Chapter 1

Introduction

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Dystrophinopathies comprise a spectrum of muscle diseases caused by mutations in the *DMD* gene which codes for the muscle protein dystrophin. The clinical spectrum ranges from mild to severe and includes asymptomatic increase of serum concentration of creatine phosphokinase (hyperCKemia), X-linked myalgia/cramps with myoglobinuria, isolated quadriceps myopathy, X-linked cardiomyopathy, Becker muscular dystrophy (BMD) and Duchenne muscular dystrophy (DMD) (Darras et al., 2008).
The *DMD* gene is located in band Xp21.2 and the inheritance of dystrophinopathies is X-linked. Mutations in the gene lead to absent or less functional dystrophin. The most common dystrophinopathy is DMD; its incidence varies between 1 in 3600 to 1 in 6000 (Emery, 1991). The incidence in the Netherlands has been estimated to be 1 in 4200 live born males in the period 1961-1974 (van Essen et al., 1992b) and 1 in 4685 in the period 1993-2002 (Helderman-van den Enden et al., 2012). The exact incidence of BMD is not known and has been estimated to be in the range of 1 in 12000 to 1 in 30000 male live births (Bushby et al., 1991; Emery, 1991).
Insufficient information is available on other dystrophinopathies. Results of immuno-histochemical analysis of the muscle biopsy on individuals with hyperCKemia showed that 8% (3/40) of those without or with minimal symptoms showed abnormal dystrophin staining, resembling Becker pathology (Dabby et al., 2006), but molecular testing to confirm dystropinopathy was not done. Abnormal dystrophin staining was also seen in 8% (8/104) of clinically normal subjects with chronic hyperCKemia, but here molecular testing did confirm a form of dystrophinopathy (Fernandez et al., 2006). On the other hand, 27% (28/104) of patients with a mild X-linked muscular dystrophy phenotype who had abnormal dystrophin in the muscle biopsy, were sub-clinical or asymptomatic (Angelini et al., 1994). There are also a number of families/patients with X-linked myalgia and cramps (Gospe et al., 1989; Sanchez-Arjona et al., 2005; Veerapandiyan et al., 2010; Helderman-van den Enden et al., 2010). At least 11 families have been published with X-linked dilated cardiomyopathy (Ferlini et al., 1999). However, only five patients with this type of dystrophinopathy were found among > 4700 mutations in the *DMD* gene reported in the Leiden DMD mutation database (Aartsma-Rus et al., 2006). No mutation was found in the *DMD* gene in 27 patients with idiopathic dilated cardiomyopathy without systemic disease (Michels et al., 1993). A recent study among 436 male patients with dilated cardiomyopathy showed a mutation in the *DMD* gene in 34 males (7,8%) (Diegoli et al., 2011). In conclusion, the incidence of other dystrophinopathies is not known as there is insufficient data, probably because many patients have not been diagnosed.

1.1 Clinical description

1.1.1 Duchenne muscular dystrophy (DMD)

DMD is the most severe form of dystrophinopathy. In general, only males are affected due to the X-linked inheritance. Occasionally girls are as severely affected as boys, usually because

of a translocation between an X-chromosome and an autosome where the normal X is preferentially inactivated. For other rare causes see Section 1.2 below: Dystropinopathy in women.

The affected boys do not show any symptoms at birth. More than half of the boys start walking only after 18 months, whereas 97% of normal children are already walking at this age (Emery and Muntoni, 2003). Most patients are diagnosed around the age of five, mainly because of delay in walking and an unsteady gait with tendency to walk on tiptoes. Some are diagnosed because a test for unrelated indications or calf pains reveals hyperCKemia or increased transaminases (Bushby et al., 1999; Emery and Muntoni, 2003). Proximal muscle weakness should be suspected if a boy has difficulties in running and climbing stairs and physical examination reveals hypertrophy of the calf muscles and a positive Gower's sign (difficulty in getting up from the floor which is solved by spreading the legs and using the hands to climb up the thighs to get to an upright position). Serum CK concentration is typically increased to at least ten times normal till about the age of six (Darras et al., 2008); it then decreases with advancing age due to progressive loss of muscle mass (Zatz et al., 1991). Most untreated DMD patients become wheelchair bound between the ages nine and twelve (Emery and Muntoni, 2003). Long-term corticosteroid therapy prolongs ambulation by two to five years and reduces the need for spinal stabilization surgery (Moxley et al., 2010). Without treatment the muscle strength deteriorates and results in death around the age of 19. The survival can be prolonged into the fourth decade with corticosteroid, cardiac, respiratory, orthopaedic and rehabilitative interventions (Bushby et al., 2010a; Bushby et al., 2010b; Eagle et al., 2002; Dreyer et al., 2010; Ishikawa et al., 2011). A typical clinical course can be seen in Figure 1.

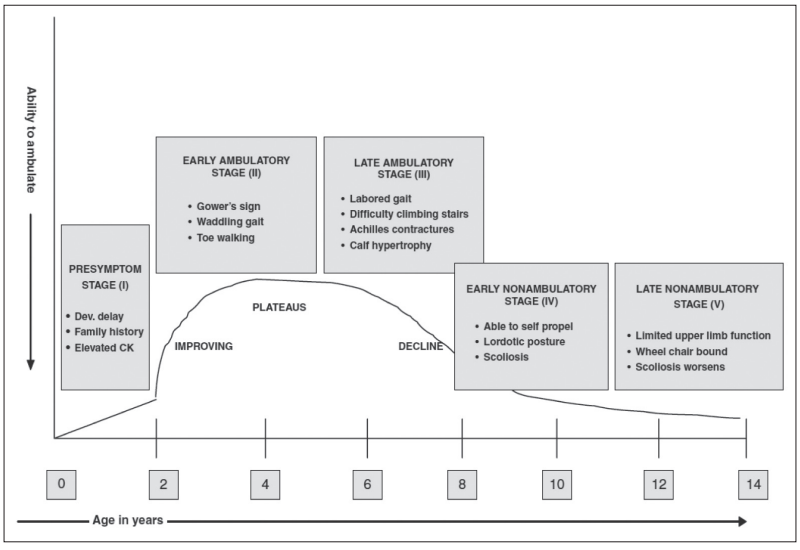

Figure 1
Musculoskeletal course in Duchenne muscular dystrophy (Verma et al., 2010) (with the kind permission of Professor Y Anziska).

When Duchenne de Boulogne first described these patients he noticed that, apart from the muscular dystrophy, some boys also had mental problems (Duchenne, 1868). Mental retardation (defined as full scale intelligence quotient below 70) has been estimated to occur in 19-35% of DMD cases (Cotton et al., 2001; Cotton et al., 2005). Cognitive impairment has been described also in patients with BMD although its frequency has not been studied systematically (Bardoni et al., 2000; North et al., 1996). In DMD patients the distribution of the IQ is shifted downward by approximately one standard deviation (Cotton et al., 2001) in comparison to the normal population. In contrast to the muscular dystrophy mental retardation is non-progressive (Anderson et al., 2002). Severe mental retardation is concordant in affected relatives (Muntoni et al., 2003), suggesting a primary role of the mutated *DMD* gene in mental retardation. Several authors have found that the loss of expression of dystrophin isoforms, especially DP140 and DP71, in the central nervous system is related to the retardation (Bardoni et al., 2000; Moizard et al., 1998; Taylor et al., 2010; Wingeier et al., 2011). The loss of DP71 is reported to result in a shift of two standard deviations of the Full Scale Intelligence Quotient (Daoud et al., 2009). If the mutation in the Dp140 isoform is located in the 5' UTR, it has less effect on full scale intelligence quotient than if it is in the promoter of protein-coding regions of Dp140 (Taylor et al., 2010).

1.1.2 Becker muscular dystrophy (BMD)

BMD is the second best known dystrophinopathy. The phenotype is less severe than DMD. As DMD, BMD is characterized by progressive symmetrical muscle weakness and atrophy, proximal greater than distal, often with calf hypertrophy. Preservation of the strength of the neck flexor muscle differentiates BMD from DMD. Wheelchair dependency, if present, occurs after the age of 16 in the natural course of the disease. However, as the corticosteroid therapy induces prolongation of the ambulation of DMD patients, the criterion of wheelchair dependency after the age of 16 does not always point to BMD. Occasional features of BMD are weakness of quadriceps femoris (sometimes the only sign), activity-induced cramping and late in the course flexion contractures of the elbows (Darras et al., 2008). Onset is usually between the ages of 5 and 15, however, it may occur later in life. The most frequent presenting symptom is calf pains, typically experienced in early teenage years, provoked by exercise and relieved by rest. Frequent falling and being slower than peers, can also be the presenting symptom (Bushby and Gardner-Medwin, 1993). Serum CK concentration is typically increased to more than five times the normal value, reaching the maximum on average between the ages of 10 and 15 (Zatz et al., 1991). The mean age of death is in the mid-40s with a large range (23-89 years) (Bushby and Gardner-Medwin, 1993) but many BMD patients with a mild phenotype who are still self supporting in their 60's or 70's have been described (Ferreiro et al., 2009; Helderman-van den Enden et al., 2010; Lesca et al., 2007; Saengpattrachai et al., 2006; Yazaki et al., 1999).

1.2 Dystrophinopathy in women

1.2.1 Carriers

Most female carriers have no symptoms of dystrophinopathy because the inheritance of DMD is X-linked. Serum CK level is significantly raised in two-thirds of the carrier women (Emery and Muntoni, 2003) but without any symptoms. In two studies 2.5 to 7.8% of the female carriers developed symptoms varying from mild muscle weakness to a rapidly progressive DMD-like muscular dystrophy (Moser and Emery, 1974; Norman and Harper, 1989).

A Dutch study of 129 female carriers reported frequent myalgia/cramps in 5% and muscle weakness in 17% (Hoogerwaard et al., 1999a). Dilated cardiomyopathy was present in 5% and left ventricle dilatation in 18% (Hoogerwaard et al., 1999b). A recent follow-up study after nine years has shown that cardiac abnormalities in these carrier women are as progressive as in DMD patients (van Westrum et al., 2011). Carrier women are advised to start cardiac examination at the age of 16 or later, at diagnosis, with follow-up examinations every five years (Bushby et al., 2003). In the United States of America only 62.9% of the carriers appeared to be aware of their risk for cardiomyopathy (Bobo et al., 2009). In Scotland the benefit of routine cardiac surveillance of all carriers was questioned after the finding that there was no significant reduced life expectancy or higher risk of cardiac death in 94 deceased carriers compared to the general population (Holloway et al., 2008). Nevertheless, there is world-wide consensus that carrier women should be tested for cardiac disease.

Symptoms in female carriers could be explained by non-random X-inactivation where the normal X-chromosome is preferentially inactivated (Azofeifa et al., 1995). It was suggested that it is useful to study the pattern of X-inactivation in carriers of DMD because women with skewed X-inactivation may show slower, yet progressive, myopathy with advancing age (Yoshioka et al., 1998). Sumita et al. have shown that a high proportion of asymptomatic carrier women (19%, 19/102) as well as normal female controls (24%, 28/117) show skewed inactivation in DNA isolated from lymphocytes (Sumita et al., 1998). They suggest that highly skewed X-inactivation pattern in blood is not enough to predict that a young DMD carrier will develop muscular weakness. X-inactivation was recently studied in 15 carriers with symptoms of DMD. Eight had exonic deletions or duplications, six had small mutations and one patient had two mutations. The X-inactivation result from one patient with a deletion was uninformative. Four of the seven with a deletion or duplication and one of the six with a small mutation showed skewed inactivation. All the rest showed a random pattern of X-inactivation. The significance of these findings depends on the definition of skewed (a value that is larger than 80:20). The authors concluded that they were not able to demonstrate a significant association between the X-inactivation pattern and progressive myopathy and that future studies with a larger number of subjects are required (Soltanzadeh et al., 2010).

1.2.2 Female dystrophinopathy patients

The following mechanisms that explain the phenotype of females with full blown dystrophinopathy have been described. All these mechanisms lead to absent or non-functional dystrophin.

- Women with translocations involving an X-chromosome with the breakpoint in Xp21 and an autosome show preferential inactivation of the normal X with the normal *DMD* allele (Greenstein et al., 1980; Jacobs et al., 1981; Lindenbaum et al., 1979; Verellen-Dumoulin et al., 1984; Zatz et al., 1981; Boyd et al., 1986).
- A mutation in the *DMD* gene in the only X-chromosome of girls with Turner syndrome results in a phenotype similar to that of affected males (Chelly et al., 1986; Ferrier et al., 1965; Sano et al., 1987).
- Uniparental disomy for the X-chromosome with a mutation in the *DMD* gene has been described once (Quan et al., 1997).
- Women with a 46,XY karyotype and DMD caused by the co-occurrence of mutations in both the dystrophin and the androgen-receptor genes have been described (Katayama et al., 2006).
- Finally, two women have been described with a normal karyotype and mutations in both *DMD* genes. One, a 14 year old girl with consanguineous parents, is homozygous for the mutation (Fujii et al., 2009). The other is a 15 year old girl with compound heterozygous mutations. DNA analysis of the mother was normal and DNA analysis of the phenotypically normal father was not possible (Soltanzadeh et al., 2010).

1.3 Diagnosing dystrophinopathies

In the absence of a family history, DMD may be suspected in a boy if he is not walking at the age of 16-18 months or if there is an unexplained increase in transaminases and certainly if he has a positive Gower's sign. If there is a positive family history, any suspicion of abnormal muscle function should lead to a diagnostic investigation (Bushby et al., 2010a). The flowchart in Figure 2 shows how the diagnosis of DMD can be confirmed. In the Netherlands, blood from almost all boys with a suspicion of dystrophinopathy is sent to the Laboratory of Diagnostic Genome Analysis at the Leiden University Medical Center. If a mutation is found in the *DMD* gene the clinical diagnosis is confirmed. If no deletion or duplication (MLPA test) is found and dystrophin in the muscle biopsy is absent, High Resolution Melting Curve Analysis (HR-MCA) is done followed by sequencing of the amplicons with abnormal melting curves (Almomani et al., 2009). cDNA sequencing (obtained from RNA) is performed in rare cases where the above mentioned tests have not revealed a DNA mutation. Once the diagnosis has been confirmed, referral to a specialized multidisciplinary team as well as genetic counselling of the patient and his family members is recommended. The patient and his family should be offered support and contact with patient organizations.

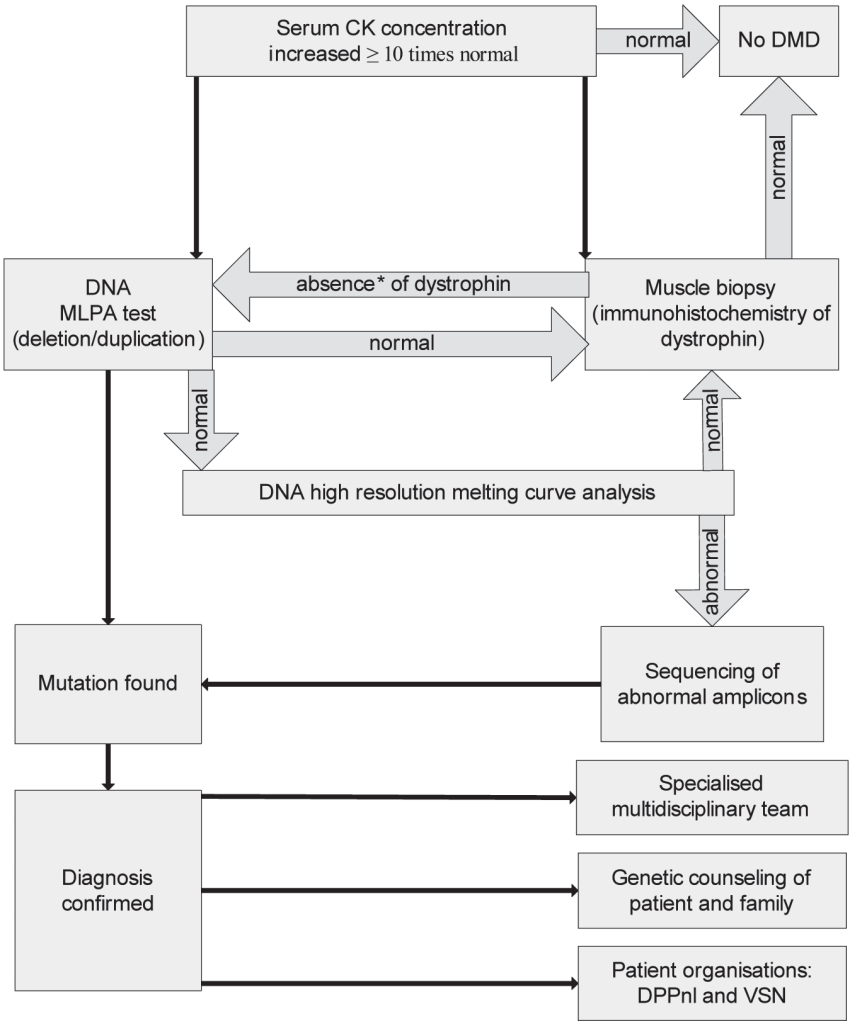

Figure 2

Confirmation of diagnosis in a patient suspected of having Duchenne muscular dystrophy.

DMD is suspected when serum CK is increased by at least 10 times the normal value. It is recommended to start with MLPA analysis of DNA from blood because this is easy to obtain and in about 60% of the patients a deletion or duplication is found. Some physicians start with a muscle biopsy. *Absence of dystrophin in the muscle biopsy is in principle enough to confirm the diagnosis. However, genetic testing to detect a mutation is a part of standard care in the Netherlands since this is indispensable for reliable carrier testing of the mother and if applicable of other female family members. Also, with the development of personalized medicine the mutation should be identified. In rare cases, genetic testing does not reveal a mutation even though dystrophin is absent in the muscle biopsy. If the diagnosis of dystrophinopathy is not confirmed by either the muscle biopsy or by genetic testing, the diagnosis of alternative muscular dystrophies, which is complex and requires specialized input, should be undertaken (Bushby et al., 2010a). However, this is outside the scope of this thesis and will not be discussed further.

In patients suspected of dystrophinopathy a muscle biopsy should be taken if genetic testing does not reveal a deletion or duplication. If it is BMD, immunohistochemistry of the muscle tissue may show reduced intensity with or without patchy staining (Hoffman et al., 1988). Western blot analysis should also be performed and if this shows an abnormal molecular weight and/or reduced quantity of the dystrophin, BMD is highly likely. In that case HR-MCA should be carried out. Less common dystrophinopathies may be suspected in the absence of a mutation but the presence of hyperCKemia and other symptoms such as cramps, myalgia, flexion contractures of elbows, wheelchair dependency after the age of 16, unexplained dilated cardiomyopathy and/or an X-linked family history with similarly affected family members. According to recent guidelines proposed for patients with unexplained hyperCKemia, a muscle biopsy should be taken if one or more of the following features are present: the level of serum CK is ≥3 times normal, the electromyogram is myopathic or the patient is younger than 25. In addition, DNA testing should be offered to women even if the level of serum CK<3 times normal. This should be done prior to a muscle biopsy because of the possibility that there is a mutation in the dystrophin gene (Kyriakides et al., 2010). As the symptoms are sometimes very mild it is possible that many patients with dystrophinopathy do not consult a doctor and are therefore not diagnosed.

1.4 Genetic counselling and prenatal testing

The family is referred for genetic counselling following the identification of a mutation in the index patient. The family members are informed that the dystrophinopathy could have occurred as the result of a *de novo* mutation or that the disease may have been inherited from the mother. In case of a *de novo* mutation the mother should be offered prenatal testing in the next pregnancy because of the risk of germ line mosaicism (see Section 1.5 below). The sisters of the patient may also request molecular testing of the familial mutation. If the dystrophinopathy is found to be inherited, further testing of first degree female family members of the mother by cascade screening is recommended. Options for having healthy offspring should be discussed with the identified carriers and cardiological surveillance should be offered (see Section 1.2: Dystrophinopathy in women).

It has been recommended that prenatal diagnosis for dystrophinopathies should be carried out only for male pregnancies. At present, it is not possible to predict whether a female heterozygote for a *DMD* mutation will manifest any signs of the disorder or not, and it is, therefore, considered to be inappropriate to offer prenatal testing for a female foetus (Abbs et al., 2010). The sex of the foetus can be determined by examining the foetal cells in the maternal serum (Lo et al., 1997). Prenatal testing for dystrophinopathy is usually performed in the 11th week of the pregnancy. A sample of chorionic villi from the developing placenta is taken either by means of a needle inserted through the abdomen of the woman or via a tube inserted through the vagina and cervix. The cells of the chorionic villi have the same genetic information as the foetus and can be used in a male foetus to test if the familial mutation has been inherited. If the foetus is affected, the parents may choose to terminate the pregnancy.

1.5 Mosaicism

Most DMD patients inherit an X-chromosome with the mutation, which is present in all cells. One in three patients has DMD as a result of a *de novo* mutation (Haldane, 1935). If a new mutation occurs during meiosis in one of the parents, the egg or the sperm will carry the mutation and will pass it on to the child who will have the mutation in all cells. If, on the other hand, a new mutation occurs during mitosis in the embryo a proportion of somatic and/or germ line cells, will carry the mutation. Such a person is a mosaic with a mixture of cells, some with and some without the mutation (Erickson, 2010). Mosaicism refers to the presence of two (or more) cell lines with different genotypes in one individual who has developed from a single fertilized egg. Somatic mosaicism has been described in several patients with dystrophinopathy (Bakker et al., 1989; Bunyan et al., 1994; Bunyan et al., 1995; Helderman-van den Enden et al., 2003; Lebo et al., 1990; Saito et al., 1995; Smith et al., 1999; van Essen et al., 2003; Voit et al., 1992; Kesari et al., 2009; Rajakulendran et al., 2010; Uchino et al., 1995). A mutation can also occur in a germ line cell in the gonad, in which case mosaicism is confined to the germ cells and a proportion of eggs or sperm carry the mutation. Such a person with germ line mosaicism, also called gonadal mosaicism, does not have the disease but can pass on the mutation to more than one child. Germ line mosaicism was reported in a number of families with dystrophinopathy in the late 80s (Bakker et al., 1987; Bech-Hansen et al., 1987; Darras and Francke, 1987). The recurrence risk due to germ line mosaicism for non-carrier females was estimated to be 7% (Bakker et al., 1989) and 10% (van Essen et al., 1992a). A recent and more reliable figure, as it is based on many more families, is 4.3% (Chapter 2.1 of this thesis) (Helderman-van den Enden AT et al., 2009).

1.6 Genetics and proteomics of the dystrophinopathies

1.6.1 The *DMD* gene

The inheritance of the dystrophinopathies is X-linked recessive. In 1983 Duchenne muscular dystrophy was found to be linked to two markers on the short arm of the X-chromosome (Davies et al., 1983). Subsequently the *DMD* gene was mapped on band Xp21 in 1985 (Ray et al., 1985) and cloned in 1987 (Koenig et al., 1987). With a size of ~2.4 Mb, it is the largest known human gene (den Dunnen et al., 1992). The *DMD* gene occupies about 1/1000 of the total human genome (Koenig et al., 1987). It has 79 exons which account for only 0.6% of the gene. The remaining part consists of large introns (Aartsma-Rus et al., 2006). The gene has seven promoters: three of them, the brain, muscle and Purkinje promoters, lead to a full length dystrophin which consists of unique first exons spliced to a common set of 78 exons (Sadoulet-Puccio and Kunkel, 1996). The size of the mRNA in the muscle is 14 kb. Four promoters (retina, brain3, schwann cells and general) lead to shorter dystrophin proteins which lack the actin binding terminus but retain the cystein rich and carboxy-terminus domains (Muntoni et al., 2003). The different promoters are named after the predominant, but not exclusive, site of expression as can be seen in Table 1.

Isoform Symbol	Isoform Name	Location of promoter/unique first exon	Protein Molecular Mass	Tissue Expression Pattern	Reference
Dp427c	Brain/Cortical-dystrophin	5' of Dp427m	427 kDa	Cortical neurones, skeletal and cardiac muscle Low levels in retina	(Nudel et al., 1989)
Dp427m	Muscle-dystrophin	5' of Dp427m exon 1	427 kDa	Skeletal and cardiac muscle and glial cells Low levels in retina	(Koenig et al., 1987)
Dp427p	Purkinje-dystrophin	Dp427m intron1	427 kDa	Purkinje cerebellar neurons Low levels in skeletal muscle	(Gorecki et al., 1992)
Dp260	Retinal dystrophin	intron 29	260 kDa	High in retina Low levels in brain and cardiac muscle	(D'Souza et al., 1995)
Dp140	B3-dystrophin	intron 44	140 kDa	Brain, retina and kidney	(Lidov et al., 1995)
Dp116	Schwann cell-dystrophin	intron 55	116 kDa	Peripheral nerves (Schwann cells) exclusively	(Byers et al., 1993)
Dp71	General-dystrophin	intron 62	71 kDa	In most tissues – brain, kidney, liver, lung, cardiac muscle Not expressed in skeletal muscle	(Lederfein et al.,

Table 1
Overview of the tissue expression of the different isoforms of dystrophin (with the kind permission of P.J. Taylor, thesis 2008 (Taylor, 2008)).

1.6.2 Mutation types in the *DMD* gene

Mutations reported in the Leiden DMD mutation database (www.dmd.nl) include deletions (72%) and duplications (7%) of one or more exons; the remaining ~ 20% of the patients have small deletions, insertions or point mutations (Aartsma-Rus et al., 2006).
In 1988 it was postulated that DNA mutations that disrupt the reading frame result in DMD while mutations that maintain the reading frame result in BMD (Malhotra et al., 1988; Monaco et al., 1988). The disrupted reading frame generates an out-of-frame messenger RNA transcript that results in a premature truncation of translation. The truncated protein that is formed lacks the cystein rich and C-terminal domains and has no or little bridge function. In BMD the reading frame remains intact; the protein is partly functional and its presence can be demonstrated in the muscles of the patients. In the more than 4700 mutations reported in the *DMD* gene in the Leiden DMD database, the reading-frame rule holds true at the DNA level in 91% of the patients; at the RNA level this percentage probably goes up to 99.5% (Aartsma-Rus et al., 2006). Non-sense mutations normally result in DMD because of the premature stop in protein translation. In rare cases a non-sense mutation is found in a BMD patient. The most likely explanation for the unexpected non-DMD phenotype is that the mutation is located in the exonic motive that is needed for the recognition of the exon by

the splicing machinery, and thus leads to exon skipping resulting in a restored reading frame (Flanigan et al., 2010; Ginjaar et al., 2000).

1.6.3 Dystrophin protein

Dystrophin is the protein encoded by the *DMD* gene; its molecular weight is 427 kDa and the number of amino acid (AA) residues, as deduced from the nucleotide sequence, is 3,685 (Hoffman et al., 1987). The dystrophin protein is absent in most muscle fibers of DMD patients. In about 50% of the DMD patients there may be some dystrophin positive fibers. The most likely explanation of these so-called revertant fibers is spontaneous in-frame splicing, for example after a second mutation (Klein et al., 1992; Mendell et al., 2010).

The full length muscle dystrophin is composed of the following four domains (Figure 3):

- The actin-binding domain is so called because the N-terminal portion is highly homologous to the N-terminal portion of α-actinin. It consists of between 232 and 240 amino acids, depending on the isoform, and has three actin binding sites (Jarrett and Foster, 1995; Koenig et al., 1987; Koenig and Kunkel, 1990).
- The central rod domain is the largest part of the protein and is composed of approximately 3000 amino acids. It is formed by a succession of 24 triple helical repeats similar to spectrin. In addition, and in contrast to the spectrin molecules, four predicted hinges separate the rod region into three sub-regions which are thought to impart flexibility to the protein (Koenig and Kunkel, 1990). A fourth actin-binding site was found between the spectrin-like repeat units 11-17 (Amann et al., 1998; Rybakova et al., 1996). The multiple spectrin-like repeats had long been thought to be largely redundant because patients with a missing part in the central rod usually had only mild symptoms (England et al., 1990). However, recent studies have shown that the spectrin-like repeats harbour sites that bind to membrane phospholipids, intermediate filaments, microtubules and neuronal nitric oxide synthase. This suggests that the central rod domain is more of a scaffolding region, rather than simply a passive link between the N-and C-terminal ends (Lai et al., 2009; Le et al., 2010).
- The cystein-rich domain has 15 cysteine residues and consists of 280 amino acids (Koenig et al., 1988). This part of the protein interacts with β-dystroglycan and has the following components: the WW domain, two EF hands and ZZ domains. The WW domain contains two conserved tryptophan (W) residues 20-23 amino-acids apart (Bork and Sudol, 1994) and is the primary site of interaction between dystrophin and the last 15 C-terminal amino acids of β-dystroglycan (James et al., 2000; Jung et al., 1995). The EF hands are putative calcium-binding sites that stabilize the WW domain and have affinity for β-dystroglycan (Chung and Campanelli, 1999; Huang et al., 2000). Finally, the ZZ domains are highly conserved widespread zinc-binding motifs that stabilize the overall complex by interacting with β-dystroglycan (James et al., 2000; Rentschler et al., 1999). The part of the ZZ domain formed by the amino acids 3326-3332, is crucial for binding to the β-dystroglycan (Hnia et al., 2007).
- The C-terminal domain consists of 420 highly conserved amino acids, with only one

cystein residue. It forms an α-helical dimeric coiled-coil structure that interacts with syntrophin (Ahn and Kunkel, 1995; Koenig et al., 1988) and dystrobrevin (Sadoulet-Puccio et al., 1997).

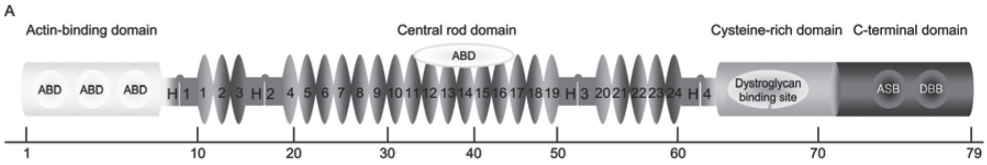

Figure 3
Schematic drawing of the dystrophin protein adapted from (Aartsma-Rus et al., 2006). The location of the different exons is shown underneath the protein (with the kind permission of Aartsma-Rus).

As mentioned above more isoforms are known in addition to the muscle dystrophin. The eight dystrophin isoforms and utrophin, a homologue of dystrophin, are depicted in Figure 4; the uppermost is the muscle isoform. The full-length dystrophins Dp427m, Dp427c and Dp427p consist of N-terminal, central rod, cysteine-rich and C-terminal domains, but each isoform has its own unique N-terminal part (which is coded by a unique first exon, depicted with ◗). The shorter isoforms lack some, or most of the N-terminal and/or central rod domains, and also have their own unique first exon (except for Dp140). Dp71 is usually alternatively spliced, which gives rise to an alternative C-terminal part. Dp40 derives from an alternative poly-adenylation signal in intron 70. The dystrophin homologue utrophin is very similar to the full-length dystrophin isoforms.

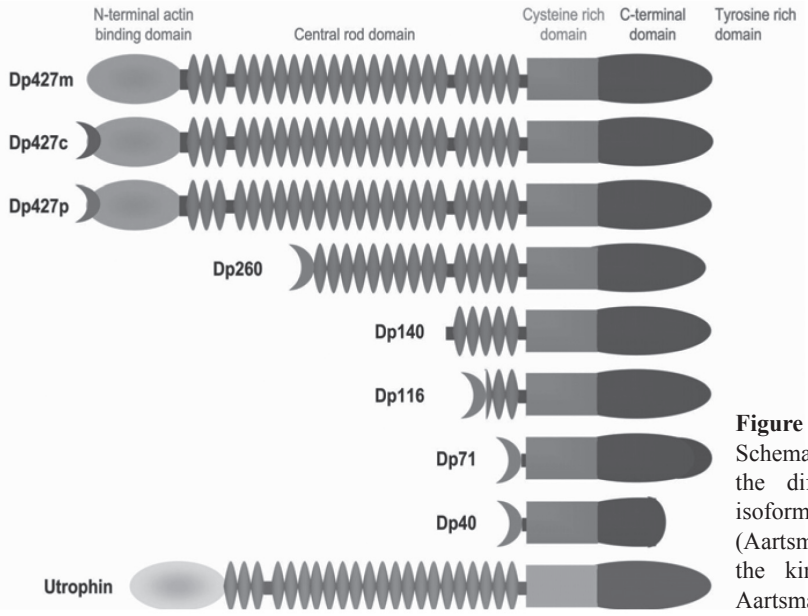

Figure 4
Schematic drawing of the different dystrophin isoforms and homologues (Aartsma-Rus, 2005) (with the kind permission of Aartsma-Rus).

1.6.4 Dystrophin and the dystrophin glycoprotein complex

Dystrophin is part of the dystrophin glycoprotein complex. This complex consists of the cytoplasmic dystrophin-containing complex, the dystroglycan complex, the sarcoglycan complex and the sarcospan as can be seen in Figure 5.

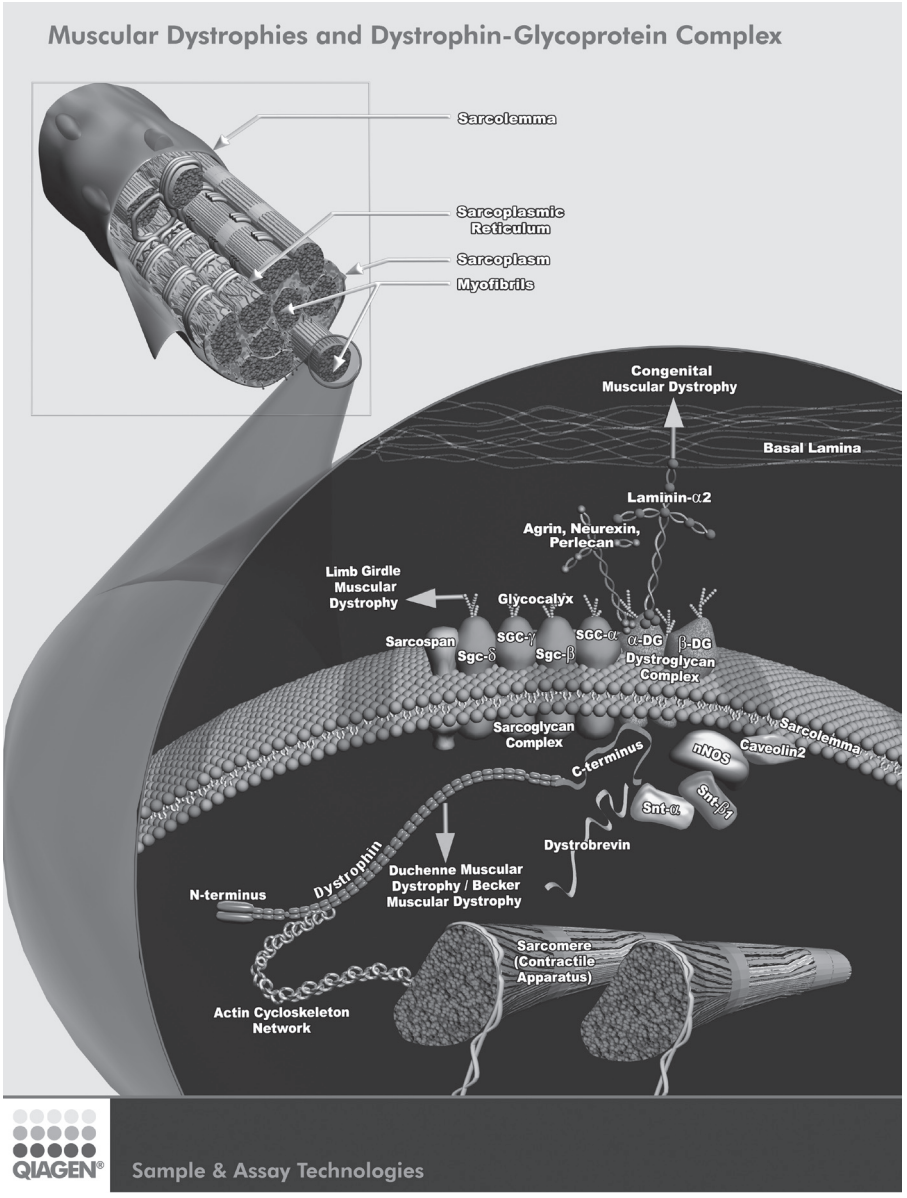

Figure 5
The dystrophin glycoprotein complex as it is located in the sarcolemma, the cell membrane of the muscle cell (with the kind permission © QIAGEN, all rights reserved).

Dystroglycan is composed of 2 subunits, α and ß, both produced from the same gene. Dystrophin binds to the tail of ß-dystroglycan. Dystroglycan binds to the extracellular matrix laminin-α2. The sarcoglycan complex is composed of multiple subunits. Mutations in the genes encoding α-, ß-, γ-, and δ-sarcoglycan lead to a phenotype similar to the one produced by mutations in the *DMD* gene and include cardiomyopathy and muscular dystrophy in humans and mice (Lapidos et al., 2004).

The dystrophin glycoprotein complex forms a mechanically strong link between the sarcolemma and actin (Rybakova et al., 2000). The muscle isoform of dystrophin serves as bolts throughout the sarcolemma stitching the sarcolemma with the intracellular actin filaments. The dystrophin bolts are more densely located at the costameres. A costamere is a protein complex located at the Z disc of the sarcomere and forms the transverse fixation system (TFS) of the intracellular desmin-vimentin intermediate filaments (DVIF) with the basal lamina (Figure 6). These dystrophin bolts protect the lipid bilayer from injury which might occur upon contraction of the muscle (Ozawa, 2010). The fact that no symptoms are present at birth in dystrophinopathy patients can be explained by the presence of utrophin, a protein with a function similar to that of dystrophin. The less densely distributed utrophin bolts appear first in the myotube stage and are later replaced by dystrophin bolts. Only when the patient starts to walk the utrophin bolts appear to be insufficiently strong to bear the muscle contractions and the lipid bilayer gets damaged (microtears) leading to a gradual atrophy and weakness of the muscle (Ozawa, 2010). Atrophy results from, on the one hand, leakage of soluble cytoplasmic enzymes and other proteins through the microtears and, on the other hand, from increased digestion of proteins through activated calpain due to leakage of Ca++ into the cytoplasm (Imahori, 1980). However, it is not known why only DMD muscles athrophy whereas also healthy muscles contain calpain and free Ca++ waxes and wanes during the contraction-relaxation cycle of the muscle (Ozawa, 2010).

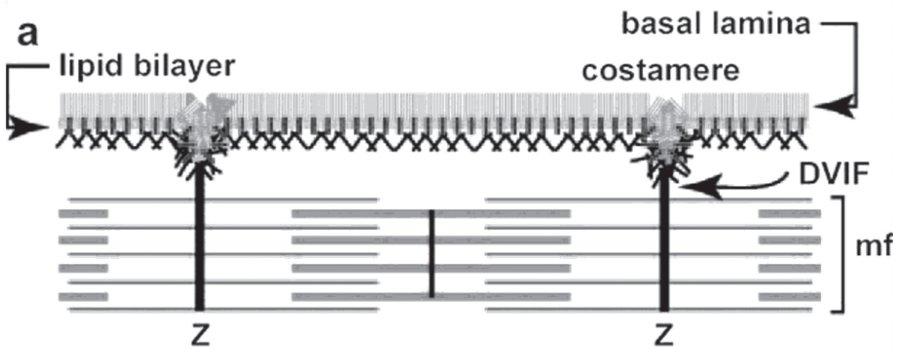

Figure 6

Transverse fixation system (TFS) (Ozawa, 2010). Adapted with kind permission of Professor E. Ozawa.

Desmin Vimentin Intermediate Filaments (DVIF) are wound around myofibrils (mf) at the level of the Z-band and connected with actin in the subsarcolemmal cytoskeleton. The small vertical bars below the lipid bilayer of the sarcolemma indicate the dystrophin bolts.

1.7 Therapy

Therapy for DMD and BMD is at present only symptomatic and should be administered by a multidisciplinary team (Bushby et al., 2010a; Bushby et al., 2010b). For optimal management, care is recommended in the following areas: pharmacology, psychosocial, rehabilitation, orthopedic, respiratory, cardiovascular, gastroenterology/nutrition, pain issues and general surgical and emergency-room precautions. The life expectancy has increased from 14.4 years in the 1960s to 25.3 in the 1990s just by treating the symptoms (Eagle et al., 2002). Some patients even reach the age of 40 or older (Rahbek et al., 2005).

Experimental therapy with the aim of restoring the absent dystrophin in the muscle has recently been focused on two treatments: antisense-mediated exon skipping and drug-induced read-through of premature stop codons (Aartsma-Rus et al., 2010). Both treatments fall under so-called personalized medicine because they depend on the specific mutation. The exon skipping treatments seem particularly promising (Goemans et al., 2011; Van Deutekom et al., 2007); a phase III trial with skipping of exon 51 has been recently started and includes 180 patients from 18 different countries (http://www.gsk.com/media/pressreleases/2011/2011_pressrelease_10016.htm). Exon skipping treatment is based on manipulating the splicing machinery with antisense oligonucleotides (AON) in a manner that one or more exons are skipped with the aim of restoring the reading frame, finally resulting in the production of BMD-like dystrophin and a milder phenotype (Figure 7).

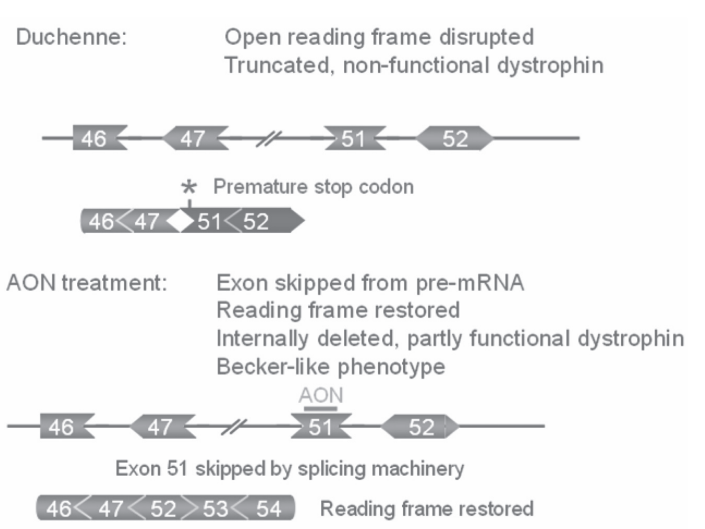

Figure 7

Exon skipping treatment (adapted with the kind permission from www.dmd.nl/gt).

An out-of-frame product is generated in which exon 47 is spliced to exon 51 in a patient with DMD with a deletion of exons 48-50. As a result, a stop codon is generated in exon 51, which prematurely aborts dystrophin synthesis. The sequence-specific binding of the antisense oligonucleotide PRO051 interferes with the correct inclusion of exon 51 during splicing so that the exon is actually skipped. This restores the open reading frame of the transcript and allows the synthesis of dystrophin similar to that in BMD patients (Van Deutekom et al., 2007).

The other treatment, the drug-induced read-through of premature stop codons is based on the finding that certain antibiotics (aminoglycosides) suppress stop codons during protein translation. In 1999 dystrophin was shown to appear at the cell membrane in mdx myotubes after in vitro exposure to gentamycin (Barton-Davis et al., 1999). A decade of further testing followed until recently when a phase 2B study during 48 weeks with Ataluren (formerly known as PTC124) was completed in 174 DMD patients. There was no measurable difference between the effect of a high dose of Ataluren and a placebo in the 6-min walk test; also the effect of a low dose of Ataluren as compared to the placebo was not significant. These results have led to a suspension of further trials http://www.duchenne.nl/976_resultsataluren.pdf.

1.8 Aims of this thesis

The focus of this thesis is on the clinical genetic aspects of dystrophinopathies.
We have investigated the following topics:

Mosaicism:
Germ line mosaicism was described by several authors in the late 80s (Bakker et al., 1987; Bech-Hansen et al., 1987; Darras and Francke, 1987). Since then the number of families in which this phenomenon has been encountered in Leiden, has increased. We were therefore able to calculate a more reliable figure for the recurrence risk (Chapter 2.1). We performed this study also because it was suggested that the published recurrence risks may be overestimates (Castagni et al., 2004).
A reliable recurrence risk is important for genetic counselling of women who have a son with dystrophinopathy as a result of a *de novo* mutation. As a part of this study we reviewed the literature on other known diseases with germ line mosaicism.
If a *de novo* mutation occurs in the *DMD* gene in one of the later divisions of the zygote, it can result in mosaicism in somatic tissues as well as in the germ line. Chapter 2.2 describes a male patient with somatic mosaicism.

Cascade screening in known families with dystrophinopathy
The study presented in Chapter 3 was prompted by the fact that women from several DMD families appeared to be unaware of their risk of being a carrier and had given birth to an affected boy. In this chapter we examine whether females at risk for being a carrier of a DMD mutation have been tested and counseled after the causative mutation was identified in an index case. Since DMD is a devastating disease for which there is no curative therapy so far, much emphasis has been put on prevention. Prevention is only possible if women are aware of this disease in their family. These women need to be informed about their risk of being a carrier, the recurrence risks, their reproductive options, the available tests and the health risks for carriers.

What has been the impact of prenatal testing for Duchenne and Becker Muscular Dystrophy in the Netherlands?
First trimester prenatal diagnosis for dystrophinopathy has been available in the Netherlands

since 1984 (Bakker et al., 1985). In Chapter 4 we show the impact of genetic counselling, the use of prenatal testing and pre-implantation genetic diagnosis on the occurrence of DMD and BMD in the Netherlands. The incidence of DMD in the birth cohort 1993-2002 was compared with the incidence in the birth cohort 1961-1974 (van Essen et al., 1992b). In order to test effectiveness of genetic studies in DMD families with regard to preventing the birth of affected boys we have also compared the proportion of first affected boys in the family between the two cohorts. A need for a change in policy has emerged.

Predicting the phenotype of DMD patients who have been treated with exon skipping therapy

Currently, new therapeutic strategies, such as antisense-mediated exon skipping, are in an early phase of clinical trials and have the potential of dramatically changing the course of the DMD disease (Goemans et al., 2011; Van Deutekom et al., 2007). Clinical trials with systemic administration of antisense oligonucleotides (AON) are taking place. If successful, therapeutic skipping using an AON that targets exon 51 can stop further muscle wasting, resulting in a less severe clinical phenotype resembling BMD. It is, therefore, useful to study the phenotype of BMD patients as it can provide information for DMD patients eligible for this new therapy. In Chapter 5 we have described the clinical phenotype in two Dutch BMD pedigrees with deletions that include exon 51 and we have reviewed the literature on this topic.

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