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Clinical genetic aspects of Duchenne and Becker muscular dystrophy in the Netherlands

Apollonia Theodora Josina Maria Helderman-van den Enden

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Clinical genetic aspects of Duchenne and Becker muscular dystrophy in the Netherlands

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For my father and grandfather

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Chapter 1

Introduction

Chapter 1 Introduction

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.

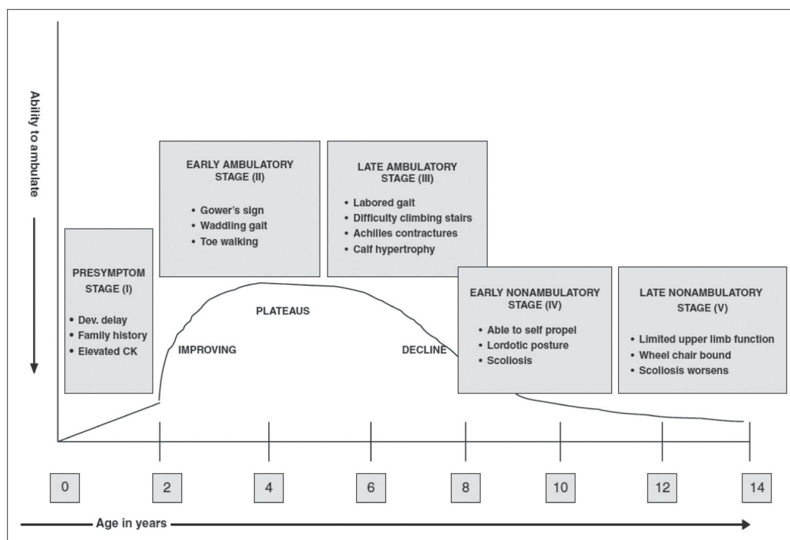

Figure 1
Musculoskeletal course in Duchenne muscular dystrophy (Verma et al., 2010) (with the kind permission of Professor Y Anziska).

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.

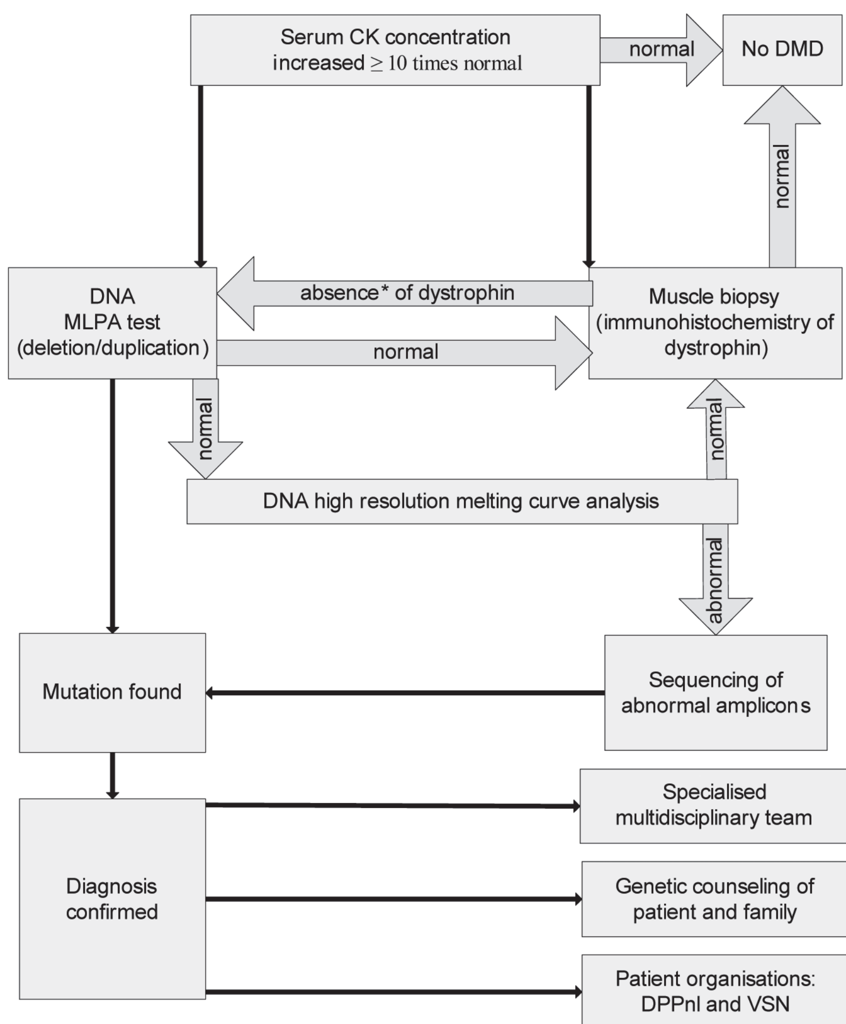

Figure 2
Confirmation of diagnosis in a patient suspected of having Duchenne muscular dystrophy.
DMD is suspected when serum CK is increased by at least 10 times the normal value. It is recommended to start with MLPA analysis of DNA from blood because this is easy to obtain and in about 60% of the patients 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).

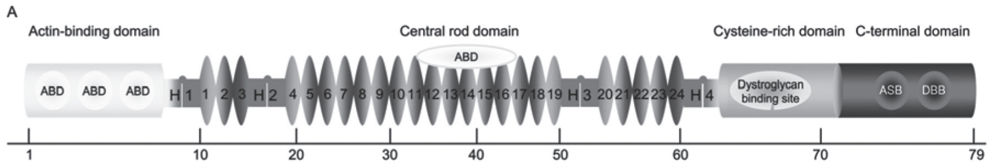

Figure 3
Schematic drawing of the dystrophin protein adapted from (Aartsma-Rus et al., 2006). The location of the different exons is shown underneath the protein (with the kind permission of Aartsma-Rus).

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.

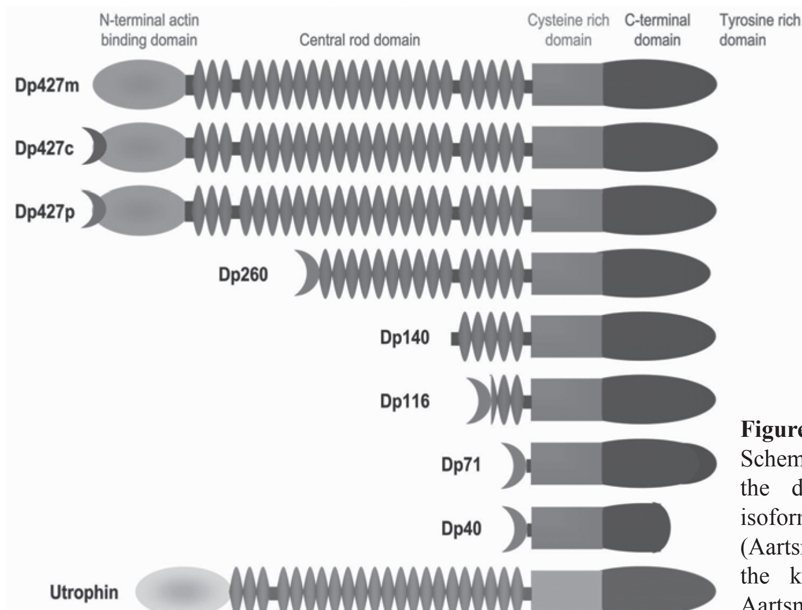

Figure 4
Schematic drawing of the different dystrophin isoforms and homologues (Aartsma-Rus, 2005) (with the kind permission of Aartsma-Rus).

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.

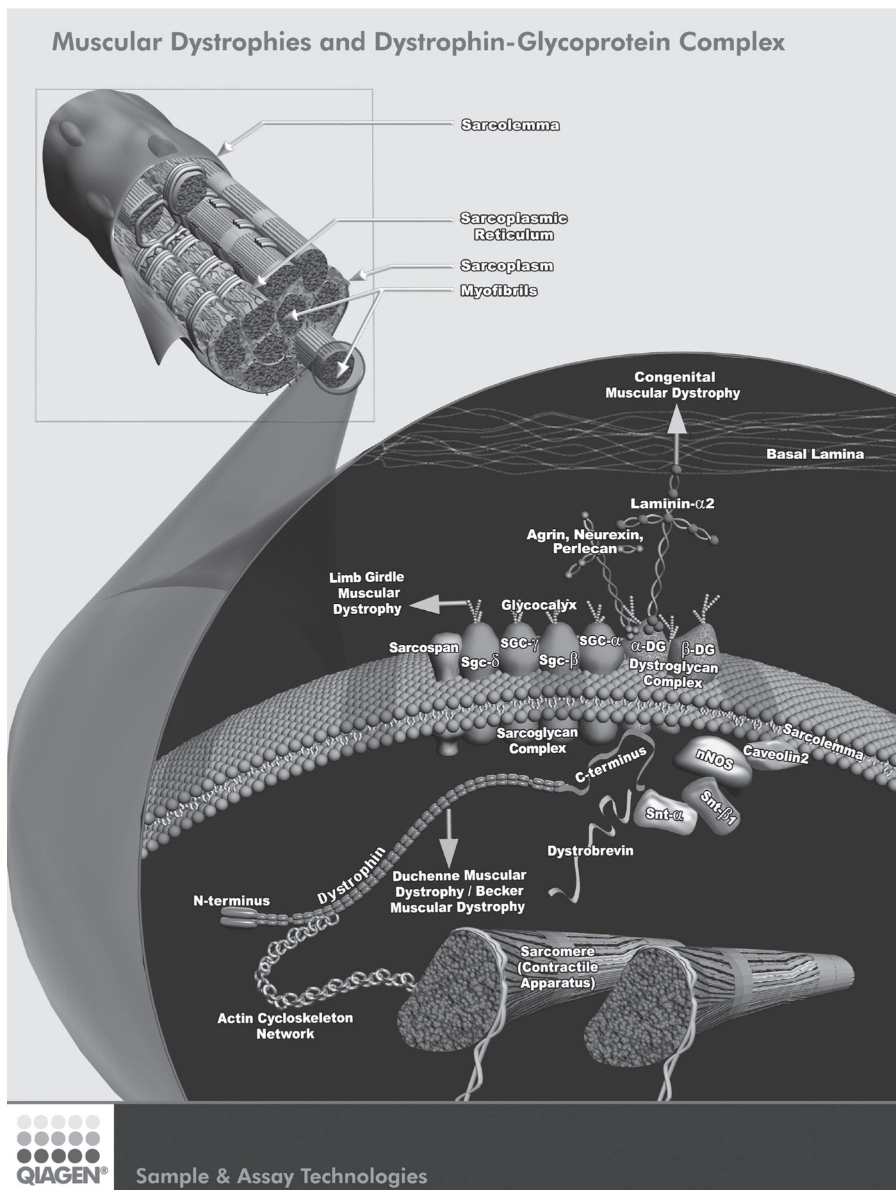

Figure 5
The dystrophin glycoprotein complex as it is located in the sarcolemma, the cell membrane of the muscle cell (with the kind permission © QIAGEN, all rights reserved).

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).

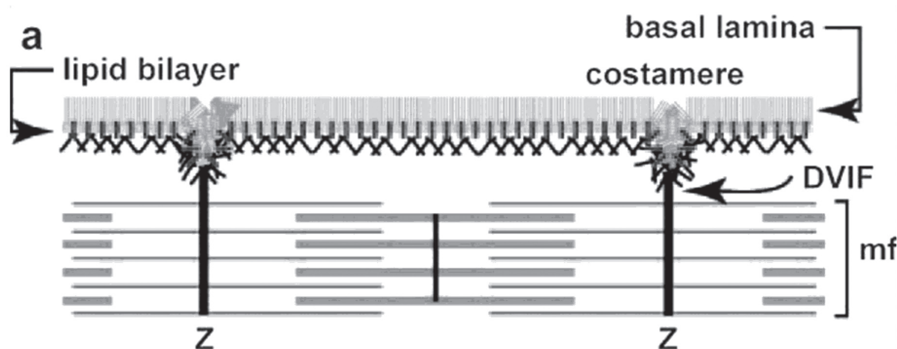

Figure 6
Transverse fixation system (TFS) (Ozawa, 2010). Adapted with kind permission of Professor E. Ozawa.
Desmin Vimentin Intermediate Filaments (DVIF) are wound around myofibrils (mf) at the level of the Z-band and connected with actin in the subsarcolemmal cytoskeleton. The small vertical bars below 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).

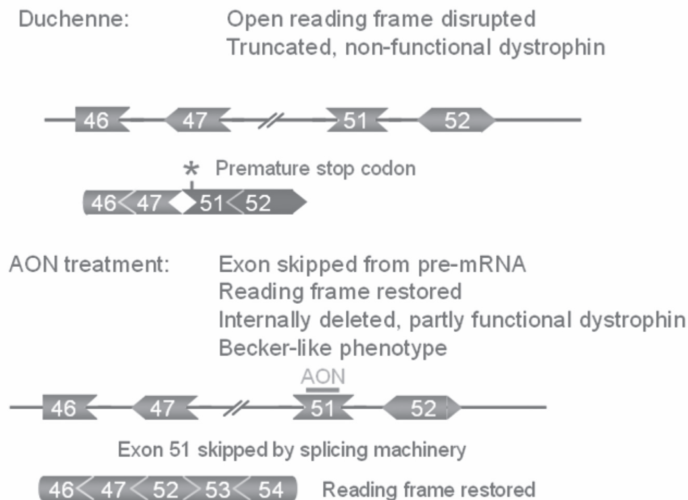

Figure 7

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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Mosaicism

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Short Report

Recurrence risk due to germ line mosaicism: Duchenne and Becker muscular dystrophy

Helderman-van den Enden ATJM, de Jong R, den Dunnen JT, Houwing-Duistermaat JJ, Kneppers ALJ, Ginjaar HB, Breuning MH, Bakker E. Recurrence risk due to germ line mosaicism: Duchenne and Becker muscular dystrophy.
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The presence of multiple affected offspring from apparently non-carrier parents is caused by germ line mosaicism. Although germ line mosaicism has been reported for many diseases, figures for recurrence risks are known for only a few of them. In X-linked Duchenne and Becker muscular dystrophies (DMD/BMD), the recurrence risk for non-carrier females due to germ line mosaicism has been estimated to be between 14% and 20% (95% confidence interval 3–30) if the risk haplotype is transmitted. In this study, we have analyzed 318 DMD/BMD cases in which the detected mutation was *de novo* with the aim of obtaining a better estimate of the 'true' number of germ line mosaics and a more precise recurrence risk. This knowledge is essential for genetic counseling. Our data indicate a recurrence risk of 8.6% (4.8–12.2) if the risk haplotype is transmitted, but there is a remarkable difference between proximal (15.6%) (4.1–27.0) and distal (6.4%) (2.1–10.6) deletions. Overall, most mutations originated in the female. Deletions occur more often on the X chromosome of the maternal grandmother, whereas point mutations occur on the X chromosome of the maternal grandfather. In unhaplotyped *de novo* DMD/BMD families, the risk of recurrence of the mutation is 4.3%.

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A genetic disease in a child with healthy non-carrier parents is usually a result of a *de novo* mutation that has taken place during cell division (mitosis/meiosis). The mutation rate for most genetic diseases is low, and hence, the risk of a second mutation in the same gene in a specific family is negligibly small. If the mutation occurs during mitosis, a large number of cells (germ and/or somatic) may carry the mutation, thus increasing the risk for a second affected child.

The presence of multiple affected offspring from apparently non-carrier parents is due to germ line mosaicism. So far, germ line mosaicism has been reported for more than 60 genetic diseases. Recurrence risk is known for only a few of these.

Duchenne muscular dystrophy (DMD) is a lethal X-linked recessive disorder and is caused by mutations in the dystrophin gene. Mutations in this gene can also lead to Becker muscular dystrophy (BMD).

An affected boy usually inherits the dystrophin gene mutation from his mother. The carrier mother has a 25% risk of having a second affected child in each pregnancy.

In 1935, Haldane (1) postulated that for X-linked lethal recessive disorders like DMD, one in three patients has the disease as a result of a *de novo* mutation.

If one in three DMD patients is affected as a result of a new mutation, then one in three mothers is not a carrier. One would expect that in these cases, the risk for a subsequent pregnancy would be zero. However, this is not the case.

Germ line mosaicism in DMD was described by several authors in the late 80s (2–4). The estimate of the recurrence risk for non-carrier females due to germ line mosaicism of transmitting the risk haplotype varies between 14% (95% confidence interval 3–25) and 20% (11–30) (5, 6). At the

meeting of the European Society of Human Genetics in 2004, Castagni et al. presented a poster on germ line mosaicism in a group of 273 Italian families. There were only two cases where the *de novo* mutation in the dystrophin gene was transmitted twice, suggesting that previous studies may have overestimated the recurrence risk (7).

In this study, we have analyzed our proven *de novo* DMD/BMD cases with the aim of obtaining a better estimate of the 'true' number of germ line mosaics and to assess the resulting recurrence risk more precisely, which is essential in genetic counseling. The recurrence risks are specified with respect to the type of the mutation and its location within the gene.

We have also traced the origin of the mutation (maternal, maternal grandmother or maternal grandfather) by haplotype analysis of *de novo* families and have investigated whether there is a relationship between the type of mutation and its origin.

Methods

Since the availability of DNA diagnostics in 1984, more than 1500 DMD/BMD patients/families have been tested in our laboratory. Mutations in most families are known and their DNA has been stored.

The records of the patients/families were selected on the basis of a mutation detected in the dystrophin gene that was proven to have arisen *de novo*. A family was considered to have a *de novo* mutation if the mother of the patient or if the parents of a carrier mother did not have the mutation in their lymphocytes.

The risk haplotype was determined in the selected *de novo* families. We then examined the frequency of the risk haplotype transmission with or without the mutation.

In a number of healthy siblings, either haplotyping was not informative (sisters without the mutation) or DNA was unavailable (healthy brothers). Yet these siblings provide valuable information about the recurrence risk. By using Bayes' theorem as described in the supplementary materials available as part of the online article at http://www.blackwell-synergy.com, we were able to compute the expected number of siblings assumed to have the risk haplotype without the mutation.

In a number of families where the mutation must have occurred in one of the grandparents, it was not possible to establish its origin. This was usually due to the non-availability of DNA of the grandfather. The probability of the origin, and hence the number of transmitted risk haplotypes, depends on F, the number of haplotyped siblings and the number of healthy siblings with no information about the haplotype. In the supplementary materials available as part of the online article at http://www.blackwell-synergy.com, this is further explained.

Results

Among 1500 DMD/BMD patients/families known in our laboratory, 318 families were identified with proven *de novo* mutations, 272 families with DMD and 46 families with BMD.

Part A: Recurrence risk due to germ line mosaicism

The mutation was transmitted more than once in 19 cases. Table 1 gives an overview of the families with germ line mosaicism and the detected mutations.

The risk haplotype without a mutation was transmitted 108 times (data not shown) to a healthy sibling. No information about the haplotype was available from 176 healthy siblings. The a priori risk that these 176 siblings received the risk haplotype is 50% (88). By using the algorithm described in the supplementary materials, 84 siblings without haplotype information could be added to the 108 siblings with the risk haplotype.

Table 1. Summary of the mutations in the 18 proven germ line mosaic families[a]

Family number	Origin of the mutation	Deletion of exon number(s)	Duplication of exon number(s)	Point mutation
DL2	Mother	5–7		
DL26	Mother	51		
DL41	Mother	48–50		
DL43	Grandmother	4–7		
DL51	Mother	45–54		
DL114	Mother	Probe 30.1		
DL154	Grandmother	8–28		
DL202	Grandmother		3–7	
DL215	Mother		2–7	
DL389	Grandmother	52–55		
BL129	Mother	45–48		
BL138	Mother		16–34	
50173	Mother	45–52		
50796	Mother	3–7		
51526	Mother	46–49		
53224	Mother			8791G>T
53435	Mother	12–19		
61447	Mother	43		

[a]Eighteen families are shown, whereas we counted 19 cases of germ line mosaicism. In family BL138, the *de novo* mutation was transmitted three times. The exon numbers of the deletion family DL114 could not be further specified because there was insufficient DNA and no new material was available.

From the families with unknown grandparental origin, we estimated that another 11 siblings are likely to carry the risk haplotype.

In total, the number of siblings with the risk haplotype is therefore 203 (108 + 84 + 11).

The recurrence risk if the risk haplotype is transmitted is:

$$19/(203+19) = 0.086(= 8.6\%)$$
$$(95\% \text{ confidence interval}: 4.8-12.2).$$

Table 2 gives an overview of the origin and the type of mutation. The most frequent type of mutation, a deletion, was present in 246/318 families. The deletions are subdivided as proximal, middle and distal to be able to calculate the specific recurrence risks for these types of deletions.

Figure 1 shows two hot spots of deletions; most deletions are found in the distal hot spot. The graph in Fig. 2 shows the distribution of deleted exons in families with proven germ line mosaicism due to a deletion. Both hot spots can be seen. However, the distal hot spot is significantly lower compared with the distal hot spot in the entire group of *de novo* deletions (Fig. 1). Table 1 shows the families with proven germ line mosaicism: six deletions proximal and eight distal. Table 2 shows the whole group of *de novo* deletion families: 53 proximal *vs* 182 distal.

The recurrence risks for the different types of mutations were calculated in the same manner as described above for the entire group. Table 3 shows the results. No recurrence risk is calculated for a middle deletion because the total number of middle deletions was too small (11 families).

Part B: Origin of the new mutation

Table 2 shows that most families (77%) had a deletion; duplications and point mutations were found in 11% and 12%. In 232 families, the mother was not a carrier; hence, the mutation has arisen in the germ line of the mother. In 40 families, the mutation originated in the grandmother and in 27 families in the grandfather. In 19 families, the origin of the mutation could not be determined (see Methods).

Discussion

Recurrence risk due to germ line mosaicism

Hall in 1988 and Edwards in 1989 speculated that every woman has several oocytes with mutations for common genetic disorders because the 6–8 million oocytes exceed the denominator of the mutation rates for these diseases (8, 9). In the male germ line, an even greater range of mutations is expected to be present.

The result of a literature search on germ line mosaicism is added as a supplement and can be viewed online at http://www.blackwell-synergy.com in the supplementary materials.

An estimate for the recurrence risk was found for only 7 of 63 diseases: 2 with an autosomal dominant and 5 with X-linked inheritance. The recurrence risks vary as follows: 0.02% [achondroplasia (10)], 5–7% [autosomal dominant osteogenesis imperfecta (11)], 11% [RETT syndrome (12), double cortex X-linked lissencephaly syndrome (13) and hemophilia B (14)] and 13% [hemophilia A (15)].

In DMD, the reported recurrence risks vary from negligible to 14–20% if the risk haplotype is transmitted (5–7). For genetic counseling, it is important to have a reliable estimate of the recurrence risk attributed to germ line mosaicism. Our study describes the largest number of families with a *de novo* mutation known to date, and we found a recurrence risk, if the risk haplotype is transmitted, of 8.6% (95% confidence interval 4.8–12.2). In the current counseling practice, information about the risk haplotype is usually not obtained because only the presence/absence of the mutation is tested in the at-risk sibling. One should use a recurrence risk of 4.3% in these families with no information about the risk haplotype.

Table 2. Overview of the origin and type of mutation[a]

Type of mutation	Maternal	Maternal grandmother	Maternal grandfather	Unknown	Total
All deletions	183	35	14	14	246 (77%)
Proximal deletion	*35*	*10*	*6*	*2*	*53*
Middle deletion	*8*	*1*	*1*	*1*	*11*
Distal deletion	*140*	*24*	*7*	*11*	*182*
Duplication	25	3	5	2	35 (11%)
Point mutation	24	2	8	3	37 (12%)
Total	232 (73%)	40 (13%)	27 (8%)	19 (6%)	318 (100%)

[a]A deletion is defined as proximal if most deleted exons are found in the proximal hot spot (exons 3–20) and as distal if most deleted exons are distal to exon 40. The other deletions are located in the middle of the gene between exons 20 and 40.

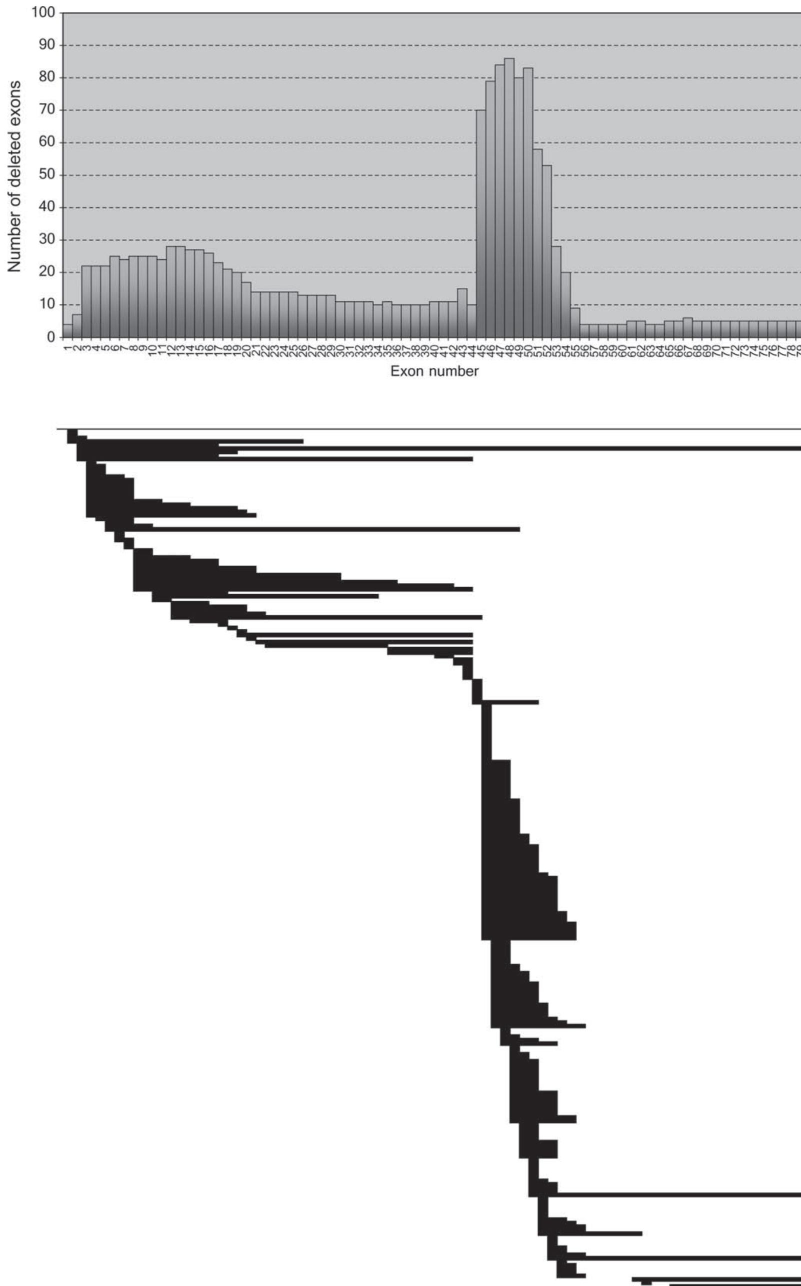

Fig. 1. Overview of the found deletions. Graphic representation of the location of 245 deletions (the border of one deletion could not be defined: DL114 del probe 30.1 because of insufficient DNA). The number of times that each individual exon is deleted is shown on the *y*-axis, and the *x*-axis shows the different exons. The lines in the lower part represent individual deletions. Summing of the individual deletions has resulted in the graph on the top.

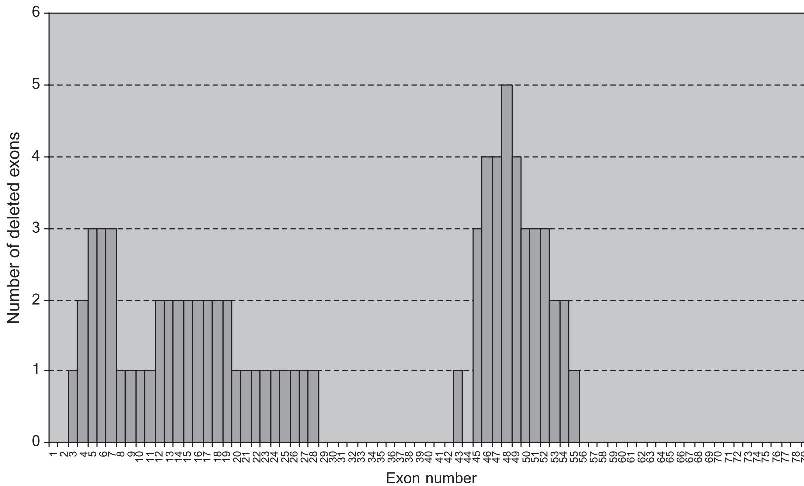

Fig. 2. Distribution of the deleted exons in 13 families with a *de novo* deletion and proven germ line mosaicism. Not included in this figure is family DL114 with a deletion of probe 30.1, this is also a proximally located deletion. The boundaries of the deletion could not be defined, however, because of insufficient DNA.

This figure is remarkably close to the 4.5% recurrence risk calculated by Van der Meulen et al. in 1995 (16). The primordial germ cell divides mitotically, so that in the *i*th generation of the germ cells, there are 2^i cells. If this process continues for a total of *n* cells (*n* may be different in females and males), then at maturity, there are 2^n germ cells. Hartl (17) showed that more complex versions of this simple model do not change recurrence risks as long as the number of gonadal generations is high enough. The recurrence risk due to germ line mosaicism can be calculated with the following formula:

$$\text{Recurrence risk} = \sum_{i=1}^{n} 1/n.(2)^{-i} \approx 1/n.$$

If the number of generations needed to form the 5–7 million oocytes is at least 22 in females (18), the recurrence risk according to this formula is 1/22 $\approx 0.045 = 4.5\%$.

In the majority of the *de novo* families, the mutation originated in the germ line of the mother (in our study 232/318) or the maternal grandmother (40/318), which might explain the fact that the theoretical calculated recurrence risk is close to our empirical risk.

The mutations with proven germ line mutations are deletions in 14/18 (77%), duplications in 3/18 (17%) and a point mutation in 1/18 (6%) families. These percentages are divided as the expected ratio of mutations in the dystrophin gene, apart from the number of point mutations, which is smaller than expected. This can be explained by the fact that these type of mutations are more difficult to locate.

Table 3. Recurrence risk due to germ line mosaicism

Type of mutation	Recurrence risk (%) if the risk haplotype is transmitted (95% confidence interval)	Recurrence risk with unknown haplotype (%)
All types together	8.6 (4.8–12.2)	4.3
All deletions	8.4 (4.2–12.6)	4.2
Proximal deletion	15.6 (4.1–27.0)	7.8
Distal deletion	6.4 (2.1–10.6)	3.2
Duplication	12.1 (1.0–23.2)	6.1
Point mutation	4.4 (0–12.7)	2.2

Different mosaicism frequencies for proximal and distal deletions

If the distribution of mutations in familial and sporadic cases was identical, no difference between these groups would be expected. Passos-Bueno et al. observed that in familial cases of DMD/BMD caused by a deletion, 47% of these were found in the proximal hot spot and 53% in the distal hot spot, whereas in sporadic cases, 28% of the deletions were found to be proximal and 72% distal (19). Furthermore, they found that germ line mosaicism for DMD was present more

often in the proximal hot spot than in the distal one. These authors calculated different mosaicism frequencies for proximal and distal DMD mutations. A distinct recurrence risk of 30% was found for proximal *de novo* mutations and 4% for distal mutations. It was speculated that proximal deletions arise earlier in embryogenesis than distal ones. This explains the higher recurrence risk because more cells would carry the mutation.

The present study confirms a difference between the recurrence risk for germ line mosaicism for proximal and distal deletions. The difference, however, is much smaller: a proximal deletion has a risk of recurrence of 15.6% whereas a distal deletion has 6.4%. Of the three families with germ line mosaicism caused by duplications, two involved also the proximal part of the gene, but it is known that duplications are found more often proximal than distal (20).

In our study, the recurrence risk due to germ line mosaicism was 12.1% for duplications and 4.4% for point mutations. These recurrence risks have relatively large 95% confidence intervals due to the small number of families in which duplications (35/318) and point mutations (37/318) were found.

We have added a flowchart (Fig. 3) for use in estimating the recurrence risk in a family with a sporadic DMD patient. To our knowledge, these recurrence risks are the most accurate at present, and this flowchart should facilitate genetic counseling. However, all 95% confidence intervals overlap, and therefore, the recurrence risks for the specific types of mutations should be used with caution.

Origin of the mutation

Since the description of the male to female ratio of mutations by Haldane in 1947 (21), many articles have been written on this subject. Usually, the ratio in DMD is assumed to be 1, which makes the risk of the mother being a carrier 2/3.

This variation of the male to female ratio of mutations depending on the type of the mutation has been described also for other diseases, for instance, in X-linked hemophilia B (22).

In 86 of the 318 DMD/BMD families (27%) in our study, the mutation arose in the grandparental germ line. This low percentage is not surprising because an unknown number of women carrying a *de novo* mutation in the dystrophin gene are

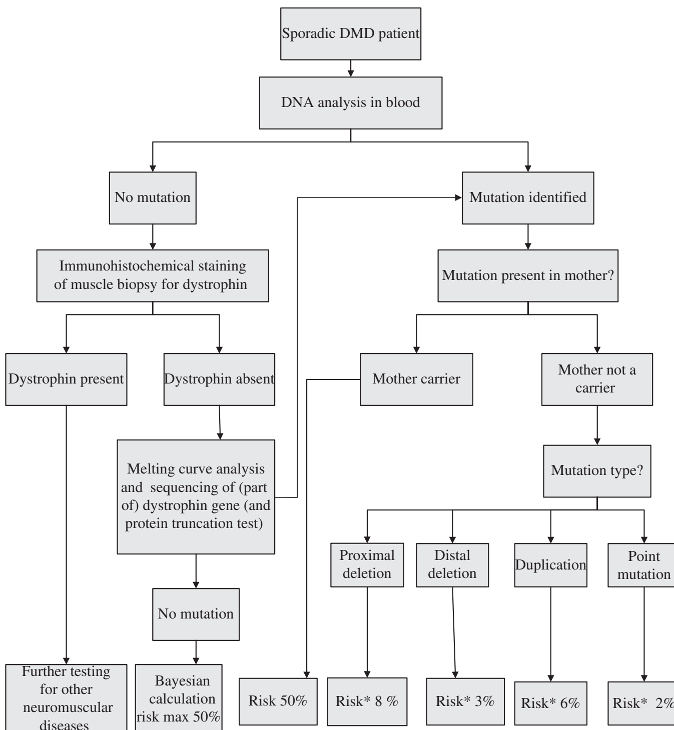

Fig. 3. Flowchart for use in counseling for the recurrence risk in a family with a sporadic Duchenne muscular dystrophy (DMD) patient. Risk* is the risk of an affected son if there is no information about the haplotype. Haplotype analysis is usually not performed because now it is possible to test for the mutation directly. The recurrence risks for the specific types of mutations should be used with caution because, as can be seen in Table 3, all 95% confidence intervals overlap.

missed if they do not pass the mutation on to a son.

Our study confirms that deletions in the dystrophin gene occur more often on the X chromosome of the maternal grandmother (35 times) than of the maternal grandfather (14 times). In a study of 81 *de novo* DMD/BMD families in 1992 (6), it was found that the mutation came from the grandmother in 49 families and from the grandfather in 32 families. The authors concluded that the mutation rate in males and females in their study did not significantly deviate from an equal mutation rate in both sexes. This study involved 97.4% deletions and only 2.6% duplications. Our study confirms this result.

Point mutations originated more often in the maternal grandfather (eight times) compared with the maternal grandmother (two times). It is in the literature a well-known phenomenon that point mutations arise more often in the male germ line. This is explained by the way germ cells in the male are formed. There are about 30 cell divisions before puberty and 1 about every 23 days thereafter. For a 30-year-old male, the number of cell divisions is 380 (23). In the female germ line, there are about 22 cell divisions (18).

At present, the mutation can be identified, and reliable testing of family members is feasible in most DMD cases. Our data indicate that in a family of a sporadic DMD patient with *unknown* mutation, the risk for a second affected boy can be as high as 8.6% if the risk haplotype is present in a subsequent male fetus. If MyoD can modify chorion villus cells in the same way as it does fibroblasts (24), we may be able to test the ability of the fetus to make dystrophin *in vitro* and thus be informed whether the fetus is affected or healthy. The couple faces a difficult decision whether to continue the pregnancy or not in case the MyoD technique fails or is not available. If they decide to terminate the pregnancy, it is important to collect muscle tissue from the fetus. Immunohistochemical staining of dystrophin should be performed on this tissue (25). If dystrophin is absent, the risk that the mother is a carrier is high (although germ line mosaicism cannot be excluded as long as the mutation is unknown) and the couple will know that their fetus was affected. If, however, dystrophin is present, the fetus was not affected with DMD. In any case, prenatal testing should be offered in a future pregnancy as there is still a recurrence risk because of possible germ line mosaicism.

Germ line mosaicism remains an important pitfall that should be considered during the counseling of families with a *de novo* mutation. The 8.6% risk of recurrence of the mutation in the risk haplotype in our large series of families indicates the need for assessing the potential DMD carrier risk for all female members of families with apparent *de novo* cases.

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Supplementary materials of the article of Helderman et al: Recurrence risk due to germline mosaicism: Duchenne muscular dystrophy. This is available as part of the online article at http://www.blackwell-synergy.com

In a number of healthy siblings either haplotyping was not informative (sisters without the mutation) or DNA was unavailable (healthy brothers). Yet these siblings provide valuable information about the recurrence risk. Bayes theorem is used as described below in the box to compute the expected number of siblings assumed to have the risk-haplotype without the mutation.

> The probability that a healthy sibling has the risk-haplotype without the mutation is
>
> $$\frac{\frac{1}{2}(1-F)}{\frac{1}{2}(1-F)+\frac{1}{2}}=\frac{1-F}{2-F}$$
>
> *Where F denotes the chance that a germ line mutation is present in the risk-haplotype and (1-F) is the chance that the risk-haplotype does not have the mutation*
>
> If F is small this probability equals ½.
>
> *The numerator gives the risk that the healthy sibling has the risk-haplotype without the mutation.*
>
> *The denominator gives the possibilities of a healthy sibling: the numerator and the possibility of receiving the non risk-haplotype (=1/2)*

Now the unknown parameter F can be estimated by using an EM algorithm which computes, based on a current value of F, the expected total number of haplotypes transmitted (E step) and then using these total number, the value of F is updated (M step). The expected total number of haplotypes is given by

$$19+108+176\frac{1-f}{2-f}.$$

Here the last term represents the healthy siblings for whom either haplotype was not informative or DNA was not available.
Then a new value of F is obtained by

$$f_{new}=\frac{19}{19+108+176\frac{1-f}{2-f}}.$$

These steps are repeated until convergence has been obtained. By using the algorithm described above, we calculated that 84 of the 176 siblings without information about the haplotype, probably carried the risk haplotype (so a little less than the apriori risk of 50%).

These formulas hold for families with known origin of mutation. For 19 (out of 86) families the origin was unknown. Also these families contain information about F. To include these families in the procedure, we formulated the posterior probability of origin as function of F and the observed family data.
Let NDC be the number of daughters carrying the mutation, NDH be the number of daughters carrying the healthy haplotype, NDU be the number of daughters without mutation and NSU be the number of sons without the mutation.

Then the probability of the family give the origin of mutation are given by
Prob(NDC, NDH, NDU | paternal origin)= $F^{NDC}\,(1\text{-}F)^{(NDH+NDU)}$

Prob(NDC, NDH, NDU, NSU | maternal origin)= $(½F)^{NDC}$ $(½-½F)^{NDH}$ $(1-½F)^{NDU+NSU}$

Using Bayes theorem and the prior probabilities of maternal origin of ⅔ and of paternal origin of ⅓, the posterior probabilities of origin can be calculated for each family.

Now given the origin, the number of transmitted haplotypes can be counted as before and the total number of transmitted haplotypes in these families is the weighted sum of the number under maternal origin and under paternal origin weighted with the corresponding posterior probabilities of origin. This total number of expected transmitted haplotypes in the family can be added to the expected total number of transmitted haplotypes given above.

In this way we estimated that in the families with unknown grandparental origin another 11 siblings are likely to carry the risk-haplotype.

The following table was published online as a supplementary file of the article of Helderman et al titled: Recurrence risk due to germ line mosaicism: Duchenne and Becker muscular dystrophy.

Overview of the literature on germ line mosaicism

Only those references were included where multiple affected offspring with a detected mutation had apparently non-carrier parents (no mutation in lymphocytes). The diseases are presented in alphabetical order, followed by the name of the gene, the type of inheritance and the reference(s). AD = autosomal dominant, AR = autosomal recessive, X-L = X-linked

Disease	Gene	AD	AR	X-L	reference
Achondrogenesis type II	COL2A1	1			(1)
Achondroplasia	FGFR3	1			(2, 3, 4)
Albright hereditary osteodystrophy	GNAS1	1			(5)
Amyloid polyneuropathy	TTR	1			(6)
Androgen insensitivity syndrome	AR			1	(7)
Angelman syndrome	UBE3A	1			(8)
Apert syndrome	FGFR2	1			(9)
Campomelic dysplasia	SOX9	1			(10)
Charcot-Marie-Tooth disease type 1 (CMT1)	MPZ/P0	1			(11, 12)
CHARGE	CHD7	1			(13)
Coffin-Lowry syndrome	RSK2			1	(14)
Craniofrontonasal syndrome (CFNS)	EFNB1			1	(15)
CRASH	L1CAM			1	(16)
Crouzon	FGFR2	1			(17)
Danon disease	LAMP-2			1	(18)
Diabetes permanent neonatal	KCNJ11	1			(19, 20)
Duchenne muscular dystrophy	dystrophin			1	(21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34)
EEC	P63	1			(35)
Epidermolysis bullosa lethal junctional (Herlitz)	LAMB3		1		(36)
Epidermolysis bullosa mild dystrophic form	COL7A1	1			(37)
Epidermolysis bullosa simplex	Keratin 5	1			(38)
Epilepsy, severe myoclonic form of infancy	SCN1A	1			(39, 40)
Fabry	alpha-gal A			1	(41)
Facioscapulohumeral myopathy	FSHD 4q35	1			(42, 43, 44, 45)
Factor X deficiency homozygous	Factor X		1		(46)
Familial Adenomatous Polyposis	APC	1			(47)
Familial hypertrophic cardiomyopathy	MYH7	1			(48)
Familial hypophosphatemic rickets (XLH)	PHEX			1	(49)
Fragile X	FraX (deletion)			1	(50)
Frontotemporal dementia with parkinsonism-17	MAPT	1			(51)
Hemoglobinopathy	beta-globin	1			(52)
Hemophilia A	Factor VIII			1	(53, 54, 55)
Hemophilia B	Factor IX			1	(56, 57, 58, 59)
Hunter	IDS			1	(60)

Disease	Gene	AD	AR	X-L	reference
Hutchinson-Gilford progeria	LMNA	1			(61)
Hyperparathyroidism–jaw tumour syndrome	HRPT2	1			(62)
Hypoparathyroidism sporadic isolated form	CASR	1			(63)
Kallmann syndrome	FGFR1	1			(64)
Lesch-Nyhan syndrome	HPRT			1	(65)
Li-Fraumeni	P53	1			(66)
Lowe syndrome	OCRL1			1	(67)
Marfan	FBN1	1			(68)
MODY 5	HNF-1beta	1			(69)
Neurofibromatosis 1	NF1	1			(70)
Ornithine transcarbamylase deficiency	OTC			1	(71)
Osteogenesis imperfecta	COL1A1/COL1A2	1			(72, 73, 73, 74, 75, 76)
Otopalatodigital syndrome (OPD) spectrum	FLNA			1	(77, 78)
Progressive external ophthalmoplegia	ANT1	1			(79)
Progressive external ophthalmoplegia	C10Orf2(Twinkle)	1			(80)
Pseudoachondroplasia	COMP	1			(81, 82)
Renal coloboma syndrome	PAX2	1			(83, 84, 85)
Resistance to thyroid hormone (RTH)	TRbeta	1			(86)
RETT	MECP2			1	(87, 88)
Subcortical band heterotopia	DCX			1	(89, 90)
Thanatophoric dysplasia type I (TDI)	FGFR3	1			(91)
Tuberous sclerosis complex	TSC1/2	1			(92, 93)
X-linked alpha thalassaemia mental retardation syndrome	ATRX			1	(94)
X-linked form of chronic granulomatous disease (CGD)	CYBB			1	(95)
X-linked dyskeratosis congenita	DKC1			1	(96)
X-linked mental retardation (XLMR)	ARX			1	(97)
X-linked mental retardation with microphthalmia and microcephaly	PQBP1			1	(98)
X-linked myotubular myopathy	MTMI			1	(99, 100)
X-linked severe combined immunodeficiency	IL2RG			1	(101, 102)

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Case Report

Somatic mosaicism of a point mutation in the dystrophin gene in a patient presenting with an asymmetrical muscle weakness and contractures

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Abstract

We describe a patient with somatic mosaicism of a point mutation in the dystrophin gene causing benign muscular dystrophy with an unusual asymmetrical distribution of muscle weakness and contractures. To our knowledge this is the first patient with asymmetrical weakness and contractures in an ambulatory patient with a dystrophinopathy.
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Keywords: Somatic mosaicism; Point mutation; Dystrophin gene; Asymmetrical muscle weakness; Contracture

1. Introduction

Duchenne muscular dystrophy (DMD) is a progressive muscular dystrophy leading to death in early adulthood. In the Netherlands the prevalence is about one in 4000 boys [1]. Signs and symptoms due to symmetrical weakness of the hip muscles and lower proximal limb muscles occur in early childhood. Before the age of 13 DMD patients are wheelchair-bound. They usually die due to cardiac arrest or respiratory failure [2] although of late a much more protracted course is observed since artificial ventilation is increasingly employed. About one third of the patients presents non-progressive intellectual impairment. The diagnosis is usually suspected in boys with symmetrical weakness and the serum creatine kinase (CK) activity of more than ten times the upper limit of normal. DMD is caused by mutations in the dystrophin gene on the X chromosome band p21. Most patients have a deletion of part of the gene (60%), about 5% have a duplication, the remaining 35% have a frameshift, a nonsense or a splice site mutation [3].

Becker muscular dystrophy (BMD) is also caused by mutations in the dystrophin gene. Usually these mutations retain the reading frame and generate a shortened and reduced amount of the protein. Mutations that disrupt the reading frame cause a premature termination and a loss of dystrophin and lead to DMD [2]. The phenotype of BMD is similar to that of DMD but in terms of skeletal muscle and cardiac involvement, the course is much milder.

One third of the patients have the disease as the result of a new mutation. Mothers of patients with apparent de novo mutations, were shown to transmit the mutation a second time, while these mothers were not carrying the mutation in lymphocytes. This phenomenon is known as germinal mosaicism. Empirical data revealed a recurrence risk for male pregnancies of around 14–20%, associated with transmission of the X chromosome of their affected son [4,5]. Somatic mosaicism has been described in a number of mothers of Duchenne patients and in one Duchenne patient and possibly in a maternal grandfather, who has three DMD grandsons from his three daughters [3,4,6–11].

Here we describe a patient with an unusual phenotype (asymmetrical muscle weakness and contractures) caused by somatic mosaicism of a point mutation in the dystrophin gene.

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2. Patient and methods

2.1. Case report

The patient was born after a normal pregnancy, birth weight was 2700 g. He walked at the age of 18 months. At 6 years of age he was diagnosed with psychomotor retardation of unknown cause. He attended a school for mildly intellectually impaired children and he never learned to read or write. Currently he lives 'semi independently' in a small group with supervision.

At the age of 8 years an abnormal gait was observed. Gradually, increasing atrophy and weakness of the muscles of the right leg, scoliosis and contractures became apparent prompting referral to a neurologist who ascribed these symptoms to a presumed perinatal trauma despite a normal electro-encephalogram (EEG) and brain computed tomography (CT).

Neurological examination at the age of 30 showed atrophy of the muscles of the upper arms, pectoralis major right more than left and the thighs, right more than left (Fig. 1). The left calf was hypertrophic (Fig. 2). There was moderate weakness of the shoulder girdle muscles and right peroneal muscles and severe weakness of the upper arm, pelvic girdle, thigh and calf muscles. Remarkably, Gowers' phenomenon was negative. He was not able to walk on tip-toe nor on his right heel. Contractures at the right shoulder, elbows (left more than right, Fig. 1) and of the finger flexors were noticed. Reflexes were negative except the left knee and left Achilles tendon. A depigmented nevus was present at the back on the left side of the chest.

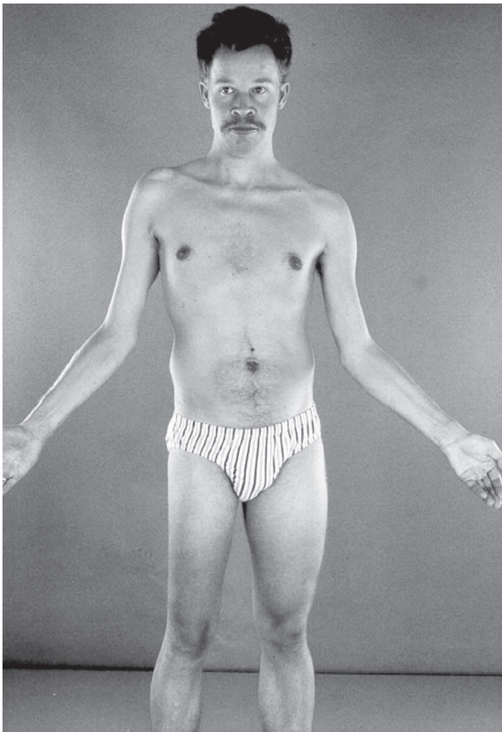

Fig. 1. Note the atrophy of the upper arms (left more than right), of the right leg and of the elbow contractures.

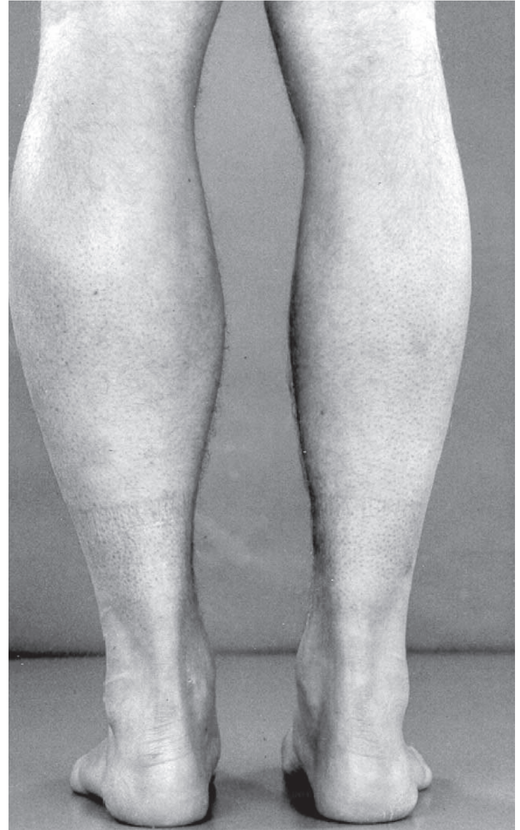

Fig. 2. Note the hypertrophy of the left calf.

Creatine kinase (CK) activity was 3000 IU/l (normal < 190 IU/l). Electromyography (EMG) showed myopathic changes. A muscle biopsy taken from the vastus lateralis muscle of the left leg showed necrotic fibers, occasional regenerating fibers and a marked variation in the size of muscle fibers. There were numerous pycnotic nuclear clumps, and atrophic fibers showed an increase in non-specific esterase activity. There was endomysial fibrosis and liposis. The histological pattern was consistent with muscular dystrophy.

The cardiologist was consulted once the diagnosis was made. Ultrasound investigation of the heart was normal. The electrocardiogram (ECG) showed an electric semivertical

heart axis, QRS time of 0.08 s with a high RS ratio right precordial and in the caudal and lateral leads rather deep Q's. This finding is compatible with abnormalities observed in patients with Becker muscular dystrophy.

Karyotyping showed a normal male pattern, 46, XY.

2.2. DNA analysis

DNA was extracted from the patient's whole blood, according to the method of Miller [12]. Multiplex polymerase chain reaction (PCR) was performed according to Chamberlain and Beggs [2] and Southern blotting was after Bakker and den Dunnen [13]. Approximately 200 ng of the PCR product generating the truncated translation product was used for sequence analysis. Sequencing was performed using the Big Dye Terminator Sequencing kit (Applied Biosystems, Nieuwekerk a/d IJssel, the Netherlands) on ABI PRISM 310. Since a PstI restriction site present in exon 64 is abolished by the mutation, the percentage of lymphocytes containing the mutation could be determined by a quantitative PCR analysis for exon 64 on genomic DNA. After quantitative PCR using a fluorescently labelled primer, the PCR product was digested with PstI followed by electrophoresis of the PCR products on an ALF DNA sequencer analyser (Amersham Pharmacia Biotech). The relative peak areas were measured.

2.3. PTT assay

RNA was isolated from muscle tissue specimens and whole blood using RNAzol (Campro Scientific, Veenendaal, the Netherlands) according to the manufacturer's protocol. Reverse transcriptase-polymerase chain reaction (RT-PCR) and the protein truncation test (PTT) was performed as described by Roest [14].

2.4. Immunohistochemistry

Serial unfixed cryostat sections of the muscle specimen of the patient were studied for dystrophin and spectrin expression. The following antibodies were used against dystrophin: NCL-DYS1, NCL-DYS3 (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK), Mandys108 [15] and spectrin (NCL-SPEC1 Novocastra). The immunohistochemical stainings were done as described previously [16].

3. Results

Immunohistochemical analysis of dystrophin revealed a mosaic pattern of positive and negative staining muscle fibers using several dystrophin antibodies (Fig. 3). Western blotting showed a reduced amount of dystrophin (not shown).

In genomic DNA extracted from lymphocytes no abnormality was found by multiplex PCR and quantitative Southern blot analysis. mRNA from the muscle biopsy was studied using the PTT analysis yielding two bands of one fragment corresponding to exons 59–68 as can be seen in Fig. 4, indicating mosaicism. Sequencing of the RT-PCR product showed a possible point mutation, in exon 64 leading to a premature stopcodon of the dystrophin gene, although the band was weak. Thereupon genomic DNA was sequenced and the mutation was confirmed at position 9554C → T; Q3116X.

The percentage of the mutated allele in DNA from lymphocytes was calculated to be 75% according to the method described in Section 2.2 of patient and methods.

DNA analysis in lymphocytes of the patient's mother did not show the point mutation.

4. Discussion

The patient reported by us is remarkable because of the peculiar phenotype and the mosaicism. As stated before, patients with BMD have progressive symmetrical muscular weakness and contractures only appear in due course, namely when patients become wheelchair-bound. Our case had asymmetric weakness and prominent contractures while he was still ambulatory. In addition, muscle biopsy showed a combination of dystrophy and neurogenic changes. Therefore the differential diagnosis was the following: facioscapulohumeral dystrophy, spinal muscular atrophy and dystrophinopathy. DNA analysis for these muscle disorders did not show any abnormality. However, only quantitative PCR and Southern blotting of the dystrophin gene were done, which only reveals large deletions or duplications. Subsequent immunohistochemical dystrophin analysis showing a mosaic of dystrophin negative and positive fibers, led way to the diagnosis.

It is known that the skeletal muscles of DMD patients may have some dystrophin positive fibers [17]. The most likely explanation of these so-called revertant fibers, is a second site in-frame deletion. In our patient the wild type allele as well as the point mutation were present both in lymphocytes and in skeletal muscle, indicating that we are dealing with a true somatic mosaicism.

About one third of DMD and BMD patients are intellectually impaired, as is our patient. The cause of the impairment is not known. A correlation was found with the loss of Dp140 regulatory sequences. Dp140 is a dystrophin isoform with predominant expression during foetal brain development. The promoter and first exon lie in the intron between exon 44 and 45 [18]. In our patient the mutation is located in exon 64. The point mutation in his dystrophin gene causes premature translation termination leading to absence of dystrophin in the affected cells, which is typical for DMD. The most likely explanation of the mental impairment in our patient is that the proportion of brain cells with the mutation is rather high. Family history and physical

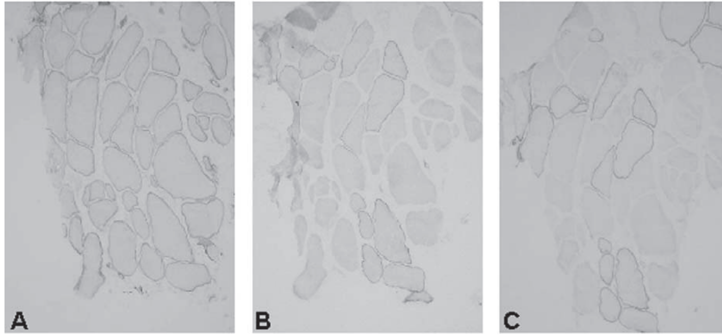

Fig. 3. Immunohistochemical labeling of spectrin and dystrophin in vastus lateralis muscle of the left leg. Serial transverse sections (10 μm) were immunostained with anti-spectrin monoclonal antibody (A) and with anti-dystrophin monoclonal antibody NCL-DYS1 (B) and Mandys108 (C) (× 100).

examination did not point to another cause for the intellectual impairment.

Somatic mosaicism has been previously described in six carriers [3,4,6–9] and in two male patients with a dystrophinopathy [10,11].

Lebo et al. [10] described a grandfather with an elevated CK, striking muscle weakness and atrophy in the right arm and shoulder in the C5–6 innervated myotomes. Each of his three daughters had a son with DMD. Molecular analysis showed the three grandsons had inherited the grand paternal X chromosome. Certainly the grandfather must have had germinal mosaicism, a somatic mosaicism is possible because of his clinical picture. Unfortunately, this could not be confirmed at the molecular-genetic level since the family was further uncooperative. Saito et al. [9] reported on a classical DMD patient who had a deletion of exon 1–7 in the lymphocytes. His mother and the patients' sister did not carry the deletion. Post mortem DNA investigation showed the presence of the deletion in ectodermal and endodermal tissues. In some mesodermal tissues (temporalis, sternocleidomastoid, diaphragm muscles and the kidney) the deletion was absent at the DNA level whereas in other mesodermal tissues the deletion was present, indicating a somatic mosaicism. Dystrophin expression in muscles showed no dystrophin in some muscles, in a few muscles a mosaic pattern was found and in the diaphragm dystrophin was detected in every fiber. They suggested that the deletion might have occurred early during embryogenesis because all cells of the ectoderm and the endoderm carried the deletion while in the mesoderm a somatic mosaicism was found.

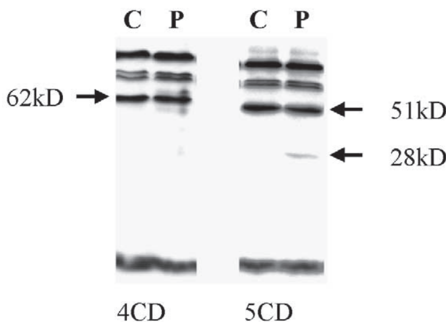

Fig. 4. Study of mRNA from the muscle biopsy with the PTT analysis yielding two bands in fragment 5 CD corresponding to exons 59–68, indicating mosaicism. C means control and P patient. The upper protein bands observed in all lanes are endogenous biotinylated proteins present in the rabbit reticulocyte lysate used for translation detection.

Our patient illustrates a somatic mosaicism of a point mutation in the dystrophin gene which made the phenotype of the dystrophinopathy (with a disruption of the reading frame), milder. The spectrum of dystrophinopathies can yet be extended to asymmetrical weakness and early contractures.

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Chapter 3

Duchenne/Becker muscular dystrophy in the family: have potential carriers been tested at a molecular level?

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Original Article

Duchenne/Becker muscular dystrophy in the family: have potential carriers been tested at a molecular level?

Helderman-van den Enden ATJM, van den Bergen JC, Breuning MH, Verschuuren JJGM, Tibben A, Bakker E, Ginjaar HB. Duchenne/Becker muscular dystrophy in the family: have potential carriers been tested at a molecular level?
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Duchenne muscular dystrophy (DMD) is the most common inherited neuromuscular disease. After identification of the mutation in the index patient, family members can be reliably investigated. Carriers should be informed about their risk of having offspring with the disease and about their own risk for cardiomyopathy for which regular cardiac surveillance is recommended. In a small country like the Netherlands with well-organized genetic services, one would expect that most DMD families are adequately informed about the above mentioned risks for carriers. We have investigated whether women at risk had been tested at a molecular level. In the national Duchenne/Becker database 311 DMD and 99 Becker muscular dystrophy (BMD) patients had been registered up to 1 July 2009. These patients were asked to give information about the number of sisters and maternal aunts of the DMD/BMD patient and anything that was known about their genetic status and that of the mother. This information was compared with the information known at the genetic laboratory. Thirty-five of 104 adult sisters/maternal aunts of DMD patients with a 50% risk of being a carrier and 45 of 148 adult women with a 4.3% risk because of germ line mosaicism for DMD had not been tested by DNA analysis. Our study indicates that about one third of the potential carriers have not been tested. Given the possible far-reaching clinical consequences of being a carrier, further studies are needed to investigate the reasons why potential female carriers have not been tested.

Conflict of interest

None declared.

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Key words: Becker muscular dystrophy – cardiomyopathy – carrier – Duchenne muscular dystrophy – risk

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Duchenne muscular dystrophy (DMD) is a progressive neuromuscular disease. Most patients become wheelchair bound before the age of 12, and their mean age of survival is 26 (1). DMD has an incidence of roughly 1 in 3500 (28.5 per 100,000) live male births (2). DMD is the most common X-linked recessive muscle disease and is caused by a mutation in the dystrophin-encoding *DMD* gene on the X chromosome which results in the absence of dystrophin in the muscles of the patient. Becker muscular dystrophy (BMD) is a less common and a milder form of dystrophinopathy. Dystrophin in the BMD muscle is present but in an aberrant and/or a reduced amount.

In two-thirds of the patients DMD is inherited from the mother, implying that the sisters of the patient have a 50% risk of being a carrier. In one third of the patients the mutation has arisen *de novo* (3). However, in the latter cases the mutation may be present in the mother as a germ line mosaicism, in which case the sisters have an estimated 4.3% risk of being a carrier (4).

Because DMD is a devastating disease for which there is no curative therapy so far, much emphasis has been put on prevention. For known DMD families, offering genetic counseling to women at risk is the first step toward prevention. The women are informed about their risk of being a carrier, the available test, the recurrence risks and their reproductive options.

Another aspect relevant to female carriers is their own health. It is estimated that about 10% of female carriers develop dilated cardiomyopathy (5). A recent study in the United States has shown that only 62.9% of the carriers are aware of their risk for cardiomyopathy (6).

At a consensus conference held in 2002, it was agreed that female carriers should be advised to start cardiologic examination at diagnosis or at the age of 16 with follow-up examinations at least every 5 years (7).

In the Netherlands most, and from 1997 onwards all, DMD DNA diagnostics is carried out in the genetic laboratory in Leiden. For most of the children with DMD, a molecular test is performed according to the best practice guidelines (8), and genetic counseling is offered to family members. The Netherlands is a small country with 17 million inhabitants and clinical genetic departments in each of the eight university hospitals. Each DMD family can have counseling within a distance of <100 km from their home. During our work as clinical geneticists (A. H-E, M. H. B) and clinical molecular geneticists (E. B, H. B. G), we have come across several DMD families in which women appear to be unaware of their risk of being a carrier. These families prompted us to carry out this study (see examples of families under the Results section). To the best of our knowledge no studies have been performed on the results of cascade screening (9) in DMD and BMD. A study on childless young women at risk for DMD in Brazil who received genetic counseling showed that the magnitude of the genetic risk did not influence the request for DNA testing (10).

In this study we have investigated whether all women at risk for carrying the familial mutation in the *DMD* gene have been tested at a molecular level.

Materials and methods

All patients/families that had been registered up to 1 July 2009, in the Duchenne/Becker database in the Netherlands (www.lumc.nl/duchenne), were asked to fill in a questionnaire about the phenotype of the patient as well as about the family history as a part of the registration procedure. The registration started during the spring of 2008.

Registrees were requested to list the number of brothers, sisters, maternal uncles and maternal aunts of the Duchenne/Becker patient. They were asked whether each of the male members was healthy or affected with DMD/BMD. They were also asked whether the mother of the DMD/BMD patients and the other female members had undergone carrier testing and if so, were they carriers.

The information from the questionnaires was compared to the information known at the genetic laboratory in Leiden. If two or more individuals from the same family were registered in the database, they were included as one family. The reasons for not including some of the families are given in Table 1.

In the investigated families where the mother was a carrier we checked whether the maternal grandmother, the sisters and the maternal aunts of the DMD/BMD patient had been tested at a molecular level at the genetic laboratory.

Subsequently we calculated the number of women who had a 50% risk of being a carrier. A woman was defined as having a 50% risk if she had not been DNA tested, was not an obligate carrier, had a brother with DMD/BMD and her mother was a proven carrier. We also calculated the number of women at a 4.3% risk because of

Table 1. Reasons for excluding 89 of 311 DMD and 36 of 99 BMD families from the study

	DMD	BMD
Family abroad	6	0
No sample at the Leiden lab[a]	27	10
No information on the questionnaire[b]	21	2
Unidentified mutation	18	9
Multiple family members	17 (out of 14 families)	15 (out of 13 families)

DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy.

[a]No blood sample received by the laboratory in Leiden for a molecular test. Not known whether the families had been tested elsewhere.

[b]Information on the phenotype of the patient given but the section on the family history was left blank on the questionnaire.

germ line mosaicism in the *de novo* families (4). Germ line mosaicism is defined as the presence of two or more genetically distinct cell lines confined to the precursor (germ line) cells of the egg or the sperm. In this study, a woman who has a brother with DMD/BMD was considered to have a germ line risk even if her mother did not have a mutation in her lymphocytes because of the possibility of a germ line mosaicism in the mother. Maternal aunts of a DMD/BMD patient were considered to have a germ line risk if the mother of the patient carried the mutation in her lymphocytes and the carrier status of the grandmother was not known. In BMD families, the maternal grandfather was not affected with BMD.

We counted the number of mothers and grandmothers tested for carrier status in families who were sent in before (old family) and after 1 January 1997 (recent family) to see if there was a possible difference in the number of women tested in the two periods.

We chose this date as it was exactly in the middle of the period between 1984, when DNA diagnostics of the *DMD* gene was started in Leiden, and 2009, the year in which most of this study was carried out.

The women at risk were divided into two groups: younger than and older than 16 years because cardiologic examination is recommended for carrier women from this age onwards (7). If the date of birth of a woman was not known, an estimate was made by considering her position in the pedigree where the ages of other family members were known.

If, in the families that were included in the study, the information on the questionnaire about one or more family members did not match the information present in the laboratory (Table 2), the information from the laboratory was used.

Results

On 1 July 2009, 311 Duchenne and 99 Becker patients had been registered at the national database. Among these, 89 DMD and 36 BMD patients were excluded from the study (Table 1), leaving 222 DMD and 63 BMD patients. The information in the questionnaire and the laboratory was concordant in 186 of 222 (84%, 95% confidence interval: 79–89%) DMD and in 43 of 63 (68%, 95% confidence interval: 57–80%) BMD cases. Table 2 shows the reasons for discordance in 36 of 222 DMD and 20 of 63 BMD families. Table 3 shows the number of women at risk in DMD families that had not had a DNA test. Eighty-six of 155 women with an *a priori* 50% risk of being a carrier had not been tested. This includes 35 of 104 (34%) women older than 16.

Seventy-seven of 180 women with a germ line risk had not been tested, including 45 of 148 (30%) women older than 16. It is noteworthy that we found germ line mosaicism in five *de novo*

Table 3. Number of women in DMD and BMD families with an *a priori* 50% risk of being a carrier and with a 4.3% risk because of a germ line mosaicism tested or not tested in the laboratory

	Women with a 50% risk			Women with a 4.3% risk		
Age group	Not tested	Tested	Total	Not tested	Tested	Total
DMD ≥16 year	35	69	104	45	103	148
DMD <16 year	51		51	32		32
Total DMD	86	69	155	77	103	180
BMD ≥16year	3	25	28	9	32	41
BMD <16 year	7		7	1		1
Total BMD	10	25	35	10	32	42

DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy.

Table 2. Families included in the study where the information about family members on the questionnaire did not match that present in the laboratory

	DMD	BMD
Conflicting data on results obtained from DNA test between laboratory and the form[a]	5/36 (14%)	3/20 (15%)
Family member ‘known’ on the form but unknown in the laboratory[b]	8/36 (22%)	2/20 (10%)
Family members not on the form but known in the laboratory[c]	8/36 (22%)	7/20 (35%)
No information on female members on the form[d]	8/36 (22%)	1/20 (5%)
Form confused, no conclusions could be drawn	7/36 (19%)	7/20 (35%)

DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy.

[a]Female family member listed as a carrier on the questionnaire but not a carrier according to the DNA test or vice versa.

[b]Female family member of an index patient in whom a mutation had been detected in Leiden, listed as a carrier or a non-carrier but never tested in Leiden.

[c]No mention of female family members on the questionnaire but female relatives tested in Leiden or had come to light via testing of other relatives.

[d]Female family member(s) mentioned on the questionnaire but without the (non)carrier status, known in the laboratory.

DMD families. The mutation was identified in other members of the family who had had a DNA test.

Table 3 also shows the number of women in BMD families who had not had a DNA test. Ten of 35 women in the 50% risk group had not been tested, including 3 of 28 women (11%) older than 16. Ten of 42 women with germ line risk had not been tested, including 9 of 41 women (22%) older than 16.

It is again worth noting that one *de novo* BMD family was found to have germ line mosaicism.

Table 4 shows the results of the DNA test of the mothers and maternal grandmothers at risk in the DMD families. In all, 4% of the mothers and 42% of the grandmothers had not been tested. There was one notable difference between old and recent families. Although 33% (95% confidence interval: 18–48) of grandmothers in the old families had not been tested, this figure was 47% (95% confidence interval: 35–59) for recent families.

Table 4 also shows the results of the DNA test of the mothers and maternal grandmothers at risk in the BMD families. In all, 22% of mothers and 57% of grandmothers had not been tested.

Examples of families

Family A illustrates the use of unreliable tests for carrier detection and failure to test the maternal grandmother

The index (IV:4), born in 1975, was diagnosed with DMD at the age of 3 (Fig. 1 pedigree A). His older brother (IV:3) who had died at the age of 4 during a light narcosis had apparently had the same pattern of walking. Around 1980 the blood (probably creatine kinase (CK)) and muscle biopsies of the maternal grandmother and a maternal aunt were tested. They were not found to be carriers. A mutation was identified in the index (IV:4) at the age of 13. At the time, only a pediatrician was consulted. The sister of the index was tested and was found not to be a carrier. However, the mother and the maternal grandmother were not tested. In 1993 DMD was diagnosed in another 4-year-old child (IV:6) who carried the familial mutation. Subsequently, information from the carrier mothers led to the confirmation of DMD in yet another child (IV:10).

When we contacted the family in 2009 for permission to publish their pedigree, we learnt that a son of a sister of the maternal grandmother (III:1)

Table 4. Results of the DNA test of the mothers and maternal grandmothers at risk of being a carrier in the DMD and BMD families

		Mothers			Grandmothers		
		All	Old <1997[a]	Recent ≥1997[a]	All	Old <1997[a]	Recent ≥1997[a]
DMD	Total number	222	83	139	114	42	72
	DNA test: carrier	114 (51%)	42 (51%)	72 (52%)	12 (11%)	3 (7%)	9 (13%)
	DNA test: not carrier	96 (43%)	36 (43%)	60 (43%)	46 (40%)	19 (45%)	27 (38%)
	No DNA test probably carrier	4[b] (2%)	3 (4%)	1 (1%)	8[c] (7%)	6 (14%)	2 (3%)
	No DNA test status unknown	8 (4%)	2 (2%)	6 (4%)	48 (42%)	14 (33%)	34 (47%)
BMD	Total number	63	33	30	21[d]	14	7
	DNA test: carrier	24 (38%)	17 (52%)	7 (23%)	5 (24%)	4 (29%)	1 (14%)
	DNA test: not carrier	18 (29%)	8 (24%)	10 (33%)	2 (10%)	1 (7%)	1 (14%)
	No DNA test probably carrier	7[e] (11%)	5 (15%)	2 (7%)	2[f] (10%)	2 (14%)	
	No DNA test status unknown	14 (22%)	3 (9%)	11 (37%)	12 (57%)	7 (50%)	5 (71%)

DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy.
[a]'Old' family: blood received before 1 January 1997; 'recent' family: blood received after 1 January 1997.
[b]Three DMD mothers had two and one had three children with a mutation.
[c]Seven maternal DMD grandmothers had an affected sib and one had three children with a mutation.
[d]Of the 24 carrier BMD mothers, three had inherited the disease from their father leaving 21 grandmothers who were at risk.
[e]Five BMD mothers had two children and two had three children with a mutation.
[f]One BMD grandmother was said to have an affected father and one had two affected brothers.

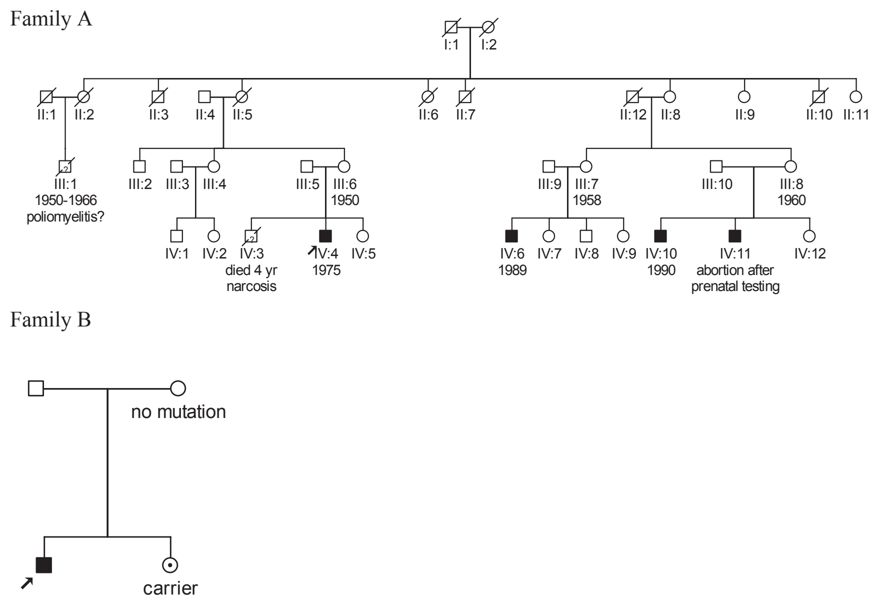

Fig. 1. Pedigrees of remarkable families.

of the index patient had probably also had DMD. He was ambulatory when he was young but later became wheelchair bound and developed scoliosis. Old photographs showed enlarged calves. He died in 1966 at the age of 16 and was thought to have been affected with poliomyelitis. We also informed the mother of the index patient that her sister (III:4) had a risk of being a carrier. This was particularly important because her daughter (IV:2) was in her 20s and did not have a family yet.

Family B, an example of germ line mosaicism

Duchenne muscular dystrophy (DMD) was diagnosed and confirmed at the molecular level in the index at the age of 7 (Fig. 1, pedigree B). The family history was negative for DMD. A DNA test on lymphocytes showed that the mother was not a somatic carrier. However, the sister was found to be a carrier of the same mutation as her brother, proving germ line mosaicism in the mother.

Family C illustrates communication problems within a family

A pregnant woman was referred for genetic counseling because she knew that her mother had had three brothers who died at a young age and were possibly affected with DMD. The mother of the pregnant woman did not cooperate, so a prenatal test could not be performed. A boy was born. At the age of 2, he was referred for molecular testing because of suspicion of DMD and a mutation was detected. Subsequent testing of the mother of the DMD patient revealed that she was the carrier.

Discussion

In 1999, Hoogerwaard et al. (5) reported the results of a study performed on 129 Dutch carriers of DMD and BMD. They showed that for these women, being a carrier not only had consequences for their offspring but also for their own health, namely an increased risk for cardiomyopathy. One would expect that in the last 10 years this knowledge would have influenced the uptake of molecular testing of potential carriers. However, our study shows that even in the Netherlands, with its well-organized genetic counseling services, there is still a large number of women in DMD/BMD families who have not been tested at the DNA level.

In the 222 of 311 registered DMD families that could be analyzed, 86 women with a 50% risk of being a carrier and 77 women with 4.3% risk because of germ line mosaicism had not been tested. In general, it is agreed that carrier testing in minors should be deferred until the child can give proper informed consent (11). So, if we consider

only women older than 16 it emerges that 34% of women with a 50% risk and 30% of the women with a germ line risk had not been tested.

In the 63 of the 99 registered BMD families that could be analyzed, 10 women with a 50% risk of being a carrier and 10 women with a germ line risk had not been tested. The percentages of women older than 16 who have not been tested in the BMD families are 11% for women at a 50% risk and 22% for the women with a germ line risk.

The above percentages of the women at risk are probably underestimates because we were able to look at only the first- and second-degree relatives of DMD/BMD patients.

There could be a variety of reasons why a woman has not been tested: she does not wish to have (more) children, she relies on the result of an earlier (unreliable) test by serum creatine kinase measurements or muscle biopsy (family A), has moral or religious objections to testing, she is unaware of her risks because of lack of communication within the family or she is not able to undergo reliable testing because her family does not cooperate (family C). Molecular testing of the maternal grandmother in DMD families, where the mother is a carrier, is important for genetic counseling. Two-thirds of these maternal grandmothers are expected to be carriers (3). In our study, the genetic status of 48 maternal grandmothers was unknown in the 114 DMD families where the mother was a carrier. More grandmothers were not tested in recent families (47%) than in old ones (33%). Besides the reasons given above for sisters and maternal aunts, there may be additional reasons why grandmothers had not been tested. These include death, anticipated guilt feelings should a mutation be detected or lack of understanding of the inheritance pattern especially if her healthy sibs have healthy sons.

Our observations illustrate the importance of drawing attention to the germ line risk of which many doctors and patients are not aware. In fact the germ line risk is 4.3% (4) and as mentioned earlier, we found proven germ line mosaicism in five DMD families and in one BMD family. In family B the healthy sister who asked for genetic counseling prior to starting a family turned out to be a carrier of the mutation, which was present in the germ line cells of the mother. With this knowledge, she and her partner can make informed choices in planning their family. Furthermore, she could be referred for periodic examination for cardiomyopathy.

Agreement between information in the questionnaire and that present in the laboratory was high for both DMD (84%) and BMD (68%) families, but more women from the DMD families were aware of their risks. This may be because some BMD patients with a very mild phenotype may not see the importance of informing their relatives.

In 16% of the DMD and 32% of the BMD families, there was a discrepancy between the information obtained via the questionnaire and that known in the laboratory. Among the reasons given for the discrepancy in Table 2, the first two are particularly worrisome. The first reason, conflicting data on results DNA test between laboratory and the form, demonstrates that in five DMD and three BMD families women think they are carrier/non-carrier whereas the result of the DNA test showed the opposite. The second reason, family member known on the form but unknown in the laboratory, shows that in eight DMD and two BMD families women think that they have been tested whereas they have not been tested. Possible explanations could be that either the respondent was not well informed or that the women had been tested elsewhere (DMD diagnosis was offered in Groningen before 1997), even though the mutation in the index patient had been detected in Leiden. The information on the questionnaire will also have been influenced by the memory and family ties of the respondents.

Our results show that even in the Netherlands genetic counseling and follow-up of families of Duchenne and Becker patients are incomplete. As a consequence, female family members are not informed of their risk of being a carrier. In some cases, this may have led to the birth of an affected boy. Adequate cascade carrier screening would have been helpful in these cases. The results indicate that our standards of care need to be substantially improved to enable us to offer a possibility of prevention to all at-risk family members.

The General Medical Council in the United Kingdom issued a guidance named 'Confidentiality' in October 2009 (12). For the first time, giving genetic information to protect the family has taken precedence over protection of the privacy of an individual (13).

According to the new advice, doctors should be told explicitly that it may be justified to reveal information in the interest of relatives even if the patient objects. Such information could, for example, alert family members with a genetic predisposition to cancer to the need for surveillance or treatment, or influence decisions on reproduction. Both these aspects are valid also for DMD/BMD families: carrier women need to undergo regular cardiac surveillance and can use the information to decide which reproductive options suit them best.

In a recent review on diagnosis and management of DMD (14, 15), genetic counseling is indicated as a part of the diagnostic process. However, so far the question of how the family should be informed has not been addressed. The question why so many women at risk have not been tested at a molecular level will be the subject of further research. For women relying on the result of an earlier unreliable test, for example serum creatine kinase measurements, it is clear that now there is a more reliable molecular test available. An ethical dilemma arises in approaching a woman who has not been tested because she was not informed about the risk of being a carrier, as a result of either lack of communication or lack of cooperation within the family. In the UK guidance of the General Medical Council, it is suggested that the patient's identity should not be disclosed in contacting and advising the family members about their risks, if practicable. These last two words are crucial for it is often impossible not to disclose the patient's identity. It is also not always possible to contact family members without the help of the patient. One form of lack of cooperation by the family members can be their refusal to provide a blood sample for identifying the mutation. This problem will be solved in the future with the availability of newer molecular techniques.

At present, in the genetic counseling practice in the Netherlands, the index case is handed a letter with which he or she can inform the family members. In this way the privacy of the index is protected because he or she can choose not to distribute the family letter. In a recent study by Van der Roest et al. (16), 88% of the family letters were distributed in the family and as a result 57% of the family members of patients with a high-risk genetic cardiac disease underwent screening. For diseases such as hereditary cancer with preventive options, on average 50% of family members undergo genetic testing (17, 18).

There is an ongoing debate on whether a more active approach of family members is justified, considering the fact that such a high percentage of family members does not undergo genetic testing. This study should help in bringing this problem under the attention of the doctors and those who care for DMD/BMD families. Thus, more women can be made aware of the implications of being a carrier, both for their offspring and for themselves, and can be offered molecular testing.

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Chapter 4

An urgent need for a change in policy revealed by a study on prenatal testing for Duchenne muscular dystrophy

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ARTICLE

An urgent need for a change in policy revealed by a study on prenatal testing for Duchenne muscular dystrophy

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Prenatal diagnosis for Duchenne muscular dystrophy (DMD) was introduced in the Netherlands in 1984. We have investigated the impact of 26 years (1984–2009) of prenatal testing. Of the 635 prenatal diagnoses, 51% were males; nearly half (46%) of these were affected or had an increased risk of DMD. As a result 145 male fetuses were aborted and 174 unaffected boys were born. The vast majority (78%) of females, now 16 years or older, who were identified prenatally have not been tested for carrier status. Their average risk of being a carrier is 28%. We compared the incidences of DMD in the periods 1961–1974 and 1993–2002. The incidence of DMD did not decline but the percentage of first affected boys increased from 62 to 88%. We conclude that a high proportion of families with *de novo* mutations in the *DMD* gene cannot make use of prenatal diagnosis, partly because the older affected boys are not diagnosed before the age of five. Current policy, widely accepted in the genetic community, dictates that female fetuses are not tested for carrier status. These females remain untested as adults and risk having affected offspring as well as progressive cardiac disease. We see an urgent need for a change in policy to improve the chances of prevention of DMD. The first step would be to introduce neonatal screening of males. The next is to test females for carrier status if requested, prenatally if fetal DNA is available or postnatally even before adulthood.
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Keywords: carrier testing; Duchenne muscular dystrophy; neonatal screening; preimplantation genetic diagnosis; prenatal diagnosis

INTRODUCTION

Duchenne muscular dystrophy (DMD), an X-linked recessive disease leading to relentless loss of muscle tissue, is clinically diagnosed around the age of five.[1] The mean age of survival is 25 years. The incidence of DMD is roughly 1 in 3500 live male births.[2] Becker muscular dystrophy (BMD), a milder variant of DMD, has an incidence of 1 in 18 450 males. *De novo* mutations account for one-third of the DMD patients.[3] The importance of prevention has been much emphasized as no curative therapy is available. Women at risk can be offered genetic counseling and carrier testing. Options for carrier women include prenatal diagnosis, preimplantation genetic diagnosis (PGD) or the use of donor eggs. With prenatal diagnosis, no further testing to determine the carrier status is done if the fetus is female. A male fetus is tested by direct analysis for a known familial mutation to determine if he is affected. If a familial mutation is not known, haplotyping is used to determine whether the fetus has a high risk of being affected with DMD or BMD. The birth of these boys can be prevented by terminating the pregnancy. With PGD, either female or unaffected male embryos can be selected and transferred to the uterus. First trimester prenatal diagnosis for dystrophinopathy has been available in the Netherlands since 1984[4] and PGD since 1995.[5] However, these strategies do not prevent the birth of boys with a *de novo* mutation. The aim of this study was to determine the effect of genetic counseling and the use of prenatal testing and PGD on the occurrence of DMD and BMD in the Netherlands.

MATERIALS AND METHODS

Patients

We have used two sets of data, a postnatal cohort 1993–2002 to determine the incidence of DMD and a prenatal cohort 1984–2009 to examine the results of prenatal diagnosis. We have also included the result of PGD in the period 1995–2009.

Incidence

Most, and from 1997 onwards all, DNA diagnostics for DMD/BMD in the Netherlands has been done in the Laboratory for Diagnostic Genome Analysis at the Leiden University Medical Center. A mutation in the *DMD* gene has been identified in more than 1000 patients in our laboratory since the start of the service in 1984. The incidence of DMD was determined from the number of DMD males born in the period 1993–2002 from our database in Leiden and from the total number of live male births obtained from Statistics Netherlands (www.cbs.nl). We chose this 10-year period, because before 1993 not all DMD patients were referred to our laboratory for DNA analysis and not all patients born after 2002 had been clinically diagnosed yet. The diagnosis of dystrophinopathy was considered certain only when a mutation that confirmed the clinical diagnosis had been identified. This was done with MLPA and if no deletion or duplication was found, high resolution melting curve analysis[6] was used.

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The incidence of DMD in the birth cohort 1993–2002 was compared with the incidence of DMD patients born in the period 1961–1974 in the Netherlands.[7] We further split the data on incidences from the 10-year period into two 5-year periods: 1993–1997 and 1998–2002 to see if there was a decline in the incidence in the second period with respect to the first. Finally, we examined the family trees for the birth cohort 1993–2002 to determine: (1) the carrier status of the mother, (2) whether the affected boy was the first affected patient in the family and (3) if the boy was not the first affected, whether the oldest affected boy was older than five at the time of the birth of the younger one. We then compared the proportion of first affected patients in this period to the proportion in 1961–1974 to determine if prenatal testing had been used by DMD families to prevent the birth of subsequent affected boys.

Pearson's χ^2-test was used to calculate the significance of difference between the incidences.

Prenatal tests and PGD

Every woman in the Netherlands with an increased risk of having a son with DMD/BMD is offered prenatal testing. PGD is done only upon request. We examined the results of all the prenatal tests done in 26 years (1984–2009) and PGD in 15 years of (1995–2009). The prenatal data are from the laboratories in Leiden and the University Medical Center Groningen. Prenatal diagnosis was done in Groningen also after 1997 in women from families wherein the mutation had been identified there earlier. We recorded the date, the type and the outcome of the test and the place of residence of the pregnant woman. Tests performed on samples from outside the Netherlands were excluded from the analysis. The PGD data on DMD/BMD cases was obtained from Maastricht University Medical Center, where all the PGD in the Netherlands are carried out.

RESULTS

Incidence

In the 10-year period (1993–2002) 216 boys with DMD were born. With the exception of one boy born in 1994, none of them had been tested prenatally. The clinical diagnosis in all the 216 boys was confirmed by postnatal DNA analysis. The incidence of DMD was about 1 in 4700 male live births (for the exact figures and confidence intervals see Table 1). The incidence reported for the period 1961–1974 is about 1 in 4200 male live births[7] (see Table 1). There was no significant difference between the incidences in these two periods ($P = 0.227$). There was also no significant difference ($P = 0.114$) between the incidences in the two 5-year periods, 1993–1997 (1:4200) and 1998–2002 (1:5200).

Percentage of first affected patients in the DMD families

Of the 216 boys born with DMD in the period 1993–2002, 189 (88%) were the first affected patient in the family (95% CI: 83–92%). Analysis of maternal lymphocytes showed that 98/189 had a carrier mother and in 91/189 the mutation had arisen *de novo*, but mosaicism was not excluded in the latter group. In 27/216 (12%) families there was an older affected family member. One of the 27 was tested prenatally, the parents accepted the 33% risk indicated by haplotyping and an affected boy was born in 1994. The remaining 26/27 families were not known to us before the birth of the second boy; in 12 of these, the older affected boy was younger than 5 years.

In the cohort of 1961–1974, 62% (95% CI: 57–66%) of the boys with DMD did not have an affected DMD relative.[7] This implies that 62% were the first affected in the family, in contrast to the above mentioned 88% in the period 1993–2002.

Table 1 Incidences of Duchenne

Period	Number of male births	DMD patients	DMD incidence (95% CI)
1993–1997	493 769	117	1:4220 (3573–5154)
1998–2002	518 210	99	1:5234 (4373–6518)
1993–2002	1 011 979	216	1:4685 (4134–5406)
1961–1974 (van Essen *et al*[7])	1 673 791	397	1:4215 (3738–4831)

Abbreviations: CI, confidence interval; DMD, Duchenne muscular dystrophy.

Prenatal diagnosis

Figure 1 shows the results of 635 prenatal samples, including 28 from Groningen, referred for DMD/BMD between 1 January 1984 and 31 December 2009. There were 322 (51%) male fetuses, 290 (46%) female and in 23 (about 4%) testing was not completed. For details see Figure 1. Figure 2 shows the number of female and male fetuses identified per year. The decreasing number of females in recent years can be attributed to the increasing use of maternal blood for fetal sex determination.

Male fetuses. Figure 1 shows that 147 of the 322 male fetuses (46%) were affected or had an increased risk of having DMD/BMD. The number of these males found per year is shown in Figure 3. Figure 4 shows that the number of tests based on haplotyping has gradually reduced over the years as the number of cases identified by direct mutation analysis has increased. However, haplotyping is still used if no DNA is available, because the index patient is deceased or if prenatal testing is done in cases without a familial mutation. The pregnancy was terminated in 145 of the 147 cases of affected or at risk fetuses and two affected boys were born. The first is the above mentioned boy born in 1994. The second, born in 1995, had BMD and the pregnancy was continued on grounds that all three affected family members were wheelchair bound only after the age of 50.

There were 175 male fetuses with no increased risk (Figure 1) and 174 unaffected boys were born. There was one false-negative result where a deletion was missed in 1989 by Southern blotting and multiplex PCR and an affected boy, born in 1990, was diagnosed in 1993. Further investigation revealed that the fetal DNA had been contaminated with 5–10% DNA from another person but we were unable to trace where or when the contamination had taken place. This was before contamination tests were implemented in our routine diagnostic laboratory in 1993.

Female fetuses. The carrier status of a female is usually determined postnatally at the age of 16 or later. In general, therefore, no molecular testing is done if a fetus is found to be a female. We divided the 290 females into two groups: those that were diagnosed before and after 1 January 1993. The 111 girls that were diagnosed before 1993 are now between 16 and 26 years old (see Figure 1). Only 24 of the 111 have been tested for the familial mutation, at an average age of 17.5 years (Figure 1). This means that 87/111 (78%) have not yet been tested. The average risk of being a carrier for these girls is 28% (Figure 1). Their mothers are either proven carriers, have an increased risk determined by haplotyping or are potential germ-line mosaics.[8]

As an exception to the rule, 6 of the 179 females diagnosed after 1993 were tested before adulthood, 3 prenatally and 3 postnatally. Two fetuses were tested because they were at a risk of being affected as they were 45,X/46,XX mosaics. The mother in the third case was afraid that she might be a germ-line mosaic and did not want a carrier daughter. One girl was tested at 2 because of delay in motor development. Two girls were tested at the request of the girls and their parents, one at the age of 11 and the other at 16.

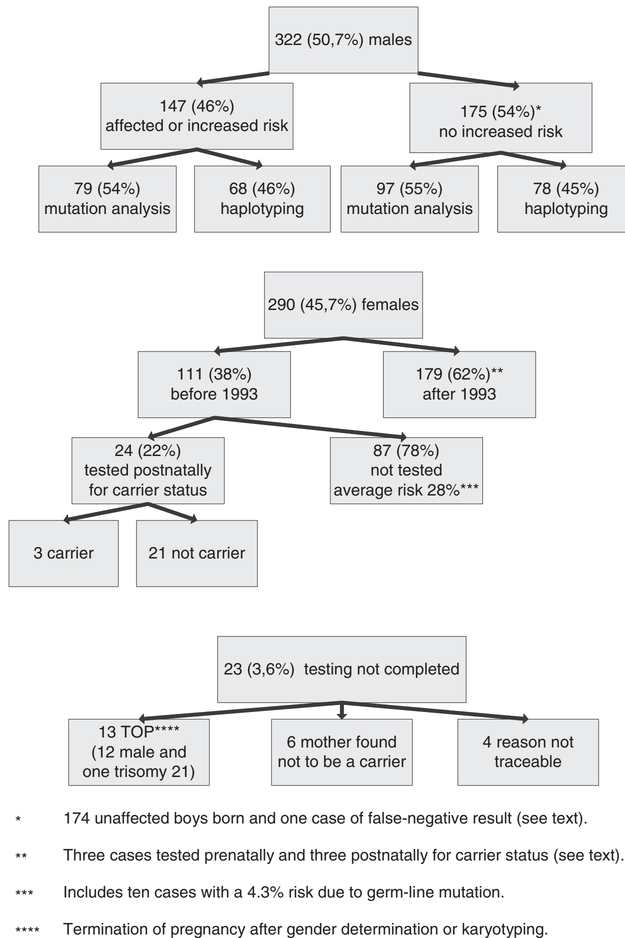

Figure 1 Results of 635 prenatal diagnoses in the period 1984–2009.

Preimplantation genetic diagnosis for dystrophinopathies
Sixty-six couples were referred for PGD for a dystrophinopathy in the period 1995–2009. Most withdrew following extensive genetic counseling; only 14 couples received the treatment with a total of 31 IVF/PGD treatment cycles. The gender was determined using fluorescence *in-situ* hybridization in all cycles and one or two female embryos were transferred to the uterus. These treatments resulted in the birth of seven healthy girls, three singletons and two sets of twins.

DISCUSSION

Effect of prenatal testing on the incidence of DMD
Our study has shown no significant difference in the incidence of DMD between the birth cohorts 1961–1974 (1:4200)[7] and 1993–2002 (1:4700), or between the two 5-year periods 1993–1997 (1:4200) and 1998–2002 (1:5200). Similar studies in Denmark and Canada have also shown no decrease in the incidence in the last three decades,[9,10] but the incidence in Australia has declined.[11]

It should be noted that the methods by which the data were obtained in the two periods were different. DMD patients in the birth cohort 1961–1974 were diagnosed at a time when molecular testing and immunohistochemical analysis of dystrophin in muscle tissue were not yet possible.[7]

Effect of prenatal testing on known families
Prenatal testing has provided women in DMD families the possibility of preventing the birth of an affected child. In the period of 26 years, 145 male fetuses that were affected or had a high risk of dystrophinopathy were aborted.

The most significant positive effect of prenatal testing in this period was the birth of 174 unaffected boys in DMD/BMD families. The parents in these cases have been spared the emotional burden of

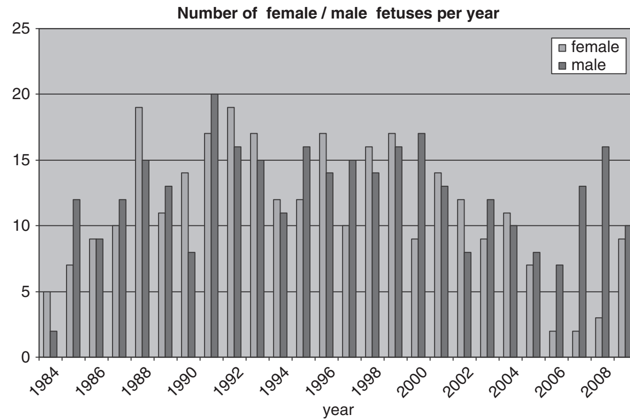

Figure 2 The number of female/male fetuses per year.

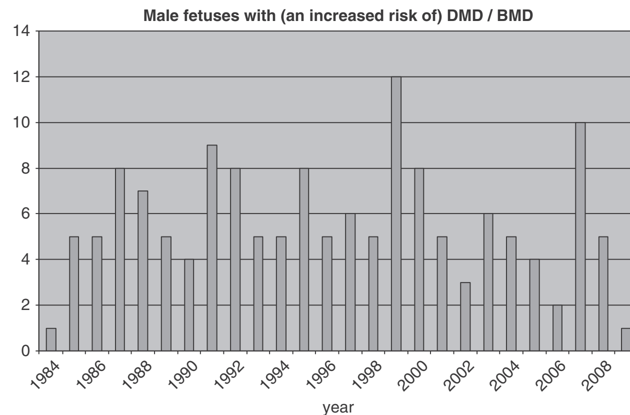

Figure 3 The number of male fetuses with or at an increased risk of having DMD/BMD per year in the period 1984–2009.

having to abort a possibly healthy boy, which was always the major drawback of prenatal diagnosis of X-linked disorders by sex determination only. PGD can offer some families the possibility of having children without the burden of any abortion at all.

Prenatal testing: no option for parents of most DMD boys

Prenatal diagnosis is offered only to women who have a known increased risk. A large proportion of women, 189/216 (88%) who gave birth to a boy in the period 1993–2002 could not make use of this preventive measure, because the affected patient was the first in the family. This high percentage can be explained as follows. One in three DMD patients is thought to have a *de novo* mutation but empirical data show the number of *de novo* deletions, the most common type of mutation in the *DMD* gene, to be as high as 57%.[11] Our own study indicates that a new mutation arose in 48%. However, even if we consider the theoretical figure of one in three, one must remember that the mutation is *de novo* not only in one-third of the patients but also in one-third of the other two-thirds of the mothers who are carriers. In theory, therefore, the total number of DMD patients who are the first affected in the family is at least 56% (1/3 *de novo* in the patient plus $1/3 \times 2/3$ *de novo* in the carrier mother). This figure is remarkably close to the empirical data from the sixties where the percentage of sporadic DMD patients was 62%.[7] Thus, the high number of sporadic DMD patients appears to be caused by the result of a relatively high rate of spontaneous mutations that have arisen in the mother, in the grandparents or in even earlier generations but which have not yet manifested in the birth of an affected offspring.

Prenatal diagnosis was also not an option for most of the remaining 12% (27/216) of the families with an older affected relative. Information about DMD in the older boy was not available at the time of birth in 26/27, in nearly half of them (12) the older boy was younger than 5 years, the age at which DMD is generally diagnosed. The parents could have opted for prenatal diagnosis had they known about the older boy. To our knowledge, three studies have addressed the question of whether neonatal screening leads to

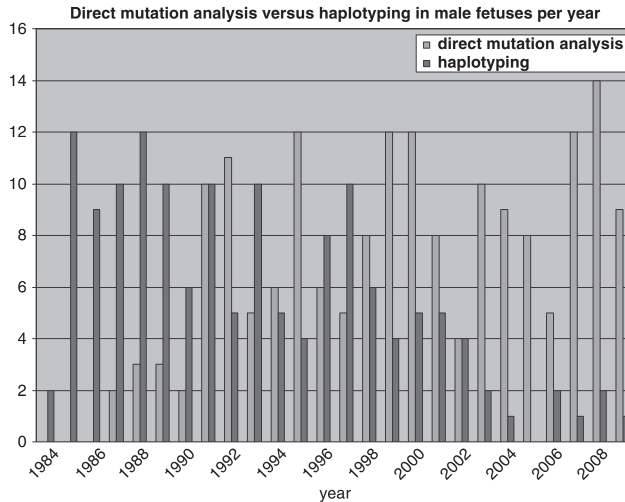

Figure 4 The number of male fetuses per year identified by direct mutation analysis and by haplotyping for an increased risk for DMD/BMD.

prenatal testing in subsequent pregnancies. A pilot study in 1993 showed that only 2/7 pregnancies of a second child were monitored by prenatal diagnosis. The authors emphasize the influence of religion and/or extremely poor social conditions in the other five families.[12] Two studies from 1998 have shown that families do assess their reproductive choices as a result of earlier diagnosis; 2/2 and 10/12 subsequent pregnancies were monitored.[13,14] Data from our prenatal cohort 1984–2009 (Figure 3) show that known DMD families do use prenatal diagnosis to prevent the birth of affected boys. A questionnaire held among DMD families in the Netherlands indicates that parents are in favor of early detection.[15] Introduction of neonatal CK screening of males would improve the chances of prevention of DMD in the future.

Increase in the percentage of first affected patients

The proportion of first affected boys in the family has increased significantly from 62% in the birth cohort 1961–1974[7] to 88% in the cohort 1993–2002. This indicates that the DMD families are more aware of their increased risk and have made use of prenatal tests to prevent the birth of a second affected boy as is shown in Figure 3. It is also possible that some families with one affected child may have decided not to have any more children.

Female fetuses and carrier testing

All professional recommendations agree that carrier testing of minors for X-linked recessive disease should ideally be deferred.[16] The guidelines emphasize that the decision to test should be made by the child when it reaches the age of maturity because in general carrier testing has implications for the future reproductive prospects of that child only and not of her parents. This view is based on the basic ethical principle of informed consent, by which an individual can freely give, without external pressure, her/his consent to be tested after being informed of the benefits, risks, procedures and other pertinent information relating to the carrier test. It is not possible to predict whether or not a female heterozygous for a mutation in the *DMD* gene will manifest any signs of the disorder. Also, being a carrier of DMD is not lethal, as it is for boys who are affected.

The current policy for DMD, therefore, is that female fetuses are not tested prenatally for carrier status.[17] The parents are informed that their daughters can be tested from the age of 16, because from that age onward carrier females should be screened every 5 years for cardiomyopathy.[18] However, in practice the vast majority of the girls (78%) who are beyond the age of 16 years have not yet been tested (Figure 1). We have also shown that one in three women at risk of carrying a mutation in DMD families have not been tested.[19] Further studies are planned to find out why potential carriers are not tested in these families.

One might say that the young potential carriers still have time to undergo carrier testing before having offspring as the average age for a woman to give birth to a first child in the Netherlands is 28 years (Statistics Netherlands, www.cbs.nl). However, that does not take into account the risk for cardiomyopathy, which is estimated to be around 10% at the age of 50 years; the youngest described carrier with cardiomyopathy in the Netherlands was 28 years.[19,20] Furthermore, a recent study has shown that the cardiac abnormalities in carrier women are as progressive as in DMD patients.[20] These women may not be aware of the risk and implications of being a carrier. For some women it may be too late as they may find out that they are carriers only after DMD is diagnosed in a son or after experiencing (sometimes severe) cardiac problems.[21] A proactive approach of using a genetic register service to contact the girls when they reach adulthood, as is done in the United Kingdom,[22] should be considered.

Parents in DMD families have great difficulty in communicating with their children about the disease and its implications for the future.[23] They may struggle with the question of how to tell their daughter that she has a risk of being a carrier. Our study has shown that the average risk of being a carrier is 28%. So, it is cruel to subject the parents to an ordeal, lasting years with this dilemma while the average chance of not being a carrier is 72%. This problem could be solved if the parents are given the choice of having their daughters tested before adulthood at a time that is convenient for them, either neonatally or later. In the families where fetal sexing has been done on chorion villi or amniotic fluid and fetal DNA is available, the possibility of carrier testing on female fetuses should be offered.

Future prospects

The majority of DMD patients are now the first in their family. So, prenatal diagnosis could not have been used as a means of prevention in these cases. Boys with a *de novo* mutation can only be detected if every pregnant woman is offered a prenatal test for the *DMD* gene. This is not realistic at present, but it may change in the future with the development of effective methods of testing the fetal genome in the maternal serum.[24] The problem of *de novo* mutations in women could be solved by testing every prospective mother for mutations in the *DMD* gene before conception, as is now increasingly possible for severe autosomal recessive diseases.[25] However, this would require huge investments and is perhaps something for the future. Although prenatal screening has been used to prevent the birth of a second affected boy in the family, this was not possible in a large number of the cases because the older boy had not yet been diagnosed as he was younger than 5 years. For these cases, neonatal screening would seem the easiest solution at present.

The difficulty is that neonatal screening for an untreatable disease is prohibited by law in the Netherlands because of the requirement that the child in question should derive real benefit from screening. The situation may be about to change as treatments for DMD are now in an advanced stage of development;[26–28] thus, neonatal screening may become feasible even under the current legal restrictions. With this expectation and the knowledge that the birth of the second affected child can be prevented perhaps a case could be made for introducing neonatal screening for boys sooner rather than later.

The most pressing matter for concern at present is that many girls have not been tested. Considering the arguments given above, we propose a change in policy, namely, that the parents should be given the choice of having their daughters tested either prenatally if possible or postnatally, also before adulthood when requested.

CONCLUSIONS

There was no significant change in the incidence of dystrophinopathies in the Netherlands as a result of prenatal testing and genetic counseling. However, we found evidence indicating that prenatal testing has been used by families to prevent the birth of a second affected boy. In the 26 years 145 male fetuses were aborted and 174 unaffected boys were born with the help of prenatal testing. Our study has revealed two problems: (1) A high proportion of families with *de novo* mutations in the *DMD* gene cannot make use of prenatal diagnosis, exacerbated by the fact that older affected boys are usually not diagnosed before the age of five; (2) Current policy dictates that carrier testing is not done in female fetuses; the girls in DMD families may be tested at 16 years. In practice, however, 78% of these girls who are 16 years or older have not been tested. A carrier woman has a risk not only of having a son with DMD but also of having progressive cardiac disease herself. These facts, together with recent developments that suggest DMD could become treatable in the near future, point to an urgent need for a change in policy to improve the chances of prevention of DMD in the future. The first is to introduce neonatal CK screening of males and the second is to test females that are at risk for carrier status prenatally if fetal DNA is available or postnatally even before adulthood if requested by the parents.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Chapter 5

Becker muscular dystrophy patients with deletions around exon 51; a promising outlook for exon skipping therapy in Duchenne patients

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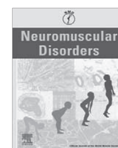

Case report

Becker muscular dystrophy patients with deletions around exon 51; a promising outlook for exon skipping therapy in Duchenne patients

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ABSTRACT

Theoretically, 13% of patients with Duchenne muscular dystrophy may benefit from antisense-mediated skipping of exon 51 to restore the reading frame, which results in the production of a shortened dystrophin protein. We give a detailed description with longitudinal follow up of three patients with Becker muscular dystrophy with in-frame deletions in the *DMD* gene encompassing exon 51. Their internally deleted, but essentially functional, dystrophins are identical to those that are expected as end products in DMD patients treated with the exon 51 skipping therapy. The mild phenotype encourages further development of exon 51 skipping therapy.

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1. Introduction

Mutations in the dystrophin-encoding *DMD* gene on the X chromosome result generally in Duchenne muscular dystrophy (DMD) if the mutation is out-of-frame and in Becker muscular dystrophy (BMD) if the mutation preserves the translational reading frame. In BMD patients an altered form of dystrophin is present, whereas in DMD patients dystrophin is virtually absent in muscle fibers. Absence of dystrophin in a muscle biopsy has an unfavorable prognosis, as DMD patients become wheelchair bound before the age of 13. The clinical phenotype in BMD is milder with a large variation in clinical severity.

Currently, new therapeutic strategies, such as antisense-mediated exon skipping, are in an early phase of clinical trials and have the potential to change the course of the DMD disease dramatically. In our recent study, intramuscular injection of an antisense oligonucleotide (AON) induced skipping of exon 51 and restored the disrupted open reading frame and therefore the production of dystrophin in four DMD patients with deletions of exons 48–50, 49–50, 50 and 52, respectively [1]. Clinical trials with systemic administration of AON are taking place. If successful, therapeutic skipping using an AON that targets exon 51 can stop further muscle wasting, resulting in a clinical phenotype like BMD. This AON can be applied in about 13% of the DMD patients [2]. Therefore, focusing on the functionality of the probable end product through studying corresponding Becker phenotypes is useful and will provide information for patients eligible for this new therapy. This would concern the in-frame deletions of exon 45–51, 47–51, 48–51, 49–51, 50–51, 51–52, 51–58, 51–61 and 51–63 that all are predicted to result in a BMD phenotype.

We describe the clinical phenotype in two BMD pedigrees in the Netherlands carrying deletions including exon 51. We also provide a review of BMD patients with these deletions reported in the literature.

2. Patients and methods

2.1. Methods

Since the availability of DNA diagnostics in 1984, more than 1500 Dutch DMD/BMD families have been tested in our laboratory. The laboratory database was searched to find patients with in-frame deletions including exon 51. After obtaining informed consent, data on clinical history and neurological examinations were extracted from clinical files and/or obtained directly from the patients by the authors (C.S. and A.H.-E.).

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Data from additional patients were collected by searching the literature and by consulting the international DMD database at the Leiden Muscular Dystrophy pages (http://www.DMD.nl) [3].

Where ever possible, additional information was obtained from the authors who have published since 1993 on patients with either a deletion of exon 45–51 or of exon 50–51 and submitted to the database.

3. Results

3.1. Description of Dutch pedigrees

In the Dutch population we found one pedigree with a deletion of exons 45–51 and one with a deletion of exons 50–51. Up until February 2009 no Dutch BMD patients had been registered with in-frame deletion of the exons 47–51, 48–51, 49–51, 51–52, 51–58, 51–61 and 51–63.

3.1.1. Family 1: deletion exons 45–51

Patient A1, born in 1962, had suffered from painful muscle cramps in his legs since childhood. Cramps were mostly provoked by exercise such as hiking or occured after he had bumped his leg. Cramps resulted in painful nodules and could last for one and a half hour. Sometimes his father had to carry him home from school. He did not participate in sports and did not like running. Neurological examination at the age of 14 showed no muscle weakness. A biopsy of the quadriceps muscle at this time showed dystrophic features, with groups of necrotic fibers and groups of regenerating fibers as well as local increase of endomysial fibrous connective tissue. In 1990 Western blotting showed a slightly reduced amount of dystrophin with a smaller molecular weight. DNA analysis identified an in-frame deletion of exons 45–51 in the *DMD* gene and confirmed the diagnosis of BMD. He now works as a truck driver and rarely suffers from cramps and is not limited in his daily activities, although he avoids jumping from his truck or climbing more than two stairs.

In childhood he had trouble concentrating, was hyperactive and attended a primary school for children with educational problems. Subsequently he succeeded in obtaining a certificate from a regular technical school.

A neurological examination in 2008 was unremarkable except for calf hypertrophy. His creatine kinase (CK) which had increased 50-fold in 1976 was only marginally increased in 2008 (243 with a reference value up to 200 U/l). Cardiac examination including echocardiography showed no abnormalities. MRI showed normal aspect of the shoulder muscles and the muscles of the leg. There were minimal fatty changes in the hip extensors (Fig. 1).

His maternal grandfather (patient A2), born 1913 and deceased 1993, carried the same mutation. He too had suffered from cramps in childhood and adolescence. At the age of 78 he was examined by the late Prof. HFM Busch, neurologist. The grandfather showed no neurological signs or symptoms of BMD and his CK values were normal.

3.1.2. Family 2: deletion exons 50–51

Patient B1, born in 1994, visited a pediatrician at the age of 8 because of hyperactive behavior. His past history was unremarkable including normal motor milestones; he was able to walk unsupported at the age of 18 months. Routine laboratory examination revealed increased transaminases and subsequently 8-fold increase in CK. Neurological examination in 2003 was unremarkable. DNA analysis showed an in-frame deletion of exons 50–51 in the *DMD* gene.

In 2008 he sporadically suffered from muscle cramps but did not complain about muscle weakness. He cycled to school every day for more than an hour each way, including riding uphill. He enjoyed climbing trees to help with pruning the branches, and played in a soccer team. Neurological examination showed normal muscle strength. There was no calf hypertrophy. ECG and echocardiography were normal. His mean time on a timed run test of 10 m was 2.6 s, (reference: mean time to run 9 m for healthy boys of 11 years is 2.5 ± 0.28 s) [4].

Behavior problems were diagnosed as attention deficit hyperactivity disorder (ADHD) by a child psychiatrist. Cognitive tests showed a subnormal IQ of 80 with performance IQ lower (77) than verbal IQ (90). For hyperactivity he was treated with methylphenidate (Ritalin) 54 mg/day, 5 days a week. He attended a primary school for special education. He now follows regular secondary education. He is still hyperactive during the weekend when he is off methylphenidate.

His mother carries the same BMD mutation. He has a healthy brother, born 1992, who has not been tested.

3.2. Patients from the DMD database at the Leiden Muscular Dystrophy pages and from published reports

The DMD gene variant database at the "Leiden Muscular Dystrophy pages, http://www.DMD.nl" [3] is a public repository of variants reported in literature or submitted directly to the database. We used

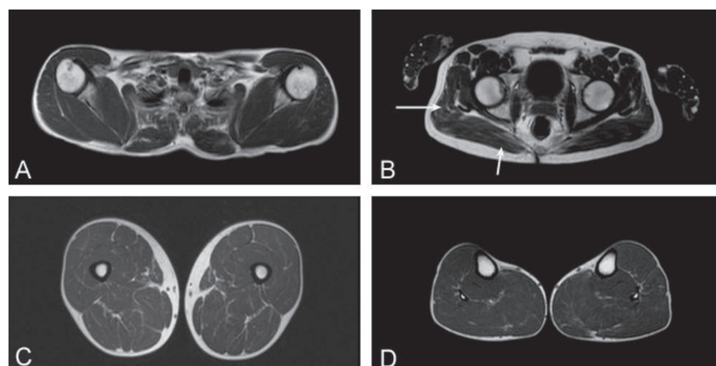

Fig. 1. MRI images of the muscles in patient A1. MRI axial T1-weighted images of the muscles in patient A1 at age 46 shows at the level of (A) the shoulder: normal volume and signal intensity of the muscles, (B) the hip joint: minimal fatty changes in the gluteal muscles (arrows), (C) upper leg: normal volume and signal intensity of the muscles and (D) lower leg: normal volume and signal intensity of the muscles.

this valuable resource to trace 57 patients with a deletion of exons 45–51 or 50–51, including our three patients. On February 3rd 2009 the DMD database listed 27 entries with a deletion of exons 45–51 (including 50 patients) and six entries with a deletion of exons 50–51(7 patients); 21 of the 33 submissions were from published reports. After contacting the authors, six patients were excluded for further analysis; in two patients further testing with MLPA (Multiplex Ligation-dependant Probe Amplification) refined the mutations originally detected by PCR to deletions of exons 45–54 and 49–51. One submitter reported that two patients could not be traced back in his database. Furthermore, two patients were excluded because they appeared to have a Duchenne phenotype; their genotype was tested with multiplex-PCR and might be not reliable enough. One submission appeared to be a monozygotic twin. All new findings were reported to the DMD database and used to update the records. In summary, 50 patients were traced using the information in the DMD gene variant database.

Our literature search identified six more patients with deletions of exons 45–51 [5–7] and six more patients with deletion exons 50–51 in the literature [8,9].

We made an overview of the 19 patients of whom we obtained clinical information (Tables 1 and 2). These 19 BMD patients came from 12 families. For 43 patients no further information was available.

4. Discussion

Our study shows that BMD patients with 45–51 or 50–51 inframe mutations have a mild phenotype.

The clinical phenotype of the three Dutch patients, including the grandfather shows certain similarities. The course of the disease is mild with muscle cramps without muscle weakness and hyper-CK-emia being the main features. As adults these patients showed no clear evidence of progression of the disease with age. None of the patients experienced major limitations in daily life. Intolerance to exercise appears to restrict patient A1. Although both patients A1and B1 have had behavioral or attention problems at primary school, they attended regular secondary school and patient A1 functions normally as an adult. Behavioral and attention

Table 1
BMD patients with a deletion of exons 45–51.

Patient number	Reason referral	Age at presentation (yr)	Last examination at (yr)	Symptoms	CK (IU/L) highest	Cardiac echography	Test method	Result muscle biopsy	Reference
A1	Muscle cramps	4	45	Cramps	2200	Normal	MLPA	Dystrophin present	Patient A1 present study
A2	Grandfather patient A1	78	78	Cramps	Normal		M-PCR		Patient A2 present study
A3	↑CK	5	5	Contractures, mild proximal muscle weakness, mild calf hypertrophy	1700		M-PCR	Dystrophin present	[7]
A4	↑CK	8	8	No	2950	Normal	M-PCR	Dystrophin present	[6]
A5	Cousin of patient A4	7	7	No	896	Normal			[6]
A6	Grandfather patient A4	67	67	No	63	Normal			[6]
A7	Pain on exercise	8	8	Pain, mild calf hypertrophy	1000	Normal	M-PCR	Dystrophin present	[21]
A8	↑CK	10	11	No			M-PCR	Dystrophin present	[5] patient B-19
A9	Myoglobinuria	14	14	No			M-PCR	Dystrophin present	[5] patient B-25
A10		10			2648		M-PCR		DMD database P.J.Taylor
A11	↑CK	4	7	Mild calf hypertrophy	3375	Normal	MLPA	No muscle biopsy	DMD data base Kekou Kanavakis
A12	↑CK	4	16	No	810	Normal	M-PCR	Dystrophin present	[22]

Abbreviations used: MLPA, Multiplex Ligation-dependant Probe Amplification; M-PCR, Multiplex Polymerase Chain Reaction; CK, Creatine Kinase.

Table 2
BMD patients with a deletion of exons 50–51.

Patient number	Reason referral	Age at presentation (yr)	Last examination at (yr)	Symptoms	CK (IU/L) highest	Cardiac echography	Test method	Result muscle biopsy	Reference
B1	↑CK	8	13	Cramps	1533	Normal	MLPA	No muscle biopsy	Patient B1 present study
B2	Limping falling	2	4	Yes	1300	Normal	M-PCR	Dystrophin present	[8]
B3	Grandfather patient B2	69	69	No		Normal	M-PCR		[8]
B4	Great uncle of patient B2	55	55	No			M-PCR		[8]
B5	Cousin of mother of patient B2	28	28	No			M-PCR		[8]
B6	Maternal cousin mother of patient B2	29–55	29–55	No			M-PCR		[8]
B7	Maternal cousin DMD patient with another mutation	18	18	No	327		M-PCR	Dystrophin present	[9]

Abbreviations used: MLPA, Multiplex Ligation-dependant Probe Amplification; M-PCR, Multiplex Polymerase Chain Reaction; CK, Creatine Kinase.

problems appear to occur more frequently in boys with Becker muscular dystrophy than in the general population [10].

Also the patients reported in the literature had a mild course of the disease. At older age they did not show any symptoms of BMD and had a normal result of cardiac examination [6,8].The low prevalence of known in-frame mutations encompassing exon 51 in the Dutch population could well be an underestimation. Patients with a comparable mild phenotype as the ones we have described might not have been recognized as a possible BMD and may never have visited a neurologist or a pediatrician.

Our study clearly shows that previous publications are not always correct regarding the extent of the deletions reported. Until recently deletions were mainly detected using methods like Southern blotting, multiplex-PCR (Chamberlain and Beggs sets [11,12] and quantitative multiplex-PCR. MLPA [13] is much more accurate and ideally should be used to confirm the deletions and, more importantly regarding clinical outcome, to reliably determine its boundaries. Furthermore, only analysis at RNA level will be able to confirm that the translational reading frame remains intact.

It should be noted that the public availability of the DMD database was an enormous help for our study. It gave a clear overview of the findings thus far and an easy resource to contact the groups having patients of interest. Of course it is unfortunate that not all data reported are correct, but the database itself is not to blame for this. To support the database we immediately reported additions and inconsistencies for inclusion or correction.

Gathering information about the phenotype of BMD patients has become relevant now that exon skipping therapies are being developed for DMD patients. Since exon skipping is mutation specific, each specific exon skip will result in a different shortened variant of dystrophin. Therefore, information on the best target exon for individual DMD patients may be found by examining BMD patients with different dystrophin variants. Since the first clinical trials are aimed at skipping exon 51, we have focused on BMD patients with a mutation encompassing this region. In fact, one of the DMD patients that was treated in the first human exon skipping trial [1] had a deletion of exon 50. After local treatment with antisense PRO051 the reading frame was restored and he produced dystrophin which should be similar to the dystrophin of patient B1 in our study. This demonstrates how one exon made the difference of being wheelchair bound at age 9 or climbing trees at age 14. The ultimate goal is to develop specific treatments to restore dystrophin production in DMD patients.

Besides, it is important to realize that these findings are mutation specific as a smaller in-frame deletion in this same area, such as a deletion of exons 48–49, results in X-linked dilated cardiomyopathy [14]. Also the in-frame deletions of exon 51–61 and 51–63 will most probably result in a moderate to severe phenotype, because they are close to the cysteine rich region (exon 64–70) which might affect the binding of dystrophin to β-dystroglycan [15]. Mice with a deletion of such a cysteine rich region have muscles with severe dystrophic pathology [16]. Furthermore, a deletion of exons 45–55 is associated with a BMD phenotype with a prognosis of a favorable outcome [17], but can also be associated with X-linked dilated cardiomyopathy depending on the exact location of the breakpoints of the deletion [18,19].

Given the many different mutations, patients will benefit highly of multidisciplinary cooperation that results in databases with information on genotype as well as on phenotype [20]. For the dystrophinopathies good databases will facilitate and accelerate the inclusion of patients in clinical trials. This underlines the importance of setting up national patient registries, which can contribute to an international database. A good example of such a database is the TREAT NMD network of excellence (http://www.treat-nmd.eu/home.php).

In conclusion, the phenotype of BMD patients with a deletion of exons 45–51 and 50–51 appears to be mild. This is encouraging for the outcome of the exon 51 skipping trials and offers hope to DMD patients who are eligible for this therapy.

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Chapter 6

Summary, discussion and future prospects

Chapter 6 Summary, discussion and future prospects

6.1 Summary and discussion

Dystrophinopathies include the well known Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD). The incidence of DMD, which is a progressive neuromuscular disease, is roughly 1 in 3,500 live male births (Emery, 1991), in the Netherlands 1 in 4685 (Helderman-van der Enden et al., 2012). Most of the patients are diagnosed around the age of five (Magri et al., 2011) and are wheelchair-bound before the age of 13. The mean age of survival is 25 (Eagle et al., 2002). The incidence of BMD, a milder variant of DMD, is 1 in 18,450 live male births (Bushby et al., 1991). The onset of BMD is usually between the ages of 5 and 15 but may also be later (Bushby and Gardner-Medwin, 1993). Dystrophinopathies show an X-linked recessive pattern of inheritance. Mutations in the *DMD* gene generally lead to DMD if the mutation is out-of-frame and to BMD if the translation reading frame is preserved (Malhotra et al., 1988; Monaco et al., 1988).

This thesis is a collection of several clinical and genetic studies on dystrophinopathies with implications for genetic counselling of patients and their families and for future therapy (e.g. personalized medicine) of patients suffering from this group of chronic progressive muscle diseases.

6.1.1 Mosaicism

6.1.1.1 Germ line mosaicism

Different frequencies have been reported for germ line mosaicism in DMD. A reliable estimate of the recurrence risk of new mutations, obtained preferably by empiric studies of large numbers of families, is important for genetic counselling.

In Chapter 2.1 we have described the largest number of dystrophinopathy families known to date with a *de novo* mutation. Germ line mosaicism is an important pitfall that should be considered when counselling families with a *de novo* mutation.

A literature search on germ line mosaicism revealed that an estimate for the recurrence risk has been calculated for only seven out of 63 diseases with documented germ line mosaicism. Two are autosomal dominant, with a risk of 0.02% for achondroplasia (Mettler and Fraser, 2000) and 5-7% for osteogenesis imperfecta (Byers et al., 1988). The other five are X-linked. The risk is 11% for double cortex X-linked lissencephaly syndrome (Gleeson et al., 2000), for RETT syndrome (Mari et al., 2005), and for haemophilia B (Ketterling et al., 1999) and 13% for haemophilia A (Leuer et al., 2001). Until recently the reported recurrence risk for DMD, if the risk haplotype is transmitted, varied from 14 to 20% (Bakker et al., 1989; van Essen et al., 1992). It is remarkable that the recurrence risk is reported more often for X-linked inherited diseases and that the risk for these diseases is higher. Most reported recurrence risks are expressed as the percentage of *de novo* families that have germ line mosaicism. To obtain a more reliable recurrence risk in our DMD families we divided the number of times the risk haplotype was transmitted with the mutation by the total number of times that the risk haplotype has been transmitted (with or without the mutation) in the *de novo* families. With this method we obtained an overall recurrence risk

of 8.6% (95% confidence interval 4.8 – 12.2%) if the risk haplotype is transmitted. In *de novo* DMD/BMD families that have not been haplotyped the recurrence risk is 4.3%.

Factors that could influence the recurrence risk in germ line mosaicism

Origin of the mutation:
In the 18 proven germ line mosaicism families in our study most mutations, 14/18 (78%) originated in the mother and the remaining 4/18 (22%) in a grandparent (all four in the grandmother). These percentages are comparable to those found in the whole group of 318 families with a *de novo* mutation (73% maternal, 27% grandparental). Of the 86 in the grandparental group, 40 occurred in the grandmother, 27 in the grandfather and in 19, the origin could not be determined. We conclude that the number of *de novo* families with proven germ line mosaicism in our study is too small to determine whether the origin of the mutation has any influence on the recurrence risk in germ line mosaicism.

Type and location of the mutation:
Among the families with proven germ line mutations, 14/18 were deletions (77%), 3/18 were duplications (17%) and 1/18 was a point mutation (6%). The percentage of deletions and duplications are comparable to the expected ratio of mutations in the *DMD* gene, i.e. 72% deletions and 7% duplications, but the number of point mutations is smaller than the expected ~20%. The number of proven germ line mutations in our study group is too small to draw any definite conclusion as to whether the type of mutation can influence the recurrence risk in germ line mosaicism.
There was a remarkable difference in the recurrence risk between deletions that were located proximally (7.8%) and those that were distally located (3.2%). It has been speculated that proximal deletions arise earlier in embryogenesis than distal ones and are therefore more likely to be found also in germ cells. (Passosbueno et al.,1992). The recurrence risk is 6.1% for duplications and 2.2% for point mutations. It appears, therefore, that the location of the mutation may play a role in the recurrence risk in germ line mosaicism.

The percentage of de novo cases:
The rate of new mutations for DMD is 33% and the recurrence risk in cases of germ line mosaicism is 4.3%. On the other hand, achondroplasia is *de novo* in 80% of cases whereas the recurrence risk due to germ line mosaicism (0.02 %) is very low (Mettler and Fraser, 2000). There appears to be no association between a high percentage of *de novo* cases and a high germ line recurrence risk. The *de novo* mutations in case of achondroplasia are apparently a result of *de novo* events during spermatogenesis in the unaffected father rather than of germ line mosaicism in the father (Francomano, 2006). It is possible that one of the factors that influence the recurrence risk due to germ line mosaicism is the proneness of the gene to undergo mutations.

In general, we conclude that more studies are needed to elucidate the factors that influence the level of the recurrence risk due to germ line mosaicism in different diseases.

6.1.1.2 Somatic mosaicism

A mutation in one of mitotic divisions of the zygote can give rise to somatic mosaicism in the patient. In Chapter 2.2, we have presented a 30-year old ambulatory patient with somatic mosaicism. The first indication that we may be dealing with mosaicism came from the immunohistochemical dystrophin analysis of the muscle biopsy, which showed a mixture of dystrophin positive and dystrophin negative fibres. DNA analysis revealed a nonsense mutation (c.9554C>T, pQ3116X) in the *DMD* gene. This mutation is described as c.9346C>T, p.Gln3116X according to the new nomenclature. It disrupts the reading frame which would normally lead to full DMD, but the phenotype of the man was milder. We considered BMD because of the mild phenotype but excluded it because patients with BMD have progressive symmetrical muscular weakness and contractures appear later, namely when patients become wheelchair-bound. Our patient had asymmetric weakness and prominent contractures while he was still ambulant. In fact, the mild phenotype of our patient was due to somatic mosaicism. To our knowledge, only two cases with somatic mosaicism of the *DMD* gene have been reported since our case was published, indicating that patients who show clinical manifestation of somatic mosaicism are rare (Deburgrave et al., 2007; Kesari et al., 2009).

6.1.2 Dystrophinopathy in the family: have potential carriers been tested?

In Chapter 3, we have shown that even in the Netherlands with its well-organized genetic counselling services, there are still a large number of potential carrier women in DMD/BMD families who have not been tested at the DNA level. In the DMD families that were analyzed, 55% of the women with a 50% risk of being a carrier and 43% of the women with 4.3% risk due to germ line mosaicism had not been tested. In general, it is agreed that carrier testing in minors should be deferred until the child can give proper informed consent (Borry et al., 2006). However, even if we consider only women older than 16, 34% with a 50% risk and 30% with a germ line risk had not been tested. Therefore, one in three potential adult carriers (sisters and or maternal aunts) in DMD families has not had a DNA test.
Given the possible far-reaching clinical consequences of being a carrier, we have planned further studies to investigate the reasons why potential female carriers have not been tested.

6.1.3 An urgent need for a change in policy revealed by a study on prenatal testing for Duchenne muscular dystrophy

In Chapter 4, we have examined the impact of 26 years of prenatal testing for Duchenne and Becker Muscular Dystrophy in the Netherlands. We analysed information on all prenatal diagnoses performed from 1984 to 2009. Of the 635 prenatal diagnoses performed in this period, 51% were males. Of these, nearly half (46%) were affected or had an increased risk of having DMD/BMD. As a result, 145 male foetuses were aborted and 174 unaffected boys were born. The most significant positive effect of prenatal testing was the birth of these 174 unaffected boys. The introduction of prenatal testing for the familial mutation has spared the parents the emotional burden of having to abort a possibly healthy boy. For a few families pre-implantation genetic diagnosis offered the possibility of having children without the burden of any abortion.

To determine the impact of prenatal testing on the incidence of DMD, we compared the incidence in the sixties to that in the nineties. There was no significant difference in incidence of DMD between the first (1961-1974) and the second period (1993-2002). We did find, however, that the percentage of first affected boys in the DMD families increased from 62% in the first period to 88% in the second period, 1993-2002. These figures show that although there was no apparent decrease in the incidence of the disease, prenatal diagnosis was used by DMD families for preventing the birth of a second affected boy.

Women who do not have an increased risk of being a carrier are not offered the possibility of prenatal testing for a dystrophinopathy. This applies to *de novo* families. It has been always assumed that one in three DMD patients has a *de novo* mutation (Haldane, 1935). In fact, this figure of 33% may be an underestimate and the real figure may be as high as 57% (Alcantara et al., 1999; Taylor, 2008). Perhaps this discrepancy can be explained by the fact that in the last decades, the families are smaller and family planning and prenatal testing are possible.
However, even if we say that the risk is 33%, the final figure of *de novo* cases is a lot higher if we consider the whole family. The mutation is *de novo* not only in 1/3 of the patients but also in 1/3 of carrier mothers. In theory, the total number of DMD patients who are the first affected in the family is at least 56% (1/3 *de novo* in the patient plus 1/3 x 2/3 *de novo* in the carrier mother). This means that a large proportion of families are unable to make use of prenatal diagnosis to prevent the birth of an affected son because the mutation has occurred *de novo*. In our study of 216 boys with DMD, born in the period 1993-2002, prenatal testing was not an option in 88% of these as they were the first affected in the family. However, it was also not an option for most of the remaining 12% because information about DMD in the older boy was not available at the time of the birth; in almost half of these, the older boy was less than five years old at the time of the birth of the second boy.
Women with a *de novo* mutation can be identified if every woman is tested pre-conceptionally for dystrophinopathy, as is now possible for a number of autosomal recessive diseases (Bell et al., 2011). Boys with a *de novo* mutation can only be detected if every pregnant woman is given a prenatal test. This is not realistic at present, but it may change in the future with the development of effective methods for testing the foetal genome in maternal serum (Lo et al., 2010).
Another important finding in our study is that 78% of the females, who were prenatally identified as potential carriers and who at the end of our study period (2009) would have been between 16 and 26 years of age, have not yet been tested for carrier status.

In short, our study has revealed that 1) a high proportion of families with *de novo* mutations in the *DMD* gene cannot make use of prenatal diagnosis, partly because the older affected boys are diagnosed at around five years, and 2) a large proportion of female potential carriers have not been tested. One can expect that there are a multitude of different obstacles hampering the cascade screening of DMD families, and the testing of potential carriers following prenatal diagnosis. At present, we do not know the most important impediments and how they can be circumvented. What we do know is that there is a need for a change in policy. We propose

that 1) neonatal screening for dystrophinopathy in males should be started in the Netherlands and 2) parents should be given the choice of having their daughters tested prenatally, in cases where foetal sexing has been done on amniotic fluid or chorionic villi, or postnatally, even before adulthood.

6.1.4 Becker muscular dystrophy patients with deletions around exon 51.

Chapter 5 zooms in on exciting developments in the field of personalized medicine for Duchenne muscular dystrophy. Theoretically, 83% of patients with DMD may benefit from exon skipping therapy, which can restore the reading frame in the patient (see 6.1 above) (Aartsma-Rus et al., 2009). Antisense-mediated skipping of exon 51 restores the reading frame and results in the production of a shortened dystrophin protein (Aartsma-Rus et al., 2009). We have given a detailed description of a longitudinal follow up of three patients with Becker muscular dystrophy with in-frame deletions encompassing exon 51 in the *DMD* gene. The internally deleted, but essentially functional, dystrophins in these BMD patients are identical to those that are expected as end products in DMD patients treated with the exon 51 skipping therapy. The mild phenotype of the three described patients encourages further development of exon 51 skipping therapy. One of our patients is included in an international follow-up study of quantification of dystrophin in 17 BMD patients with deletions around exon 51. The results indicate that the three types of internally deleted dystrophins assessed in the study have the functional capability of providing a substantial clinical benefit to DMD patients (Anthony et al., 2011).

6.2 Future prospects

At present, the most common dystrophinopathies are still serious life-shortening diseases for which curative therapy is not yet available. Therefore, much emphasis has been placed on prevention. This is only possible through adequate genetic counselling and prenatal testing of known families.

In this thesis (see 6.1.2 and 6.1.3 above) we have shown that we need to address two points: 1) early detection of boys with DMD caused by *de novo* mutations with a view to preventing the birth of subsequent affected boys in the family and also for possible therapy in the future (see 6.2.1 below). 2) DNA testing of potential carriers (see 6.2.2 below).

6.2.1 Early detection of *de novo* DMD boys: neonatal population screening for dystrophinopathy

We have shown (see 6.1.3 above) that the birth of a second affected boy could have been prevented if DMD had been diagnosed early in an older affected boy in the family. The percentage of boys with an older affected relative varies in the literature: for example 13% (16/122) in North-East England (Gardner-Medwin et al., 1978) and 33% (61/181) in Iowa (Zellweger et al., 1982). In our cohort of 216 boys born between 1993 and 2002, the birth of 27 affected boys (12%) could have been prevented by means of prenatal testing if the older affected boy had been detected by neonatal screening.

In general, minors are not tested for a genetic disease unless it has consequences for the

child at the time of testing (preventive surgery or early therapeutic intervention) (Borry et al., 2008). Although prospective parents in the Dutch population seem to be interested in neonatal screening for untreatable childhood-onset disorders (Plass et al., 2010), DMD is not included in the neonatal screening program in the Netherlands at present. This is because so far it is considered to be a non-treatable disease (The Dutch Health Council: Neonatal Screening, 2005). We expect that with developments in personalized medicine, such as exon skipping therapy (Aartsma-Rus, 2010; Goemans et al., 2011; Guglieri and Bushby, 2010; Van Deutekom et al., 2007), the options for early treatment of dystrophinopathies will become available in the near future. If that happens, early diagnosis will become essential also for the patient.

While preparing this thesis, we counselled a Dutch family with a history that illustrates the importance of neonatal screening for the prevention of the birth of subsequent affected children in a family.

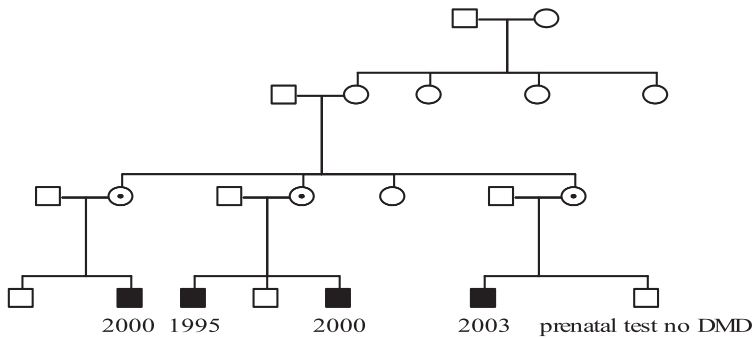

Figure 1:
Pedigree of a Dutch family illustrating the importance of neonatal screening for the prevention of the birth of subsequent affected children (with the kind permission of the family).
□ *male,* ○ *female,* ■ *affected male,* ⊙ *carrier female*

The first patient was born in 1995 but it was only in 2002, at the age of seven, that DMD was diagnosed; the delay was due to other health problems.

After his diagnosis, it became clear that one of his younger brothers and a cousin, both born in 2000, were also affected. In 2003, some months after the diagnosis of the index patient, another affected cousin was born in the family. According to their respective parents (personal communications) if the index patient had been diagnosed by neonatal screening the three subsequent affected boys would most probably not have been born.

A questionnaire among Dutch Duchenne families showed that most parents are in favour of neonatal screening. All the parents wished they had known the diagnosis earlier, preferably before the child was two years of age. They regretted having treated their child the way they did during the period in which they were unaware of his disorder. Early diagnosis would have enabled parents to treat their child in a way most suited to his condition ('good parenting'). This need for 'good parenting' expressed by the parents by far outweighed the possibility of enjoying a carefree period with their child (Plass et al., 2010).

Table 1 shows the published results from neonatal screening for dystrophinopathies in various countries (including those where a pilot has been performed); the percentage of affected neonates is very similar.

Country	Period	number of tested male neonates	number of affected (percentage)	Incidence	Reference
Belgium (Antwerp)	1979-2003	218214	49 (0.02%)	1:4453	(Eyskens and Philips, 2006)
Germany	1977-2010	500000	200 (0.04%)	1:2500	(Scheuerbrandt, 2011)
United States of America (Ohio)	2005-2007	4983	2 (0.04%)	1:2492	(Al-Dahhak et al., 2008)
United Kingdom (Wales)	1990-2011	300000	50 (0.02%)	1:6000	(Meeting discusses newborn screening for Duchenne muscular dystrophy, 2011)
Cyprus	1992-1997	30014	5 (0.02%)	1:6003	(Drousiotou et al., 1998)
Canada (Manitoba)	1986-1987	18152	5 (0.03%)	1:3630	(Greenberg et al., 1988)

Table 1
The number and percentage of DMD patients identified during neonatal screening in different countries.

In 2004 a working group of experts in the field of neonatal screening and dystrophinopathies met in Atlanta, USA and agreed on the following key points about newborn screening for DMD (Newborn Screening for Duchenne Muscular Dystrophy Workgroup: Lay Report, 2004):

- An earlier age at diagnosis of DMD might improve the quality of life of families.
- There are no universal early signs or symptoms by which all children with DMD can be recognized by paediatricians at an early age.
- The current newborn screening standards do not justify mandatory newborn screening for DMD given the lack of evidence of medical benefit to the child.
- Voluntary newborn screening for DMD, as a supplement to mandatory newborn screening programs, might be problematic because (1) the period immediately before or after birth is a difficult time to obtain true informed consent, and (2) parents might have trouble distinguishing voluntary testing for DMD from other mandatory newborn screening tests.
- Mandatory and voluntary newborn screening tests ideally should be separated in time and space.
- False or transient-positive screening results might have a negative impact on families.
- Screening later in infancy will reduce the number of false or transient-positive screening results.

- Screening later in infancy might introduce inequities into the system because not all families have equal access to routine paediatric services.
- Identification of appropriate personnel and protocols for informing parents of test results is critical.

It is important that the parents should be informed about the pros and cons of testing in a manner that is analogous to antenatal screening: so that a decision can be made without any feelings of obligation (Plass, 2007).

In principle, the CK screening test could also be offered for female newborns because some female carriers have a high CK. If the CK is high, molecular testing can be done to detect a mutation and to confirm that the girl is a carrier. In this way girls who are carriers of a *de novo* mutation or who have inherited the mutation from a mother who was unaware of being a carrier, can be identified. The birth of affected boys in these families can be prevented with the help of genetic counselling. However, we need more studies before we can introduce neonatal screening for the detection of carrier status in females, since not all carriers are picked up with the CK screening test. Furthermore, it is not possible as yet to predict whether a female carrier will develop any symptoms (Newborn Screening for Duchenne Muscular Dystrophy Workgroup: Lay Report, 2004).

In any case, testing for dystrophinopathies in newborn boys on a voluntary basis could start as soon as possible in the Netherlands.

6.2.2 DNA testing of potential carriers in known families

We have shown that a high proportion of women who are potential carriers have not been tested to determine whether they are carriers or not. These are adult family members of affected males (Chapter 3) or women who were identified prenatally and are now 16 years or older (Chapter 4). It is important to test potential carriers and to inform them of their risk of having an affected son as well as the risk of developing heart problems themselves. A study in the United Kingdom among 33 families with six different genetic diseases has shown that children with DMD receive less information than those with other diseases and that the daughters in the DMD families are not informed about potential carrier risk until they are about 16. The parents of DMD patients carry a heavy burden of having to inform their children, which can be difficult and stressful (Plumridge et al., 2010).

Early testing of potential carriers would greatly reduce the anxiety of parents. We have shown that the average risk of a girl in a DMD family being a carrier is 28% (Helderman-van den Enden et al., 2012). This means that almost three-quarters of the parents struggle for nearly two decades with the dilemma of how best to tell their daughter, while the chance that she is not a carrier is 72%. The problem could be avoided if the parents are given the choice of having their daughters tested before adulthood. Also, families with a daughter who is a carrier will probably be able to prepare themselves better, both emotionally and practically, for informing their daughters (Campbell and Ross, 2005).

In current practice, the consensus on not testing minors (see 6.2.1 above) also holds for carrier testing of girls (Borry et al., 2008). Since the screening for cardiomyopathy should start at

the age of 16, carrier testing of girls at this age is logical (Bushby et al., 2003; Hermans et al., 2010). In the absence of compelling reasons, therefore, carrier testing is deferred until adulthood when the person can give informed consent.

Incidentally obtained genetic information

According to two guidelines on genetic testing, information on carrier status obtained incidentally (e.g., after screening or prenatal diagnosis) should not be disclosed to parents or to third parties (American Medical Association: genetic testing of childeren, 1995; Committee for Public Relations and Ethical Issues of the German Society of Human Genetics: Statement on Genetic Diagnosis in Children and Adolescents, 1995). They recommend that this information should be discussed with the child when he or she reaches reproductive age. This is in contradiction with the recommendations made by the British Medical Association (British Medical Association.Human Genetics: Choice and Responsibility, 1998) and by the American Academy of Pediatrics (Committee on Bioethics, 2001), which state that results obtained incidentally on the carrier status should be conveyed to the parents.
At present, with the introduction of DNA tests in neonatal screening, the doctors are frequently confronted with incidentally obtained results. Some couples may find themselves in a difficult situation because of the above-mentioned contradictory policies. For example a couple may receive information about the carrier status of one child following neonatal screening, but are counselled to delay carrier testing for another child until this child has reached reproductive age (Borry et al., 2007). These situations illustrate the importance of a uniform way of handling.
On the one hand the parents are responsible for discussing the possibilities of carrier testing with their children and on the other hand genetic service providers are supposed to follow up the genetic risk of a family. This could lead to situations where a minor is never informed about his/her genetic risk. Borry et al (2006) emphasize that such situations should be avoided but do not suggest how this should be done.

We suggest that when counselling anyone from a known dystrophinopathy family the possibility of other eligible members for counselling and carrier testing should be discussed. Genetic counsellors should re-contact the family every few years to discuss these possibilities. The parents and their potential carrier daughters should be informed about the pros and cons of testing before adulthood.

6.2.3 Recommendations

- Testing for dystrophinopathies in newborn boys on a voluntary basis should start as soon as possible in the Netherlands. This will reduce the chance of the birth of subsequent affected boys in the family and will also help in early introduction of possible therapy when this becomes available.
- Parents should be given the choice of having their daughters tested prenatally, if foetal DNA is available. They should also be encouraged to have their daughters tested, even before adulthood. This will not only considerably reduce the incidence of DMD but will also ease

the burden for the parents who have to inform their daughter of their future risks.
- Genetic counsellors should have a more active approach to cascade screening and informing the patients and their families of their risks and possibilities.

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6.3 Samenvatting

Dystrofinopathieën zijn geslachtsgebonden erfelijke spierziekten die veroorzaakt worden door mutaties in het *DMD* gen. Dit gen codeert voor het spiereiwit dystrofine.
Voornamelijk jongens/mannen zijn ziek omdat zij maar één kopie hebben van het *DMD* gen op het X-chromosoom. Vrouwen hebben twee X-chromosomen met elk een *DMD* gen. Een mutatie in één van de twee genen geeft meestal geen klachten, omdat er nog een normaal gen aanwezig is.
De meest bekende dystrofinopathie is Duchenne spierdystrofie (DMD). De ziekte komt voor bij 1 op 4685 pasgeboren jongens in Nederland. Jongens met deze ziekte missen het eiwit dystrofine in hun spiercellen. Meestal wordt dit veroorzaakt door een leesraam-verschuivende mutatie in het *DMD* gen, waardoor er geen of nauwelijks dystrofine wordt gesynthetiseerd. De eerste tekenen uiten zich soms al voor de leeftijd van twee jaar in de vorm van laat gaan lopen, de neiging om op de tenen te lopen en vaak vallen. Bij de meeste patiënten wordt de diagnose gesteld rond het vijfde jaar. Opvallend is de kuithypertrofie. Er is toenemende symmetrische spierzwakte waardoor patiënten voor het 12e jaar rolstoelafhankelijk worden. De ziekte is momenteel niet te genezen. Met de huidige behandeling is de gemiddelde levensverwachting ongeveer dertig jaar. Patiënten overlijden aan de gevolgen van niet goed kunnen ademen (door aangedane ademhalingspieren) en/of een niet goed pompend hart.
Becker spierdystrofie is ook een dystrofinopathie. De ziekte heeft een milder beloop dan DMD. Becker patiënten worden per definitie niet rolstoelgebonden voor het 16e jaar en een groot aantal van hen komt ook nooit in een rolstoel terecht. Wel kunnen zij hartklachten krijgen zoals bij DMD. Soms treden de hartklachten zelfs meer op de voorgrond dan de spierzwakte.
In de spiercellen van Becker patiënten is wel (gedeeltelijk) functioneel dystrofine aanwezig, maar meestal is het dystrofine eiwit kleiner dan normaal en in kleinere hoeveelheden aanwezig. In de regel wordt dit veroorzaakt door een mutatie in het *DMD* gen die het leesraam intact laat.
Vrouwen die draagster zijn van een mutatie in het *DMD* gen krijgen meestal geen spierklachten. Wel hebben zij een kans van 10% om gedurende het leven hartklachten te ontwikkelen. Om die reden komen zij in aanmerking voor regelmatige cardiologische controles vanaf de leeftijd van 16 jaar.

In dit proefschrift worden een aantal studies beschreven die van belang zijn voor het geven van erfelijkheidsadvies aan patiënten en hun familieleden en één studie die relevant is voor de naar verwachting op niet al te lange termijn in te voeren *exon skipping* therapie.

Mozaïcisme

Kiemcelmozaïcisme

Kiemcellen zijn geslachtscellen, zaadcellen bij de man en eicellen bij de vrouw. Bij kiemcelmozaïcisme heeft een persoon meerdere soorten geslachtscellen: normale

geslachtscellen en geslachtscellen met een mutatie. Indien een geslachtscel met een mutatie bevrucht wordt, kan de bevruchte eicel uitgroeien tot een kind met een erfelijke ziekte.
De kans voor elke vrouw op een zoon met DMD is ongeveer 1 op 10.000, terwijl de kans op een DMD zoon voor een vrouw die draagster is van een mutatie in het *DMD* gen 50% is. Bij ongeveer een derde tot de helft van de Duchenne patiënten is de ziekte ontstaan als gevolg van een nieuwe mutatie in het X-chromosoom in de eicel van de moeder. De moeder is dan geen draagster van deze mutatie in haar bloedcellen. Soms blijkt dat zij wel meerdere eicellen heeft met deze mutatie naast normale eicellen zonder de mutatie. Voor de groep vrouwen met een zoon met een nieuw ontstane mutatie, is de kans op een tweede zoon met Duchenne hoger dan het populatie risico. In 1989 werd in 28 Nederlandse families met een nieuwe mutatie berekend dat de kans om de mutatie een tweede keer door te geven ongeveer 7% was.
In **hoofdstuk 2.1** wordt de grootste groep dystrofinopathie patiënten uit 318 verschillende families beschreven bij wie de mutatie nieuw is ontstaan. Hierdoor was het mogelijk om de kans voor een niet-draagster moeder op een tweede zoon met DMD te berekenen: 4,3%. Dit percentage heeft een kleiner betrouwbaarheidsinterval dan eerdere percentages en kan sinds de publicatie van dit artikel in 2009, gebruikt worden bij het geven van erfelijkheidsadvies in deze families.

Somatisch mozaïcisme

Somatisch mozaïcisme betekent dat in een deel van de lichaamscellen van een persoon een mutatie aanwezig is, terwijl de overige cellen normaal zijn. In **hoofdstuk 2.2** beschrijven we een 30-jarige man met een dystrofinopathie veroorzaakt door een somatisch mozaïcisme. De eerste aanwijzing voor het somatisch mozaïcisme was de observatie van spiervezels met en zonder dystrofine in zijn spierbiopt. Daarna toonde DNA-onderzoek een leesraam-verschuivende mutatie in het *DMD* gen in een deel van zijn cellen aan. Deze mutatie zou normaliter leiden tot DMD. De patiënt had echter een veel milder klinisch beeld. Het beeld paste evenmin bij Becker spierdystrofie omdat daar symmetrische spierzwakte en contracturen ontstaan op het moment dat patiënten rolstoelgebonden raken. Onze patiënt had asymmetrische spierzwakte en contracturen terwijl hij nog ambulant was.
Zijn relatief milde ziektebeeld bleek verklaard te kunnen worden door somatisch mozaïcisme. Na de publicatie van onze patiënt in 2003 zijn er, voor zover wij konden nagaan, slechts twee andere patiënten met somatisch mozaïcisme in het *DMD* gen beschreven, beiden in 2007. Patiënten met klachten veroorzaakt door somatisch mozaïcisme in dit gen zijn zeldzaam.

Duchenne/Becker spierdystrofie in de familie, zijn vrouwen getest met behulp van DNA-onderzoek?

In **hoofdstuk 3** beschrijven we dat, ondanks de aanwezigheid van acht klinisch genetische centra in een relatief klein land, een groot aantal vrouwen uit Nederlandse Duchenne/Becker families die een risico hebben om draagster te zijn, geen DNA-onderzoek hiernaar hebben laten doen.

De onderzochte vrouwen in deze studie zijn zussen van patiënten en zussen van de moeders van patiënten. Uit onze studie bleek dat 55% van deze vrouwen met een 50% kans op dragerschap en 43% van deze vrouwen met een kans van 4,3% (op basis van kiemcelmozaïcisme) geen moleculair onderzoek heeft laten uitvoeren. Meestal wordt DNA-onderzoek bij jonge meisjes niet verricht tenzij het consequenties heeft voor het kind op het moment van de test. Bij dragerschap onderzoek naar Duchenne/Becker spierdystrofie wordt gewacht tot het meisje volwassen is, zodat ze goed geïnformeerd zelf een beslissing kan nemen. Ook als we alleen de vrouwen ouder dan 16 jaar selecteerden bleek 34% met een 50% kans en 30% met een 4,3% kans geen DNA-onderzoek te hebben laten uitvoeren.
We concluderen dat één op de drie volwassen vrouwen met een verhoogde kans op dragerschap geen DNA-test heeft gehad. Verder onderzoek is gepland om de redenen te achterhalen waarom zoveel vrouwen niet onderzocht zijn ondanks de verhoogde kans op een zoon met DMD en de kans van 10% op hartklachten.

Wat heeft 26 jaar prenataal onderzoek naar Duchenne spierdystrofie opgeleverd? Een overzicht waaruit blijkt dat een tweetal wijzigingen van het huidige beleid zinvol zijn teneinde de incidentie van DMD te verlagen.

In **hoofdstuk 4** geven we een overzicht van 26 jaar (1984-2009) prenataal DNA-onderzoek naar Duchenne en Becker spierdystrofie in Nederland. Er waren 635 DNA-onderzoeken tijdens de zwangerschap verricht; in 51% bleek het ongeboren kind mannelijk. Bijna de helft (46%) van de jongens was aangedaan of had een verhoogd risico op Duchenne/Becker spierdystrofie. Dit leidde tot het afbreken van 145 zwangerschappen, daarnaast werden 174 niet-aangedane jongens geboren. Het invoeren van DNA-onderzoek tijdens de zwangerschap heeft er voor gezorgd dat ouders in geval van een jongen niet meer voor de moeilijke keuze van een mogelijke abortus staan, zoals voor 1984, waarbij er een kans was een gezonde jongen te aborteren. Voor een klein aantal families bood pre-implantatie genetische diagnostiek (embryoselectie) de mogelijkheid om kinderen te krijgen zonder een kans op abortus.
Om de vraag te kunnen beantwoorden of prenataal DNA-onderzoek naar dystrofinopathie de incidentie heeft beïnvloed, hebben we de incidentie van DMD in de jaren 60 vergeleken met die in de jaren 90. Er was geen significant verschil tussen de eerste (1961-1974) en de tweede (1993-2002) onderzoeksperiode. Wel bleek dat het percentage families waarin de Duchenne patiënt de eerste was in de familie toenam van 62% in de eerste periode naar 88% in de tweede periode. Er was dus geen afname van de incidentie maar de Duchenne families maakten wel gebruik van prenataal onderzoek om de geboorte van een tweede aangedane jongen in de familie te voorkomen. De meeste DMD patiënten die geboren werden tussen 1993 en 2002 waren de eerste met deze ziekte in de familie.
In 12% van de families was er al een oudere aangedane DMD patiënt. Er was echter geen gebruik gemaakt van prenatale diagnostiek door het zwangere paar, meestal omdat zij niet wisten dat dit mogelijk was. In bijna de helft van de paren werd dit veroorzaakt door de nog niet gestelde diagnose in het oudere aangedane familielid, omdat de patiënt jonger dan vijf jaar was.

Als DMD screening in de hielprik wordt opgenomen, wordt de diagnose wel eerder gesteld. In Nederland is tot nog toe DMD screening niet opgenomen in de hielprik met als één van de belangrijkste redenen dat deze ziekte nog niet te genezen is. In 2011 is echter wel taaislijmziekte screening opgenomen in de hielprik, terwijl dit ook een nog niet te genezen ziekte is.

Als tweede belangrijke punt vonden we in onze studie van 26 jaar prenataal onderzoek naar Duchenne spierdystrofie het volgende:
Ongeveer 78% van de geboren meisjes met verhoogde kans op dragerschap voor DMD, die ten tijde van onze studie (2009) tussen de 16 en 26 jaar oud waren, hadden zich nog niet gemeld voor DNA-onderzoek naar dragerschap. De gemiddelde kans op dragerschap van deze nog niet moleculair onderzochte meisjes was 28%. Dit percentage is lager dan 50% onder andere omdat tien meisjes een kleinere kans hadden van 4.3% op dragerschap vanwege mogelijk kiemcelmozaïcisme. Volgens de huidige richtlijn kunnen meisjes zich pas laten testen als ze volwassen zijn. Dit betekent, dat bijna driekwart van de ouders twee decades nadenken over hoe en wanneer zij hun dochter het beste kunnen vertellen over hun kans op dragerschap voor DMD. Terwijl door sneller testen al veel eerder zou kunnen blijken dat meeste dochters geen DMD draagster zijn. Deze niet wenselijke huidige situatie kan voorkomen worden door de ouders de keuze te geven hun dochters al eerder te laten testen op dragerschap, dus voordat zij volwassen zijn.
Een ander argument dat pleit voor het loslaten van het geldende beleid is:
De hielprik bij pasgeborenen is in de laatste jaren in Nederland uitgebreid van drie naar achttien ziekten. Bij twee ziekten kan de uitslag zijn dat de pasgebore drager is van een ziekte (erfelijke bloedarmoede en taaislijmziekte). In zo'n situatie zou het kunnen gebeuren dat ouders wel horen dat hun pasgeborene draagster is terwijl van oudere kinderen in het gezin gezegd wordt dat het beter is om te wachten tot het kind volwassen is en zelf kan beslissen of het dit wil weten.
Het is invoelbaar dat ouders in die situatie vragen om testen op dragerschap van het andere kind.

Becker spier dystrofie patiënten met een deletie rondom exon 51

Hoofdstuk 5 gaat in op de veelbelovende ontwikkelingen op het gebied van de (op de patiënt afgestemde) therapie bij DMD. Theoretisch gezien kan 83% van de Duchenne patiënten baat hebben bij exon skipping therapie, het skippen van uitsluitend exon 51 kan toegepast worden bij 13% van de patiënten. Gedetailleerde beschrijvingen worden gegeven van drie Becker spierdystrofie patiënten met niet-leesraam-verstorende mutaties rond exon 51. Het spiereiwit dystrofine van deze patiënten mist een deel maar is wel functioneel. Daarbij is het identiek aan het eiwit dat ontstaat na behandeling van Duchenne patiënten met de exon 51 skipping therapie. Het milde beeld van deze drie Becker patiënten stimuleert de verdere ontwikkeling van deze therapie.

Aanbevelingen in dit proefschrift

Dystrofinopathieën zouden op vrijwillige basis moeten worden aangeboden in de hielprik van pasgeboren jongens. Hierdoor wordt de kans kleiner dat er een tweede aangedane jongen geboren wordt in de familie. Voor de jongen zelf is een vroege diagnose in de toekomst ook van belang. Hij kan dan starten met therapie op maat (bijvoorbeeld exon skipping therapie) voordat hij symptomen heeft, waardoor het resultaat, naar verwachting, beter zal zijn.
Ouders moeten de keuze krijgen om hun dochters op draagsterschap te laten testen tijdens de zwangerschap als er DNA van de foetus aanwezig is. Ook moeten ouders aangemoedigd worden om hun dochters te laten testen, eventueel al voor ze volwassen zijn. Hiermee kan de incidentie van DMD gereduceerd worden en het zal voor de ouders makkelijker zijn om de dochters te informeren over de toekomstige risico's.
Klinisch genetici en genetisch consulenten moeten actiever cascadescreening in dystrofinopathie families aanbieden waardoor patiënten en familieleden beter geïnformeerd worden over de kans op dragerschap en over de implicaties van dragerschap.

Chapter 7

Publications
Curriculum vitae
Acknowledgements

7.1 Publications

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7.2 Curriculum vitae

Paula Helderman-van den Enden was born on 15 October 1962 in Monster as the third child of Arie van den Enden and Plony Dobbe. She graduated from high school (VWO-atheneum) in 1981 at the Thomas More College in the Hague. After one year at the Agricultural University in Wageningen (propedeuse plant cultivation) Paula started her medical training at the Erasmus University in Rotterdam. As a student she did two internships of four months each. The first was in 1986 at the Christian Medical College in Vellore, Tamil Nadu, India. The second was in 1989 at the department of medical genetics, under the supervision of Prof. Dr. V.A. McKusick, in the Johns Hopkins University in Baltimore, USA. Paula obtained her medical degree in 1989.

From 1990 to 1992 she worked as a junior resident at the department of internal medicine and cardiology in the St. Joseph Hospital in Kerkrade. This was followed by a year as a staff member at the department of medical genetics in Ghent, Belgium. From 1993 to 1997 she completed her training as a clinical geneticist under the supervision of Prof Dr. J.J.P. van de Kamp at the Leiden University Medical Center where she subsequently worked as a clinical geneticist under the supervision of Prof. Dr. M.H. Breuning. Here she participated in the long standing research on Duchenne muscular dystrophy. In 2007 she accepted a position as a clinical geneticist in the Maastricht University Medical Center but continued working part time in Leiden to complete the research reported in this thesis.

Paula is married to Kees Helderman and they have three children, Arie (19), Bert (14) and Leontien (10).

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