Spectrum of mutations in Duchenne Muscular Dystrophy (DMD) and clinical phenotype correlation in Sri Lankan population.

By

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DECLARATION

I declare that the contents of this dissertation are my own work, except for that detailed below, for which I would like to thank the following person:

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ABSTRACT

Duchenne Muscular Dystrophy (DMD), which affects 1 in 3500 newborn male, is one of the most common fatal neurodegenerative disorders in children. It is an X-Linked recessive disorder and the most severe form of dystrophopathy affecting newborn males. It is also the most common muscular dystrophy in all parts of the world.

Kunkel and his colleagues cloned the gene, which was named as dystrophin gene. Dystrophin gene is localized to chromosome Xp21 and is composed of 79 exons. It is the largest gene in the human genome. Dystrophin gene produces dystrophin protein which is a component of the sarcolemmal cytoskeleton network. Deficiency of dystrophin expression affects formation of the DAPC (dystrophin associated protein complex) and disrupts the molecular bridge. These effects can lead to two consequences: first, cell membranes become more fragile which can be mechanically damaged during muscle contraction; and second, membrane proteins, especially mechanic-sensitive ion channels get deregulated. The calcium overloading results in activation of ca^{2+} dependent protease and contributes to muscle fiber degeneration. Hyperactivation of signaling cascades also promotes an inflammatory response by increasing expression of inflammatory mediators and chemoattractants in dystrophin deficient muscle.

Exon deletions and duplications are the most common molecular defect causing the disease accounting for approximately 65% and 6% of mutations respectively. Point mutations accounts for the rest. Approximately 30% of DMD patients have unidentified mutations or point mutations in the dystrophin gene. Deletions, and more rarely duplications, can occur anywhere; however two deletion hotspots are known – one located towards the central part of the gene and the other towards the 5’ end. The former is most commonly mutated and includes exons from 45 to 55 while the 5’ end hotspot includes exons from 2 to 19. The present study was aimed at

investigating the spectrum of deletions/duplications in a cohort of Sri Lankan children with DMD and to study the correlation with the clinical phenotype.

We selected 50 clinically diagnosed children with DMD. Written informed consent was taken from parents as all the children were under 18 years of age. Clinical data were obtained by examining all the children and reviewing their records. We applied Multiple Ligation-binding Probe Amplification (MLPA) to analyze the spectrum of deletions and duplications involving the DMD gene in the Sri Lankan population. This was performed by SALSA MLPA kit (MRC Holland, Netherlands) according to the manufacturer's protocol (version MDP-v001). Data analysis was done by using the coffalyser software program from MRC-Holland.

The genotyping showed deletions in 40 (80%) children and duplications in 4 (8%) children. Deletion/ duplication were not detected in 6 (12%) children. The most common deletion was an eight exon deletion ranging from exons 45 to 52 which was seen in 6 (12.0%) children. The next common exon deletion was single exon 45 deletion which was seen in 4 (8.0%) children. When exons involved in deletions or duplications were looked, single exon involvement was seen in 8 (16%) children. Two exons involvement was seen in 3 (6%) children, three exons involvement was seen in 6 (12%), four exons involvement was seen in 1 (2%) child and more than four exons involvement was seen in 26 (52%) children.

The 79 exons of the dystrophin gene were divided into four regions in order to correlate with the clinical phenotype. The first group ranged from exon 1 to exon 20, the second group ranged from exon 21 to exon 44, the third group ranged from exon 45 to 55 and the fourth group ranged from exon 56 to exon 79. In this study, the most frequent mutant region fell within exons 45 to 55 (52%) in *DMD* gene, followed by exons from 21 to 44 (26%) and exons from 1 to 20 (26%) and

the least common region fell within exons 56 to 79 (4%). We found that the most common region in our study was ranged from exon 45 to 55 and the least common region was from exon 56 to 79. In this study the position of deletion/duplication did not correlate with the clinical phenotype.

In conclusion, the deletion/duplication pattern seen in the Sri Lankan population was similar to that of other global populations but there was no correlation between the genotype and the clinical phenotype. We suggest a large scale study including all the clinical parameters as well as all types of mutations such as point mutation to find a correlation between genetic mutations and phenotypes.

PUBLICATION AND ABSTRACTS

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1. Introduction:

This dissertation describes the genetic etiology and phenotypic spectrum of Duchenne Muscular Dystrophy (DMD) in Sri Lankan population using clinical as well as molecular genetics aspects. The introductory chapter begins with background information about the disease, its clinical progression, staging of the disease, diagnostic criteria and its histological characteristics. It briefly describes about the gene and the protein which are the main basis of this disease. It also describes briefly on known pathophysiological aspects of the disease. The next section of introduction is a review of scientific literature on the current knowledge of genetic mutations and the disease causing mechanisms. The next section describes the systemic involvement with this disease and genetic treatment approaches in DMD. The introductory chapter concludes with the objectives of the dissertation. All the sections of the introductory chapter include review of a scientific literature on the current state. The second section describes about the methodology used in this research and dissertation. It describes a description of the recruitment of the subject, the phenotyping methods, the molecular genetics methods and the statistical methods used for these investigations. Results are reported in the third section and fourth chapter will discuss these results. Finally the dissertation will be summarized with conclusion.

1.1 Background and Justification:

Duchenne muscular dystrophy (DMD) is an X-Linked recessive disorder. It is the most severe form of dystrophopathy affecting 1 in 3500 newborn males and is the most common muscular dystrophy in all parts of the world (Monckton *et al.* 1982). It is characterized by proximal muscle weakness and calf hypertrophy. Initially the child presents with motor delay or abnormal gait. The child can also present with difficulty in running, getting up from ground, frequent falls and toe walking. Most of the children present between 3 to 5 years of age.

1.2 Clinical progression:

DMD children are normal at birth although the serum levels of the muscle isoform of creatine kinase are elevated. Usually the child presents with delayed motor milestones including a delay in walking, unsteady gait and difficulty in running. Subsequently, appearance of pseudohypertrophy of calf muscles, proximal muscle weakness and Gower's sign (the use of child's arms to climb up his body when going from a lying to standing position) suggest diagnosis of DMD. The affected child may have proximal weakness affecting the lower extremities before the upper one, leading to distal lower and then upper limb weakness. Weakness of neck flexors is often present and most affected children with DMD have difficulty in jumping. Affected children have waddling gait, calf enlargement and lumbar lordosis which disappear on sitting position. It is a progressive disorder which eventually leads the child to wheelchair by the age of 11-12 years due to decreased lower limb muscle strength and joint contractures (Yiu *et al.* 2008). Weakness of the arms occurs later with progressive kyphoscoliosis. They can also have cardiac disease with dilated cardiomyopathy due to cardiac fibrosis and disturbances in rhythm and conduction. Cardiomyopathy is apparently seen after 10 years of age. It affects one third of children by the age of 14 years and present in almost all patients over 18 years of age (Nigro *et al.* 1990). Atrial and ventricular arrhythmias including premature ventricular beats and complex ventricular ectopy may increase with age and degree of ventricular dysfunction (Chenard *et al.* 1993). Children with DMD may present with respiratory complication. DMD causes a progressive impairment of muscle function leading to hypercapnic respiratory failure. Chronic respiratory insufficiency due to restrictive lung disease is inevitable in all patients. Vital capacity increases until age around 10 years, after that it starts to decrease at a rate of 8-12% per year. When vital capacity reaches less than 1 liter the risk of death within the

next one to two years is relatively high (Phillips *et al.* 2001). The most common cause of sleep disordered breathing is obstructive sleep apnea in the first decade of life. In the second decade of life, it is complicated with hypoventilation (Suresh *et al.* 2005). Intellectual disability is seen in 30% of cases with DMD. Verbal IQ is more impaired than performance IQ. Intellectual impairment, especially verbal impairment is associated with DMD but it is non-progressive and does not affect every child. A high rate of emotional disturbance is also associated with the disease (Leibowitz *et al.* 1981) . Scoliosis occurs in almost all children with DMD. This scoliosis impacts on respiratory vital capacity (Smith *et al.* 1989). Progression of this becomes significant after loss of ambulation (Rodillo *et al.* 1988). Long bone fractures are commonly seen due to frequent falls, affecting 21-40% (McDonald *et al.* 2002). Osteoporosis can be seen in most of the children with DMD. Bone mineral density begins to decrease even when children are still ambulant and continues to diminish with age (Larson *et al.* 2000; Aparicio *et al.* 2002). Affected children suffer each day with increasing disability physically and mentally and eventually leading to death in early teen age which also causes psychological and social distress to the family.

1.3 Staging and Diagnosis of DMD:

There are five stages of DMD (Bushby *et al.* 2010).

1. Presymptomatic: In presymptomatic stage, child will come with a family history of DMD and raised creatinine kinase. He might present with developmental delay but there will not be any gait disturbance.
2. Early ambulatory: In early ambulatory stage, the child will present with Gower's sign, waddling type of gait. He might be with toe walking but will be able to climb stairs.

3. Late ambulatory: In late ambulatory, the child will present with increasingly labored gait and will be losing ability to climb stairs and rise from floor.
4. Early non-ambulatory: In early non-ambulatory stage, the child might be able to self propel for some time and able to maintain posture but he might develop scoliosis.
5. Late non-ambulatory: In late non-ambulatory stage, there will be limitations in upper limb function and postural maintenance.

1.4 Diagnostic Criteria:

This diagnostic criteria is based on a report originally published in Neuromuscular Disorder in the year 1991 (Jennekens *et al.* 1991).

Elements

1. Symptoms are present before the age of 5 years.
2. Clinical signs comprise progressive symmetrical muscle weakness; proximal limb muscles more than distal muscles; initially only limb muscles. Calf hypertrophy is often present.
3. Exclusions: Fasciculations, loss of sensory modalities.
4. Loss of unassisted ambulation before the age of 13 years.
5. There is at least a 10-fold increase in serum creatinine kinase (SCK) activity (in relation to age and mobility).
6. Muscle biopsy: Abnormal variation in diameter of the muscle fibers (atrophic and hypertrophic fibers), (foci of) necrotic and regenerative fibers, hyaline fibers, increase of endomysial connective and fat tissue.

7. Muscle biopsy: Almost no dystrophin demonstrable, except for an occasional muscle fiber (less than 5% of fibers).
8. DNA: Duchenne-type mutation within the dystrophin gene, identical haplotype, involving closely linked markers, as in previous case in the family.
9. Positive family history, compatible with X-linked recessive inheritance.

Assessment

The diagnosis is definite when

A. The first case in a family:
    a. Age < 5 years: (2), 3, 5, 6, 7, (8) all present.
    b. Age 5-12 years: 1, 2, 3, 4, 5 (at least once), 6, 7, (8) all present.
    c. Age > 12 years: (1), 2, 3, 4, 5 (at least once), 8, (or 6 and 7) all present.
B. Another case in the family (according to element 9) complies with the criteria under A:
    a. Age < 5 years: 5 and 9 present
    b. Age 5-12 years: 1, 2, 3, 5 (at least once) all present.
    c. Age > 12 years: (1), 2, 3, 4, 5 (at least once) all present.

The diagnosis is possible when:

a. Age < 5 years: (2), 3, 5, 6, all present
b. Age 5-12 years: 1, 2, 3, (4), 5 (at least once), 6, all present.

Note: When family history is positive (according to element 9) and B is not valid, one should rule as specified under A.

1.5 Histological features:

Normally skeletal muscle consists of evenly spaced, angular and uniform sized muscle fibers. It is multinucleated with nuclei located at the periphery of the fiber. Necrotic or degenerating muscle fibers are seen in almost all DMD muscle biopsies even before appearance of muscular weakness. Muscle biopsies from DMD children show inflammatory cells at perimysial and endomysial sites because necrotic fibers are subject to phagocytosis. A secondary sign of muscle fiber necrosis is the active regeneration of muscle to replace or repair damaged fibers. Ultimately, regenerative capacity of the muscle is lost and muscle fibers are gradually replaced by adipose tissue and fibrous connective tissue which clinically appears as pseudohypertrophy which is followed by atrophy. Eventually progressive fibrosis and muscle fiber loss leads to muscle wasting and muscle weakness (Blake *et al.* 2002).

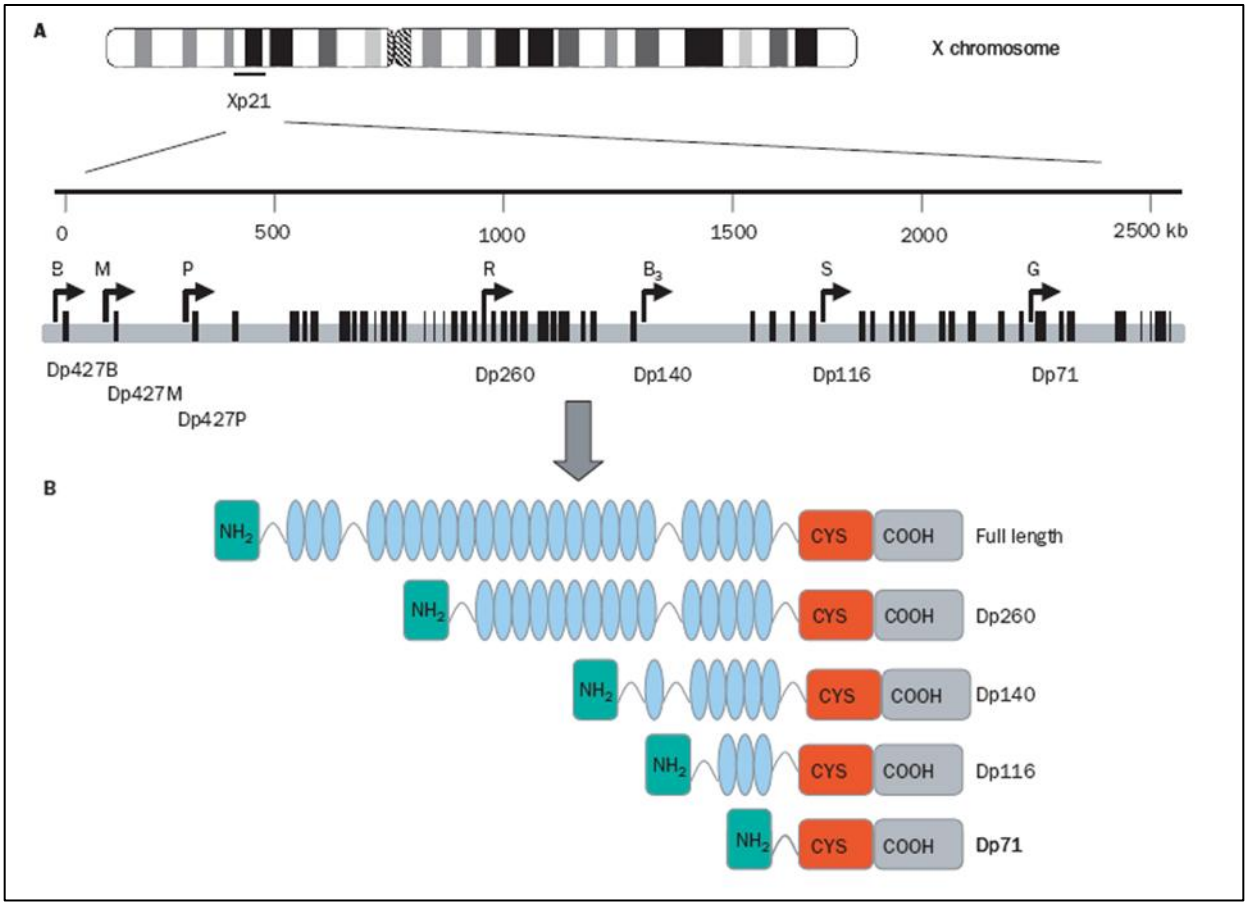
1.6 Dystrophin Gene and protein:

In 1987, about 150 years after Duchene muscular dystrophy (DMD) was described, the gene responsible for it, the dystrophin gene, was cloned by Kunkel and colleagues. Most pathological features of DMD were described in the 19^{th} century. By middle of 20^{th} century, the milder type of DMD, namely, Becker muscular dystrophy was described. Presence of aldolase at higher levels in serum in patients with muscular dystrophy was described by Lehninger in 1949. Ebashi and Sugita discovered high serum creatinine kinase (CK) level in 1959. In 1987, Kunkel and his colleagues cloned the gene, which was then named as dystrophin gene. Dystrophin gene is localized at chromosome Xp21, its size is 3 megabases, occupying approximately 1/1000 of total genome size and is composed of 79 exons and 8 tissue specific promoters. It is one of the largest

known genes in the human genome. The size of the mRNA is 14kb. The number of amino acid (AA) residues in dystrophin is 3,685 and it has a molecular weight of 427Da (Ozawa 2010).

1.6.1 Tissue specific promoters:

Expression of the full length dystrophin transcript is controlled by three independently regulated promoters. The brain (B), muscle (M) and purkinje (P) consists of unique first exons spliced to a common set of 78 exons. These three full length isoforms that have the same number of exons but which are derived from three independent promoters in the brain, muscle and purkinje cerebella neurons (figure 1) are named according to their site of expression. The brain promoter drives expression primarily in cortical neurons and the hippocampus of the brain whereas purkinje promoter is expressed in the cerebellar purkinje cells and skeletal muscle. The muscle promoter derives expression in skeletal muscle and cardiomyocytes and also in some glial cells in the brain. Dystrophin gene also produces many isoforms generated from splicing events. These splice variants are formed either by exclusion of some exons known as exon skipping or by subversion of the reciprocal order of exons known as exon scrambling (Sadoulet-Puccio *et al.* 1996; Blake *et al.* 2002). The dystrophin gene has at least four internal promoters that give rise to shorter dystrophin proteins lacking the actin binding terminus but retaining the cysteine rich and carboxy-terminus domains which contains the binding site for dystroglycan, dystrobrevin and syntrophin. Each of these internal promoters uses a unique first exon that splices into exons 30, 45, 56, and 63 to generate protein products of 260kDa (Dp260), 140kDa (Dp 140), 116kDa (Dp 116) and 71kDa (Dp 71) (D'Souza *et al.* 1995; Lidov *et al.* 1995). Dp 260 is expressed in the retina; Dp 140 is expressed in brain, retina and kidney. Dp 116 is expressed in peripheral nerves. Dp 71 can be found in brain, retina, kidney, liver and lung (Muntoni *et al.* 2003)



Source: Francesco Muntoni, Silvia Torelli, and Alessandra Ferlini. THE LANCET Neurology Vol 2 December 2003

Figure 1: Genomic organization of the dystrophin gene, located in Xp21. The black vertical lines represent the 79 exons of the dystrophin gene distributed over about 2·5 million bases. The arrows indicate the various promoters: in particular are brain (B), muscle (M), and Purkinje (P) promoters; R, B3, S, and G represent the Dp260 (retinal), Dp140 (brain3), Dp116 (Schwann cells), and Dp71 (general) promoters. B: The domain composition of the various dystrophin proteins is indicated. The amino-terminal domain is followed by the spectrin like domain, the cysteine rich, and the carboxyl-terminal domain.

1.6.2 The dystrophin protein:

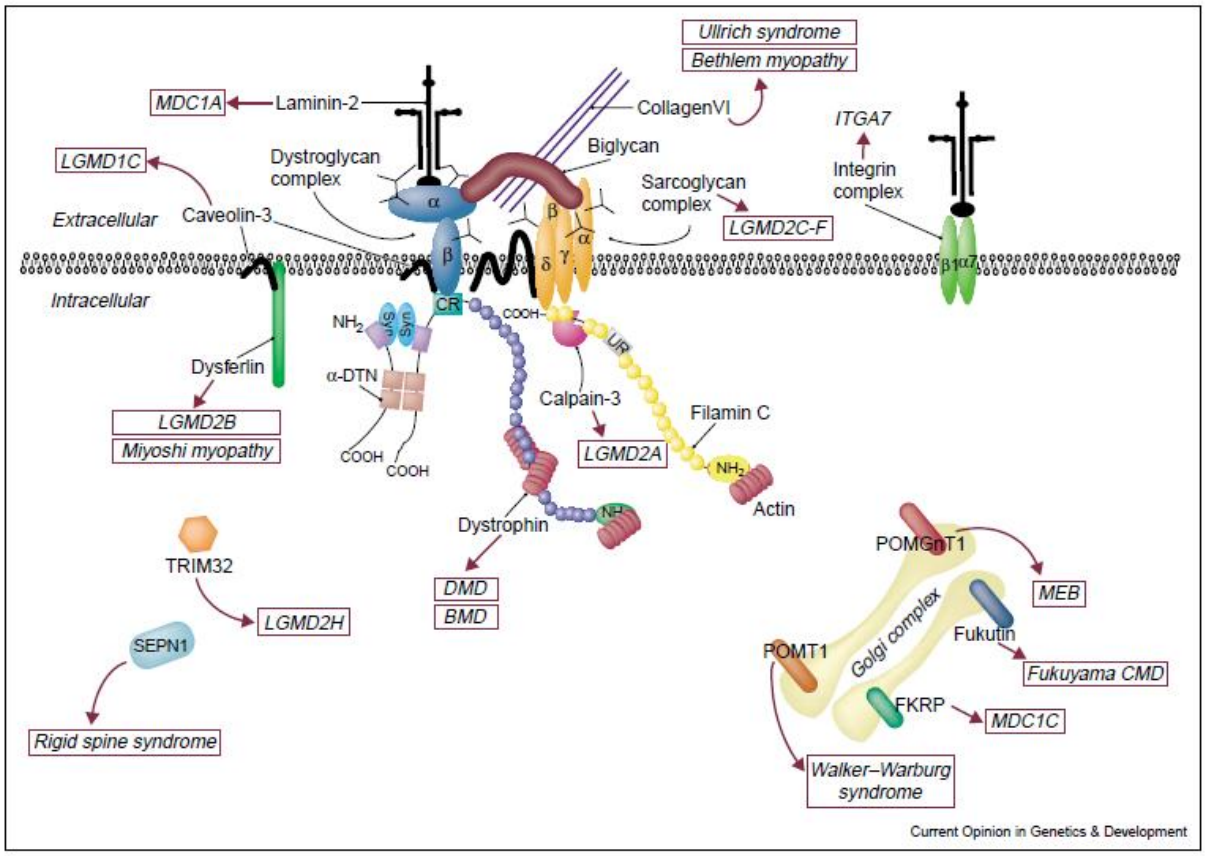
Dystrophin is 427-kDa cytoskeleton protein belonging to β-spectrin/α-actinin protein group. This group is characterized by an NH_2-terminal actin binding domain followed by a variable number of repeating units known as spectrin like repeats. Dystrophin can be organized into four regions based on sequence homologies and protein binding capabilities. These are actin-binding domain

at the NH_2 terminus, the central rod domain, the cystein rich domain and the COOH-terminus domain (Den Dunnen *et al.* 1989).The rod domain is composed of 24 repeating units which are similar to the triple helical repeats of spectrin. This repeating units accounts for the majority of the dystrophin protein and is thought to give the molecule a flexible rod like structure similar to β spectrin (Koenig *et al.* 1990). Dystrophin is a component of the sarcolemmal cytoskeleton network. Sarcolemma is used to define the basal lamina, lipid bilayer and subsarcolemmal cytoskeleton network together. The lipid bilayer is the main layer among the sarcolemma which serves as the barrier to maintain the constant intracellular environment, because ionized substances are impermeable. The basal lamina is a thick and tough layer that extracellularly attaches to the lipid bilayer, but is permeable to various molecules including proteins. The subsarcolemmal cytoskeleton networks present beneath the lipid bilayer of muscle fibers which contain a number of ɣ-actin filaments. ɣ-actin filaments are connected to desmin by plectin to form the main components of the networks that form the lining of the lipid bilayer (Dalkilic *et al.* 2003). Knowledge of actin-binding dystrophin protein and its localization to the muscle cell membrane led to researches on dystrophin and dystrophin-associated protein complex. There are sarcolemmal and subsarcolemmal proteins that form the dystrophin associated protein complex (DAPC) summarized below (figure-2).

1.6.3 Dystrophin associated protein complex (DAPC):

The dystrophin-associated protein complex (DAPC) was identified because dystrophin was found to be enriched in muscle membrane fractions from a wheat germ agglutinin (WGA) column (Blake *et al.* 2002). The DAPC can be subdivided into three subgroups: the dystroglycans, the dystrobrevins and syntrophins and the sarcoglycan-sarcospan complex. There

are two types of dystroglycans named as β-dystroglycan and α-dystroglycan. β-dystroglycan binds directly to the C terminus of dystrophin inside the muscle cell whereas α-dystroglycan is a highly glycosylated protein that bridges β-dystroglycan to extracellular matrix proteins such as laminin-2 (Williamson *et al.* 1997). Apart from the link between the cytoskeleton and extracellular matrix, dystroglycans are important in signaling pathway via interaction with other proteins known to have signaling functions such as Grb-2 (adaptor protein which interacts with syntrophins in the cytoplasm) (Oak *et al.* 2001). Dystrobrevins and syntrophins are the second subgroup. There are multiple isoforms of α and β-dystrobrevin and three syntrophin isoforms intracellularly which binds to dystrophin (Jung *et al.* 1995). Third subgroup is sarcoglycan-sarcospan complex. These are transmembrane proteins which interacts strongly with one another (Dalkilic *et al.* 2003). The DAPC destabilizes in the absence of dystrophin, due to which there is diminished level of the membrane proteins. This leads to progressive fiber damage and membrane leakage (Straub *et al.* 1997). The DAPC has a signaling role, the loss of which also contributes to pathogenesis (Blake *et al.* 2002). The main function of dystrophin is to stabilize the plasma membrane. There are many consequences of membrane instability on the function and homeostasis of myofiber.



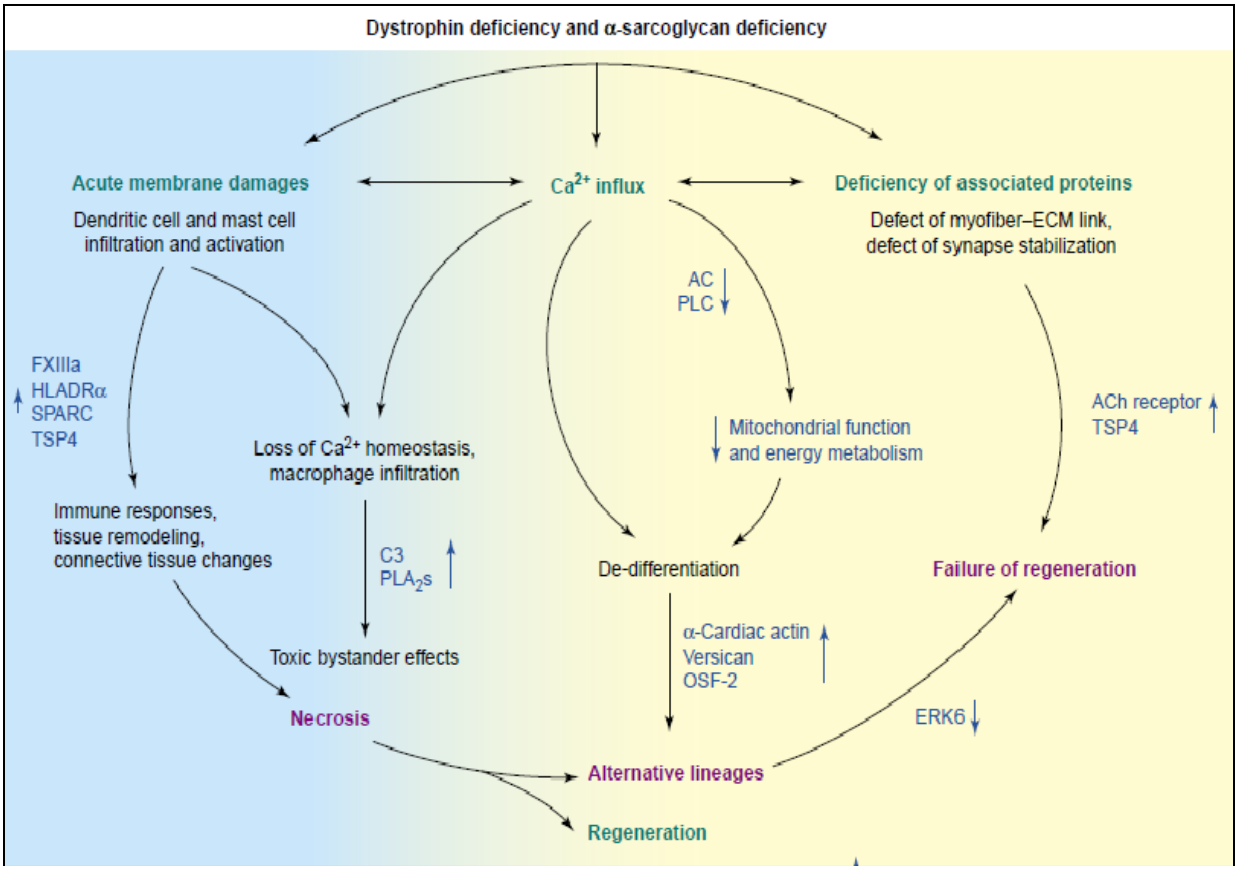
Source: Isin Dalkilic, Louis M Kunkel. Current Opinion in Genetics & Development 2003, 13:231–238

Figure 2: Muscular dystrophies and the membrane and enzymatic proteins they are associated with. This schematic diagram shows the location of various membrane and enzymatic proteins associated with muscular dystrophies. The diseases these molecules cause when mutated are shown in boxes. Dystrophin, via its interaction with the dystroglycan complex, connects the actin cytoskeleton to the extracellular matrix. Intracellularly, it interacts with dystrobrevin (a-DTN) and syntrophins (Syn) [shown in blue]. Extracellularly, the sarcoglycan complex (orange) interacts with biglycan, which connects this complex to the dystroglycan complex and the extracellular matrix collagen. Intracellularly, d- and g-sarcoglycans interact with filamin C.

1.7 Pathophysiology:

Dystrophin is present in normal individual from fetal life in skeletal, heart, smooth muscle and some neuronal cells. Deficiency of dystrophin expression affects formation of the DAPC and disrupts the molecular bridge (Blake *et al.* 2002). These effects can lead to two consequences: first, cell membrane becomes more fragile and can be mechanically damaged during muscle

contraction; and second, membrane proteins, especially mechanic-sensitive ion channels get deregulated (Iwata *et al.* 2003; Kumar *et al.* 2004). One of the primary concerns is the unregulated influx of ca^{2+} break in membrane. Abnormal ca^{2+} content and abnormal handling of ca^{2+} by dystrophin deficient cell leads to ca^{2+} mediated cellular toxicity. The calcium overloading results in activation of ca^{2+} dependent protease and contributes to muscle fiber degeneration. Hyperactivation of signaling cascades also promotes an inflammatory response by increasing the expression of inflammatory mediators and chemoattractants in dystrophin deficient muscle (Zhou *et al.* 2006). Dystrophin also binds to a series of proteins which are lacking in dystrophin-deficient muscle. The pathway involves through neuronal nitric oxide synthase (nNOS) which binds to the carboxy-terminus of dystrophin through α-syntrophin. Dystrophin is responsible to localize nNOS to the plasma membrane where it responds to physiological changes in muscle activity and regulate blood flow. The lack of dystrophin leads to deficiency of nNOS at the membrane and inability of the muscle to regulate vascular perfusion accurately (Hoffman *et al.* 2001). All these pathophysiology is shown in figure 3. There are other cellular factors which may be involved in the pathophysiology of DMD. Aquaporin-4 (AQP4) is a protein enriched in the sarcolemma of normal skeletal muscle and helps in accommodating the rapid changes in the cell volume and hydrostatic force that occur during contraction. Absence of AQP4 in dystrophin deficient muscle suggests that there is a possible association with the pathophysiological process (Wakayama *et al.* 2002)



Source: Eric P. Hoffman, Devin Dressman. TRENDS in Pharmacological Sciences Vol. 22, No. 9, September 2001

Figure 3: Pathophysiological flow chart of the muscular dystrophies showing the pathological cascades initiated by dystrophin deficiency. Both cell-autonomous (intrinsic to the muscle fiber; right-hand side of figure) and non-cell-autonomous (occurring in the external microenvironment of the myofibers; left-hand side of figure) abnormalities are shown. Most of the downstream consequences of dystrophin deficiency shown were derived from expression profiling studies using microarrays on human Duchenne muscular dystrophy (DMD) muscle.

1.8 Mutations in dystrophin gene:

The frequency of DMD coupled with a high new mutation rate ($1*10^{-4}$genes/generation) has led to the characterization of several independent mutations. Mutations causing DMD results in the absence or much reduced levels of dystrophin protein whereas BMD patients generally make partially functional protein (AMALFITANO A *et al.* 1997). Since the cloning of the gene in 1987, exon deletions and duplications have been found to be the most common molecular defect

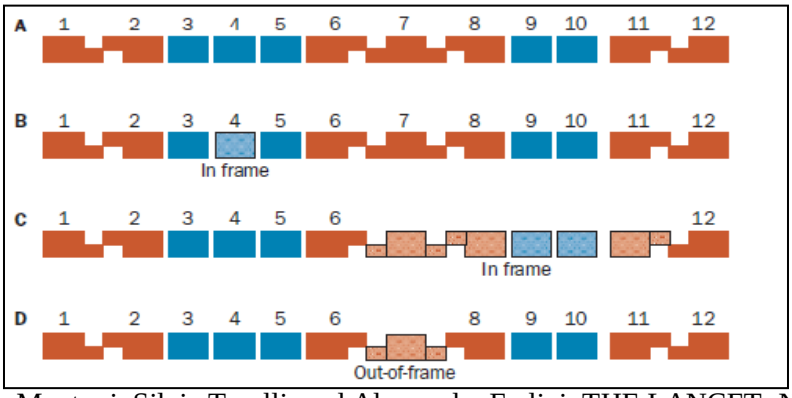
causing the disease accounting for approximately 65% and 6% of mutations respectively. Point mutations accounts for the rest. The most common mutations are intragenic deletions, which accounts for 65% of dystrophin mutations. Deletion and, more rarely duplications, can occur anywhere; however two deletion hotspots are known – one located towards the central part of the gene and the other towards the 5' end. The former is most commonly mutated and includes exon 45-55 with genomic breakpoints lying within intron 44, whereas the 5' end hotspot includes exons 2-19 with genomic breakpoints commonly found in introns 2 and 7. The clusters of these two hotspots are basis for the multiplex PCR technique, which enables identifying 98% of deletions by screening only 19 exons (Den Dunnen *et al.* 1989; Beggs *et al.* 1990; Nobile *et al.* 1995).

1.8.1 The Leiden DMD mutation database:

There is open access database for all the DMD mutations called The Leiden DMD mutation database. This is an open access database in which all mutations that have been reported in literature are stored. According to this database, intragenic deletions of one or more exons are most common (72%) of all mutations reported so far. The most common deletions are those of exon 45 (reported 80 times) and exons 45-47 (reported 72 times). Most of the deletions in the database cluster in the major "hotspot" region, which spans exons 45-53. Single or multiexon duplication are found in 7% of patients. The most common duplication is a single exon duplication of exon 2 (reported 20 times). Small deletions and insertions or point mutations involved in 20% of the collected mutation (Aartsma-Rus *et al.* 2006).

1.8.2 Disease severity and frame shift hypothesis:

There is no clear relation between the size of the deletion and clinical phenotype. As for example, the deletion of small exon like exon 44, results in classic DMD. However large deletions which may involve nearly 50% of the gene, have been described in patients with BMD (England *et al.* 1990; Love *et al.* 1990; Love *et al.* 1991). Therefore the effects on the phenotype depend, not much on the extent of a deletion (same with duplications) but whether or not it disrupts the reading frame (Monaco *et al.* 1988). Another observation is that very different deletions (in terms of size and position) may have similar severe phenotype. The reason for this effect might be the occurrence of nonsense-mediated RNA decay (Kerr *et al.* 2001). Mutation that maintains the reading frame (in-frame) results in abnormal but partly functional dystrophin and is associated with BMD. In children with DMD, deletions and duplications disrupts the reading frame (frame-shift), resulting in unstable RNA that eventually leads to the production of negligible concentration of truncated proteins (figure 4). This reading frame hypothesis holds for over 90% of cases and is commonly used for diagnostic confirmation as well as for differential diagnosis of DMD and BMD (Nicholson *et al.* 1990). There are exceptions to the reading frame rule which includes patients with BMD carrying frame-shift deletions or duplications and patients with DMD with in-frame deletions or duplications.



Source: Francesco Muntoni, Silvia Torelli, and Alessandra Ferlini. THE LANCET. Neurology Vol 2, December 2003.

Figure 4: Effects of different genomic deletions on the reading frame of the dystrophin gene (A). The removal of exon 4 (B) and of exon 7-11 (C) maintains the open-reading frame. The deletion of exon 7 leads to the loss of the open-reading frame (D).

Approximately 30% of DMD patients have unidentified mutations in the dystrophin gene. Analysis of these small or point mutations requires sequencing of all 79 exons and the 8 promoters. There is no common point mutation or point mutation hotspots, and each affected family may carry a unique mutation in the gene. Most of these mutations are nonsense, frame shift or splice site mutations; missense mutations are extremely rare. A study done in Brazilian families showed 19 point mutations and a 1 bp insertion (Table 2). All the mutations were randomly distributed along the dystrophin gene, 20% in the first quarter of the gene, 30% in the second quarter of the gene, 30% in third quarter of gene and 20% in the last quarter. They also investigated whether the patient has a *denovo* mutation or whether the mother was a carrier. They also identified six polymorphisms (Table 1) (Dolinsky *et al.* 2002). Similarly, a study carried out by Mendell and colleague for diagnosis of DMD by enhanced detection of small mutations in 2001 showed small mutations in DNA samples from 93 patients. In this study Sixty-eight (73%) patients were found to have small mutations, including 34 (36.5%) nonsense mutations, 27(29%) micro deletions or insertions, and 7 (7.5%) splice site mutations (Mendell *et al.* 2001).

DMD Polymorphism

Table 1: Polymorphism seen in Brazilian family.

Exon	DNA	RES[a]	Protein	Frequency
17	2201 – 37 G>T	–Taq I		5/20
17	2376 + 13C>T			4/20
21	2853 G>A	+Bgl II	Gly 882 Asp	15/20
31	4442 – 13 A>G			1/20
37	54442 G>A	– Sac II	Arg 1745 His	6/20
51	7750 + 13 A>G			1/20

Table 2: Mutations seen in DMD (Brazilian families)

Family number	Exon	DNA change	Protein	De novo mutation
DMD_01	39	5669 G>T	Glu 1821X	No
DMD_02	24	3404 G>T	Glu1066X	No
DMD_03	21	2879 A>T	Lys891X	No
DMD_04	04	401 G>T	Glu65X	Yes
DMD_05	30	4295 A>T	Lys1363X	No
DMD_06	44	6644 A>T	Lys2146X	No
DMD_07[b]	45	6668 C>T	Gln2154X	No
DMD_08	67	9956 G>T	Glu3250X	Yes
DMD_09	77	11177 G>T	Glu3657X	No
DMD_10	48	7262 G>T	Glu2352X	No
DMD_11	08	881 A>T	Lys225X	No
DMD_12	24	3482 A>T	Arg1092X	No
DMD_13	72	10487 C>T	Gln3427X	No
DMD_14	20	2615 C>T	Gln803X	No
DMD_15	59	8894 A>T	Arg2896X	No
DMD_16	51	7547 C>T	Gln2447X	Yes
DMD_17	35	5060 C>T	Gln1618X	F[c]
DMD_18	69	10280 G>T	Glu3358X	F
DMD_19	57	8722_8723insT	Pro2839fsX2848	F
DMD_20	14	1841 A>T	Arg545X	F

1.9 Systemic involvement:

Mutations in dystrophin gene were also noticed to have systemic involvements specifically cardiovascular, brain and retinal involvement.

1.9.1 Cardiovascular:

X-linked cardiomyopathy (XLDC) belongs to the genetically heterogeneous group of the dilated cardiomyopathies and it has been demonstrated to be an allelic disorder to DMD and BMD. The mutations can be broadly classified into two categories so far: mutations similar to those found in patients with DMD or BMD phenotype and secondly unusual gene defects not found in patients with typical DMD or BMD. There are two main regions of dystrophin gene which are most commonly involved in XLDC: the 5' end of the gene and the central hot-spot region around exons 48-49. Rearrangement in the muscle promoter (M) and adjacent intron 1 have been described with XLD (Muntoni *et al.* 1993; Yoshida *et al.* 1998). Two other 5' end mutations were reported in XLDC families which consist of a duplication involving exon 2-7. An unusual mutation caused by insertion in intron 11 of the dystrophin gene has also been reported. It is found that this rearrangement affects the splicing of exons 11 and 12 in the heart, abolishing the normal transcript in the cardiac muscle only. This results in absent dystrophin production in the cardiac muscle, while in the skeletal muscle there was significant residual expression of the protein as a correct splicing of exons 11 and 12 (Ferlini *et al.* 1999). Another splicing mutation has been reported in exon 29 caused by the exon 29 skipping both in the cardiac and skeletal muscle (Franz *et al.* 1995). Mutation in the spectrin-like region typically gives rise to BMD but patients affected by XLDC have been reported to be carrying in-frame deletions of exons 49-51, 48-49, and 48 (Muntoni *et al.* 1997). The findings of different mutations and different protein

expressions in all these cases suggest the presence of multiple mechanisms for the cardiac involvement in XLDC cases.

1.9.2 Nervous system:

The brain is affected by the lack of dystrophin. However, the range of abnormalities found, is very significant. There have been several studies of IQ in affected boys, the results of which are summarized by Anderson and colleagues. In this review, they have compared the DMD affected boys with genetically most relevant animal model of DMD, the dystrophin-deficient mdx mouse. In DMD affected children, there was evidence of disordered CNS architecture, abnormalities in dendrites and loss of neurons, all associated with neurons that normally express dystrophin. In mdx mouse, there have been reports of a 50% decrease in neuron number and neural shrinkage in the region of cerebral cortex and brain stem. At the biochemical level, DMD children were also having abnormal CNS bioenergetics and so as mdx mouse. There were histological evidence as well, showing the decrease in density of $GABA_A$ channel clusters in purkinje cells and hippocampal CA1 neurons (Anderson *et al.* 2002). The severity and frequency of mental retardation increases with the successive loss of functional distal isoforms. Mutations that affect the 5' end of the gene will only result in disruption of the three full length isoform (brain, muscle, and purkinje) but will leave the transcription of the isoforms on the 3' side of the mutation intact. Mutations located at the 5'end of the gene are not generally associated with mental retardation, which, when present, is mild in most patients even though the mutations affect full length CNS isoforms (Rapaport *et al.* 1992; Bushby *et al.* 1995) . Out of frame deletion of exon 45, will exert its effect not only on the three 5' full-length isoforms but also on the Dp260 and Dp 140 isoforms, leaving the expression of Dp 116 and Dp71 unaffected. Finally,

a deletion located on the 3' side of exon 63 will affect all dystrophin isoforms (figure 1). Disruption of two of the shorter isoform seems to have the greatest effect on IQ in the DMD. A study done by Bresolin and co-workers showed significant contribution of deletions involving the Dp140 isoform to mental retardation. This has been shown by comparing the effect of deletions in the Dp140 gene region with deletions not altering Dp140 expression (Bresolin *et al.* 1994). Distal deletions were associated with cognitive impairment. Mutations that extend 3' of exon 63 have greater effect on IQ and these affect the transcription of the Dp71 isoform. In patients with these mutations the expression of any isoform in the brain is affected and the patients have severe mental retardation. As mutations that extend beyond 63 are rare, this severe cognitive impairment is not commonly seen. However, all patients with mutation affecting Dp71 reported so far had severe mental retardation (Muntoni *et al.* 2003).

1.9.3 Retinal involvement:

Although affected boys have normal visual acuity, electroretinography has shown significant defects in several patients. Different dystrophin isoforms are expressed in the retina but do not overlap in their distributation, which suggests non-redundant different functional roles. The main retinal isoform is the Dp260, which localizes to the outer plexiform layer together with Dp427 (Howard *et al.* 1998). Mutations in the 5' end of the gene do not result in the phenotype, whereas mutation in the central and 3' portions of the gene that affect dystrophin isoform Dp260 results in a scotopic electroretinography b wave (Pillers 1999; Pillers *et al.* 1999). The abnormal retinal electrophysiology in patients with different deletions and in mice, with targeted inactivation of individual isoforms illustrates the complexity and multivariate nature of the events secondary to dystrophin deficiency.

1.10 Therapeutic approaches:

There are various management approaches for Duchenne Muscular Dystrophy including pharmacological approach, physiotherapy, occupational therapy and gene therapy. This study is particularly based on genetic research, so, some of the gene therapy and new treatment strategies in DMD are elaborated. Many different strategies for DMD gene therapy have been studied and improved over the years as shown in table 3.

Table 3: Strategies for Duchenne Muscular Dystrophy gene therapy

Overview of strategies for Duchenne muscular dystrophy gene therapy

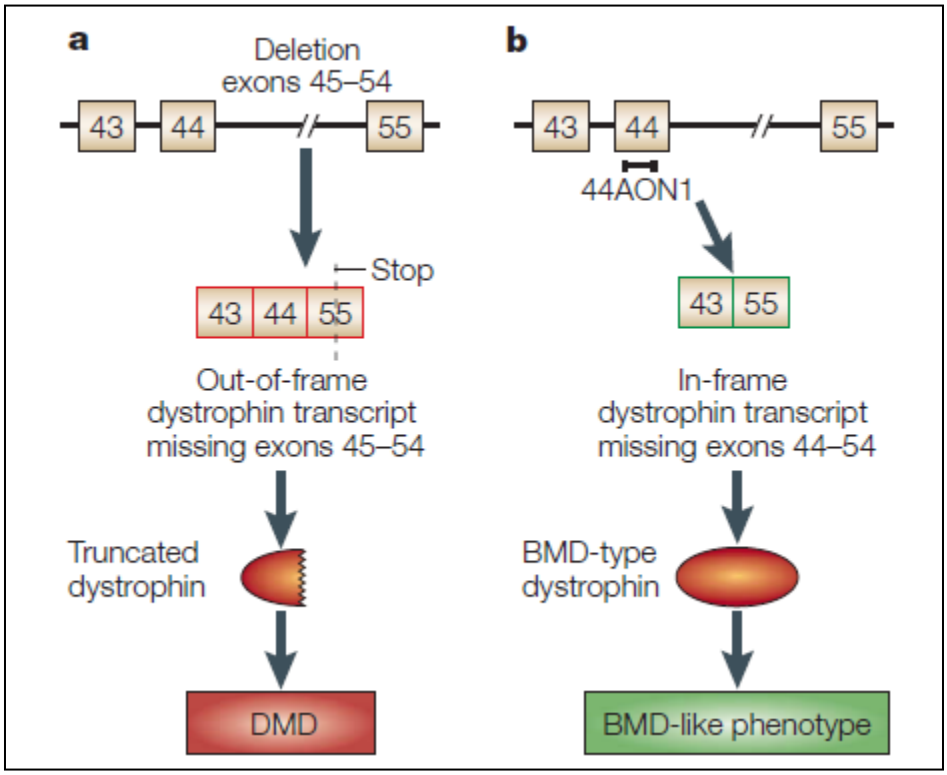
Strategy	Action/effect	Advantages	Disadvantages
Adenoviral vectors	Full-length dystrophin cDNA transfer	High transduction levels in regenerating muscle, expression of fully functional dystrophin	Viral immune response, limited persistence of transgene expression, maturation dependent
Herpes simplex viral vectors	Full-length dystrophin cDNA transfer	High transduction levels in regenerating muscle, expression of fully functional dystrophin	Viral toxicity and immune response, limited persistence of transgene expression, maturation dependent
Plasmid vectors	Full-length dystrophin cDNA transfer	Synthetic, non-infectious, relatively safe, flexible, simple engineering	Large molecule, delivery requires efficient transfection method
Myoblast transplantation	Introduce dystrophin-producing cells	Non-infectious, relatively safe	Low efficiencies, immune suppression required
Stem-cell therapy	Introduce dystrophin-producing cells	Conventional treatment, relatively safe	Low efficiencies, immune suppression required
Chimeric oligonucleotides	Correction of mutation at the DNA level	Cumulative, permanent effect	Low *in vivo* efficiencies
Gentamicin therapy	Ribosomal read-through of stop codons in mRNA	Conventional drug	Low reproducibility, risk of nonspecific adverse effects
rAAV vectors*	Mini- or micro-dystrophin cDNA transfer	High transduction efficiencies in muscle, non-pathogenic minimal immune responses	Unable to deliver full-length dystrophin, laborious production systems
Antisense oligonucleotides*	Splicing modification of pre-mRNA	Synthetic, small-molecule drug, relatively safe, restores all isoforms	Repeated administrations and (targeting) delivery reagent needed, mutation specific
Utrophin upregulation*	Replacement of dystrophin	Small-molecule drug, no immune response, relatively safe	No effective specific compound identified as yet

Source: Judith C. T. van Deutekom and Gert-Jan B. van Ommen. *NATURE REVIEWS GENETICS,* Volume 4, October 2003, 775.

Few of the new strategies are discussed in this section. One of the most upcoming new strategy is antisense-induced exon skipping.

1.10.1 Antisense-induced exon skipping:

Becker muscular dystrophy has relatively mild phenotypes which are caused by some large deletions or nonsense mutations showing other possible gene therapy strategies in DMD. This was possible by skipping an exon during pre-mRNA splicing to enlarge a DMD deletion, so that it becomes it's nearest in frame BMD counterpart.



Source: Judith C. T. van Deutekom and Gert-Jan B. van Ommen. *NATURE REVIEWS GENETICS*, Volume 4, October 2003, 775

Figure 5: a) In Duchenne Muscular Dystrophy (DMD), patients with a deletion of exons 45-54, out of frame transcripts are generated in which exon 44 is spliced to exon 55. Due to frame shift, a stop codon occurs in exon 55, which prematurely aborts dystrophin synthesis. b) Using an exon-internal antisense oligonucleotide (AON) in exon 44, the skipping of this exon can be induced in cultured muscle cells. Accordingly, the transcript is back in frame and a Becker muscular dystrophy (BMD) – like dystrophin can be synthesized.

A study done on a Japanese DMD patient showing 52 – bp frame-disrupting deletion in exon 19 was found to cause exon skipping from the dystrophin transcripts (Matsuo 1991). It was thought

that this region might contain an exon recognition site (ERS), which is purine rich sequence that is required for the correct splicing of exons with weak splice- site consensus sequences (K 1994; Cartegni 2002). The feasibility of applying antisense oligonucleotides (AON) to induce potentially therapeutic exon skipping from DMD pre- mRNA was shown in *mdx* mouse model (Dunckley 1998). The first evidence for human therapeutic exon skipping in muscle cells from a study targeting exon 46. A single exon 45 deletion is (app. 7%) the single most frequent DMD-causing mutation reported in the DMD database. Specific AON- induced skipping of exon 46 was seen in muscle cells from two exon 45 deletion patients. With skipping efficiency of only 15% (app.), the reading frame was restored and dystrophin synthesis induced in more than 75% of transfected myotubes (van Deutekom *et al.* 2003). An assessment of the spectrum of mutations causing DMD shows that the skipping of any given exon could be an effective gene therapy for many different mutations as shown in table 4. For example, skipping exon 51 would restore the reading frame in patients that carry deletions of exons 45-50, 47-50, 48-50, 49-50, 50-52 or 52-63, which accounts upto a total of 17.5% of all DMD patients. If we consider theoretically, the skipping of only 12 different exons could correct almost 75% of all deletions (van Deutekom *et al.* 2003).

Table 4: Duchenne Muscular Dystrophy (DMD) - causing deletions reported in the Leiden DMD (LDMD) database.

Overview of therapeutic exon skipping for a series of DMD-causing deletions

Skippable exon	Therapeutic for DMD deletions (exons)	Percentage of deletions in LDMD database
2	3–7, 3–19, 3–21	2.9
8	3–7, 4–7, 5–7, 6–7	4.5
17	12–16, 18–33, 18–41, 18–44	1.8
43	44, 44–47, 44–49, 44–51	3.7
44	14–43, 19–43, 30–43, 35–43, 36–43, 40–43, 42–43, 45, 45–54	7.8
45	12–44, 18–44, 44, 46–47, 46–48, 46–49, 46–51, 46–53, 46–55	11.2
46	21–45, 45, 47–54, 47–56	5.6
50	51, 51–53, 51–55	5.2
51	45–50, 47–50, 48–50, 49–50, 50, 52, 52–63	17.5
52	51, 53, 53–55	4.0
53	10–52, 45–52, 46–52, 47–52, 48–52, 49–52, 50–52, 52	7.5
55	45–54, 48–54	1.8
Total	12 AONs	73.5

Source: Judith C. T. van Deutekom and Gert-Jan B. van Ommen. *NATURE REVIEWS GENETICS*, Volume 4, October 2003, 775

The therapeutic potentials of AONs were shown in cultured muscle cells from DMD patients that were affected by several different deletions. The targeted exon was specifically skipped in all cases at relatively high level upto 90%. This induced the synthesis of significant levels of dystrophin in more than 75% of treated cells. These dystrophin located appropriately to the sarcolemma and restored the dystrophin-glycoprotein complex. This was a indication of functional restoration (Aartsma-Rus *et al.* 2003). Progress in understanding the gene and its function has pointed several innovative therapeutic strategies. Considering the efficiency and relatively simplicity, the antisense approach seems the most promising therapy for clinical trials.

1.11 Objective:

- To identify the spectrum of mutations (Deletions/Duplications) found in patients with DMD.
- To co-relate the clinical phenotype with mutations.

2. Methodology:

In this chapter, all the methods used in this investigation are described in detail. The first consideration was obtaining ethical clearance to conduct investigations and it is described at the beginning of the chapter. It was followed by a description of the recruitment of subjects and the inclusion criteria which were used in the recruitment. The detailed description of the molecular genetics methods is also mentioned. The chapter concludes with a description of the statistical methods, databases and software tools used for this investigation.

2.1 Ethical considerations:

The study was conducted according to the Declaration of Helsinki (2008) which received ethical clearance from the Ethics Review Committee (ERC) of the Faculty of Medicine, University of Colombo, Sri Lanka and the Hospital Ethics Committee of the Lady Ridgeway Hospital for Children (LRH), Colombo. The study was built on collaborative links, the HGU has established with patients with DMD, local Paediatricians and foreign experts in the field. The study has social value because it contributes to generalized knowledge on the molecular genetics mechanisms involved in DMD. This will be further useful in the future practice of clinical genetics and genetic counseling in Sri Lanka. The study was designed appropriately to ensure scientific validity. The study was open to all patients with DMD, so there was fair participant selection. Appropriate measures were taken to ensure that consent was obtained in an ethical manner from all participant's parents or legal guardians because all the participants were under 18 years of age. Written informed consent was obtained from all individual's parents or legal guardians. Written informed consent was obtained from all study participants using consent forms in Sinhala, Tamil and English languages. Each participant was given an information sheet

which included details about the study and a consent form for reading and signing before participating in the study. All study participants or their parents/guardians were able to read and understand the information and make a voluntary decision to participate in the study. The patient with their family was interviewed privately in the genetic counseling room to ensure privacy and was able to discuss the study privately with the investigators without the presence of others. Informed written consent was obtained after providing the necessary information and giving them time to make a decision in private. The data collection booklet was designed to ensure confidentiality of information gathered. Soon after collecting the personal information, the identification page was removed and filed separately. The only identification number in the rest of the booklet was a coded subject study number which cannot be linked to an individual without the page containing the personal information. The information was kept by the principal investigator under lock and key. The electronic database containing the data had only the subject study number thus ensuring confidentiality. The database and the computer containing the database were password protected. These measures ensured that loss of confidentiality was minimized. No information by which subjects can be identified were released or published. The data were not used in a way that the subjects would be identified in any public presentation or publication.

The minimal risk of pain or minor bruising at the venepuncture site was minimized by venepuncture being performed under aseptic conditions by a trained nurse or a doctor. There was a direct benefit to the study participants by participating in this study. They became aware of their genetic mutation status and associated genetic risk. As genetic testing for these disorders was not freely available in Sri Lanka, this knowledge was valuable for the patients. In addition, participants and parents/guardians received genetic counseling based on the results of the genetic

tests and referred for further long term management when needed. This study added further input to the already existed scanty knowledge on the molecular genetics mechanisms involved in Sri Lankan patients with DMD. In addition, there was also the benefit of identifying a genotype-phenotype correlation in patients with DMD that would be useful in the future practice of clinical genetics and genetic counseling. After the study, when the common genetic defects causing DMD in Sri Lankan patients is known, it would be possible in future to develop cheap genetic tests and clinical trials suitable for Sri Lanka. This would be further helpful for the diagnosis and management of this devastating disorder. The result of genotyping was also given to the patients during post test counseling. The samples and data were stored for future use after the completion of the study for further studies on DMD. Appropriate consent was obtained for this purpose and any future studies would be subject to ethics review prior to commencement.

2.2 Recruitment of subjects and the study protocol:

This was a descriptive study; therefore, patients with a clinical diagnosis of DMD were recruited both prospectively and retrospectively. The recruited patients were identified from the clinical records of the patient database maintained at the HGU, Faculty of Medicine, University of Colombo from January 2006 onwards and the Professorial Ward (Wd1, LRH) as well as from other patients referred to Human Genetics Unit, Faculty of Medicine, University of Colombo. Patients, who have been given a clinical diagnosis of DMD, were recalled and included in the study after obtaining their informed consent from their parents. They were contacted via phone/mail. Those who were willing to participate and who gave their informed consent were recruited in this study. The prospective recruited patients were identified from those referred to the Unit from November 2011 to March 2012. In addition, patients with a clinical diagnosis of

DMD from the Professorial Ward (Wd1, LRH), between October 2011 and March 2012, were also recruited in the study.

2.3 Study Population:

The study population comprised a total of 50 participants. Since most of the related international standard scientific publications have included smaller samples; we believe that this sample size would be sufficient to effectively describe the genotype-phenotype correlation in this study group.

2.3.1 Study Participants:

All clinically diagnosed patients were recruited in the study.

2.4 Clinical Evaluation:

In the recruitment study, participants or children's parents/guardians were personally interviewed by the principal investigator to obtain demographic and clinical data (please see the data collection booklet). Complete medical history was obtained from each participant. Additional clinical data was also gathered by examining the participant's medical records. The family pedigree, including familial background up to 3 generations, was also taken whenever possible.

2.5 Biological sample and Genetic testing:

At recruitment, a sample of 3ml of venous blood was obtained from participants. These blood samples were stored at -80 °C. DNA was extracted from the sample and molecular genetic

testing was performed. This test involves the testing for DMD mutations. Initially, samples were tested for deletions/duplications which were most common cause of DMD. The samples, where genetic diagnosis was not confirmed and further testing could not be established by contacting with a suitable laboratory abroad. The left over samples (blood/DNA) after genotyping was stored in a -80^0C freezer under the supervision of the supervisor of this study indefinitely for future genetic research of DMD. Appropriate consent was obtained for this purpose.

2.6 DNA Extraction:

DNA extraction was done using Wizard® Genomic DNA purification kit from Promega according to the manufacturer's protocol. The promega Wizard® DNA purification procedure was carried out as follows: Tubes containing blood sample was gently rocked until thoroughly mixed and 300 µl of sample was transferred into a sterile 1.5 ml microcentrifuge tube containing 900 µl of Cell Lysis Solution. The tube was inverted 5-6 time to mix and incubated for 10 minutes at room temperature to lyse red blood cells. The tube was then centrifuged at 14,000 rpm for 20 seconds at room temperature and maximum amount of supernatant was removed and discarded without disturbing the visible white pellet; approximately 10-20 µl of residual liquid remained. The tube was vortexed vigorously until white blood cells were resuspended. Nuclei Lysis Solution (300 µl) was added to the tube and pipetted 5-6 times to lyse the white blood cells. The solution became very viscous. RNase Solution (1.5 µl) was added to the nuclear lysate, mixed by inverting the tube 2-5 times and incubated at 37°C for 15 minutes and then cooled to room temperature. Protein Precipitation Solution (100 µl) was added to the nuclear lysate and vortexed vigorously for 10-20 seconds and centrifuged at 14,000 rpm for 3 minute. A dark brown pellet was seen. Room temperature isopropanol (300 µl) was taken into a clean 1.5 ml

microcentrifuge and the supernatant was transferred into it. Solution was gently mixed by inversion until white thread-like strands of DNA formed a visible mass. Tube was centrifuged at 14,000 rpm for 1 minute and DNA was visible as a small white pellet. Supernatant was decanted and 300 µl of 70% ethanol was added to the DNA and tube was gently inverted several times to wash the DNA pellet and the sides of the microcentrifuge tube. Again, the tube was centrifuged at 14,000 rpm for 1 minute and ethanol was aspirated by a pipette. The tube was inverted on a clean absorbent paper and pellet was air dried for 10-15 minutes. DNA Rehydration Solution (80 µl) was added to the tube and DNA was rehydrated by incubating overnight at 4^0C. The tubes were labeled and stored at -20 °C after rehydration. Backup blood samples taken from the patients were stored in a -80 °C freezer under the supervision of the supervisor of this study indefinitely for future research into genetics of DMD.

The extracted DNA was used in Multiplex Probe Amplification (MLPA) for detection of copy number change in Duchenne muscular dystrophy (DMD).

2.7 Multiplex Ligation Probe Amplification (MLPA):

Multiplex Ligation Probe Amplification (MLPA) is a method for the detection of unusual copy of number of changes in genomic sequences. The principle of MLPA is based on the identification of target sequences by hybridization of pairs of MLPA probes that binds to adjacent sequences and is joined by a ligation reaction. In order to make one copy of each sequence, specific MLPA probes are added to nucleic acid sample for each of the sequence. The sequences are then simultaneously amplified with the use of only one primer pair, resulting in a mixture of amplified products, in which each PCR product of each MLPA probe has a unique length. One PCR primer is fluorescently labeled so that the MLPA reaction products can be

visualized, when electrophoresed on capillary sequencer. Resulting chromatograms show size-separated fragments ranging from 130 to 490 bp. The peak area of each amplified product reflects the relative copy number of that target sequence. Comparison of the electrophoresis profile of the tested sample with the control sample enables the detection of deletions or duplications of genomic regions.

Procedure: SALSA MLPA kit (MRC Holland, Netherlands) according to the manufacturer's protocol (version MDP-v001).

2.7.1 MLPA Reaction:

All the processes of MLPA reactions are listed in detail below.

2.7.1.1 DNA Denaturation:

This was the first step.

- 0.2 ml tubes were labeled.
- 5 µl of DNA sample was added to each tube.
- Tubes were placed in a thermocycler (ABI 2720) and MLPA thermocycler programme was started. Denaturation of DNA sample was done for 5 minutes at 98^{o}C and cooling of samples was paused at 25^{o}C.

Table 5: Thermocycler programme for the MLPA reaction

1) DNA denaturation			
1.	98 ^{o}C	*5 minutes*	
2.	25 ^{o}C	*pause*	
2) Hybridization reaction			
3.	95 ^{o}C	*1 minutes*	
4.	60 ^{o}C	*pause*	
3) Ligation reaction			
5.	54 ^{o}C	*pause*	
6.	54 ^{o}C	*15 minutes*	
7.	98 ^{o}C	*5 minutes*	
8.	20 ^{o}C	*pause*	
4) PCR reaction			
9.	35 cycles	95 ^{o}C	*30 seconds*
		60 ^{o}C	*30 seconds*
		72 ^{o}C	*60 seconds*
10.	72 ^{o}C	20 *minutes*	
11.	15 ^{o}C	*pause*	

After denaturation, hybridization reaction was carried out by the method given below.

- MLPA probe mix and viscous MLPA buffer were vortexed before use.
- For each reaction, preparation of hybridization master mix was done, containing: 1.5 µl MLPA buffer (yellow cap) + 1.5 µl probe mix (black cap). The hybridization master mix was mixed well by pipetting or vortexing.
- After DNA denaturation step, 3 µl of the hybridization master mix was added to each sample tube at 25 ^{0}C. Mixed well by pipetting up and down and then, the mixture was heated for 1 minute at 95 ^{0}C followed by 16 hrs at 60 ^{0}C.

2.7.1.2 Ligation reaction:

- The two Ligase buffer were vortexed before use.
- Ligase-65 master mix was prepared. For each reaction mix: 25 µl dH_2O + 3 µl Ligase buffer A (transparent cap) + 3 µl Ligase buffer B (white cap). Then 1 µl Ligase-65 enzyme (green cap) was added. Mixed well by pipetting gently up and down.
- After 16 hrs incubation, thermocycler program was reduced and pause at 54 ^{o}C.
- When the samples were at 54 ^{o}C, 32 µl of the ligase master mix was added to each reaction tube.
- Thermocycler program was continued: 15 minutes incubation at 54 ^{o}C (for ligation), followed by 5 minutes at 98 ^{o}C for heat inactivation of the ligase-65 enzyme and then pause at 20 ^{o}C. Then the tubes were removed from thermocycler.

2.7.1.3 PCR Reaction:

- SALSA PCR primer mix was vortexed before use. Polymerase was warmed for 10 seconds in hand to reduce viscosity.
- For each reaction, a polymerase master mix was prepared by adding: 7.5 µl dH_2O + 2 µl SALSA PCR primer mix (brown cap) + 0.5 µl SALSA polymerase (orange cap). Mixed well by pipetting up and down.
- At room temperature, 10 µl polymerase mix was added to each ligated products. Mixed well by pipetting up and down and thermocycler program was continued for 35 cycles: 30 seconds at 95 ^{o}C, 30 seconds at 60 ^{o}C and 60 seconds at 72 ^{o}C. The reaction was ended with 20 minutes incubation time at 72 ^{o}C and then paused at 15^{o}C.

2.7.1.4 Fragment separation by Capillary electrophoresis: ABI-3130

- MLPA PCR primer dye: FAM.
- Recommended capillaries: 36
- Injection mixture: 0.7 µl PCR reaction + 0.2 µl LIZ size standard + 9 µl formamide.
- The chosen filter set was compatible with the fluorescent dye of the standard size.
- Initial settings 36 cm capillaries: injection voltage: 1.6 kv; injection time: 15 seconds; run voltage: 10 kv; polymer: POP7.
- Methods: injection plates were sealed and incubated for 2 minutes at 80 ^{o}C and cooled rapidly.

2.7.2 Peak pattern evaluation:

Prior to probe ratio calculation, evaluation of the quality of MLPA experiments was done by inspecting MLPA peak patterns (electropherograms). Evaluation of raw data as well as size-called data was done.

2.7.3 Analysis:

Data analysis was done by coffalyser software program from MRC-Holland (Coffalyser Stand alone Alpha version). The coffalyser analyzed the raw data files produced by capillary electrophoresis devices. The raw data allows a better estimation of the quality of the MLPA run which was not supported with fragment data files. This data contains the fluorescent measurement traces of a sample containing the PCR products of a MLPA reaction and a size marker. FSA raw data files with an .fsa extension were collected. All the files of the runs, which were performed in one experiment with the same MLPA mix, were selected. Machine type was

recognized. The wizard was also selected from the main form. Now, MLPA kit number was selected in the product box. When the available lots appeared in the lot box, specific lot number was selected, which was followed by the selection of size marker. Then, the size marker dye color was also selected (dye stream). Dye colors always showed the standard MLPA dye in blue (FAM) and the size marker in orange (LIZ). Reference runs were also selected and then, analysis was done. Soon after analyzing, the data were exported in the form of PDF file as shown in figure 6.

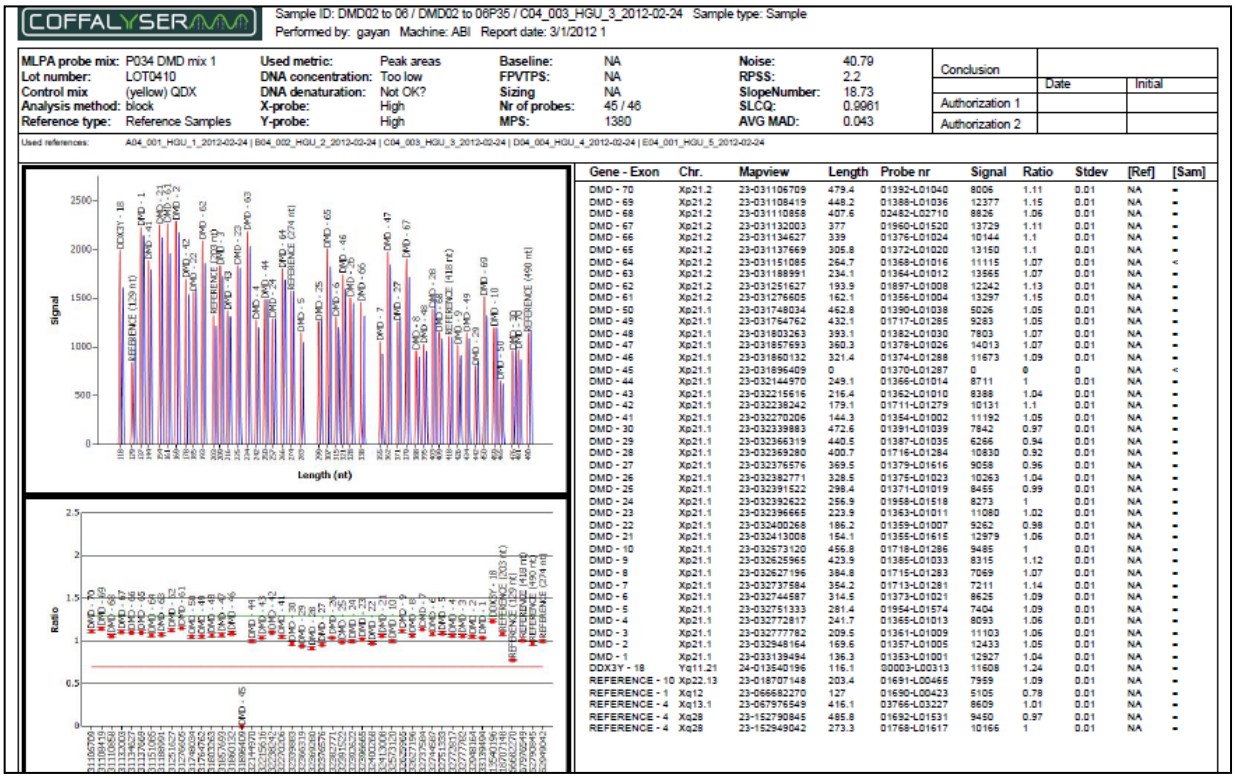


Figure 6: Coffalyser data analysis sheet

2.8 Software tools and electronic database information:

In order to organize the data, data were entered into an electronic database. Personal data were collected to keep track of the study participants for further follow-up. In order to maintain confidentiality of the study participants, their personal data were kept separate from the database and were not entered into the electronic database. Blood and DNA samples of patients were stored in Human Genetics Unit using subject numbers. The electronic database and stored biological samples were kept under the care of the supervisors at the end of the study.

2.9 Statistical methods:

Phenotyping data, which were entered directly into an electronic database, were manually checked for errors. In the phenotyping analysis, summary values for numerical data were presented as mean [standard deviation], when data were normally distributed. Summary values for categorical data were presented as percentage distributions. Genotype and phenotype were compared to establish genotype-phenotype correlation. Genotype–phenotype correlation was established by statistically significant test. ANOVA test was done to identify the correlation between numerical and categorical data. Statistically significant values were presented as ‘p’ value.

3. Results:

In this chapter, the demographic characteristics, clinical phenotype and genetic etiology of the 50 clinically diagnosed DMD cases were analyzed. Detailed discussion and comparison of the results of this study with previously published data will be carried out in the following chapter under the discussion section.

3.1 The DMD phenotype:

This section was aimed at analysis of the phenotype of the 50 Sri Lankan children with Duchenne muscular dystrophy. All the children were clinically diagnosed.

3.2 Demographic characteristics:

The demographic characteristics of DMD children are listed in table 6. All the children were considered for age, height, weight and BMI. All these measurements were taken by measuring tape. The mean age of the children was 8.94 years (SD ± 3.84) whereas most common age group ranges between 7 years to 10 years. While measuring the height of the children, it was possible to take height in 41 children, whereas for 9 children, it was not possible as they were wheelchair bound. The mean height was 114.29 cm (SD ± 16.17). In case of height, it was possible to take weight of 43 children whereas, for 7 children, it was not possible because of their wheelchair dependence. The mean weight was 21.1 kg (SD ± 9.1). Thereafter, mean Body Mass Index (BMI) was calculated which was 15.13 (SD ± 3.56). The maximum BMI was 24.10 and minimum was 11.30.

Table 6: Demographic characteristics of Sri Lankan Children with DMD.

		Sri Lankan Children
Mean Age (Years)	(SD)	8.94 (3.84)
Mean Height (Cm)	(SD)	114.29 (16.176)
Mean Weight (Kg)	(SD)	21.1 (9.1)
Body Mass Index (BMI)	(SD)	15.13 (3.56)

SD: standard deviation.

3.3 Clinical phenotype characterization:

Detailed clinical history was taken to establish a clinical phenotype. When considering the age of onset of disease, it was found that the most common age of onset was 3 years. The mean age of onset was 3.85 (SD ± 2.0). The age of onset is shown in form of bar diagram in figure 7.

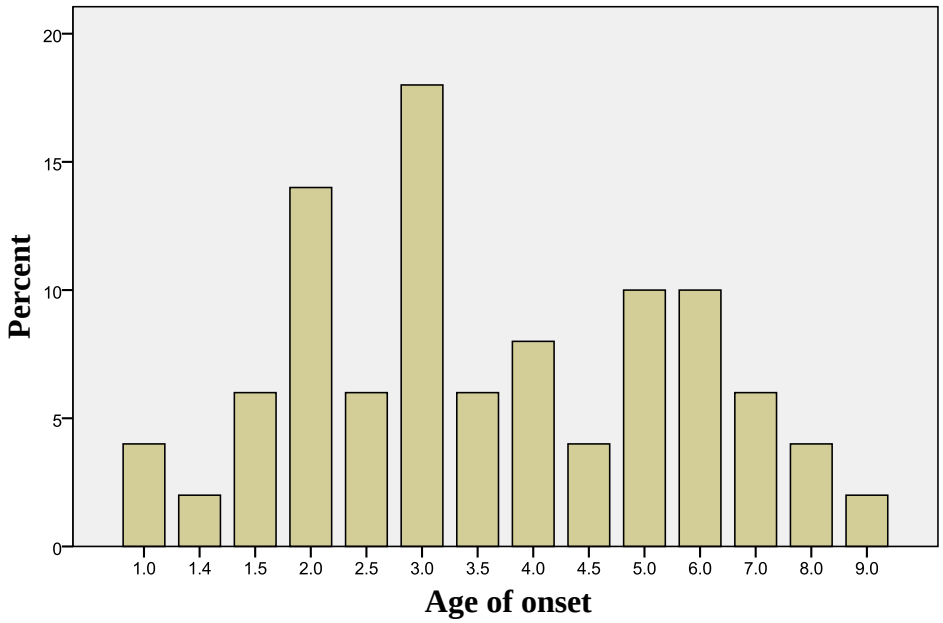


Figure 7: Bar diagram showing frequency distribution of Age of onset in Sri Lankan children with DMD.

There are five variables for clinical symptomatology which were mostly seen in DMD children. The frequencies of these variables are shown in table 7. The first symptom was frequent fall which was seen in 12 (24%) children. The second symptom was difficulty in walking, which was seen in 28 (56%) children. The third was difficulty in climbing, which was seen in 32 (64%) children. The fourth symptom was difficulty in standing up from ground, which was seen in 25 (50%) children. The last clinical symptom was proximal muscle weakness, which was seen in 15 (30%) children. Each of these percentages listed above, are among the 50 children of this study. According to above data, most common symptom among these children was difficulty in climbing followed by difficulty in getting up from the ground. Frequency of symptoms is shown in table 7.

Table 7: Presenting symptoms among the Sri Lankan DMD children.

Symptoms	**Sri Lankan children**
Frequent fall	12 (24%)
Difficulty in walking	28 (56%)
Difficulty in climbing	32 (64%)
Difficulty in getting up	25 (50%)
Proximal muscle weakness	15 (30%)

* Each of the percentage mentioned in table 7, was among 50 children (N = 50 in for each symptoms)

Different stages of the disease were also considered in this study. While considering the age of diagnosis of the disease, it was seen that the mean age of diagnosis was 5.25 years (SD ± 2.26). The most common age of diagnosis was at the age of 3 years. The frequency graph for age of diagnosis is shown in figure 8.

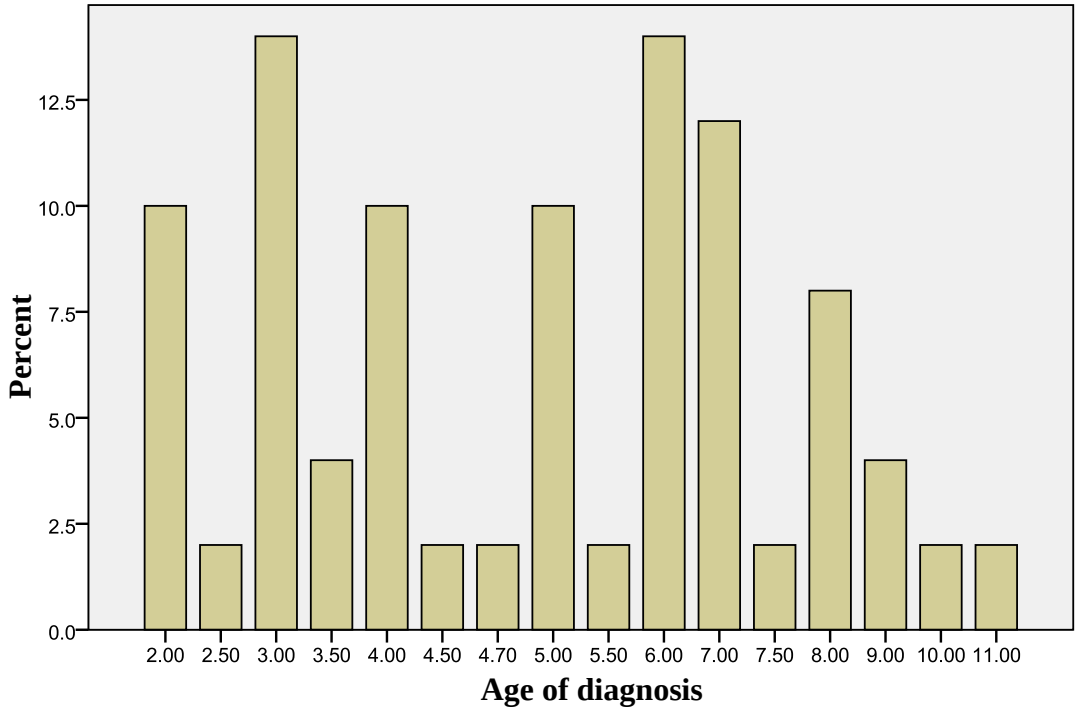


Figure 8: Bar diagram showing frequency distribution of Age of diagnosis in Sri Lankan children with DMD.

Another variable on aspect of developmental effect of the disease was age of first walk of the child. The mean age of the first walk of the child with DMD was 1.42 years (SD ± 0.441). The most common age of first walk was at one year of age which was seen in 20 children (40%) followed by age of 1.5 years (30%) children. The frequency diagram of age of first walk is shown in figure 9.

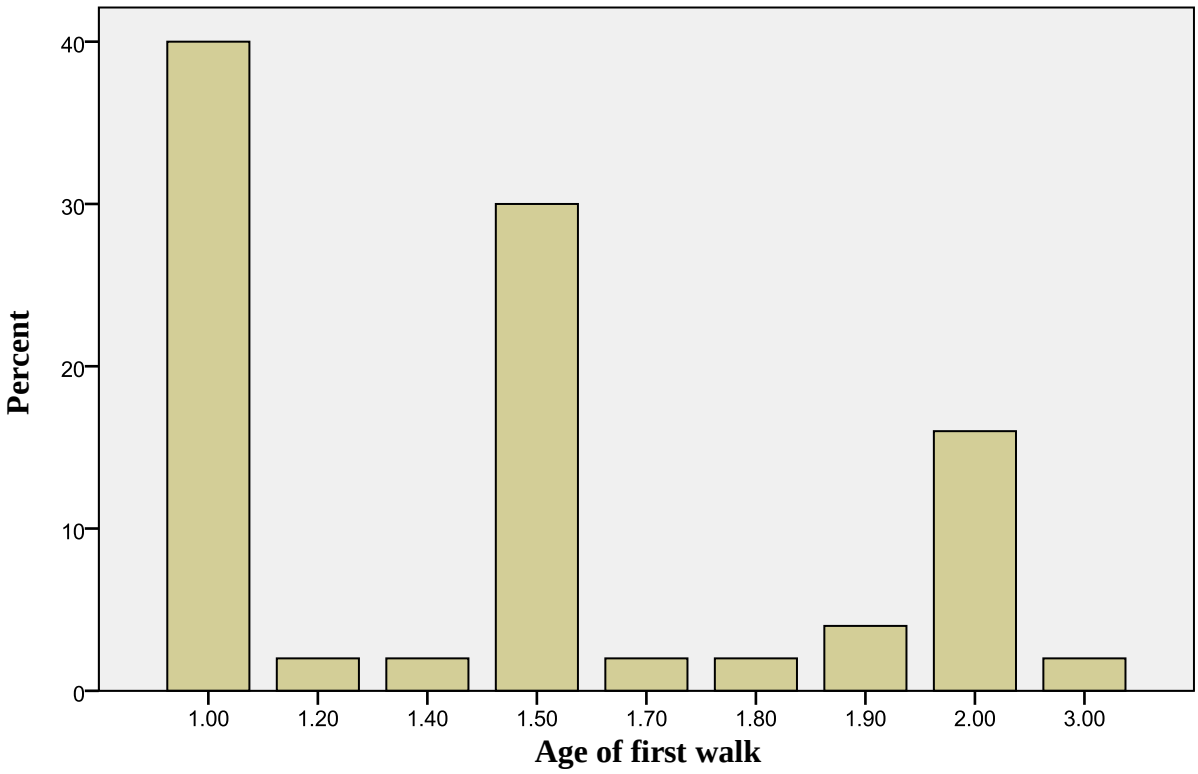


Figure 9: Bar diagram showing frequency distribution of Age of first walk in Sri Lankan children with DMD.

There were only few children, who have lost ambulation in this study. Their age of loss of ambulation as well as age of wheelchair bound was also taken into consideration. Among 50 children, there were only 12 children who have lost ambulation. The mean age of loss of ambulation among these children was 8.83 years (SD ± 2.58) and the most common age of being non-ambulatory was 10 years. The frequency diagram is shown in figure 10.

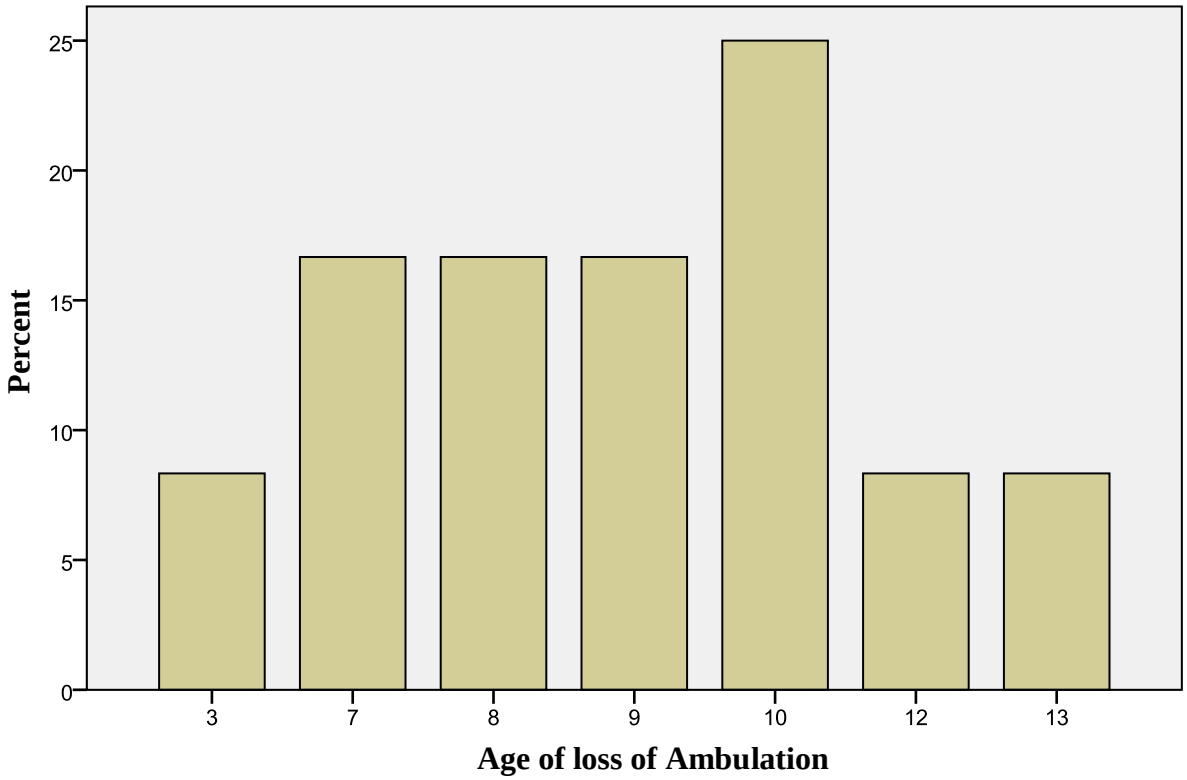


Figure 10: Bar diagram showing frequency distribution of Age of loss of ambulation in Sri Lankan children with DMD.

There were 6 children, who have become wheelchair bound. The mean age of becoming wheelchair bound was 11.33 years (SD ± 3.55). There was no specific or most common age of becoming wheelchair bound among these children.

3.4 Clinical examination and investigation:

Calf hypertrophy was an important examination for diagnosing DMD. In this study, bilateral calf measurements were taken with measuring tape from all the patients. The mean size of bilateral calf was found 25.90 (SD ± 5.1). The minimum calf size of the right leg was 17 cm whereas maximum calf size was 38. Similarly, the minimum calf size for left leg was 17 and maximum 40 cm (Table 8).

Table 8: Bilateral calf measurements in Sri Lankan children with DMD.

		Sri Lankan Children N = 50
Mean Calf size (Rt)	(SD)	25.90 (5.1)
Mean Calf size (Lt)	(SD)	25.94 (5.07)
Minimum Calf size	(CM)	17.00
Maximum Calf size	(CM)	38.00

SD: Standard deviation.

CM: Centimeters.

Gower's sign was positive among all the children. There were three major investigations which were taken into consideration. One of the most important biochemical markers for DMD is serum creatinine kinase (CPK) enzyme. Every child in this study had raised CPK levels. We were able to retrieve CPK level reports in 47 children. The mean CPK level was 12426.62 (SD ±

7723.6). The minimum CPK level was 1006, whereas the maximum CPK level was 41101. The second investigation was Echocardiography for dilated cardiomyopathies. Only one patient showed regional cardiomyopathy with previous history of myocarditis. Electromyography (EMG) and muscle biopsy was also noted in these children. Among 50 patients, EMG was done in 25 (50%) patients with results suggestive of myopathic features. Muscle biopsy was available only in 5 (10%) children. The frequency diagram is shown in the form of pie chart in fig 11.

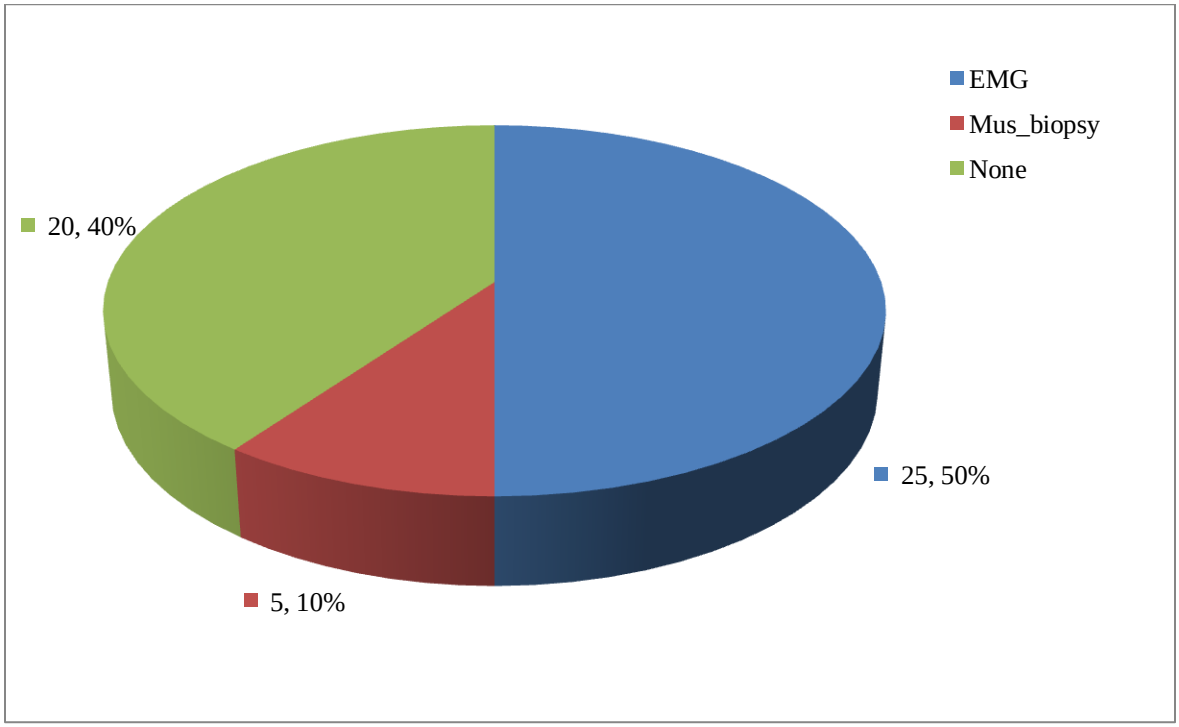


Figure 11: Pie diagram showing frequency of EMG and muscle biopsy in Sri Lankan children with DMD.

3.5 Family history:

Since this disease is an X-linked recessive disorder, family history was very important factor to be considered. A three generation family pedigree was drawn. Among 50 children, 14 (28%) of children had family history of this disease and 36 (72%) did not have any family history. There

were 13 (26%) families with more than one child affected with DMD. There were 5 families without a family history of DMD with more than one affected child. Second degree relatives were also checked for affected status. Among 50 children, 10 (20%) had affected second degree relatives.

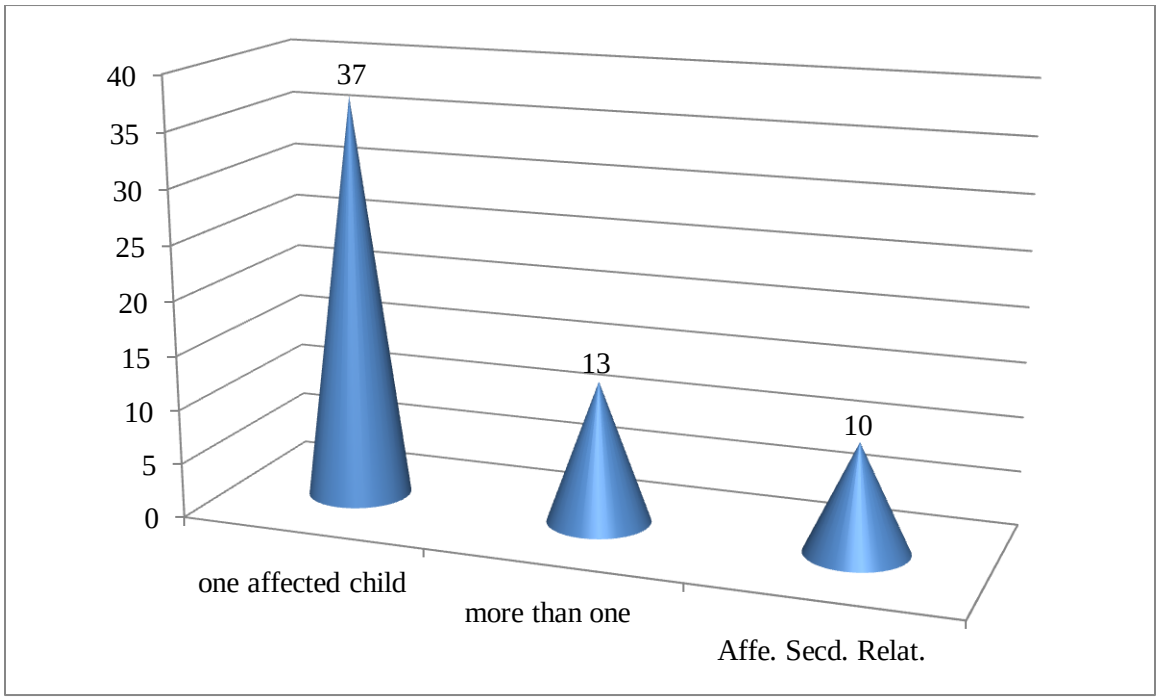


Figure 12: Cone diagram showing frequency distribution of affected individual in the family with DMD in Sri Lanka.

3.6 Molecular genetic testing:

Genotyping was performed to analyze deletion/duplication mutations in DMD gene. Deletions were found in 40 (80%) children whereas duplications were only in 4 (8%) children. Deletion/duplication was not detected in 6 (12%) children. These results are shown in pie chart in figure 13. Focusing among deletions, there were single exon deletion as well as multi exon deletions. The most common deletion was eight exon deletions ranging from exon 45 to 52 which was seen in 6 (12%) children. The next common exon deletion was a single exon 45

which was seen in 4 (8.0%) children. Duplications were seen in 4 (8%) children. When exons involved in deletions or duplications were examined, single exon involvement was seen in 8 (16%) children. Likewise, two exons involvement were seen in 3 (6%) children, three exons involvement were seen in 6 (12%), four exons involvement were seen in 1 (2%) child and more than four exons involvement were seen in 26 (52%) children. These results are shown in table 9.

Table 9: Frequency of exon deletions and duplications in children with DMD in Sri Lanka.

	Percent	**Sri Lankan Children with DMD**
Deletions	80%	40
Duplications	8%	4
Negative result	12%	6
Single exon	16%	8
Two to four exons	20%	10
More than four exons	52%	26

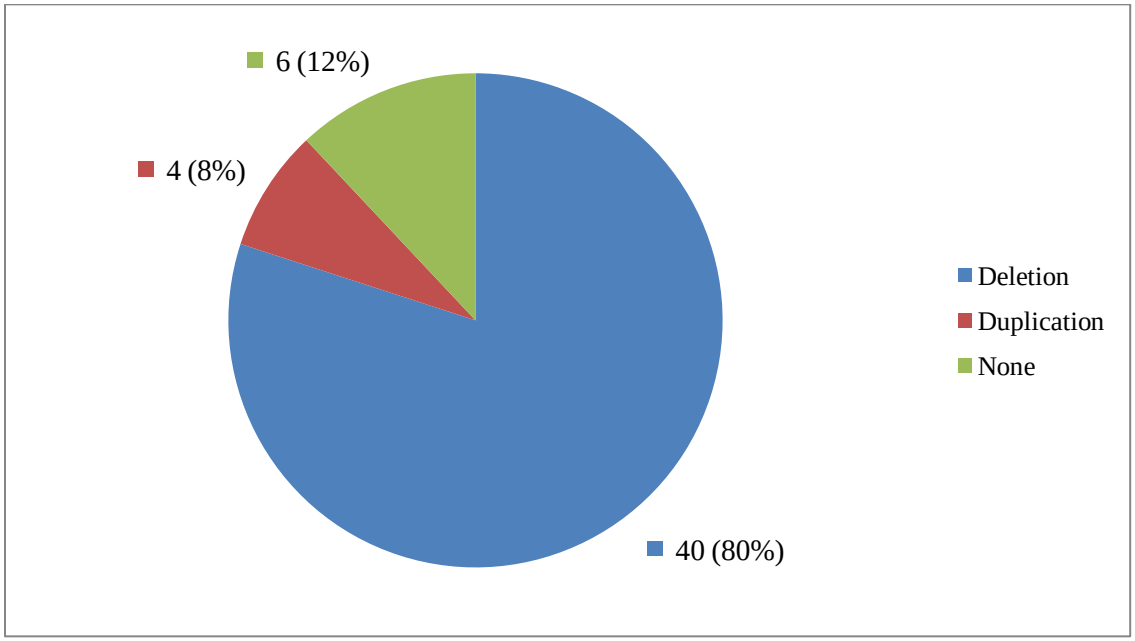


Figure 13: Pie diagram showing deletion/duplication frequency in Sri Lankan children with DMD.

3.7 Mutation profile in children with DMD in Sri Lanka:

Table 10: Mutation profile in children with DMD in Sri Lanka

Patient no.	Deletion/Duplications in Sri Lankan children
1	No
2	Duplication from exon 3 to exon 29
3	Deletion from exon 44 to exon 50
4	Deletion exon 45
5	Deletion from exon 49 to exon 50
6	Deletion from exon 3 to exon 11
7	Duplications from exon 7 to exon 9
8	Duplications from exon 7 to exon 9
9	Deletions from exon 45 to exon 52
10	No
11	Duplications from exon 7 to exon 9
12	Deletions from exon 45 to exon 52
13	Deletions from exon 48 to exon 50
14	Deletions from exon 8 to exon 44
15	Deletion exon 45
16	Deletion from exon 6 to exon 7
17	Deletions from exon 49to 50, exon 6 to 7, exon 68 and exon 30
18	Deletions from exon 35 to exon 43
19	Deletions from exon45 to exon 52om exon
20	No
21	Deletions from 48 to exon 50
22	Deletions from exon 10 to exon 11
23	Deletions from exon 49 to exon 54
24	Deletions from exon 46 to exon 50
25	Deletion exon 26
26	Deletion exon 26
27	Duplications from exon 14 to exon 27
28	Deletions from exon 44 to exon 60
29	Deletion from exon 45 to exon 52
30	Deletions from exon 45 to exon 52
31	Deletion exon 43
32	Deletions from exon 27 to exon 43
33	Deletions from exon 45 to exon 54
34	Deletions from exon 46 to 52
35	Deletions from exon 8 to exon 13
36	Deletion exon 45
37	Deletions exon 45
38	Deletions from exon 51 to exon 54
39	Deletions from exon 46 to exon 52
40	Deletions from exon 45 to exon 52
41	Deletions from exon 46 to exon 52
42	No
43	No
44	Deletions from exon 3 to exon 7
45	No
46	Deletion exon 44
47	Deletions from exon 8 to exon 13
48	Deletions from exon 46 to exon 52, exon 31
49	Deletions from exon 49 to exon 50, exon 31
50	Deletions from exon 45 to exon 50

The 79 exons of the dystrophin gene were divided into four groups. The first group ranged from exon one to exon twenty, the second group ranged from exon 21 to exon 44, the third group ranged from exon 45 to 55 and the fourth group ranged from exon 56 to exon 79. In this study, the most frequent mutant region fell within exons 45 to 55 (52%) in DMD gene, followed by exons 21 to 44 (26%) and exons 1 to 20 (26%) and the least common region fell within exons 56 to 79 (4%). Most common region in this study, was from exon 45 to 55 and least common region was exons 56 to79.

Table 11: Deletion/duplications involved mutant region in dystrophin gene in children with DMD in Sri Lanka.

Mutant region	**Sri Lankan Children (%) N = 50**
Exons 1 – 20	26 %
Exons 21 – 44	26%
Exons 45 – 55	52%
Exons 56 – 79	4%

3.8 Clinical phenotype co-relation:

Correlation between the genotype and phenotypic characteristics was done in this study. All clinical parameters important for the disease diagnosis and its clinical progression were included but extensive clinical phenotypes were not taken into consideration. The dystrophin gene was divided into four groups as mentioned above (table 11) and the most common mutant region was from exon 45 to exon 55. So, this region was co-related with the clinical phenotype by doing statistical analysis. Age of onset, age of first walk, age of diagnosis, Body Mass Index (BMI), and CPK level were compared with the most common mutant region as well as most common mutations (deletions/duplications) present in this study. Age of onset, age of first walk, age of diagnosis, BMI, and CPK level are numerical data (continuous data). Most common mutant region (exon 45 to exon 55) and the most common mutations (exon 45 to exon 52) were categorical data. The variables were correlated by doing statistically significant testing. There was no correlation found between age of onset and the most common mutant region ($p>0.8$), there was no correlation with age of diagnosis ($p>0.32$) as well. Age of first walk was also compared with the most common mutant region and interestingly these two variables were found to be correlated ($p<0.002$). Body Mass Index (BMI) was not correlated with the most common mutant region ($p>245$). The CPK level was also not correlated with the most common mutant region ($p>0.79$). These variables were also compared with the most common mutation present in this study but there were no co-relation found between these variables and most common mutation.

4. Discussion:

This study was carried out to determine the spectrum of mutations in Duchenne Muscular Dystrophy (DMD) and clinical phenotype co-relation in a cohort of Sri Lankan patients with Duchenne Muscular Dystrophy. This study was first to report deletion/duplication pattern in Sri Lankan children with DMD.

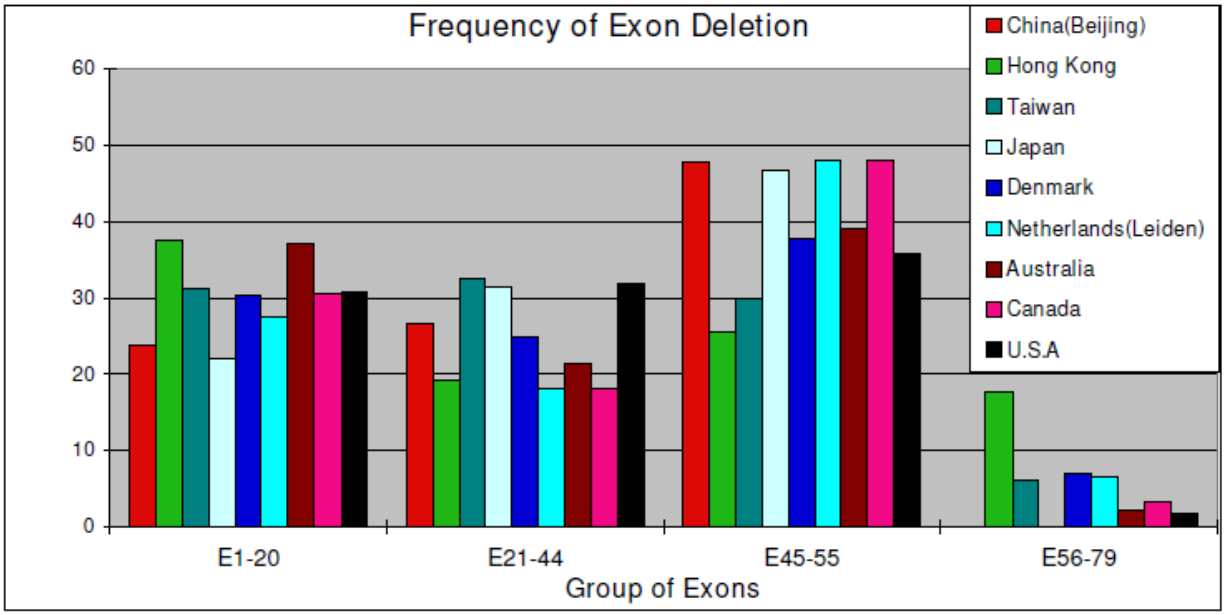
Multiplex Ligation Probe Amplification (MLPA) method was used in the present study to detect these deletions/duplications. This method analyzed all the 79 exons in the dystrophin gene, which can be used to identify duplications as well as carrier status in females (Li *et al.* 2009). This method was a qualitatively better method compared to multiplex polymerase chain reaction (mPCR) (Lai *et al.* 2006). Multiplex PCR was a method of choice for initial screening as it was sensitive and cost effective to assess any of the common 32 exon deletions (Beggs *et al.* 1990; Nadkarni *et al.* 2008) but it misses the other exon deletions and duplications.

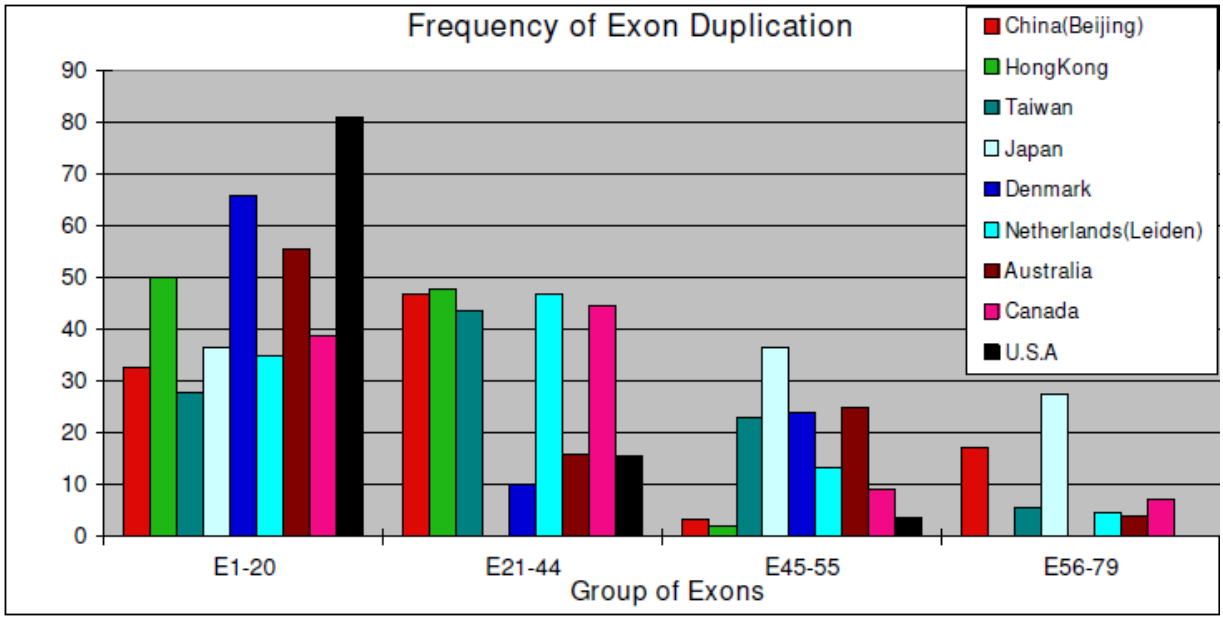
The deletions were seen in 80% of the children, the duplications were seen in 8% and there were no deletions/duplications in 12%. The most common mutant region in the DMD gene was within exons 45 to 55 (52%) and the least common region was within exon 56 to 79 (4%). These data were in agreement with other global population.

Previous study carried out in Sri Lanka in 24 DMD patients, showed a deletion frequency of 62.5%. This was detected by multiplex DNA amplification using the polymerase chain reaction, (Welihinda *et al.* 1993). In contrast, our study could able to detect 80% deletions as well as 8% duplications. This also showed that MLPA was an advanced technique than the mPCR.

Similarly, study carried out in 179 unrelated Chinese patients, showed 66.25% deletions and 6.25% duplication which were then compared to global populations. The region 45 to 50 was the

most common deleted region which was similar to our findings. The results compared with the mutations in the global populations is shown in the graph below (figure 14 and 15) (Wang *et al.* 2008).



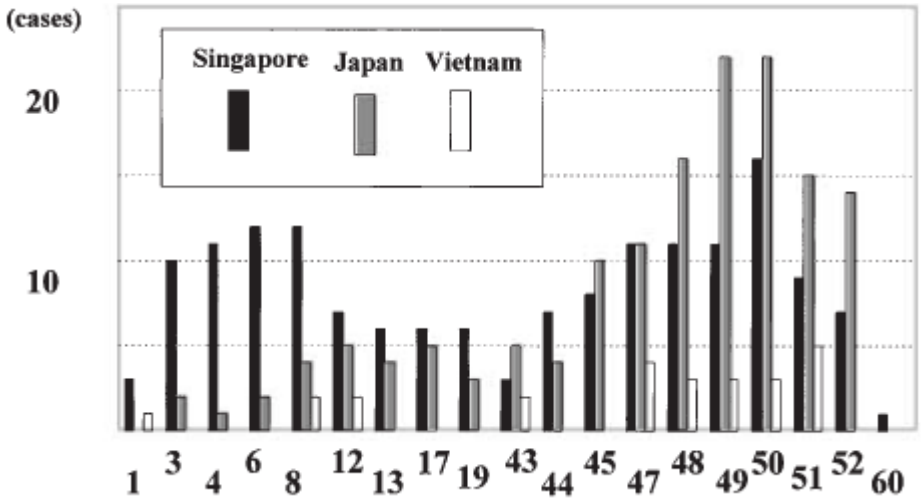


Source: Xiaozhu Wang, Zheng Wang, Ming Yan, Shangzhi Huang, Tian-Jian Chen and Nanbert Zhong. *Behavioral and Brain Functions.* 2008, 4:20

Figure 14 and 15: Comparison of exon deletion (A) and duplication (B) among global populations. All these data showed that exons 45–55 are the most frequent deletion region of DMD gene. Deletion and

duplication were not common in region of exons 56–79, although these data were derived from various methodologies (MLPA for Hong Kong, Taiwan, Denmark, Australia, and the current study; quantitative multiplex PCR for Canada; and multiplex PCR and Southern blotting for Japan and USA).

Focusing on Asian Populations, a comparative study done by Poh-San Lai and collogue in 2002 among Japanese, Vietnamese and Singaporeans showed that the most commonly deleted exons was in central deletion hotspot. The most commonly deleted exons were exon 50 (16 patients) in Singaporeans, exon 49 to 50 (22 patients) in the Japanese and exon 51 (5 patients) in Vietnamese which also comes within the range of our most common deleted exons (45 to 50). The screening of 19 deletion-prone exons of the dystrophin gene in 105 Singaporean, 86 Japanese and 34 Vietnamese patients showed deletions in 42 (40.0%), 44 (51.2%) and 11 (32.4%) patients respectively. At the proximal hotspot, the most commonly deleted exons were exon 6 and 8 in Singaporeans, 12 and 17 in the Japanese and 8 and 12 in the Vietnamese (Lai *et al.* 2002).



Source: Lai, P. S., Y. Takeshima. J *Hum Genet*. 47(10): 552-555.

Figure 16: Histogram showing distribution of deletion among 19 exons in different populations.

In a study done in Southern India in 112 children, exon deletions were present in 82%. In this study, they also showed single exon deletion in 17 (20%), distal exon 45 deletion in 30 (36%), exon 47 deletion in 29 (35%), exon 49 deletion in 28 (33.7%) and exon 50 deletion in 32 (38.5%) (Swaminathan *et al.* 2009). In eastern Indian study, the deletion rate was 65.7%. There were 38 deletions (82.61%) located in the hotspot region extending from exon 42 to exon 53, five (10.87%) deletions at the proximal hotspot (exon 1 to exon 20) and 3 deletions (6.52%) between exon 21 to 42 (Basak *et al.* 2006) which was similar to our findings.

We did not consider ethinicity as an important factor in mutation in the DMD gene. A study done in India among 160 children to see the ethnic difference supported this fact which showed 64.4% deletions. Most of the deletions involved exon 45-51. The authors argued that there was likely to be no ethnic difference with respect to deletions in the *DMD* gene (Banerjee *et al.* 1997).

In our study there were five families with no family history of DMD but having more than two affected children. The presence of multiple affected offspring from apparently non-carrier parents is caused by germ line or gonadal mosaicism. We were not able to confirm this by genetic testing of mother but this study can be further extended to identify the germ line mosiacism in Sri Lankan cohort.

The recurrence risk for non-carrier females due to germ line mosiacism has been estimated between 14% and 20%, if the risk haplotype is transmitted. In 138 children, study done by Enden and collogue in DMD and BMD showed risk of 8.6% if the risk haplotype was transmitted. There was a remarkable difference between the proximal (15.6%) and distal (6.4%) deletions (Helderman-van den Enden *et al.* 2009).

Clinical phenotype correlation was difficult in this study due to some limitations. We were not able to correlate genotype with clinical phenotype in this cohort but this study showed important aspects of DMD research in Sri Lanka. The finding of no correlation between genotype and phenotype in this study was also supported by number of other studies as well including the followings.

A study done by Magri and colleagues in 320 DMD/BMD patients showed genotype – phenotype characterization in a large dystrophinopathic cohort with extended follow-up. According to this study, in DMD, mutation type did not influence clinical evolution but mutation located in distal regions (irrespective to their nature) was more likely to be associated with lower IQ levels (Magri *et al.* 2011).

Our study was further supported by Bastaki 1999, who demonstrated that there was no significant correlation between the size/site of dystrophin deletions and clinical severity in patients with Dystrophinopathy (50 DMD patients and 2 BMD patients) (Bastaki 1999).

This type of study has not been done previously, so there was lack of information of DMD status at Sri Lanka. This study gave genetics diagnostic approach to DMD which was very important for diagnosis, carrier testing, genetic counseling, prenatal diagnosis, management, new treatment strategies and further research. However, there were few limitations in this study. We were unable to establish diagnosis in 6 (12%) patients. These patients might have point mutation as discussed earlier in this dissertation. Sequencing of whole gene is needed to identify the molecular cause among these patients. We were not able to do so, due to time constrains and funding difficulties.

The result of this study opened a pathway for further large scale new research in DMD, which can be followed up for a longer period. Therefore, the study would also be useful for finding out the point mutation associated with DMD in Sri Lanka in longer term. As this study also showed a similar pattern of mutation as compared to the global population, new ongoing therapeutic clinical trials can also be considered in near future for further research.

5. Conclusion:

In conclusion, this study was conducted to identify the deletion and duplication pattern in a cohort of Sri Lankan children with DMD. Deletions and duplications are the most common cause of mutations involved in DMD in global population. The finding of this study corresponds to the other global population as well. This study was not able to establish diagnosis of 6 (12%) patients which are thought to be point mutation, since it is known that approximately 30% of DMD is caused by point mutation. This study was inconclusive for clinical phenotype correlation with mutation, so, we suggest further large scale study. The children and parents recruited for this study were counseled pre test and post test, which was very beneficial for the families. This study also revealed that 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. The diagnosis of the DMD is based on clinical, biochemical and histopathological studies and further confirmed by molecular analysis. However, genetic studies should be the investigation of choice in DMD.

The dystrophin gene is a large and fascinating gene with a complexity in transcriptional regulation, function and protein-protein interactions. Diagnosis and genotype-phenotype correlation are particularly important to understand the pathways and mechanism that regulates

the expression pattern. Improvement in knowledge about these features opens a way for a future treatment for this devastating disorder.

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APPENDIX 1

Reagents used (SALSA MLPA kit components provided by the company):

1. SALSA MLPA Buffer (Yellow cap)	KCL, Tris-HCL, EDTA and PEG-6000. Ph 8.5
2. SALSA Ligase-65 (Green cap)	Glycerol, BRIJ (0.05%), EDTA, Beta-Mercaptopurine (0.1%), KCL, Tris-HCL, Ph 7.5, Ligase-65 enzyme (bacterial origin)
3. Ligase Buffer A(Transparent cap)	NAD (bacterial origin), Ph3.5
4. Ligase Buffer B (White cap)	Tris-HCL, non-ionic detergents, $MgCl_2$. pH 8.5
5. SALSA PCR Primer mix (Brown cap)	Synthetic oligonucleotides, one of which is fluorescently labeled, (FAM), dNTPs, Tris-HCL, KCL, EDTA, BRIJ (0.04%).Ph 8
6. SALSA Polymerase (Orange cap)	Glycerol, BRIJ (0.5%), EDTA, DTT (0.1%), KCL, Tris-HCL, Polymerase enzyme (bacterial origin), Ph 7.5
7. Probemix (Black cap)	Synthetic oligonucleotides purified from bacteria, Tris- HCL, EDTA, Ph 8.0