

**INVESTIGATING THE DISEASE-CAUSING MUTATIONS IN DUCHENNE
MUSCULAR DYSTROPHY**

by

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INVESTIGATING THE DISEASE-CAUSING MUTATIONS IN DUCHENNE MUSCULAR DYSTROPHY

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University of Pittsburgh, 2009

Duchenne muscular dystrophy (DMD) is a progressive, degenerative muscle disease. It is caused by a large variety of mutations in the dystrophin gene. Studies of new therapies that are based on specific genotypes are generating a high level of interest among both researchers and patients. This investigation examines the mutations reported in patients with DMD by the large international academic research group, the Cooperative International Neuromuscular Research Group (CINRG). It also compares the types of mutations reported in two large mutation databases, Leiden DMD mutation database and the French Universal Mutation Database-Duchenne Muscular Dystrophy (UMD-DMD), to those reported in DMD patients from three CINRG studies.

Diagnostic, strength, and medical history data were reviewed retrospectively for 374 DMD patients from 20 CINRG centers worldwide. The frequency of each type of mutation found in the CINRG data was compared with similar information abstracted from the Leiden and UMD-DMD mutation databases. On an exploratory basis, the distribution of DMD-causing lesions in the CINRG data was also compared to data from the patient registry, DuchenneConnect.

The distribution of dystrophin mutations within the CINRG database is similar to the two large published databases and the patient registry data collection. The immediate results improve understanding of the many mutations in the dystrophin gene. These results suggest the need for

more rigorous and harmonized genetic screening as well as the continued collection of global data in easily accessed, searchable databases. The results of this work have a public health importance because DMD is the most common form of muscular dystrophy. Furthermore, the creation and improvements to existing disease databases can advance the standard of care for all patients and families with muscular dystrophy over diverse geographies and cultures. Harmonization of mutation data collection for DMD studies will benefit clinical trials and ultimately enhance pairing of eligible patients to specific molecular-based treatments.

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PREFACE

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1.0 INTRODUCTION

Duchenne Muscular Dystrophy (DMD) is an X-linked recessive condition characterized by progressive muscle weakness leading to premature death. DMD has an incidence of 1 in 3,500 newborn males. Two-thirds of dystrophin mutations are inherited and the remaining one-third of mutations are caused by *de novo* mutations. The Cooperative International Neuromuscular Research Group (CINRG) was established to conduct clinical research on DMD. This project was undertaken to investigate the different mutations reported in patients with DMD in several studies carried out by CINRG and to compare the distribution of those mutations to those in two published databases and one patient registry.

While there have been reports of some ethnic or regional variations in the various mutations of the dystrophin gene in DMD patients, it is generally accepted that the distribution of mutations is approximately the same worldwide. With the establishment of several large databases, it is possible to analyze whether the patient population examined by CINRG is representative of the DMD patient population at large.

This investigation will provide information on the types of data collected and stored by CINRG as well as that in two large DMD databases, Leiden DMD mutation database, abbreviated as Leiden, and the French Universal Mutation Database-Duchenne Muscular Dystrophy abbreviated as UMD-DMD. This investigation will highlight the similarities and differences found. It will also identify the strengths and weaknesses of each database and

identify areas for improvement, cooperation and even future harmonization. This investigation will provide information on ways to enhance the collection of demographic and mutation-related data as well as identify some of the relevant clinical data that might be included in databases to facilitate genotype/phenotype correlations.

As new treatments for DMD become available, information on the specific mutation present in a patient's dystrophin gene is likely to be required to select or exclude individuals in certain clinical trials. Therefore, the way such data are collected and reported will be crucial to physicians, genetic counselors, and parents as they make recommendations and decisions on patient care. As the quality, quantity, interpretability, and access to large national and international databases of genetic and clinical information of DMD patients improves so does our understanding of the disease and opportunities to identify new approaches to its treatment and care.

2.0 HYPOTHESIS AND SPECIFIC AIMS

2.1 HYPOTHESIS

If the distribution of mutations causing DMD is similar worldwide, then the distribution of mutations in the CINRG database, representing an international DMD clinical trials network, will be similar to the distribution of mutations in two large international databases, the Leiden and UMD-DMD databases.

2.2 SPECIFIC AIMS

- Aim 1: To characterize the DMD gene mutations in a collection of participants included in CINRG DMD treatment and observational studies.
- Aim 2: To compare the types of DMD gene mutations carried by CINRG study participants to those reported in two large international databases of DMD mutation data.

Plan: Plot the distribution of mutations carried by CINRG study participants and compare this distribution to the Leiden and UMD-DMD databases. On an exploratory basis, compare the distribution of DMD-causing mutations in the CINRG database with the distribution of DMD-causing mutations in the Parent Project Muscular Dystrophy DuchenneConnect registry generated by patient/parent reporting.

- Aim 3: To compare the muscle strength data to the distribution of mutation type in the CINRG population.

Plan: Analyze muscle strength data for a subset of CINRG study participants as a function of mutation type and other variables, such as treatment with corticosteroids, and test for statistical associations.

3.0 BACKGROUND AND SIGNIFICANCE

3.1 DUCHENNE MUSCULAR DYSTROPHY

Duchenne muscular dystrophy (DMD) is a relentlessly progressive muscular dystrophy causing severe disability and ultimately death. Although the disease was named after the French neurophysiologist C.B. Duchenne de Boulogne, Edward Meryon presented the first clinical description to the Royal Medico-Chirurgical Society on December 9, 1851. The cases he presented illustrated a disease involving the muscle with post-mortem granular degeneration (Meryon 1851). In January of 1868, in the *Archives Generales de Médecine*, C.B. Duchenne de Boulogne describes the case of a 9-year-old boy who could not walk because of muscle wasting and proposed the name ‘paralysie pseudo-hypertrophique’ translated as ‘pseudo-hypertrophic paralysis’ (Duchenne 1868). He later described the male predominance, progressive course, weakness in the lower limbs, loss of ambulation by adolescence and premature death.

William Richard Gowers proposed the hereditary basis in 1886 stating: ‘the disease is thus transmitted by women who are not themselves its subjects, thus the congenital tendency is exclusively due to the maternal element in the embryo. This is also shown by another fact, that the children of the same women, by different husbands, have been affected’ (Gowers 1886). In 1955, Becker and Kiener described the same disease with a milder clinical course (Becker and Kiener 1955). This milder allelic disorder has been named Becker muscular dystrophy (BMD).

DMD is the most common form of muscular dystrophy, affecting 1 in 3500 male births worldwide. One hundred years after the observations of Duchenne and Gower, the gene was identified on the X chromosome (Kunkel, Beggs et al. 1989). The X-linked dystrophin gene defect causes absence of the muscle cytoskeletal protein dystrophin (Hoffman, Brown et al. 1987). A high spontaneous mutation rate of roughly 30% in the large dystrophin gene complicates disease eradication.

Affected boys become symptomatic at 3 to 5 years of age with proximal leg weakness that impairs mobility, decreases the ability to get up from a squat, and prevents the normal ability to run. By 8 years of age, some affected boys begin to lose the ability to walk and need to resort to a wheelchair. This shift from an ambulant to a non-ambulant phase occurs in most boys with a diagnosis of DMD by age 12 years (Engel 2004). A pattern of respiratory insufficiency results from weakness of the diaphragm and other accessory muscles of respiration and deformities of the thorax caused by progressive spinal scoliosis. Pre-clinical cardiac disease is present in 25% of DMD patients under 6 years of age with an increase to 59% by age 10. A clinically apparent dilated cardiomyopathy develops in an increasing proportion of patients with increasing age beginning at age 10 (Nigro, Comi et al. 1990). Treatment strategies to date for DMD have been largely restricted to supportive aids and surgical approaches to ameliorate the effects of joint contractures and scoliosis (Engel 2004). Corticosteroids are widely used to prolong the period of ambulation (Mendell, Moxley et al. 1989).

3.2 GENETICS OF DUCHENNE MUSCULAR DYSTROPHY

3.2.1 Inheritance

DMD is inherited in an X-linked recessive pattern. The effect of this inheritance pattern is different in men and women. If a woman is hemizygous for a mutation, there is a 50% chance of transmitting the DMD mutation in each pregnancy. If a son inherits the mutation, he is affected. Daughters who inherit the mutation are carriers. There have been no reports of any males with DMD reproducing. Males with BMD may reproduce; all of their daughters will be carriers and none of their sons will inherit the mutation if the mother is mutation free.

3.2.2 Dystrophin Gene

The dystrophin gene is the largest human gene, measuring 2.4 Mb and containing 79 exons. The gene represents 0.1% of the entire human genome and is approximately 1.5% of the X-chromosome. The gene was identified through positional cloning on the p-arm of chromosome X at position Xp21.2 (Monaco, Neve et al. 1986; Koenig, Hoffman et al. 1987). It is transcribed into a 14-kb mRNA, and the 11-kB coding sequence encodes a 3,685 amino acid protein of 427 kDa (Appendix A). The protein represents approximately 0.002% of the total striated muscle protein (Hoffman, Brown et al. 1987). Figure 1 is a schematic representation of the human muscle narrowing down to the location of the muscle fibers and dystrophin-glycoprotein complex.

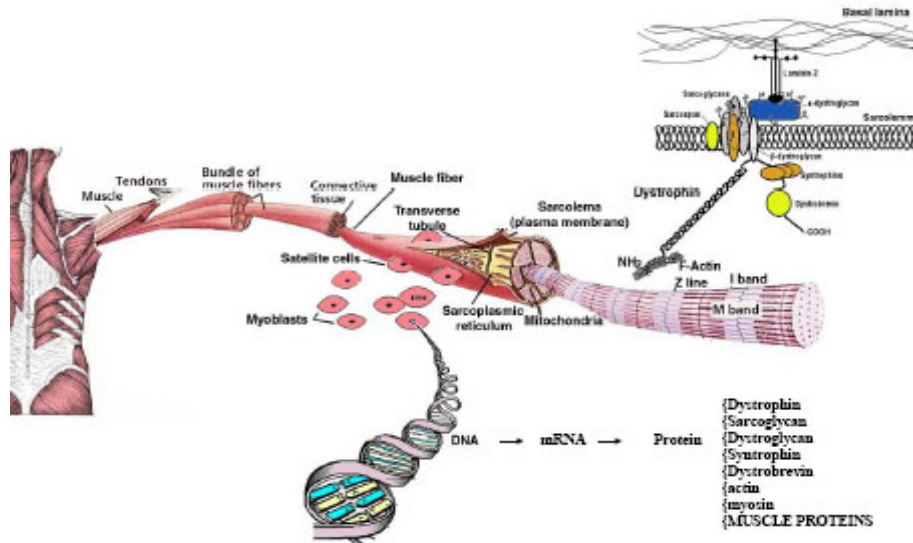


Figure 1: Schematic of the Human Muscle and Location of the Dystrophin-Glycoprotein Complex (ActionDuchenne 2007) Localization diagram of the human muscle, muscle fiber, plasma membrane (sarcolemma), and dystrophin-glycoprotein complex.

Ahn and Kunkel determined that the gene has at least five independent promoters that specify the transcription of their respective alternative first exons (Ahn and Kunkel 1993). The promoters have been named according to their molecular weights. Three tissue-specific promoters (Dp427b, Dp427m, and Dp427p) located in the 5' region of the gene control the expression of the full-length dystrophin. Four additional promoters (Dp260, Dp140, Dp116, and Dp71) are located further downstream; each is responsible for a unique first exon that splices into exons 30, 45, 56, and 63, respectively. Furthermore, this complex gene contains two-polyA sites and the dystrophin RNA is differentially spliced. All these events generate further protein diversity (Byers, Lidov et al. 1993; Lederfein, Yaffe et al. 1993; D'Souza, Nguyen et al. 1995; Lidov, Selig et al. 1995). Figure 2 illustrates the 7 promoters and 4 isoforms.

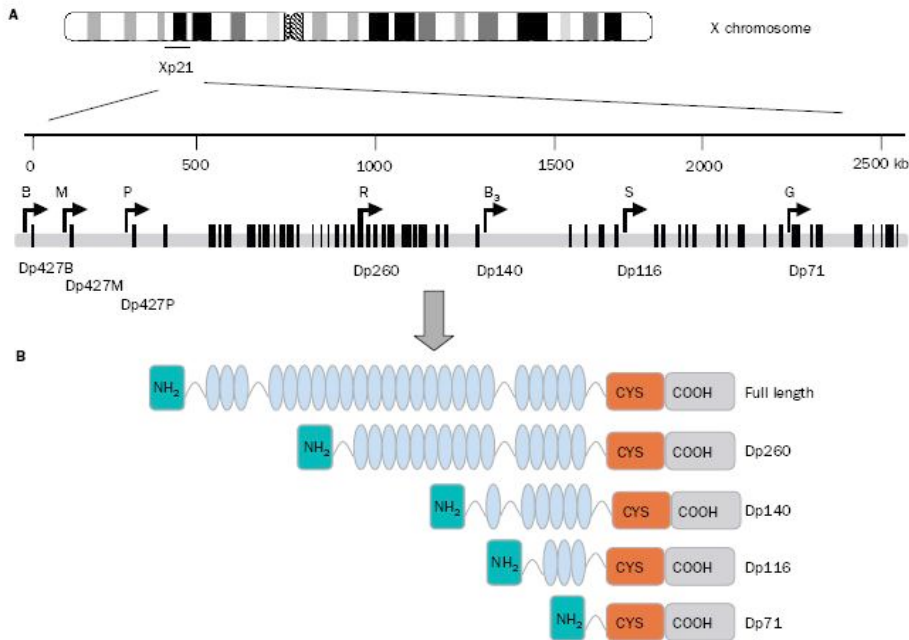


Figure 2: Dystrophin Gene and Isoforms (Muntoni, Torelli et al. 2003)

A. Genomic organization of the dystrophin gene, located in Xp21. The black lines represent the 79 exons.

B. The domain composition of the dystrophin protein and various protein isoforms.

Dystrophin belongs to a large network of proteins called the dystrophin-glycoprotein complex. The complex forms a bridge across the skeletal muscle membrane (sarcolemma) and basal lamina of the extracellular matrix (Muntoni, Torelli et al. 2003). The role of dystrophin is not fully understood. It is thought to play a role in stabilizing the sarcolemma and in protecting muscle fibers from contraction-induced damage and necrosis (Davies and Nowak 2006). Dystrophin can be separated into four domains:

1. Actin binding domain, located at the 5' end covers amino acids 14 through 240 (exons 2 – 8). This region was discovered due to its homology with chicken alpha-actinin (Hammonds 1987).
2. Central rod domain, which covers amino acids 253 through 3,040 (exons 8 – 61), is formed by 24 spectrin-like helical elements with homology to alpha-actinin and spectrin (Koenig and Kunkel 1990).

3. Cysteine-rich domain, which covers amino acids 3,080 through 3,360 (exons 63 – 69), was found to be homologous to the C-terminal domain of the slime mold alpha-actinin (Bies, Caskey et al. 1992).
4. Carboxy-terminal domain, which covers amino acids 3,361 through 3,685 (exons 70 – 79), has not been shown to be homologous to any proteins other than the dystrophin related proteins.

Both the C-terminus and the actin-binding regions of the protein have been found to play a critical role in its function. However, the central rod-domain may be shortened and some function of the protein is conserved explaining the three distinct allelic diseases: DMD, BMD, and X-linked cardiomyopathy (Muntoni, Cau et al. 1993).

3.2.3 Animal Models

Homologues of DMD have been identified in several species including: dogs, cats, mice, fish, and invertebrates (Collins and Morgan 2003). The most commonly studied models are the *mdx* mouse and the golden retriever muscular dystrophic (GRMD) dog. The models are used to study potential pharmacological interventions as well as gene and cell replacement therapy.

3.2.3.1 Mouse Model

The *mdx* mouse is the most widely used animal model for DMD. The *mdx* mouse has a nonsense mutation in exon 23 of the dystrophin gene causing premature chain termination of the dystrophin protein (Sicinski, Geng et al. 1989). The complete absence of dystrophin protein expression in skeletal muscle in this model results in progressive degeneration of the tissue.

3.2.3.2 Canine Model

A canine X-linked muscular dystrophy has been identified in a line of golden retrievers (GRMD) (Cooper, Valentine et al. 1988). The GRMD mutation is a point mutation in the splice site within intron 6 causing an exon 7 deletion that is out-of-frame (Sharp, Kornegay et al. 1992). The GRMD represents the most clinically relevant model for DMD since these dogs lack dystrophin and present with a very similar phenotype. It includes severe weakness and muscle atrophy at about six to eight weeks of age.

3.3 MUTATION CATEGORIES

The mutation rate of the dystrophin gene is higher (1×10^{-4}) than the estimated average gene mutation rate (1×10^{-5} to 1×10^{-6}) in humans (Emery 1991; Tuffery, Chambert et al. 1998). The overall dystrophin mutation rate appears to be equal in males and females (Tuffery, Chambert et al. 1998). Two-thirds of dystrophin mutations are inherited and the remaining one-third of mutations are caused by *de novo* mutations. The variation of mutations is broad and can be divided into several categories based on size and complexity.

3.3.1 Large Lesions

Large lesions, which include intragenic deletions and duplications, account for the majority of changes in the dystrophin gene ranging from 60-70% (Koenig, Beggs et al. 1989; White, Kalf et al. 2002; Muntoni, Torelli et al. 2003). Large lesions are defined as deletions or duplications of more than one exon. These mutations are clustered at two hotspots: 30% at the hotspot in the

proximal part of the gene and the remaining 70% at a more distal hotspot (Oudet, Hanauer et al. 1992). The proximal hotspot of the gene spans from exons 2 through 22 and the distal hotspot of the gene spans from exons 45 through 55 (Beggs, Koenig et al. 1990; Oudet, Hanauer et al. 1992; Nobile, Galvagni et al. 1995; Sironi, Pozzoli et al. 2003; Gualandi, Rimessi et al. 2006).

3.3.2 Small Lesions

Small lesions, which account for approximately 20-30% of the dystrophin mutations, include small mutations and point mutations. Small mutations are changes affecting less than an exon but more than a single base pair, whereas point mutations refer to a change affecting a single base pair. Point mutations can be further divided into missense and nonsense mutations.

3.3.3 Other Mutations

The third subdivision of mutations includes mutations in an intron or complex rearrangements. These types of mutations account for approximately 2% of the dystrophin mutations and require high density-comparative genomic hybridization array and RNA analysis in order to be confirmed (Bovolenta 2008).

3.4 GENOTYPE AND PHENOTYPE CORRELATIONS

Monaco et al. were the first to postulate an explanation for the phenotypic differences observed between DMD and BMD patients (Monaco, Bertelson et al. 1988). In DMD patients, a disruption

of the open reading-frame causes a complete absence of the dystrophin protein, whereas BMD patients carry an in-frame mutation that allows a stable expression of dystrophin with a smaller molecular size that may be expressed at a lower quantity. The reading-frame rule has been confirmed by several additional studies (Baumbach, Chamberlain et al. 1989; Gillard, Chamberlain et al. 1989). This reading-frame hypothesis explains over 90% of BMD and DMD cases. It is often used for the differential diagnosis of BMD and DMD (Muntoni, Torelli et al. 2003).

Figure 3 represents the dystrophin gene exon map. Each numbered block represents an exon from exon 1 through exon 79. Rectangular blocks which include exons: 3-5, 9-10, 13-16, 23-42, 47-49, 60 encompass exons that start and stop at full codons. The deletion of any of these exons results in an in-frame mutation. Blocks starting or ending with an arrow which include exons: 1-2, 6-8, 11-12, 17-22, 43-46, 50-59, 61-79 represent exons that start and/or stop with split codons. Arrows pointing to the left represent codons that are split at the third nucleotide and arrows pointing to the right represent codons that are split at the second nucleotide. The deletion of any of these exons results in an out-of-frame mutation.

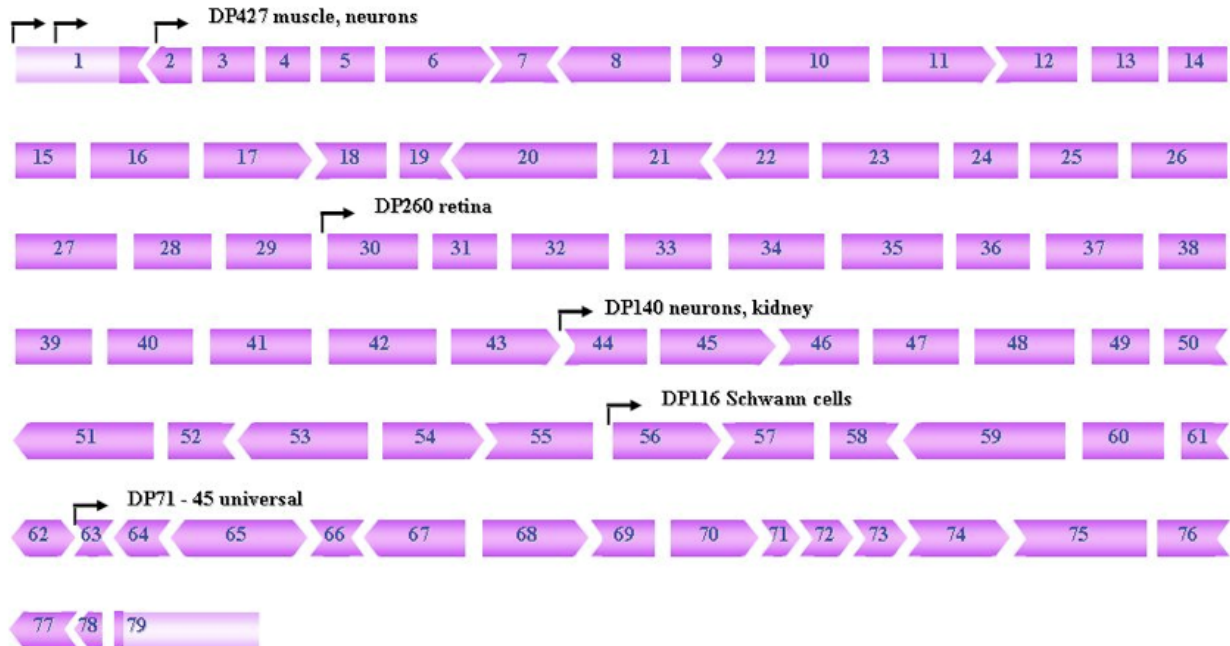


Figure 3: Dystrophin Gene Exon Map

Numbered blocks represent each exon (1 through 79). Rectangular blocks (such as exon 3, 4, and 5) represent exons that start and end with full codons. Blocks starting and/or ending in an arrow, pointed to the right or left, represent exons that start or end at nucleotide 2 and 3 respectively. For example, the deletion of exon 42 alone would cause an in-frame mutation, whereas the deletion of exon 52 would cause an out-of-frame mutation. The black arrows indicate the location of the alternative promoters and protein isoforms.

There are, however, exceptions to the reading-frame hypothesis in both BMD and DMD patients. There exist several well-characterized regions of the dystrophin gene where frameshift mutations are associated with some dystrophin production. These patients tend to have a BMD phenotype due to the production of dystrophin. It is thought that the most common event that allows the production of dystrophin is exon skipping, which occurs via alternative splicing (Arahata, Beggs et al. 1991; Muntoni, Torelli et al. 2003). There are also exceptions in which an in-frame mutation may result in a DMD phenotype (Arahata, Beggs et al. 1991).

In a 1993 paper by Hoffman, the complexity of the dystrophin gene highlights the challenges faced in studying genotype and phenotype correlations (Hoffman 1993). He stated that ‘the plethora of mutations of the massive and complex dystrophin gene are known to cause a dramatic range of clinical disorders, and the X-linked recessive expression enables study of

solitary loss-of-function or change-of-function mutations in hemizygous males, and in the mosaic situation caused by X-inactivation in heterozygous females.’ At the time, it was speculated that point mutations, which in 1993 were challenging to identify, would yield critical information about genotype and phenotype correlations (Hoffman 1993).

3.5 RISK ASSESSMENT

Genetic risk assessment is typically performed in the context of genetic counseling. The Genetic Counseling Definition Task Force of the National Society of Genetic Counselors developed a new definition in 2006. Fraser published the original definition in 1974 in the *American Journal of Human Genetics* (Fraser 1974). The new definition states: ‘Genetic Counseling is the process of helping people understand and adapt to the medical, psychological and familial implications of genetic contributions to disease. This process integrates the following:

- Interpretation of family and medical histories to assess the chance of disease occurrence or recurrence
- Education about inheritance, testing, management, prevention, resources and research
- Counseling to promote informed choices and adaptation to the risk or condition.’ (Resta, Biesecker et al. 2006).

It is important for families with a child suspected to have muscular dystrophy to have a risk assessment. The risk will depend on the origin of the mutation, as DMD may be inherited or occur as a new mutation. The risk is highest for sons of carrier mothers with a 50% risk that each son will be affected. The risk may be reduced for sons of mothers with germline mosaicism, estimated to be around 15% recurrence. The risk is not increased over the general population risk

for children of mothers who have a son with a new mutation. Beyond the risk assessment, genetic counseling can also provide psychological support for families with a child affected with a progressive and fatal disease. Genetic counselors can offer resources and additional support that provide assistance through the difficult times ahead of them.

3.6 TESTING STRATEGIES

There exist several different methods of diagnosing and confirming DMD. However, there is currently no consensus on the route of confirming the diagnosis. The diagnosis of DMD may be suspected when a male child presents with abnormal muscle function and elevated muscle enzymes in the blood.

3.6.1 Screening

Creatine kinase (CK) is an enzyme expressed by various tissue types and is used clinically as a marker of myocardial infarction, rhabdomyolysis, and muscular dystrophy. An elevation in serum CK indicates muscle damage. Normal values range from 25 to 200 U/L. In individuals with DMD, CK levels are greater than 10 times the upper limit of normal (Hoffman, Fischbeck et al. 1988; Zatz, Rapaport et al. 1991). This blood test is often performed as a first screening test. A review of the CK levels for DMD, BMD, X-linked cardiomyopathy, and female carriers is presented in Table 1 (GeneTests 1993-2009).

Table 1: Serum CK Concentrations

	Phenotype	% of Affected Individuals	Serum CK concentrations
Males	DMD	100%	Greater than 10 times the normal range
	BMD	100%	Greater than 5 times the normal range
	X-linked cardiomyopathy	Most individuals	2 – 10 times the normal range
Female Carriers	DMD	~ 50%	2 – 10 times the normal range

The discovery of elevated transaminase enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), may also be an indication of the presence of muscle degeneration as these enzymes are also produced by the muscle and are released into the bloodstream in the setting of muscle destruction. Because AST and ALT are more commonly associated with production by the liver, their elevation in the serum may incorrectly lead to further testing for liver disease in DMD patients.

3.6.2 Diagnostic

Since the discovery of the gene, molecular testing to detect large deletions and duplications has been fairly straightforward. It has allowed for nearly two-thirds of the affected patients to have a confirmed molecular diagnosis. The remaining third have been more challenging due to the large size of the gene and the widespread distribution of small or point mutations across the gene. With the more recent availability of complete gene sequencing, a molecular diagnosis can be confirmed for nearly all DMD patients.

Based on information available through the Laboratory Directory page of the online GeneTests resource, Deletion/Duplication Analysis, which includes the original Chamberlain

PCR methodology (Chamberlain, Gibbs et al. 1988), is currently the testing strategy most widely available. Different testing strategies that are performed around the world are summarized in Table 2 from the Gene Tests Online resource (GeneTests 1993-2009).

Table 2: Summary of Diagnostic Testing Strategies Around the World

	Sequencing of the entire coding region	Sequencing of select regions	Mutation scanning of the entire coding region	Linkage	FISH	Deletion/Duplication Analysis	Immunohistochemistry	Prenatal	Carrier testing	Total number of laboratories offering testing
USA	4	1	2	3	1	16	0	11	11	49
International	4	1	5	10	0	18	1	19	17	75
Total	8	2	7	13	1	34	1	30	28	124

3.6.2.1 DNA Testing

The most common method used to identify large deletions and duplications in the dystrophin gene is multiplex polymerase chain reaction (PCR). The original PCR testing was developed by Chamberlain and included probes for 9 exons (Chamberlain, Gibbs et al. 1988). Then Beggs et al. added several additional probes (Beggs, Koenig et al. 1990) to be able to detect 98% of the deletions in the dystrophin gene. Newer versions of the PCR-based dystrophin gene deletion test typically include probes for approximately 30 exons. Some laboratories use a combination of multiplex PCR and the Southern blot method to be able to determine the exact endpoints of the deletion or duplication. Knowledge of the deletion endpoints is important for determination of the effect of the deletion on the dystrophin reading frame.

Southern blot was the first method used for genetic confirmation of a DMD diagnosis. This method can directly detect large deletions and duplications by use of full-length dystrophin

cDNA clones to generate probes (Prior and Bridgeman 2005). Each cDNA probe covers approximately 10 exons and is used to detect the exact site of the mutation. Since the majority of mutations are located in two hotspots, most mutations are detected by four cDNA probes: cDNA 1-2a, cDNA 2b-3, cDNA 5b-7, and cDNA 8. The deletions and duplications are detected by analyzing the Southern blots for the presence or absence of exon containing genomic restriction fragments that hybridize to the cDNA probe. Limitations of the Southern blot technique include a high labor requirement, the need for radioactive isotopes and for high molecular weight DNA for analysis.

Newer techniques such as multiplex amplifiable probe hybridization (MAPH) and multiplex ligation-dependent probe amplification (MLPA) are able to detect deletions and duplications more effectively and efficiently. MAPH is based on the quantitative recovery of probes, after their hybridization to immobilized DNA (White, Kalf et al. 2002). The advantage of MAPH over Southern blotting and PCR are the relative simplicity, speed, and coverage of all 79 exons. Although PCR is able to detect up to 98% of deletions, it is not always able to determine the exact breakpoints. MLPA is a similar method to MAPH. However, it has the advantage of requiring a lower amount of DNA and can be completed as a one-tube assay (Lalic, Vossen et al. 2005).

Other methods, including single-condition amplification internal primer sequencing (SCAIP) and denaturing gradient gel electrophoresis (DGGE), have been developed with the aim of detecting large lesions in a semiautomatic, rapid, accurate and economical fashion (Flanigan, von Niederhausern et al. 2003; Hofstra, Mulder et al. 2004).

The newest method, comparative genomic hybridization (CGH) array, uses a microchip technology and can detect large deletions and duplications in all 79 exons in affected patients

and carriers. CGH can identify not only deletions and duplications, but also previously unidentified deep intronic mutations. It also allows testing for female carriers (Hegde, Chin et al. 2008).

Other less common methods have also been studied. These include fluorescence *in situ* hybridization (FISH), single strand conformation polymorphism (SSCP), and denaturing high-performance liquid chromatography (Calvano, Memeo et al. 1997; Muscarella, Piemontese et al. 2007). Linkage analysis is also used in cases where no mutations are identified and there is a family history of muscular dystrophy (Hodgson, Walker et al. 1987).

If the routine testing for deletions and duplications yields no detectable deletion or duplication, some centers then proceed to test methods that are able to detect small lesions, such as gene sequencing or chip-based technology.

3.6.2.2 Muscle Biopsy

A muscle biopsy may be performed if the Deletion/Duplication testing is negative. The tests performed on the muscle sample for DMD are immunohistochemistry and immunoblotting. Table 3 summarizes the typical findings for males with BMD and DMD and female carriers (Nicholson, Johnson et al. 1993; Nicholson, Johnson et al. 1993; Nicholson, Johnson et al. 1993; Muntoni, Torelli et al. 2003).

Table 3: Muscle Biopsy Findings in the Dystrophin Protein

	Phenotype	Western Blot		Immunohistochemistry
		Molecular Weight (normal: 427-kb)	Dystrophin Quantity	
Males	DMD	Non detectable	0-5%	Complete absence
	BMD	Normal Abnormal	20-50% 20-100%	Normal appearing or reduced intensity
Females Carriers	DMD random X-inactivation	Normal/ Abnormal	>60%	Mosaic pattern
	DMD skewed X-inactivation	Normal/ Abnormal	<30% on average	Mosaic pattern

3.6.3 Prenatal Testing

Prenatal testing is available for at-risk pregnancies to determine if a fetus is affected with DMD. Fetal cells can be obtained through chorionic villus sampling (CVS) between the 10th and 12th week of pregnancy. During the CVS, a small sample of cells from the placenta is removed by either inserting a thin catheter through the cervix or by inserting a thin needle into the mother's abdomen. Fetal cells may also be obtained through amniocentesis, a procedure performed during or after the 15th week of pregnancy. During this procedure a thin needle is inserted into the amniotic sac to remove a sample of the amniotic fluid that surrounds the baby and contains cells from the baby. Both CVS and amniocentesis are associated with a similar risk of miscarriage. The risk for miscarriage from either procedure is approximately one in every thousand procedures performed. The fetal cells obtained from either procedure are then analyzed by one of the molecular methods described above.

3.6.4 Carrier Testing

There are carrier tests for women who have a family history of DMD whether or not they exhibit symptoms of weakness. Genetic carrier testing is currently the most accurate way to identify carriers. Genetic carrier testing allows for family planning and can provide a risk assessment for other relatives. The method used for genetic carrier testing will depend on whether a mutation is known in the family. If a mutation has been identified in an affected individual, the carrier testing should be targeted to the specific known mutation. If testing indicates that the mother is not a carrier, there is still a risk of having an affected son and having a carrier daughter due to germline mosaicism (Prior and Bridgeman 2005). Muscle biopsy is not typically recommended unless a woman is symptomatic; muscle biopsy can detect a mosaic pattern of muscle fibers that are both positive and negative for dystrophin expression. Except in the setting of skewed X-inactivation, carriers generally have sufficient levels of dystrophin in the muscle for normal function. In some families with no known mutation, linkage analysis may be an option to identify carrier females. This method requires blood samples from multiple family members as the method relies on co-inheritance of the disease gene with DNA variations known to be located very close to or within the disease gene (Prior and Bridgeman 2005).

3.7 MANAGEMENT AND TREATMENT RECOMMENDATIONS

Since there is currently no cure for DMD, the goal of care is to provide the best quality of life through all stages of life. To date, the treatments have been aimed at optimizing strength and

function through the use of pharmacological interventions, physical therapy and assistive and adaptive devices.

3.7.1 Pharmacological Interventions

At present, glucocorticoids are the only pharmacological intervention that is able to slow the decline of strength and function in DMD patients. The two main corticosteroids used in DMD are prednisone and deflazacort. Daily oral administration of prednisone or deflazacort stabilizes or improves strength and prolongs ambulation. Drachman et al. first reported this finding in an open trial of prednisone at a dose of 2mg/kg/day (Drachman, Toyka et al. 1974). The therapeutic benefit of prednisone for the ambulant DMD patient was confirmed in subsequent open design trials (Brooke, Fenichel et al. 1987), and then, through the collaboration of the Clinical Investigation of Duchenne Dystrophy (CIDD) investigators, in double-blind, placebo-controlled trials (Mendell, Moxley et al. 1989; Fenichel, Florence et al. 1991; Griggs, Moxley et al. 1993). Doses of 1.5mg/kg/day and 0.75mg/kg/day were equally effective and the lower dose was associated with fewer side effects. A beneficial effect was detected by strength measures as early as 10 days and peaked at 3 months after initiation of treatment (Griggs, Moxley et al. 1991). The mechanism by which prednisone is beneficial in dystrophin deficiency is likely multifactorial, and is not solely related to the immunosuppressive effects of prednisone since other immunosuppressants have not shown similar benefits (Griggs, Moxley et al. 1993).

Studies using deflazacort have also shown clinical benefits for patients with DMD (Mesa, Dubrovsky et al. 1991). In 2005, the American Academy of Neurology issued a practice parameter regarding corticosteroid treatment in DMD and recommended that corticosteroids should be offered as treatment (Moxley, Ashwal et al. 2005). However, glucocorticoids are

associated with side effects including cushingoid features, adverse behavioral changes, obesity, growth retardation, increased risk for bone fractures, gastritis, delayed puberty, cataracts, hypertension, glucose intolerance, susceptibility to infection, and masking of response to stress (Manzur, Kuntzer et al. 2008).

In addition to the side effects of glucocorticoids on bone health, it has been reported that DMD patients naïve to steroids also have reduced vitamin D levels (Bianchi, Mazzanti et al. 2003). Therefore, many centers around the world are also placing DMD patients on daily supplementation with vitamin D and calcium whether they are placed on corticosteroids or not.

Many other agents have been studied in the *mdx* mouse and in DMD patients. To date, none have shown any significant ability to improve quantitative or manual strength. These include anti-inflammatory drugs, anti-cytokines, nutritional supplements, and angiotensin-converting enzymes (ACE) inhibitors (Strieter, Remick et al. 1988; Folkers and Simonsen 1995; Nguyen, Broussas et al. 1998; Vary, Dardevet et al. 1999; Duboc, Meune et al. 2005; Escolar, Buyse et al. 2005; Buyse, Goemans et al. 2007; Duboc, Meune et al. 2007; Escolar 2008; Tesi-Rocha 2008).

Studies of new therapies that are based on specific genotypes are generating a high level of interest among both researchers and patients. Small molecules that can read through nonsense mutations could potentially impact approximately 10% of DMD patients (Hamed 2006). Exon skipping, which uses short stretches of DNA-like molecules to alter the splicing pattern of the gene, is designed to bring out-of-frame deletions into frame. This new type of treatment could potentially correct more than 85% of mutations in DMD patients (Hoffman 2007; van Deutekom, Janson et al. 2007; Pennisi 2008). The full characterization of DMD patient mutations will be crucial to fully realize these novel therapies as they are developed.

3.7.2 Physical Therapy

The goal of physical therapy is to preserve strength and flexibility. Due to the rapid progression of the disease, some therapies may be carried out with the use of assistive devices (Sussman 2002).

3.7.3 Assistive Devices

Ankle-foot orthoses (AFOs) may help prevent plantar flexion contracture (Sussman 2002). Many European pediatric orthopedic surgeons have encouraged prophylactic tendon release or lengthening to prolong ambulation (Rideau, Duport et al. 1995). Although some studies have shown that tendon release surgery prolongs the period of independent walking (up to 1.2 years), it is hard to determine whether this surgery is the sole reason for continued ambulation as there are many confounding factors (parent motivation, environmental influences, and surgery). Night splinting of the ankles in dorsiflexion has also been advocated; however, no studies have been performed to demonstrate their efficacy (Sussman 2002).

Due to the progression of the disease, patients will eventually require a power wheelchair, which requires an adapted home and van. The transition to full-time wheelchair use is often temporarily correlated with a rapidly worsening spinal deformity. Approximately 90% of patients with DMD will develop scoliosis (Kinali, Messina et al. 2006). The spinal curvature may be severe enough to require surgery and the placement of rods. Pulmonary function will also continue to deteriorate and lead to the use of assistive ventilation devices, which initially includes cough-assist and non-invasive night ventilation using bi-level positive airway pressure.

As the disease progresses, upper and lower extremity function becomes more limited and many patients are no longer able to operate a power wheelchair by using a hand control. To maintain independence, computer voice-activated devices and head controls for wheelchairs may be used (Sussman 2002). Some patients may also require full-time non-invasive or invasive ventilation.

3.8 CINRG

CINRG was formed in 1999 as the clinical research arm of the Duchenne Muscular Dystrophy Research Center (DMDRC) and the Research Center for Genetic Medicine at the Children's National Medical Center (CNMC) in Washington, DC. As of 2009, CINRG comprised 21 sites in 10 countries. Each site has a study site Principal Investigator (PI), clinical coordinator (CC) and clinical evaluator (CE) (Appendix B). The CE at each site is a trained physical therapist or physician who performs the manual muscle, quantitative muscle, respiratory and timed function outcome measures that are integral to muscular dystrophy clinical research. Each center has the same quantitative muscle testing equipment, including computer hardware and software, and is supported by a central gold standard CINRG CE. Central CE training and inter-rater reliability testing is performed initially and re-certified annually.

The impetus to develop the CINRG network was to fill an unmet need for clinical trials that would benefit muscular dystrophy patients. CINRG's vision is that patients, families and parent-led foundations are the true 'stakeholders' of this research and; as such, are central to CINRG's mission. This objective was borne out in the method of recruiting study sites to CINRG. Highly committed physicians leading large muscular dystrophy clinics, self-selected or

identified through interpersonal networks, have demonstrated their commitment to patients with muscular dystrophies and their families.

3.8.1 CINRG Quantitative Measurement System

CINRG developed a standardized muscle strength testing system called the CINRG Quantitative Measurement System (CQMS). CQMS is a modification of the Tufts Quantitative Neuromuscular Testing Equipment designed for ALS clinical trials. This quantitative muscle testing (QMT) technique collects measurements of force using a load cell while performing a maximum voluntary isometric contraction. This set-up measures changes in strength of 0.25 pounds and thereby provides accurate and sensitive force generation measurement in children. In effort dependent tests, it is important to provide motivation to yield the best effort by participants. To increase reliability and sensitivity in children, the QMT system is interfaced with audiovisual feedback. In essence, the audiovisual feedback turns the strength measurement process into a “video game,” leading to increased compliance and effort in DMD children providing optimal reliability. The system also integrates timed function tests, manual muscle testing, and pulmonary function testing. CINRG showed that both total QMT scores and individual QMT assessments were highly reliable (Escolar, Henricson et al. 2001; Mayhew, Florence et al. 2007).

4.0 EXPERIMENTAL DESIGN AND METHODS

The University of Pittsburgh Institutional Review Board approved the study reported in this thesis (Appendix C). De-identified records from baseline visits of past and ongoing CINRG studies were obtained through the CINRG Coordinating Center. The data obtained from CINRG was collected from 20 CINRG centers (Appendix B) between January of 2004 and September of 2008. Two published DMD mutation databases (Aartsma-Rus, Van Deutekom et al. 2006; Tuffery-Giraud, Beroud et al. 2009) and one survey compiled by patient/caregiver data were reviewed (DuchenneConnect 2007). Information regarding DNA testing was abstracted from these three sources and categorized by mutation types described below.

4.1 DATA

4.1.1 CNMC0601 Study Data

The data used from the CNMC0601 study entitled: *A Randomized Study of Daily versus High Dose Weekly Prednisone Therapy in Duchenne Muscular Dystrophy* was collected between January of 2004 and December of 2007. The study was designed to help determine whether a high-dose weekly course of prednisone therapy is safer than and at least as effective as daily dose therapy for individuals with DMD. In order to be eligible for this study all participants were

steroid-naïve and must have received genetic confirmation of DMD by the central CINRG genetic counselor. Seventy-seven participants were screened and sixty-four participants between the ages of 4 and 10 years old were randomized to either a daily regimen of prednisone of 0.75mg/kg/day or weekend dose of prednisone of 10mg/kg/weekend. Data collected at the screening visits was used for fifty-four participants and includes demographic, diagnostic, and strength function data.

4.1.2 CNMC0705 Study Data

The data used from the CNMC0705 study entitled: *A Double-Blinded Randomized Placebo Controlled Study of Daily Pentoxifylline as a Rescue Therapy in Duchenne Muscular Dystrophy* was collected between September of 2005 and December of 2007. The study was designed to help determine if pentoxifylline when added to patients receiving corticosteroids improves or stabilizes strength. In order to be eligible for this study all participants were receiving prednisone, prednisolone, or deflazacort for at least 12 months. All participants were required to have genetic confirmation of diagnosis in order to be randomized into the study. Seventy-three participants were screened and sixty-four participants over the age of 8 years old were randomized to either placebo or daily pentoxifylline. Data collected at the screening visits was used for sixty-six participants and includes demographic, diagnostic, and strength function data.

4.1.3 UCD0305 Study Data

The data used from the UCD0305 study entitled: *Longitudinal Study of the Relationship between Impairment, Activity Limitation, Participation and Quality of Life in Persons with Confirmed*

Duchenne Muscular Dystrophy is taken from the screening visit only from participants that were enrolled between February of 2006 and September of 2008. In order to be eligible for this study all participants were required to have a genetic confirmation of DMD by the central CINRG genetic counselor. The study is a 5-year observational study that is projected to be completed in January of 2014. Data collected at the screening visits was used for two hundred and fifty-four participants and includes demographic, diagnostic, and strength function data.

4.1.4 CINRG General Guidelines for Inclusion and Exclusion

4.1.4.1 Genetic Confirmation

In all CINRG studies at least one of the following criteria must be met in order to be enrolled or randomized:

- A muscle biopsy with dystrophin immunofluorescence and/or immunoblot showing complete dystrophin deficiency, and clinical picture consistent with typical DMD
- Gene deletion test positive in the central rod domain (exons 25 through 60) of the dystrophin gene, where the reading-frame can be predicted as ‘out-of-frame’, and clinical picture consistent with typical DMD
- Complete dystrophin gene sequencing showing an alteration (point mutation, duplication, or other mutation resulting in a stop codon mutation) that can be definitely associated with DMD, and clinical picture consistent with typical DMD
- Positive family history of DMD confirmed by one of the criteria listed above in a sibling or maternal uncle, and clinical picture consistent with typical DMD.

4.1.4.2 Cognitive Aptitude

The CINRG trials that use muscle strength assessments as primary endpoints exclude participants unable to follow simple commands. Participants must be able to reproduce strength test values obtained over two consecutive days that lie within 15% of each other. Both the CNMC0601 and CNMC0705 studies included quantitative muscle strength assessments as primary outcomes. Although the UCD0305 study is a natural history study that is intended to enroll any participants with DMD, it may not capture the full spectrum of patients with DMD. CINRG studies do not include any IQ testing.

4.2 METHODS

4.2.1 Review of Charts from CINRG Studies

All de-identified data was reviewed and entered into an Excel spreadsheet. The demographic information includes: participant five-digit identifier, participant month and year of birth, and participant race and ethnicity. The diagnostic data includes the study case report form as well as a de-identified copy of the actual diagnostic report from the laboratory that performed the testing. The strength function data includes information on steroid use (1= Yes, the participant has or is using steroids and 0= No, the participant has never used steroids), ambulatory status (1= Walking or 0= Wheelchair use for greater than 50% of the day), and results obtained from the screening CQMS assessment (age at test, time to walk or run 10 meters, time to climb 4 standard stairs, time to stand from the lying position, quantitative strength in pounds for leg, arm, and grip

muscles as well as the average quantitative strength, and functional evaluation of upper and lower body strength).

The CINRG diagnostic data was sorted according to the mutation categories defined below. These categories have been adapted from the ones reported from the literature described in Section 3.3. The DNA and muscle biopsy testing was coded as ‘1’ for those who had either DNA, muscle biopsy testing or both and ‘0’ for those who had neither DNA nor muscle biopsy testing. For those who had DNA testing, the data was further subdivided into those who had large lesions versus those who had small lesions. Individuals with small lesions were further subdivided into those who had small deletions and those who had point mutations.

4.2.2 Review of the Published Literature

Two large DMD mutation databases were reviewed. The Leiden database was created in July of 1997 as an open-access database in which all mutations that have been reported in the literature are stored (Fokkema, den Dunnen et al. 2005). The Leiden database homepage www.dmd.nl states that the pages are intended to be used by ‘scientist performing research and/or diagnosis in Duchenne and Duchenne-like muscular dystrophies.’ By 2006, more than 4,700 mutations had been reported in their databases (Aartsma-Rus, Van Deutekom et al. 2006).

The Leiden database is divided into two databases: large lesions that affect one exon or more and small lesions that affect less than one exon. In order to determine the number of large lesions, an advanced search of the large lesions repository was performed for deletions and duplications. To ascertain the number of small lesions, multiple searches were performed for each subcategory: substitutions, insertions, deletions, and duplications. To differentiate between the nonsense and missense point mutations, separate searches, which required the addition of

information on the protein change description, were performed. In addition, the publications based on the Leiden database were reviewed and data was abstracted into a table format (Aartsma-Rus, Van Deutekom et al. 2006).

The second database used in this project is the Universal Mutation Database-Duchenne Muscular Dystrophy (UMD-DMD), which was developed as a joint national effort through the network of diagnostic laboratories to provide up-to-date information about mutations of the DMD gene identified in patients with dystrophinopathies in France. The UMD-DMD database homepage www.umd.be/DMD states that the database has a goal of ‘making the information readily accessible to anyone (geneticists, clinicians or researchers) interested in the genetic variations of the DMD gene, the clinics of dystrophinopathies, or the development of new therapeutic approaches.’ The data presented within the UMD-DMD website was abstracted into a table format.

On an exploratory basis, data was obtained from Parent Project Muscular Dystrophy DuchenneConnect www.duchenneconnect.org, which was ‘created to serve as a central hub linking the resources and needs of the Duchenne/Becker muscular dystrophy community: young men with Duchenne; their families and caregivers; and the provider community: clinical care providers, policymakers, industry professionals and the medical research fields.’ The DuchenneConnect data was abstracted from surveys completed by parent and/or caregiver data available through a password protected provider portal.

4.2.3 Data Analysis

For specific Aim 1, the data was sorted according to CINRG study participants’ diagnostic testing method as follows: DNA testing alone, muscle biopsy testing alone, DNA testing and

muscle biopsy testing, or family history information with no diagnostic test. DNA testing information was further divided into the following groups:

- Large Lesions (greater than one exon)
 - Large deletions
 - Large duplications
- Small Lesions (less than one exon)
 - Small mutation (greater than one base pair and less than one exon)
 - Point mutation
 - Missense
 - Nonsense
- No Detected Mutation

For specific Aim 2, the parent surveys and the mutation information reported by the two large DMD databases were sorted according to the above groups. Chi-square analysis was used to compare the CINRG mutation proportions to the two large databases and parent surveys. Chi-square tests were performed by hand and verified with an online calculator from the University of Kansas (Preacher 2008). A p-value of 0.05 or higher was considered significant for these analyses.

For specific Aim 3, the CINRG data was divided according to age into two cohorts. The first cohort includes CINRG participants ages 7 through 9 when they completed the strength assessment and the second cohort includes CINRG participants ages 10 through 20. The first cohort, labeled ambulant, represents a cohort in which all CINRG participants were ambulant and the second cohort, labeled mixed, includes a mixed population of ambulant and non-ambulant participants.

Three analyses were performed for the ambulant cohort. Two separate ANOVA tests were performed using the SPSS® 17.0 statistical software package to look for significant differences between time to walk 10 meters and the use of corticosteroids or type of mutation. A linear regression was performed using the SPSS® 17.0 statistical software package. The time to walk 10 meters was set as the dependent variable and the use of corticosteroids and mutation types served as independent variables for this analysis. A p-value of 0.05 or lower was considered significant for these analyses.

One analysis was performed for the mixed cohort. A binary logistic regression was performed using the SPSS® 17.0 statistical software package. The ambulation status was set as the dependent variable and the use of corticosteroids, mutation types, and age served as covariates. A p-value of 0.05 or lower was also considered significant for this analysis.

5.0 RESULTS

5.1 CHARACTERIZATION OF CINRG DATA

5.1.1 Demographic Analysis

The CINRG data is summarized in Table 4 by site. A total of 374 participants from 20 CINRG sites were included in the Demographic Analysis. The average age of the participants was 11.6 years with a median age of 9.8 years. Two hundred and forty-seven participants (66%) reported the ability to walk at the initial muscle testing visit. The age at the loss of ambulation was not available at the time of data request. Two hundred and thirty-nine participants (63.9%) reported the use of corticosteroids either at the initial muscle testing visit or at some point prior. Two hundred and ninety-four participants (78.6%) had DNA testing. For 161 (54.8%) of these, DNA testing was the only method used to confirm diagnosis. Muscle biopsy data was available for 204 participants (54.5%). For 71 (34.8%) of these, muscle biopsy was the only method used to confirm diagnosis.

Participants who did not undergo DNA or muscle biopsy were included in the three CINRG studies on the basis of confirmation testing performed on siblings. A total of 9 participants fell into this category. Due to the lack of direct patient DNA confirmation testing, these participants were not included in the mutation data analysis or genotype-phenotype cohort

analyses. All 294 participants who underwent DNA testing were included in the mutation analysis (Figure 4).

Table 4: Demographic Analysis of CINRG Data

The demographic information (average age, ambulation status, steroid use, and diagnostic methodology) for all 374 CINRG study participants is presented by CINRG site

Site	Number of Participants	Average Age	Number of Participants:							
			Ambulant	Steroid Users	Biopsy Testing	DNA Testing	DNA and Biopsy Testing	Large Lesion	Small Lesion	No Detected Mutation
Buenos Aires	22	14.2	16	18	19	4	3	3	0	1
Calgary	29	11	20	16	14	25	11	17	0	8
Chennai	31	9.2	18	26	10	31	10	26	0	5
Edmonton	10	10.2	9	9	3	8	1	5	2	1
Göteborg	19	12.2	10	14	18	19	18	12	5	2
Houston	5	14.7	2	1	2	4	1	4	0	0
Jerusalem	19	9.5	15	12	17	6	4	3	2	1
Melbourne	24	6.3	24	9	7	19	2	16	2	1
Memphis	14	12.3	9	7	9	13	8	10	0	3
Milan	16	10.1	15	14	16	16	16	11	1	4
Minneapolis	1	8.9	1	1	0	1	0	1	0	0
Pittsburgh	16	14.6	7	13	10	13	7	12	0	1
Richmond	7	12.3	4	2	4	3	0	3	0	0
Rochester	14	10.1	7	7	5	10	2	9	1	0
Sacramento	49	12.3	25	18	24	43	20	32	7	4
St. Louis	16	14.5	8	13	10	14	9	10	3	1
San Juan	5	16.7	0	0	2	5	2	3	0	2
Sydney	10	8.6	8	9	3	9	2	9	0	0
Toronto	28	15.2	16	23	8	24	6	22	0	2
Washington, D.C.	39	8.9	33	27	23	27	11	19	6	2
TOTAL	374	11.6	247	239	204	294	133	226	30	38

The majority of CINRG study participants (87%) that had DNA testing were found to have a mutation that could be classified as either small or large. Figure 4 illustrates the proportion of all DNA test results by category, including those who were not found to have an identifiable mutation, for the 294 CINRG study participants. Most CINRG study participants were found to have a large deletion (72%). The next most common category comprised those participants who had muscle biopsy to confirm the diagnosis of DMD, but did not have an identifiable mutation by genetic testing and therefore no mutation was reported (13%). The final categories were those with point mutations (8%), large duplications (5%), and small deletions (2%).

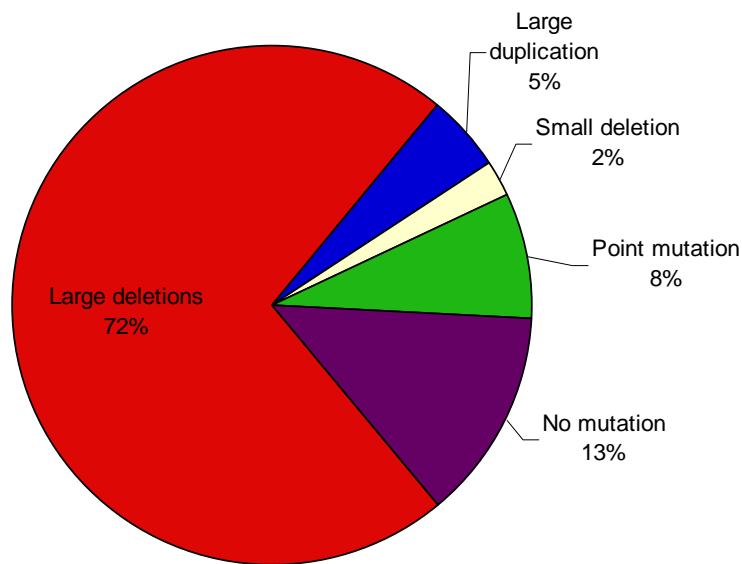


Figure 4: Representation of all CINRG Study Participants' DNA Test Results
Pie chart representation of the types of dystrophin gene mutations carried by the 294 CINRG study participants that had DNA test results.

The cohort of CINRG study participants limited to those found to have an identifiable mutation were subdivided into mutation type as shown in Figure 5. CINRG study participants found to have point mutations were further divided into nonsense and missense mutations,

illustrated in the smaller pie chart of Figure 5. Of the CINRG study participants with point mutation, the majority were found to have nonsense mutations (70%). The analysis to generate Figure 5 was performed to permit comparison with the data reported by the Leiden and UMD-DMD databases; their databases only include patients with known mutations.

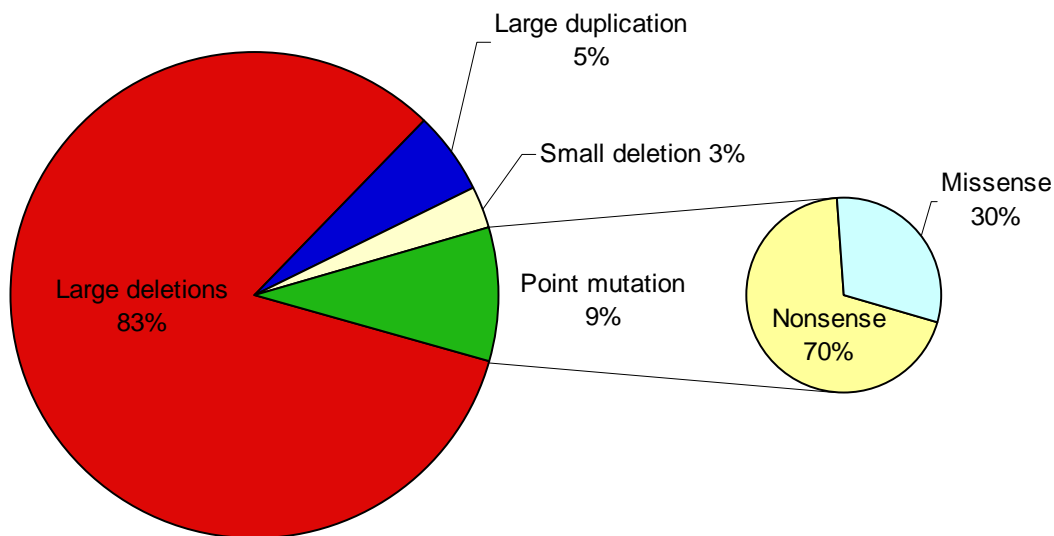


Figure 5: CINRG Study Participants with Identified Mutations
Pie chart representation of the types of dystrophin gene mutations carried by the 256 CINRG study participants that had an identifiable mutation by DNA testing. The subdivision of point mutations is represented in the smaller pie chart.

5.1.2 Large lesions

In the analysis of CINRG participants with large lesions, the specific mutations were further divided into large duplications and large deletions (Figures 6 – 8). Large duplications (Figure 6) and deletions (Figures 7 and 8) are displayed according to their position (shaded area) and their frequency (number in the first column). A total of 14 CINRG study participants have a duplication of one or more exons. The most common duplicated area is exon 2, with a total of 3 participants (21.4% of CINRG study participants with duplications). The largest duplication reported spans 18 exons (exon 8 through 25). All duplications are out-of-frame mutations.

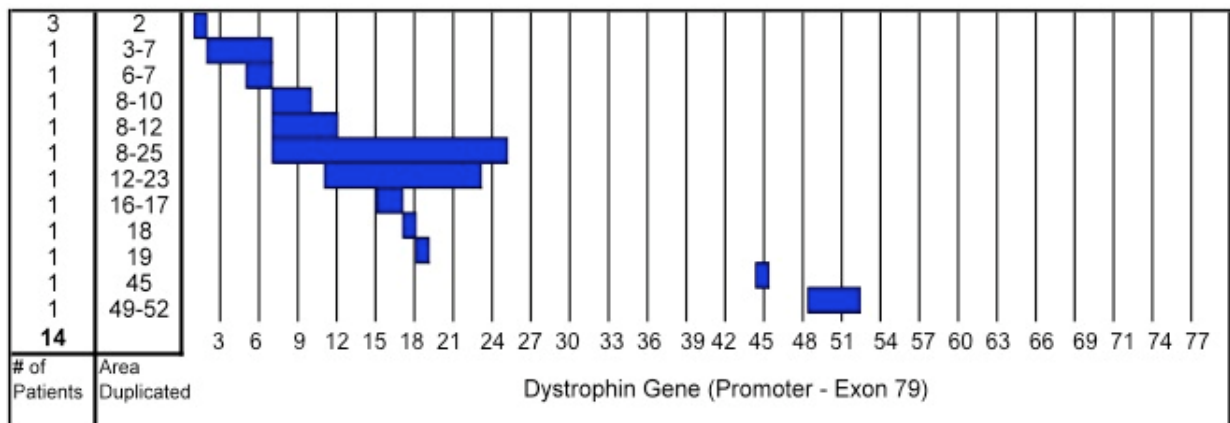


Figure 6: Schematic of CINRG Study Participants with Dystrophin Gene Duplications
 The figure illustrates the number of CINRG study participants with large duplications between the promoter and exon 79. The blue area indicates the region of exons that are duplicated. For example, three CINRG study participants were reported to have a duplication of exon 2.

The representation of CINRG study participants with large deletions are displayed in two figures according to the position of the deletion. The first figure (Figure 7) represents the deletions that occur in the proximal region of the gene and the second figure (Figure 8) represents the deletions that begin in the distal region of the gene. A total of 92 different deletions (43.4%) were reported in the CINRG deletion dataset. The majority of mutations cluster in the two reported hotspots. The proximal hotspot, which includes the region of exon 2 through 20, represents 17.9% of the deletions. The distal hotspot, which includes the region of exon 45 through 55, represents 68.4% of CINRG study participants with large deletions.

Large deletions that include exons from both hotspots were identified in 9 participants (4.2%). The deletions affecting the regions outside of these two hotspots are not as common. Eight participants (3.7%) have deletions lying in exons 21 through 43 and 3 participants (1.4%) have deletions starting after exon 55.

Forty-eight participants (22.2%) have deletions beginning between the promoter and exon 38 (Figure 7). The most common deletion in this region is a deletion of exons 3 through 7,

which was reported in 5 participants. In this proximal region 12 CINRG study participants have in-frame, large deletions.

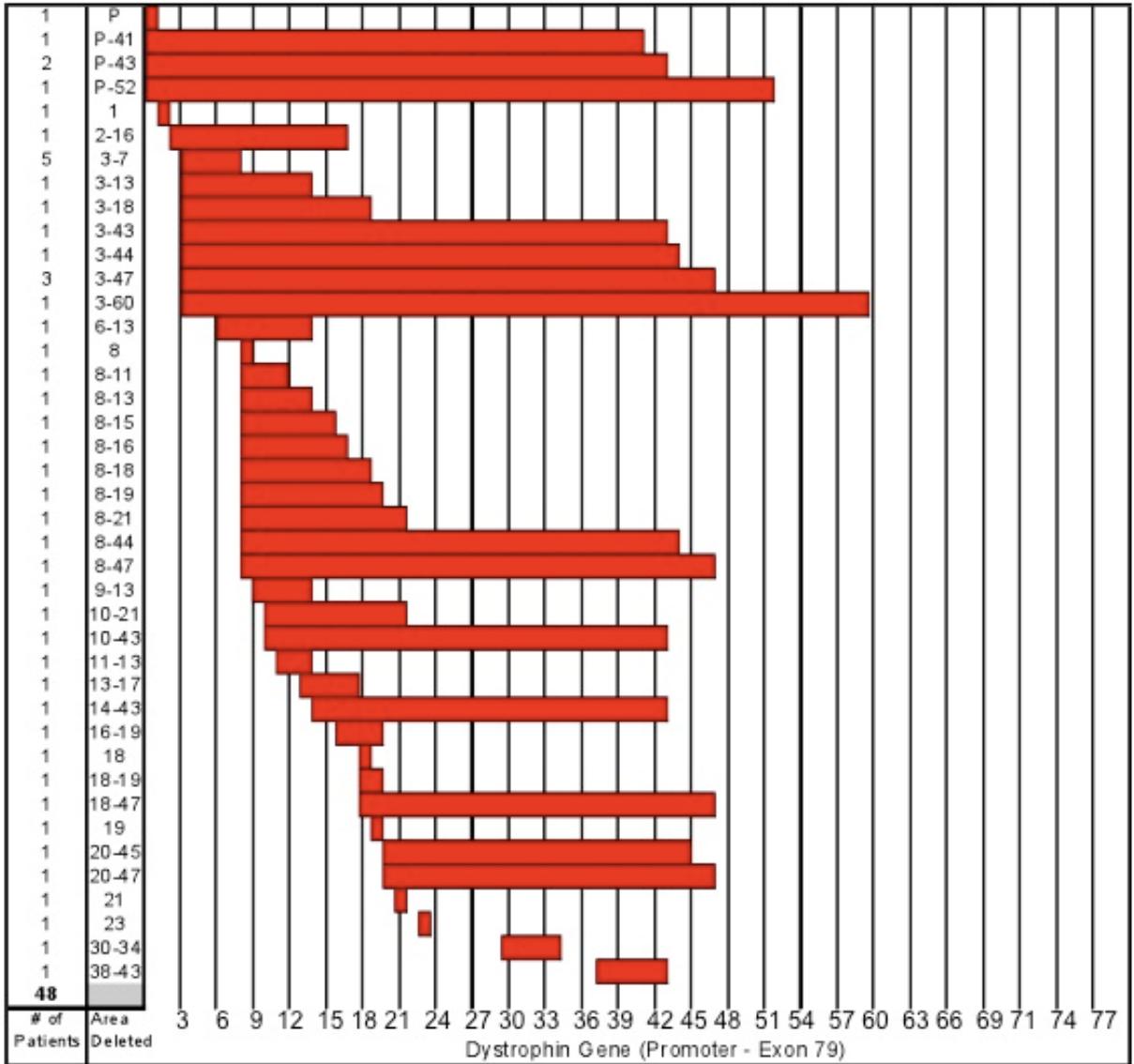


Figure 7: Schematic of CINRG Study Participants with Dystrophin Gene Deletions Starting in the Proximal Region of the Gene

The figure illustrates the number of CINRG study participants with large deletions that start in the proximal region of the dystrophin gene. The red area indicates the region of exons that are deleted. For example, five CINRG study participants were reported to have a deletion of exons 3 through 7.

Figure 8 displays the large deletions occurring in the distal region of the gene. One hundred and sixty-four participants (77.8%) have deletions beginning at exon 43. The most common large deletion is of exon 45, accounting for 7% of all large deletions. The second most common large deletion (6.6%) identified spans exons 48 through 50. In this distal region 12 CINRG study participants have in-frame, large deletions, bringing the total percentage of in-frame large deletions to 11.3%. In the CINRG dataset the reading-frame rules can be applied in 88.7% of cases.

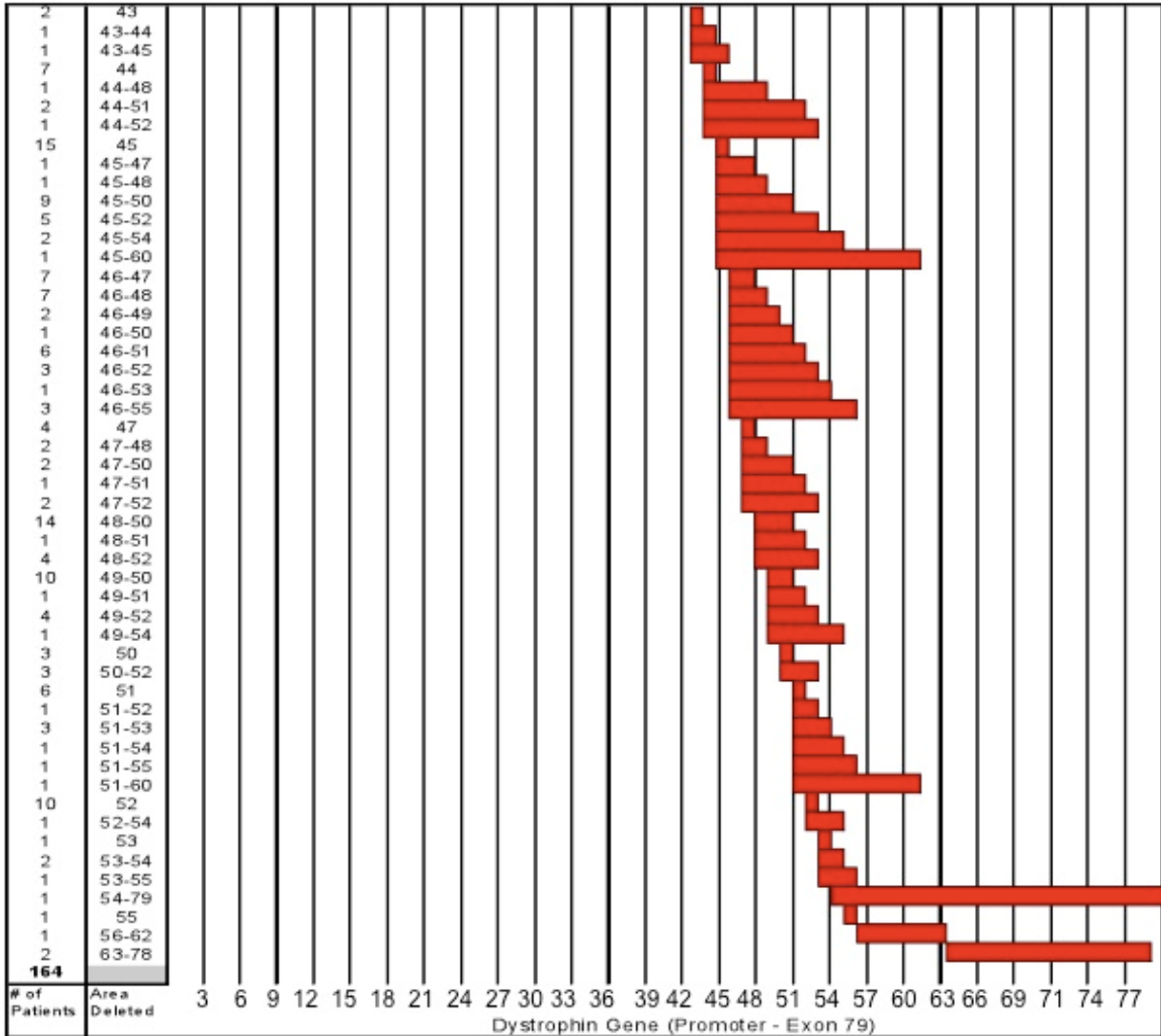


Figure 8: Schematic of CINRG Study Participants with Dystrophin Gene Deletions Starting in the Distal Region of the Gene

The figure illustrates the number of CINRG study participants with large deletions that start in the distal region of the dystrophin gene. The red area indicates the region of exons that are deleted. For example, ten CINRG study participants were reported to have a deletion of exon 52.

5.1.3 Small Lesions

Small lesions were identified in 30 CINRG participants who had DNA testing. The small lesions were further divided into small deletions and point mutations. In participants with small lesions (11.7% of the participants with identified mutations), nonsense mutations are the most common mutation. They represent 53.3% of participants with small lesions and 6.25% of all participants with an identified mutation. Frameshift and missense mutations each account for 23.3% of the small lesions.

Six different small deletions leading to a frameshift were identified. The small deletions involved 2 to 11 base pairs within 6 different exons. There is no apparent pattern within these small deletions. One small deletion was reported in two siblings. The six different mutations lie within the following exons: 6, 18, 36, 40, 41, and 68. Both the Leiden and UMD-DMD databases were searched to see if these six different small deletions were reported by either or both databases. Neither database reported all of the CINRG small lesions. In the CINRG database, one small deletion found in exon 4 at nucleotide position 412 was reported in the UMD-DMD database and not in the Leiden database. Three other small mutations in the CINRG database were found in the Leiden database. The Leiden database also provides information on the origin of the reported sequence variant and all three sequence-variants were reported in the USA, as were the ones in our dataset. Therefore, these could be the same reported sequence variants as the Leiden database is open to the public for reporting.

Seven different missense mutations were identified. Although a possible hotspot for missense mutations has been reported (Tuffery-Giraud, Beroud et al. 2009), the CINRG dataset does not suggest a missense mutation hotspot. The missense mutations are reported within exons: 4, 5, 14, 15, 17, 54, and 56. None of the missense mutations in our dataset are reported in the

UMD-DMD database. Two mutations, c.8390 G>C and c.2060 C>T, are also present in the Leiden database. The c.8390 is likely to be the same mutation as they are both reported from the USA. The other mutation, however, is reported in the USA for our dataset but it is from an Indian patient in the Leiden database.

Nonsense mutations, which account for the majority of the small lesions in the CINRG dataset, represent 53.3% of all identified small lesions. All 16 identified nonsense mutations are unique and were identified in exons: 13, 14, 20, 21, 29, 30, 31, 33, 35, 45, 54, 55, 56, 59, and 69. Two nonsense mutations were identified within exon 20 but were located at different nucleotides within exon 20. One mutation, c.2611 A>T, was reported in both the Leiden and the UMD-DMD databases. Neither database had information on the country of origin for this particular mutation. Five other mutations were found in the Leiden database. Based on the country of origin, it is likely that one of them is common to our dataset, c.1594C>T. The other four mutations are from different countries of origin. The C to T substitution is the most common reported event, occurring in 50% of the nonsense mutations.

The CINRG database does not include any participants with splice site mutations or mid-intronic lesions.

5.2 PUBLISHED LITERATURE MUTATION ANALYSIS

The Leiden database information is summarized in Table 5. Information on a total of 4,939 mutations was abstracted from the two Leiden databases for large and small lesions. The majority of mutations are large lesions (72.8%) and small lesions represent 25.9% of their

reported mutations. Information on splice site mutations was not readily available; and therefore, is not reported in Table 5.

Table 5: Leiden Database Mutation Summary

Total	4939	100%
Large lesions	3594	72.8%
Large deletions (> 1 exon)	2992	60.6%
Large duplication (>1 exon)	602	12.2%
Small lesions	1277	25.9%
Small deletions (<1 exon)	168	3.4%
Small insertions (<1 exon)	35	0.7%
Splice sites (<10 bp)	Not reported	Not reported
Point Mutation	1074	21.7%
Nonsense	955	19.3%
Missense	119	2.4%
Mid-intronic lesions	68	1.4%

The UMD-DMD database information is summarized in Table 6. This table was directly taken from their database website and includes a total of 2,411 mutations (Leturcq and Tuffery-Giraud 2009). The majority of mutations are large lesions (78.3%) and small lesions represent 21.0% of their reported mutations.

Table 6: UMD-DMD Database Mutation Summary

Total	2411	100%
Large lesions	1887	78.3%
Large deletions (> 1 exon)	1638	67.9%
Large duplication (>1 exon)	249	10.3%
Small lesions	507	21.0%
Small deletions (<1 exon)	118	4.9%
Small insertions (<1 exon)	44	1.8%
Splice sites (<10 bp)	132	5.5%
Point Mutation	213	8.8%
Nonsense	204	8.5%
Missense	9	0.4%
Mid-intronic lesions	17	0.7%

The DuchenneConnect data taken from the providers search performed on February 18, 2009 is summarized in Table 7. Information on a total of 617 mutations was abstracted from the search profile. The majority of mutations are large lesions (81.4%) and small lesions represent 18.6% of their reported mutations. Information on splice site lesions and mid-intronic lesions was not readily available and therefore is not reported in Table 7.

Table 7: DuchenneConnect Survey Mutation Summary

Total	617	100%
Large lesions	502	81.4
Large deletions (> 1 exon)	429	69.5
Large duplication (>1 exon)	73	11.8
Small lesions	115	18.6
Small deletions (<1 exon)	19	3.1
Small insertions (<1 exon)	11	1.8
Splice sites (<10 bp)	Not reported	Not reported
Point Mutation	85	13.8
Nonsense	80	13.0
Missense	5	0.8
Mid-intronic lesions	Not reported	Not reported

5.3 COMPARISON OF MUTATION DATA WITH THE CINRG DATASET

The comparison analyses for the CINRG dataset with the two published databases are presented in Tables 8 and 9. The comparison analysis with the parent report is presented in Table 10. A summary bar graph, which includes the data from CINRG, Leiden, UMD-DMD, and DuchenneConnect, is presented in Figure 9.

Although the Leiden database mutation summary (Table 5) includes numbers for mid-intronic lesions, they were excluded for the purpose of the comparison analysis (Table 8) as the CINRG dataset does not contain any study participants with mid-intronic lesions. The

contribution of mid-intronic lesions in the Leiden database represented a small proportion (1.4%) of the entire Leiden mutation data.

Table 8: Comparison of the CINRG Mutation Categories to the Leiden Database

	Large lesions	Small lesions	Total
CINRG	226 (88.3%)	30 (11.7%)	256
Leiden	3594 (76.9%)	1074 (23.1%)	4668
Total	3820	1104	4924

A chi-square analysis was carried out to assess the significance of differences between the frequency of the mutation categories in the CINRG dataset and the ones reported in the Leiden database. Results indicated that the proportion of CINRG study participants with large and small lesions was statistically different from those reported in the Leiden database. The chi-square value is 17.78 with 1 degree of freedom and a p-value of 0.00002. Therefore the null hypothesis is rejected and these two databases, the CINRG and Leiden, are statistically different with regards to large and small mutations.

For the comparison of reported mutations between the CINRG dataset and the UMD-DMD database (Table 9), mid-intronic lesions reported by the UMD-DMD database were excluded as the CINRG dataset does not contain any study participants with mid-intronic lesions. The contribution of mid-intronic lesions in the UMD-DMD database represented a very small proportion (0.7%) of the entire UMD-DMD mutation.

Table 9: Comparison of CINRG Mutation Categories to the UMD-DMD Database

	Large lesions	Small lesions	Total
CINRG	226 (88.3%)	30 (11.7%)	256
UMD-DMD	1887 (78.8%)	507 (21.2%)	2394
Total	2213	537	2650

A chi-square analysis was completed to assess the significance of difference between the frequency of the mutation categories in the CINRG dataset and the ones reported in the UMD-DMD database. Results indicated that the proportion of CINRG study participants with large and small lesions was statistically different from those reported in the UMD-DMD database. The chi-square value is 12.80 with 1 degree of freedom and a p-value of 0.00034. Therefore the null hypothesis is rejected and these two databases, the CINRG and UMD-DMD, are statistically different with regards to large and small mutations.

The comparison of mutations between the CINRG dataset and DuchenneConnect is presented in Table 10.

Table 10: Comparison of CINRG Mutation Categories to DuchenneConnect

	Large lesions	Small lesions	Total
CINRG	226 (88.3%)	30 (11.7%)	256
DuchenneConnect	502 (81.4%)	115 (18.6%)	617
Total	728	145	873

A chi-square analysis was completed to assess the significance of difference between the frequency of the mutation categories in the CINRG dataset and the ones reported in the DuchenneConnect registry. Results indicated that the proportion of CINRG study participants with large and small lesions was statistically different from those reported in the DuchenneConnect registry. The chi-square value is 6.25 with 1 degree of freedom and a p-value of 0.0124. Therefore the null hypothesis is rejected and these two databases, the CINRG and DuchenneConnect, are statistically different with regards to large and small mutations.

A graphical representation of the percentage of large and small mutations is presented in Figure 9. The difference between the CINRG database and the other databases, Leiden, UMD-DMD and DuchenneConnect is statistically significant. The difference is not very large, with a difference of 11.4% with the Leiden database, 9.5% with UMD-DMD, and 6.9% with DuchenneConnect.

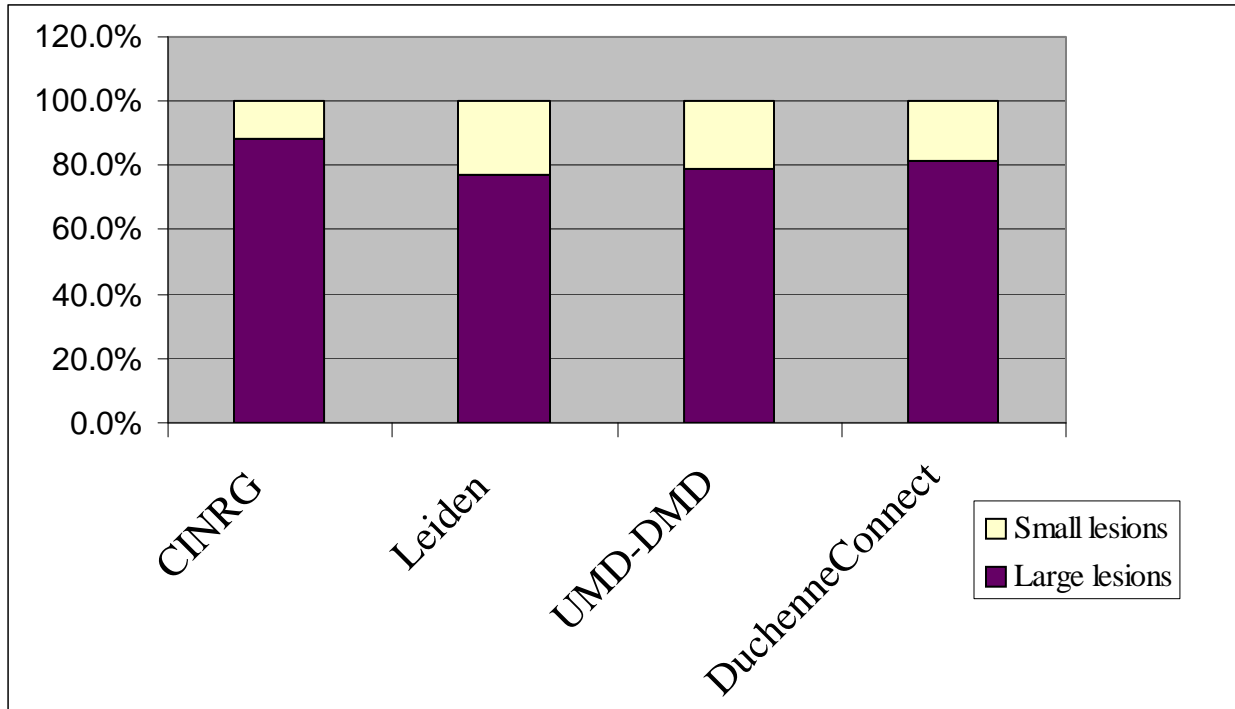


Figure 9: Comparison of the Percentages of Large and Small Lesions in all Four Datasets
 The percentage of large lesions are represented in the dark purple colored bars and the percentage of small lesions are represented in the cream colored bars. All four datasets: CINRG, Leiden, UMD-DMD, and DuchenneConnect are included in the bar graph representation with the datasets in the x-axis and percentages in the y-axis.

5.4 GENOTYPE-PHENOTYPE ANALYSIS

For the genotype-phenotype analysis, the CINRG data was divided into two particular cohorts.

5.4.1 Ambulant Cohort Analysis

The ambulant cohort included 71 participants taken from all three CINRG studies. The demographic analysis of this cohort is summarized in Table 11. The average age of the cohort is 8.4 years. The majority of CINRG study participants in this cohort had large lesions (87.3%). The average time to walk 10 meters for all CINRG study participants in this cohort is 7 seconds. The average quantitative muscle testing (QMT) total score, which is based on the average of the QMT arm, leg and grip measurements, is 10.9 pounds of force for this cohort. Most CINRG study participants (90.1%) in this cohort are currently taking or have used corticosteroids.

Table 11: CINRG Ambulant Cohort Genotype-Phenotype

Genotype	Total number of participants	Average age of participants	Phenotype		
			Number of steroid using participants	Average Time to walk 10 meters in seconds	Average QMT total score
Large lesions	62	8.4	56	6.8	11.0
Deletions	60	8.4	54	6.9	10.9
- Out-of-frame	53	8.3	49	6.7	11.0
- In-frame	7	9.0	5	9.0	10.5
Duplications (all out-of-frame)	2	8.8	2	4.0	2.2
Small lesions	9	8.2	8	8.1	9.8
Small deletions	2	8.4	2	8.6	6.8
Point mutations	7	8.1	6	7.9	10.7
Missense	2	8.2	1	9.9	10.0
Nonsense	5	8.1	5	7.2	11
TOTAL	71	8.4	64	7.0	10.9

Two ANOVA tests were performed for this ambulant cohort. The first test was performed to look for significant differences between the time to walk 10 meters and the mutation type (large versus small lesions). The p-value for this test was not found to be statistically significant ($p = 0.435$, mean time for participants with large lesions: 6.8 seconds and mean time for participants with small lesions: 8.1 seconds). The second ANOVA test performed to look for significant differences between the time to walk 10 meters and corticosteroid use was also not found to be statistically significant ($p = 0.671$, mean time for participants using corticosteroid: 6.9 seconds and mean time for participants never exposed to corticosteroids: 7.6 seconds).

The linear regression, which used the time to walk 10 meters as the dependent variable and the use of corticosteroids and mutation type as independent variables, confirmed the results from the two ANOVA tests. The mutation type did not have an effect on the time to walk 10 meters in this cohort ($p = 0.441$ and coefficient value $B = -1.209$). The use of corticosteroids did not have an effect on the time to walk 10 meters ($p = 0.681$ and coefficient value $B = -0.719$).

5.4.2 Mixed Cohort Analysis

The mixed cohort included 100 participants taken from all three CINRG studies. The demographic analysis of this cohort is summarized in Table 12. The average age of the cohort was 14.2 years. The majority of CINRG study participants in this cohort had large lesions (88.0%). Most CINRG study participants in this cohort were non-ambulatory (62%). The average quantitative muscle testing (QMT) total score, which was based on the average of the QMT arm, leg, and grip measurements, was 8.9 pounds of force. Most CINRG study participants (64.0%) in this cohort were using or had used corticosteroids.

Table 12: CINRG Mixed Cohort Genotype-Phenotype

Genotype	Total number of participants	Average age of participants	Phenotype		
			Number of steroid using participants	Number of ambulant participants	Average QMT total score
Large lesions	88	14.1	57	32	8.7
Deletions	84	14.2	55	32	8.9
- Out-of-frame	71	14.0	46	29	9.4
- In-frame	13	15.2	9	3	6.4
Duplications (all out-of-frame)	4	13.8	2	0	5.3
Small lesions	12	14.8	7	6	9.9
Small deletions	3	16.8	2	1	13.3
Point mutations	9	14.2	5	5	8.7
Missense	1	14.5	5	0	9.0
Nonsense	8	14.1	0	5	8.7
TOTAL	100	14.2	64	38	8.9

The results of the binary logistic regression, which set the ambulation status as the dependent variable and the use of corticosteroids, mutation type, and age at testing as covariates, indicated that the use of corticosteroids and the age of the CINRG study participants had a statistically significant effect on the ambulation status. The p-values for corticosteroid use and age were 0.002 and <0.001, respectively. The mutation type did not have a statistically significant effect on the ambulation status for this cohort ($p = 0.120$).

6.0 DISCUSSION

6.1 CHARACTERIZATION OF CINRG MUTATIONS

The goal of the first aim was to characterize the dystrophin gene mutations of CINRG study participants from one observational and two treatment studies. The majority of CINRG study participants (87%) underwent DNA testing for diagnosis confirmation (Result Section 5.1, Table 4). The data obtained from the analysis of the gene mutations, observed in the CINRG studies, illustrates the heterogeneity of the disease-causing mutations in the dystrophin gene. Among CINRG study participants with large lesions, 92 unique deletions and 12 unique duplications were identified (Result Section 5.1, Figures 6-8). For small lesions, 29 different mutations were identified, of which only 11 were previously reported in the Leiden and UMD-DMD databases (Aartsma-Rus, Van Deutekom et al. 2006; Tuffery-Giraud, Beroud et al. 2009).

Two dystrophin gene mutation hotspots have been well characterized in the literature. The proximal hotspot spans from exons 2 through 22 and the distal hotspot spans from exons 45 through 55 (Beggs, Koenig et al. 1990; Oudet, Hanauer et al. 1992; Nobile, Galvagni et al. 1995; Sironi, Pozzoli et al. 2003; Gualandi, Rimessi et al. 2006). The majority of the CINRG mutations, representing 86.3%, fell into the two dystrophin gene mutation hotspots.

There is currently no known relationship between the size of the deletion and the clinical phenotype. The effects of the phenotype depend on whether or not the reading-frame is disrupted

(Muntoni, Torelli et al. 2003). As discussed in Section 3.4, in DMD patients, a disruption of the open reading-frame causes a complete absence of the dystrophin protein, whereas BMD patients carry an in-frame mutation that allows the stable expression of dystrophin with a smaller molecular size that may be expressed in lower quantities than normal. This reading-frame hypothesis holds true in approximately 90% of DMD patients (Monaco, Bertelson et al. 1988; Koenig, Beggs et al. 1989; Muntoni, Torelli et al. 2003). In the CINRG dataset, the reading-frame hypothesis is similar with 88.7% to that reported in the literature.

The demographic data, analyzed while characterizing the mutations reported by CINRG, demonstrated that the method of DNA confirmation used varied between CINRG centers. As was discussed in Section 3.6.2, the most common diagnostic method used worldwide is Deletion/Duplication analysis. By far, this was also the most common method of diagnostic testing performed in the different CINRG sites around the world, but the specific technique with which it was performed varied between the different CINRG sites. Some centers still perform deletion screening by using the original Chamberlain protocol with PCR primers for 9 exons (Chamberlain, Gibbs et al. 1988). It is therefore possible that some of the 38 CINRG study participants with no detectable mutation may indeed have some form of lesion, (likely small) that was not detected by the Deletion/Duplication analysis. The Deletion/Duplication analysis detects 98% of large deletions and does not detect small lesions (Beggs, Koenig et al. 1990).

The review of the CINRG demographic data by site suggests that there are differences in the way the diagnosis of DMD was confirmed. For instance, DNA and muscle biopsy data was obtained for all 16 participants in the site in Milan, Italy; while the site in Rochester, MN, with a similar sample size and population age, had only 2 of their participants with diagnostic information on both DNA and muscle biopsy. The majority of CINRG participants from the

Rochester, MN site had diagnostic confirmation of DMD done by DNA testing alone. Review of the CINRG data by study site highlights the variability in the methods used to confirm the diagnosis of DMD. The means used for confirmation probably varies based on the individual site's access to rapid and reliable testing. There are currently no published studies that examine the clinical utility of the different testing strategies used to confirm diagnoses in affected DMD and BMD patients (Taylor, Maroulis et al. 2007). Although CINRG has not established guidelines on the ideal way to confirm the diagnosis of DMD within its network, such an organization is well suited to carry out the studies required to determine the most clinically efficient and scientifically productive means of diagnostic confirmation.

The variation in the methods used to confirm the diagnosis of DMD is a limitation in the attempt to characterize the mutations reported by CINRG. In this characterization of mutations, CINRG study participants whose affliction with DMD was confirmed solely by muscle biopsy were excluded from the analysis (71 CINRG participants). Since they had no genetic testing, it is impossible to determine what type of mutation they might have. Unfortunately, this represents nearly 20% of the entire CINRG dataset used in this project. It is likely that if these CINRG study participants had also had genetic testing, they may have shifted the proportion of large and small mutation categories in the CINRG dataset.

Another limitation of the CINRG dataset is its more stringent guidelines for the information that can be used to confirm the diagnosis of DMD as discussed in the Method Section 4.1.4. To ascertain that all participants enrolled in CINRG studies have DMD, CINRG has established strict guidelines regarding confirmation of the DMD diagnosis. The guidelines restrict study inclusion to participants with a positive gene mutation test result and/or complete absence of dystrophin on a muscle biopsy.

The results of the investigation of the dystrophin gene mutations in the CINRG study participants shows that the mutations observed approximately match the percentages of mutation type and mutation location reported in the published literature. Depending on the population sample, large lesions are reported anywhere from 60 to 80% (Koenig, Beggs et al. 1989; Haider, Bastaki et al. 1998; White, Kalf et al. 2002; Muntoni, Torelli et al. 2003), which matches closely to CINRG's 72%. The number of small lesions in the CINRG dataset seems to be slightly lower than those in the published literature. However, unlike the literature reports, CINRG study participants with no detectable mutations were included in the DNA testing data analysis (Result Section 5.1, Figure 4). The percentage of small lesions may be slightly lower in the CINRG dataset than in the general literature due to the kinds of DNA testing methodologies used as was discussed above.

6.2 COMPARABILITY OF CINRG MUTATIONS TO THE PUBLISHED DATABASES

The goal of the second aim was to compare the types of dystrophin gene mutations carried by CINRG study participants to those presented in two large international databases. The hypothesis was that if the distribution of mutations causing DMD is similar worldwide, then the distribution of mutations in the CINRG dataset will be similar to the distribution of mutations in two large international databases, the Leiden and UMD-DMD databases. The results obtained from the chi-square test (Result Section 5.1) showed that the distribution of mutations causing DMD in the CINRG database were statistically different from those in the Leiden and UMD-DMD databases, with p-values of 0.00002 and 0.00034, respectively. However, the statistically significant

differences found are unlikely to be scientifically significant. The rationale for this conclusion is discussed further below.

On an exploratory basis, the distribution of DMD-causing mutations in the CINRG dataset was also compared to the distribution of mutations from the Parent Project Muscular Dystrophy DuchenneConnect registry, which is generated by parent/caregiver reports. This analysis also determined that the distribution of mutations in the DuchenneConnect registry was statistically different from the CINRG dataset with a significant p-value of 0.012.

In the literature, the report of dystrophin causing mutations has largely been presented as a percentage of the population under study. Depending on the reports, the range of variations of reported percentages for some of the mutation types may be as high as 20% (Koenig, Beggs et al. 1989; White, Kalf et al. 2002; Muntoni, Torelli et al. 2003). The proportion of CINRG large and small mutations is within 10 to 15% of those found in the Leiden and UMD-DMD databases. Thus, it is unlikely that the differences observed between the CINRG dataset and the three different data sources, Leiden and UMD-DMD databases and the DuchenneConnect registry are scientifically significant. In the Leiden and UMD-DMD databases, the reading-frame hypothesis is reported as 91% and 96%, respectively (Aartsma-Rus, Van Deutekom et al. 2006; Tuffery-Giraud, Beroud et al. 2009), which is very similar to the 88.7% found in the CINRG dataset. There are several explanations that could explain the statistical differences found.

6.2.1 Limitations of Chi-Square Test

Although the chi-square test can determine if two categorical variables (database and mutation type) are significantly related, it does not address the meaning of the relationship. The chi-square test does not provide us any information about the strength of the relationship or its substantive

significance in the population (Bohannon 1986). In addition, the chi-square test is sensitive to sample size. The size of the calculated chi-square is directly proportional to the size of the sample, independent of the strength of the relationship between the variables (Bohannon 1986; Walker 1999). The CINRG dataset contains much smaller numbers (N=256) than the Leiden database (N=4,668) or the UMD-DMD database (N=2,394).

6.2.2 CINRG Inclusion and Exclusion Criteria

6.2.2.1 Genetic Confirmation

As previously discussed in Section 4.1.4, CINRG has established guidelines for including participants with positive gene mutation test result and/or complete absence of dystrophin on a muscle biopsy. This particular inclusion criterion may be a limitation for the CINRG dataset. The inclusion criterion specifies that participants with a complete absence of dystrophin on a muscle biopsy may be included in CINRG trials without having had DNA testing. In the CINRG dataset used in this project, 80 participants (21%) did not have any records that they had any DNA testing. Although only CINRG participants with identified mutations were included in the comparisons with the Leiden and UMD-DMD databases, the absence of DNA testing results on 21% of the CINRG participants may have shifted the distribution of large and small lesions in the CINRG dataset.

6.2.2.2 DMD Spectrum of Clinical Symptoms

The two databases, Leiden and UMD-DMD, and the DuchenneConnect registry are likely to include patients with the full spectrum of clinical signs associated with DMD, as these databases do not specify any particular inclusion or exclusion criteria. In the CINRG research

trials, especially those that investigate pharmacological agents, such as CNMC0601 and CNMC0705 described in Section 4.1, participants that are unable to follow simple commands during the CQMS testing are excluded. The total number of participants that may have been excluded from these two trials because of this criterion is unknown. The only genetic data of CINRG participants with mild to moderate mental retardation that could have been included in the CINRG dataset are from the CINRG *UCD0305 Longitudinal study*. The inclusion of these participants may also have resulted in a different distribution of DMD-causing mutations in the CINRG dataset.

6.2.3 Ethnic or Regional Differences in Predisposition to Dystrophin Mutations

The hypothesis for the second aim was that if the distribution of mutations causing DMD is similar worldwide, then the distribution of mutations in the CINRG dataset will be similar to the distribution of mutations in two large international databases, the Leiden and UMD-DMD databases. It is therefore possible to speculate that since the CINRG dataset and the Leiden and UMD-DMD databases were not statistically similar, then the distribution of mutations causing DMD is not similar worldwide. This possibility has been investigated in smaller ethnically diverse groups. Lo et al. reported in a retrospective review of 67 Hong Kong Chinese patients that the distribution of DMD large deletions was significantly lower than the literature reports (Lo, Lai et al. 2006). In their small cohort, the proportion of mutation type was divided as follows: 34.3% had large lesions, 7.5% had large duplications, 34.3% had small mutations, and 23.9% had no DMD gene mutation. Their findings matched several other Chinese studies of DMD gene mutations (Soong, Tsai et al. 1991; Yang 1991; Zeng, Chen et al. 1991; Ko, Tseng et al. 1992; Zhang 1993; Yang, Yang et al. 1994).

In a small study of 89 Taiwanese patients, 32 (35.9%) were identified with large deletions, 22 (24.7%) with large duplications, and 35 (39%) were not found to have a mutation by using a combination of MLPA and PCR methodologies (Hwa, Chang et al. 2007). A study in South Indian DMD patients identified 41 patients (62.1%) with large deletions. Their study only used PCR methodology to detect the DMD-causing mutations (Mallikarjuna Rao, Hussain et al. 2003). In their discussion, they did however; present a summary table of the deletion frequency in different Asian populations. This table has been abstracted and slightly modified from their paper and is presented in Table 13.

Table 13: Summary of Deletion Frequency in Different Asian Populations (Katayama, Takeshita et al. 1993; Shomrat, Gluck et al. 1994; Dincer, Topaloglu et al. 1996; Banerjee and Verma 1997; Singh, Sinha et al. 1997; Haider, Bastaki et al. 1998; Yuge, Hui et al. 1999; Mallikarjuna Rao, Hussain et al. 2003)

Population (n)	Mode of Analysis	Frequency of deletions (%)	Frequency of deletions at proximal region (%)	Frequency of deletions at distal region (%)	Most frequent deletion break point	Reference (Year)
Chinese (138)	PCR and Southern blot	62.3	26.7	68.6	Not included	Yuge et al (1999)
Japanese (30)	PCR and Southern blot	52	21.3	78	Intron 44	Katayam et al (1993)
Israel (62)	PCR	37	22	78	Not included	Shomrat et al (1994)
Arab (25)	PCR	86	8	52	Not included	Haider et al (1998)
Turkey (57)	PCR	60	31	69	Intron 44	Dincer et al (1996)
North India (121)	PCR	73	15.9	81.8	Intron 44	Singh et al (1997)
India (160)	PCR	64.4	30.3	70	Not included	Banerjee et al (1997)
South India (66)	PCR	62.1	22	78	Intron 44	Mallikarjuna (2003)

Upon subsequent analysis, the distribution of large lesions by CINRG sites also appears to vary geographically. Figure 10 presents the percentage of large lesions in CINRG study

participants from sites located in the United States and Figure 11 presents those found in CINRG study participants from International sites.

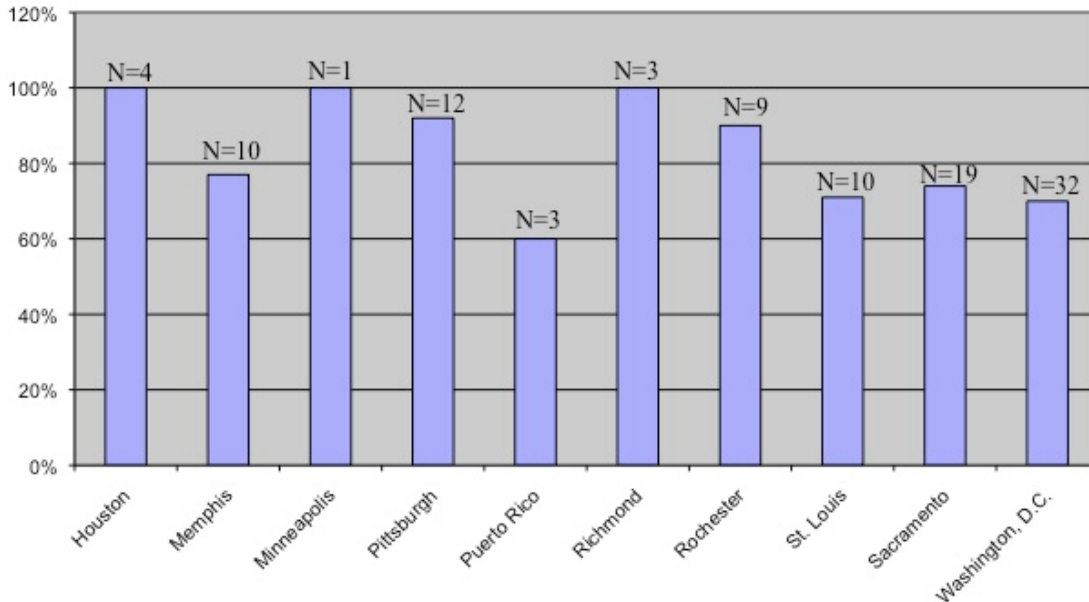


Figure 10: Representation of Large Lesions in CINRG Sites from the United States
 Each bar represents the percent of individuals by CINRG site in the United States with large lesions. For example, the site in St. Louis has 10 study participants (71%) with large lesions.

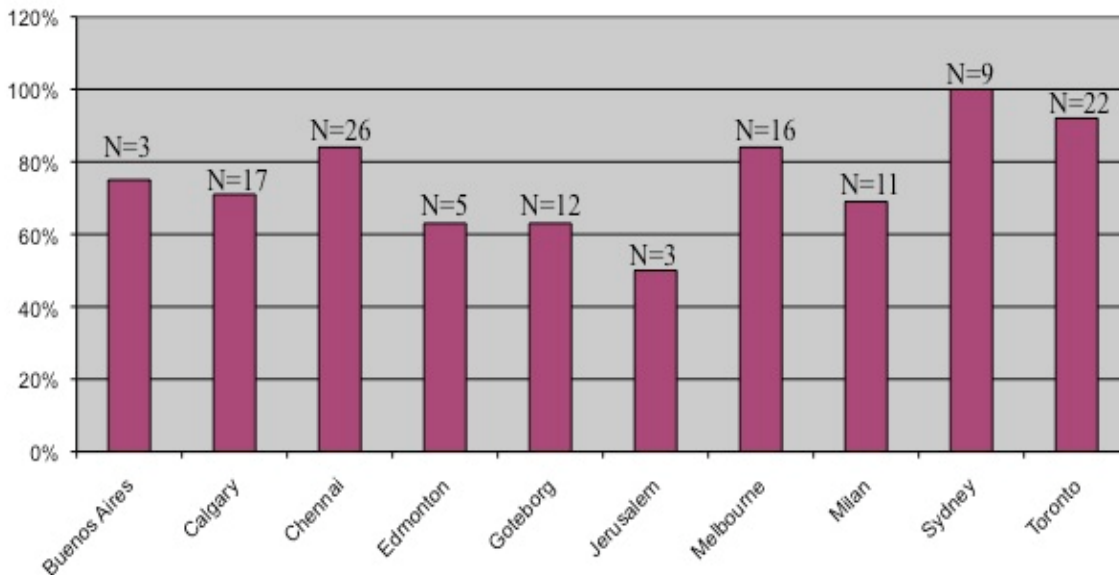


Figure 11: Representation of Large Lesions in International CINRG Sites
 Each bar represents the percent of individuals by CINRG site in countries outside of the United States with large lesions. For example, the site in Goteborg, Sweden has 12 study participants (63%) with large lesions.

Figures 10 and 11 illustrate the range of percentage of large lesions amongst the 20 different CINRG sites. Although there is a sample size bias for some sites, with less than 5 CINRG study participants having had DNA testing (sites include: Houston N=4, Minneapolis N=1, Richmond N=3, Buenos Aires N=4), the percentage of large lesions ranges from 50%, for the site in Jerusalem, to 100% for the sites in Houston, Minneapolis, Richmond, and Sydney. This additional finding may strengthen the argument regarding ethnic or regional difference in predisposition to dystrophin mutations.

6.2.4 Strengths and Limitations of the Different Databases

The Leiden database is the largest DMD database. As of March 5, 2009, a total of 16,630 variants (large and small) had been entered that include 3,077 unique variants. The Leiden database is also the only database to collect information on non-pathogenic variants. As of March 5, 2009, the Leiden database included 684 variants with no known phenotype and 102 variants with unknown pathogenicity. Neither of these groups of variants was included in this investigation. With the increased use of complete gene sequencing assays, the reporting of these types of variants will help differentiate DMD-causing genetic alterations from those that are not associated with disease. The Leiden database is accessible to any individual and allows scientists to report their individual mutation findings.

The French UMD-DMD database contains detailed mutation information for 2,046 male patients and 38 manifesting carrier females with a genetic diagnosis of dystrophinopathy. The UMD-DMD is the first country-specific database. By having a more controlled environment, the UMD-DMD database creates a national resource that may allow researchers to better determine the prevalence and incidence of the disease. The UMD-DMD database is also planning to expand

by including clinical data. This database creates a model for other countries and may lead to international DMD patient registries.

The Leiden and UMD-DMD databases are the two datasets with the largest sample size in this project. To test their comparability, a chi-square test was performed using the abstracted data on large and small lesions from Tables 8 and 9, in the Results Section 5.1. The chi-square value obtained was 3.049 with a p-value of 0.08. In this exploratory analysis, the null hypothesis is not rejected and therefore these two databases are statistically similar. This illustrates the importance of the sample size as well as the data content that is contained in these databases. They both include information on subjects with identifiable mutations; and, in the case of the Leiden database, their data is especially geared to report small or less common mutations.

The DuchenneConnect registry is the first comprehensive registry in the United States for patients and families with DMD. This type of database bridges the gap between the patient community and scientists. DuchenneConnect allows for patients and families to report their unique experiences with the disease. Demonstrating that the information from patient/parent reporting is comparable to a controlled clinical trials group dataset highlights the importance of patient/parent reporting and the strength and apparent accuracy of that data. DuchenneConnect is able to include phenotype information as well as quality of life assessments.

There are particular limitations that can be addressed for both the Leiden and UMD-DMD databases as well as the DuchenneConnect registry. Although all three sources are managed by central curators, the curation process is critical for maintaining high-quality data (Tuffery-Giraud, Beroud et al. 2009). In the Leiden database, Dr. Johan den Dunnen is in charge of the two databases (large and small lesions). The disclaimer states: ‘The allelic variants listed here are a collection of those we have found in the literature and which were submitted directly

to the database. When you notice that we have missed allelic variants or when you detect mistakes, please let us know.’ As of March 4, 2009, the website listed a total of 11,588 reported variants. It is therefore possible that some of the reported variants may be duplicated or entered with errors that have not yet been identified.

Additionally, because of the manner in which the Leiden database lists its variants, a possible limitation associated with the abstraction of the data was that a separate search of each category of mutation had to be carried out. The assessment of nonsense and missense mutations had to be performed by examining at the protein product described. Therefore, errors leading to some differences in mutation category assignments may have occurred. This could be avoided had the data been presented or reported directly in the Leiden database as it was in the UMD-DMD database. This would have avoided possible interpretation errors by this author.

For the UMD-DMD database, the curation guidelines are more rigorous. Drs. Leturcq and Tuffery-Giraud are the two curators in charge of quality control for the database. They have established specific guidelines; and also control, to some extent, the diagnostic testing methods used to ascertain the DNA lesions presented in their patients (Tuffery-Giraud, Beroud et al. 2009). All of the diagnostic data entered into the UMD-DMD database comes from 14 specific molecular laboratories that are likely to follow similar testing guidelines.

6.3 GENOTYPE-PHENOTYPE CORRELATIONS

The goal of the third aim was to compare the strength data in two particular cohorts of CINRG study participants to their mutation type. As discussed in Section 3.4, the majority of genotype-phenotype correlation studies have been focused on BMD vs. DMD and studying the effect on

the dystrophin reading-frame (Monaco, Bertelson et al. 1988; Baumbach, Chamberlain et al. 1989; Gillard, Chamberlain et al. 1989; Muntoni, Torelli et al. 2003). In this study, an analysis of two defined age cohorts was examined to evaluate strength measures of DMD patients with large and small dystrophin gene mutations. The hypothesis was that CINRG study participants with large or small lesions will have different strength data measurements.

In the first cohort (age 7 through 9 years, as set out in the demographic data presented in Table 11), two ANOVA tests and a linear regression test were performed to look for significant differences between the time to walk 10 meters and mutation type. The analyses did not show any significant difference between the time to walk 10 meters and mutation type; with p-values for the ANOVA and linear regression of 0.580 and 0.774, respectively. In the second cohort (age 10 through 20 years, as set out in the demographic data presented in Table 12), a binary logistic regression was performed to look for significant differences between ambulation status and mutation type. The analysis did not show any significant difference between the ambulation status and mutation type; with a p-value of 0.12.

There were many limitations to the data from both cohorts that likely contributed to the results observed. In the ambulant cohort, the number of participants with small lesions was much smaller compared to those with large lesions, 12% vs. 88%. A total of five participants were excluded from the ambulant cohort, as they were unable to perform the 10-meter walk test. Three of these participants were reported to have never used corticosteroids. Most CINRG study participants (90%) reported the use of corticosteroids. Corticosteroid use may present a confounding factor as the use of corticosteroids is the only medication to date that has been shown to slow the decline in strength in DMD (Drachman, Toyka et al. 1974; Brooke, Fenichel et al. 1987; Mendell, Moxley et al. 1989; Fenichel, Florence et al. 1991; Griggs, Moxley et al.

1991). In order to take into account the use of corticosteroids, a separate ANOVA test was performed on the ambulant cohort to compare the effect of corticosteroid use on the time to walk 10 meters.

In this particular cohort of participants, aged 7 through 9 years old, the use of corticosteroids was not found to have a statistically significant effect on the time to walk 10 meters ($p = 0.671$). This result was also confirmed by including corticosteroids as an independent variable in the linear regression analysis ($p = 0.681$).

Similar limitations with the data should be noted in the second mixed cohort. The proportion of those with small lesions was much smaller as compared to those with large lesions 12% vs. 88%. The majority of CINRG study participants reported the use of corticosteroids (64%). In this broader aged cohort (ages 10 through 20), the use of corticosteroids was shown to have a positive effect on the ambulation status with a p-value of 0.002. Due to the mixed age in this particular cohort, ranging from 10 to 20 years, age was also included in the binary logistic regression as a covariate. Age was also found to have a significant effect, p-value <0.001 , on the ambulation status, which is expected due to the progressive nature of the disease (Engel 2004).

A recent publication from a research group in France explored the clinical heterogeneity of the presentation of DMD symptoms (Desguerre, Christov et al. 2009). Their study included 75 DMD patients with comprehensive clinical data, who had been evaluated longitudinally by the same team over a mean follow-up period of greater than 10 years. Their findings clearly indicated the presence of clinical heterogeneity among DMD patients. They report four distinctive clusters based on intellectual and motor outcomes. The four groups proposed are presented in Table 14.

Table 14: Four Distinctive DMD Clinical Subsets (Desguerre, Christov et al. 2009)

Clinical Clusters	Clinical description and selective indicators for patient classification
A: Early infantile DMD	Very poor intellectual and motor outcome Indicators: psychomotor delay as first symptom, clinical onset < 2 years + mental retardation
B: Classical DMD	Intermediate intellectual and poor motor outcome Indicator: no clear indicator
C: Moderate pure motor DMD	Normal intelligence and delayed motor impairment Indicators: loss of ambulation >11 years, lower limb manual muscle score > 6 at age 8 + normal or borderline cognitive status
D: Severe pure motor DMD	Normal intelligence and poor motor outcome Indicator: no clear indicator, IQ may be used to differentiate between cluster B and D.

The researchers also performed a genotype-phenotype correlation by using the particular clusters defined in Table 14. They found that clinical classification partially correlated with mutations before exon 30. The proportion of patients with a mutation upstream to exon 30 increased from group A to D, with group A: 18%, group B: 29%, group C: 40%, and group D: 55%. The frequency of mutations before exon 30 correlated well ($p < 0.003$) with IQ measurements. They did not find any correlation with motor parameters (Desguerre, Christov et al. 2009). This study illustrates the importance of looking at the location of the mutation (proximal vs. distal) as opposed to the particular mutation type (large vs. small) as well as the collection and report of clinically relevant data on DMD symptoms.

Their study also suggests that the correlation of motor parameters with mutation type may be difficult to assess. Even in a very controlled setting, the authors were not able to observe

any significant correlation between genotype and phenotype for changes in muscle strength. The study used specific diagnostic confirmation protocols that included both DNA and muscle biopsy testing. The same medical team carried out consistent clinical assessments and controlled corticosteroid use by excluding corticosteroid users. However, their report does raise another possible explanation for the observable clinical heterogeneity in DMD patients. Patients in cluster A had the most severe striated muscle involvement with the poorest motor, respiratory and cardiac outcome. Patients in cluster C had better muscular status than any other cluster (Desguerre, Christov et al. 2009). One could therefore speculate that there may be a different phenotype effect of DMD mutations on different muscle groups. Cluster A includes the patients with the earliest onset of cardiac dysfunction. This research raises the question, could different genotypes affect the smooth, skeletal, and cardiac muscles differently, or at different rates?

Due to the design of the different datasets used in this project, the genotype-phenotype analysis could only be performed in the CINRG dataset and, furthermore, in only two small subsets of data and using only information on muscle strength. Although the Leiden database contains some information on phenotype (BMD vs. DMD), it is not yet set up to report clinical classifications of motor, respiratory and cardiac involvement or cognitive issues. The recent publication of the UMD-DMD database (Tuffery-Giraud, Beroud et al. 2009) does state that they are in a developmental stage for clinical data collection. DuchenneConnect is currently the only other dataset in this project that includes some phenotype-related information. The DuchenneConnect registry includes general questions on age of onset of symptoms, age of diagnosis, age of ambulation loss, and use of ACE inhibitor therapy for cardiac involvement. Due to the format of the DuchenneConnect report, a genotype-phenotype analysis was not feasible.

The genotype-phenotype analysis in the study participants from three CINRG studies was also further limited by the heterogeneity of the large lesions and multiple confounding factors of the data. As this CINRG data largely represents natural history data, it includes data on CINRG study participants that may be on additional pharmaceutical interventions (e.g. nutritional supplements, ACE inhibitors), participants with different physical therapy interventions, and very different environments due to the diversity of CINRG sites.

Finally, genotype-phenotype studies may be limited by the observation that apparently identical exonic deletions are likely to have different genomic breakpoints (Muntoni, Torelli et al. 2003). Genotypes may therefore be different even within patients with the same exon deletion due to the theory of different intron splicing. Gene-gene interactions have not yet been proposed as an additional limitation to these types of studies. However, with the elucidation of the human genome project, it is likely that these types of interactions will play a role in complex diseases, such as DMD (Namkung, Elston et al. 2009).

6.4 FURTHER RESEARCH OPPORTUNITIES

Given the results and observations of this project, many opportunities for future research exist. It would be useful to expand the CINRG dataset and create a database that could be accessible to the public. It would be beneficial to create a central repository that could merge the information from all the different databases and registries that are forming around the world. The creation of the Treat-NMD group (Treat-NMD) enhances the possibility of database consolidation in Europe. In addition, it would be advantageous for the various individuals currently involved with the databases investigated in this study to explore how they might harmonize various aspects of

their data collection, such as DNA testing methods used for confirmation of the DMD diagnosis. This group of curators and representative data submitters would be an appropriate group to discuss studies of the DNA testing tools used to confirm DMD diagnosis and the clinical parameters that might be reported to them to enable better genotype/phenotype correlations. While maintaining their independence and unique attributes, improved harmonization would improve the applicability of their information in many ways.

In order to improve and expand the CINRG dataset and create interactions with other networks, databases, and registries, it would be useful to have someone in CINRG who would be responsible for central data curation.

Characterizing the different genotypes has become critical in this era of personalized medicine. This study suggests that more detailed genetic testing may be recommended for individuals without identifiable mutations as these results may allow them to be assigned to and participate in more specific clinical trials. For example, identification of the genetic mutation type carried by a DMD patient is critical for new trials, such as *Phase 2a Extension Study of PTC124 in DMD* which specifically targets DMD patients with nonsense mutations or *Safety and Efficacy Study of Antisense Oligonucleotides in DMD*, which specifically targets DMD patients with large deletions that could alter the reading-frame by skipping a particular exon of interest (ClinicalTrials.gov). As new, more genetically-based treatments become available, detailed data on mutation type will be invaluable to physicians and counselors in order to provide relevant information to patient families.

The study performed by Desguerre emphasizes the importance of the conduct and report of consistent clinical assessments (Desguerre, Christov et al. 2009). In order to further study genotype-phenotype correlations, it is critical to establish gold-standard clinical assessment tools

that can be applied to multiple age groups. The CINRG network is currently the only research group to use the CQMS tool. Furthermore, it is essential to establish practice parameter guidelines for the diagnostic methodologies. Tools to assess cardiac, respiratory, nutritional, and cognitive parameters are needed. The addition of such data might enable further investigations of the genotype effects on the different muscle groups and onset of disease specific symptoms (specifically pulmonary and cardiac).

7.0 CONCLUSION

This investigation compared the DMD-causing mutations in the dystrophin gene of patients from several CINRG clinical studies with those abstracted from the Leiden and UMD-DMD mutation databases. The distribution of dystrophin mutations within the CINRG dataset was found to be similar to the Leiden and UMD-DMD databases.

In addition, the strength data in two particular cohorts of CINRG study participants were compared to their mutation type. With no current cure for DMD, corticosteroids are the only pharmacological intervention that is able to slow the decline of strength and function in DMD patients. Until now, the beneficial effects of corticosteroids have exclusively been demonstrated in the setting of controlled clinical trials. While this study was not able to draw any conclusions on the effect of specific dystrophin mutation types reported within the CINRG dataset and muscle strength, this is the first report that indicates that the use of corticosteroids improves the length of ambulation in a non-controlled study environment.

The results of this study demonstrate that harmonization of means by which mutation data is collected from DMD patients and subsequently reported has the potential to benefit clinical trials and ultimately enhance pairing of eligible patients to specific molecular-based treatments. Some of the limitations encountered while analyzing the data and interpreting the results of this study point to the need to develop mutually-agreed standards for diagnosis and assessment of the clinical signs of DMD in patients.

Through the interaction of international academic research groups like CINRG and curators of mutation databases and patient registries, a global repository of genetic and clinical information can be created. This will lay the ground for larger genotype/phenotype correlations and help us further understand many of the unanswered questions about the natural history of Duchenne muscular dystrophy.

APPENDIX A: DYSTROPHIN GENE SEQUENCE

exon 01		tcct	-241
	ggcatcagttactgtgttgactcactcagtggtgggatcactcactttcccctacagga		-181
	ctcagatctgggaggcaattaccttcggagaaaaacgaataggaaaaactgaagtgttac		-121
	tttttttaagctgctgaagtttggtggtttctcattgtttttaagcctactggagcaat		-61
	aaagtttgaagaacttttaccaggttttttttatcgctgccttgatatacacttttcaaa		-1
	ATGCTTTGGTGGGAAGAAGTAGAGGACTGTT <u>2</u> ATGAAAGAGAAGATGTTCAAAAGAAAACA		60
	M L W W E E V E D C Y E R E D V Q K K T		20
	TTCACAAAATGGGTAAATGCACAATTTTCTAAG <u>3</u> TTTGGGAAGCAGCATATGAGAACCTC		120
	F T K W V N A Q F S K F G K Q H I E N L		40
	TTCAGTGAACCTACAGGATGGGAGGCGCCTCCTAGACCTCCTCGAAGGCCTGACAGGGCAA		180
	F S D L Q D G R R L L D L L E G L T G Q		60
	AAACTG <u>4</u> CCAAAAGAAAAGGATCCACAAGAGTTCATGCCCTGAACAATGTCAACAAGGCA		240
	K L P K E K G S T R V H A L N N V N K A		80
	CTGCGGGTTTTGCAGAACAATAAT <u>5</u> GTTGATTAGTGAATATTGGAAGTACTGACATCGTA		300
	L R V L Q N N N V D L V N I G S T D I V		100
	GATGGAAATCATAAACTGACTCTTGGTTTGATTGGAATATAATCCTCCACTGGCAG <u>6</u> GTC		360
	D G N H K L T L G L I W N I I L H W Q V		120
	AAAAATGTAATGAAAAATATCATGGCTGGATTGCAACAAACCAACAGTGA AAAAGATTCTC		420
	K N V M K N I M A G L Q Q T N S E K I L		140
	CTGAGCTGGGTCCGACAATCAACTCGTAATTATCCACAGGTTAATGTAATCAACTTCACC		480
	L S W V R Q S T R N Y P Q V N V I N F T		160
	ACCAGCTGGTCTGATGGCCTGGCTTGAATGCTCTCATCCATAGTCATAG <u>7</u> GCCAGACCTA		540
	T S W S D G L A L N A L I H S H R P D L		180

TTTGACTGGAATAGTGTGGTTTGCCAGCAGTCAGCCACACAACGACTGGAACATGCATTC 600
 F D W N S V V C Q Q S A T Q R L E H A F 200

AACATCGCCAGATATCAATTAGGCATAGAGAACTACTCGATCCTGAAG | 8 ATGTTGATACC 660
 N I A R Y Q L G I E K L L D P E D | V D T 220

ACCTATCCAGATAAGAAGTCCATCTTAATGTACATCACATCACTCTTCCAAGTTTTCCT 720
 T Y P D K K S I L M Y I T S L F Q V L P 240

CAACAAGTGAGCATGAAGCCATCCAGGAAGTGAAATGTTGCCAAGGCCACCTAAAGTG 780
 Q Q V S I E A I Q E V E M L P R P P K V 260

ACTAAAGAAGAACATTTTCAGTTACATCATCAATGCACTATTCTCAACAG | 9 ATCACGGTC 840
 T K E E H F Q L H H Q M H Y S Q Q | I T V 280

AGTCTAGCACAGGGATATGAGAGAACTTCTTCCCCTAAGCCTCGATTCAAGAGCTATGCC 900
 S L A Q G Y E R T S S P K P R F K S Y A 300

TACACACAGGCTGCTTATGTCACCACCTCTGACCCTACACGGAGCCCATTTCTTACAG 960
 Y T Q A A Y V T T S D P T R S P F P S Q 320

| 10
 | CATTTGGAAGCTCCTGAAGACAAGTCATTTGGCAGTTTCATGATGGAGAGTGAAGTAAAC 1020
 | H L E A P E D K S F G S S L M E S E V N 340

CTGGACCGTTATCAAACAGCTTTAGAAGAAGTATTATCGTGGCTTCTTTCTGCTGAGGAC 1080
 L D R Y Q T A L E E V L S W L L S A E D 360

ACATGCAAGCACAGGAGAGATTCTAATGATGTGGAAGTGGTGAAGACCAGTTTCAT 1140
 T L Q A Q G E I S N D V E V V K D Q F H 380

ACTCATGAG | 11 GGTACATGATGGATTGACAGCCATCAGGGCCGGTGGTAAATATTCTA 1200
 T H E | G Y M M D L T A H Q G R V G N I L 400

CAATTGGGAAGTAAAGCTGATTGGAACAGGAAAATTATCAGAAGATGAAGAACTGAAGTA 1260
 Q L G S K L I G T G K L S E D E E T E V 420

CAAGAGCAGATGAATCTCCTAAATCAAGATGGGAATGCCTCAGGGTAGCTAGCATGGAA 1320
 Q E Q M N L L N S R W E C L R V A S M E 440

AAACAAAGCAA | 12 TTTACATAGAGTTTAAATGGATCTCCAGAATCAGAAACTGAAAGAGTTG 1380
 K Q S N | L H R V L M D L Q N Q K L K E L 460

AATGACTGGCTAAACAAAAACAGAAGAAAGAACAAGGAAAATGGAGGAAGAGCCTCTTGA 1440
 N D W L T K T E E R T R K M E E E P L G 480

CCTGATCTTGAAGACCTAAACGCCAAGTACAACAACATAAG | 13 GTGCTTCAAGAAGATCTA 1500
 P D L E D L K R Q V Q Q H K | V L Q E D L 500

GAACAAGAACAAGTCAGGGTCAATCTCTCACTCACATGGTGGTGGTAGTTGATGAATCT 1560

E Q E Q V R V N S L T H M V V V V D E S 520

AGTGGAGATCACGCAACTGCTGCTTTGGAAGAACAACCTTAAG | ¹⁴ GTATTGGGAGATCGATGG 1620
S G D H A T A A L E E Q L K | V L G D R W 540

GCAAAATCTGTAGATGGACAGAAGACCGCTGGGTTCTTTTACAAGACATCCTTCTCAA 1680
A N I C R W T E D R W V L L Q D I L L K 560

TGGCAACGTCTTACTGAAGAACAG | ¹⁵ TGCCTTTTAGTGCATGGCTTTCAGAAAAAGAAGAT 1740
W Q R L T E E Q | C L F S A W L S E K E D 580

GCAGTGAACAAGATTCACACAACCTGGCTTTAAAGATCAAAATGAAATGTTATCAAGTCTT 1800
A V N K I H T T G F K D Q N E M L S S L 600

CAAAAATGGCC | ¹⁶ GTTTTAAAAGCGGATCTAGAAAAGAAAAAGCAATCCATGGGCAAACTG 1860
Q K L A | V L K A D L E K K K Q S M G K L 620

TATTCACTCAAACAAGATCTTCTTTCAACACTGAAGAATAAGTCAGTGACCCAGAAGACG 1920
Y S L K Q D L L S T L K N K S V T Q K T 640

GAAGCATGGCTGGATAAATTTGCCCGGTGTTGGGATAAATTTAGTCCAAAAACTTGAAAAG 1980
E A W L D N F A R C W D N L V Q K L E K 660

AGTACAGCACAG | ¹⁷ ATTCACAGGCTGTCACCACCACCTCAGCCATCACTAACACAGACAAC 2040
S T A Q | I S Q A V T T T Q P S L T Q T T 680

GTAATGAAACAGTAACTACGGTGAACCACAAGGGAACAGATCCTGGTAAAGCATGCTCAA 2100
V M E T V T T V T T R E Q I L V K H A Q 700

GAGGAACTTCCACCACCACCTCCCAAAAAGAAGAGGCAGATTACTGTGGATTCTGAAATT 2160
E E L P P P P P Q K K R Q I T V D S E I 720

AGGAAAAG | ¹⁸ GTTGGATGTTGATATAACTGAACTTCACAGCTGGATTACTCGCTCAGAAGCT 2220
R K R | L D V D I T E L H S W I T R S E A 740

GTGTTGCAGAGTCTGAATTTGCAATCTTTTCGGAAGGAAGGCAACTTCTCAGACTTAAAA 2280
V L Q S P E F A I F R K E G N F S D L K 760

GAAAAAGTCAAT | ¹⁹ GCCATAGAGCGAGAAAAAGCTGAGAAGTTCAGAAAACCTGCAAGATGCC 2340
E K V N | A I E R E K A E K F R K L Q D A 780

AGCAGATCAGCTCAGGCCCTGGTGAACAGATGGTGAATG | ²⁰ AGGGTGTAAATGCAGATAGC 2400
S R S A Q A L V E Q M V N E | G V N A D S 800

ATCAAACAAGCCTCAGAACAACCTGAACAGCCGGTGGATCGAATTCTGCCAGTTGCTAAGT 2460
I K Q A S E Q L N S R W I E F C Q L L S 820

GAGAGACTTAACTGGCTGGAGTATCAGAACAACATCATCGCTTTCTATAAATCAGCTACAA 2520
E R L N W L E Y Q N N I I A F Y N Q L Q 840

CAATTGGAGCAGAT TG ACAACACTACTGCTGAAAACCTGGT TG AAAATCCAACCCACCACCCCA	2580
Q L E Q M T T T A E N W L K I Q P T T P	860
TCAGAGCCAACAGCAATTAAAAGTCAGT TAAAA ATTTG TAA G 21 GATGAAGTCAACCGGCTA	2640
S E P T A I K S Q L K I C K D E V N R L	880
TCAGGTCTTCAACCTCAAAT TGA ACGAT TAAAA ATTCAAAGCAT AG CCCT TGA AAGAGAAA	2700
S G L Q P Q I E R L K I Q S I A L K E K	900
GGACAAGGACCCATGTTCTCGGATGCAGACTTTGTGGCCTTTACAAATCATT TTA AGCAA	2760
G Q G P M F L D A D F V A F T N H F K Q	920
GTCTTTTCTGATGTGCAGGCCAGAGAGAAAGAGCTACAGACAA 22 TTTT TG ACACTTTGCCA	2820
V F S D V Q A R E K E L Q T I F D T L P	940
CCAATGCGCTATCAGGAGACCAT TG AGTGCCATCAGGACATGGGTCCAGCAGTCAGAAACC	2880
P M R Y Q E T M S A I R T W V Q Q S E T	960
AAACTCTCCATACCTCAACT TAG TGTCACCGACTATGAAATCATGGAGCAGAGACTCGGG	2940
K L S I P Q L S V T D Y E I M E Q R L G	980
GAATTGCAG 23 GCTTTACAAAGTTCTCTGCAAGAGCAACAAAGTGGCCTATACTATCTCAGC	3000
E L Q A L Q S S L Q E Q Q S G L Y Y L S	1000
ACCACT TG AAAGAGATGTGGAAGAAAGCGCCCTCTGAAAT TAG CCGAAATATCAATCA	3060
T T V K E M S K K A P S E I S R K Y Q S	1020
GAATT TGA AGAAAT TG AGGGACGCTGGAAGAAGCTCTCCTCCAGCTGGT TG AGCATTGT	3120
E F E E I E G R W K K L S S Q L V E H C	1040
CAAAGCT TAG AGGAGCAAAT TGA ATAAACTCCGAAAAATTCAG 24 AATCACATACAAACCC TG	3180
Q K L E E Q M N K L R K I Q N H I Q T L	1060
A AGAAATGGATGGCT TGA AGTTGATGTTTTTCT TGA AGGAGGAATGGCCTGCCCTTGGGGAT	3240
K K W M A E V D V F L K E E W P A L G D	1080
TCAGAAATTC TAAAA AGCAGCT TGA AACAGTGCAGA 25 CTTT TAG TCA TG ATATT CAG ACA	3300
S E I L K K Q L K Q C R L L V S D I Q T	1100
ATT CAG CC CAG TC TAAA ACAGTGTCAAT TGA AGGTGGGCAGAAGAT TAA AGAA TGA AGCAGAG	3360
I Q P S L N S V N E G G Q K I K N E A E	1120
CCAGAGTTTGCTTCGAGACT TG AGACAGA AACTCAA AGAACT TAA CACTCAGTGGGATCAC	3420
P E F A S R L E T E L K E L N T Q W D H	1140
ATGTGCCAACAG 26 GTCTATGCCAGAAAGGAGGCCT TGA AGGGAGGTTTGGAGAAA ACTGTA	3480
M C Q Q V Y A R K E A L K G G L E K T V	1160
AG CCTCCAGAAAGATCTATCAGAGATGCACGAATGGAT TG ACACAAGCT TGA AGAAGAGTAT	3540

S L Q K D L S E M H E W M T Q A E E E Y 1180

CTTGAGAGAGATTTGAATATAAACTCCAGATGAATTACAGAAAGCAGTTGAAGAGATG 3600
L E R D F E Y K T P D E L Q K A V E E M 1200

27
AAG | AGAGCTAAAGAAGAGGCCCAACAAAAAGAAGCGAAAGTGAAACTCCTTACTGAGTCT 3660
K | R A K E E A Q Q K E A K V K L L T E S 1220

GTAATAGTGTCTAGCTCAAGCTCCACCTGTAGCACAAAGAGGCCTTAAAAAGGAAGT 3720
V N S V I A Q A P P V A Q E A L K K E L 1240

GAACTCTAACCCCAACTACCAGTGGCTCTGCACTAGGCTGAATGGGAAATGCAAGACT 3780
E T L T T N Y Q W L C T R L N G K C K T 1260

TTGGAA | 28
| GAAGTTTGGGCATGTTGGCATGAGTTATTGTCTACTTGGAGAAAGCAAACAAG 3840
L E | E V W A C W H E L L S Y L E K A N K 1280

TGGCTAAATGAAGTAGAATTTAACTTAAAACCACTGAAAACATTCCTGGCGGAGCTGAG 3900
W L N E V E F K L K T T E N I P G G A E 1300

GAAATCTCTGAGGTGCTAGAT | 29
| TCACTTGAAAATTTGATGCGACATTCAGAGGATAACCCA 3960
E I S E V L D | S L E N L M R H S E D N P 1320

AATCAGATTGCGCATATTGGCACAGACCCTAACAGATGGCGGAGTCATGGATGAGCTAATC 4020
N Q I R I L A Q T L T D G G V M D E L I 1340

AATGAGGAACTTGAGACATTTAATTCCTGTTGGAGGGAAGTACATGAAGAG | 30
| GCTGTAAGG 4080
N E E L E T F N S R W R E L H E E | A V R 1360

AGGCAAAAGTTGCTTGAACAGAGCATCCAGTCTGCCAGGAGACTGAAAAATCCTTACAC 4140
R Q K L L E Q S I Q S A Q E T E K S L H 1380

TTAATCCAGGAGTCCCTCACATTCATTGACAAGCAGTTGGCAGCTTATATTGCAGACAAG 4200
L I Q E S L T F I D K Q L A A Y I A D K 1400

GTGGACGCAGCTCAAATGCCTCAGGAAGCCAG | 31
| AAAATCCAATCTGATTGACAAGTCAT 4260
V D A A Q M P Q E A Q | K I Q S D L T S H 1420

GAGATCAGTTTAGAAGAAATGAAGAAACATAATCAGGGGAAGGAGGCTGCCCAAAGAGTC 4320
E I S L E E M K K H N Q G K E A A Q R V 1440

CTGTCTCAGATTGATGTTGCACAG | 32
| AAAAAATACAAGATGTCTCCATGAAGTTTCGATTA 4380
L S Q I D V A Q | K K L Q D V S M K F R L 1460

TTCCAGAAACCAGCAATTTTGGAGCAGCTCTACAAGAAAGTAAGATGATTTTAGATGAA 4440
F Q K P A N F E Q R L Q E S K M I L D E 1480

GTGAAGATGCACTTGCTGCATTGAAACAAAGAGTGTGGAACAGGAAGTAGTACAGTCA 4500
V K M H L P A L E T K S V E Q E V V Q S 1500

CAGCT AAAT CATTGTGTG ³³ AACTTGTATAAAAAGTCTGAGTGAAGTGAAGTCTGAAGTGGAA	4560
Q L N H C V N L Y K S L S E V K S E V E	1520
ATGG TGATAA AGACTGGACGTCAGATTGTACAGAAAAAGCAGACGGAAAATCCCAAAGAA	4620
M V I K T G R Q I V Q K K Q T E N P K E	1540
CTTGATGAAAGACT AA CAGCTTT GAA ATTGCATTATAATGAGCTGGGAGCAAAG ³⁴ GTAACA	4680
L D E R V T A L K L H Y N E L G A K V T	1560
GAAAGAAAGCAACAGTTGGAGAAATGCT TGA AATTGTCCCGT AA GATGCGAAAGGAAAT G	4740
E R K Q Q L E K C L K L S R K M R K E M	1580
AAT GTCT TGAC AGAATGGCTGGCAGCTACAGATATGGAAT TGAC AAAGAGATCAGCAGTT	4800
N V L T E W L A A T D M E L T K R S A V	1600
GA AGGAATGCCT AGTAA TTTGGATTCT GAA GTGCCTGGGGAAAG ³⁵ GCTACTCAAAAAGAG	4860
E G M P S N L D S E V A W G K A T Q K E	1620
ATTGAGAAACAGAAGGTGCACCT GAA AGATATCACAGAG TAG GAGAGGCCT TG AAAACA	4920
I E K Q K V H L K S I T E V G E A L K T	1640
GTTTTGGGCAAGAAGGAGACGTTGGTGGAAAGATA AA ACTCAGTCTTCT TGA ATAGT AA CTGG	4980
V L G K K E T L V E D K L S L L N S N W	1660
ATAG CTGTACCTCCCGAGCAGAAGAGTGGT TAA ATCTTTTGTG ³⁶ GAATACCAGAAACAC	5040
I A V T S R A E E W L N L L L E Y Q K H	1680
ATGGAAACTTTT GAC CAGAATGTGGACCACATCAAAAGTGGATCATT CAGGCTGAC ACA	5100
M E T F D Q N V D H I T K W I I Q A D T	1700
CTTTTGGAT GAA TCAGAGAAAAAGAAACCCAGCAAAAAGAAGACGTGCT TAA G ³⁷ CGTT TA	5160
L L D E S E K K K P Q Q K E D V L K R L	1720
AAG GCAGAACT TGA ATGACATACGCCAAAGGTGGACTCTACACGT GAC CAAGCAGCAAAC	5220
K A E L N D I R P K V D S T R D Q A A N	1740
TGAT GGCAAACCGGGT GAC CACTGCAGGAAAT TAGT AGAGCCCAAATCTCAGAGCTC	5280
L M A N R G D H C R K L V E P Q I S E L	1760
AACCATCGATTTGCAGCCATTT CAC ACAGAAT TAA GACTGGAAAG ³⁸ GCCTCCATTCTTT G	5340
N H R F A A I S H R I K T G K A S I P L	1780
AAG GAATTGGAGCAGTT TAA CTCAGATATACAAAAATTGCT TGA ACCACTGGAGGCT GAA	5400
K E L E Q F N S D I Q K L L E P L E A E	1800
ATTCAGCAGGGG TGA ATCT TGA AAGAGGAAGACTTCAATAAAGATAT G ³⁹ AATGA AGACAAT	5460
I Q Q G V N L K E E D F N K D M N E D N	1820

GAGGGTACTGTAAAAGAATTGTTGCAAAGAGGAGACAACTTACAACAAAGAATCACAGAT 5520
 E G T V K E L L Q R G D N L Q Q R I T D 1840

GAGAGAAAGCGAGAGGAAAATAAGATAAAAACAGCAGCTGTTACAGACAAAACATAATGCT 5580
 E R K R E E I K I K Q Q L L Q T K H N A 1860

CTCAAG | 40 .
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 L K | D L R S Q R R K K A L E I S H Q W Y 1880

CAGTACAAGAGGCAGGCTGATGATCTCCTGAAATGCTTGGATGACATTGAAAAAAAAATTA 5700
 Q Y K R Q A D D L L K C L D D I E K K L 1900

GCCAGCCTACCTGAGCCAGAGATGAAAGGAAAAATAAG | 41 .
 GAAATTGATCGGGAATTGCAG 5760
 A S L P E P R D E R K I K | E I D R E L Q 1920

AAGAAGAAAGAGGAGCTGAATGCAGTGCCTAGGCAAGCTGAGGGCTTGTCTGAGGATGGG 5820
 K K K E E L N A V R R Q A E G L S E D G 1940

GCCGCAATGGCAGTGGAGCCAACTCAGATCCAGCTCAGCAAGCGCTGGCGGAAATTGAG 5880
 A A M A V E P T Q I Q L S K R W R E I E 1960

AGCAAATTTGCTCAGTTTCGAAGACTCAACTTTGCACAAATT | 42 .
 CACACTGTCCGTGAAGAA 5940
 S K F A Q F R R L N F A Q I | H T V R E E 1980

ACGATGATGGTGAATGACTGAAGACATGCCTTTGGAAATTTCTTATGTGCCTTCTACTTAT 6000
 T M M V M T E D M P L E I S Y V P S T Y 2000

TTGACTGAAATCACTCATGTCTCACAAAGCCCTATTAGAAGTGAACAACCTTCTCAATGCT 6060
 L T E I T H V S Q A L L E V E Q L L N A 2020

CCTGACCTCTGTGCTAAGGACTTTGAAGATCTCTTTAAGCAAGAGGAGTCTCTGAAG | 43 .
 AAT 6120
 P D L C A K D F E D L F K Q E E S L K | N 2040

ATAAAAGATAGTCTACAACAAAGCTCAGGTCGGATGACATTATTCATAGCAAGAAGACA 6180
 I K D S L Q Q S S G R I D I I H S K K T 2060

GCAGCATTGCAAAGTGCAACGCCTGTGGAAAGGTTGAAGCTACAGGAAGCTCTCTCCAG 6240
 A A L Q S A T P V E R V K L Q E A L S Q 2080

CTTGATTTCCAATGGGAAAAAGTTAAACAAAATGTACAAGGACCGACAAGG | 44 .
 GCGATTTGAC 6300
 L D F Q W E K V N K M Y K D R Q G | R F D 2100

AGATCTGTTGAGAAATGGCGGCGTTTTTCATTATGATATAAAGATATTTAATCAGTGGCTA 6360
 R S V E K W R R F H Y D I K I F N Q W L 2120

ACAGAAGCTGAACAGTTTCTCAGAAAGACACAAATTCCTGAGAATTGGGAACATGCTAAA 6420
 T E A E Q F L R K T Q I P E N W E H A K 2140

TACAAATGGTATCTTAAG | 45 .
 GAACTCCAGGATGGCATTGGGCAGCGGCAAACCTGTTGTGAGA 6480

Y K W Y L K | E L Q D G I G Q R Q T V V R 2160
 ACAT**TGA**ATGCAACTGGGGAAGAA**ATA**ATTCAGCAATCCTCAAAAACAGATGCCAGTATT 6540
 T L N A T G E E I I Q Q S S K T D A S I 2180
 CTACAGGAAAAATGGGAAGCCT**TGA**ATCTGCGGTGGCAGGAGGTCTGCAAACAGCTGTCA 6600
 L Q E K L G S L N L R W Q E V C K Q L S 2200
 GACAGAAAAAAGAG | **46** GCT**TGA**AGAACAAGAAATATCTTGTGAGAATTTCAAAGAGATTT**TA** 6660
 D R K K R | L E E Q K N I L S E F Q R D L 2220
AATGAATTTGTTTATGGTTGGAGGAAGCAGATA**AA**CATTGCT**AG**TATCCC**ACTTGA**ACCT 6720
 N E F V L W L E E A D N I A S I P L E P 2240
 GGAAAAGAGCAGCAACT**TAA**AAGAAAAGCTT**GAG**CAAGTCAAG | **47** TTACTGGTGGAAAGAGTTG 6780
 G K E Q Q L K E K L E Q V K | L L V E E L 2260
 CCCCTGCGCCAGGGAATTCTCAAA**CAATTA**AA**TGA**AACTGGAGGACCCGTGCTT**GTA**AGT 6840
 P L R Q G I L K Q L N E T G G P V L V S 2280
 GCTCC**ATA**AGCCCAGAAGAGCAAGATA**AA**ACT**TG**AAAATA**AG**CTCAAGCAGACAAATCTC 6900
 A P I S P E E Q D K L E N K L K Q T N L 2300
 CAGTGG**ATA**AG | **48** GTTTCCAGAGCTTTACCT**TG**AGAAACAAGGAGAAAT**TGA**AGCTCAA**ATA** 6960
 Q W I K | V S R A L P E K Q G E I E A Q I 2320
AAAGACCTTGGGCAGCTT**TGA**AAAAAAGCTT**TGA**AGACCTT**TGA**AGAGCAGTT**AA**ATCATCTG 7020
 K D L G Q L E K K L E D L E E Q L N H L 2340
 CTGCTGTGGTTATCTCCTATT**AG**GAATCAGTTGAAAATTT**TATA**ACCAACCAAACCAAGAA 7080
 L L W L S P I R N Q L E I Y N Q P N Q E 2360
 GGACCATT**TG**ACGTT**CAG** | **49** GAAACT**TGA**AA**TAG**CAGTT**CA**AGCT**AAA**CAACCGGATGTGGAA 7140
 G P F D V Q | E T E I A V Q A K Q P D V E 2380
 GAGATTTTGTCT**AA**AGGGCAGCATT**TG**TACAAGGAAAAACCAGCCACTCAGCCAG**TGA**AG 7200
 E I L S K G Q H L Y K E K P A T Q P V K 2400
 | **50** AGGAAGTT**TAG**AAGATCT**TGA**GCTCT**TG**AGTGGAAAGGCG**TAA**ACCGTTTACTT**CA**AGAGCT**TG** 7260
 | R K L E D L S S E W K A V N R L L Q E L 2420
 AGGGCAAAGCAGCCT**TG**ACCT**TAG**CTCCTGGACT**TG**ACCACTATTGGAGCCT | **51** CTCCTACTCAG 7320
 R A K Q P D L A P G L T T I G A S | P T Q 2440
 ACTGTTACTCTGG**TG**ACACAACCTGTGGTTACT**TAG**GAAACTGCCATCTCCAAACT**TAG**AA 7380
 T V T L V T Q P V V T K E T A I S K L E 2460
 ATGCCATCTTCCT**TG**ATGTTGGAGGTACCTGCTCTGGCAGATTT**CA**ACCGGGCTTGGACA 7440
 M P S S L M L E V P A L A D F N R A W T 2480

GAACCTACCGACTGGCTTTCTCTGCTTGATCAAGTTATAAAATCACAGAGGGT GT ATGGTG	7500
E L T D W L S L L D Q V I K S Q R V M V	2500
GGT GACCTTG AGGATATCAACGAGAT GT ATCATCAAGCAGAAG 52	7560
G D L E D I N E M I I K Q K A T M Q D L	2520
GAACAGAGGCGTCCCAGTTGGAAGAACTCATTACCGCTGCCCAAATTT G AAAAACAAG	7620
E Q R R P Q L E E L I T A A Q N L K N K	2540
ACCAGCAATCAAGAGGCT AGA ACAATCATTACGGATCGAA 53	7680
T S N Q E A R T I I T D R I T G AAAGAATTCAGAATCAG	2560
TGGGAT G AAGTACAAGAACACCTTCAGAACCGGAGGCAACAGTT G AATGAAATGT TAA AG	7740
W D E V Q E H L Q N R R Q Q L N E M L K	2580
GATTCAACACAATGGCTGGAAGCT AA GGAGAAGCT G AGCAGGTCT TAG GACAGGCCAGA	7800
D S T Q W L E A K E E A E Q V L G Q A R	2600
GCCAAGCT TG AGTCATGGAAGGAGGGTCCCTATACAG TAG ATGCAATCCAAAAGAAAATC	7860
A K L E S W K E G P Y T V D A I Q K K I	2620
ACAGAAACCAAG 54	7920
T E T K CAGTTGGCCAAAGACCTCCGCCAGTGGCAGACAAATGT TAG ATGTGGCA	2640
Q L A K D L R Q W Q T N V D V A	
AAT G ACTTGGCCCT G AAACTTCTCCGGGATTATTCTGCAGAT G ATACCAGAAAAGTCCAC	7980
N D L A L K L L R D Y S A D D T R K V H	2660
ATGATAA CAGAGAATATCAATGCCTCTTGGAGAAGCATT CATA AAAG 55	8040
M I T E N I N A S W R S I H K R G GT GAGT GAGCGA	2680
V S E R	
GAGGCTGCTTTGGAAGAAA ACTCATAG ATTACTGCAACAGTTC CCCC TGGACCTGGAAAAG	8100
E A A L E E T H R L L Q Q F P L D L E K	2700
TTTCTGCCTGGCTTACAGAAGCT G AAACA ACTG CCAATGTCTTACAGGATGCTACCCGT	8160
F L A W L T E A E T T A N V L Q D A T R	2720
AAG GAAAGGCTCCT TAGA AGACTCCAAGGGAG TAAA AGAGCT GATG AAACAATGGCAA 56	8220
K E R L L E D S K G V K E L M K Q W Q D GAC	2740
CTCCAAGGT G AAAT TGA AGCTCACACAGATGTTTATCACAACCTGGAT G AAAACAGCCAA	8280
L Q G E I E A H T D V Y H N L D E N S Q	2760
AAAATCCT TG AGATCCCTGGAAGGTTCCGAT G ATGCAGTCTGT TACA AAAGACGTTTGGAT	8340
K I L R S L E G S D D A V L L Q R R L D	2780
AACATG AACTTCAAGTGGAGT G AACTTCGGAAAAAGTCTCTCAACATT AG 57	8400
N M N F K W S E L R K K S L N I R G TCCCATTTG	2800
S H L	

GAAGCCAGTTCTGACCAGTGAAGCGTCTGCACCTTTCTCTGCAGGAAGTTCTGGTGTGG E A S S D Q W K R L H L S L Q E L L V W	8460 2820
CTACAGCTGAAAGATGATGAATTAAAGCCGGCAGGCACCTATTGGAGGCGACTTTCCAGCA L Q L K D D E L S R Q A P I G G D F P A	8520 2840
GTTCAGAAGCAGAACGATGTACATAGG 58. V Q K Q N D V H R GCCTCAAGAGGGAATTGAAAACATAAAGAACCT	8580 2860
GTAATCATGAGTACTCTTGAGACTGTACGAATATTTCTGACAGAGCAGCCTTTGGAAGGA V I M S T L E T V R I F L T E Q P L E G	8640 2880
CTAGAGAAACTCTACCAGGAGCCAGAG 59 L E K L Y Q E P R E AGCTGCCTCCTGAGGAGAGAGCCAGAATGTC	8700 2900
ACTCGGCTTCTACGAAAGCAGGCTGAGGAGGTCAATACTGAGTGGGAAAAATTGAACCTG T R L L R K Q A E E V N T E W E K L N L	8760 2920
CACTCCGCTGACTGGCAGAGAAAAATAGATGAGACCCTTGAAAGACTCCAGGAACCTCAA H S A D W Q R K I D E T L E R L Q E L Q	8820 2940
GAGGCCACGGATGAGCTGGACCTCAAGCTGCGCCAAGCTGAGGTGATCAAGGGATCCTGG E A T D E L D L K L R Q A E V I K G S W	8880 2960
CAGCCCGTGGGCGATCTCCTCATGACTCTCTCCAAGATCACCTCGAGAAAGTCAAG 60. Q P V G D L L I D S L Q D H L E K V K GCA	8940 2980
CTTCGAGGAGAAATTGCGCCTCTGAAAGAGAACGTGAGCCACGTCAATGACCTTGCTCGC L R G E I A P L K E N V S H V N D L A R	9000 3000
CAGCTTACCACTTTGGGCATTCAGCTCTCACCGTATAACCTCAGCACTCTGGAAGACCTG Q L T T L G I Q L S P Y N L S T L E D L	9060 3020
AACACCAGATGGAAGCTTCTGCAG 61 N T R W K L L Q GTGGCCGTCGAGGACCGAGTCAGGCAGCTGCATGAA	9120 3040
GCCACAGGGACTTTGGTCCAGCATCTCAGCACTTTCTTTCCA 62 A H R D F G P A S Q H F L S T CGTCTGTCCAGGGTCCC	9180 3060
TGGGAGAGAGCCATCTCGCAAACAAGTGCCTACTATATCAA 63 W E R A I S P N K V P Y Y I N CCACGAGACTCAAACA	9240 3080
ACTTGCTGGGACCATCCCAAAATGACAGAGCTCTACCAGTCTTTAG 64 T C W D H P K M T E L Y Q S L A CTGACCTGAATAAT	9300 3100
GTCAGATTCTCAGCTTATAGGACTGCCATGAAACTCCGAAGACTGCAGAAGGCCCTTTGC V R F S A Y R T A M K L R R L Q K A L C	9360 3120
T 65 L TGATCTCTGAGCCTGTGAGTGCATGTGATGCCTTGGACCAGCACAACCTCAAGCAA	9420 3140
L D L L S L S A A C D A L D Q H N L K Q	

AATGACCAGCCCATGGATATCCTGCAGATTATTAATTGTT TG ACCACTATTTATGACCGC	9480
N D Q P M D I L Q I I N C L T T I Y D R	3160
CTGGAGCAAGAGCACAACAATTTGGTCAACGTCCCTCTCTGCGTGGATATGTGTCT TGAAC	9540
L E Q E H N N L V N V P L C V D M C L N	3180
TGGCTGCT TGA ATGTTTATGATAC 66 GGGACGAACAGGGAGGATCCGTGTCTCTTTT TAAA	9600
W L L N V Y D T G R T G R I R V L S F K	3200
ACTGGCATCATTTCCCTGTGT TAA AGCACATTTGGAAGACAAGTACAGAT 67 ACCTTTT CAAG	9660
T G I I S L C K A H L E D K Y R Y L F K	3220
CAAGTGGCAAGTTCAACAGGATTTTGT TGA CCAGCGCAGGCTGGGCCTCCTTCTGCATGAT	9720
Q V A S S T G F C D Q R R L G L L L H D	3240
TCTATCCAAATTC AAGACAGTTGGGT TGA AGTTGCATCCTTTGGGGCAG TAA CATTGAG	9780
S I Q I P R Q L G E V A S F G G S N I E	3260
CCAAGTGTCCGGAGCTGCTTCCAATTT 68 GCT TAA TAAATAAGCCAGAGATCGAAGCGGCCCTC	9840
P S V R S C F Q F A N N K P E I E A A L	3280
TTCT TAG ACTGGAT TGA ACTGGAACCCAGTCCATGGTGTGGCTGCCCGTCCCTGCACAGA	9900
F L D W M R L E P Q S M V W L P V L H R	3300
GTGGCTGCTGCAGAAACTGCCAAGCATCAGGCCAAATGT TAA CATCTGCAAAGAGTGTC	9960
V A A A E T A K H Q A K C N I C K E C P	3320
ATCATTGGATT CAG 69 GTACAGGAGTCT TAA AGCACTTTAATTATGACATCTGCCAAAGCTGC	10020
I I G F R Y R S L K H F N Y D I C Q S C	3340
TTTTTTTCTGGTCGAGTTGCAAAAGGCCATA TAA ATGCACTATCCCATGGTGGAAATATTGC	10080
F F S G R V A K G H K M H Y P M V E Y C	3360
ACTCCG 70 ACTACATCAGGAGAAGATGTTTCGAGACTTTGCCAAGTACT TAA AAAAACAAATTT	10140
T P T T S G E D V R D F A K V L K N K F	3380
CGAACCAAAAGGTATTTTGCGAAGCATCCCCGAATGGGCTACCTGCCAGTGCAGACTGTC	10200
R T K R Y F A K H P R M G Y L P V Q T V	3400
T TAG AGGGGGACAACATGGAAAC 71 TCCCGTTACTCT TG ATCAACTTCTGGCCAG TAG ATTCT	10260
L E G D N M E T P V T L I N F W P V D S	3420
GC 72 GCCTGCCTCGTCCCCTCAGCTTTACACGATGATACTCATTACGCAT TGA ACATTAT	10320
A P A S S P Q L S H D D T H S R I E H Y	3440
GCT TAG CAG 73 GCT TAG CAGAAATGGAAAACAGCAATGGATCTTATCT TAA ATGATAGCATCTCT	10380
A S R L A E M E N S N G S Y L N D S I S	3460
74	

CCTAATGAGAGCAT AGATGATGAACATTTGTAA TCCAGCATTACTGCCAAAGTT TGAAC	10440
P N E S I D D E H L L I Q H Y C Q S L N	3480
CAGGACTCCCCCT TGAG CCAGCCTCGT AGT CCTGCCAGATCT TGAT TTTCCT TAGAGAGT	10500
Q D S P L S Q P R S P A Q I L I S L E S	3500
GAGGAAAGAGGGGAGCT TAG AGAGAATCC TAG CAGATCTT GAG GAAAGAAAACAG 75 GAATCTG	10560
E E R G E L E R I L A D L E E E N R N L	3520
CAAGCAGAATATGACCGTCT TAA AGCAGCAGCACGAACATA TAA AGGCCTGTCCCCACTGCCG	10620
Q A E Y D R L K Q Q H E H K G L S P L P	3540
TCCCCTCTG AAATG ATGCCACCTCTCCCCAGAGTCCCCGGGATGCT GAG CTCATTGCT	10680
S P P E M M P T S P Q S P R D A E L I A	3560
GAGGCCAAGCTACTGCGTCAACACAAAGGCCCGCCTGGAAGCCAGGATGCAAATCCTGGAA	10740
E A K L L R Q H K G R L E A R M Q I L E	3580
GACCACAATA AA CAGCTGGAGTCACAGTTACACAGGCT TA AGGCAGCTGCTGGAGCAA 76 CCC	10800
D H N K Q L E S Q L H R L R Q L L E Q P	3600
CAGGCAGAGGCCAAAG TGA ATGGCACAACGGTGTCTCTCTCTTCTACCTCTCTACAGAGG	10860
Q A E A K V N G T T V S S P S T S L Q R	3620
TCCGACAGCAGTCAGCCTATGCTGCTCCGAGTGGTTGGCAGTCAAACCTTCGGACTCCATG	10920
S D S S Q P M L L R V V G S Q T S D S M	3640
G 77 GT GAG GAAAGATCTTCTCAGTCTCCCCAGGACACAAGCACAGGGT TAG AGGAGG TGATG	10980
G E E D L L S P P Q D T S T G L E E V M	3660
GAGCAACTCAACAACCTCCTTCCCT AG TTCAAGAG 78 GAAGAAATACCCCTGGAAAGCCA ATG	11040
E Q L N N S F P S S R G R N T P G K P M	3680
AGAGAG 79 GACACAATGTAG	11058
R E / D T M *	3685
H N V G	

APPENDIX B: CINRG SITES

National CINRG Sites	
Children’s National Medical Center Washington, DC	University of Puerto Rico San Juan, PR
Children’s Hospital of Virginia Richmond, VA	Mayo Clinic Rochester, MN
Children’s Hospital of Pittsburgh Pittsburgh, PA	University of Minnesota Minneapolis, MN
University of Tennessee Memphis, TN	Washington University St. Louis, MO
Texas Children’s Hospital Houston, TX	University of California, Davis Sacramento, CA
International CINRG Sites	
Children’s Hospital University Hospitals K.U. Leuven Leuven, Belgium	University of Alberta Edmonton, Alberta, Canada
Hadassah, Hebrew University Hospital Jerusalem, Israel	Bloorview MacMillan Medical Center Toronto, Canada
Centro Clinico NeMO Hospital Niguarda Cà Granda Milano, Italy	Royal Children’s Hospital Melbourne, Australia
Queen Silvia Children’s Hospital Goteborg, Sweden	The Children’s Hospital of Westmead Sydney, Australia
Alberta Children’s Hospital Calgary, Alberta, Canada	Dr. Rangarajan Memorial Hospital Chennai, India
Unidad de Enfermedades Neuromusculares Buenos Aires, Argentina	

APPENDIX C: IRB APPROVAL LETTER



University of Pittsburgh
Institutional Review Board

3500 Fifth Avenue
Pittsburgh, PA 15213
(412) 383-1480
(412) 383-1508 (fax)
<http://www.irb.pitt.edu>

Memorandum

To: MS. LAUREN HACHE
From: SUE BEERS, PhD, Vice Chair
Date: 12/10/2008
IRB#: [PRO08100407](#)
Subject: Characterization of the mutations reported in Duchenne muscular dystrophy: Do genotype-phenotype correlations exist?

The above-referenced project has been reviewed by the Institutional Review Board. Based on the information provided, this project meets all the necessary criteria for an exemption, and is hereby designated as "exempt" under section 45 CFR 46.101(b)(4).

Please note the following information:

- If any modifications are made to this project, use the " **Send Comments to IRB Staff**" process from the project workspace to request a review to ensure it continues to meet the exempt category.
- Upon completion of your project, be sure to finalize the project by submitting a "**Study Completed**" report from the project workspace.

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.

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