischemic injury by exacerbating the formation of vasogenic edema.

**EXPERIMENTAL STRATEGY**

A permanent MCAO protocol will be implemented to induce cerebral ischemia in mice (Longa et al., 1989). In summary, mice will be anesthetized with 2% isoflurane via nosecone and their temperature maintained with a heating pad. Ischemia will be
induced by inserting and tying a monofilament distal to the carotid bifurcation. The cervical wound will be closed with sutures and the animal will be allowed to recover. In the sham surgery, the carotid artery will be maneuvered as in MCAO, however the arteriotomy and filament ligation will not be executed. Studies will be performed on the following groups of 3 month old mice (n=10 for each group):

i. Sham-operated C57/BL6 control
ii. MCAO C57/BL6 control
iii. Sham-operated mdx
iv. MCAO mdx

The infarct size as a result of MCAO will be quantified in each group using a T₂-weighted image 24 hours after the surgery. A DWI protocol will be implemented and images will be acquired at the following time points after MCAO/Sham surgery: 1, 4, 8, 24, and 72 hours. In addition, a neurologic deficit score adapted from (Hunter et al., 2000) will be assigned 24 hours after MCAO by an observer blinded to the genotype and surgical procedure. Once imaging studies are completed, brains will be fixed for histologic analysis (n=3 each group) by transcardiac perfusion of paraformaldehyde (4% in 0.1M sodium phosphate buffer), immediately stored at 4°C in fixative overnight and paraffin embedded for sectioning. Slices will be stained with hematoxylin/eosin, 2,3,5-triphenyltetrazolium chloride (TTC), or glial fibrillary acid protein (GFAP) using the avidin-biotin peroxidase complex method (Garcia et al., 1993) to assess morphology and infarct size in each group. The infarct size (as measured by both histology and MRI) and mean ADC will be statistically compared
between the control and mdx groups using standard ANOVA methods with the appropriate adjustments.

**INTERPRETATION**

I anticipate that the mdx group will experience a significantly worse prognosis following MCAO as measured by diffusion MRI, histology, and neurologic deficit scoring. I expect to find an increase in cerebral edema and slower resolution of fluid accumulation in the mdx group following an ischemic insult. Together, the results would suggest that dystrophin-mediated BBB integrity is an essential component of the tissue response to ischemic injury in the mouse.

**POTENTIAL CONCERNS**

Defining the pathophysiologic role of dystrophin in ischemic stroke is a complex task. Dystrophin is necessary to anchor AQP4 to the astrocytic foot processes (Nicchia et al., 2008a, 2008b; Vajda et al., 2002b). *Aqp4* homozygous null mice do not exhibit a phenotype under baseline conditions and have similar brain water content as compared to wild-type in the absence of pathology (Tait et al., 2010). Under conditions of cytotoxic edema, either by cerebral ischemia or water intoxication, the absence of AQP4 is protective and reduces injury in both pathologic models (Manley et al., 2000). In contrast, AQP4 deletion worsens cerebral swelling and neurologic function following vasogenic edema due to the inability to remove water from the tissue (Papadopoulos et al., 2004).
We described a decrease in cerebral ADC of mdx mice that likely represents cellular edema as a result of BBB disruption in our prior work (Goodnough et al., 2014). While it has been shown that AQP4 facilitates the clearance of vasogenic edema as a result of BBB disruption (Papadopoulos et al., 2004), the lack of properly functioning AQP4 channels at the BBB in mdx mice (Adams et al., 2008; Frigeri et al., 2002) may prevent the removal of water from the cerebrum and therefore worsen cellular edema.

Ischemic stroke is first characterized by cytotoxic edema, and vasogenic edema causes further cell damage at later time points (Simard et al., 2007). I anticipate that there is vasogenic edema in mdx mice prior to MCAO that will exacerbate and accelerate the ischemic injury since the homeostatic fluid balance is already compromised. This result would suggest the dystrophin has a role in maintaining the BBB outside of its role for anchoring proteins for AQP4. However, if I observe an improvement in edema formation (particularly in the acute phase) after MCAO, it would imply that the main function of dystrophin is the anchoring of AQP4 in the astrocytic endfeet. In either outcome, the interpretation of dystrophin’s role in maintaining the BBB during acute stroke will be novel.

4) Functional consequences of hypoperfusion in DMD

RATIONALE

It was observed early on that DMD boys have lower IQs than their age-matched controls (Karagan, 1979); in fact, Duchenne described cognitive impairment in his initial description of the disease (Duchenne, 1868). Similarly, there is also
evidence of cognitive impairment in the mdx murine model, particularly passive
avoidance learning (Muntoni et al., 1991) and newly learned information (Vaillend et
al., 1995). There is evidence of cortical atrophy and ventricular dilation in DMD
patients (Septien et al., 1991; Yoshioka et al., 1980), however there has not been a
study to establish the link between cognitive impairment and gross cerebral
abnormalities. In addition, there is evidence of hypo-metabolism in DMD (Bresolin et
al., 1994b; Tracey et al., 1995, 1996b) where there is a significant increase in the
cerebral Pi/ATP ratio in DMD patients and mdx mice.

Our prior work suggests that cerebral perfusion is compromised in mdx mice
(Goodnough et al., 2014). Although it has not yet been studied in the brain, there is
also evidence of vascular dysfunction in the skeletal muscle of DMD. Functional
muscle ischemia has been shown in mdx mice, which is due to a deficiency of nNOS
deficiency and thus contraction-induced modulation of sympathetic vasoconstriction
(Sander et al., 2000). Previously, enhanced arteriogenesis was observed in the
hindlimbs of mdx mice (Straino et al., 2004). Similarly, an increase of VEGF and
VEGFR2 expression results from deletion of dystrophin via enhanced expression of
matrix-metalloproteinase (MMP)-2 and -9 (Nico et al., 2006). In addition to
angiogenesis, increased VEGF activity may also contribute towards an opening of
tight junctions at the BBB and enhanced vascular permeability (Schoch et al., 2002;
Zhang et al., 2002).

EXPERIMENTAL STRATEGY

The aim of this study is to correlate decreased perfusion in mdx mice with
cognitive functional deficits. I will correlate deficits in cerebral perfusion and
metabolism with the onset cognitive function over an extended time course in mdx mice compared to wild-type (WT) controls. Mdx mice will be compared to WT mice at (n=8 each group): 2 and 4 weeks, 2, 3, 4, 6, 8 and 10 months. At each time point, the cerebral perfusion will be measured as described in (Gao et al., 2014), as well as the cerebral $P_i$/ATP ratio using magnetic resonance spectroscopy (Tracey et al., 1996b). Prior to beginning the study, I will complete a full battery of cognitive testing (Figure 6.1, (Rodriguiz and Wetsel, 2006) on 10 month mdx mice (n=5) to determine which paradigm of cognitive exams I will choose for the course of the study. Although it has been shown that passive avoidance is diminished in mdx (Muntoni et al., 1991), a broad range of cognitive abilities and functions remain untested in mdx mice.

**INTERPRETATION**
I anticipate a gradual decline in perfusion, metabolism, and cognition in the mdx mice as compared to control. We observed a decrease in cerebral perfusion from 2 to 10 months in mdx mice, and the mean ADC was already decreased at 2 months in mdx compared to WT (Goodnough et al., 2014). It is unclear if defects in perfusion will precede hypometabolism or vice versa. I predict that perfusion and metabolism are unrelated and will demonstrate independent time courses. Since adequate perfusion and metabolism are necessary for cognition, it is likely that their effects will be additive on cognitive function testing.
CHAPTER 6 REFERENCES


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