Duchenne muscular dystrophy

Duchenne muscular dystrophy: The NIMHANS experience

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I dedicate this Thesis to my MOTHER who always encouraged us daughters and every woman to achieve the highest academic level in their life and also to my CHILDREN who understood my busy schedule and have allowed me to pursue my ambitions

Abbreviations

CK	Creatine Kinase
CNS	Central Nervous system
DMD	Duchenne Muscular Dystrophy
DNA	De-oxy Ribonucleic acid
IQ	Intelligence Quotient
mPCR	Multiplex Polymerase Chain Reaction
MLPA	Multiplex Ligation Probe Amplification
MRI	Magnetic Resonance Imaging
NGS	Next Generation Sequencing
RNA	Ribonucleic Acid

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CHAPTER 1

Introduction, aim and outline of the thesis

Introduction

The X-linked Duchenne muscular dystrophy (DMD) occurs due to mutations in the DMD gene, which consists of 79 exons encoding the dystrophin protein. DMD gene is the largest gene in human beings 2.5 million base pairs, comprising 0.1% of total human genome and 1.5% of X- chromosome 427-kDa protein (99% introns) provides structural stability connecting Actin and the Dystrophin associated glycoprotein complex (DAG) (Figures 1 and 2). DMD has a commonly cited incidence of 1 in 3500 live male births (Emery AE. 1991) but a recent survey of published data from a variety of newborn screening studies shows the reported incidence to range from 1:3802 to 1:6291 (Mendell JR., 2012).

Classically out-of-frame deletions result in a truncated reading frame and DMD is nearly always associated with a complete absence of dystrophin and a severe phenotype of dystrophinopathy. One of the primary roles of dystrophin is to link the cytoskeleton to the extracellular matrix, via the transmembrane dystroglycan protein and its associated protein complex including the sarcoglycans. Dystrophin binds to cytoskeletal actin via its N-terminal actin-binding domain 1 (ABD1) and to β-dystroglycan via its C-terminal domain. The central rod domain, consisting of 24 spectrin-like repeats, is located in between the two binding sites (Figure 2). In the absence of dystrophin, the muscle membrane is susceptible to damage and muscle fiber deterioration occurs, resulting in cycles of regeneration and degeneration that result in fibrosis and fatty replacement of muscle. The partially functional nature of internally truncated dystrophin provides a route for novel therapies.

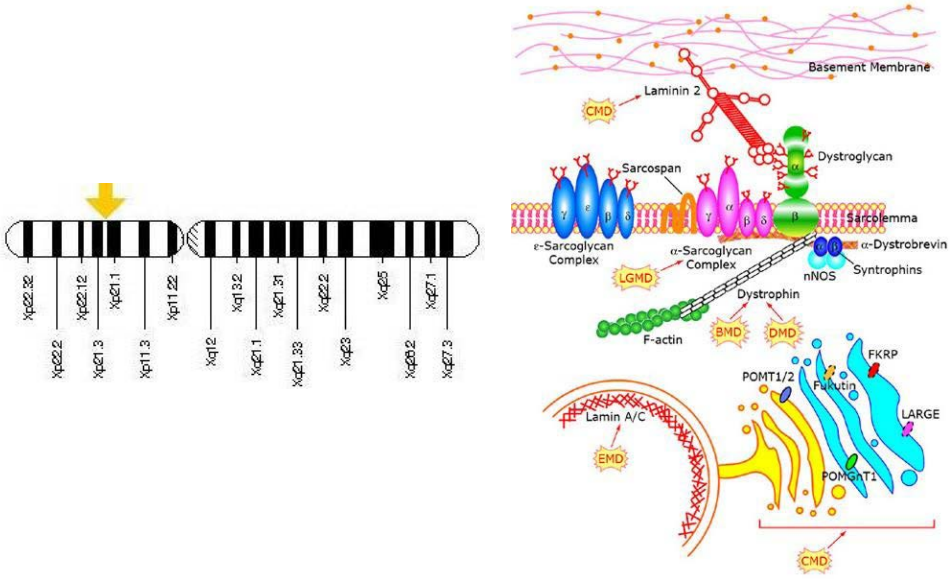

Figure 1. Dystrophin associated glycoprotein complex (DAG). Syntrophin, Sodium channel TRPC channel, calcium homeostasis, cell signalling. Dystrophin is a 427 kDa sub-membrane cytoskeletal protein, associated with the inner surface membrane and incorporated in a large macromolecular complex of proteins, the dystrophin associated glycoprotein complex (DAG). In addition to dystrophin the DAPC is composed of dystroglycans, sarcoglycans, sarcospan, dystrobrevins and syntrophin.

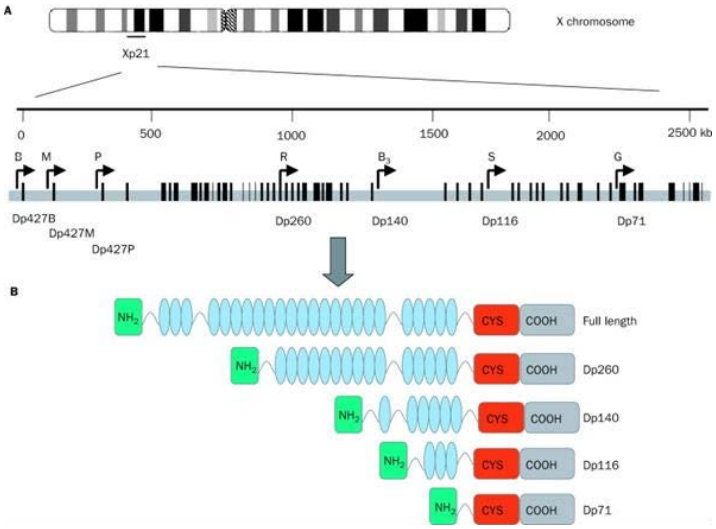

Figure 2. Schematic diagram depicting the Dystrophin Isoforms. Different DMD/dystrophin proteins (**Dp**) is based on their length in kiloDaltons (Dp**427**, Dp**260**, etc.), the tissue of expression (Dp427**m** from muscle, Dp427**p** from Purkinje cells, etc.) and the differential splicing pattern generating them (Dp71**a**, Dp71**b**, etc.)

Clinical findings

Boys with DMD are typically brought to the hospital between the ages of 2 and 5 years. Delayed gait is sometimes described, but alteration of gait is the most common presenting symptom. Toe walking often leads to referral to physical therapists or orthopedic surgeons before recognition of DMD. However, recent studies show that motor function is impaired in the infantile phase of DMD (Connolly AM et al., 2013) and assessment of serum creatine kinase (CK) is recommended as part of the routine screening of all infants with motor delay (Noritz GH et al., 2013). Cognition is also affected, and language development is delayed (Cyrulnik SE et al., 2007). Intelligence quotient (IQ) is diminished by one standard deviation, although verbal IQ improves with age (Cotton SM et al., 2005; Cotton S et al., 2001). There is an increased risk of autism, attention deficit hyperactivity disorder, and obsessive-compulsive disorder in DMD boys (Hendriksen JG et al., 2008).

Proximal weakness is typically evident to the pediatrician / neurologist at the time of presentation, seen as difficulty in climbing stairs, hopping, or arising from the floor with classical Gower maneuver. Muscle enlargement, particularly of the calves, is common; although classically called pseudohypertrophy (Duchenne GB. 1868), and attributed to fibrosis and fatty replacement, true hypertrophy of contractile mass is also present (Kornegay JN et al., 2012).

Tightness of the Achilles tendon and mild lordosis are frequently seen at diagnosis and along with gait disturbance. The mean age at which in retrospect the first symptoms were noted is in between 3 and 4 years whereas mean age at diagnosis is close to 5 years (Ciafaloni E et al., 2009).

DMD usually follows a predictable clinical course (Brooke MH et al., 1983; Brooke MH et al., 1981; McDonald CM et al., 1995; Nicholson LV et al., 1993). In the absence of steroids, the loss of independent ambulation occurs by the age of 12 years. Thereafter, arm function declines, but the major determinant of morbidity is progressive respiratory insufficiency. Forced vital capacity declines after loss of ambulation,(McDonald CM et al., 1995; Tangsrud S et al., 2001), and scoliosis is frequent. Prevalence of Cardiomyopathy increases with age, and without ventilatory intervention, death in the absence of steroid therapy typically occurs by the age of 20 years.

Diagnosis

Biochemical assays

Serum CK is universally elevated in DMD, presumably due to increased permeability of the sarcolemmal membrane, and this is always the initial diagnostic test performed. In DMD it is often 50 to 100 times normal values (Zatz M et al.,1991). Serum CK is elevated at birth, providing the opportunity for newborn screening (Drummond LM et al., 1979). Although not a specific finding, the transaminases aspartate aminotransferase and alanine aminotransferase are also elevated (Morse RP et al., 1993). This finding on several occasions leads to multiple investigations to look for liver disorders including unnecessary liver biopsies. Gamma-glutamyl transferase level is instead a useful marker of liver injury (Rosales XQ et al., 2008). In this population, assessment of renal function should make use of cystatin C rather than serum creatinine or creatinine clearance, both of which are diminished in DMD (Viollet L et al., 2009).

Muscle biopsy

Although advances in molecular diagnostics (see below) have diminished the role of muscle biopsy in the diagnosis of DMD, muscle biopsy is still in relatively common use. Absent or altered dystrophin expression in a muscle biopsy specimen remains a gold standard of diagnosis, and it is important to remember that current methods of genomic analysis detect only around 93% to 96% of mutations (Dent KM et al., 2005). The remainder consists of mRNA rearrangements, including pseudoexon insertions (Gurvich OL et al., 2008) that require analysis of muscle-derived mRNA to detect.

Histopathology

The characteristic histopathology changes seen in DMD muscle result from deficiency of dystrophin. The resulting loss of muscle fiber integrity leads to myofiber necrosis, muscle fibrosis, and failure of regenerative capacity. The chronic and severe myopathic changes that lead to the description of dystrophic findings include fibrosis and fatty replacement, which increase with time, leading to end-stage myopathic change.

Assessment of dystrophin expression

Histopathologic changes seen in DMD are not pathognomonic as they may occur in many muscular dystrophies, especially limb-girdle muscular dystrophies due to sarcoglycan mutations. DMD diagnosis from muscle biopsy therefore depends on analysis of dystrophin expression which can be performed by immunohistochemical (IHC) or immunofluorescent (IF) analysis of muscle sections or by immunoblot (IB) of muscle homogenates. In the clinical laboratory, IHC or IF is performed using antibodies directed toward the N-terminal, the central rod, and the C-terminal domains. The use of rod domain antibodies alone may result in a mistaken interpretation of dystrophin absence, if an internally truncated BMD-associated protein lacks the epitope toward which the antibody is directed.

If appropriate antibodies are used, a complete absence of dystrophin reliably predicts a DMD phenotype. However, in intermediate forms of DMD, up to 50% of DMD patient muscle biopsies frequently show small foci or clusters of fibers with significant dystrophin expression (Arechavala-Gomeza V et al., 2010; Nicholson LV et al., 1989) under standard staining. These fiber clusters are revertant fibers, due to secondary alterations in the gene such as altered pre-mRNA splicing that allow expression of some dystrophin. However, these fiber clusters do not have a clear association with disease severity.

In contrast to IF or IHC staining, IB provides information on both the size and the amount of dystrophin. Although significant differences in methodology among laboratories make an absolute threshold difficult to establish, values of less than 3% are still used in some laboratories as indicative for DMD diagnosis.

Rating scales for DMD disease severity

A variety of outcome measures have been used in the clinical trial setting or natural history settings, including muscle strength assessment by modified Medical Research Council grading of up to 34 muscles (Brooke MHet al., 1983), quantitative muscle testing (Lerario A etal., 2012), and the 6-minute walk test, which has become a de facto standard in trials over the past several years (McDonald CM et al., 2013). Recently, the DMD-specific North Star Ambulatory Assessment has demonstrated robustness and sensitivity for change in boys with DMD (Mazzone ESet al., 2009). Among tests commonly performed in the clinic, the 10-m walk test (time to walk 10 m) is the only one that has been shown to have prognostic value: a time of greater than 12 seconds predicts wheelchair use within 1 year in 100% of patients (McDonald CM et al., 1995).

Mutational analysis

Complete mutational analysis of the DMD gene from blood lymphocyte-derived DNA is now a readily available and affordable genetic test. In the appropriate clinical setting, it is typically ordered after a clinical suspicion and elevated serum CK level. Detailed characterization of the mutation now is considered the standard of care, because it allows the confirmation of the diagnosis of dystrophinopathy and provides information essential to counseling the family. Further-more, mutation class–specific molecular therapies are in or are nearing clinical trials, and it is imperative to consider the suitability of the patient for these.

The size of the gene has historically presented a challenge for complete molecular diagnosis. The DMD gene is the largest gene known, consisting of 79 exons spread across 2.4 million nucleotides on the X chromosome. Deletions of one or more exons account for approximately 65% of DMD and BMD mutations (Flanigan KM et al., 2009). Early genetic testing for mutation detection were directed toward the detection of deletions that were within or ended within 2 deletion "hot-spots" near the N-terminus and within the central rod region. For many years, the standard clinical test was the economical multiplex polymerase chain reaction (mPCR) test, which checked for the presence of around 25 exons by use of multiple primer sets (Beggs AH et al., 1990). Useful in males who are hemizygous at the DMD locus, this test was not useful for detection of carriers or patients with duplications or point mutations. This mPCR test has been superseded by methods that test the copy number of every exon, including multiplex ligation- dependent probe amplification (Janssen B et al., 2005) and comparative genomic hybridization using either custom or commercial microarrays (del Gaudio D et al., 2008; Hegde MR et al., 2008). These tests readily identify exon duplication mutations (6% of all mutations), define the extent of contiguous exon deletions that extend out of the "hot-spot" exons, and diagnose female carriers.

The remainder of mutations require sequence analysis for detection, including nonsense, small subexonic insertions or deletions, missense, or splice site mutations. A variety of methods are in clinical use, including traditional Sanger sequencing of exons as well as next-generation sequencing approaches. It is important that sequencing must be performed over the entire

DMD coding region (ie, all exons), because nearly all point mutations are "private" and do not occur in hotspots. The distribution of DMD mutation classes varies slightly depending on the sample tested. In an unselected clinical cohort of 68 index cases, exonic deletions accounted for 65% of mutations; exonic duplications for 6%; nonsense mutations for 13%; missense mutations for 4%; and small insertions/deletions for 3% (Dent KM et al., 2005). These numbers differ somewhat for reports from referral laboratory settings. For example, in a large cohort enriched for nondeletion patients, the distribution of mutation classes for all DMD and BMD patients was as follows: exon deletions 42.9%, nonsense mutations 26.5%, small frameshifts 11.4%, duplications 11.0%, splice site mutations 5.8%, and missense mutations 1.4% (Flanigan Km et al., 2009).

Patients with no mutation detectable by modern genomic methods but who have either an X- linked family history or biopsy evidence for a dystrophinopathy frequently have pseudoexon mutations in which a deep intronic mutation creates a cryptic splice donor or acceptor site, resulting in the inclusion of intronic sequencing into the mature mRNA (Gurvich OL et al., 2008). Sequencing of the DMD cDNA derived from muscle mRNA is required for this diagnosis; the same test will identify unusual mutations such as exon inversions, which may go undetected by standard analyses (Bettecken T et al., 1989; Madden HR et al., 2009).

Consistent with the Haldane rule for an X-linked lethal disorder, one-third of DMD cases are *de novo*. Carrier testing should always be considered for mothers of DMD boys, although it is not required in the setting of a clear X-linked history consistent with an obligate carrier status.

Genetic counseling should be provided and address the risk of germline mosaicism in mothers with negative DNA tests, because it may occur in up to 10% (Grimm T et al., 1990).

Table 1. DMD gene and the range of different transcripts encoding various dystrophin isoforms, proteins of varying lengths containing different segments of the basic dystrophin sequence

Name	Synonyms		Protein length	Promoter located at	Expression site
Dp427c	cortical dystrophin	brain or C-dystrophin	427 kDa	5′ Dp427m	brain
Dp427m	muscle dystrophin	M-dystrophin	427 kDa	5′ ofgene	muscle
Dp427p	Purkinje dystrophin	P-dystrophin	427 kDa	3′ Dp427m	Purkinje cells
Dp260	retinal dystrophin	R-dystrophin	260 kDa	intron 29	retina
	Dp260-1	R-1			retina
	Dp260-2	R-2			retina
Dp140			140 kDa	intron 44	central nervous system and kidney
Dp116	apo- dystrophin 2	S-dystrophin	116 kDa	intron 55	Schwann cells
Dp71	apo- dystrophin 1	liver or G-dystrophin	71 kDa (70.4)	intron 62	ubiquitous, central nervous system
	Dp71b		72.2 kDa		ubiquitous
	Dp71a		68.9 kDa		ubiquitous
	Dp71ab		70.8 kDa		ubiquitous
Dp40	apo- dystrophin 3		40 kDa	intron 62	ubiquitous

A - types miss exon 71, **b** - types miss exon 78, **ab** - types exons 71 and 78, **c** - types exons 71-74, etc. **Synonym:** alternative names used in literature. **Protein length:** length of the dystrophin isoform in kiloDalton. **Amino acids:** protein length in amino acids. **mRNA:** length of the mRNA in kilo basepairs. **Expression:** tissues in which the isoform is expressed

Imaging

Imaging has historically been of little use in the diagnosis of DMD, but very recent work has highlighted the sensitivity of muscle magnetic resonance imaging (MRI) and in particular T2 signal and lipid fraction to longitudinal progression of disease, raising the possibility that MRI may become a robust outcome measure for clinical trials (Arpan I et al., 2013; Forbes SC et al., Willcocks RJ et al., 2014).

Diagnostic dilemmas

The diagnosis of DMD can be made on clinical grounds with essentially complete certainty in the presence of an X-linked family history, but even in its absence the classic presentation and the prevalence of the disorder allows diagnosis with very high reliability. The classic mimic of DMD includes the severe forms of the sarcoglycanopathies, those autosomal recessive limb- girdle muscular dystrophies (LGMD2s) that occur due to mutations in the a-sarcoglycan (SGCA; LGMD2D), b-sarcoglycan (SGCB; LGMD2E), g-sarcoglycan (SGCG; LGMD2C), and d-sarcoglycan (SGCD; LGMD2F) genes. In their severe form, historically called severe childhood autosomal recessive muscular dystrophy, (Matsumura K et al., 1992) they can be indistinguishable from DMD except for the pattern of inheritance (which allows girls to be affected). Also mutations in the FKRP gene that lead to LGMD2I can result in a Duchenne like phenotype, including cardiomyopathy (Brockington M et al., 2001). In the setting of negative DMD mutational analysis, these syndromes can be diagnosed by muscle biopsy characterization of sarcoglycan expression and a-dystroglycan glycosylation or by mutational analysis of all of these genes.

Exceptions to the reading frame rule

One diagnostic challenge is that the reading frame rule is accurate 90% of the time, making it necessary for clinicians to recognize that exceptions to it exist. Examples include large in-frame deletions that affect the N-terminal dystrophin actin-binding domain 1 and extend into the central rod domain, thereby often resulting in DMD. Conversely, 14% of nonsense mutations (as predicted by genomic analysis) are associated with Becker Muscular Dystrophy (BMD), rather than the predicted DMD (Flanigan KM et al., 2011). Typically, this occurs due to altered pre-mRNA splicing within the flanking in-frame context of exons 23–42, resulting in an open reading frame and continued protein translation (Flanigan KM et al., 2011). Other classic examples include the relatively common out-of-frame deletion of exons 3–7, which results in both DMD and BMD phenotypes due to downstream translational Initiation (Gangopadhyay SB et al., 1992). The aforementioned examples highlight that prognostication, or phenotypic classification, cannot rely solely on the predicted reading frame; the entire clinical picture must be taken into account, including age at presentation, consistency of examination findings with predicted phenotype, and results of dystrophin expression studies.

Management

Management of DMD involves the multiple systems affected in patients; the significant psychosocial and familial stressors induced by the disease; the burdens of proposed interventions; and the complications induced by the sole therapy to date, corticosteroids.

Management may ideally be provided within a multidisciplinary care setting, which can be organized to provide the components of universal care standards (Bushby K et al., 2010). At a minimum, patients should be seen yearly by a neurologist or rehabilitation physician, cardiologist, pulmonologist, physical and occupational therapists, and nutritionist. Other specialists and consultants often play critical roles in the management of the DMD patient, including endocrinologists, orthopedic surgeons, and social workers, among others.

Management strategies and goals in DMD

DMD children need a standard-of-care protocol and this should be followed diligently to have a good quality of life in all children. In general, points of interest in this protocol are:

- Preservation of strength and ambulation
- Minimization of steroid complications
- Obesity
- Delayed puberty
- Osteoporosis
- Cataracts
- Prevention of complications including contractures
- Minimization of injury risk from falls
- Preservation of ventilatory function
- Prophylaxis or treatment of cardiomyopathy
- Avoidance or treatment of scoliosis
- Appropriate school environment
- Treatment of family stressors

Glucocorticoids can improve muscle function

In DMD, the only medications that have been conclusively demonstrated to affect muscle function are the glucocorticoids prednisone and deflazacort. Their mechanism of action in DMD is unclear, but multiple studies have confirmed a beneficial effect. The original trial established a prednisone dose of 0.75 mg per kg per day as the standard dose, resulting in improved muscle strength at 6 months of therapy (Mendell JR et al., 1989). Comparable efficacy results are found with deflazacort, 0.9 mg per kg per day, with potentially fewer complications and in particular less pronounced weight gain (Bonifati MD et al., 2010). Multiple trials have confirmed positive effects, leading to a consensus that treatment may result in prolongation of ambulation by 1 to 3 years compared with no treatment (Escolar DM et al., 2011).

The side effects of corticosteroid therapy included small stature, weight gain (due to both fat accumulation and increased appetite), increased osteoporosis, an increased risk of cataracts, delayed puberty, and a tendency toward behavioral disturbance. These side effects frequently require careful monitoring (Bushby K et al., 2010). Nutritional input is critical, and bone health screening is essential, with vitamin D supplementation where appropriate and DEXA scans yearly while on steroids.

Current recommendations for initiation suggest starting glucocorticoids therapy between the age of 2 and 5 years if a boy has plateaued in strength. In case strength is declining, it is "highly recommended" to start glucocorticoids (Bushby K et al., 2010). There is general consensus that it should be initiated during the phase of functional motor plateau, whenever that occurs, although treatment by age 5 years is common.). No consensus exists as to whether corticosteroid therapy should be stopped at loss of ambulation, and although this practice is common, some retrospective data suggest that use of low-dose steroids in nonambulant boys results in less scoliosis and in improved ventilation (Flanigan KM et al., 2013).

Cardiac care

Cardiomyopathy is a nearly universal feature of DMD, with an estimated incidence as high as 25% by age 6 years and 59% by age 10 years, (Nigro G et al., 1990), although other studies suggest the median age of onset is around 14 to 15 years (Connuck DM et al., 2008). Although cardiac fibrosis and diastolic dysfunction may precede systolic dysfunction, the systolic dysfunction that is commonly detected on standard clinical echocardiograms may be the earliest feature. Screening echocardiograms are recommended at diagnosis or by age 6 years; every 2 years up to 10 years; and yearly after 10 years. Treatment of cardiomyopathy typically begins with afterload reduction using angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, which likely have similar efficacy (Allen HD et al., 2013). The timing of initiation of these medications remains unclear, although it is suggested that they should be initiated before the identification of systolic dysfunction. Cardiac conduction disturbance is frequent, requiring Holter monitoring for assessment (Corrado Get al., 2002) these may precede systolic dysfunction and necessitate treatment.

Pulmonary care

Ventilatory insufficiency eventually occurs in all cases of DMD and is the major cause of mortality (Birnkrant DJ et al., 2010). Forced vital capacity declines after loss of ambulation because the diaphragm weakens, thoracic capacity declines, and pulmonary morbidity increases. However, the use of a mechanical insufflator/exsufflator decreases the frequency of respiratory infections, and early acquisition of and training with this device should be considered after loss of ambulation. The second crucial intervention is the recognition and treatment of nocturnal ventilatory insufficiency. Historically before the arrival of nocturnal ventilation, the percentage of DMD patients surviving to age 25 years was 13.5%. An elegant study from Italy (Passamano L., 2012) evaluated the percentage of survivors in the different decades. They studied 835 DMD patients followed at the Naples Centre of Cardiomyology and Medical Genetics from 1961 to 2006. Patients were divided, by decade of birth, into 3 groups:

DMD born between 1961 and 1970; 2) DMD born between 1971 and 1980; 3) DMD born between 1981 and 1990; each group was in turn subdivided into 15 two-year classes, from 14 to 40 years of age. A significant decade on decade improvement in survival rate was observed at both the age of 20 (increased from 23.3% of patients in group 1 to 54% of patients in group 2 and to 59,8% in patients in group 3 ($p < 0.001$) and at the age of 25 (increased from 13.5% of patients in group 1 to 31.6% of patients in group 2 and to 49.2% in patients in group 3 ($p < 0.001$).

The causes of death in the Italian cohorts were both cardiac and respiratory, with a prevalence of the respiratory cause of death till 1980s. The overall mean age for cardiac deaths was 19.6 years (range 13.4-27.5), with an increasing age in the last 15 years. The overall mean age for respiratory deaths was 17.7 years (range 11.6-27.5) in patients without a ventilator support while it increased to 27.9 years (range 23-38.6) in patients who could benefit of mechanical ventilation.

A retrospective study among 197 patients showed that early and appropriate nocturnal ventilation resulted in an increase of this percentage to 53%. Snoring, morning headaches,

or excessive daytime sleepiness should lead to a polysomnogram, which may be repeated yearly in nonambulant patients, because their risk for nocturnal hypoventilation increases each year. Patients with either obstructive apnea or hypercapnia are typically treated with bilevel continuous positive airway pressure ventilation.

Scoliosis

The risk of scoliosis increases once ambulation is lost and boys become wheelchair-bound. Scoliosis ultimately affects up to 77% of boys, although it may be delayed by proper wheelchair fitting and by use of steroids (Kinali M et al., 2007). Once scoliosis is clinically evident on examination of the nonambulant boy, yearly spinal radiographs are warranted to assess progression and the need for orthopedic intervention. Orthopedic consultation is advised for curves past 20 degrees.

Novel therapies

Novel therapies are directed toward a variety of gene corrective, gene replacement, and surrogate gene approaches.

Nonsense suppression

Nonsense suppression therapies induce "readthrough" of nonsense mutations, such that instead of translational termination, an amino acid is inserted into the nascent peptide chain and translation continues. The nonsense suppression agent ataluren has shown promise in preclinical studies in the standard DMD mouse model, mdx, which carries a nonsense mutation in exon 23,(Welch EM et al., 2007), as well as in an early phase human trial (Finkel RS et al., 2013). Other agents directed toward the same mechanism are undergoing preclinical studies.

Exon skipping

Exon skipping therapy makes use of the clinical observations inherent in the reading frame rule. Antisense oligonucleotides (AONs) complementary to exon definition elements within a target exon induce the ribosome to ignore an exon during pre-mRNA splicing. Though the resulting mRNA has a larger deletion, it is now an in-frame deletion that can be translated through the C- terminus. Targeting of one exon would theoretically be able to correct multiple DMD mutations. Skipping of exon 45 would correct both deletions of exons 46–47 and exons 46–48, among others. Because of this, targeting a limited number of exons (44, 45, 51, and 53) would be expected to be beneficial to around 35% of all DMD patients and skipping of 2 exons could theoretically be beneficial to 83% (Aartsma-Rus A et al., 2013).

Two different AON chemistries directed toward exon 51 skipping have been used in DMD patient trials: 20O-methyl phosphorothioate AONs and phosphorodiamidate morpholino oligomers. Initial results for each appear to be promising (Cirak S et al., 2011). However, in a the recent small trial (n 512 cell stage) on the use of morpholino, it is shown that after 48 weeks of treatment the distance walked by DMD patients was significantly larger for patients that were immediately treated as compared to the distance walked by those initially treated with placebo. (Cirak S et al., 2011) There were also essentially no side effects, which is a very promising result. If confirmed, this approach may be extended to

other exons as well as to other mutation classes such as single exon duplications, where skipping would restore a wild- type rather than a BMD-like mRNA.

Gene transfer
Current gene transfer approaches in clinical development use adeno-associated viruses (AAVs) to deliver a transgene of interest to muscle. Since AAVs can only carry a transgene of around 5 kb, smaller than the DMD cDNA of nearly 14 kb, current strategies make use of micro- or mini- dystrophin transgenes that contain only 4 spectrin-like repeats in the central rod domain and do not have the C-terminus. Expression in an initial trial was poor, but a trial of an improved vector is planned, and other vectors are in preclinical development (Wang Z et al., 2012).

Summary of current knowledge on Duchenne muscular dystrophy

In short the salient features of clinical, genetic and therapeutic aspects of DMD are:

- Duchenne muscular dystrophy is due to mutations in the DMD gene.
- Mutational analysis of blood samples can lead to the diagnosis of a dystrophinopathy in around 95% of cases.
- Muscle biopsy may still be required in selected cases negative by genetic testing.
- The corticosteroids prednisone and deflazacort are the mainstays of therapy, which should be initiated by the age of 5 years.
- Management of the side effects of corticosteroids is a significant challenge.
- Optimal management of DMD requires multidisciplinary care including neurology, cardiology, pulmonary, physical medicine and rehabilitation, nutrition, physical therapy, and occupational therapy.
- A variety of novel and promising therapies are on the horizon and have entered clinical trials.

Aim and outline of the thesis

This thesis is a compilation of the experience of progress in research on Duchenne Muscular Dystrophy (DMD) over the last 15 years from a multidisciplinary Neuromuscular Disorders clinic (NMD) at a single Quarternary referral Neurological center in India. At the outset from clinical and biochemical diagnosis, to histopathological confirmation, we have evolved through simple genetic to complex genetic analysis, genotype-phenotype correlation, neuropsychological features, muscle MR imaging characteristics, mutations in familial forms of DMD to the latest Quantitative Brain Imaging and Next generation Sequencing (On-going) in DMD.

In the year 2002 we could start mPCR testing for DMD thanks to support by a National Level Project. At that time, only a few laboratories across India performed mPCR testing for DMD because costs were prohibitive. At NIMHANS, e.g., we only used muscle biopsy with immunohistochemistry as diagnostic test for DMD. However, though immunohistochemistry could confirm the diagnosis of DMD it did not provide genetic

diagnosis and, hence, counseling was hampered. In **Chapter 2** we describe this first study on the use of mPCR testing for DMD at NIMHANS. The objectives were to have a systematic assessment of the clinical presentation of DMD and the mutation pattern as tested by mPCR technique in 112 cases of DMD.

Since the beginning of muscle immunohistochemistry in the mid-1990's, the diagnosis of DMD at NIMHANS was confirmed for a large number of children. All these cases were stringently followed up at the neuromuscular disorders clinic at NIMHANS. We performed mPCR testing for all consecutive DMD cases by an NGO and collected many patients with genetic confirmation. From the year 2012 on, MLPA testing commenced at NIMHANS and we further collected this group of cases. In addition, we collected all data of the diagnosed children with respect to their motor abilities at last follow-up as recorded in the medical case records. For those who were lost for follow-up, we posted a simple Questionnaire with return paid envelopes (n= 600) and got back information with regard to their ambulatory status and also death. This data was compiled to study the natural history / disease progression in DMD children evaluated between 1998 and 2014 in an observational study from southern India population, the results of which are presented in **Chapter 3**.

We had a large number of DMD children confirmed by mPCR, MLPA and also muscle biopsy. There were no studies comparing the sensitivity of mPCR and MLPA findings in the same cohort of children. In **Chapter 4** we describe the results of a study in which we performed a comparative analysis of 83 selected cases that had both mPCR and MLPA testing and also muscle biopsy in the negative cases. This was the first study from India and possibly in English literature, to have such a comparative analysis.

At NIMHANS a large number of MLPA confirmed cases were seen over the years and the clinical characteristics, biochemical assay results and genetic data were available. In **Chapter 5** we describe the study that we took up to have a robust genotype-phenotype correlation study on a large sample size. It is very important to have a genotype–phenotype correlation for a given population so that any intervention studies will depend on the genotype and its particular phenotype. Also prognostication is more accurate if this information is available.

From the first description of DMD it is well known that these children have significant cognitive and intellectual disabilities. In **Chapter 6** we describe our study on the Identification of the pattern of behavioural and cognitive disturbances for different population children.

Information on this topic is important so as to have the appropriate evaluation and intervention protocols. Our children mostly came from low socio-economic status that also had features of poor nutrition and low environmental influences. In this first study from India we evaluated the neuropsychological findings in an elaborate systematic manner but in a small group of children. The neuropsychological profile of Duchenne muscular dystrophy helped us to understand the cognitive defects as part of non-motor manifestations in DMD.

Muscle Imaging is a fast emerging non-invasive investigative tool for all types of muscle diseases. Advanced MRI of the whole body to evaluate the pattern of muscle involvement and also quantification of the fat content is being utilized to study the pattern of muscle affliction in different forms of muscle diseases as well as for follow-up studies with drug intervention and to study its effect on the muscle bulk. In the study described in **Chapter 7** we subjected 50 MLPA confirmed children for muscle MRI without any sedation or general anesthesia to describe the pattern of muscle involvement and also to correlate the findings with functional Muscular Dystrophy Functional Rating Scale (MDFRS) as well as muscle strength measurements.

There are no dedicated studies reporting on the clinical and mutation spectrum in familial forms of Duchenne muscular dystrophy. This is the first study on the mutational spectrum in a large cohort of DMD children with an emphasis to compare the mutations in familial and sporadic forms (**Chapter 8**). MLPA identified significantly larger mutations in sporadic than in familial cases. Through NGS, nonsense mutations were more common in familial than in sporadic cases. The familial group reported an earlier onset of disease as compared to sporadic cases. Thus, MLPA could identify mutations in a high percentage of our DMD children. The preponderance of small mutations was noted to be distinctly higher in the familial group. These facts could help in deciding the diagnostic test to be selected in familial forms of DMD.

In our concluding chapter (**Chapter 9**) we present a general discussion where we look at the connection between the studies in Chapters 2 – 8 as well as their possible implications to the field of DMD

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CHAPTER 2

2

Duchenne muscular dystrophy: A clinical, histopathological and genetic study at a neurology tertiary care center in southern India

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Abstract

Background: Duchenne muscular dystrophy (DMD) is the most common muscular dystrophy that affects young boys and the dystrophin gene on the X chromosome has been found to be associated with the disorder.

Materials and methods: In this prospective study, 112 clinically diagnosed DMD patients had muscle biopsy and were tested for exon deletions. Genotyping was also carried out at STR44, STR45, STR49 and STR 50 markers in 15 families.

Results: Of the 112 clinically suspected DMD patients, the diagnosis of DMD was confirmed by histopathology and/or genetics in 101 patients. The mean age of onset was 3.1 6 1.44 years (1-6 years) and the mean age at presentation was 8.0 6 3.1 years (1.1-18.0 years). Delayed motor milestones were present in 63(62.3%) patients. The mean creatine kinase value was 11822.64 6 8206.90 U/L (1240-57,700). Eighty-four patients had muscle biopsy and immunohistochemistry was done in 60 muscle samples, all of which demonstrated absence of dystrophin staining. Of the 60 dystrophin-negative cases, 73% showed deletion of at least one exon. Single exon deletion was found in 20.4%. Distal hotspot Exons 45, 47, 49 and 50 were the commonly deleted xenons and

Conclusions: the deletion rates were 36%, 35%, 33.7% and 38.5% respectively. Conclusions: In this study population in south India the deletion rate was 73% and were more frequent in the distal end exon. With the availability of genetic analysis, the first investigation of choice in DMD should be genetic studies and muscle biopsy should be considered only if the genetic tests are negative or not available.

Key words: Duchenne muscular dystrophy, dystrophin gene, exon deletions

Introduction

Duchenne muscular dystrophy (DMD) is the most common form of all muscular dystrophies with an incidence rate of 1:3500 live male births.[1] Earlier, histopathology was the most widely accepted method of distinguishing the types of muscular dystrophies.[1] Availability of genetic tests make it possible to diagnose these disorders early and also avoid invasive procedures like muscle biopsy. The gene responsible for DMD is one of the longest genes, spanning a length of 2.3 MB and contains 79 exons.[2] The disease is caused by mutations in the dystrophin gene, which leads to the loss of function of the protein.[3] Deletions account for 60-65% cases in DMD; duplications for 5-6% and point mutations for the remaining cases.[4-7] Using primers targeting 18 hotspot exons in the dystrophin gene, 98% of deletions can be detected. The proximal hotspot encompasses Exons 3-7 and the distal hotspot Exons 45-51.[8] The genetic diagnosis for DMD involves multiplex polymerase chain reaction analysis of 27 exons which include these hotspots. Dinucleotide repeat polymorphism-based genetic analysis at short tandem repeats loci within the gene and near the deleted exon can ascertain carrier status in the majority of female relatives.[4,9] The present study reports the analysis of exonic deletions in 112 clinically suspected DMD patients.

Materials and methods

The study was approved by the Institutional Ethics Committee. A prospective study was performed on 112 definite or probable DMD patients. Diagnosis was based on clinical characteristics, elevated serum creatine kinase (CK) and electromyographic features. Other evidences for the diagnosis of DMD prior to genetic analysis included: The absence of dystrophin staining in muscle biopsy and X-linked inheritance pattern of a myopathy clinically compatible with DMD. General intelligence was tested using Binet Kamath Test of Intelligence.[10] Based on the intelligence quotient they were classified as mild mental retardation(38-63), borderline intelligence (63-74), dull normal (75-86), average intelligence (87-112), and bright normal (113-124).

Muscle biopsy

Muscle biopsies were obtained by the open method from quadriceps or biceps muscles. Immunostaining with monoclonal antibodies to dystrophin (1,2,3) (Novocastra, UK)., a-Sarcoglycan and a-2 Laminin (Merosin) as primary and HRP tagged LSAB as secondary was carried out.

Genetic analysis

After written informed consent, genomic DNA was isolated from blood by the salting out method as described by Miller et al.[11] Multiplex polymerase chain reactions (PCR) were carried out for 27 exons according to Chamberlain et al., and Beggs et al.[8,12] PCR products were resolved on 9% PAGE gels or 2% Agarose gels, and the gels were analyzed for exonic deletions by the presence or absence of a corresponding band.

Microsatellite analysis

Genotyping was carried out at STR 45, STR 49 and STR 50, highly polymorphic dinucleotide (CA)n loci in the dystrophin gene.[9] The FAM-labeled products were electrophoresed on a 5% ABI377 sequencing gel.

Results

Clinical findings

Of the 112 clinically suspected DMD boys, the diagnosis of DMD was confirmed by histopathology and immunohistochemistry and/or genetic studies in 101 boys. The clinical details are described in Table 1. All boys presented with progressive proximal muscle weakness particularly of the lower limbs and the majority (90%) complained of calf muscle hypertrophy.

Table 1. Salient clinical features of the patients (N = 101) with Duchenne muscular dystrophy (percentage/range)

Features	N = 101
Mean age of onset (years)	3.1 ± 1.44 (1-6)
Mean age at presentation (years)	8.0 ± 3.1 (1.1-18.0)
Consanguinity	19
Delayed motor milestones	63
Family history [sibling(s)]	21
Progressive lower limb weakness	101
Calf hypertrophy	91
Repeated falls	87
Toe walking	45
LL proximal muscle weakness	101
Contractures	
Ankles	89
Hamstrings	24
Iliopsoas	20
Biceps	5
Hypertrophy	
Deltoid	34
Triceps	16
Quadriceps	19
Calf	91
Extensor digitorum brevis	49
Wasting	
Shoulder girdle	53
Thigh muscles	25
Legs	12
Neck muscles	
Flexors	101

Features	N = 101
Extensors	60
Weakness -upper limbs	
Deltoid	90
Pectorals	101
Biceps	90
Triceps	95
Distal	30
Weakness-lower limbs	
Gluteus maximus/iliopsoas	101
Hip abductors/adductors	85
Quadriceps	100
Hamstrings	70
Tibialis anterior	67
Gastrocnemius	30
Investigations	
CK (units/L)	11822.6 ± 8206.9 (1240-57,700)
Mothers (n 5 57)	270.3 ± 417.3 (52-2679)
Electrocardiography (n = 70)	
Abnormal q-waves	23 (32.8)
Right ventricular dominance	24 (34.3)
2D- Echocardiography (n = 52)	
Normal	50 (96.1)
Bicuspid aortic valve	1 (2.0)
Dilated cardiomyopathy	1 (2.0)
Electromyography (n = 56)	
Myopathic process	56 (100.0)
Intelligence quotient (n = 81)	
Mild mental retardation (38-63)	12 (14.8)
Borderline intelligence (63-74)	27 (33.3)
Dull normal (75-86)	22 (27.1)
Average intelligence (87-112)	16 (19.7)
Bright normal (113-124)	4 (4.9)

Investigations

Details of the investigation findings are given in Table 1. The mean CK value in 57 mothers was 270.3 ± 417.3 U/L (52-2679) and in 20 mother the values were above the upper limit of normal (170U/L) with four individuals having more than 1000 U/L. Of the 56 sisters of the probands tested for serum CK, two had values of 7420 and 23,490 U/L and in the remaining the values were normal. Intelligence Quotient (IQ) done in 81 patients showed varying degrees of mental subnormality. Of the 101 patients for whom the genetic data was available, 84 (83.2%) patients had muscle biopsy. In 60 (71.4%) of these patients immunohistochemistry was done, all of them demonstrated absence of dystrophin staining. Among the 60 dystrophin-negative cases, 44 (73%) patients had deletion of at least one exon.

Genetic findings

The deletion pattern is given in Table 2. Distal hotspot Exons 45, 47, 49 and 50 were common deletions [Figure 1]. Correlation of deletions with immunohistochemistry results is depicted in Figure 2. Mild mental retardation was seen 15% of patients and all of them had at one deletion; 32.1% had borderline intelligence and 80.8% of them had at least one deletion; 27.2% had dull normal IQ and 81.8% of them had deletions; 19.8% had average intelligence and 75% of them had deletions, 5% were with bright normal IQ and 75% of them had deletions.

Table 2. Details of the pattern of Duchenne muscular dystrophy gene deletion

DMD exon deletion pattern	n = 83	%
Single	17	20.4
Distal exon 45	30	36.0
Distal exon 47	29	35.0
Distal exon 49	28	33.7
Distal exon 50	32	38.5
Two or more consecutive exons	49	59.0
Three or more consecutive exons	28	33.7
Proximal hotspot	34	41.0
Distal hotspot	69	83.0

DMD: Duchenne muscular dystrophy

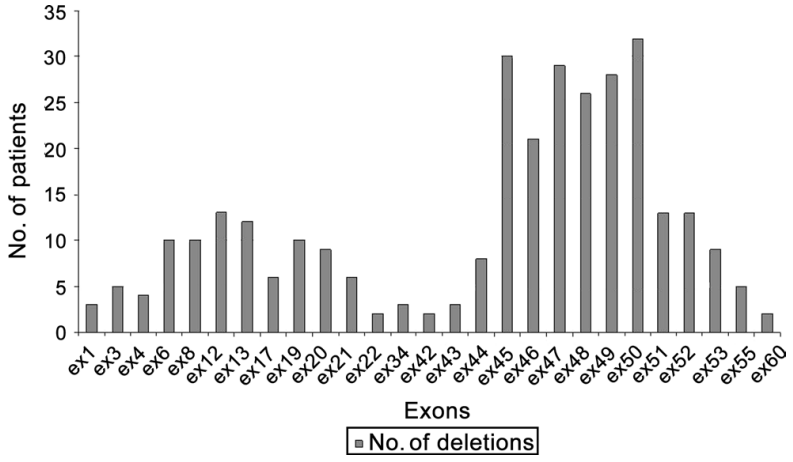

Figure 1: Graph representing exonic deletion pattern in Duchenne muscular dystrophy patients

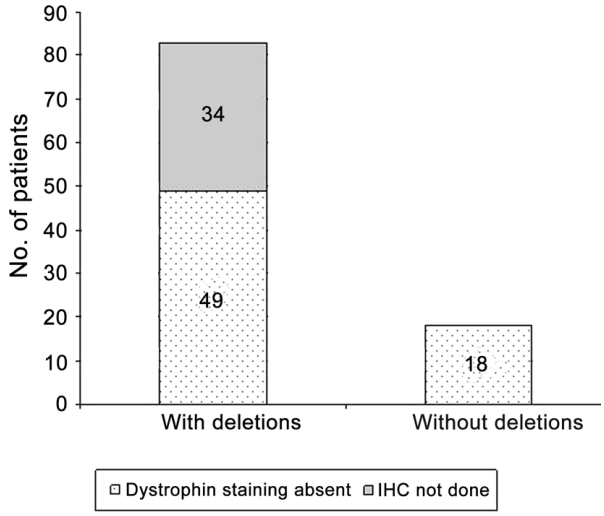

Figure 2. Graph analyzing the histopathological and deletion status among Duchenne muscular dystrophy patients

Carrier testing

Genotyping was undertaken at four short tandem repeat markers reported to have high heterozygosity viz. STR44, STR45, STR49, STR50 in the hotspot region of the dystrophin gene for 'mother and son' samples from 15 families. Six mothers were found to be carriers based on homozygosity at these marker loci. In these six families, positive family history was present in three and in the remaining three the deletions were probably new. Carrier status determination was used to study a DMD family where there was apparent paternal inheritance.[13]

Discussion

This study presents the clinical, histopathological, immunohistochemical and molecular findings in a large cohort of 101 patients with DMD from south India. Random distribution of age of onset was noted in the present study similar the earlier studies.[14] The distribution of motor weakness was similar to that described earlier.[15] Inter-individual variability in the clinical severity was observed among the patients and also with the families similar to the other studies.[16-19]

The reported frequency of the dystrophin gene deletions have been 22% to 86%.[6,20-26] and dystrophin can be demonstrated by immunohistrochemistry in about 98% of DMD patients.[27] In our cohort the diagnosis of DMD was confirmed by either the absence of dystrophin staining only (18%) and/or exon deletions (82%). Correlation between exonic deletion and certain clinical features like ambulation, mental retardation, and histological findings have been observed Mental retardation is seen in about one-third of DMD patients.[23,28] In our cohort mental retardation was seen in 48% of the patients, The lack of correlation between exonic deletions and immunohistochemical findings in 26% of

the patients may be related to deletions in any of the other exons, mosaicism, or point mutation which we have not studied. Such genetic abnormalities have been documented in about 30% of cases.[4-7]

In most of the studies, 80% to 91% of deletions occurred in the distal region of the dystrophin gene[6,24,25] and the deletion rates can be low, 42% to 52%.[6,20,22,24] In the study of 160 Indian patient population from all over the country, the deletion rate was 64.4% and 69.7% of which was in the distal hotspot region. This study did not find any ethnic differences in the deletion patterns of the dystrophin gene.[29] In the eastern Indian study the deletion rate was 63% and 79% of which was in the distal hotspot region.[30] Singh et al., reported a deletion rate of 73% in a north Indian patient population which included both DMD and Beckers muscular dystrophy.[25] Among a southern Indian DMD population the reported deletion rate was 62.1% and 78% of which was located in the distal hotspot region.[31] The authors of this study concluded that the lower deletion rate in their population when compared to the north Indian population may be related to the ethnic differences in the two populations. The deletion rate reported among 25 western Indian DMD patients was 72% mostly located at the 3' hotspot region.[32] The deletion rate in our study was about 73% and was similar to the frequency reported from the other parts of India.[33] The reported frequency of dystrophin gene deletions in other countries in Asia is quite variable: 40.7% in Pakistan,[34] and 66.25% in China.[35] In Egypt, an African country, it was 61.1%.[36]

The diagnosis of DMD is based on clinical, biochemical and histopathologic studies and further confirmed by molecular analysis. However, genetic studies should be the investigations of choice in DMD and muscle biopsy should be limited to the cases where genetic studies are not informative. Carrier state assessment and prenatal diagnosis are essential for counseling and can be offered only after the possible mutation has been identified in the proband.

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CHAPTER 3

3

Natural history of a cohort of Duchenne muscular dystrophy children seen between 1998 and 2014: An observational study from South India

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Abstract

Background: Duchenne muscular dystrophy (DMD) is the most common muscular dystrophy. There are no large studies describing its natural course from India.

Materials and methods: Immunohistochemically/genetically confirmed DMD patients diagnosed between 1998 and 2014 were ambispectively included. The main aim was to study the natural course of motor milestones, i.e., age at onset of wheelchair status, bedbound state, and age at death, which were considered as primary outcome measures. We also correlated the DMD genotype with the motor milestones and other phenotypic features.

Results: A total of 500 DMD patients were included and 275 participated in the study. The mean age at symptom onset was 3.7 ± 1.9 years, mean age at presentation was 8.1 ± 2.5 years, and mean duration of illness was 4.4 ± 2.6 years. On following them over 15 years, 155 (56.4%) had attained at least one of the primary outcome measures. Wheelchair status was attained in 124 (45.1%) [mean age: 10.4 ± 1.6 years] and bedbound state in 24 (8.7%; mean age: 11.8 ± 2.2 years) patients. Seven patients (2.6%) died during the follow-up period (mean age: 15.2 ± 2.4 years). There was no significant impact of the genotypic or phenotypic features on the primary outcome.

Conclusion: The pattern of major motor milestones (primary outcome measures) in this large cohort is comparable with that of the Western population despite variability in medical care. The genotypic pattern was also similar to other large studies, which suggests that DMD is a more homogeneous disorder with limited ethnic variability in its geno-phenotypic expression.

Key words: Duchenne muscular dystrophy, India, natural history

Key Message: The study of natural history of Duchenne muscular dystrophy (DMD) and its survival pattern in a large cohort of patients from India revealed that the age at onset, age at loss of ambulation and death are comparable to the Western literature, despite variability in the medical care provided. The genotype is also similar to the global trends seen in this disease, suggesting a more homogeneous geno-phenotypic presentation of DMD. Oral steroid administration helps in delaying the loss of ambulation and disability arising from this disease.

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive muscular dystrophy and affects 1 in 3600 live male births.[1] It results from mutations in the dystrophin gene at the short arm (locus 21.2) of X chromosome.[2] Its diagnosis is by genetic testing using either multiplex polymerase chain reaction (mPCR) or multiplex ligation-dependent probe amplification (MLPA) techniques to identify exon deletions/duplications. Next generation sequencing (NGS) is helpful in identifying deletion/duplication negative patients, while muscle biopsy with immunohistochemistry or western blot still remains the gold standard technique for genetically unconfirmed cases.

The majority of patients are diagnosed around 5 years of age when their physical ability starts differing significantly from their peers.[3] Muscle weakness, due to ongoing loss of skeletal muscle, results in loss of ambulation at around 10 years of age. As the disease progresses, respiratory, orthopedic, and cardiac complications ensue. Without intervention, the mean age at death is 19 years.[3]

There are no long-term follow-up studies from India to describe the course of the disease and the survival pattern in this population. The present work was an attempt to study the natural history of the disease and its survival pattern in a large cohort of DMD population from India.

Materials and methods

Study design, patient selection, and data collection

This was an observational study with retrospective and prospective patient recruitment from the neuromuscular clinic of a quaternary care university hospital. Patients were identified retrospectively by retrieving case records of those diagnosed with DMD either genetically (mPCR/MLPA or both) or by muscle immunohistochemistry (biceps or quadriceps biopsy) between March 1998 and February 2013. Prospective inclusion was from March 2013 to April 2014.

The study protocol was approved by the institutional ethics committee. All patients or their legal representatives provided written informed consent for participation in the study. A total of 500 patients who had furnished details of postal address were sent a simple questionnaire in English and local language along with return paid post. The clinical details were collected till April 2014 and entered in a pre-designed proforma.

Outcome analysis

Primary outcome measures included age at onset of attaining wheelchair status (loss of ambulation), bedbound (additional truncal weakness) stage, and age at death. Secondary outcome included correlation of severity of intellectual disability, cardiac dysfunction, and degree of spine deformity with the primary outcome measures. Intelligence was assessed by a qualified neuropsychologist using the Binet-Kamat scale.[4]

Statistical analysis

The demographic data is expressed using descriptive statistics {mean ± standard deviation [SD] (range)}. When the data was skewed, median and range are provided. The presenting clinical features at initial evaluation are presented as the number of patients demonstrating individual clinical features. Primary outcome is expressed as the number and percentage of patients with age as mean ± SD (range). Univariate analysis was used for correlating a variable with the primary outcome measure. Wilcoxon log rank test was used for group differences of nonparametric data. Kaplan Meir survival curves are presented for primary outcome measures. The statistical analysis was done using the Statistical Package for the Social Sciences for Windows version 16.0 (SPSS, Chicago, IL, USA). The significance level was fixed at a = 0.05.

Results

Demographic and clinical characteristics of the Duchenne muscular dystrophy population

Among 500 patients, 275 responded or came for follow-up, 31% by completing the questionnaire, and 69% by completing both the questionnaire and attending the outpatient clinic. The demographic features are shown in Table 1.

The mean age at onset of symptoms was 3.7 ± 1.9 (range, 1 to 8) years; 41% of the children had an age of onset less than 3 years. A delay in acquisition of milestones was seen in 57%, and a delay in mental milestones in 14% of the children. The mean age at presentation was 8.1 ± 2.5 (2–15) years. The mean duration of symptoms was 4.3 ± 2.5 years, and the mean duration of follow up was 2.6 ± 2.8 years.

All the cases had lower limb symptoms; more than one-third had associated truncal weakness with difficulty in rising from the supine position, and approximately one-third had upper limb weakness with difficulty in raising arms above the head [Figure 1].

Calf hypertrophy was present in 93.3% and tendoachilles contractures in 70% of the patients. Winging of scapula was seen in 34.5% of the patients and kyphoscoliosis in 13.5% of them. Intelligence assessment revealed average intelligence in 37 (42.0%), dull normal intelligence in 27 (30.6%), borderline intelligence in 14 (15.9%), and mild mental retardation in 9 (10.2%) of the patients. One (1.1%) patient was classified as bright normal. Cardiac evaluation done in 127/275 patients showed an abnormal electrocardiogram (ECG) and/or two-dimensional echocardiogram in 2.9% of the cases.

Primary outcome measures

During the study period of 15 years, 155/275 (56.3%) children had attained at least one of the primary outcome measures [Table 2]. Wheelchair bound status was attained by 124 children at a mean age of 10.3 ± 1.6 years [Figure 2]; and bedbound status was attained by 24 children at a mean age of 11.8 ± 2.1 years [Figure 3]. In 7 patients who died, the mean age at death was 15.2 ± 2.4 (12–19) years. The exact cause of death was unclear as the patients had died at home/local hospital with no clinical details being available.

Table 1. Demographic details of 275 patients with DMD

Variable (*n*=275)	Parameters Mean±SD (range) years
Age at symptom onset	3.7±1.9 (1--8)
Age at presentation	8.1±2.5 (2-15)
Duration of illness	4.3±2.5 (0.5-12)
Duration of follow-up	2.6±2.8 (0.1-13)

n = Number; SD = Standard deviation

Table 2. Statistical data of the primary outcome measures of 155 boys with DMD

Outcome	*n*# (Percentage)	Mean age (in years)*
Wheelchair bound	124 (45.0%)	10.3±1.6 (5-15)
Bedbound	24 (8.7%)	11.8±2.1 (9-17)
Death	7 (2.5%)	15.2±2.4 (12-19)

#*n*, number of patients, *Values are mean±SD (range)

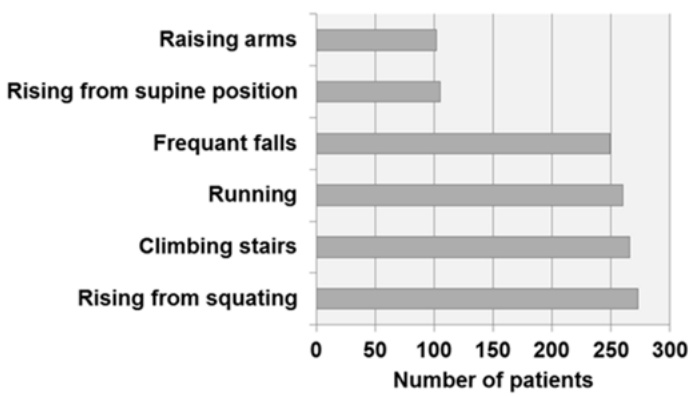

Figure 1. The clinical features in DMD patients at the initial evaluation

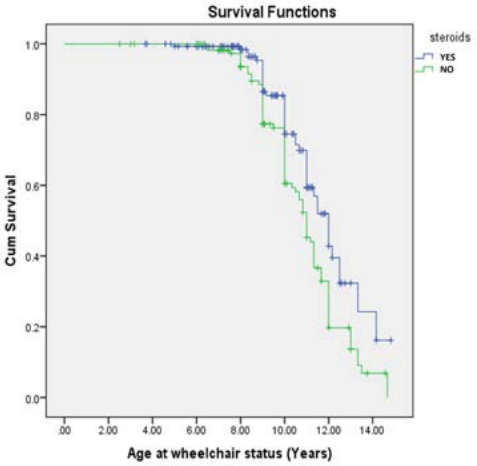

Figure 2. Kaplan Meier survival analysis: Age at onset of wheelchair bound status with and without oral steroid

Molecular and histological characteristics of the Duchenne muscular dystrophy cohort

The genetic data was available in 206/275 boys. Both mPCR and MLPA techniques were used; however, the current hospital policy is only to perform MLPA testing. Gene deletion was present in 204 patients and duplication in 2 patients. Of the 206 boys with gene deletion, 36 had proximal, 158 had distal, and 10 had proximal and distal deletions. Among the 2 duplications, one each had proximal and distal duplication [Table 3]. The type of deletion/duplication had no bearing on any of the primary outcome measures. Immunohistochemical confirmation with total absence of dystrophin antibody staining for all 3 domains was available for 114 patients (in some children, subsequent genetic confirmation was done for genetic counseling).

Treatment and clinical improvement

Around 54.5% of the children were on a regular oral prednisolone dose of 0.75 mg/kg/day whereas others did not agree to take steroids or chose to take alternate forms of therapy/medicines (ayurveda, homeopathy, yoga, etc.), or stopped their medication without medical consultation. In the subgroup of patients on steroids, 50% showed reduction and 5.3% showed stabilization in the frequency of falls. Approximately 37.3% showed improvement in the speed of walking, whereas stabilization of walking difficulty was observed in 11.3% of the children. Approximately 13.3% showed improvement in their ability to run and 10% maintained a stable disability. Improvement in their ability to climb stairs was observed in 12% children, and this symptom stabilized in 12.6% of the children. 6% of the patients improved in their ability to rise from the floor and 12% had clinically stable deficit.

Correlation analysis

Correlation analysis [Table 4] showed a significant effect of older age at presentation, longer duration of illness, late toe walking, and intellectual disability, on early loss of ambulation. These factors were identified as significant predictors among the bedbound patients. Cardiac abnormality, kyphoscoliosis, and the gene deletion/duplication pattern did not show any significant effect on the time-to-loss of ambulation. No significant correlation was observed with other parameters. Correlation analysis was not possible between the expired patients and other groups due to the small number of patients present in each of the subgroups. Hence, only their descriptive parameters are presented.

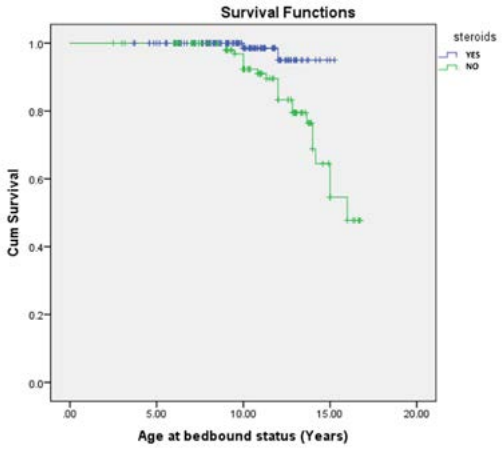

Figure 3. Kaplan Meier survival analysis: Age at onset of bed bound status with and without oral steroid

Table 3. Molecular characteristics of the DMD patients by multiplex polymerase chain reaction (mPCR) or multiplex ligation-dependent probe amplification (MLPA)

Genotype (deletion/ duplication pattern)	Number of patients (*n*=206)	Percentage of patients
Deletion (*n*=204)		
Proximal	36	17.5
Distal	158	76.7
Combined	10	4.9
Duplication (*n*=2)		
Proximal	1	0.5
Distal	1	0.5

Table 4. Correlation analysis of clinical parameters with primary outcome measures

Variable	Wheelchair bound mean±SD; [median, range] (*n*)	Non-wheelchair bound mean±SD; [median, range] (*n*)	P	Bed bound mean±SD; [median, range] (*n*)	Non-bed bound mean±SD; [median, range] (*n*)	P
Age at onset (years)	3.6±1.8; [3, 1-8] (124)	3.7±2.0; [3, 1-8] (151)	0.71	3.3±1.4; [3, 1-7] (24)	3.7±1.9; [3, 1-8] (251)	0.93
Age at onset (years)						
<3	55 (124)	58 (151)	0.57	12 (24)	101 (251)	0.64
3-5	45 (124)	58 (151)		8 (24)	95 (251)	
>5	24 (124)	35 (151)		4 (24)	55 (251)	
Age at presentation (years)	8.6±2.7; [8, 3-15] (124)	7.6±2.2; [8, 2-15] (151)	0.003	8.4±3.3; [8, 3-15] (24)	8.0±2.4; [8, 2-15] (251)	0.04
Duration of illness (years)	4.9±2.5; [5, 0.7-12] (124)	3.8±2.4; [3.5, 0.1-11] (151)	0.001	5.1±3.2; [4, 1-12] (24)	4.2±2.4; [4, 0.1-12] (251)	0.002
Delayed motor milestones	69 (124)	88 (151)	0.71	16 (24)	141 (251)	0.22
Delayed mental milestones	27 (124)	13 (151)	0.003	4 (24)	36 (251)	0.47
Frequent falls	116 (124)	133 (151)	0.15	23 (24)	226 (251)	0.31
Age at onset of falls (years)	4.8±2.4; [5, 1-16.5] (115)	4.5±1.9; [5, 1-9] (133)	0.30	4.9±1.8; [5, 2-9] (23)	4.6±2.1; [5, 1-16.5] (225)	0.65
Toe walking	88 (124)	43 (151)	0.001	19 (24)	112 (251)	0.001
Age at onset of toe walking	6.0±2.0; [1.5-10] (82)	5.8±2.4; [6, 1-12] (40)	0.06	5.6±1.8; [6, 3-9] (19)	5.9±2.2; [6,1-12] (103)	0.58
(years)						
Intellectual ability (IQ ≥90)	6 (124)	39 (151)	0.001	0 (1)	29 (71)	-
Cardiac abnormality	4 (49)	4 (73)	0.41	0 (8)	8 (114)	-
Thoracic kyphoscoliosis	19 (124)	18 (151)	0.48	5 (24)	32 (251)	0.21
Family history	24 (124)	38 (151)	0.31	4 (24)	58 (251)	0.33
Genotype						
Deletion size	34 (64)	75 (139)		6 (11)	103 (192)	0.95
≥5	30 (64)	64 (139)	0.91	-	-	
Deletion site						
Proximal	13 (57)	23 (137)	-	2 (10)	34 (184)	0.90
Distal	44 (57)	114 (137)	0.32	8 (10)	150 (184)	

*Figure in parenthesis indicate number of patients, Bold values: *P* value <0.05 is significant; IQ: Intelligence quotient

Discussion

Demography and diagnostic delay

In our cohort, the onset of symptoms was approximately at 3.7 years of age, which is consistent with other studies published from India and elsewhere.[5-7] The duration from the symptom onset-to-diagnosis was 4.3 years, which is also similar to the studies published in the Western literature.[8-10] This could be due to a lack of awareness of the existence of the disease by the parents, or due to a diagnostic delay by the primary care physicians/ pediatricians, especially when the phenotype of the patients is skewed toward mental or language delay. Mohamed et al., reported the presence of nonmotor phenotype as an important contributor to the late diagnosis of the disease.[11] Recent reports also highlight an unchanged pattern of diagnostic delay over the last 2 decades.[12,13] In addition, lack of testing for creatine kinase (CK) levels in children with motor/ developmental delay also contributed to the diagnostic delay.[9,10] We encountered a few patients who underwent exhaustive diagnostic work-up for liver pathology due to raised alanine transaminase (ALT) and asparate transaminase [AST] levels (one patient even underwent liver biopsy twice). Similar instances have been reported in literature resulting in delayed diagnosis.[10] To circumvent this issue, neonatal screening for DMD has been proposed; however, at present, establishing the utility of these approaches and their implications need further studies.[14,15]

Our cohort consisted mainly of children hailing from a lower socioeconomic status and usually residing in rural areas. They had less access to medical facilities and neurologists, which could be the main reason for the delayed diagnosis. In addition, the low literacy rate among parents and caretakers might have led to the delay in seeking medical advice. On instances, we have observed poor clinical suspicion by the primary care physicians resulting in delayed referral/diagnosis. They tend to consider the delay in motor milestones as a constitutional delay or ascribe it to other nonspecific causes unless there is an affected child in the family.

Clinical features

The history of developmental delay was observed in 57% of the children, and this frequency is similar to an earlier study from India wherein 62.3% children had delayed milestones.[5] In a study by Cyrulnik et al., the authors reported developmental delay in nearly two-third of the children with DMD, which is similar to the findings of the present series.[16] Lower limb symptoms dominated in our cohort, with gait change, running difficulty, frequent falls, and difficulty in rising from squatting position being the most common symptoms. Squatting on the floor is a common practice in Indian culture, often bringing the motor problem into focus. Lower limb predominant phenotype is well in line with the earlier published literature.[7]

The majority of patients with DMD exhibit pseudohypertrophy of the calf muscles. Reporting on calf hypertrophy can be subjective, resulting in variable rates in different studies. In our study, calf hypertrophy was observed in 93.3% of the patients, which is comparable to the study by Pradhan et al., who observed this sign in 94% of the DMD patients.[17] Beenakar et al., used ultrasound as an objective tool for measuring calf

circumference and found a very low prevalence of this finding, which was present in only one-third of the patients.[18] A late presentation allowing for an easier clinical detection of pseudohypertrophy of the calf muscles may be the reason for a better diagnosis of this sign in a larger percentage of patients in our cohort.

Kyphoscoliosis was seen in 13.5% of patients at the time of evaluation in the present study. The age of onset of scoliosis in boys with DMD is related to the age at which they lose their ability to ambulate, which is generally between 10 and 14 years. When they are wheelchair bound, the spinal curves are known to progress and evolve to include the entire thoracic and lumbar spine with potentially dangerous increases in the pelvic obliquity.[19,20] Some studies suggest no beneficial effect of scoliosis surgery on declining respiratory function and no increased life expectancy.[21] None of our patients underwent scoliosis surgery as it is not widely practiced in India.

Cardiac disease in DMD most often manifests as a cardiomyopathy and/or cardiac arrhythmias. Cardiac involvement was seen in a small number of children in our study. Approximately 2.9% had abnormalities detectable either on electrocardiogram (ECG) and/or echocardiography. All these children had hypertrophied cardiomyopathy and abnormal ECG findings. In the present cohort, none had cardiac failure or was on a pacemaker. Gulati et al., studied 30 patients of DMD for evidence of cardiac involvement, and noted that the onset of cardiac dysfunction was usually seen after the age of 10 years.[22]

Cognitive impairment is an important manifestation of dystrophinopathies, especially DMD, and might present challenges while managing these children. Fortunately, severe cognitive impairment is not common in DMD. The mean intelligence quotient (IQ) score in our cohort was 86, which is comparable with the mean score of 83.2 as reported by Magri et al.[23] Among the Egyptian DMD children, the average IQ was more than 80.[24] Emery et al., in their analysis of 721 children reported that the mean IQ score was 82, which is similar to our findings.[25]

Motor milestones

Among the primary outcome measures, the mean age at wheelchair bound stage was 10 years. Despite ethnic variability, DMD patients lose the ability to walk at a median age of 10 years with some losing this ability either earlier than 10 years or after 12 years of age.[26] Kohler et al., studied 43 patients with DMD, and the mean age at which the patients lost their ambulation was 9.4 years.[27] They became dependent on a wheelchair at 14.6 years and had a median survival of 35 years. Parker et al., in their report on patients surviving into adulthood also reported similar observations, with the median age at loss of ambulation of approximately 10 years and wheelchair dependency at 11 years of age.[28] In a study by Rao et al., from India, 38 of 81 patients were wheelchair bound at 13 years of age.[6]

Treatment and course

The most common cause of death in DMD children is respiratory infection.[28,29] Seven patients in our cohort died. with their age ranging between 12 and 19 (median: 15.2) years. However, the cause of death could not be ascertained because they died either at home or at a local hospital and medical details of the cause of death were not available.

The follow-up was particularly difficult in our cohort (as is seen in most of the progressive neuromuscular disorders without definitive treatment), as most of the patients hailed from distant places and villages from where regular communication was difficult.

In 1974, Drachman et al., reported a positive outcome in DMD using steroid medication, which were subsequently confirmed in other studies and meta-analyses.[30-32] Approximately 54.5% of our children received oral daily regimen of prednisolone at 0.75 mg/kg. There was significant improvement in clinical parameters with a good outcome as well as reduction in the frequency of falls, improvement in walking, running, climbing stairs, and ability to rise from the floor.[33] Survival analysis showed an improvement in the survival pattern with steroid therapy along with prolongation of the period of ambulation and delay in the attainment of bedbound status by approximately 21 and 36 months, respectively. These findings are similar to those reported by Desilva and Mendell.[34,35] Griggs showed the dose response and time course of improvement where DMD children with 0.75 mg/kg of prednisolone were significantly stronger at follow-up than those treated with 0.3 mg/kg.[33] Many of the parents/caregivers preferred only physiotherapy or alternative medicine because of personal preferences, local beliefs, cultural biases, and an undue apprehension towards the adverse effects of medications, and the lack of efficacy of majority of medicines utilized in treating this disease.

Predictors of motor progression

The age at presentation of symptoms, duration of illness, delay in mental milestones, toe walking, and intellectual ability all showed significant impact on the ambulatory status, though no significant association was observed with age at onset, cardiac abnormality, scoliosis, and genotype. Mirski et al., found that delay in the onset of walking in boys with DMD is strongly associated with a cognitive delay; however, the impact of the latter on loss of ambulation was not discussed.[36]

Conclusion

In conclusion, this is the first study from India describing the natural history of DMD in a large cohort of genetically and/or immunohistochemically confirmed cases. The patterns of major DMD milestones, including the age at onset, age at loss of ambulation and death in our cohort is comparable to that of the Western cohort despite variability in the medical care. The genotype also parallels global trends suggesting a more homogeneous geno-phenotypic presentation of DMD. Oral steroids delay the loss of ambulation and probably add quality years to the life of the patients suffering from DMD. This study also highlights the current scenario of obstacles involved in establishing the diagnosis of DMD and its management.

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Conflicts of interest

There are no conflicts of interest.

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CHAPTER 4

A comparative study of mPCR, MLPA, and muscle biopsy results in a cohort of children with Duchenne muscular dystrophy: A first study

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Abstract

Background: Multiplex ligation-dependant probe amplification (MLPA) is a highly sensitive and rapid alternative to multiplex polymerase chain reaction (PCR). Muscle biopsy should be reserved for mutation-negative cases.

Materials and methods: An attempt was made to compare the sensitivity and pattern of mutations by mPCR and MLPA testing in a cohort with suspected Duchenne muscular dystrophy (DMD). Eighty-three children with DMD were enrolled for mPCR and MLPA testing. MLPA-negative cases underwent muscle immunohistochemistry (IHC) for dystrophin.

Results: Mean age of onset was 45.3 ± 25.2 months; and mean duration of illness was - 53.3 ± 30.8 months. About 11.9% patients had delayed mental milestones. Mean creatine kinase (CK) value was 12136.1 ± 8591.1 LU/L. mPCR detected deletions in 60/83 (72.3%). Proximal deletions were found in 8 (8.6%), distal deletions in 51 (54.8%), and, both proximal and distal deletions were found in 1. Majority of the deletions were <5 exons [34(36.6%)]; two showed large deletions of >10 exons (2.2%). Deletions in hot spot region occurred in 83.3%. MLPA in the same 83 samples detected deletions in an additional six cases and duplications in 6 (6.5%). Combined detection rate of deletion was 79.5%. Duplications were found in 7.2% of the whole sample. MLPA showed 14 (15.1%) proximal and 57 (61.3%) distal deletions, and proximal and distal deletion in 1. Large deletions (>10 exons) were seen in 6.5%, and single deletions were observed in 24 (36.4%). Most common multiple exon deletion was seen at 45–52 region in 7 samples (10.6%). Longest duplication extended from exon 60 to 66. In the 11 MLPA-negative cases, IHC confirmed dystrophinopathy in 36.36%, sarcoglycanopathy in 36.36%, and no deficiency in 27.27%.

Conclusions: This is the first study from India and possibly in English literature, comparing the sensitivity and pattern of mutations by both mPCR and MLPA in the same cohort of DMD. It further validates that 36.4% of MLPA-negative cases were confirmed to have DMD by IHC. The clinical accuracy has been very high in our cohort. MLPA-negative samples should be subjected for next-generation sequencing before contemplating a biopsy.

Key words: Duchenne muscular dystrophy; immunohistochemistry; multiplex polymerase chain reaction (mPCR); multiplex ligation dependant probe amplification; muscle biopsy

Introduction

Duchenne muscular dystrophy (DMD) is a progressive X-linked recessive neuromuscular disorder caused by mutations in the dystrophin gene located at Xp21 chromosome.[1] With an incidence of 1 in 3500–5000 live born males, DMD is the most common lethal muscle disorder in children.[2] Dystrophin gene is the largest known human gene consisting of 79 exons, which encodes a 14kb mRNA.[1,3] Although immunohistochemistry/western (lc w) blotting remains the gold standard for diagnosing DMD[4], lack of any effective treatment has emphasized the need for prenatal diagnosis and carrier detection. With biopsy, one can only make a definitive diagnosis, but this does not help in genetic counseling. Multiplex PCR technique as described by Chamberlain et al. and Beggs et al. has offered a rapid and less-invasive screening tool for detecting deletions in the central and 5′ end hot spot regions of the dystrophin gene.[5,6] Deletions account for about two-thirds of the mutations in dystrophin gene and mPCR allows detection of 98% of those deletions.[5-7] This technique detects large deletions in about 60–65% patients, has largely replaced biopsy, and has become the preferred method of diagnosis in many developing countries like India.[8] However, it is qualitative and does not detect duplications, which account for 6% of mutations in DMD gene.[5,6,9] Multiplex ligation-dependant probe amplification (MLPA), originally developed by Schouten et al., offers a reliable quantitative method to detect deletions and duplications in all 79 exons of the dystrophin gene and also carrier testing.[10,11] MLPA adds another 10–15% positive cases to mPCR. Many neurologists, particularly in India, still perform muscle biopsies to diagnose children with DMD, and this should be performed only after available genetic testing is negative for the mutation.

In this prospective study, we describe the comparative mutational findings by both mPCR and MLPA testing in 83 suspected children of DMD followed by muscle biopsy to confirm the diagnosis of DMD in mutation-negative cases. It is important to consider more advanced genetic testing such as next-generation sequencing (NGS) to diagnose the MLPA-negative cases and, thus, further reduce/avoid the invasive muscle biopsy procedure, which does not assist in genetic counseling.

Materials and methods

Ethical approval for this study was obtained from the institute ethics committee. Written informed consent from the parents/guardian and assent forms were obtained before recruiting the children for the study. This is a prospective study conducted over a 12-month period during 2012–2013, wherein 83 non-random, clinically suspected cases of DMD were recruited from the neurology services/neuromuscular disorders clinic for initial genetic analysis. The collected blood samples of 3 ml each were sent for genetic analysis first by mPCR technique followed by MLPA. Comparative analysis was done to determine the sensitivity and mutational pattern in the two genetic testing methods and also muscle biopsy in MLPA-negative cases as tools for diagnosing DMD.

Multiplex PCR: Genomic DNA was isolated from peripheral blood leukocytes and the test was performed as described by Chamberlain et al. and Beggs et al. for 30 exons corresponding to the hot spot regions.[5,6]

Multiplex ligand-dependant probe amplification (MLPA): The MLPA reaction was performed to screen exons of the dystrophin gene using the SALSA MLPA probe sets P034 and P035 (MRC-Holland, Amsterdam, the Netherlands), according to the manufacturer's instructions. The MLPA samples consisted of approximately 200 ng of genomic DNA. Ligation and amplification were carried out on an ABI 9600 Thermal Cycler. All amplified fragments were separated using capillary electrophoresis on an ABI PRISM 3130 Genetic Analyzer.

Muscle biopsy

Open method was used for obtaining the samples from either the biceps or quadriceps muscle. Immunohistochemical staining was carried out on fresh frozen sections to monoclonal antibodies against dystrophin (1,2,3), sarcoglycans (a,b,d,g), merosin (a2 laminin) as primary, and HRP tagged NOVO-linked secondary antibody (Novocastra Laboratories, Newcastle, UK).

Statistical analysis

Descriptive results were expressed as mean and standard, standard deviation for continuous variables and as the frequency (percentage) for categorical variables. Non-parametric Spearman correlation coefficients were calculated to study the correlation between parameters. All tests were two-sided, and the level of significance was fixed at 0.05.

Results

The mean age of onset was 45.3 ± 25.2 months. The mean duration of illness was 53.3 ± 30.8 months. Majority of the children had delayed acquisition of motor milestones. About 11.9% patients had a history of delayed mental milestones. The predominant symptoms were difficulty in rising from the floor or low chair, climbing stairs and frequent falls. There was a history of consanguineous marriage in 18%. Similar family history was elicited in 32.0% of patients. On physical examination, calf hypertrophy was noted in 94.1% of patients. Other hypertrophied muscles were brachioradialis (9.9%), extensor digitorum brevis (EDB] (10.9%), deltoid (8.9%), quadriceps (4%), and tongue (10.4%). Toe walking was noted in 31.2% and weakness of the pectoral girdle in 25.2% of patients. Winging of the scapula was observed in 85(42.1%) patients. Contractures were common and present at tendoachilles in 75.7%, iliopsoas in 14.9%, hamstrings in 17.3%, and biceps in 4%. The mean creatinine kinase (CK) value was 12136.1 ± 8591.1 LU/L.

Of the 83 samples tested, mPCR detected deletions in 60 cases accounting for 72.3% positivity. Among these 60 deletions, 8 (8.6%) were proximal, 51 (54.8%) were distal, and 1 showed proximal and distal deletions [Figure 1]. While majority of the mutations involved less than 5 exons [34 (36.6%)], mPCR could identify 2 large deletions (2.2%) involving greater than 10 exons [Figure 2]. Deletion in the hot spot region of 44–55 exons was found

in 50 (83.3%) cases. There were 22 single exon deletions (36.6%) in total. Most common deletions were of exon 45 in 10 cases (16.6%) followed by exon 51 and 45 – 52 exons in 6 cases each (10%).

All the 83 samples were further subjected to MLPA analysis, which detected deletions in an additional six cases and duplications in six (6.5%) mPCR-negative samples, thus increasing the combined detection rate of deletions to 79.5%. The rate of duplication detection was 7.2% for the whole cohort. MLPA detected 14 (15.1%) proximal and 57 (61.3%) distal deletions while, in one case, both proximal and distal deletions were found [Figure 1]. As with mPCR, the majority of mutations involved less than 5 exons [41 (44.1%)], but MLPA was able to detect 6 (6.5%) large mutations that involved more than 10 exons [Figure 2]. About 54 mutations were picked up in the 44–55 region accounting for 75% of all mutations detected by MLPA. Single exon deletions were found in 24 cases (36.4%) of which exon 45 was most commonly deleted (10 cases -15%), and exon 51 was deleted in 6 cases. Most common multiple exon deletions were at the 45–52 region in 7 cases (10.6%). Duplications accounted for 6.5% of all the mutations. Of the six duplications detected by MLPA, three were single exon duplications of exons 2, 45, and 52 [Table 1]. Longest duplication extended from exon 60 to 66, i.e., outside the hot spot region.

Muscle biopsy performed in the 11 MLPA-negative cases showed evidence of dystrophic features on routine histology in all these patients. IHC showed absent dystrophin staining in 4 (36.36%), sarcoglycan deficiency in 4 (36.36%), and in the remaining 3 (27.27%) cases, no deficiency was detected [Figure 3].

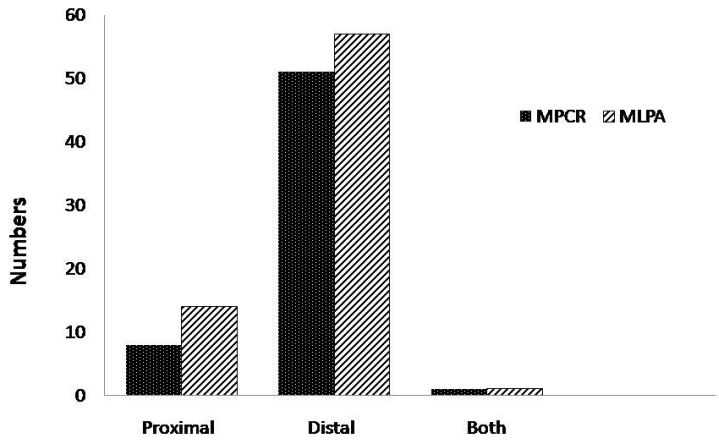

Figure 1. Frequency of proximal and distal mutations as detected by MLPA and multiplex PCR techniques

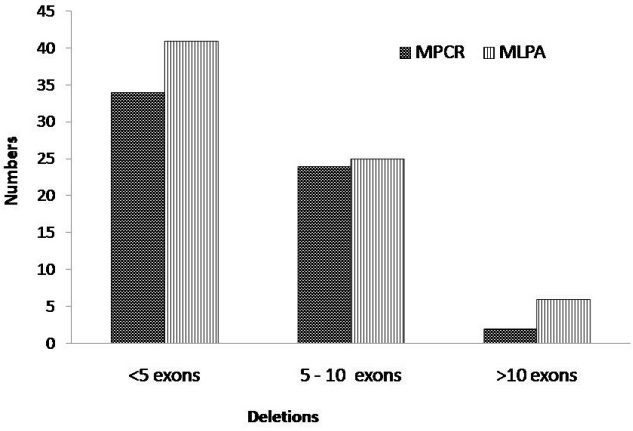

Figure 2. Comparison of small and large mutations as detected by multiplex PCR and MLPA techniques

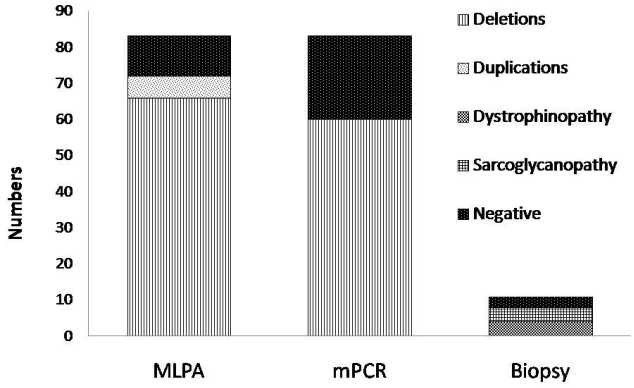

Figure 3. Comparison of the sensitivity and pattern of mutations by mPCR and MLPA methods for diagnosing DMD

Table 1. Details of frequency of exon duplications as detected by MLPA assay

Case no.	Exon duplication	No. of exons duolicated
6	8,9	2
23	52	1
32	60-66	7
35	2	1
63	45-49	5
88	45	1

Discussion

This is the first study from India and possibly in English literature to have compared the sensitivity of detection of mutations by mPCR and MLPA techniques in the same cohort of children with clinically and biochemically suspected cases of DMD and also to have looked at the detection rate by the gold standard method of IHC on muscle biopsy among the MLPA-negative cases.

By both methods, the deletions accounted for 79.5% (66/83) and duplications accounted for 7.22% (6/83), which differed from that observed among other Asian populations where duplication rates were considerably higher and to the extent of 27.3% in Korean[12] and 24.7% in Taiwanese patients,[13] as compared to deletions. Our findings were comparable to that seen those observed among among Caucasians where 63.4% deletions and 7.3% duplications were reported[11,14] and North Chinese populations, which showed 66.2% deletions and 6.25% duplications.[15]

Compared to mPCR positivity, the deletion detection rate by MLPA increased by 7.2% among our cohort as compared to 5.7% among other Asian populations.[12,13] The overall detection rate of our samples by MLPA was 86.8%, and thus, an increase by 14.5% was noted by combining both deletion and duplication rates, and this is similar to that observed among European patients where the improvement in detection rate was about 13%.[11]

Deletions and duplications are known to happen almost anywhere in the dystrophin gene. However, one location toward the central part of the gene (exons 44–55) and the other site toward the 5′ end (exons 2–20) have been reported as the two hotspot regions.[1,6] In our study, the percentage of deletions restricted to the hotspot region decreased from 83.3% with mPCR to 75% with MLPA, suggesting that the overall high detection rate by MLPA could be attributed in part to the detection of additional mutations outside the hot spot region.

It is well known that the underlying mutation is not detectable in at least 4% of cases with available genetic testing,[16,17] due to a possible discrepancy between mutation study and the clinical phenotype. A muscle biopsy subjected for IHC and western blotting is indicated in patients without a detectable mutation.[16,17]

Biopsy done for MLPA-negative patients in our study showed that 36.4% had absent staining for dystrophin antibody, thus confirming the diagnosis of DMD by the gold standard method in more than one-third of the MLPA-negative cases. However, we will not be able to offer genetic counseling without mutation analysis. Identification of Duchenne cases by IHC among the MLPA-negative patients is a clear indication of the possibility of detecting more unidentified mutations, and thus, if there are a high clinical suspicion and positive family history suggestive of DMD, subjecting the mutation-negative cases to novel methods like custom high-density comparative genomic hybridization array (CGH), which analyzes copy number variation across the entire dystrophin gene, and NGS of whole genomic DNA should be considered. Further, this is feasible as these tests are now

available in India.[18-20] NGS has the added advantage of detecting complex rearrangements and large- scale intronic alterations, thus offering a higher mutation detection rate than MLPA and other exon-based tests.[20] However, it is apparent from the majority of genetic studies that all mutations cannot be identified with standard molecular analysis. In these small number of cases, a muscle biopsy may be helpful for protein studies and muscle RNA analysis to establish an accurate diagnosis.

Based on our observation in the suspected cohort of 83 DMD children, genetic study for the dystrophin gene analysis should begin with quantitative screening with MLPA testing, followed by full sequence analysis from genomic DNA. In the recent reports, MLPA-based array analysis system is a simple, rapid, and automated system, providing high resolution and speed. With the costs of advanced genetic tests becoming affordable, MLPA analysis should be enhanced by future technological improvements to further increase mutation detection and avoid the invasive muscle biopsy, which stops at a diagnosis and no further intervention.

The costs of MLPA and mPCR are comparable, and MLPA testing is even cheaper in certain laboratories. MLPA is a highly sensitive and rapid alternative to multiplex PCR. It can be used on blood samples, chorionic villi, and paraffin-embedded tissue. The ease of detection of duplications and the application for female carrier analysis are clearly the main advantages of the method. In our cohort, 36.36% of MLPA-negative cases still had DMD, and this emphasizes the need to consider more advanced genetic testing and consider performing a muscle biopsy only if it becomes mandatory for the diagnosis.

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Conflict of Interest: None of the authors have any financial relationships or other competing interests that could be perceived as biasing this study.

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CHAPTER 5

5

Duchenne muscular dystrophy and Becker muscular dystrophy confirmed by multiplex ligation-dependent probe amplification: Genotype-phenotype correlation in a large cohort

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Abstract

Background and purpose: Studies of cases of Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) confirmed by multiplex ligation-dependent probe amplification (MLPA) have determined the clinical characteristics, genotype, and relations between the reading frame and phenotype for different countries. This is the first such study from India.

Methods: A retrospective genotype-phenotype analysis of 317 MLPA-confirmed patients with DMD or BMD who visited the neuromuscular clinic of a quaternary referral center in southern India.

Results: The 317 patients comprised 279 cases of DMD (88%), 32 of BMD (10.1%), and 6 of intermediate phenotype (1.9%). Deletions accounted for 91.8% of cases, with duplications causing the remaining 8.2%. There were 254 cases of DMD (91%) with deletions and 25 (9%) due to duplications, and 31 cases (96.8%) of BMD with deletions and 1 (3.2%) due to duplication. All six cases of intermediate type were due to deletions. The most-common mutation was a single-exon deletion. Deletions of six or fewer exons constituted 68.8% of cases. The deletion of exon 50 was the most common. The reading-frame rule held in 90% of DMD and 94% of BMD cases. A tendency toward a lower IQ and earlier wheelchair dependence was observed with distal exon deletions, though a significant correlation was not found.

Conclusions: The reading-frame rule held in 90% to 94% of children, which is consistent with reports from other parts of the world. However, testing by MLPA is a limitation, and advanced sequencing methods including analysis of the structure of mutant dystrophin is needed for more-accurate assessments of the genotype-phenotype correlation.

Key words: Duchenne muscular dystrophy, Becker muscular dystrophy, genotype, phenotype.

Introduction

Duchenne muscular dystrophy (DMD) is a severe, progressive X-linked disease affecting 1 in 3,600–6,000 live male births.[1] Becker muscular dystrophy (BMD), which has a milder phenotype, has an incidence of 1 in 18,450 live male births,[2] with the patients experiencing a later onset and longer survival. In the intermediate phenotype (which represents outliers), patients may continue to walk until they are 16 years of age. All three patterns are caused by mutations in the Xp21DMD gene, which encodes a 427-kDa cytoskeletal protein called dystrophin.[3-6]

The DMD gene is the largest human gene, spanning 79 exons. The clinical severity of DMD depends on the disruption or maintenance of reading frames. Out-of-frame deletions usually cause DMD with a complete absence or very low level of dystrophin protein,[7] while deletions maintaining reading frames produce a protein of abnormal low molecular weight that results in the BMD phenotype,[8,9] though there are exceptions to this rule. The reading-frame rule was found to hold for 90% of cases.[10]

Deletions account for 60–65% of cases of BMD and DMD, while duplications cause another 10–15% and the remainder may be due to point mutations.[6] Most of the deletions occur in hot-spot exons 45–53, while the duplications occur mainly in minor hot-spot exons 2–20.[10] Various diagnostic techniques are available, with the main one being multiplex ligation-dependent probe amplification (MLPA). The small mutations that might not be identified by MLPA include small insertions/deletions within an exon, missense/nonsense mutations, and splice-region variants.[6]

Studies of mutation characteristics and the genotype-phenotype correlation have been performed in various countries.[11] The present study is the first large-scale one aimed at identifying the MLPA-based genotype-phenotype correlation in an Indian population.

Methods

This retrospective study involved 317 patients with MLPA- confirmed DMD or BMD who visited the neuromuscular clinic of a quaternary hospital in southern India between 2013 and 2016. Clinical data on the age at onset, presentation, loss of ambulation, and family history were collected. Data on IQ (as assessed using the Binet Kamat scale) and cardiac evaluations were also obtained. The institutional ethics committee approved the study.

The MLPA reaction was carried out to screen the exons of the dystrophin gene using SALSA MLPA P034 and P035 probe sets (MRC Holland, Amsterdam, The Netherlands). The procedure was performed according to the manufacturer's instructions. Amplified products were separated using a genetic analyzer (ABI 3500 XL, MRC Holland) and data were analyzed using Coffalyser software (MRC Holland). Normal healthy individuals were used as controls and included in every run.

Results

Demographic and clinical profile of patients

The 317 patients comprised 279 cases of DMD (88%), 32 cases of BMD (10.1%), and 6 cases of intermediate phenotype (1.9%). Most of the boys exhibited delays in attaining motor milestones. Around 50.8% of them had achieved independent walking after 1.5 years of age, with 59% of them starting to walk after 2 years of age. The age at the onset of illness for the children with DMD was 1.5 to 8 years, and their age at evaluation ranged from 3 to 14 years. Around 9.1% of DMD cases were wheelchair-dependent at evaluation, with this state appearing at a mean age of 9.5 years (range 7 to 12 years). The age range at the evaluation for BMD patients was 16 to 46 years.

Deletions accounted for 91.8% of cases, while duplications caused the remaining 8.2%. The mutation pattern among the 279 cases of DMD comprised 91% deletions and 9% duplications, while the 32 cases of BMD comprised deletions in 96.8% and duplications in 3.2%. The six cases of intermediate type had deletions. Thus, among 291 probands with deletions, 254 had DMD, 31 had BMD, and 6 had the intermediate phenotype, while among the 26 probands with duplications, 25 had DMD and 1 had BMD.

Deletion pattern in the DMD gene

The deletions among the 317 probands were as follows: those in a single exon was the most frequent (24.2%), followed by 3 (15.1%), 2 (9.7%), 5 (8.2%), 6 (7.2%), and 4 (4.1%) exons.

Thus, deletions of six or fewer exons constituted 68.8% of cases. Deletions of more than 10 exons comprised only 8.2% of cases. Among the BMD cases, the most frequent deletion was that of exons 45–47 (37.5%), followed by exons 45–48 (18.7%). The largest deletion found in BMD was that of exons 14–42.

Single-exon deletions constituted in 26.4% of all 291 deletions: exon 45 (29/77) and exon 51 (10/77). Among the 291 cases, exon 50 was most frequently deleted (118/291), followed in order by deletions of exons 48, 49, 46, 47, and 45. Of the 214 cases with multiexon deletions, the 8-exon deletion involving exons 45–52 was the most common (*n*=19), followed by the 3-exon deletion involving exons 45–47 (*n*=18) and a 6-exon deletion involving exons 45–50 (*n*=12). The most-common proximal deletion was from exons 10–17.

Distal deletions constituted 81.7% of all deletions, proximal deletions constituted 15.4%, and proximodistal deletions constituted 2.9%. The largest deletion involved exons 8–47. None of the deletions involved exons 58–63 or 65–79. The frequencies of the deletions are shown in Fig. 1, while Table 1 summarizes the clinical features of common multiexon deletions.

Table 1. Summary of clinical features of the prominent multi exonic deletions

Exons deleted	Number	Mean age at onset (years)	Mean age at presentation (years)	Children with low IQ (*n*)	Families with affected sibling (s)	Number of children who lost ambulation (mean age at loss of ambulation)
45–52	19	4.13	8.9	2	2	6 (9 years)
45–50	12	4.9	7	1	2	-
45–54	8	4.8	8.7	-	-	1 (10 years)
46–51	7	4.4	5.7	1	-	-
10–17	3	2.8	6	-	2	-
10–43	2	3.2	8	-	-	-
48–52	8	5	9.5	-	4	1 (10 years)
8–47	1	2	8	-	4	-

Duplications in the DMD gene

Duplications were identified in 26 children, with the most common involving exons 8, 9, and 60–66 (*n*=2 for each). Only one patient with BMD showed the involvement of exons 4–6 and 61–69. One case of DMD also showed the involvement of exons 45–55 and 63. Single-exon involvement occurred in 23.1% of cases, while six or fewer exons were involved in 50%. Large duplications involving 10 or more exons were found in eight cases (30.7%). Exons 8 and 9 were the most commonly affected (*n*=8 for each, 30.7%). The largest duplication involved exons 3–25. Proximal duplications were seen in 46.1% of cases. None of the duplications involved exons 1, 30–42, and 75–79. The child with the largest duplication of exons 3–25 (an in-frame duplication) exhibited symptom onset at 7 years of age and had presented at 8 years of age. Another large duplication was that of exons 8–29 in a child with symptom onset at 4 years of age. Two other cases of proximal in-frame duplications (involving exons 3–12 and 3–18) experienced symptom onset at 7 and 2 years, respectively. Two patients with duplications had lost ambulation: 1) in-frame duplication of exons 50–55 with symptom onset at 3 years of age and loss of ambulation at 7 years of age, and 2) out-of-frame duplication of exons 18–21 with symptom onset at 3 years of age and loss of ambulation by 12 years of age. The frequencies of duplications are shown in Fig. 2.

Association with reading frames

Among the 279 cases of DMD, 31 cases (11.1%) were due to in-frame deletions or duplications. Of the 291 deletions in the entire cohort, 254 resulted in DMD, 228 were out-of- frame deletions, and 25 (8.9%) were in-frame deletions, of which 12 were proximal deletions: 2 pairs of siblings had deletions of exons 21–23 and 3–34. Two children with DMD also had deletions of exons 45–49, with symptom onset at 3 years of age and presentation for evaluation at 5 years of age. The larger in-frame deletions involved exons 11–31 (onset at 4 years of age), 11–41 (onset at 5 years of age), 3–34 (siblings with onset at 3 years of age), and 21–39 (onset at 3 years of age). One DMD child showed deletion of exons 1 to 29, whose effect may be difficult to predict using the Leiden reading-frame checker. This child experienced symptom onset at 2 years of age and was evaluated at 7.5 years of age.

In-frame deletions contradicting the reading frame were almost equally distributed between the 5′ and 3′ ends of the DMD gene. Two patients with BMD had out-of-frame deletions: one with deletion of exons 42 and 43, symptom onset at 10 years of age, and presentation at 23 years of age, and the other with deletion of exons 45–50, symptom onset at 5 years of age, and presentation at 29 years of age.

There were 26 duplications, of which 25 resulted in DMD: 19 were out of frame and 6 were in frame. These duplications were also equally distributed toward the 5′ and 3′ ends of the dystrophic gene. Out of 6 cases of intermediate dystrophinopathy, 2 had out-of-frame deletions of exons 51–55 and exons 42 and 43, and the remaining 4 were due to in-frame deletions.

Thirty of the 32 cases of BMD were due to in-frame deletions or duplications, with the other 2 comprising out-of- frame deletions of exons 45–50 and exon 50.

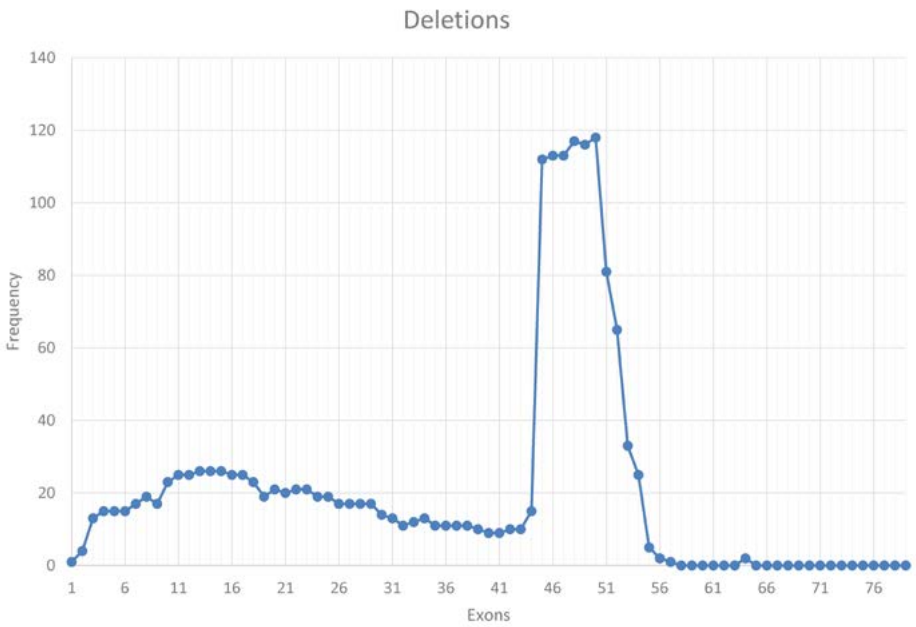

Figure 1. Frequencies of deletions of exons in DMD and BMD. BMD: Becker muscular dystrophy, DMD: Duchenne muscular dystrophy.

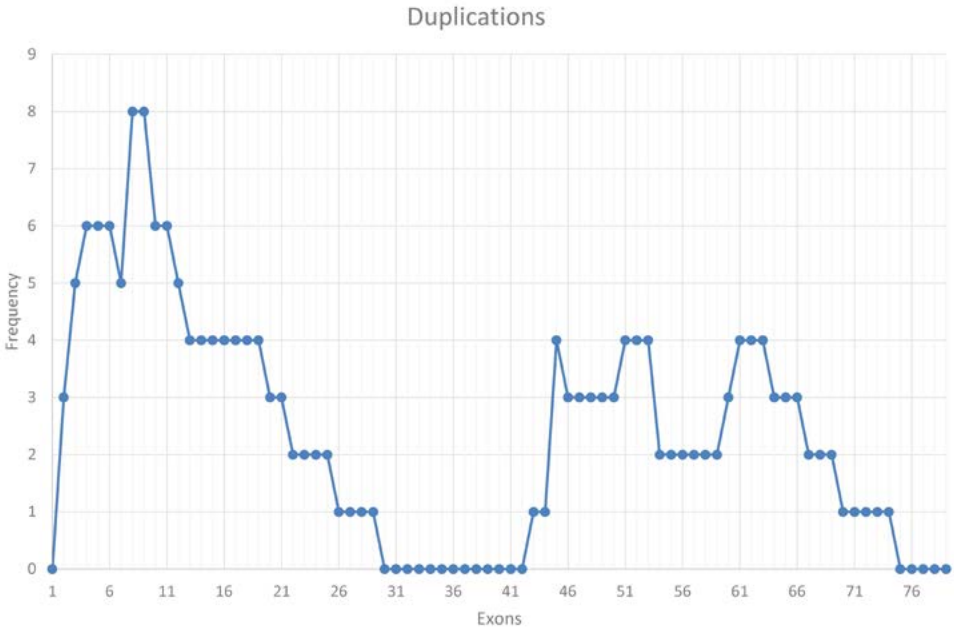

Figure 2. Frequencies of duplications of exons in DMD and BMD. BMD: Becker muscular dystrophy, DMD: Duchenne muscular dystrophy.

Other clinical parameters

IQ tests performed in 29 DMD children showed average intelligence (90–109) in 48.3%, below-average intelligence in 17.2%, borderline (70–79) intelligence in 13.8%, mild mental retardation in 10.3%, and moderate mental retardation in 3.4%, while 6.8% had above-average intelligence. The children with below-average intelligence had deletions toward the distal part of the gene.

Cardiac abnormalities such as dilated cardiomyopathy and left ventricular dysfunction were found in five of the BMD patients. Their age ranged from 22 to 30 years, two of them had deletions of exons 45–48, while one each had deletion of exons 45–49, exon 50, and exons 10–18. Two of the DMD children had congenital heart disease: one had a bicuspid aortic valve and the other had an atrial septal defect with right ventricular volume overload and trivial aortic regurgitation. Two DMD patients also had mild mitral regurgitation and tricuspid regurgitation.

At the time of the last evaluation, 29 children with DMD were wheelchair-dependent, with a mean age at loss of ambulation of 9.6 years. Twenty-seven of them had deletions, most of which were of exons 44–55; the other two had deletions of exons 20–29 and 34–54. Two patients had duplications of exons 50–55 and 18–21. Twenty-one of the 29 DMD patients were not receiving steroids prior to becoming wheelchair-dependent. Six patients had lost ambulation before 8 years of age, two patients had deletions of exons 45–52, one each had deletions of exons 46 and 47, exons 48–50, and exon 50, and one had duplication of exons 50–55.

Discussion

MLPA is a simple, rapid, and reliable screening tool that analyzes all 79 exons of the DMD gene, and can reportedly be used to identify deletions and duplications with a sensitivity ranging from 63% to 79.5%.[11-13] The present study included MLPA-positive cases in the analysis and correlated the findings with various phenotypic presentations/outcomes in dystrophinopathies. More than 50% of our patients exhibited delays in the acquisition of milestones. A study involving cases from eastern India found that 46.9% experienced delays in attaining milestones,[14] which is similar to the present proportion. The mean ages at onset and presentation in our study were 3.4 and 9.5 years, respectively; these ages are very similar to the findings of a previous study from the same institute and also those of other studies from eastern and western India.[14-17]

A family history of DMD or BMD was present in 18.5% of our cohort, which is also comparable to previous findings for the prevalence of family histories of 20–27%.[11,14-16] Despite having a positive family history, in many cases medical attention was sought late,[11] with some of the DMD children already having lost ambulation by the time they visited a neurologist. The most-common phenotype resulting from mutations in the dystrophin gene is DMD. In our study also, 88% of the rearrangements in the dystrophin gene caused DMD. Studies conducted in other parts of India and China have showed similar proportions of around 92%.[11,16]

In our entire cohort, 91% of the cases resulted from deletions. Previous studies from China have found similar rates (84–90%) among genetically confirmed cases, with an overall positivity of 64–67% among all probands suspected of having DMD.[11,18] The reported deletion rate for MLPA-confirmed cases of DMD or BMD has varied from 80% in an Argentinian population[12] to 89% in a Korean population;[19] these proportions are very close to that for our cohort. In contrast, only 59% of MLPA-confirmed children with DMD in a Taiwanese cohort[20] and 67.4% in the Universal Mutation Database-Duchenne muscular dystrophy (UMD-DMD) showed deletions.[21]

In the current cohort, single-exon deletions were most common (26%), and deletions of six or fewer exons constituted 68.7% of cases. Similarly, Magri et al.[22] found that single-exon deletions in the DMD gene constituted 24% of cases. Exon 45 was the most frequently deleted single exon in the present study, while exon 50 was most commonly affected overall. Among multiexon deletions in our study, exons 45–52 were the most commonly involved. Similarly, Yang et al.[11] found that the most-prevalent single-exons deletions involved exons 45 and 51 in a Chinese population. Exons 45–50 were predominantly involved in multiexon deletions, although those of exons 45–52 (as found in our study) were also common. The deletion of exons 45–52 has also been the most common finding in other studies, with single-exon deletions being less common.[11,22]

Most of the deletions were centered in a distal hot-spot region, constituting 81.8% of our cohort. Distal deletions constituted 74% of the deletions in the UMD-DMD,[21] and three patients in that database exhibited deletion of all exons, with one case showing spread of the deletions outside the DMD gene to also involve the contiguous glycerol kinase gene. The largest deletion found in our study extended from exons 8 to 47.

Most of the duplications also resulted in DMD. The duplications of six or fewer exons constituted more than 50% of the present cases. The most-common duplication found in the DMD gene in Western studies is that of exon 2,[21-23] in contrast to our finding of duplications involving exons 8 and 9 being the most frequent. Similar to us, Yang et al.[11] found exons 8 and 9 to be affected in duplications. This suggests that there is a preferential pattern of the involvement of exons 8 and 9 among Asians relative to Caucasians. Single-exon duplications were found in 23% of duplications in our study, while other studies have found prevalence rates of around 20%[11] to 36%.[22] Moreover, multiexon duplications were reported to be frequent in the UMD-DMD, also with involvement of proximal hot spots in 64%.[21] Duplications are said to occur due to unequal crossing over of sister chromatids, interchromosomal events, or tandem duplications due to non- homologous end joining.[23-25] We found one case each of DMD (exons 45–55 and 63) and BMD (exons 4–6 and 61–69 due to two nontandem duplications. Two cases of non-contiguous duplications were found in the UMD-DMD: one of noncontiguous deletion and one of exon triplication. Such duplications involving exons in two separate fragments of the gene may be due to two tandem duplications occurring in the same patient.[25]

No deletions were found in exons 58–63 and 65–79 in our cohort. A Chinese study[11] and the UMD-DMD[21] include cases involving the deletion of all exons. Exons 1, 30–42, and 75–79 were not involved in duplications in our cohort. Similarly, exons 72–79 were not involved in a study from China.[11] The reading-frame rule held for 89% of DMD and 93.7% of BMD patients in our study. Previous studies found that 87.6%, 89.6%, and 96% of DMD patients had out-of-frame deletions or duplications.[11,18,21] There are known exceptions to the reading-frame rule, and there are case reports of relatively benign cases of dystrophinopathy with absent dystrophin expression in muscle fibers.[26] Exon-48 deletions in asymptomatic male patients with ages ranging from 8–58 years in a single family have also been reported.[27] Deletions of exons 3–7, which are predicted to shift the reading frame, are the most-common exceptions to the reading-frame rule and are associated with a wide range in clinical severities from DMD to BMD. Winnard et al.[28] postulated that some amount of dystrophin production could be due to the new initiation of ATG at exon 8 or to a second somatic mutation. Other proposed mechanisms include ribosomal frame shift, cryptic promoter, alternate splicing, and RNA editing, which helps in maintaining the reading frame and a certain amount of dystrophin production.[29,30]

The maintenance of the triple coil structure of wild-type dystrophin requires the constituent amino acids to have a heptad pattern. In-frame deletions that respect the heptad pattern permit for formation of hybrid repeats, and triple coiling may occur. The alpha helix may form in fractional repeats, but triple coiling does not occur due to the absence of a heptad pattern.[31] These differences in structure of mutant truncated dystrophin might account for the variability in the phenotypes of BMD and DMD. A study that analyzed deletions of exons 45–48, 45–51, 45–49, and 45–47 found that proteins corresponding to deletions 45–48 and 45–51 had a similar structure of hybrid-repeat-like wild-type dystrophin compared to fractional repeats with an unrelated structure in another two deletions.[32] That study found that dystrophin levels were moderately reduced in all patients. Fractional repeats have a higher molecular surface hydrophobicity and slower refolding dynamics, which is reflected clinically in the early development of cardiomyopathy and earlier wheelchair dependence

in patients with deletions of exons 45–47. We found that two of the five BMD patients with cardiomyopathy had deletions of exons 45–48, while one had deletions of exons 45–49. The presence of the hybrid repeat alone does not ensure better dystrophin function—this may also depend upon mRNA stability and protein-protein interactions of mutant dystrophin protein. However, the large size of dystrophin makes it difficult to investigate the consequences of all mutations.[32]

Cardiac involvement in DMD and BMD is attributable to replacement of the myocardium by connective tissue or fat at the left ventricular posterobasal part or lateral walls.[33] Cardiac involvement and a low ejection fraction were found in five of our patients with BMD who were aged 22–30 years. Magri et al.[22] reported cardiac dysfunction in DMD patients aged around 15 years. None of those in our DMD cohort had overt cardiac involvement. Further, only a few patients with BMD had evidence of cardiomyopathy, and we could not identify a definite correlation between the mutation type and cardiac involvement. Previous studies have suggested that early cardiac involvement is associated with proximal deletions[22] and deletions of exons 48 and 49.[34] Exon 48 was involved in three of our patients (two with deletions of exons 45–48 and one with deletions of exons 45–49), and one had a proximal deletion. Cardiac involvement is known to have more severe in BMD patients with deletions than in those with duplications,[22] which is consistent with all of our BMD patients with cardiac involvement having deletions.

The IQ as assessed in 29 of our patients indicated average or below-average intelligence in 93% of them, with a low IQ being more pronounced in those with distal deletions. This finding is consistent with the findings of Magri et al.[22] In contrast, a study from Kuwait and Egypt did not find any correlation between IQ and the site or frequency of deletions, although a slightly lower IQ was identified in the presence of deletions of exons 12, 45, and 48.[35]

Loss of ambulation in our patients occurred at a mean of about 9.5 years, which is broadly consistent with previous findings of a mean age of around 10.3 years.[22] Most of our patients who lost ambulation exhibited distal hot-spot region involvement. However, most of them presented late and were not on steroids, and hence a definitive correlation could not be determined. Another study found no definite associations between age at onset, age at wheelchair dependence, and deletion mutations in the DMD gene.[22]

In conclusion, the results in our study are comparable with those obtained in other countries. The reading-frame rule held in more than 90% of the present cases. Though a general trend toward early wheelchair dependence and low IQ was observed in the presence of distal deletions, a definite correlation was not identified. Further studies including analyses of the structure of mutant dystrophin and the definite functional role of other dystrophin isoforms may be needed to identify a definite genotype-phenotype correlation. We acknowledge the limitation that only MLPA-confirmed cases of DMD or BMD were included in this study, and hence future studies that include cases of point mutations will further enhance our understanding of the genotype-phenotype correlation in dystrophinopathies.

Conflicts of interest: The authors have no financial conflicts of interest.

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CHAPTER 6

6

Neuropsychological profile of Duchenne muscular dystrophy

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Abstract

Duchenne muscular dystrophy (DMD) is an inherited myogenic disorder characterized by progressive muscle wasting. DMD is a fatal X-linked recessive disorder with an estimated prevalence of 1 in 3,500 male live births. This disease has long been associated with intellectual impairment. Research has shown that boys with DMD have variable intellectual performance, indicating the presence of specific cognitive deficits. The aim of the study was to use a battery of intelligence, learning, and memory tests to identify a neuropsychological profile in boys with DMD. A total of 22 boys diagnosed with DMD in the age range of 6 to 10 years old were evaluated using the Wechsler Intelligence Scale for Children-Third Edition, Rey's Auditory Verbal Learning Test, and the Memory for Designs Test. The data were interpreted using means, standard deviations, percentages, and percentiles. Normative data were also used for further interpretation. The results showed that boys with DMD had a significantly lower IQ (88.5). Verbal IQ (86.59) was found to be lower than Performance IQ (92.64). There was evidence of impaired performance on the Processing Speed, Freedom From Distractibility, and Verbal Comprehension Indexes. Specific deficits in information processing, complex attention, immediate verbal memory span, verbal working memory, verbal comprehension, vocabulary, visuoconstruction ability, and verbal learning and encoding were observed. However, perceptional organization, general fund of information, abstract reasoning, visual discrimination and acuity, visual learning and memory, and verbal memory were adequate. The neuropsychological findings support the hypothesis that these children have specific cognitive deficits as opposed to a global intellectual deficit.

Key words: cognitive functions, Duchenne muscular dystrophy, intelligence, neuropsychological profile

Introduction

Duchenne muscular dystrophy (DMD) is a neurogenetic developmental disability. It is the commonest of muscular dystrophies with an estimated prevalence of 1 in 3,500 male live births (Emery, 1991). Children with DMD show difficulties in motor abilities such as walking, running, climbing, and jumping by the age of 3 (Gowers, 1879/1991). This is followed by worsening of gait, pseudohypertrophy, falling down and difficulty in standing up, lumbar lordosis, and heel contractures. Weakness of the upper limbs begins by 5 years of age. Ambulation becomes impossible and children are wheelchair-bound at a mean age of 9.5 years (Van Essen et al., 1997). Eventually, patients with DMD die from respiratory insufficiency or from cardiac arrhythmia before 25 years of age (Mukoyama, Kondo, Hizawa, & Nishitani, 1987). Mutations of the dystrophin gene located on the X chromosome cause DMD, thus affecting boys, while girls can be carriers. Approximately one third of all new cases of DMD result from spontaneous mutations (Barbujani et al., 1990). The first writers in the field, Duchenne (1861/1975), Gowers (1879/1991) and Erb (1891/1975),

based on clinical observations studied the relationship between intellectual deficits and DMD. A meta-analysis of intellectual functioning and DMD was conducted using four decades of research (Cotton, Voudouris, & Greenwood, 2001). IQ scores for children with DMD were normally distributed around a mean of 80.2, 1 standard deviation below the normal population. However, researchers argued that children with DMD encounter specific problems in cognitive functioning. A lower Verbal IQ (VIQ) than Performance IQ (PIQ) has been replicated numerous times. However, these discrepancies are not demonstrated by all children with DMD (Cotton et al., 2001).

Impairments have been reported in confrontational naming, verbal fluency, reading, phonological and graphological production, receptive language, expressive language, and verbal learning. Billard, Gillet, Barthez, Hommet, and Bertrand (1998) analyzed the reading abilities and processing of children with DMD, children with spinal muscular atrophy (SMA), and children receiving normal education. Learning disabilities were related to VIQ, similarities and arithmetic subtests, phonological abilities, oral word repetition, and digit span scores. The reading impairment of children with DMD was significant for reading nonwords, suggesting a reading disability similar to dysphonetic-dysdeidetic dyslexia. Cotton, Crowe, and Voudouris (1998) studied the cognitive deficits of boys with DMD who were matched for age, verbal intelligence, and depression to healthy controls. The children with DMD demonstrated significant difficulty for verbal fluency, complex attention, and memory for faces. Hinton, De Vivo, Nereo, Goldstein, and Stern (2000) evaluated the verbal and memory profile of 92 boys with DMD in comparison to their healthy siblings. The verbal and nonverbal test scores were ranked from least to best. Boys with DMD did have a specific cognitive profile; they scored significantly lower on digit span, comprehension, and story recall, irrespective of the level of intelligence. Hinton, De Vivo, Nereo, Goldstein, and Stern (2001) studied the neuropsychological profile of 41 boys with DMD compared to 41 unaffected siblings. The DMD group showed impairment for verbal comprehension of complex items, story recall, digit span, auditory comprehension, and academic achievement. The profile was indicative of selective impairment in

verbal working-memory skill. Hinton, De Vivo, Fee, Goldstein, and Stern (2004) further established that verbal span significantly influenced academic scores, even though vocabulary and home and education environments were comparable. Wicksell, Kihlgren, Melin, and Olofsson (2004) studied memory, information processing/learning ability, and executive functions in children with DMD in comparison to normally developing age-matched individuals. The DMD group showed significant difficulty in memory, learning, and executive functions. Analyses showed that shortterm memory impairment was most apparent. The type of stimulus did not impact the nature of deficit; both verbal-auditory and visuospatial tests showed impairment. Hinton, Fee, Goldstein, and De Vivo (2007) examined the verbal skills and verbal memory skills in boys with DMD. They compared probands to two comparison groups—unaffected siblings and boys with cerebral palsy. The deficits were related to poor verbal span, and not verbal recall.

Research suggests that children with DMD do have specific cognitive deficits, rather than a global intellectual impairment. In the Indian context, there have been studies looking at the neurological, genetic, and biomedical aspects of DMD. However, there is a dearth of research attempting to determine the neuropsychological profile of these children. Jamuna, Taly, and Nalini (2006) assessed the intellectual functioning of 115 boys with DMD. Seventy-two percent of children with DMD had subnormal intelligence, and the mean IQ was 71.86 (*SD* = 18.37). The profile indicated poor social intelligence and verbal and nonverbal reasoning. Further research in identifying a neuropsychological profile would help evaluate the cognitive strengths and deficits of children with DMD. The aim of this study is to examine the neuropsychological profile of DMD. The information obtained from the neuropsychological profile can be used to devise cognitive rehabilitation programs for the identified cognitive deficits. By doing so, the level of functioning and quality of life of these children may be improved, thus enabling the children and their families to better cope with the cognitive and physical disabilities.

Methods

Participants

After obtaining clearance from the ethical committee, boys diagnosed with DMD were selected from the Neuromuscular Dystrophy Clinic in the Department of Neurology at the National Institute of Mental Health and Neurosciences (NIMHANS). A total of 22 schoolgoing boys aged 6 to 10 years old with an IQ greater than 70 were selected for the neuropsychological assessment. Informed consent was obtained. The mean age of the sample was 8 years old (*SD* = 1.24). Mean education was 3rd standard (*SD* = 1.51). All boys were right-handed. All children were ambulatory and did not have any comorbid medical or neurological problems.

Measures

A *sociodemographic data sheet* was used to collect information such as age, education, academic performance, family history, developmental milestones, socioeconomic status, and other relevant clinical information. The *Edinburgh Handedness Inventory* (Oldfield, 1971) was used for assessing handedness of the child. The test consists of 10 items regarding the preference for the left or right hand on various tasks, for which a laterality quotient is obtained. *The Functional Disability Inventory-Parent Form* (Walker & Greene, 1991) is a 15-item inventory used to assess the level of disability an illness has on the child's physical and psychosocial functioning. The response to each item ranges from "0" (no trouble) to "4" (impossible).

The *Wechsler Intelligence Scale for Children-Third Edition* (WISC-III; Wechsler, 1991) is an individually administered tool for assessing intellectual ability in children. The test is for children aged 6 to 16 years and 11 months old. The child's performance on the subtests can be summarized into three composite measures, namely the VIQ, PIQ, and Full-Scale IQ (FSIQ). It also provides four optional factor-based index scores. The WISC-III is also used in neuropsychological evaluation, specifically with regard to brain dysfunction. Large differences in verbal and nonverbal intelligence may indicate specific types of brain damage. The current version of the WISC, the WISC-III, consists of 13 subtests and takes 50 min to 75 min to complete. For the purpose of the present study, the Indian standardized version (Panicker, 2005) was used. There are 6 verbal subtests in the WISC-III. The *Information* subtest consists of a series of orally presented questions that tap the child's knowledge about common events, objects, places, and people. In the *Similarities* subtest, the child is asked how two stimulus words are similar. The stimulus words are objects or concepts, and the child has to respond orally. In the *Arithmetic* subtest, the child is asked to solve a series of arithmetic word problems mentally. In the *Vocabulary* subtest, a word is read to the child, for which a verbal definition is to be given. In the *Comprehension* subtest, the child is asked to respond orally to a series of questions that deal with solutions to everyday problems or understanding social rules and concepts. In the *Digit Span* subtest, the examiner reads a series of number sequences to the child at the rate of one digit per second, and the child is required to repeat each sequence in either the same order (digits forward) or the reverse order (digits backward). There are seven performance subtests: The *Picture Completion* subtest involves a set of colored pictures of common objects and scenes, in which the child has to identify an important part that is missing. In the *Coding* subtest, the child copies symbols that are paired with simple geometric shapes or with numbers. Using a key, the child draws each symbol in its corresponding shape (Coding A) or under its corresponding number (Coding B). Coding Level A is administered for children aged 6 to 7 years and Level B is administered for those aged 8 years and older. The *Picture Arrangement* subtest includes sets of colored pictures that depict a story. The cards are presented in a faulty order, and the child has to arrange the cards in a logical manner. In the *Block Design* subtest, a set of modeled and printed two-dimensional geometric patterns are presented, and the child has to replicate them manually using similarly colored cubes. The *Object Assembly* subtest consists of several pieces of a puzzle, which if put together, will make the shape of a common object. The child is required to assemble the pieces within the time limit. For the *Symbol Search* subtest, the child must visually scan two groups of shapes and indicate by marking a box whether any shapes are common to

both of the groups. The *Mazes* subtest requires the child to solve a series of mazes. In each maze, the child is required to draw a line from the center to the exit without entering any blind alleys or crossing through walls. The time taken and errors committed are recorded. The four factor-based index scores are the Verbal Comprehension Index (VCI), which is a sum of the scaled scores of the Information, Similarities, Vocabulary, and Comprehension subtests; the Perceptual Organization Index (POI), which is the sum of the scaled scores obtained on the Picture Completion, Picture Arrangement, Block Design, and Object Assembly subtests; the Freedom From Distractibility Index (FDI), which is the sum of the scaled scores obtained on the Arithmetic and Digit Span subtests; and the Processing Speed Index (PSI), which is obtained by the summing the scaled scores obtained on the Coding and Symbol Search tests.

Tests of Learning and Memory. Additional neuropsychological tests for learning and memory were selected from the NIMHANS Neuropsychological Battery for Children (Kar, Rao, Chandramouli, & Thennarasu, 2004). Verbal learning and memory were assessed using *Rey's Auditory Verbal Learning Test* (World Health Organization, University of California, Los Angeles Version; Maj, D'Elia, Satz, & Janssen, 1993). It measures immediate memory, acquisition of new learning, retention, primacy and recency effect, and susceptibility to proactive and retroactive interference. It consists of two lists, A and B, of 15 words each. *The Memory for Designs Test* (Jones-Gotman & Milner, 1986) was used to measure visual learning and memory. It consists of 18 abstract designs. The number and complexity of designs vary for different age groups.

Informed consent was obtained from the family members, and participants were informed about participation being voluntary. Twenty-two children diagnosed with DMD were evaluated individually. The duration of overall assessment was approximately 3 hrs; the children were given adequate breaks to avoid fatigue. Feedback was given to the participants after assessments were completed. Behavioral counseling was provided to the family when required.

Analysis

Descriptive statistics such as means, standard deviations, percentages, and Pearson's correlation were used to understand the demographics and neuropsychological measures. A comparison of means was done using the independent *t* test to determine if the neuropsychological measures differed significantly from the normative scores of the respective tests.

Results

Demographics

The mean age of the sample was 8 years (*SD* = 1.24). All the boys were right-handed. Eighteen percent of the sample reported being from a lower socioeconomic status, 46% from a middle socioeconomic status, and 36% from an upper socioeconomic status. The mean education was 5.09 years (*SD* = 1.51). Fifty percent of the sample reported average academic performance, 36% reported above-average performance, and 14% reported below-average performance. Eighty-two percent of the boys did not have a family history of the disorder. From the Functional Disability Scale, it was found that the mean disability was 13.4 (*SD* = 9.1). The demographic characteristics of the sample are presented in Table 1.

Table 1. Demographics of the DMD Sample

	Mean (SD)
Age (years)	8 (1.24)
Education (years)	5.09 (1.51)
Socioeconomic Status (SES)	
Lower SES (%)	18
Middle SES (%)	46
Upper SES (%)	36
Academic Performance	
Above Average (%)	36
Average (%)	50
Below Average (%)	14

Table 2. Means, Standard Deviations, and Range of the DMD Sample on the Neuropsychological Measures

WISC-III Verbal Subtests	**Mean (SD)**	**Range**
Information	9.23 (3.15)	5–14
Similarities	7.00 (3.79)	2–19
Arithmetic	8.50 (2.94)	5–14
Vocabulary	6.82 (3.39)	1–12
Comprehension	9.05 (2.65)	4–14
Digit Span	7.05 (3.03)	4–16
Performance Subtests		
Picture Completion	11.36 (2.32)	7–15
Coding	7.32 (3.26)	2–15
Picture Arrangement	8.36 (2.11)	5–14
Block Design	8.77 (2.98)	4–15
Object Assembly	9.36 (3.11)	4–16
Symbol Search	6.86 (3.44)	0–12
Mazes	10.18 (3.66)	5–19
Factor Indexes		
Verbal Comprehension	86.73 (16.08)	62–123
Perceptual Organization	96.18 (12.15)	76–122
Freedom From Distractibility	85.59 (15.90)	69–125
Processing Speed	81.41 (21.47)	51–154
Rey's Auditory Verbal Learning Test		
Total Learning	44.00 (10.77)	21–60
Delayed Recall	10.14 (2.80)	4–14
Long-Term Percent Retention	86.86 (17.04)	44–125
The Memory for Designs Test		
Total Learning	45.86 (13.48)	21–74
Delayed Recall	11.05 (3.27)	5–17
Long-Term Percent Retention	99.41 (18.51)	58–140

Performance on the WISC-III

The mean scores, *SD*, and ranges obtained are presented in Table 2. On the WISC-III, the mean FSIQ of the DMD sample was 88.5 (*SD* = 13.18, range = 70–116). The mean FSIQ of the DMD sample differed significantly ($t = -5.858$, $p = .000$) from the mean FSIQ (105.01, *SD* = 13.90) of the normative sample (Panicker, 2005). The results showed that 32% of the participants demonstrated borderline intelligence, 27% demonstrated low-average intelligence, 36% demonstrated average intelligence, and 5% demonstrated above-average intelligence. The mean VIQ was 86.59 (*SD* = 16.81) and is significantly different from the normative mean ($t = -4.425$, $p = .000$), indicating low-average verbal intelligence. The mean PIQ was 92.64 (*SD* = 11.81), indicating average performance intelligence, and was significantly different from the normative sample ($t = -5.420$, $p = .000$). The mean VIQ–PIQ was –6.05; 59% of the sample had a VIQ less than the PIQ. However, the mean VIQ–PIQ did not differ significantly from that of the normative sample ($t = 1.642$, $p = .116$). The participants obtained the highest scores on the POI. From the percentiles obtained on each of the factor indexes, it may be noted that 73% of the sample had obtained <15th percentile on the PSI, 55% obtained <15th percentile on the FDI, 54% obtained <15th percentile on VCI, and 14% obtained <15th percentile on POI. Age was correlated using Pearson's correlation with the FSIQ, VIQ, PIQ, and the four factor indexes. There was no significant correlation with any of the measures. Performance on WISC-III was compared to academic performance using the independent *t* test; the values are presented in Table 3. The sample was divided into Group 1, which included children with above-average academic performance, and Group 2, which included children with average or below-average academic performance. Group 1 performed better on the FSIQ ($t = -3.842$, $p = .001$), VIQ ($t = 2.410$, $p = .026$), and PIQ ($t = 4.314$, $p = .000$). Three factor indexes of VCI ($t = -2.978$, $p = .007$), POI ($t = -2.355$, $p = .029$), and FDI ($t = 5.806$, $p = .000$) also showed significant difference. However, the groups did not differ on the PSI. The mean scores obtained by the DMD group on the WISC-III were compared to the mean values from the normative data using the *t* test (Panicker, 2005). The difference in means was significant for the FSIQ, VIQ, PIQ, and the following subtests: Arithmetic, Vocabulary, Comprehension, Coding, Picture Arrangement, Block Design, Object Assembly, Symbol Search, Mazes, and Digit Span. However, the performance of the DMD group on the Information, Similarities, and Picture Completion subtests was comparable to the normative sample. The comparison of mean scores from the DMD sample and normative sample are provided in Table 4.

Performance on Rey's Auditory Verbal Learning Test

On *Rey's Auditory Verbal Learning Test,* from a total of 75 words, which were presented over five trials, the mean number of words totally learned was 44.0 (*SD* = 10.77). Of the 15 words on the delayed trial, the mean number of words recalled was 10 (*SD* = 2.8). The mean long-term percent retention was 86.86% (*SD* = 17.04%). For the scores obtained for Total Learning and Delayed Recall, the corresponding percentiles were obtained from the normative data. It was found that 27% of the participants obtained below the 15th percentile for both Total Learning and Delayed Recall of verbal material. The scores obtained for Total Learning and Delayed Recall did not show any significant correlation to age of the participants. The mean scores for Total Learning and Delayed Recall did not

differ significantly between those boys with above- average academic performance and those with average or below-average academic performance.

Performance on the Memory for Designs Test

On the *Memory for Designs Test,* from a total of 90 designs, which were presented over five trials, the mean number of designs totally learned was 45.86 (*SD* = 13.48). Of the 18 designs on the delayed trial, the mean number of designs recalled was 11.05 (*SD* = 3.27). The mean long- term percent retention was 99.41% (*SD* = 18.51%). The percentiles obtained from the normative data indicated that 14% of the sample obtained below the 15th percentile for Total Learning and 18% obtained below the 15th percentile for Delayed Recall of visual material (refer Table 2). The scores obtained for Total Learning and Delayed Recall did not show any significant correlation to age of the participants. The mean scores for Total Learning and Delayed Recall did not differ significantly between those boys with above-average academic performance and those with average or below-average academic performance.

Table 3. Comparison of Academic Performance and Neuropsychological Measures

	Group 1	Group 2		Sig.
	Mean (SD)	Mean (SD)	t Value	(Two-Tailed)
WISC-III FSIQ	100.38 (10.18)	81.79 (9.46)	–3.842	.001*
VIQ	100.75 (13.06)	78.50 (13.07)	–2.410	.026*
PIQ	99.88 (9.43)	88.50 (11.25)	–4.314	.000*
Factor Indexes VCI	98.25 (14.57)	80.14 (13.24)	–2.978	.007*
POI	103.50 (10.32)	92.00 (11.38)	–2.355	.029*
FDI	101.88 (13.53)	76.29 (7.32)	–5.806	.000*
PSI	90.00 (31.29)	76.50 (12.08)	–1.456	.161
Rey's Auditory Verbal Learning Test Total Learning	44.50 (8.37)	43.71 (12.23)	–0.161	.874
Delayed Recall	9.88 (3.18)	14.79 (17.53)	0.777	.446
The Memory for Designs Test Total Learning	42.00 (12.41)	48.07 (14.01)	1.017	.321
Delayed Recall	10.50 (2.56)	11.36 (3.67)	1.053	.567

FSIQ = Full-Scale IQ; VIQ = Verbal IQ; PIQ = Performance IQ; VCI = Verbal Comprehension Index; POI = Perceptual Organization Index; FDI = Freedom From Distractibility Index; PSI = Processing Speed Index.

*Significance $p < .05$.

Table 4. WISC-III: Comparison of Means of the DMD Sample and the Normative Sample

WISC-III Subtests	DMD Sample Mean (SD)	Normative Sample Mean (SD)	df	Statistic t	p Value
Information	9.23 (3.15)	10.47 (3.69)	21	–1.853	.078
Similarities	7.00 (3.79)	7.78 (4.98)	21	–0.965	.346
Arithmetic	8.50 (2.94)	13.63 (3.92)	21	–8.815	.000*
Vocabulary	6.82 (3.39)	15.30 (5.54)	21	–11.737	.000*
Comprehension	9.05 (2.65)	10.67 (5.98)	21	–2.880	.009*
Digit Span	7.05 (3.03)	13.28 (3.06)	21	–9.647	.000*
Picture Completion	11.36 (2.32)	11.31 (4.20)	21	0.108	.915
Coding	7.32 (3.26)	41.75 (10.15)	21	–49.585	.000*
Picture Arrangement	8.36 (2.11)	12.31 (6.96)	21	–8.792	.000*
Block Design	8.77 (2.98)	21.25 (10.66)	21	–19.672	.000*
Object Assembly	9.36 (3.11)	12.75 (5.76)	21	–5.108	.000*
Symbol Search	6.86 (3.44)	18.27 (6.68)	21	–15.55	.000*
Mazes	10.18 (3.60)	12.85 (5.44)	21	–3.420	.003*

*Significance $p < .05$.

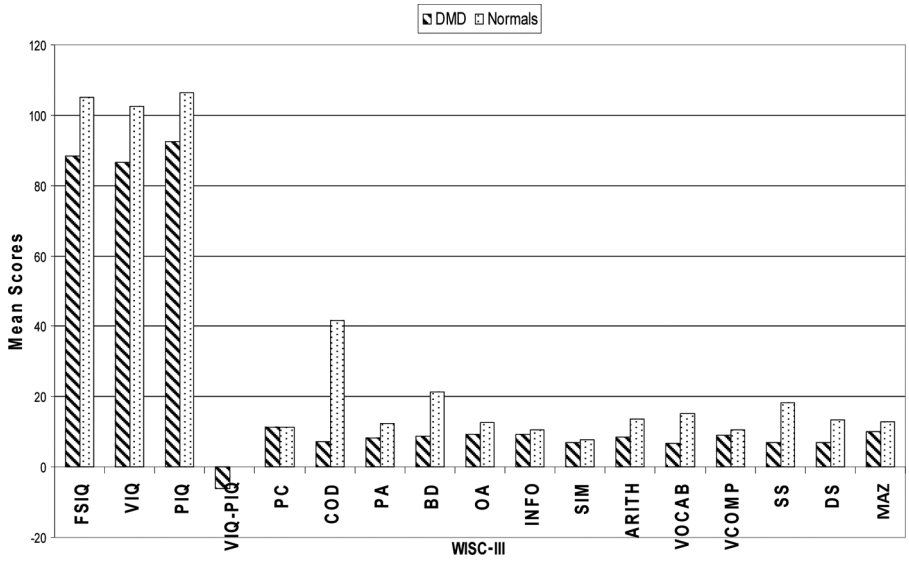

Figure 1. Comparison of mean scores of the DMD sample and the normative sample. FSIQ = Full-Scale IQ; VIQ = Verbal IQ; PIQ = Performance IQ; PC = Picture Completion; COD = Coding; PA = Picture Arrangement; BD = Block Design; OA = Object Assembly; INFO = Information; SIM = Similarities; ARITH = Arithmetic; VOCAB = Vocabulary; VCOMP = Verbal Comprehension; SS = Symbol Search; DS = Digit Span; MAZ = Mazes.

Discussion

The findings of this study show that boys with DMD have a mean FSIQ of 88.5 (*SD* = 13.18), which indicates low-average intelligence. This correlates with other studies of DMD, where the IQ has been found to be 80.2 (Cotton et al., 2001). A study conducted in an Indian sample (Jamuna et al., 2006) showed that boys with DMD had a mean IQ of 71.86. The low IQ scores were attributed to genetic heterogeneity, brain dysfunction, poor socioeconomic status, or lack of environmental stimulation. However, in the present study, boys with an IQ lower than 70 were excluded, which may have contributed to the higher mean FSIQ. There was no correlation between the age and composite IQ measures or factor indexes. However, there was a significant difference between boys with above-average academic performance and average or below-average academic performance on the FSIQ, VIQ, and PIQ measures, as well as the POI, VCI, and FDI. With regard to the PSI, it may be noted that both groups showed difficulty in information processing, irrespective of having above-average or average/below-average academic performance. Hinton et al. (2004) established that children with DMD had significantly poorer academic scores, and verbal span significantly influenced academic scores, even though vocabulary and home and education environments were comparable. In spite of having a low-average IQ, most children (50%) in this study reported average academic performance. However, academic performance was assessed based on the parents' report of how they faired in school; obtaining an objective measure such as school grades, teacher report, or an academic achievement measure may have yielded more useful results.

The mean scores for the Arithmetic, Vocabulary, Comprehension, Coding, Picture Arrangement, Block Design, Object Assembly, Digit Span, Symbol Search, and Mazes subtests were significantly lower than those of the normative sample, as shown in Figure 1. However, performance on the Information, Similarities, and Picture Completion subtests was comparable to the normative data. It may be inferred that boys with DMD have an adequate general fund of information and recall as demonstrated by performance on the Information subtest. Performance on the Similarities subtest indicates that the participants in this study have adequate abstract reasoning and semantic knowledge. Results on the Picture Completion test indicate good visual discrimination and attention to detail; performance was comparable to the normative sample. Hinton and colleagues (2001) found that boys with DMD as a group did not perform differently on the test of Gestalt closure, picture completion, and spatial relations. Children with DMD have performed better on the POI, and 86% of the sample scored above the 15th percentile. Inadequate performance on the PSI is suggestive of impaired mental speed (73% obtained < the 15th percentile). The Coding test requires visuomotor coordination, motor persistence, sustained attention, and response speed (Lezak, 1995). The boys with DMD were observed to be slow in their performance across testing. Cotton et al. (1998) had used the Symbol Digit Modality Test, and the results indicated impaired processing of information. Dixon et al. (2007) suggested that the rate of cognitive performance could reflect underlying neural integrity and could indicate the extent to which neural resources are available to support higher-level cognitive processing. Similarly, on the Symbol Search test, the DMD sample performed significantly worse compared with the normative sample. This task requires adequate visual attention and search, which also requires efficient information

processing. Inferior performance was noted on the FDI indicative of impaired sustained attention (55% obtained scores below the 15th percentile). Digit Span is used to assess attentional capacity. The digits forward measures freedom from distractibility. The digits backward requires perceptual tracking and mental manipulation of information and assesses immediate verbal working memory. The results indicate that boys with DMD have a shorter span for verbal material and have impaired verbal working memory when compared with normally developing boys. Hinton and colleagues (2001) used the Digit Span test while assessing the neuropsychological profile of children with DMD and found that they had a significantly shorter digit span for both forward and backward tasks, suggesting immediate verbal working-memory deficits. The Arithmetic subtest also involves mental manipulation of information, as the arithmetic problems were presented orally and the children had to mentally determine the solutions. Thus, verbal working-memory problems may have contributed to poor performance on this subtest. Billard and his colleagues (1998) noted that boys with DMD perform poorly on the Arithmetic subtest, which they interpreted as a difficulty in verbal symbol manipulation and considered as a subtle language deficit. The boys with DMD showed impaired performance on the VCI (54% obtained scores below the 15th percentile). The Vocabulary subtest assesses verbal and language ability; this test also measures general mental ability. Billard et al. found that the vocabulary of boys with DMD was comparable to that of children with SMA. Verbal comprehension assesses verbal reasoning; the results indicate that boys with DMD have performed significantly below normal. Hinton et al. (2001) evaluated verbal comprehension using the Token Test and found that boys with DMD performed poorly on the complex items when compared with their siblings, suggesting that as the auditory load increased, they had selective difficulty with auditory/syntactic comprehension. Boys with DMD have significant difficulty in attending to and comprehending relatively complex verbal material. The participants in this study have performed inadequately on the Object Assembly and Block Design tests, indicating impaired visuospatial analysis, visuoconstructive ability, and visual problem solving. Performance of boys with DMD on the Picture Arrangement test differed significantly from that of the normative sample, indicating difficulty in planning, logical thinking, and social thinking. However, the Object Assembly, Block Design, and Picture Arrangement tests are timed tests that require rapid and efficient cognitive performance, and difficulty in processing speed may also have contributed to impaired performance.

From the results of verbal learning and memory, it was found that 27% of the sample obtained scores below the 15th percentile for total words learned and for Delayed Recall. However long-term percent retention for the learned words was adequate (M = 86.86%). Hence, difficulty on the Delayed Recall may be a result of poor learning of verbal material rather than impaired retention. On the Memory for Designs Test, 86% of the sample obtained scores above the 15th percentile for Total Learning of abstract designs and 82% obtained scores above the 15th percentile for Delayed Recall, indicating adequate learning and recall of visual material. Long-term percent retention for designs was adequate. With respect to learning and memory, the results indicate adequate recall and retention, but Total Learning appears to be impaired. Children with DMD show adequate verbal or nonverbal memory but have a deficit in learning and encoding, predominantly on the verbal learning test. Cotton et al. (1998), in their study, documented impaired memory

for faces, but memory for verbal material was found to be intact. In contrast, Hinton and colleagues (2000) found impaired story recall in boys with DMD, but other measures of verbal learning, nonverbal learning, and nonverbal memory were adequate. Wicksell et al. (2004), in their study of the specific cognitive deficits associated with DMD, found that children performed lower on all tests of learning, memory, and executive functions. Short-term memory was more significantly affected than long-term memory. A similar pattern was seen in learning; there was a significant interaction effect when comparing learning ability to short-term memory.

In conclusion, boys with DMD have an IQ that is lower than that of normally developing children (approximately 1 *SD* below normal), and there is an indication that their VIQ is less than their PIQ. The neuropsychological findings support the hypothesis that these children have specific cognitive deficits as opposed to a global intellectual deficit. The boys with DMD demonstrated specific deficits in information processing, complex attention, immediate verbal memory span, verbal working memory, verbal comprehension, vocabulary, visuoconstruction ability, and verbal learning and encoding. However, perceptional organization, general fund of information, abstract reasoning, visual discrimination and acuity, visual learning and memory, and verbal memory were found to be adequate. The findings from the current study provide a better understanding of the neuropsychological profile of boys with DMD. Parents and caregivers of children with DMD may be educated and made aware of their neuropsychological strengths and weaknesses. It would be useful to understand that these children may have problems with processing large amounts of information, and they would benefit from breaking information and tasks into concise and simple steps. Use of visual cues may be useful as visual learning and memory appear to be superior to verbal learning for these children. Maintaining consistent routines and repetition may help, as boys with DMD appear to have adequate recall once learning occurs. In addition, engaging these children in tasks and activities that enhance attention, information processing, and working memory would probably help in improving neuropsychological functioning and reduce the impact of cognitive disability.

However, this study did have several limitations. A larger sample with a matched control group would have yielded more meaningful and reliable results. The battery of tests chosen for this study was based on the availability of normative standardized data. However, if there had there been a matched control group, more current and specific neuropsychological measures could have been utilized to provide a better understanding of the neuropsychological profile. In addition to the cognitive deficits, an assessment of the psychosocial impact of the illness would have been useful to educate and counsel the children and their families. Further research into the nature of the neuropsychological profile may prove useful in determining the use and effectiveness of cognitive rehabilitation and retraining for children with DMD.

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CHAPTER 7

7

Muscle MRI in Duchenne muscular dystrophy: Evidence of a distinctive pattern

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Abstract

The purpose of this study was to describe the pattern of muscle involvement using MRI findings and correlate with functional as well as muscle strength measurements. Fifty genetically confirmed DMD children with a mean age of 7.6 ± 2.8 (4–15 years) underwent muscle MRI and qualitative assessment was done for muscle changes using Mercuri staging for fibro-fatty replacement on T1 sequence and Borsato score for myoedema on STIR sequence. Detailed phenotypic characterisation was done with Manual muscle testing (modified MRC grading) and Muscular Dystrophy Functional Rating Scale (MDFRS). Mercuri scoring showed severe fibro-fatty changes in Gluteus medius, minimus and Adductor magnus followed by moderate to severe changes in Gluteus maximus and Quadriceps muscles. Total sparing of Gracilis, Sartorius and Semimembranosus muscles was observed. Superficial posterior and lateral leg muscles were preferentially involved with sparing of deep posterior and anterior leg muscles. Myoedema showed significant inverse correlation with fatty infiltration in thigh muscles. Similarly, significant inverse correlation was observed between Mercuri scores and MRC grading as well as MDFRS scores. A direct linear correlation was observed between duration of illness and fibro-fatty changes in piriformis, quadriceps and superficial posterior leg muscles. There was no correlation between MRI findings and genotypic characteristics. However, this specific pattern of muscle involvement in MRI could aid in proceeding for genetic testing when clinical suspicion is high, thus reducing the need for muscle biopsy. Fibro fatty infiltration as measured by Mercuri scoring can be a useful marker for assessing the disease severity and progression.

Key words: Duchenne muscular dystrophy; Magnetic resonance imaging; Muscle imaging

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder with a reported incidence ranging from 1:3802 to 1:6291 live male births [1]. DMD is the most common and severe form of progressive muscle disease affecting boys [2,3]. Dystrophin gene is the largest gene described in humans and encodes the sarcolemmal protein dystrophin [4,5]. In the majority of children, the clinical diagnosis is confirmed by genetic testing. Muscle immunohistochemical analysis still remains the gold standard in genetically negative cases [6]. Over the past three decades, neuromuscular imaging has become an important adjunct to clinical diagnosis in detecting degree and pattern of muscle involvement. Muscle MR imaging with its high soft tissue contrast and ability to assess deeper muscle groups has become the technique of choice in differentiating inherited muscle disorders including DMD [7,8]. Semi-quantitative assessment of transverse images of lower limbs at hip, thigh and calf levels is a simple and reliable tool for identifying pattern and progression of muscle involvement especially in children [7]. Recent studies have successfully demonstrated variation of fatty infiltration among different age groups of children affected with DMD and its correlation with duration of illness [9].

In the present study we describe in detail the pattern of fatty infiltration and myoedema in lower limb muscles in a cohort of genetically confirmed children with DMD and correlate the severity of muscle involvement with scores of manual muscle testing (MMT) and Muscular dystrophy functional rating scale (MDFRS).

Materials and methods

This was a cross-sectional prospective study done at the Neuromuscular disorders clinic, department of Neurology at a national referral centre for neurological disorders. It was conducted between 1 April 2013 and 31 December 2014. Ethical clearance for data collection and for conducting the investigations was obtained from the institute. Written informed consent was taken from the parents/guardians and assent from the children. The inclusion criteria were: (1) MLPA (Multiplex Ligation-dependent Probe Amplification) confirmed cases of DMD; (2) Parents willing to subject their children for MR Imaging; (3) Children who cooperated for MRI testing.

Methodology

Fifty genetically confirmed DMD children were included in the study. A detailed phenotypic characterisation was done and an exhaustive proforma with muscle involvement pattern was completed for all patients. Manual muscle examination score (modified MRC Grading) [10] and MDFRS [11] (permission obtained) were applied for all. Liver enzymes (SGOT, SGPT), creatine kinase (CK) and lactic dehydrogenase (LDH) levels were estimated in all. Genetic testing was performed by MLPA technique for all 79 exons of the Dystrophin gene.

Muscle MR imaging protocol

Muscle MRI of both lower limbs extending from the iliac crest up to the lateral malleoli was performed on 1.5 Tesla machine (SIEMENS) as a multi-sequence imaging protocol and included T1-weighted (T1W) and T2-weighted (T2W) (turbo) spin echo as well as fat-suppressed (short tau inversion recovery or spectral fat suppression techniques) and T2-weighted sequences (T2WFS). The image acquisition was performed in the axial plane with a slice thickness of 5–7 mm. When necessary, additional images in other anatomical planes (coronal or sagittal) were done. Skeletal muscles were analysed for signs of muscle degeneration and edema. Scans were examined for various abnormalities and scored. The presence of muscle edema was assessed on the fat suppressed T2-weighted images (STIR). The following muscles were evaluated:

At pelvis: Gluteus maximus, medius and minimus, Piriformis and Iliopsoas.

At thigh: Vastus medialis, intermedius and lateralis, Gracilis, Sartorius, Adductor muscles, Tensor fasciae latae, Rectus femoris, Semimembranosus, Semitendinosus and Biceps femoris muscles.

At leg: Tibialis anterior, Extensor hallucis longus, Peronei, Soleus, Popliteus, Extensor digitorum longus, medial and lateral heads of Gastrocnemius and Tibialis posterior muscles.

The fibro-fatty replacement was evaluated on T1 sequences by applying Mercuri score [12,13]. Myoedema was evaluated on STIR sequences by using a specific new Borsato myoedema score [13]. The findings were analysed independently by an experienced neuroradiologist and a neurologist. MRI findings were correlated with the gene mutation spectrum, MRC score and MDFRS.

Statistical analysis

Data were analysed using the statistical package for social science (SPSS 17). Median scores of Mercuri and Borsato staging were calculated for individual muscles/muscle groups to identify the pattern of involvement. Pearson's correlation was employed for correlation analysis between cumulative scores of MRI findings, MRC grading and MDFRS. $P < 0.05$ was considered for statistical significance.

Results

We recruited 60 children clinically suspected to have DMD. Ten cases were eliminated [four children showed no mutation, 6 did not cooperate for MRI]. Finally 50 MLPA confirmed cases completed the study protocol.

Clinical characteristics

The mean age at evaluation of the 50 children was 7.6 ± 2.8 years. The mean age at symptom onset was 46.9 ± 26.8 months; mean duration of illness was 46.3 ± 29.6 months. Delayed acquisition of motor milestones was reported in 29 (58%) children and delay in attaining mental milestones in 4 (8%). The predominant symptoms at onset were difficulty in rising from the floor or low chair, climbing stairs and frequent falls. 42 children (84%) were independently ambulant at the time of study. Positive family history was present in 16 (32%) patients. In addition to calf muscle hypertrophy other muscles like Deltoid, Biceps, Triceps, Brachioradialis, Tibialis anterior and Extensor digitorum brevis were hypertrophied in the majority. Contractures of Tendoachilles were present in 42 (84%), Hamstrings in 9 (18%) and Iliopsoas muscle in 6 (12%). Thoracic scoliosis was seen in 10 (20%) cases. Mean serum CK level was 14,100.4 ± 9800.8 IU/L with mean LDH of 1334.9 ± 587.2 IU/L and liver enzymes SGOT, SGPT measuring 238.7 ± 108.8 U/L and 321.8 ± 156.2 U/L respectively. There was regular follow-up in 49 children and all were on oral prednisolone therapy at 0.75 mg/kg/day as recommended by Dubowitz et al. [14].

Molecular characteristics

All 50 patients in our cohort were genetically confirmed to have DMD by MLPA technique. Among these 47 (94%) showed deletion and 3 (6%) showed duplication. Proximal deletion was present in 14 (28%), distal deletion in 35 (70%) and 1 (2%) child showed both proximal and distal deletion.

The number of exon(s) deletion was: Single exon in 27.7%, 5 in 17%, >10 in 12.8% of patients. Exon no. 51 showed highest deletion rate of 46% while exons 56–79 did not show any deletion. Those with less than 5 exons deletion were 46.8% and more than 5 exons were 53.2% (n = 47, as 3 patients showed duplication).

Table 1. Fatty infiltration and myoedema scores among affected lower limb muscles.

Muscle name	Mercuri score						Myoedema score					
	0 N	1 N	2a N	2b N	3 N	4 N	0 N	1 N	2a N	2b N	3a N	3b N
Gluteus maximus	0	7	6	8	19	10	19	9	5	4	6	7
Gluteus medius	0	2	10	4	12	22	30	5	2	10	3	0
Gluteus minimus	0	2	9	5	11	23	29	5	1	2	9	4
Piriformis	4	6	5	5	15	15	27	3	6	4	9	1
Iliopsoas	21	13	5	8	3	0	11	9	6	15	8	1
Vastus medialis	1	4	11	10	15	9	13	6	6	4	7	14
Vastus intermedius	4	3	9	12	11	11	12	6	2	10	6	14
Vastus lateralis	0	3	8	13	16	10	11	8	4	5	6	16
Rectus femoris	5	9	3	12	13	8	19	9	4	6	9	3
Gracilis	48	2	0	0	0	0	15	4	1	13	9	8
Sartorius	41	4	4	1	0	0	15	2	1	17	7	8
Adductor longus	15	12	10	6	3	4	9	4	2	9	11	15
Adductor magnus	0	2	2	8	15	23	30	5	1	1	8	5
Semimembranosus	44	6	0	0	0	0	16	1	3	10	11	9
Semitendinosus	13	9	10	13	4	1	13	3	2	6	15	11
Biceps femoris long head	4	7	4	16	8	11	27	4	2	1	5	11
Biceps femoris short head	28	9	8	5	0	0	30	4	2	8	2	4
Tibialis anterior	33	9	2	4	2	0	24	8	5	6	3	4
Soleus	2	7	12	21	8	0	2	2	2	9	4	31
Medial head of gastrocnemius	3	9	9	25	4	0	1	3	4	12	7	23
Lateral head of gastrocnemius	3	8	12	18	8	1	4	3	1	6	9	27

Muscle MRI characteristics [Table 1, Figure 1]

At pelvis level

All glutei muscles showed fatty infiltration. Gluteus medius and minimus muscles showed maximum scores with Mercuri grade 3 and above in 68%. Gluteus maximus muscle was more involved than Iliopsoas muscle in 96%. Piriformis muscle showed severe fatty infiltration with grade 3 and above in 60%. Myoedema scores were less in Gluteus medius and minimus muscles compared to Gluteus maximus and Iliopsoas muscles.

At thigh level

Thigh muscles showed significant selectivity with more uniform involvement of anterior group. In the anterior compartment Vastii muscles and Rectus femoris showed higher Mercuri scores. All Vastii muscles were equally affected. Sartorius muscle was spared from fatty infiltration with only 10% showing grade 2 fatty infiltration, but mild to moderate intra fascicular myoedema was present in 66%. In the posterior compartment long head of Biceps femoris muscle showed greater fatty infiltration with Mercuri stage 2b and above in 70% of patients, whereas only 10% had 2b score in short head. Semimembranosus muscle showed minimal while Semitendinosus had mild to moderate fatty infiltration scores. However, both these muscles had higher myoedema scores.

In the medial compartment Adductor magnus muscle was most affected with 76% showing severe fatty infiltration (Mercuri stage 3 and greater). Adductor longus, Adductor brevis and Obturator muscles were less severely involved. Gracilis muscle was totally spared. Myoedema was more in Adductor longus muscle with stage 2b and higher in 70%. Gracilis and Obturator muscles also had significant myoedema.

At leg level

Superficial part of posterior compartment of leg was most severely affected followed by lateral compartment with >50% showing moderate to severe fatty infiltration in Gastrocnemius, Soleus and Peronei muscles. Anterior leg compartment muscles were relatively spared with Tibialis anterior showing absent fatty changes in 66% and deep posterior compartment muscles were least affected with ≥90% showing no infiltration in Tibialis posterior and Popliteus muscles. Borsato myoedema scoring was greatest in superficial posterior compartment of leg with Soleus and lateral head of Gastrocnemius muscles showing severe intra fascicular myoedema in 70% and 72% respectively.

Correlation findings

Cumulative MRC score of lower limb muscles showed significant negative correlation with fatty infiltration on MRI (Mercuri T1) (Pearson $r = -0.382$, $p < 0.01$) [Fig. 2(a)].

There was negative correlation between fatty infiltration (Mercuri T1) and individual domain scores as well as total MDFRS score (Pearson $r = 0.608$, $p < 0.001$) [Fig. 2(b)].

Total Mercuri score showed no correlation with either age of onset or duration of illness. But there was significant linear correlation between duration of illness and fatty infiltration of individual muscles, in Piriformis ($p < 0.05$), Quadriceps ($p < 0.05$) and superficial posterior leg muscles ($p < 0.01$).

Although there was no correlation between sums of scores, significant inverse relation was observed between myoedema and Mercuri scores in thigh muscle groups ($p < 0.05$). However, myoedema did not show correlation with any of the clinical/ functional parameters studied. MRI findings showed no association with genotypic findings including location of mutation and number of exons involved.

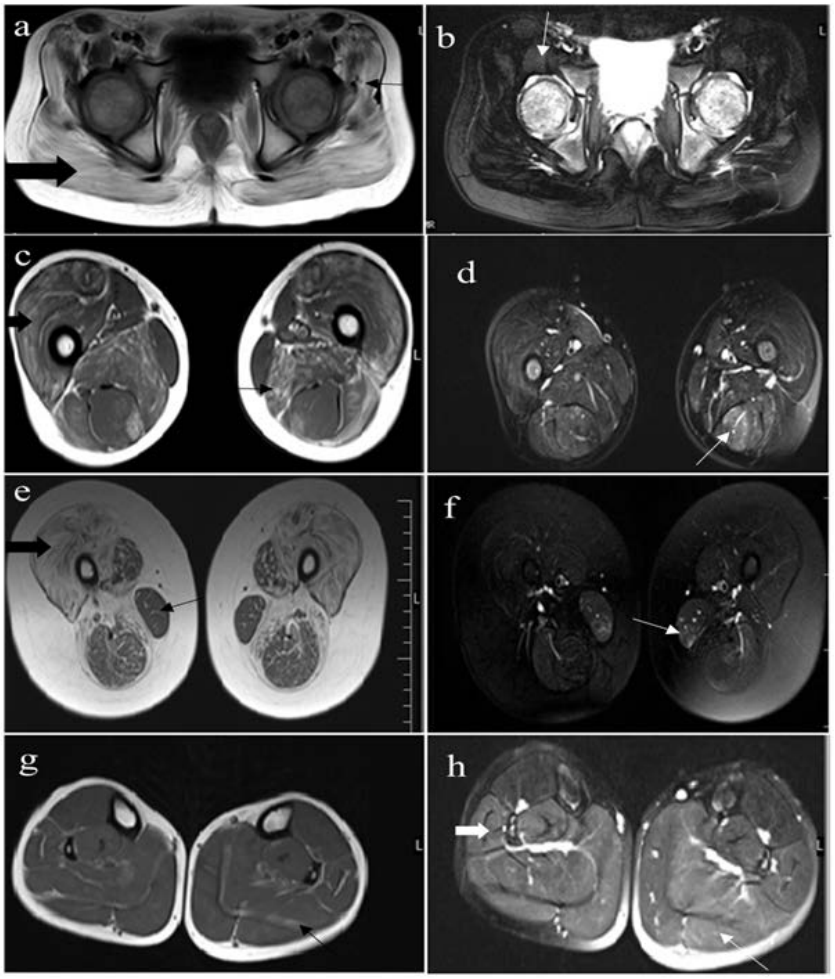

Figure 1. Muscle MRI showing fatty infiltration and myoedema with characteristic pattern. (a) T1 sequence at pelvis level shows gross fatty infiltration of Gluteus maximus (block arrow) and Gluteus medius (line arrow). (b) STIR sequence at same level as (a) shows myoedema of Iliopsoas muscle (line arrow). (c) T1 sequence at mid-thigh level shows fatty infiltration of Vastus lateralis (block arrow) and Adductor magnus (line arrow). (d) STIR sequence at same level shows myoedema of posterior thigh muscles (line arrow). (e) T1 sequence at mid-thigh level in a different patient shows advanced fatty infiltration of Quadriceps (block arrow) with sparing and hypertrophy of Gracilis muscle (line arrow). (f) STIR sequence at same level shows myoedema in Gracilis (line arrow). (g) T1 sequence at mid-leg level shows early fatty changes of Gastrocnemius and Soleus muscles (line arrow). (h) STIR sequence at same level showing myoedema in lateral (block arrow) and posterior (line arrow) leg muscles.

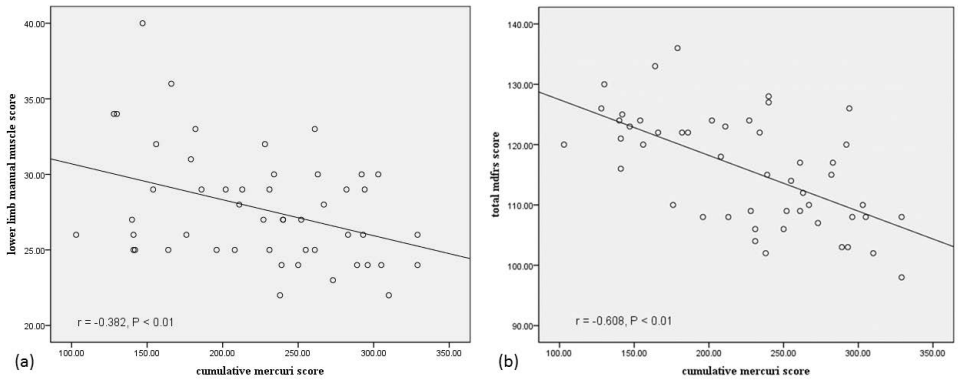

Figure 2. Inverse correlation of lower limbs Mercuri scoring (fatty infiltration) with muscle strength (a) and MDFRS score (b).

Discussion

Muscle MRI plays an important role in demonstrating the degree and pattern of involvement of muscles with high soft tissue contrast. It is a non-invasive test and does not have any side effects of ionising radiation. T1-weighted images show a high diagnostic accuracy in detecting early fibro-fatty infiltration in affected muscles [7,15]. Fat suppressed T2 image allows edema detection in the early stages of muscle degeneration [13,16]. The earliest findings in muscle MRI in DMD include patchy edema and inflammation followed by intramuscular fatty infiltration with a characteristic differential pattern [17]. Mercuri staging provides a simple yet reliable scoring system of fatty infiltration and has been useful in identifying patterns of muscle involvement in various inherited muscle disorders [7]. Few studies utilised a modification of a 5-point staging as described by Lamminen [18–20]. Other scoring methods like Goutallier, Patte, and Warner MRI classification systems are being predominantly used in orthopaedic disorders and not widely described in muscle diseases [21]. However, no consensus on the correlation between different classifications has been observed [22].

In the present study among our 50 children with DMD a distinctive pattern of skeletal muscle involvement was consistently observed irrespective of the age at evaluation. MRI showed features of dystrophic process involving both proximal and distal musculatures. The features were more severe in the pelvic girdle and thigh muscles as compared to leg muscles.

According to Kim et al., Gluteus maximus muscle showed maximum fatty infiltration among pelvic girdle muscles in DMD [23,24]. This preferential and early involvement of Gluteus maximus muscle has also been previously reported [7,16,17]. However, interestingly in our study, Gluteus medius and minimus muscles showed highest Mercuri scores followed by Gluteus maximus and Piriformis muscles. This pattern of less severe involvement of Gluteus maximus muscle compared to Gluteus medius has been reported in only two

BMD patients [25]. We observed moderate degree of fatty infiltration of Piriformis muscle on imaging, however, it is difficult to clinically assess the involvement of this muscle.

Among the thigh muscles, Adductor magnus showed highest fatty infiltration score followed by Quadriceps and long head of Biceps femoris muscles, which is similar to previous studies [17,24,26]. Gracilis, Sartorius, Semimembranosus and Semitendinosus muscles are known to be relatively spared in DMD [23,27–29]. However, mild to moderate fatty infiltration of these muscles has been described in older children from 7 to 8 years of age [9]. We found an almost similar pattern in our cohort with relative sparing of Gracilis, Sartorius and Semimembranosus muscles, while Semitendinosus, short head of Biceps femoris and Obturator muscles had mild infiltration with edema. Even in older children above 7 years of age, Gracilis and Semimembranosus muscles did not show significant fatty change. Sartorius muscle showed mild fatty infiltration in children above 9 years of age.

Prominent involvement of superficial posterior compartment and Peronei muscles have been observed in previous studies in DMD [26,30,31]. In one study selective sparing of Tibialis posterior muscle is mentioned [31]. Similar to these reports in our patients the leg muscles including Soleus, Gastrocnemius and Peronei muscles were more significantly affected as compared to the deep posterior and anterior leg muscles.

The characteristic pattern of muscle involvement in MRI could help in distinguishing DMD from other limb girdle muscular dystrophies with similar phenotype such as LGMD2C-F (Sarcoglycans) and LGMD2I (FKRP). Although few previous studies report more involvement of anterior thigh muscles in LGMD2C-F, similar to DMD, posterior leg muscles were relatively spared when compared to DMD [8,25].

In LGMD2I (FKRP), Gluteus maximus is reported to be involved early and more severely when compared to other Glutei with significant involvement of posterior and medial thigh muscles. Anterior thigh muscles are relatively spared until advanced disease stage unlike DMD [8,25,32]. Hence, these features could help in differentiating DMD from LGMD2I (FKRP).

Correlation of MRI findings with manual muscle testing showed inverse linear relation with fatty infiltration. This correlation was even more significant with MDFRS functional scores. Such correlation studies in DMD either with MRC grading or MDFRS are not described earlier. However, previous studies have shown correlation between MRI findings and other standard functional measures. Wren et al. noted significant correlation of fatty infiltration with grading on a functional ability scale developed by Brooke et al. [33,34]. MR grading system of muscle developed by Liu et al. showed correlation with clinical functional score (CFS) [28]. Positive correlation between T2 mapping and functional assessments using CFS, timed Gower score, time to run 30 feet was reported by Kim et al. [23].

In a similar study on LGMD2A (CAPN3) and LGMD2B (DYSF) by Borsato Carlo et al., a significant inverse linear correlation between the degree of fibro-fatty changes and MRC score was observed whereas a direct linear correlation was found with GSGCA (Gait, Stairs, Gower's, Chair, Arms functional tests) scale [13].

Previous studies reported significant correlation between duration of illness and MRI findings [28]. Li et al. observed significant variation in fatty infiltration of thigh muscles among different age groups in a large cohort of 171 DMD patients [9]. Our findings show significant progression of fatty infiltration in Piriformis, Quadriceps, Gastrocnemius and Soleus muscles with increasing duration of illness. Involvement of lower leg muscles in later stages of disease has been previously described and MRI findings of these muscles can be important markers of prognostication and disease severity [26,31].

Myoedema findings unlike fatty infiltration did not correlate with functional and clinical findings in our study. However, inverse correlation between edema and fat infiltration in thigh muscles was noticed. This inverse distribution of edema and fibrofatty changes was described by Borsato Carlo et al. in other dystrophies like LGMD2A (CAPN3) and LGMD2B (DYSF) [13]. Myoedema measures inflammation secondary to muscle fibre injury during the early stages of dystrophic process before complete fibrofatty replacement leads to loss of structural integrity in the muscle [13]. Although not effective for assessing functional decline, myoedema can be a suitable marker for active dystrophic process in the early stages of disease.

We attempted to analyse the impact of mutation patterns with muscle MRI findings. There was no correlation of location of deletion/duplication with Mercuri or Borsato scores. Further, the number of exons deleted had no correlation with muscle MRI scoring. Studies are limited in this aspect; however, our findings were consistent with the observations made in a recent study from China [27].

Acquisition time and claustrophobia while performing MRI contributed largely for the 10% dropout among children in our study. Muscle ultrasound is a reliable technique, has an advantage over MRI in such cases, particularly in younger children, as it is faster and can be done at bedside. Recent studies suggest quantitative assessment of echo intensity in muscles by ultrasound which can be a useful objective measure of severity and progression in DMD [35,36]. However, ultrasound lacks the ability to visualise deeper muscles in comparison to MRI and is highly operator dependent. We did not perform muscle ultrasound in these children owing to nonavailability of normative data and technical expertise.

Assessment of muscle atrophy and hypertrophy was not performed for our cohort. We also acknowledge that constraining to qualitative methods of muscle grading is another limitation of this study. Due to inherent inter-observer variability and poor sensitivity and specificity, particularly in LGMDs with overlapping spectrum of muscle involvement, pattern recognition by muscle imaging is often unreliable for routine clinical use [37]. Recent advances in quantitative MRI methods like T2 relaxation time measurements, muscle fat quantification using the 3-point Dixon technique, magnetic resonance spectroscopy and perfusion imaging are highly objective and carry good inter-observer reliability in detecting even small pathological changes and progression of muscle involvement [8,32]. These methods are challenging in terms of technique and expertise, require longer sequencing time and may not be essential for pure diagnostic purposes [38,39]. Quantitative imaging can be a superior marker for longitudinal studies

and therapeutic trials. Furthermore, imaging limited to lower limbs is often inadequate and whole body MRI carries higher diagnostic value, especially in patients with advanced disease, where involvement of upper limb and axial muscles is significant [40].

In conclusion muscle MRI is a non-invasive tool to demonstrate degree and pattern of muscle involvement. It also gives information about the selectivity of muscle involvement in DMD patients which is highly consistent and distinctive. Evaluation of the degree of fibro-fatty changes by using Mercuri score definitely helps us to confirm the topography and severity of muscle damage. The inflammatory aspects as evaluated by myoedema score could be useful to monitor early and asymptomatic dystrophic process in these muscles. The severity of muscle involvement as measured by these MRI scores could be useful as a marker for predicting the course of the illness and perhaps prognostication.

When routine MLPA testing is negative, muscle MRI findings along with clinical phenotype could help in proceeding to next level genetic testing.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.nmd.2016.09.002.

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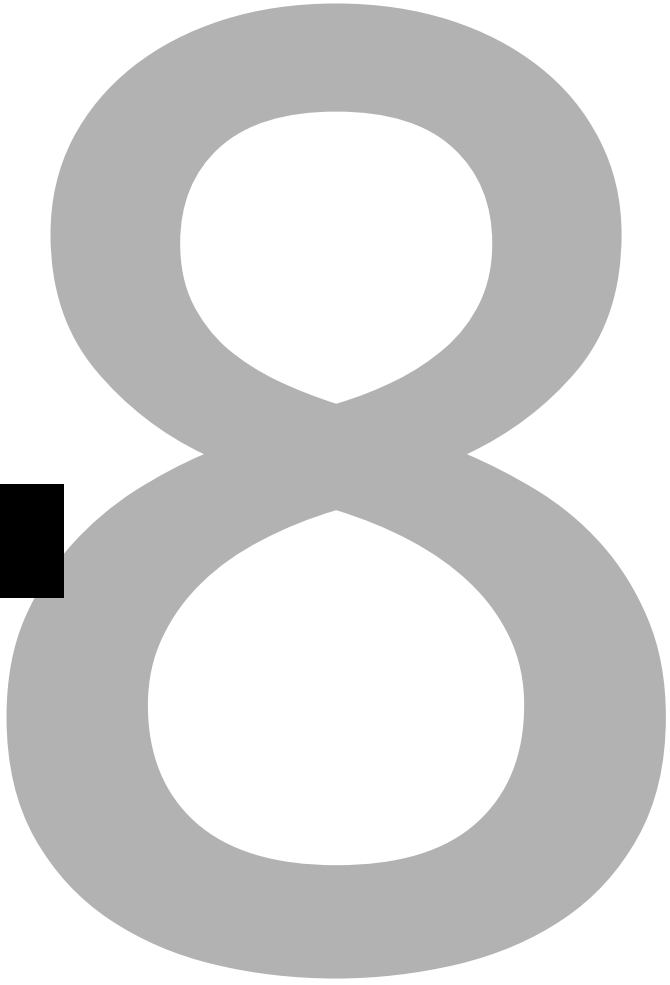
CHAPTER 8



Mutation pattern in 606 Duchenne muscular dystrophy children with a comparison between familial and non-familial forms: A study in an Indian large single-center cohort

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Abstract

Introduction: Duchenne muscular dystrophy (DMD) is induced by a wide spectrum of mutations such as exon deletions, duplications and small mutations in the dystrophin gene. This is the first study on the mutational spectrum in a cohort of DMD children from India, with an emphasis to compare the mutations in familial and sporadic forms.

Results: Multiplex ligation-dependent probe amplification (MLPA) and next-generation sequencing (NGS) identified 525 and 70 cases of DMD, respectively, while 11 cases showed absent dystrophin staining with no mutations detected. Families with two or more affected males contributed to 12% of the entire cohort. The mutations comprised of exonic deletions in 492/606 (81.2%), duplications in 33/606 (5.4%) and small mutations (point mutations and INDELs) in 70/606 (11.5%) cases. MLPA identified significantly more larger mutations in sporadic (88.2%) than in familial cases (75.3%). The mutations in NGS were: [nonsense = 40 (57.1%); frameshift = 17 (24.3%); splice variant = 12 (17.1%)]. Nonsense mutations were more common in familial than in sporadic cases: 17.8% vs 10.7%. The familial group reported an earlier onset of disease (2.8 ± 1.7 years) as compared to sporadic cases (3.8 ± 1.6 years).

Conclusion: MLPA could identify mutations in a high percentage of our DMD children. The preponderance of small mutations was noted to be distinctly higher in the familial group. Intriguingly, the familial form of DMD formed a small percentage of the entire cohort. The reasons could be increasing awareness among parents and physicians with early identification of DMD cases, genetic counseling and prenatal testing.

Key words: Duchenne muscular dystrophy · Familial · Mutation · India

Introduction

Duchenne muscular dystrophy (DMD) is a common X-linked recessive neuromuscular disorder with an universal prevalence of 1in 3500 newborn boys [1]. Clinically, DMD is characterized by rapidly progressive limb girdle muscle weakness with calf muscle hypertrophy with the diagnosis generally being made before the age of 5 years. The dystrophin gene is the largest human gene, comprising of 79 exons occupying a genomic region of 2.3 Mb on X chromosome short arm [2]. DMD is the severe form of Xp21 mutation disorder either from a shift in the translational reading frame or a premature stop codon resulting in loss of functional dystrophin [3, 4]. The majority of DMD cases are caused by deletion (65%) or duplication (5% to 8%) of single or multiple exons. [5]. About 25-30% cases are attributable to point mutations, and small insertion/deletions (INDELs) [4, 6].

Multiplex ligation-dependent probe amplification (MLPA) is a proven diagnostic tool for detection of exon deletions and duplications in DMD. MLPA is also extensively used in carrier detection and for prenatal diagnosis [5, 7, 8]. However, smaller mutations (point mutations and INDELs) require either direct sanger sequencing of DMD gene or Next Generation Sequencing (NGS) based testing [4]. Although mutations in DMD have been compiled in worldwide databases and studied elaborately in a few large cohorts (>500 patients), information on mutational pattern in familial DMD is rarely described [1, 9–13]. Early genetic diagnosis and subsequent carrier analysis and genetic counseling can play a key role in prevention of new cases in the immediate family members and relatives. Although there are simple, reliable and affordable genetic tests available to diagnose DMD, the factors contributing to occurrence of familial forms need to be studied and measures to reduce the societal disease burden is critical. We have previously reported on various aspects including mutational aspects identified by mPCR (Multiplex Polymerase Chain Reaction) and MLPA, genotype-phenotype correlation, muscle MRI (Magnetic Resonance Imaging) patterns, natural history and disease progression in hospital based studies of Dystrophinopathy (DMD andBMD) patients from India [14–18]. In the current study, we have described and compared the pattern of mutations in sporadic cases and those with positive family history in a large cohort of 606 Indian DMD children who have undergone genetic testing by MLPA and NGS in a tertiary neurology hospital from South India.

Materials and methods:

This is a retrospective study where patients were selected from the records of 804 suspected cases of dystrophinopathy registered at the Neuromuscular disorders' clinic at a national referral center for neurological disorders. The study period was between October 2012 and May 2018. The case files contained detailed information on clinical history, neurological findings with muscle strength scoring chart and mutation data obtained by MLPA / NGS / muscle biopsy. The first molecular assay that is undertaken for all suspected cases of DMD is MLPA of the 79 DMD exons. If MLPA fails to identify any mutation the sample is taken for NGS for genomic DNA coding regions and / or muscle biopsy for dystrophin staining.

MLPA Methodology

Exon deletion and duplication detection was performed using SALSA MLPA kit (MRC-Holland) with probe sets P034 and P035 for 79 exons of DMD gene as per the manufacturer protocol. Coffalyser MLPA analysis software (MRC-Holland) was used for analysis of the data.

NGS Methodology

Sequencing for MLPA negative cases was performed on HiSeq and NextSeq 500 platforms (Illumina) following target capture with Sureselect XT method using custom DMD probe design (Agilent). Raw data analysis with sequence alignment and variant annotation was done using SureCall (Agilent) software.

Results

Entire DMD cohort

Among the 804 suspected cases, DMD diagnosis was confirmed in 606 children based on clinical features, raised CK level and a confirmatory genetic or immunohistochemical result. MLPAidentified mutations in the DMD gene in 525 (86.6%) samples and was negative in 81 (13.4%) cases. The pattern of mutations was: exonic deletions in 492/606 (81.2%) cases with hot spot (45-54) region deletions in 365/492 (74.2%). Duplications occurred in 33/606 (5.4%) cases (Fig 1). Out-of-frame mutations were found in 481/525 (91.6%) and in-frame in 44/525 (8.4%). There were 150 different deletion / duplication patterns identified. While exon 50 was most frequently deleted in 234/492 (47.6%), the most common mutation in the overall group was deletion of exon 45 in 38/525 (7.2%) cases. Single exon deletions and duplications were observed in 120/492 (24.4%) and 7/33 (21.2%) patients respectively. NGS confirmed small mutations were seen in 70/606 (11.5%) [Nonsense = 40 (57.1%); frameshift = 17 (24.3%); splice variant = 12 (17.1%)] cases (Fig 1). Only one DMD patient in our entire cohort had a missense mutation. Small mutations showed random distribution with no hot spot predilection. Both MLPA and NGS did not identify any mutation in coding regions of DMD gene in 8 patients (1.3%), and 3 patients did not undergo NGS but biopsy showed loss of dystrophin staining. Novel mutations were seen in 43/70 (61.4%) of cases. No hotspot region for duplications or small mutations was identified.

Familial group

Among the entire cohort of DMD cases there were 73 families with more than one affected child. According to the history, a total of 117 affected family members were reported / identified: brothers of probands (n=33), maternal uncles (n=55), nephews (n=7) cousins (n=14), grand maternal uncles (n=8). The 73 probands of the familial group comprised of 12% (73/606) of our total DMD cohort. Among this familial group, MLPA showed mutations in the DMD gene in 55/73 (75.3%) samples, while it was negative in 18/73 (24.7%) cases. Deletions were noted in 48/73 (65.7%) and duplications in 7/73 (9.6%) cases (Fig 1). Thirty-three different deletion patterns were found (Fig. 2) in this cohort. Deletions in hot-spot region were identified in 34/48 (70.8%) (Fig. 3)cases. All seven duplications were unique and uniformly distributed along the DMD gene (Table 1). Exon 50 was the most frequently deleted exon (24/48 =50.0%) and single exon 50 deletion was the single most common mutation occurring in 4/73 families. Single exon deletion and duplication was present in

11/48 (22.9%) and 1/7 (14.3%) respectively. Out-of-frame mutations were present in 48/55 (87.3%) and in-frame in 7/55 (12.7%). NGS confirmed mutations in 13/73 (17.8%) (Fig 1) cases. Novel mutations occurred in 7/13 (53.8%) cases. Among the remaining 5 cases, all showed absent dystrophin staining; all were MLPA negative, two were NGS negative and 3 did not undergo NGS testing. The mean age at presentation was 6.8 ± 2.5 (2-12) years and age at onset was 2.8 ± 1.7 (1-7) years.

Non-familial group:
There were 533 non-familial cases and among this group MLPA was positive for mutations in the DMD gene in 470 (88.2%) and was negative in 63 (11.8%) samples. The pattern of mutation was: exonic deletions in 444/533 (83.3 %) and exonic duplications in 26/533 (4.9%) cases (Fig 1). There were 110 different deletion patterns and 24 duplication patterns (Supplementary Table. 1A and 1B). One deletion (Exons 1-29 and 40) and one duplication (Exons 45-55, 63) were of non-contiguous pattern. Hot spot (45-54) deletions were present in 331/444 (74.5%), while no significant hot spot regions were identified for duplications (Fig. 4). Similar to the familial group, the most common exon deleted was exon 50 in 210/444 (47.3%), while exon 45 deletion was the most commonly repeated mutation (36 times). Single exon deletion and duplication was present in 109/444 (24.5%) and 6/26 (23.1%) cases respectively. Out-of-frame mutations were observed in 433/470 (92.1%) patients while 37/470 (7.9%) had in-frame mutations. NGS confirmed presence of small mutations in 57/533 (10.7%) and no mutation in 6 (1.1%) samples (Fig 1). Novel mutations were seen in 36/57 (63.1%) cases. The mean age at presentation was 7.6 ± 2.1 (3-14) years with onset of symptoms at 3.8 ± 1.6 (1-8) years.

Discussion

Comparison of familial and sporadic cases [Table 2]
In the current comparative study, in a large cohort of DMD children, we observed that identification of mutation by MLPA technique was significantly higher in the non-familial DMD group as compared to the familial group (p=0.0025). Exonic deletions were significantly more frequent among the sporadic cases (p=0.0003) while the proportion of duplications were more in the familial group. Subsequently, point mutations and INDELs identified by NGS were comparatively higher in the familial DMD group (~18%). This difference would have been greater considering that three of our MLPA negative familial cases with dystrophin loss on muscle biopsy did not undergo NGS due to non-availability of DNA samples. Point mutations are considered to be derived during spermatogenesis resulting in increased carrier frequency for these mutations in comparison to large deletions (≥1 exon) which can be predominantly de novo arising during oogenesis [19]. The large deletion pattern, hot spot involvement and single exon mutations were comparable in both groups. The age at onset and presentation were significantly earlier in the familial group (p<0.005) and could possibly be attributed to parents / family members identifying the motor disabilities at an early age, given the experience with previously affected family members. In comparison the age at presentation of our DMD children is later than that of patients from western countries (4-5 years), and the delay increases further among sporadic cases (mean 7.6 years) [20, 21]. A common belief and also

presumptions by the parents / guardians / general practioners that the delayed motor milestones may be a normal variant, leads to delay in investigating such boys for muscle diseases. In the current study we found that positive family history was present in 12% of our entire DMD cohort, which is significantly less compared to the percentages of familial cases in the two large cohort studies (n>500) that have reported the proportion of familial cases in DMD [11, 13]. Notably, comparative mutational spectrum in familial cases is lacking even in global DMD mutation database studies [1, 10]. Only an Italian study (n=184) has presented a comparable percentage of familial cases. Studies from the USA and Asia mention percentages ranging from 21 to 43% [11, 13, 22–24] (Table 3). A study (n=128) from South China [23] and a small cohort (n=32) from Taiwan [24] have reported high percentage of familial forms of DMD, i.e 31% and 43% respectively. Yang et al., have reported 27.5% of familial cases in a large Chinese cohort of 1053 patients, but their study includes both DMD and BMD patients [13]. Our cohort comprised of only DMD patients. BMD cases are considered to have a higher positive family history due to transmission of mutations from affected males to daughters resulting in higher carrier frequency [19]. Yang et al., attributed the high incidence of familial cases to the lack of knowledge of DMD/BMD among primary care physicians, insufficient scientific information, and minimal awareness towards genetic counseling and prenatal testing in families with an affected boy and particularly with small DMD mutations [13]. In the western population, a study from Italy reported 9.2% patients out of 184 probands having a positive family history and this finding is similar to our cohort [22]. Flanigan et al., from the USA reported 203 (21%) familial cases in a large cohort of 1111 dystrophinopathy cases (from 967 families) including all phenotypes. However, they reported a selection bias as the study population was enriched with small mutations [11].

Our low percentage of familial cases might originate from the following aspects. On the one hand it might be caused by under-reporting because there might have been a few families who for unknown reasons did not reveal about other affected members. However, detailed, minimum 3-generation pedigree diagrams are part of the clinical work up and missing out on familial forms is less likely. On the other hand, we could surmise from our findings that DMD cases were diagnosed early in our cohort and that swift genetic counseling helped in preventing subsequent cases in the same family. Also, as majority of our patients are from joint families, grandparents tend to recognize motor disability at a very early age and thus consult physicians while children are at a very young age. An earlier study from India indicates that prenatal diagnostic testing using mPCR method has been available since past two decades [25]. We are also aware that several centres in India are utilizing MLPA for prenatal diagnosis (PND) since it emerged as a routine screening tool for DMD patients and carriers [15, 26]. Although NGS has been undertaken for the last few years in India, its availability for DMD diagnosis and prenatal testing for our patients has been utilized form 2014 onwards. This could be considered as one of the reasons for the apparently increased number of point mutations in our familial cases. However, data on the usage of various tests for PND in India under the current scenario is lacking.

Other plausible factors that contributed to the low number of familial cases in our cohort would include easy accessibility to pediatricians and neurologists, as there is no referral system in India to consult specialists. It is also possible that our DMD cases may be more

due to de-novo mutations without mothers being carriers. Unfortunately, we could not perform carrier testing for this cohort.

Comparison with other large DMD cohorts [Table 3]

We have compared the mutational pattern of our entire DMD cohort with ten other large published global/country-wide studies and databases [1, 9–13, 22, 23, 27, 28] (Table 3). TREAT-NMD and Leiden DMD mutation databases are the largest global DMD registries with >7000 and 4700 entries respectively [1, 10]. In our group, the percentage of large deletions (81.2%) is higher, while duplications (5.4%) and small mutations (11.5%) are much lower compared to all other studies. Similar to the global registries and UMD database of French DMD mutations, exon 45 deletion is the most common exon deletion in our cohort [1, 9, 10]. In contrast, our familial group has exon 50 deletion as the most common mutation. The Remudy database from Japan [12], which includes a significant number of BMD cases (n=295), has reported an in-frame deletion of exons 45-47 as the most common deletion while studies from Spain and Italy have found exon 51 and exons 45-52 deletions as most frequent mutations respectively [12, 22, 28]. Deletion of exons 3-7 is the most common proximal deletion in our patients and this is similar to the French study [9]. While 74% of deletions occurred in the distal hot-spot region between exons 45-54, we did not find any segregation of deletions and duplications in proximal exons as reported by previous studies [9, 10, 12]. Wang et al., have reported similar findings in 128 DMD patients from South China with no proximal hot-spot for large rearrangements, but a distal hot-spot for deletions at exons 45-54 was noted [23]. Exon 2 has been reported as the single most common duplication pattern in most of the databases available (Table 3). Interestingly, we did not identify isolated exon 2 duplication in any of our patients and only two patterns (duplication of exons 3-6 and 8-9) occurred more than once. The reading frame rule concordance is considered to be present in ~90% of DMD mutations [4]. In our entire cohort, out-of-frame mutations accounted for 91.6% which decreased slightly to 87.3% in the familial group. The significantly lower percentage of point mutations as observed in our patients is difficult to explain. It may be surmised that availability of NGS for the last 4 years only may be one of the reasons for detection of lesser number of point mutations. While the proportion of small mutations is significantly low in our patients in comparison to other studies, nonsense mutations resulting in stop codon constituted 57.0% of point mutations which increased to 61.5% when only familial cases were considered. Small INDELs resulting in frameshift accounted for 24.3% which decreased to 15.4% (2 patients only) in familial cases. Nonsense and frameshift mutations in previous large global studies accounted for about ~50.0% and ~32-34% of small mutations respectively (Table 3). Truncating mutations in particular are considered to be more frequent in DMD than in BMD [9, 29]. The global mutational databases also include BMD cases which may explain the difference in the proportion of nonsense mutations in comparison to our cohort. Flanigan et al., have reported the highest proportion of nonsense mutations (65.0%) in a biased sample study group from USA and the lowest percentage (42.5%) was seen from a study from South China [11, 23]. The prevalence of splice-site and missense mutations seen in our cohort was comparable to previous studies. We did not find any hot spot region for small mutations unlike large deletions. Our overall mutation detection rate was 98.2% (595/606) with combined MLPA and NGS. In eight of our NGS negative cases, further data analysis and RNA sequencing might be

required to look for any deep intronic mutations resulting in introduction of 'pseudo-exon' or other complex rearrangements / transpositions [9].

Novel therapeutic implications

An attempt was made to look for the number of DMD cases in our cohort who may be eligible for novel mutation-based therapies. While exon 51 skipping drug Eteplirsen has received accelerated approval by USFDA for further confirmatory studies, skipping agents of exons 53 and 45 are currently in Phase 3 trials [30]. Skipping of single exons 51, 53 and 45 have been theoretically considered to be effective in 13%, 8.1% and 7.7% respectively [31]. We identified that 38.6% (234/606) of our DMD patients are amenable for the top 3 single exon skipping strategies [Table 4]. Additionally, 40 patients (6.6%) with nonsense mutations resulting in stop codons can benefit from nonsense suppression treatment by compounds like Ataluren which enables read-through of premature stop codon [32]. This group of patients will be considered for Ataluren phase 3 clinical trials in India [Clinicaltrials.gov; CT/67/17-DCG(I)].

Conclusion

The results from this Indian single center large cohort forms one of the largest well-established mutation databases. The current report is one among the very few reports in English literature that has studied the prevalence of familial forms of DMD and their mutation pattern. We have demonstrated distinct mutation pattern differences as compared to other Asian and Western populations. Our findings may have important implications in the genetic testing approach when familial cases are encountered in the Indian sub-continent. Although MLPA is considered to be the most powerful single technology to identify mutations in the huge dystrophin gene, it might be contemplated to use NGS as the first screening test in familial forms of DMD because of the higher possibility of obtaining a negative MLPA result when familial cases are encountered.

We also suggest that disease awareness in general population and primary physicians, introduction of new born screening for DMD in India would lead to early identification of sporadic cases with possibility of genetic counselling and subsequent pre-natal testing. In the light of upcoming novel therapies where early treatment is the key factor, early mutational screening is the need of the hour in India.

Conflicts of interest: The authors have no conflict of interest.

Ethical standards: Approval from 'Institutional Ethics Committee' has been obtained.

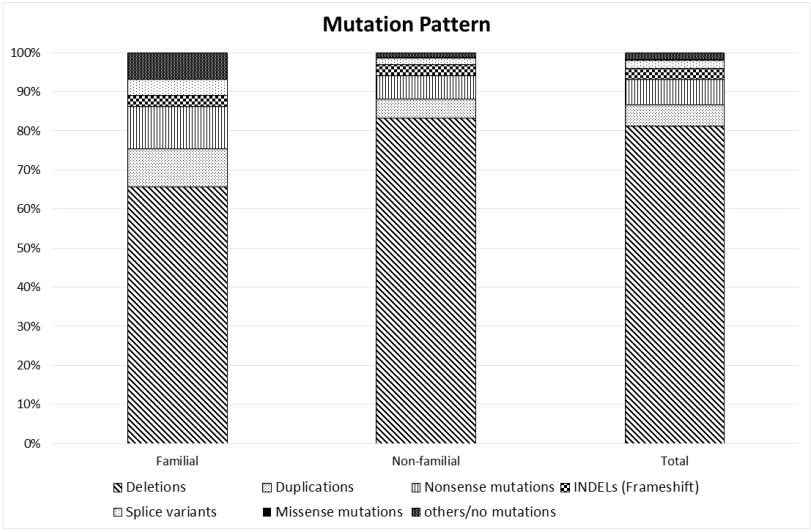

Figure 1. Bar diagram showing the comparative mutational spectrum in DMD cases

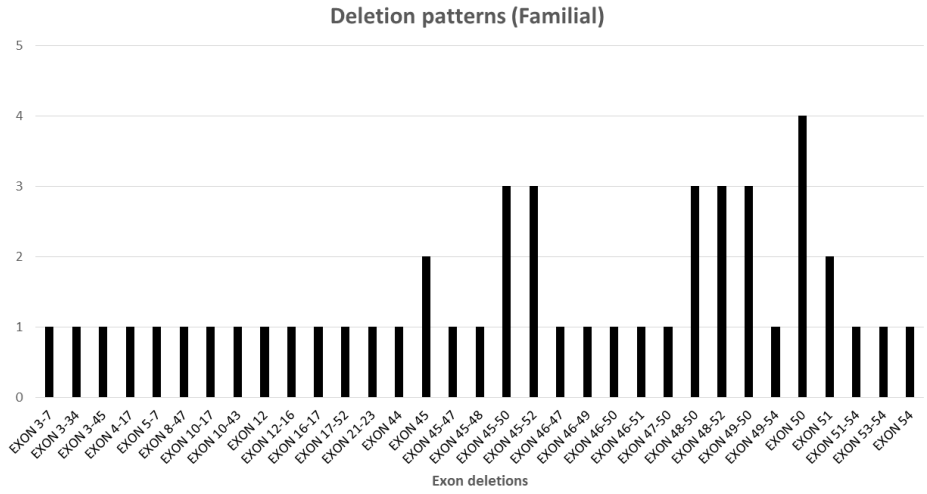

Figure 2. Bar diagram representing the deletion patterns in familial DMD cases

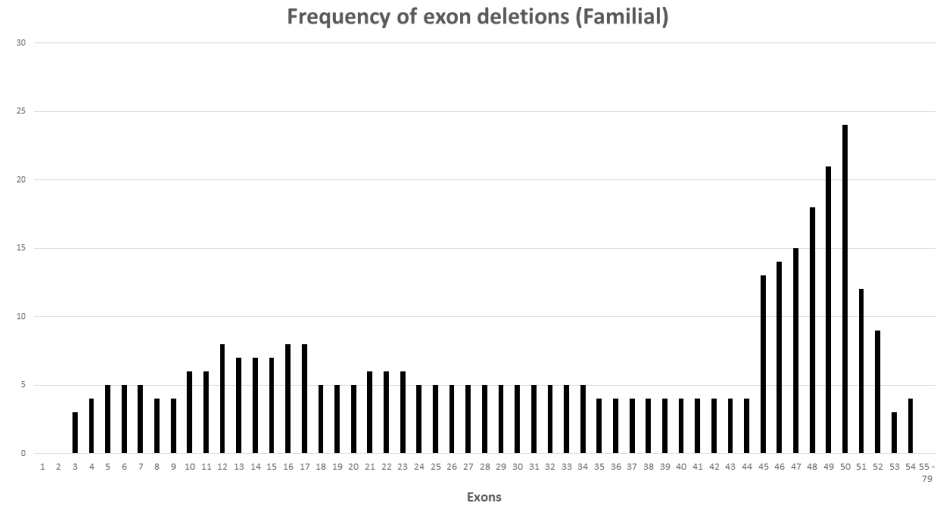

Figure 3. Bar diagram depicting the frequency of exon deletions in hot spot regions in familial DMD cases

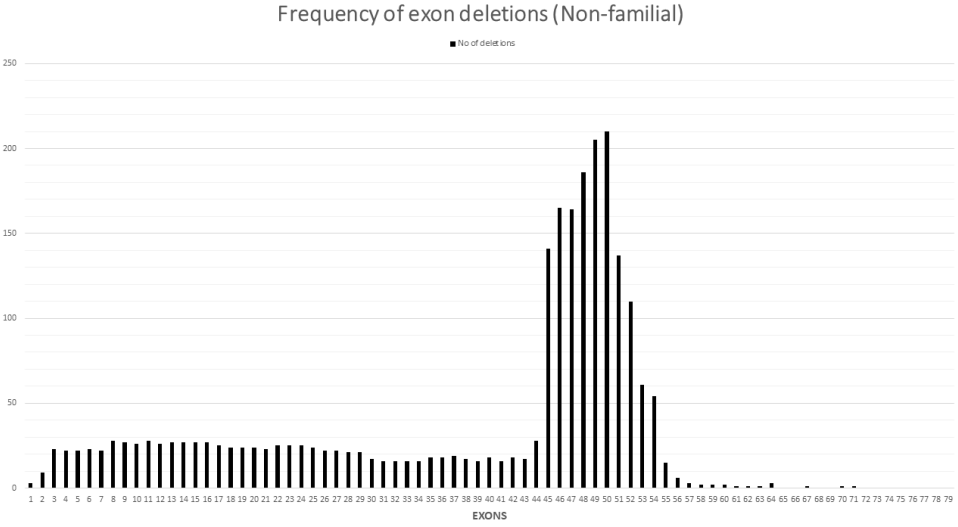

Figure 4. Bar diagram illustrating the frequency of exon deletions showing hot spot region in sporadic DMD cases

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Table 1. List of exon duplications in familial cases

Exon 2
Exon 2–7
Exon 3–25
Exon 21–30
Exon 46–49
Exon 56–62
Exon 60–66

Table 2. Comparison of findings in familial and non-familial DMD groups in a total of 596 cases (where percentage is not applicable the expression is indicated beside the variable)

Variable	Total group (*n*=606)	Familial group (*n*=73)-12.05%	Non-familial group (*n*=533)-87.95%	p value (familial vs. non-familial)
MLPA Positive	525 (86.63%)	55 (75.34%)	470 (88.18%)	0.0025
MLPA negative	81 (13.37%)	18 (24.66%)	63 (11.82%)	
Deletions	492 (81.18%)	48 (65.75%)	444 (83.30%)	0.0003
Single-exon deletions	120 (24.39%)	11 (22.92%)	109 (24.55%)	0.8029
Deletion patterns	119	33	110	
Non-contiguous deletions	1	0	1	
Duplications	33 (5.44%)	7 (9.59%)	26 (4.88%)	0.0966
Single-exon duplications	7 (21.21%)	1 (14.28%)	6 (23.08%)	0.6186
Duplication patterns	31	7	24	
Non-contiguous duplications	1	0	1	
Out-of-frame	481 (91.61%)	48 (87.27%)	433 (92.13%)	0.2188
In-frame:	44 (8.39%)	7 (12.73%)	37[a] (7.87%)	
Most common exon deleted	50 (47.56%)	50 (50%)	50 (47.30%)	
Most common mutation	Del 45 (7.24%)	Del 50 (7.27%)	Del 45 (7.66%)	
Hot spot region for deletions	45–54 365/492 (74.19%)	45–54 34/48 (70.83%)	45–54	0.5762
NGS confirmed small mutations	70 (11.55%) 40 (nonsense), 17 (frameshift), 12 (splice variant), 1 (missense)	13 (17.81%) 8 (nonsense), 2 (frameshift), 3 (splice variant)	57 (10.69%) 32 (nonsense) 15 (frameshift), 9 (splice variant), 1 (missense)	0.0745
No mutation identified/sequencing not performed	11 (1.81%)	5 (6.85%)	6 (1.12%)	
Biopsy confirmed in MLPA negative DMD	58/81 (71.60%)	12/18 (66.66%)	46/63 (73%)	
Age at onset:	3.7 ± 1.8 (1-8)	2.8 ± 1.7 (1-7)	3.8 ± 1.6 (1-8)	p < 0.0001
CK [U/L]:	14256.8 ± 7148.7	14165.7 ± 8594.0	14273.7 ± 6576.8	

[a]In three cases with in-frame deletions, there is a change in junctional codon resulting in premature stop codon in 1 case (2–19 deletion) and amino acid change from alanine to valine in 2 cases (64 deletion) [9]

[b]Sequencing not performed due to non-availability of DNA samples (blood) in three familial cases

Table 3. Comparison with global and country-based DMD mutational studies

Variable	TREAT-NMD	Leiden (Global) [1]	France [9]	Japan [12]	Brazil [27]	Spain [28]	USA [11]	South China [23]	China [13]	Italy [22]	Our Cohort (India)
Number of patients	7149*	4700*	2046* 1315#	1497* 1167#	576* 367#	284	967* 642#	128	1053* 951#	184	606
Positive family history			-		-		21%*	31.3%	27.5%*	9.2%	12.0%
Deletions	68%*	72%*	61.5%	61%	66%	46.1%	44.2%	57.8%	60.6%	65.8%	81.2%
Most common deletion	Exon 45*	Exon 45*	Exon 45*	Exon 45-47*		Exon 51*				Exon 45-52	Exon 45; Exon 50 (familial)
Most common Proximal deletion			3 to 7 (17)							-	3 to 7 (10)
Proximal Hot-spot deletions	14% (2-20)*	-	15% (2-20)*	28% (3-21)*						-	-
Distal Hot-spot deletions	66% (45-55)*	-	74% (45-55)*	42% (45-52)*							74.2% (45-54)
Number of deletion patterns		-	260*							-	119
Duplications	11%*	7%*	13%	13*	11%	19.7%	13.5%	10.9%	11.1%	13.6%	5.4%
Most frequent duplication	Exon 2*	Exon 2*	Exon 2	Exon 2*		Exon 2				Exon 2	Exon 3 – 6 & 8-9
Proximal Hot-spot duplications			50% (2-20)*	47% (3-25)*							
Distal hot-spot duplications			15% (45-55)*								
Number of duplication patterns			120*							-	31
Reading-frame rule concordance	93%	91%	96%		90.4%	85%			86.4%	94.6%	91.6% 87.3% (Familial)
Small mutations	20%*	20%*	26%	24.8%*	23%	34.2%	42.2%	31.2%	20^	17.9%	11.5%
Nonsense mutations	50%*	50%*	48%	50%*	56.1%	50.5%	64.9%	42.5%	8^	51.5%	57.1% 61.5% (Familial)
Splice site mutations	14%*		19%	16%*	15.7%	10.3%	8.1%	10%	6^	21.2%	17.1%
Frameshift	34%*		32%	32%*	24.1%	37.1%	25.5%	45%	1^	27.3%	24.3%
missense	2%*	0.5%*	1%	2%*	3.6%	2.1%	0.73%	2.5%	5^		1.4%

*Inclusive of all dystrophinopathy phenotypes. #Patients with DMD phenotype.

^Sequencing for point mutations was performed only in 20 out of 310 MLPA negative patients. Hence, percentages will not be representative.

Table 4. DMD patients with

	Exon 51 Skipping		Exon 53 skipping		Exon 45 skipping	
	Mutation	**No.**	**Mutation**	**No.**	**Mutation**	**No.**
Familial	Del 45-50	3	Del 45-52	3	Del 44	1
	Del 47-50	1	Del 17-52	1	Del 46-47	1
	Del 48-50	3	Del 48-52	3	Del 46-49	1
	Del 49-50	3			Del 46-51	1
	Del 50	4				
Sporadic	Del 45-50	35	Del 45-52	31	Del 44	16
	Del 47-50	1	Del 48-52	11	Del 46-47	18
	Del 48-50	24	Del 49-52	3	Del 46-48	6
	Del 49-50	20	Del 50-52	3	Del 46-49	3
	Del 50	15			Del 46-51	12
	Del 52*	8			Del 46-53	1
					Del 46-55	2
Total	117		55		62	
Total % of All Patients Deletions	19.31% 23.78%		9.07% 11.18%		10.23% 12.6%	

*Exon 52 deletion is amenable for treatment with skipping of either 51 or 53 exons. Hence, it is not included in list of mutations eligible for exon 53 skipping to avoid repetition.

Supplementary Table 1A

Exon deletions	Frequency
1-2	1
1-29, 40	1
1-44	1
2-3	1
2-17	1
2-19	1
2-37	1
2-43	2
3-4	1
3-5	1
3-7	9
3-11	1
3-16	1
3-17	1
3-44	1
5-7	1
6	1
6-19	1
8-9	5
8-16	2
8-17	2
8-25	1
8-29	1
8-30	2
8-33	1
8-41	1
10-11	1
10-15	1
10-43	1
10-44	1
11-40	1
11-41	1
13-17	1
16-17	1
17	1

Supplementary Table 1A

Exon deletions	Frequency
18	1
18-20	1
18-27	1
18-29	1
18-42	1
18-47	1
19-25	1
20-25	1
20-29	1
20-38	1
22-24	1
22-30	1
31-43	2
34-54	1
35-43	2
40-43	1
42-43	2
42-45	1
43	1
44	16
44-47	1
44-48	1
44-51	1
44-52	1
45	36
45-46	1
45-47	3
45-48	2
45-49	6
45-50	35
45-51	3
45-52	31
45-54	15
45-56	1
46-47	18

Supplementary Table 1A

Exon deletions	Frequency
46-48	6
46-49	3
46-50	12
46-51	12
46-52	7
46-53	1
46-55	2
47-50	1
48-49	2
48-50	24
48-51	1
48-52	11
48-54	7
49	3
49-50	20
49-52	3
49-54	2
50	15
50-52	3
50-54	1
51	19
51-53	3
51-54	4
51-55	6
52	8
52-53	1
52-54	3
53	2
53-54	6
53-55	1
53-56	1
53-57	1
54	1
55	1
55-60	1

Supplementary Table 1A

Exon deletions	Frequency
56	1
61-64	1
64	2
67	1
70-71	1

Supplementary Table 1B

Exon duplications	Frequency
2-11	1
3-6	2
3-7	1
3-12	1
3-18	1
6-21	1
8-9	2
8-17	1
8-29	1
14-17	1
18-21	1
19	1
26-50	1
43	1
44-53	1
45	1
45-55, 63	1
49-63	1
50-55	1
51	1
51-60	1
52	1
52-76	1
53	1

CHAPTER 9

9

General discussion

General discussion

The X-linked Duchenne Muscular Dystrophy (DMD) is named after Duchenne de Boulogne who described the disease in a series of papers in the 1860s (Emery AEH et al., 1995). DMD is the most common childhood neuromuscular disease, with an estimated incidence of roughly 1 in 4000 to 6000 (Mendell JR, 2012). This leaves a total of 3798 newly diagnosed DMD patients each year in the Indian subcontinent with a tremendous societal and economic burden.

The experience and progress in investigative and treatment strategies as well as in comprehensive research that evolved over the last 15 years at NIMHANS in Duchenne Muscular Dystrophy is decribed in this thesis. From the level of clinical and biochemical diagnosis, to histopathological confirmation, we have evolved through simple genetic to complex genetic analysis, genotype-phenotype correlation, cognitive features, muscle MR imaging characteristics, mutations in familial forms of DMD onwards to the recent Quantitative Brain Imaging studies and the latest Next generation Sequencing. Thus, keeping in trend with the contemporary technology of Next Generation Sequencing (NGS), we are presently performing NGS for all Multiplex Ligation Probe Amplification (MLPA) negative cases. The small percentage of cases found to be negative by NGS testing are taken up for muscle biopsy and immunostaining for confirming or rejecting the clinical diagnosis of DMD. Furthermore, we perform mRNA-based studies in muscle tissue to identify the mutation in the very few samples that are negative for dystrophinopathy by both MLPA and NGS methods but positive by immunohistochemistry. These mRNA based studies are presently not performed in any other laboratory in India.

We have thus steadily evolved to our current state-of-the-art clinical and genetic testing that is at par with any leading international advanced center for neuromuscular diagnosis and treatment. However, we still lack in several aspects of care for DMD children as well as for DMD carriers and care givers.

The study described in **chapter 2** comprises of the results of histopathology and multiplex Polymerase Chain Reaction (mPCR) findings in a cohort of DMD children. mPCR offers a rapid and less-invasive screening tool for detecting deletions in the central and 5′ end hot spot regions of the dystrophin gene (Beggs AH et al., 1990; Chamberlain JS et al., 1988). This was our first paper to be published on deletion pattern in DMD from NIMHANS. At this stage mPCR, a technique that examines proximal and distal hot spot regions, just became available. The negative cases of DMD were diagnosed by the gold standard muscle immunohistochemistry for dystrophin expression. Genetic counseling was feasible for the mPCR positive cases and a guarded counseling for the biopsy proven families. In this prospective study, 112 clinically diagnosed DMD patients had muscle biopsy and were tested for exon deletions. The diagnosis of DMD was confirmed by histopathology and/or genetics in nearly 90% of patients. The majority of the 60 IHC confirmed cases showed deletion of at least one exon. The predominant deletions were observed in the distal hot spot region similar to other reports. The definitive diagnosis of DMD thus could be genetically confirmed in a large number of patients. Our results are similar to other published reports where deletions account for about two-thirds of the mutations in

dystrophin gene and mPCR allows detection of 98% of those deletions (Koenig M et al., 1987; Forrest SM et al., 1988; Koenig M et al., 1989). mPCR technique has largely replaced muscle biopsy, and has become the preferred method of diagnosis in many developing countries including India, although the trend now is to more often perform MLPA as the screening tool to diagnose new cases of DMD.

In order to understand the natural history of DMD in children seen at NIMHANS, we subsequently studied more than 600 patients diagnosed to have DMD (**chapter 3**). We posted a simple questionnaire to know the motor abilities of the child who were lost for follow-up.

Among 500 postages, 275 parents responded by letter or came for follow up to the clinic, 31% by completing the questionnaire, and 69% by completing both the questionnaire and attending the outpatient clinic. The data obtained from follow-up notes in medical records of patients maintained at the Neuromuscular disorders clinic were entered in a pre-designed proforma.

The disease progression with time to loss of independent ambulation and time to attain wheel chair bound state were recorded. Any difference in the outcome was compared among children treated / not treated with steroids. None of the children had received life supportive measures. Several factors were studied including the socio-economic status, urban / rural background.

This is the first study from India describing the disease progression and natural history of DMD in a large cohort of genetically and/or immunohistochemically confirmed cases. The patterns of major DMD milestones, including the age at onset, age at loss of ambulation and death in our cohort is comparable to that of the Western cohort (Kohler M et al., 2009; Parker AE et al., 2005) despite variability in the medical care and despite ethnic variability. Having the natural history curve and disease progression pattern will help in future for comparison when specific mutation based drug treatment becomes available for DMD. The genotype also parallels global trends suggesting a more homogeneous geno-phenotypic presentation of DMD. Recent reports also highlight an unchanged pattern of diagnostic delay over the last 2 decades (Parsons EP et l., 2009). This could be due to a lack of awareness of the existence of the disease by the parents, or due to a diagnostic delay by the primary care physicians/pediatricians, especially when the phenotype of the patients is skewed toward mental or language delay. Mohamed et al., reported the presence of nonmotor phenotype as an important contributor to the late diagnosis of the disease (Mohamed K et al., 2000). Our cohort consisted mainly of children hailing from a lower socioeconomic status and usually residing in rural areas. They had less access to medical facilities and neurologists, which could be the main reason for the delayed diagnosis. In addition, the low literacy rate among parents and caretakers might have led to the delay in seeking medical advice. On instances, we have observed poor clinical suspicion by the primary care physicians resulting in delayed referral/diagnosis. They tend to consider the delay in motor milestones as a constitutional delay or ascribe it to other nonspecific causes unless there is an affected child in the family. Oral steroids delay the time to loss of ambulation and probably adds quality years to the life of the patients suffering from DMD. Typically, DMD children without steroid

therapy attain wheelchair bound by age 12. However, steroid therapy (Biggar WD et al., 2006; Daftary AS et al., 2007; Markham LW et al., 2008) and advanced ventilatory support (Eagle M et al., 2002) has changed this situation by significantly improving quality of life and prolonging the life expectancy of the affected boys. Sadly, there are no dedicated facilities in any set up in India to provide life supporting care particularly with ventilatory support, although at a personal level parents who can afford make use of non- invasive ventilation which again comes at a high cost.

In **chapter 4** our study on the mutation detection rate in DMD by mPCR, MLPA and muscle biopsy in the same cohort of DMD children is described. The research question in this paper was to compare the sensitivity and pattern of mutations by both mPCR and MLPA in the same cohort of DMD. The clinical accuracy has been very high in our cohort. This is the first study from India and possibly in English literature to have compared the sensitivity of detection of mutations by mPCR and MLPA techniques in the same cohort of children with clinically and biochemically suspected cases of DMD and also looked at the detection rate by the gold standard method of IHC on muscle biopsy among the MLPA negative cases.

It is well known that the underlying mutation is not detectable with available genetic testing in at least 4% of cases, due to a possible discrepancy between mutation study and the clinical phenotype. For this reason, a muscle biopsy subjected for IHC and western blotting is indicated in patients without a detectable mutation. Biopsy done for MLPA negative patients in this study showed that 36.4% had absent staining for dystrophin antibody thus, confirming the diagnosis of DMD by the gold standard method in more than one third of the MLPA negative cases.

Identification of Duchenne cases by IHC among the MLPA negative patients is a clear indication of the possibility of detecting more unidentified mutations. Conceivably, mutation-negative cases should possibly be subjected to novel Next generation sequencing (NGS) or whole genome sequencing (WGS) in case of a high clinical suspicion and positive family history suggestive of DMD. This is a feasible strategy as NGS and WGS are now available in India. NGS has the added advantage of detecting complex rearrangements and large scale intronic alterations, thus offering a higher mutation detection rate than MLPA and other exon-based tests. However, it is apparent from the majority of genetic studies that not all mutations can be identified with standard molecular analysis. In the very small genetically negative group, a muscle biopsy may be helpful for protein studies and muscle RNA analysis to establish an accurate diagnosis. By both methods, in our study, the deletions accounted for 79.5% (66/83) and duplications accounted for 7.22% (6/83), which differed from that observed among other Asian populations where duplication rates were considerably higher and to the extent of 27.3% in Korean (Lee BL et al., 2012) and 24.7% in Taiwanese patients (Hwa HL et al., 2007), as compared to deletions. Our findings were comparable to that observed among Caucasians where 63.4% deletions and 7.3% duplications were reported [White SJ et al., 2006) and North Chinese populations, which showed 66.2% deletions and 6.25% duplications (Wang X et al., 2008). Complex rearrangements and deep intronic changes account for approximately 2% of DMD cases (Stockley TL et al., 2006; del Gaudio D et al. 2008; 2008; Abbs S et al., 2010). Though we did not study the presence of complex rearrangements or deep intronic

mutations among the 2% of genetically negative group, the facility will soon be available at our institute. Having a genetic diagnosis in 100% of suspected DMD cases is imperative to offer the all important genetic counseling and pre-natal testing. Finally, because at NIMHANS we currently can go through all stages of genetic testing, we can compete with most international advanced standards of genetic testing and care in DMD.

With the costs of advanced genetic tests becoming affordable, MLPA analysis should be enhanced by future technological improvements to further increase mutation detection and avoid the invasive muscle biopsy which stops at a diagnosis and no further intervention. The costs of MLPA and mPCR are comparable and MLPA testing is even cheaper in certain laboratories. MLPA is a highly sensitive and rapid alternative to multiplex PCR. It can be used on blood samples, chorionic villi and paraffin-embedded tissue. The ease of detection of duplications and the application for female carrier analysis are clearly the main advantages of the method. In our cohort 36.3% of MLPA negative cases still had DMD and this emphasizes the need to consider more advanced genetic testing and consider performing muscle biopsy only if indispensable. The overall high detection rate by MLPA could be attributed in part to the detection of additional mutations outside the hot spot region.MLPA negative samples should be subjected for Next Generation sequencing to identify point mutations before contemplating a biopsy.

Chapter 5 of this thesis is related to the MLPA positive cases and Genotype-Phenotype correlation. In India there were no publications on genotype-phenotype correlate in large cohorts of DMD. Characterization of clinical phenotype and particular mutations helps in prognostication and also in choosing the appropriate treatment strategies. Our study included MLPA-positive cases in the analysis and correlated the findings with various phenotypic presentations/outcomes in dystrophinopathies. More than 50% of our patients exhibited delays in the acquisition of milestones. A study involving cases from eastern India found that 46.9% experienced delays in attaining milestones, which is similar to the present proportion (Dey S et al., 2015). The mean ages at onset and presentation in our study were 3.4 and 9.5 years, respectively; these ages are very similar to the findings of a previous study from our institute and also those of other studies from eastern and western India (Swaminathan B et al., 2009;

Rao MV et al., 2014; Manjunath M et al., 2015). In our study 88% of the rearrangements in the dystrophin gene caused DMD. Studies conducted in other parts of India and China have shown similar proportions of around 92% [Yang J et al., 2013]. In our entire cohort, 91% of the cases resulted from deletions. Single-exon deletions were most common (26%), and deletions of six or fewer exons constituted 68.7% of cases. Similarly, Magri et al. found that single-exon deletions in the DMD gene constituted 24% of cases (Magri F et al., 2011). Exon 45 was the most frequently deleted single exon in the present study, while exon 50 was most commonly affected overall. Among multiexon deletions in our study, exons 45–52 were the most commonly involved. Similarly, the duplications of six or fewer exons constituted more than 50% of the present cases. Loss of ambulation usually occurs around the age of 10.3 years. One study found no definite associations between age at onset, age at wheelchair dependence, and deletion mutations in the DMD gene (Magri F et al., 2011).

The IQ as assessed in 29 of our patients indicated average or below-average intelligence in 93% of them, with a low IQ being more pronounced in those with distal deletions. In contrast, studies from Kuwait and Egypt did not find any correlation between IQ and the site or frequency of deletions, although a slightly lower IQ was identified in the presence of deletions of exons 12, 45, and 48 (Bastaki LA et al., 1999). Reasons for more common presence of low IQ in our cohort could be that our DMD children are deprived from sources due to their, on average, low socio-economic status; have co-existing malnutrition; lack schooling from young age; and lack cognitive stimulation and motivation. The majority are dropouts due to motor disabilities. Further, our schools are not disabled-friendly and transportation for disabled children is lacking. In conclusion, the results in this study are comparable with those obtained in other countries except for more frequently lower IQ among our children. The reading-frame rule was held in more than 90% of the present cases. Though a general trend toward early wheelchair dependence and low IQ was observed in the presence of distal deletions, a definite correlation was not identified.

As a subsequent step we studied in **chapter 6** the cognitive functions in DMD, an extension of the genotype phenotype correlate study. As early as 1868, Duchenne de Boulogne in his initial description of DMD, first noticed the presence of cognitive deficits in DMD (Duchenne G. 1968). This was followed by many dedicated human studies focusing on the central nervous system involvement in DMD (Desai AD et al., 1969; Cohen HJ et al., 1968), and the presence of intellectual disability was confirmed further in many subsequent studies (Nicholson LV et al., 1993; Cotton S et al., 2001). Thus, children with DMD are well known to have low IQ and several other cognitive impairments. The low IQ scores were attributed to genetic heterogeneity, brain dysfunction, poor socioeconomic status, or lack of environmental stimulation. However, it is now clear that there is dystrophin deficiency in cerebral neurons and mutation specific brain function abnormalities have been established. Certain of the cognitive and learning disabilities as well as neurobehavioral disorders, are associated with disruption of dystrophin isoforms. Mutations affecting Dp260 isoform and 5'untranslated region of Dp140 are known to occur in 60% of patients with learning disability, in 50% in case s with intellectual disability, in 77% of those with autism spectrum disorders, and in 94% of DMD patients with anxiety. (Banihani R et al., 2015) In our research work a total of 22 school-going boys aged between 6 to 10 years old with an IQ greater than 70 were selected for the neuropsychological assessment. The findings from our study provide a better understanding of the neuropsychological profile of boys with DMD in Indian population. Parents and caregivers of children with DMD may be educated and made aware of their neuropsychological strengths and weaknesses. It would be useful to understand that these children may have problems with processing large amounts of information, and they would benefit from breaking information and tasks into concise and simple steps. Use of visual cues may be useful as visual learning and memory appear to be superior to verbal learning for these children. Maintaining consistent routines and repetition helps, as boys with DMD appear to have adequate recall once learning occurs. In addition, engaging these children in tasks and activities that enhance attention, information processing, and working memory would probably help in improving neuropsychological functioning and reduce the impact of cognitive disability. Further, many of these children cannot compete with their normal peers in all spheres of learning and would need special opportunistic schools to adjust

to their learning disabilities and mental subnormality. Thus, current knowledge gained shows that there is definite evidence of autistic features, obsessive-compulsive disorder, and attention deficit hyperactivity disorder (ADHD) in children with DMD. All these studies represent an increased association of neuropsychiatric problems in DMD along with the well- known motor disabilities in these boys. Thus, the frequent association of cognitive impairments in DMD emphasizes the need to identify neurobehavioural disorders in the predominantly muscle disease. Enhanced psychology testing to include both cognitive and neurobehavioral disorders is recommended in all children with DMD (Bushby K et al., 2010). This facility is well established at NIMHANS and we offer advanced neuropsychological testing as well as cognitive retraining for DMD cases with neurobehavioural symptoms.

Chapter 7 of this thesis comprises of MR imaging of the lower limb muscles to identify any stereotyped pattern of muscle involvement in DMD. We also assessed whether MRI-findings can serve as a non-invasive biomarker for DMD, when simple genetic tests are negative. Over the past three decades, neuromuscular imaging has become an important adjunct to clinical diagnosis in DMD. Muscle MRI plays an important role in demonstrating the degree and pattern of involvement of muscles in DMD with high soft tissue contrast. It is a non-invasive test and does not have any side effects of ionizing radiation. T1-weighted images show a high diagnostic accuracy in detecting early fibro fatty infiltration in affected muscles. Fat suppressed T2 image allows edema detection in the early stages of muscle degeneration. In our study we consistently observed a distinctive pattern of skeletal muscle involvement among the 50 children studied, irrespective of the age at evaluation. MRI showed features of dystrophic processes involving both proximal and distal musculature. The features were more severe in the pelvic girdle and thigh muscles as compared to leg muscles.

Muscle MR imaging with its high soft tissue contrast and ability to assess deeper muscle groups has become the technique of choice in differentiating inherited muscle disorders including DMD. (Mercuri E et al., 2007; Wattjes MP et al., 2010). Recent studies have successfully demonstrated variation of fatty infiltration among different age groups of children affected with DMD and its correlation with duration of illness (Li W et al., 2015). Thus, muscle MRI has attained an important role in demonstrating the degree and pattern of involvement of muscles with high high soft tissue contrast. When simple genetic tests are negative, muscle MRI could lead the way in choosing the appropriate gene test to confirm DMD before embarking on the expensive NGS testing.

Quantitative imaging including muscle fat quantification with 3-point Dixon technique, magnetic resonance spectroscopy and perfusion imaging are highly objective and carry good inter- observer reliability in detecting even small pathological changes and progression of muscle involvement and can be a superior marker for longitudinal studies and therapeutic trials. We have just undertaken a small study on serial Quantitative MR imaging of muscle in DMD to understand the evolution of muscle degeneration over time. In conclusion muscle MRI is a non- invasive tool to demonstrate degree and pattern of muscle involvement. It also gives information about the selectivity of muscle involvement in DMD patients which is highly consistent and distinctive. Evaluation of the degree of

fibro-fatty changes definitely helps us to confirm the topography and severity of muscle damage. The severity of muscle involvement as measured by these MRI scores could be useful as a marker for predicting the course of the illness and perhaps prognostication. When routine MLPA testing is negative, muscle MRI findings along with clinical phenotype could help in proceeding to next level genetic testing.

Concluding remarks on DMD diagnostic work-up

It is imperative that for all suspected cases of DMD the minimum level of diagnostic testing to be undertaken is a screening test that detects the majority of exonic deletions. The most basic method still in regular use involves multiplex PCR of the exons involving the hot spot regions. The advantage of this method is its relative simplicity. However, it does not detect duplications, does not characterise all deletion breakpoints, and cannot be used for carrier testing of females. Nevertheless, in remote areas of India with lack of advanced facilities, this method can definitely identify a major portion of the cases. Mothers with only male children and who have completed their family may not actually need genetic counselling unless the child has maternal female cousins.

Newer quantitative genetic tests can fully delineate the exon boundaries of detected mutations and are able to detect mutations in carrier females. Of these methods MLPA is now the most widely used and we are following this for the last 7 years and diagnosed more than 700 cases by this method. A more recent technique utilises oligonucleotide-based array comparative genomic hybridisation (CGH) which analyses copy number variation across the entire gene, including intronic and 3' and 5' flanking regions. CGH has the added advantage of detecting complex rearrangements and large scale intronic alterations and delineating mutation breakpoints much more closely. The CGH method also avoids false positive results.

Unfortunately, the facility to perform CGH does not exist in India. To summarise, the optimum molecular testing strategy for DMD, i.e. current best practice that best balances technical and patient considerations, is an initial screen, preferably quantitative, to detect deletions/duplications, followed by full sequence analysis from genomic DNA. If these are still negative, a muscle biopsy should be performed to enable protein studies like immunohistochemistry and western blotting, both of which are performed at NIMHANS. mRNA analysis could be done if warranted. But this method is not available in India.

The best clinical outcomes for DMD patients are likely to result from early application of treatments that restore expression of the various tissue-specific dystrophin isoforms. To date, both nonsense mutation read-through and induced exon skipping have shown potential to deliver this outcome. Therapies that induce ribosomal read-through of premature termination codons allow production of a full-length functional protein, and Ataluren (PTC124) has undergone clinical evaluation. We presently have begun this drug trial on our nonsense mutation positive patients at NIMHANS.

Another more promising approaches to therapy for DMD is antisense oligonucleotide-induced exon skipping, where the cellular machinery is fooled into by-passing an exon containing a disease-causing point mutation or altering a deletion or duplication, such that a null mutation is no longer generated and DMD is converted to a milder BMD phenotype. Theoretically, 83% of DMD children could be treated with anti-sense oligonucleotides (Aartsma-Rus A et al., 2009).

Antisense oligomer-induced exon skipping demands tailored therapies for different mutations. This is predicated upon accurate genetic diagnosis of young patients at an early stage in the disease, as the benefit will be greatest before substantial muscle is lost. It is essential that we consider preclinical development of many different therapeutic compounds for DMD and deliver exon skipping as a personalised genetic medicine.

As stated in above discussion, best practice therapy for DMD requires early identification of affected boys because this allows implementation of treatment before muscle tissue is permanently lost. Early treatment includes steroid therapy where there is some evidence that using these drugs in an early state produces highly beneficial results (Kinali M et al., 2002; Merlini L et al., 2003) . Thus, it would be imperative to identify DMD cases at an early stage to start treatment and prevent irrevocable muscle damage, but the majority of DMD cases do not have a family history - 33% of affected boys and 33% of mothers have de novo mutations and hence there is frequently a delay in identification of DMD cases, unlike in the familial forms where the motor deficits in the next affected child are recognised early. Nevertheless, potential delay in the diagnosis could still occur with the non-motor forms of DMD.

Screening methods to identify DMD children at an early age is important for management of the motor and non-motor disabilities. Globally, researchers and clinicians have adopted different methods to determine DMD at an early age but the average age at diagnosis still remains high with a wide range from 1 year and 4 months to 8 years and 3 months (Bushby KM et al., 1999). Even now the age at presentation of DMD children at NIMHANS continues to be above the age of 5 years. This delayed consultation in a tertiary care center has not changed over the last 2 decades. Many children are brought to NIMHANS after loss of ambulation due to lack of awareness among parents and primary care physicians. This late arrival to specialised centers like NIMHANS is generally noted in patients coming from remote rural areas. Presently, the best way to successfully identify DMD patients early is through population screening which is currently only performed in a few places in the world. One such successful program running over 2 decades is in Wales, done through newborn screening for Duchenne, using high serum creatine kinase levels as the screening tool. Newborn screening as practised in some countries would detect the first affected boy born in a family shortly after birth, but this may lead to immense emotional strain and anxiety and hopelessness on the new parents. Hence, it has been argued whether it could be done at a later age and not at birth. Implementation of population screening programs for DMD would definitely change the pattern of best practice diagnosis, but it would still depend on the same diagnostic tools and allow early intervention.

Another major effect of early population screening for Duchenne is that it can allow early genetic counselling and the opportunity to avoid secondary cases. This could unequivocally / decidedly promote the reduction of a significant percentage of DMD cases in society. However, this is not possible when early screening is not in place, resulting in families having multiple DMD children. The situation is similar in many families in India as there is no system to detect DMD at an early age. Due to lack of any effective treatment for DMD, all efforts including prenatal diagnosis and carrier detection are mandatory to catch DMD cases early in the course and take appropriate measures.

Future perspectives for DMD care at NIMHANS and beyond

Based on the outcome from this thesis, we envision the following future perspectives to improve patient care:

- To create a large data base of genetically confirmed cases of DMD, segregate them based on the mutation, and categorise them into different intervention groups. With genetic confirmation being routinely performed on all suspected cases of DMD, currently we have a huge number of patients with mutation specific data who are trial- ready for EXON skipping. We have commenced the ATALUREN trial in nonsense mutation DMD children with the candidate as principal investigator.
- Serve as many of DMD cases at NIMHANS and make genetic testing affordable for all patients. NIMHANS being a nodal center for diagnosis and management of neuromuscular disorders and particularly muscle diseases, it is imperative that we take the lead in all aspects and spread the message of standard of care for muscle diseases with special reference to DMD.
- Offer genetic counselling to all potential carriers by services of a clinical geneticist which is available now at NIMHANS.
- Aspire to commence and perform prenatal testing for DMD which is presently not available at NIMHANS.
- Advice regarding prenatal genetic diagnosis (PGD) as a potential screening method for DMD just as is done for cystic fibrosis.
- To propagate the benefits of newborn screening for DMD (Mendell JL et al, 2012)
- Perform quantitative longitudinal muscle MRI and study the pattern of evolution of changes which could be a potential biomarker both for diagnosis and treatment response.
- To establish an Indian Neuromuscular network (INN) to provide co-ordinated molecular and pathological diagnosis for DMD along with all other neuromuscular disorder.
- To overcome our limitations regarding palliative care facilities. We want to provide life supporting therapies such as non-invasive / invasive ventilation that prolong survival and improve the quality of life. Another important facility to strive for is to perform surgery for scoliosis, a common complication after loss of independent ambulation. To perform carrier status of mothers and female relatives of DMD children as a routine procedure and not only in selective mothers and sisters and few centers in India. This

routine screening needs to be considered seriously to reduce the burden of DMD in the society at the institute as well as national level.

- Awareness programs for primary care physicians / pediatricians / rehabilitation specialists is an important aspect to detect children with DMD at an early stage and we with expertise on neuromuscular disorders will have to take the initiative to drive across the need for early confirmatory diagnosis of DMD.

To conclude, the work described in this thesis provided a solid base for NIMHANS to function as an expertise centre for Duchenne Muscular Dystrophy that offers state-of-the-art diagnosis and management at par with many international centers in the world. With the current facilities and infrastructure at NIMHANS, we aim to create a new set of pediatric / adult neuromuscular specialists through our training program and post-doctoral fellowships. Eventually we aspire to provide best palliative care for end stage DMD cases and also have the most crucial pre-natal treating, identification of all potential female carriers as well as counselling for DMD at NIMHANS.

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APPENDICES

A

Appendices

A1

Valorisation

In this thesis we have investigated various aspects of Duchenne Muscular Dystrophy (DMD).

Here we would like to indicate the impact of our research on the standard of diagnosis and management of children with DMD, their family members and care takers as well as future directions.

The Dystrophin (DMD) gene is the largest gene described in human beings. It is made up of more than 2.5 million base pairs (bp), which comprises about 0.1% of the total human genome and 1.5% of the entire X chromosome (Kunkel LM et al., 1989). The properties of the Dystrophin (DMD) gene offer several challenges that include the large size of the gene, frequent new mutations and de novo mutations and problems in identifying its optimal expression level and the target tissue. Due to the huge size of the DMD gene, vector and vector delivery programs have met with hurdles in effective gene therapy. Despite these difficulties, new potential forms of genetics-driven therapeutic strategies are being tested and implemented, e.g. stem cell therapy, virus-based gene therapy, and exon skipping by antisense oligonucleotides or morpholinos. DMD is a rapidly progressive debilitating disease with skeletal and cardiac muscles being the most commonly affected tissues in DMD. However, brain is also a major site of dystrophin expression with several neuropsychiatric manifestations. Presently there is no specific cure for DMD and the lack of any effective treatment has emphasized the need for prenatal diagnosis and carrier detection. Genetic testing for dystrophinopathy is highly sensitive and specific, however identifying a proband often leads to implications for several relatives at risk for cardiomyopathy, weakness, or anesthetic reactions which is a huge ethical challenge. There is currently no genetic screening program implemented in India. The findings of this thesis can be used to design genetic testing which must be sideguarded by genetic counseling before and after the genetic testing. The translation of our research work into standard of care for all patients with DMD including the latest availability of disease modifying agents is the next application of this thesis.

Currently, several genetics-driven therapeutic approaches to cure DMD are being investigated, which can be categorized into two groups: therapies that aim to restore dystrophin expression, and those that aim to compensate for the lack of dystrophin. Therapies that restore dystrophin expression include read-through therapy, exon skipping, vector-mediated gene therapy, and cell therapy. Of these approaches, the most advanced are the read-through and exon skipping therapies. In 2014, Ataluren, a drug that can promote ribosomal read-through of mRNA containing a premature stop codon, was conditionally approved in Europe. Presently, we are part of the global Ataluren trial in DMD cases with nonsense mutation. This is an example of the genetic-pharmacology that is possible through the stringent geno-phenotype analysis that is presented in this thesis.

Improving care through knowledge sharing

The data in this thesis has not only been presented at national and international meetings and conferences, but also in several continuing medical education and muscular dystrophy awareness programs. We also participated in patient – parents programs and shared our research data with peers, students and teachers. All previous data has been published

in peer-reviewed journals and are accessible for reading. Without knowledge sharing through publications as well as conference deliberations on DMD, the less common phenotypic presentations of DMD (predominant cognitive form, cardiomyopathy form) would only be appreciated by neuromuscular specialists and not by general physicians and pediatricians. Every year, we host rotations at NIMHANS for medical personnel to improve their knowledge and understanding in DMD and all other neuromuscular disorders. The collected and acquired data is shared with the public and particularly the parents and relatives of DMD children. We also have prepared dedicated manuals on DMD for the patient and caregivers in English, Hindi, and Kannada. All patients registered at NIMHANS receive a copy of this.

The role of muscle MR imaging and its potential capacity to act as a non-invasive biomarker is emphasized as a highly interesting application of this thesis. Children with high clinical suspicion of DMD but negative by MLPA testing could undergo muscle MRI to look for the characteristic findings and, if positive, then proceed with more expensive genetic testing for DMD. Thus, this could lead to avoiding the painful invasive muscle biopsy in some cases. Moreover, in clinically challenging cases with childhood muscular dystrophies such as sarcoglycanopathy and Fukutin related protein (FKRP) disorder with Duchenne like phenotype, the DMD-specific pattern of muscle involvement on MRI could aid in proceeding for advanced targeted genetic testing, further reducing the need for muscle biopsy. Thus, our findings of the distinct MRI pattern in DMD will aid in this decision. Furthermore, the degree of fibro-fatty infiltration could be a useful marker for assessing the disease severity and progression as well as the response to treatment. Thus, muscle MRI could be utilized as an "adjunct and localized biomarker" for DMD.

Patterns of symptom clustering with Attention Deficit Hyperactivity Disorder, Autistic Spectrum Disorder, and mental subnormality as identified in this thesis suggest that DMD is a major neuropsychiatric syndrome that requires prompt evaluation and early intervention by child psychiatrist / psychologist. In this thesis we used a battery of intelligence, learning, and memory tests to identify the neuropsychological profile in boys with DMD. We identified specific cognitive deficits like significantly lower IQ (88.5) with verbal IQ (86.59) lower than performance IQ (92.64). We also found impaired performance on the processing speed, freedom from distractibility, and verbal comprehension indexes. Specific deficits in information processing, complex attention, immediate verbal memory span, verbal working memory, verbal comprehension, vocabulary, visuoconstruction ability, and verbal learning and encoding were also observed. However, perceptional organization, general fund of information, abstract reasoning, visual discrimination and acuity, visual learning and memory, and verbal memory were adequate. Our findings were opposed to the more frequently reported global intellectual deficit in DMD. Cognitive disturbances have been identified even in infants and children less than 5 years of age with DMD. Enhanced psychology testing to include both cognitive and neurobehavioral disorders is nowadays recommended for all children with DMD. The results of this thesis can be used to develop a program to help DMD-patients with cognitive disorders.

Special cognitive training modules could be developed fot the DMD children based on the impairment as identified at school or in special education centers during the elementary school years itself.

We suggest that the standard evaluation for young boys with global developmental delay includes an inexpensive but sensitive serum creatine kinase test to capture undiagnosed cases of DMD at an early stage. Early diagnosis and genetic counseling can prevent mothers from having a second child with DMD. The program to address the non-motor cognitive deficits in DMD should include early identification of the various disturbances as well as appropriate measures including behavioural therapy, special school admission and cognitive training.

Neurologists and child psychiatrists should evaluate for ADHD in DMD children with behavioral concerns (e.g., inattention, hyperactivity, impulsivity) or poor academic progress using validated assessment tools with observers from several settings (home, school, community) and self-observation. Behavioral treatments are recommended for preschool-aged children and may be helpful at older ages. Effective behavioral therapies include parent training, classroom management, and peer interventions. While there is no cure for specific learning disorders in DMD, we can help them to improve their reading, writing, and mathematics skills. They would require both strengthening the skills and developing a learning strategy tailored to take advantage of the child's strengths. Treatment for specific learning disorder often also involves multimodal teaching. A learning specialist can identify the learning disabilities in the DMD child and help determine the special services a child might benefit from at school. Psychotherapy, cognitive behavior therapy in particular, may also be helpful in treating the emotional and behavioral problems in DMD. Ultimately, the goal of treatment is to improve symptoms, optimize functional performance, and remove behavioral obstacles. Finally, the aim is that all these interventions would lead to the improvement in the quality of life for DMD patients and their families. We at NIMHANS are now in a strong position to identify all forms of mutations, to perfom genotype-phenotype correlation and to offer early diagnosis. This will result in early intervention with respect to cognitive disturbances and will provide a base for appropriate counseling for schooling and vocational training.

Major components of the thesis work presented here were directly and clinically applicable and resulted in a successful standard of care for all cases of DMD, which was agonizingly lacking in our country. A confirmatory and reliable genetic diagnosis of DMD children was not available till a decade ago, leading to lack of genetic counseling and prenatal testing. There were no means to advise / test regarding carrier status and mothers took a risk with future male births. Further, as a result of the continued knowledge deficit among the illiterate / literate families and physicians there are families with multiple members affected in multiple generations leading to profound emotional and economic burden on the already poor families.

Therefore, the long term goals of this research data is to collaborate with the knowledge users such as the government, philanthropists, social and humanity related individuals. Financial support and commitment of governmental and non-governmental agencies is of utmost importance to continue with clinical and genetic work. National policies need to be framed that will have resource allocation for research and improvement of clinical care on such rare diseases because families with DMD children silently suffer.

India needs more specialists in neuromuscular disorders to take care of the large numbers of these patients although they are rare disorders. Informal collaborations that are common among scientists need to be made more formal between institutions. Our research consists of expertise, experience and knowledge gathered over several decades of research on DMD, beginning with humble clinical studies to the latest and high technology genetic studies.

Future perspectives

DMD research needs "transdisciplinary research" and management strategies. Multidisciplinary specialists comprising of pediatricians, neurologists, neuromuscular specialists, clinical geneticist, neuropsychologists, rehabilitation specialists, counselors, special teachers and social workers, need to come together to provide the best care for all patients and their families. In our research work we have involved different specialities in the armamentarium of DMD management which can be translated to other neurology centers in the country. In addition, we have learnt that there is a need for participatory transdisciplinary research in DMD. The fact that the knowledge gained through our DMD research enabled us to be part of an "advisory transdisciplinary" group for other neurodegenerative diseases may be the utmost application of the work presented in this thesis.

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Summary

The X-linked Duchenne muscular dystrophy (DMD) occurs due to mutations in the Xp21 gene, which consists of 79 exons encoding the dystrophin protein. Children with DMD typically manifest between the ages of 2 and 5 years with symptoms of delayed attainment of motor milestones. Universally they have highly elevated serum creatine kinase levels and liver enzymes. Progressive proximal weakness with calf hypertrophy occurs in all. DMD children generally lose independent ambulation by 12 years of age. Prevalence of cardiomyopathy increases with age, and without ventilatory intervention, death typically occurs by 20 years of age. Non-motor manifestations include cognitive impairment, delayed language development, diminished intelligence quotient (IQ), autism, attention deficit hyperactivity disorder, and obsessive-compulsive disorder.

Until molecular diagnosis became available, muscle biopsy with immunohistochemistry and western blotting was the GOLD standard procedure. Current genomic analysis detects around 93% to 96% of mutations. The remainder consists of mRNA rearrangements that require analysis of muscle-derived mRNA. Complete mutational analysis of the DMD gene is now a readily available and affordable genetic test and is considered the standard of care. Further-more, mutation specific molecular therapies are in or are nearing clinical trials, and it is imperative to consider the suitability of the patient for these.

Deletions account for approximately 65% of DMD. The simple multiplex polymerase chain reaction (mPCR) test was widely used but is not useful for detection of carriers or patients with duplications or point mutations. Multiplex ligation-dependent probe amplification is the test of choice, which identifies exon duplication mutations, defines the extent of contiguous exon deletions and detects carriers. Currently, next-generation sequencing approaches are the best. Management is ideally provided at a multidisciplinary care setting and Glucocorticoids remain the mainstay treatment.

This thesis comprises of a compilation of articles reporting on the experience of progress in research on DMD over the last 15 years at a multidisciplinary Neuromuscular Disorders (NMD) clinic at the National Institute of Mental Health and Neurosciences (NIMHANS) in Bangalore, Karnataka, India. From a clinical and biochemical diagnosis with histopathological confirmation, we have evolved through simple genetic to complex genetic analysis, genotype-phenotype correlation, cognitive assessment, muscle MR imaging, mutations in familial forms of DMD to the latest Quantitative Brain Imaging and Next Generation Sequencing in DMD.

The first progress at NIMHANS in the genetic diagnosis of DMD was in the year 2002 with mPCR testing for DMD. In this prospective study (chapter 2), 112 DMD patients had muscle biopsy and were tested for exon deletions. The diagnosis of DMD was confirmed by histopathology and/or genetics in 101 patients. Here, we concluded that this high deletion rate by mPCR (90%) was possibly due to good phenotyping.

For the purpose of understanding the natural history in DMD, confirmed cases seen between 1998 and 2014 were contacted through letter / telephone / follow-up visit (chapter 3). Around 275 cases out of 500 families participated, 31% by completing the questionnaire and 69% by completing the questionnaire and attending the clinic.

Wheelchair status was attained in 124 (45.1%) boys at a mean age of 10.4 years and bedbound state in 24 at a mean age of 11.8 years. Seven patients (2.6%) had died at a mean age of 15.2 year. This is the first study from India describing the disease progression of DMD. The patterns of major DMD milestones, including the age at onset, age at loss of ambulation and death in our cohort is comparable to that of the Western cohorts.

In the subsequent study (chapter 4), we compared the sensitivity of mPCR and MLPA testing in 83 DMD cases. MLPA-negative cases underwent muscle immunohistochemistry (IHC). mPCR detected deletions in 60 (72.3%), while MLPA detected deletions in an additional six cases (7.2%) and duplications in 5 (6.5%). IHC confirmed dystrophinopathy in 30 (36.1%), sarcoglycanopathy in 30 (36.3%), and no deficiency in 23 (27.7%). This is the first comparative study of its kind in India (and possibly in the world) to study the sensitivity and pattern of mutations by both mPCR and MLPA in the same cohort.

Later on we studied the genotype-phenotype pattern in 317 patients with dystrophinopathy confirmed by MLPA method (chapter 5). There were 279 cases of DMD, 32 of BMD and 6 of intermediate phenotype. In this cohort 88% of the rearrangements in the dystrophin gene caused DMD.The reading-frame rule was present in 90% of DMD and in 94% of BMD cases. A tendency toward a lower IQ and earlier wheelchair dependence was observed with distal exon deletions.

It is well known that DMD children have a high incidence of neuropsychiatric manifestations. In chapter 6 we describe our study in which a total of 22 school-going DMD boys aged between 6 to 10 years old were neuropsychologically assessed by means of Wechsler Intelligence Scale for Children-Third Edition, Rey's Auditory Verbal Learning Test, and the Memory for Designs Test. They had a significantly lower IQ (88.5). Verbal IQ (86.59) was found to be lower than Performance IQ (92.64). The neuropsychological findings supported the hypothesis that these children have specific cognitive deficits as opposed to a global intellectual deficit. Based on these results we offer advanced neuropsychological testing as well as cognitive retraining for DMD cases at NIMHANS.

Clinicallly DMD children have a particular pattern of muscle weakness and to correlate this we performed lower limb muscle MRI and identified a distinct pattern in all cases irrespective of the age at evaluation (chapter 7). Muscle MRI could help as an imaging biomarker for directing genetic testing and, if serially performed, could help in assessing drug response.

In our latest report (chapter 8) we have studied 606 cases of DMD and compared various parmeters between familial and non-familial forms of DMD. The mutations comprised of exonic deletions in 81.2%, duplications in 5.4% and small mutations (point mutations and INDELs) in 11.5% of cases. Families with two or more affected males contributed to 12% of the entire cohort. MLPA identified the larger mutations more often in sporadic (88.2%) than in familial cases (75.3%), while nonsense mutations were more common in familial (17.8%) than in sporadic cases (10.7%). The familial group reported an earlier onset of disease (2.8 ± 1.7 years) as compared to sporadic cases (3.8 ± 1.6 years). MLPA could identify mutations in a high percentage of our DMD children. The preponderance

of small mutations was noted to be distinctly higher in the familial group. Intriguingly, the familial form of DMD formed a small percentage of the entire cohort. The reasons could be increasing awareness among parents and physicians with early identification of DMD cases, genetic counseling and prenatal testing.

The above studies on DMD depict the journey we have experienced and the outcome of the research over the last many years at NIMHANS. In Chapter 10, all findings of the various chapters were put into broader perspective. Moving from clinical and biochemical diagnosis in the 1980's we now have reached the status of center of excellence and advanced care for DMD at par with leading international centers. However, we still lack in several palliative care aspects and end-of-life care for DMD children. Also, more serious attempts to identify all DMD carriers followed by genetic counseling are required.

The roughly 4000 newly diagnosed DMD patients each year in the Indian subcontinent have a tremendous societal and economic burden. Every attempt is necessary to diagnose DMD at an early stage and offer genetic counseling and pre-natal testing.

Mutation-negative cases should be subjected to novel NGS or Whole Genome Sequencing (WGS) in case of high clinical suspicion and positive family history. NGS has the added advantage of detecting complex rearrangements and large-scale intronic alterations, thus offering a higher mutation detection rate than MLPA and other exon-based tests. A small number will require mRNA studies.

Having a genetic diagnosis in 100% of suspected DMD cases is imperative to offer the important genetic counseling and prenatal testing. Finally, because at NIMHANS we currently can go through all stages of genetic testing, we can compete with most international advanced standards of genetic testing and care in DMD.

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Acknowledgements

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During the visit of **Prof. Tammo Delhaas** to NIMHANS in December 2016, I was introduced to the possibility to pursue a PhD at Maastricht University. I was immediately excited and took this opportunity under his guidance. Within a week I was sent a long list of criteria for my thesis and although I was not sure of being successful, I did take a plunge into this fresh assignment and now I have only pleasant memories to cherish. As part of this PhD thesis I was to have a joint publication. I recollect that I would send drafts to Professor Delhaas and would get an overnight reply with abundant suggestions and comments. This fast reply encouraged me to do the same and I would sit up in the early hours to revise the write-up and try and send back the same morning. I immensely learnt from him the art of writing and admire his goal of perfection. **Prof. Boris Kramer**, my second supervisor, took extraordinary efforts to give a good write-up of the thesis despite being an overwhelmingly busy neonatologist.

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About the author

CURRICULUM VITAE

A. Nalini was born on February, 7th 1964 in Bangalore, India. She completed her schooling in Bethany High School in April 1981, followed by her two years Pre-University studies in science subjects at the Jyoti Nivas womens college in 1983.

In the same year August she entered Medical school at the Bangalore Medical College and successfully passed with honors in the year 1988 and then completed her internship in April 1989. In May 1989, she competed in the National level entrance examination of the premier National Institute of Mental Health and Neurosciences NIMHANS, Bangalore and was selected for the post MBBS DM in Neurology training program in September 1989. This was a great achievement as she was one among 4 students who were selected for the course. Her DM neurology thesis was entitled "Evaluation of Superoxide Dismutase levels in cerebrospinal fluid and trial of Intravenous Cyclophosphamide in ALS". She successfully completed the 5 year Neurology training program and received the GOLD medal for best outgoing resident in the final examination. Thereafter, as a continuation of her thesis research work, Dr Nalini worked as a senior scientific officer in the department of Neurophysiology at NIMHANS, granted by the Council for Scientific and Industrial Research. During this period she worked on animal models of ALS and learnt the basic techniques of tissue culture. She later joined as faculty in the department of neurology at the MS Ramaiah Medical College and Teaching hospital, Bangalore and worked at this place till December 1999 when she was selected as Assistant Professor of Neurology at NIMHANS, her Alma Mater.

For the last 2 decades Dr. Nalini is relentlessly working on clinical and basic research aspects of acquired and inherited Neuromuscular disorders including ALS and Hirayama disease. She has to her credit around 145 publications in peer reviewed journals and her seminal work is on the clinical and genetic aspects of Duchenne muscular dystrophy in which she has made great strides. Dr Nalini has described several phenotypic variants of Hirayama disease which were hitherto considered as degenerative disorders. She has been a guide to several DM Neurology students and also PhD scholars. She and her team conduct the only kind of multidisciplinary Neuromuscular disorders clinic at NIMHANS and have registered more than 10,000 cases from its inception in the year 1991. She has created large cohorts of genetically confirmed inherited Neuromuscular disorders which is the only kind from India.

Recognizing her contributions to medical research in Neuromuscular disorders, Dr Nalini, was awarded the prestigious SIR CV Raman award for Young Medical Scientist in the year 2008. In the year 2013 she was invited as visiting Professor to the University of Massachusetts General Hospital by Professor Robert Brown. Currently she is an Executive Board member for the Asia Oceanian Myology center and invited member for the Asia Oceanian Inherited Neuropathy Consortium. She is immensely contributing to the clinical phenotyping and genetic diagnosis and management of Neuromuscular disorders for patients in India.