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DYSTROPHINOPATHIES

heterogeneous clinical aspects of

Becker and Duchenne muscular dystrophy

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DYSTROPHINOPATHIES

heterogeneous clinical aspects of
Becker and Duchenne muscular dystrophy

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On ne voit bien qu'avec le coeur.
L'essentiel est invisible pour les yeux.
le Petit Prince

Contents

Chapter 1 Diagnostic considerations in dystrophinopathies 9
Aim of this thesis 29

PART I Becker muscular dystrophy 33

Chapter 2 Diagnosis of Becker muscular dystrophy: results of re-analysis of DNA samples. *(submitted)* 35

Chapter 3 Becker muscular dystrophy patients with deletions around exon 51; a promising outlook for exon skipping therapy in Duchenne patients *(Neuromuscular Disorders 2010, 20:251-4)* 47

PART II Duchenne muscular dystrophy 59

Chapter 4 Reduced grey matter volume and altered white matter in boys with Duchenne muscular dystrophy *(Annals of Neurology 2014, Epub July 10th)* 61

Chapter 5 Temporal muscle hypertrophy and skull eccentricity in Duchenne muscular dystrophy *(Journal of Child Neurology 2014, Epub March 19th)* 77

Chapter 6 Prednisone 10 days on / 10 days off in patients with Duchenne muscular dystrophy *(Journal of Neurology 2009;256:768-73)* 87

Chapter 7 Summary and future perspectives 99
Samenvatting in het Nederlands 111

Appendices 123
Supplementary Table A 125
Supplementary Table B 130
References 135
Curriculum vitae 149
List of publications 151
Dankwoord 155
List of abbreviations 159



1

Diagnostic considerations in dystrophinopathies

Dystrophinopathies can be distinguished in two classic phenotypes, Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD).[1] Both disorders are well known for the muscle symptoms and weakness, which first affects the lower limbs and subsequently the upper limbs. Historically, the clinical difference between BMD and DMD was defined by the progression of the muscle weakness. Early natural history studies have reported that in general boys with DMD would become wheelchair-bound before the age of 13 whereas in boys with BMD ambulation should be preserved at the age of 16 years.[1] Since the pathogenesis of dystrophinopathy has been discovered, immunological and molecular-genetic studies have provided better insight in the difference between both phenotypes. Although the two disorders have many similarities the heterogeneity of both disorders is intriguing. For example, the severity of muscle weakness and the age of onset in BMD is much more heterogeneous than in DMD and may result in different presentations and a larger differential diagnosis in the diagnostic work up. Other heterogeneous clinical aspects of dystrophinopathies are cognitive and behavior problems and cardiac involvement; rarely these are presenting symptoms.[2-6]

Diagnosis in historical perspective

DMD was described in the 19th century by the French neurologist Guillaume Duchenne de Boulogne and the English physician Edward Meryon separately.[1] The latter reported a familial trait of the disease with predilection in males and he described from autopsies that a pathological substrate for the paralysis could be found in destroyed muscle fibres with specific involvement of the sarcolemma.[1] Duchenne was able to obtain muscle specimens in life by means of a needle-harpoon and he observed different stages of the disease pathologically.[1, 7] BMD was first described by Kostakow in 1937, and recognized as a novel X-linked muscular dystrophy distinct from Duchenne muscular dystrophy by the German neurologist and geneticist Peter E. Becker and the psychologist Franz Kiener in 1955.[1, 8] They provided detailed clinical descriptions of affected males with a X-linked inheritance pattern among multiple generations within large families; the age of onset was between 12 -25 years with proximal weakness in the lower limbs which was slowly progressive and extended to the arms usually 5-10 years after the first symptoms had started in the legs.[8] Since the identification of dystrophin in 1987 disturbed dystrophin synthesis could be assessed by means of immunological investigation of muscle tissue, which enabled distinguishing a dystrophinopathy from other limb girdle muscular dystrophies. In DMD dystrophin is virtually absent and in BMD dystrophin is present but in a decreased amount and/or in altered size.[9] Genetically, DMD and BMD are X-linked and allelic, and are caused by a mutation in the *DMD* gene.[10] In most cases mutations that lead to a disturbed

reading frame cause lack of dystrophin production which results in a DMD phenotype.[11] In general, mutations that leave the reading frame intact result in synthesis of a shortened or longer, but still functional, dystrophin and a BMD phenotype. At present, diagnosis of dystrophinopathies still starts with a good description and interpretation of the clinical data .

Clinical characteristics

Muscle symptoms in Duchenne muscular dystrophy

Among the muscular dystrophies DMD is the most frequent variant with an incidence of 10.71 to 27.78 per 100,000 live born males and a prevalence of 4.78 (95% confidence interval 1.94-11.81) per 100,000 males.[12] The onset is typically in childhood and should be present before the age of five years according to the European Neuromuscular center (ENMC) diagnostic criteria .[13] The mean age at which first symptoms of DMD were noticed is 2,5 years (SD 1.4, range 0,2-6.1 years).[14] The boys often have an unsteady waddling gait with tiptoe walking. They may also be delayed in starting to walk; one third of the boys started to walk after 19 months, but in general all DMD patients will be able to walk before the age of three years.[1] Enlarged calves, caused by hypertrophy of the calf muscles may be an early sign for the clinician. However, the reported mean age at diagnosis was around 4.9 years [14, 15] which suggests that early symptoms are not always recognised. A positive family history may be helpful for early diagnosis but in approximately 30% of the patients the mutation is *de novo* and then the family history is negative. The muscle weakness in DMD is symmetrical and progressive with the proximal muscles more affected than the muscles in the distal limbs.[13] An important early sign of weakness in the proximal leg muscles is the Gowers' sign, in which the child uses his arms climbing up his legs when he raises from sitting on the floor to standing position. Fasciculations and sensory disturbances suggest a neurogenic disorder and are exclusion criteria.[13] Although muscle strength usually improves in toddlers with DMD they will not be able to run. Following a plateau phase, which is usually reached at age 4-8 years,[16] muscle weakness progresses, not only in the upper legs but also in muscles of the lower legs, proximal arm and neck muscles. During the progressive phase ambulation will be lost, in historical series in or before adolescence. Since the introduction of steroid treatment in the 1990's, loss of ambulation can be delayed for at last a year. In puberty, there is a risk to develop scoliosis due to weakness of the spinal muscles in combination with fast length growth. At a later stage of the disease respiratory muscles become weak resulting in reduced pulmonary function. Often the first sign is nocturnal hypoventilation and hypercapnia.

Muscle symptoms in Becker muscular dystrophy

Clinically, muscle symptoms in BMD are more heterogeneous than in DMD, ranging from very mild, such as exercise induced myalgia or cramps, to severe, like progressive weakness in the upper legs.[13] Often calf hypertrophy is present, but this is not a specific sign[17] and may be lacking.[18] Weakness of the quadriceps femoris muscles can be the only sign for a long time. Usually at a later stage the upper limbs become affected as well. In sporadic cases with proximal weakness the differential diagnosis should include limb girdle muscular dystrophy (LGMD)[19, 20] or spinal muscular atrophy (SMA).[21] Presence of fasciculations or sensory disturbances excludes BMD.[13] As in DMD a positive family history of X-linked inheritance may be helpful for the diagnosis. However in a patient with a *de novo* mutation the symptoms will interpreted as a sporadic case. The estimated birth incidence of BMD is about one-third to one-fifth of the incidence of DMD.[22, 23] A recent meta-analysis estimated that the prevalence among males worldwide is 1.53 (95% confidence interval 0.26-8.94) per 100.000.[12] BMD seems to be difficult to recognize in some patients as it was reported that though 90% had symptoms at age 20, diagnosis was often made at an older age; only 50% was diagnosed at the age of 15 years and 90% diagnosed at age 35.[24] Rarely, progressive weakness presented in the third until even the sixth decade.[25-28]

Intermediate dystrophinopathy

Sometimes the phenotype is milder than expected for DMD because weakness is less progressive and/or ambulation is preserved longer but not as good as one expects in BMD. Michael Brooke introduced in 1983 the term "outliers" in a natural history study of 114 DMD patients. Ten patients of 8 years and older were performing better on climbing stairs than the rest.[29] It was suggested that dystrophinopathies could represent a spectrum with several phenotypes or that these outliers might be genetically different. Indeed when levels of dystrophin in muscle biopsies were related to the clinical phenotype, three groups could be distinguished: DMD patients who had little or no dystrophin (< 3%), BMD patients who were having more qualitative than quantitative abnormalities in dystrophin and a third group with intermediate dystrophy (outliers); these patients had reduced amounts of dystrophin (3-60 % of normal).[9] In addition, patients with milder phenotypes have been described in relation to specific mutations such as deletions of exon 3-7 of the DMD gene.[30] In clinical studies the terms *outlier* and *intermediate dystrophinopathy* apparently have not got much support as very few publications on this topic can be found.

Female carriers of dystrophinopathy

In two-third of the patients with dystrophinopathy the mutation has been transmitted by the mother, who is carrier, and in one-third the dystrophinopathy is due to a new *(de novo)* mutation in the affected male. In the latter the mother is not a carrier somatically, although

she may have germ line mosaicism.[31] If the mother is carrier of a mutation in the *DMD* gene her daughters have a 50% risk to carry the same mutation and if so to transmit the mutation to their offspring. Also her sisters and maternal female family members may be carriers. In a recent study one-third of the adult potential carriers had not been tested for carrier status.[32] Female carriers are often asymptomatic but can have muscle symptoms like myalgia, hyperCKemia, and muscle weakness. In a large Dutch cohort of 129 female carriers 22% was symptomatic (26% of the DMD carriers; 13% of the BMD carriers) of whom 17% had mild to moderate muscle weakness.[33]

Cardiac involvement in dystrophinopathy

Cardiac muscles may be involved in all dystrophinopathies and, if so, this can result in cardiac arrhythmias and/or dilated cardiomyopathy. In DMD cardiac involvement is usually diagnosed around the age that ambulation is lost.[34] Often it is asymptomatic but progressive. Currently screening with yearly ECG/echocardiography is advised at young age aiming at pharmacological intervention when first abnormalities are detected in order to prevent heart failure.[34, 35]

In BMD heart failure can be the presenting symptom,[5, 36] and cardiomyopathy occurs in the majority of the BMD patients during the course of the disease.[36] Dystrophin amounts of 30 % quantified by Western Blotting analysis seem to be sufficient to avoid weakness in skeletal muscles, as is found in families with in-frame mutation in the DMD gene, but did not protect against severe cardiac pathology.[37]

Concerning female carriers, the majority had cardiac abnormalities on ECG and/or echocardiography. [38, 39] Left ventricular dilatation occurred in 18% of dystrophinopathy carriers (19% of DMD, 16% of BMD carriers).[39] During follow up dilated cardiomyopathy was found in 10%.[38] Regular cardiac screening is advised for all adult DMD/BMD carriers [40] but only two-third of the carriers was aware of their cardiomyopathy risk when questioned.[41]

Cognition and behavior problems in dystrophinopathy

Besides muscle involvement in DMD it is known that the prevalence of cognitive deficit is higher in DMD. The mean full-scale IQ is one SD below the IQ in the normal population. Approximately 30% of the boys with DMD have mental retardation with an IQ < 70.[42] In contrast to the progressive nature of muscular weakness in DMD the cognitive deficit seems to be stable. In addition learning problems especially with reading and spelling occur more frequently in DMD.[43] The reading age and reading performance was significantly lower in DMD boys of 8.5-13.5 years old compared to aged matched patients with SMA and healthy controls.[44] DMD is also associated with several behavioral disorders. Firstly, the reported

prevalence of attention-deficit hyperactivity disorder (ADHD) is 12-33%.[45, 46] which is two to five fold the prevalence in a general school population.[47] Secondly, about 3% was reported to be diagnosed with an autism spectrum disorder which is also increased compared to 0.16% in the general population.[45, 48] Finally, in almost 5% an obsessive-compulsive disorder (OCD) was reported, twice as high as compared to the general population.[45]

In patients with BMD limited studies on cognition have been performed. Mental retardation has been reported as a presenting symptom in combination with elevated CK levels.[6] The four patients described were aged 15-42 years and all had a IQ below 70; besides calf enlargement in one and occasional muscle cramps and a mild scoliosis in another, none of them had muscle weakness on neurological examination. CK levels were elevated, with levels of 2550, 7500, 896 and 730 U/l (normal < 240 U/l) in respectively the patients aged 15, 17, 19 and 42 years old. In addition, a mean full scale IQ (FSIQ) of 83 (± 25) has been reported in a series of 115 patients with BMD.[49] The low IQ was confirmed recently in a small series of 13 BMD patients (11 adults) with a mean FSIQ of 86 (± 10.4).[50] In contrast, in a series of 24 Australian BMD patients (median age 14.2 y; range 6-43.2) in whom IQ and neuropsychological profile was investigated, the mean FSIQ was reported to be normally distributed with a mean of 95.6 (± 23.3);[51] in twelve of them (52%) the scores on the FSIQ ranged between 85-115. However in three others (13%) the FSIQ was below 70 and in two others ≥ 130. The FSIQ was not related to severity of clinical symptoms but a correlation with socioeconomic status was found; a lower IQ was found in patients with a lower socioeconomic status. Moreover, in this study, 21% of the patients had reading problems and 32% had spelling difficulties. Two patients were autistic. Eleven parents completed a checklist on behavior disorders and they reported behavior compatible with ADHD in four children. In general, clinically relevant behavior problems were reported in eight children. This seems a high number, compared to reported frequency of 14.1% in the general (Australian) population.[51, 52]

Resuming, cognition as well as reading performance and behavior appear to be heterogeneous features and demonstrate a broad spectrum in dystrophinopathies.

Creatine kinase

Introduction

The enzyme creatine kinase (CK) plays an important role in cellular energy generation in the muscles, because it enables the (reversible) phosphorylation of creatine to creatine-phosphate. Creatine-phosphate serves as an energy reserve in muscles as it can donate a phosphate group to adenosine diphosphate (ADP) to form adenosine triphosphate

(ATP) and creatine during intense muscular activity. The serum CK level is the standard laboratory test for creatine kinase and is often used as a marker of functional status of muscle tissue. The total serum CK level reflects three iso-enzymes: CK-MM (predominantly present in skeletal muscles), CK-MB (present in cardiac muscle), and CK-BB (present in brain).[53] High CK-MB levels are related to myocardial damage such as acute myocardial infarction or cardiomyopathy. Elevated CK-BB can be found following epileptic seizures or following stroke. Elevated CK-MM is associated with neuromuscular diseases and in this chapter the focus will be on CK-MM which, if elevated in serum, will result in an increased total serum CK level. Increased CK levels can be a parameter for muscle disorders, like muscular dystrophies, hereditary, inflammatory and toxic myopathies. In addition, CK is often mildly elevated, to 5 times the upper limit of normal, in neurogenic disorders like hereditary spinal muscular atrophy (SMA), motor neuron disease (MND) and axonal neuropathy. In daily practice an increased level can be important whereas a low CK is not clinically relevant. Therefore only the upper limit of normal range is used to discriminate pathological from physiological levels. The measured value can vary intraindividually on different test moments. Each diagnostic laboratory has its own population based reference values of the normal range. For example, in the Leiden University Medical Center the upper limit is 170 U/l in males and 140 U/l in females. Gender and racial differences have been described as the upper limit of normal range is higher in males and almost doubled in black people.[54]

Rule of the thumb is that CK is always increased in dystrophinopathy. Elevated CK level can be the sign which is noticed first in dystrophinopathy. In DMD CK levels can exceed the upper limit of normal more than 100 times especially at young age. In progressive disease fat replaces muscle tissue and thus functional muscle tissue is lost; the serum CK levels can be lower then, but will still be elevated. In BMD the CK level is also increased, but lower levels have been described than in DMD.[55] In adult males with more muscle weakness the tendency is that CK levels decrease. Besides, in families with mild BMD youngsters had an increased CK and adults had normal levels.[56] In asymptomatic female DMD carriers the CK level can also be mildly increased.[33] Elevated CK level can serve as an important clue in diagnosis of dystrophinopathy, but is not specific for this disorder; moreover hyperCKemia is frequently observed in patients with a wide range of muscle disorders. Therefore hyperCKemia will be discussed from different perspectives in the next paragraphs.

HyperCKemia not related to neuromuscular disorders

The prevalence of incidentally elevated CK in a large Norwegian cohort of 12828 subjects (age range 30-87y) who participated in a population based health survey (Tromsø study), was reported 5.3% which decreased to 1.3 % following a second measurement with a standardised control test three days after refraining from exercise and alcohol. Because statin

use or known disorders could explain the hyperCKemia in 46% of the cases with persistent hyperCKemia, the prevalence of idiopathic hyperCKemia in the general population was estimated at 0.71%.[57] A frequently reported cause of acquired hyperCKemia is statin treatment for hyperlipidaemia, in cardiovascular or cerebrovascular disease, which can result in asymptomatic elevated serum CK levels or symptomatic hyperCKemia with muscular symptoms such as myalgia, myopathy but also life threatening rhabdomyolysis. The mechanism is not clearly elucidated and a genetic susceptibility is suggested.[58] In patients who are evaluated clinically after discontinuation of the statin rarely a neuromuscular disorder was diagnosed; for example myotonic dystrophy, McArdle's disease, mitochondrial myopathy and Kennedy´s disease, which apparently had been subclinical before the start of statin treatment.[59, 60] Elevated CK has also been reported in patients treated with other medication including beta-blockers, retinoic acid, antiretroviral treatment and clozapine as well as following substance abuse like alcohol, cocaine and heroin.[53] Traumatic muscle injury, strenuous exercise, heavy manual labour or intramuscular injections are other acquired causes of hyperCKemia in otherwise healthy people.[61] In healthy people a 50-fold increase has been found in post-exercise CK levels;[62] this was more pronounced in males than in females and occurred more frequently in black people and in untrained people. Military recruits in whom exertional rhabdomyolysis was observed following basic military training were often asymptomatic.[63] A single muscle cramp can result in a threefold increased CK within 6 hours and nearly fivefold at 30 hours.[64] Exercise or cramp related hyperCKemia will decrease spontaneously after a resting period of several days. However, persistent hyperCKemia is described in asymptomatic healthy subjects[65] and in athletes.[66] A possible underestimated phenomenon is the macro-CK which represents CK variants with a molecular mass > 200 kDa. This is much higher than the molecular mass of 80 kDa of the "common " CK iso-enzymes.[67] The prevalence of macro-CK in hyperCKemia is around 4%.[68] Two types of macro-CK are known: type 1 is a benign complex of CK iso-enzymes with either immunoglobulin IgG or IgA which will increase the serum half-life due to the high molecular weight. Its reported prevalence in the general population is 0.43-1.2% and it is associated with autoimmune myositis.[53] Type 2 has a prevalence of 0.5-3.7% in the general population but is prognostic unfavourable because it is associated with liver tissue necrosis or malignancies. This type of macro-CK encompasses oligomeric mitochondrial CK. Macro-CK can be determined with electrophoresis[69] but not all laboratories perform this diagnostic analysis.

Idiopathic hyperCKemia

The term idiopathic hyperCKemia was introduced in 1980 to describe patients with persistent elevated CK levels despite normal neurological examination and diagnostic work up including EMG and muscle biopsy.[53, 70] In addition, familial idiopathic hyperCKemia has been reported.[71] Forty-one subjects of 13 families had elevated CK 1.2-7.7 times. The family trait of

hyperCKemia in most pedigrees was autosomal dominant with a higher penetrance in men and male-to-male transmission in eight families. Muscle biopsies showed no abnormalities or non-specific features such as increased variance in fibre size. The prognosis was favourable in all families without progression to weakness. Also long term prognosis in sporadic idiopathic hyperCKemia seems to be favourable.[57, 72-74] In 31 patients (87% males) with idiopathic hyperCKemia, who all had had muscle biopsy at referral, follow up was possible with a mean interval of 7.2 years (range 4-18 years) in 23 cases and none showed clinical deterioration besides one patient who had developed axonal polyneuropathy.[74] In another series of 55 predominantly male cases out of originally 93 cases with undiagnosed hyperCKemia, with a mean interval between referral and clinical follow up of 7.5 years (range 4-15 years) the yield was one subject with limb girdle weakness, three cases with thyroid abnormalities, one dystrophinopathy carrier and one possible SMA carrier.[73] Interestingly, 78% had persistent hyperCKemia but in all but one subject the CK level at follow up had decreased compared to the initial CK level. In one female the CK had increased from the range of 194-554 U/l at referral to a value in the range of 555-1038 U/l at follow up; subsequently, neurological examination revealed that she had weakness with a limb girdle pattern. In a case-control follow up study of the aforementioned population based Tromsø study 120 subjects (66 women, 54 men) with persistent hyperCKemia participated as case.[75] Muscle symptoms like cramps and muscle pain or stiffness were reported in 71% of these cases compared to 54% in controls (p=0.017). In only five cases hyperCKemia could be related to a muscle disorder although muscle biopsies were not part of the study protocol. Three cases were newly diagnosed as unclassified myopathies, one female was known with the diagnosis facioscapulohumeral dystrophy (FSHD) and one male suffered from polymyositis. This indicates that hyperCKemia in a general population is rarely associated with a neuromuscular disorder.

HyperCKemia and the risk of dystrophinopathy

Is dystrophinopathy excluded in asymptomatic hyperCKemia?

Retrospective evaluation of 114 asymptomatic predominantly male patients aged between 3-70y (mean age 33) with increased CK (mean 1410 units/l, range 246-9082) resulted in diagnosis of a neuromuscular disorder in 21 patients (18,4%) by means of muscle biopsy and/or EMG.[76] Five cases (one adult with myalgia and four asymptomatic children) were diagnosed with dystrophinopathy, two of them had normal histology and immunohistochemical dystrophin expression in muscle samples. Immunoblotting however was not performed and the dystrophinopathy was confirmed by DNA analysis.

Often CK and biopsy findings are combined when diagnostic yield was reviewed. Dabby et al.[77] evaluated muscle biopsies of 40 cases with hyperCKemia with mild or no symptoms (male to female ratio 3:1, age 7-67 years), and observed non-specific histopathological ab-

normalities in 22 patients. Subsequently, immunohistochemical staining for dystrophin antibodies could classify three asymptomatic patients in whom abnormal dystrophin was found, which was compatible with BMD. Fernandez et al.[78] also reviewed muscle biopsy specimens of 104 patients with persistent CK levels > 500 units/l and no or mild symptoms. The mean age was 40 years (range 4-79y) and 71% of the cases was male. In 57 cases a diagnosis could be established: seven males with dystrophinopathy (not specified) and one carrier of dystrophinopathy. Dystrophinopathy represented 14% of the confirmed diagnoses and was established in 7.7% of the cohort. The probability of a diagnosis was increased if CK levels were > 10 times elevated and in cases younger than 15 years.[78] Notably, dystrophinopathy can present with mild symptoms like post-exertional myalgia and cramps,[79,80] cardiac failure,[5,81] or non-muscular symptoms like behavior problems,[82] speech delay[4] or cognitive problems.[3,6] Often the combination with hyperCKemia may raise the suspicion of an underlying muscular dystrophy and further analysis will be performed. However normal serum CK levels as well as normal histology in muscle biopsies have been described in BMD cases. Also female carriers of DMD often have a mildly elevated CK.

In conclusion, CK is an important signal in early diagnosis, and asymptomatic hyperCKemia will not rule out BMD neither will a normal CK in adult patients with mild muscles symptoms.[56] The question is which patients with mild symptoms will benefit from extensive diagnostic work up.

Differential diagnosis and diagnostic algorithms in hyperCKemia

Several diagnostic algorithms for asymptomatic /pauci-symptomatic hyperCKemia were proposed by different groups. The Italian Association of Myology approved an algorithm in 2006.[83] One of the co-authors also contributed to the European Federation of Neurological of Societies (EFNS) guidelines published in 2010.[84] More recently an American paper reviewed the state of the art in hyperCKemia and proposed a diagnostic flow-chart.[53] The algorithms have much in common, such as the first step in all is exclusion of non-neuromuscular causes of hyperCKemia including macroCK. In the EFNS guidelines a redefinition is proposed to define hyperCKemia as values above 1.5 times the upper limit of normal CK and correct for gender and race. It will increase the specificity of the test to screen for neuromuscular disorders with an acceptable reduction in sensitivity.[85] The American diagnostic flow-chart adopted this approach. Confirmation of the elevated CK is advised at least once following a period of 7 days in which the person refrains from exercise. Preferably the interval between the two measurements is one month.

A difference between the algorithms is the priority for forearm exercise testing *(see framed text)* because the American review proposed this as the next step to perform in cases with

marked exercise intolerance. If abnormal, metabolic testing for glycogen and lipid storage disorders was advised and the mitochondrial respiratory chain enzymes. For the latter, respiratory chain enzyme analysis can be performed on fresh or snap-frozen muscle tissue in specialized centers.[86] Of note, exercise tests will not pick up Pompe's disease or carnitine palmitoyltransferase II (CPT2) deficiency, for which the diagnostic screening tests of choice are measurement of acid maltase enzyme activity in leucocytes and tandem mass spectrometric measurement of serum/plasma acylcarnitines respectively. In contrast, the EFNS guidelines incorporate a diagnostic test for McArdle's disease in the set of histochemical investigations advised for the muscle biopsy specimen. Furthermore, the EFNS stresses the importance of enquiring the family history of neuromuscular disorders, familiar hyperCKemia or malignant hyperthermia *(see framed text)*.

Both algorithms for asymptomatic and pauci-symptomatic hyperCKemia propose EMG studies before taking a muscle biopsy. It was previously reported that the sensitivity of needle EMG was 69% to pick up abnormalities that correlate with underlying pathology in the biopsy specimen and that its specificity was 54%. The positive predictive value of an EMG to predict an abnormal muscle biopsy is only 51% and the negative predictive value of normal EMG to predict normal biopsy result is 74%.[76] Nevertheless, the EFNS guidelines advise EMG including nerve conduction studies in all patients with persistent hyperCKemia without further explanation on the usefulness of nerve conduction studies for hyperCKemia. Notably, the 25 reported cases with neurogenic diagnoses all had endured a muscle biopsy

Exercise test for glycogenoses

Exercise tests can demonstrate a defect in glycogenolysis in most muscle glycogenoses (except deficiency of acid maltase,[106] phosphorylase b kinase or branching enzyme) as ammonia levels increase normally following exercise but lactate levels fail to raise because production is reduced or absent. In myoadenylate deaminase deficiency lactate levels increase normally but ammonia levels remain at baseline. McArdle described in 1951 the lack of lactate rise in a patient with myophosphorylase deficiency following ischemic exercise of forearm muscles.[107] Ischemic exercise bears the risk of necrosis. For detection of McArdle's disease the non-ischemic forearm test is a good alternative for the potentially hazardous ischemic forearm test.[108]

Exercise test for mitochondrial myopathy

Venous oxygen desaturation during non-ischemic forearm exercise can be used as diagnostic screening test for mitochondrial myopathy(MM). In MM exercise-induced venous O_2 desaturation was limited compared with healthy subjects.[109] Notably, both tests are time consuming, requiring some expertise of the executor to perform properly. On the other hand a muscle biopsy is, though invasive, a procedure useful for multiple purposes as the tissue can be used for histology, histochemistry, immunological studies and even metabolic tests and RNA analysis if requested.

that showed neurogenic atrophy.[84] Thus in the majority of reviewed studies the EMG appeared not the primary tool to reveal neurogenic disease. In the EFNS algorithm myopathic changes on EMG are used as one of the screening criteria for muscle biopsy. If myopathic changes are found this is sufficient reason to take a biopsy independently from the serum CK level, symptoms or age. But if EMG is normal in males > 25 years of age without exercise related symptoms and serum CK is mildly elevated (< 3 times normal) muscle biopsy is not indicated according to the EFNS guidelines. The American review considers the role of the EMG to be limited in hyperCKemia, and mainly useful to exclude motor neuron disease and myotonic dystrophy. Apparently only needle EMG is required according to this algorithm.

Resuming, investigation of muscle tissue is indicated in all cases with hyperCKemia, when neurogenic or myotonic findings are excluded with an EMG, unless the case is male > 25 years without exercise related symptoms and having a serum CK < 3 times normal.

Muscle biopsy according to the EFNS guidelines should include histology (Haematoxylin & eosin staining) and histochemistry (Gomori trichome, Oil red O, period acid-schiff, ATP-ase (9.4, 4.2 and 4.6), SDH, NADH, cytochrome c oxidase, myophosphorylase, and acid phosphatase as well as immunohistochemical studies with antibodies against dystrophin, α, β, γ and δ sarcoglycans, dysferlin, caveolin-3, Major histocompability complex 1 (MHC-1) and α-dystroglycan.[84] The muscle biopsy appearance may also direct further (optional) diagnostic tests such as Western Blotting analysis (WB) of muscle tissue as well as enzymology (e.g. acid maltase enzyme activity in leucocytes) and mitochondrial DNA analysis.

In **Malignant hyperthermia (MH**) patients are asymptomatic during normal daily activities but the condition manifests itself during general anaesthesia when the body temperatures raises to very high levels, potentially with fatal outcome.[110] It is a severe and hazardous condition related to mutations in the RYR1 gene and sometimes also to myopathy with central cores.[111, 112] In symptomatic patients and in relatives a positive in vitro contracture test on fresh muscle tissue is used to confirm malignant hyperthermia susceptibility. Only one report has reported that 51% of the cases with hyperCKemia had a positive *in vitro* contracture test.[113] In contrast, in other studies on idiopathic hyperCKemia malignant hyperthermia susceptibility was found to be a rare condition.[89, 114] Moreover, in the study of Weglinski the majority of the patients with a positive contracture test and asymptomatic hyperCKemia did not get complications when they experienced general anaesthesia.[113]
Moreover, the consequences of MH testing are not settled and this invasive test is not generally available (e.g. in the Netherlands the contracture test is available in one expert center, Canisius-Wilhelmina Hospital in Nijmegen). Thus, for practical reasons it is advised that patients with idiopathic hyperCKemia inform their physicians that they have a potential risk of MH and that appropriate anaesthetic guidelines should be followed.[53, 84]

The American review analysis on muscle tissue shares the previously proposed similar immunohistochemical analysis but also includes Western blot analysis of dystrophin, dysferlin, calpain-3, caveolin-3 in the standard work up. Diagnostic tests for Pompe's disease and mitochondrial disorders are not mentioned.

Concerning the likelihood that muscle biopsy and/or EMG resulted in a definitive diagnosis was calculated at 28.1% or 125/445 cases in nine studies.[53] However, the two diagnostic algorithms reviewed in total 11 studies and only one study performed routinely WB analysis in the work up for hyperCKemia.[78] On the other hand studies that focussed on long term follow up have found that the prognosis of hyperCKemia was favourable.[73, 74] For the daily practice it is important to prevent unnecessary diagnostic tests, especially invasive procedures like taking a muscle biopsy. As mentioned before, hyperCKemia should be defined as values above 1.5 times the upper limit of normal.[84] It will increase the specificity of the test to screen for neuromuscular disorders with an acceptable reduce in sensitivity.[85] Before the diagnosis idiopathic hyperCKemia is made, careful history taking, including family history, neurological examination and diagnostic tests such as muscle biopsy and or DNA analysis should be considered to exclude subclinical myopathy.[61, 72, 76, 84]

The final step in the algorithms is genetic analysis in which the American review advises to consider DNA analysis of five muscular dystrophy genes namely dystrophin, dysferlin, calpain-3, caveolin-3 and fukutin-related protein (FKPR). Of note, the anoctamine-5 (ANO-5) gene was not yet mentioned, and neither is suggested in which order DNA analysis of the different genes could be performed. Unfortunately the algorithm does not indicate whether all undiagnosed cases should receive DNA testing or how elaborate DNA analysis should be, such as including sequencing of the *DMD* gene. Remarkably in the EFNS guidelines DNA analysis of muscular dystrophy genes is not mentioned except for the *DMD* gene to screen for possible carrier status in females. The EFNS guidelines advise that all women with increased CK at any level should receive further work up, first by means of DNA analysis of the *DMD* gene which will detect 70% of the carriers, and, in case no mutations are found, by means of a muscle biopsy.

In patients with asymptomatic or paucisymptomatic hyperCKemia in whom a neuromuscular disorder is considered the level of CK in combination with clinical features may help to guide diagnostic work up. Neuromuscular diagnoses that can present with hyperCKemia are summarized in a table (**Supplementary Table**, pages 125-133); table A reports the CK level and age of onset of the different disorders and table B gives information on specific weakness patterns, disease progression and expected results of diagnostic tests. The main reference for this overview is the neuromuscular home page[87]; when the information

on the various disorders are summarised in a table it is clear that CK levels are reported quite differently in the literature: some authors use the absolute measured CK level in units per litre, some the level in a ratio to the upper limit of normal (e.g. 15x normal) and others prefer a qualitative description like "slightly elevated" or " very high" CK level. This suggests that also laboratory assessments are subject to interpretation; measured levels may increase or decrease the probability of a specific disorder they but should be used in relation to clinical features in the patient.

Dystrophinopathy in asymptomatic or pauci-symptomatic hyperCKemia

The aforementioned algorithms on asymptomatic or pauci-symptomatic hyperCKemia also reported the yield of diagnostic work up in seven series dating from 1988-2008 concerning 348 cases with hyperCKemia of whom 261 were males (75%).[72, 76-78, 88-90] Dystrophinopathy was confirmed in 17 patients with asymptomatic or pauci-symptomatic hyperCKemia and in one carrier. This means that 6.5% of the males was diagnosed with dystrophinopathy, which accounted for 14% of all diagnoses in these combined patients series. Following polymyositis, dystrophinopathy was the second most frequently confirmed diagnosis among the participants in these series. Nevertheless, in 3/17 males (18%) with diagnosed dystrophinopathy the biopsy was not conclusive, but the diagnosis was confirmed by DNA analysis.[76, 78] One of these patients was included in the study where WB was performed.[78] This indicates that dystrophinopathy should be considered in persistent hyperCKemia and, if indeed suspected, diagnostic work up should include immunological studies on muscle tissue and/or DNA analysis of the *DMD* gene.

EMG

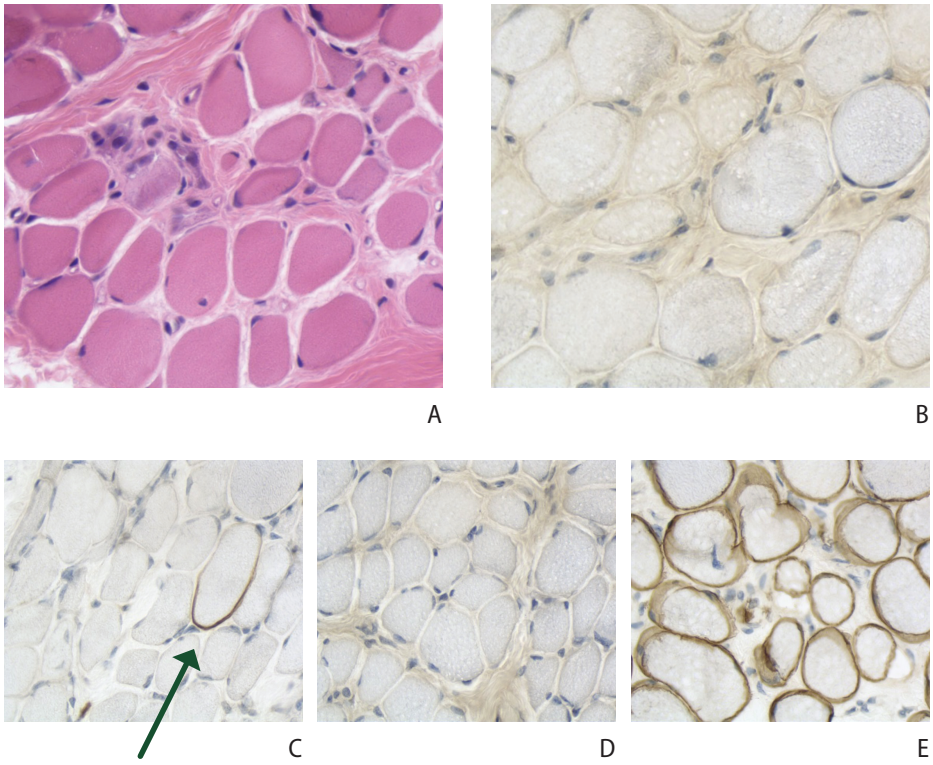
The role of the electromyography (EMG) in the work up for possible dystrophinopathy is considered to be limited, and mainly useful to exclude, if clinically relevant, motor neuron disease and myotonic dystrophy. Both disorders have a different phenotype, course and inheritance than dystrophinopathy but in the first stage they have features in common with muscular dystrophy including a mildly elevated serum CK. EMG might distinguish the different disorders. Motor neuron disease represents a broad spectrum: the spinal muscular atrophies (SMA) which are inherited as an autosomal recessive, X-linked or dominant trait. This in contrast to progressive spinal muscular atrophy (PSMA) and amyotrophic lateral sclerosis (ALS) which occur sporadically in ninety percent of the cases. The latter two disorders will not be easily confused with an underlying muscular dystrophy because the presenting clinical symptom in those is painless weakness, often asymmetrical and distally located with atrophy and fasciculations. Hereditary SMA is caused by homozy-

gous mutations in the *SMN1* gene and besides congenital (type 1), infantile (type 2) and childhood/juvenile onset (type 3) late onset is described which is classified as SMA type 4. The clinical features proximal weakness and mildly elevated CK in hereditary SMA types 3 and 4 may resemble muscular dystrophy and particularly dystrophinopathy in males. Other SMA's with possible limb girdle weakness are Kennedy's syndrome (X-linked bulbar spinal muscular atrophy) and autosomal dominant proximal SMA with adult onset. Fasciculations are often not prominently present and calf muscles have normal size. Neurogenic abnormalities on EMG will disclose the underlying neurogenic disorder and this excludes dystrophinopathy. However in a study that compared EMG and biopsy results in 114 cases with hyperCKemia the biopsy showed 14 cases with neurogenic atrophy.[76] In 13 cases of this group EMG was available which confirmed neurogenic abnormalities only in five. The definite diagnosis was not reported, neither whether genetic tests were performed, but this might indicate that a biopsy could be more sensitive to find neurogenic abnormalities than EMG in adult onset SMA. However the EMG is expected to be more specific and neurogenic abnormalities will direct towards different work up e.g. DNA analysis of the *SMA genes* instead of muscular dystrophy genes. In myotonic dystrophy specific myotonic discharges can be found on needle EMG. The next step will be DNA testing of the *DMPK* gene. If symptomatic, the phenotype in DM1 is often quite typical with myopathic face, ptosis, and atrophy of temporal muscles and weakness is more prominent in distally located muscles in hands and feet and DM1 will not easily be confused with muscular dystrophy with a limb girdle pattern. Asymptomatic or pre-symptomatic family members however could present with hyperCKemia. Myotonic dystrophy type 2 (DM2 or proximal myotonic myopathy, PROMM) however can present with proximal weakness of the legs in which pain is often prominent. Calves can be enlarged as well. An EMG in DM2 will not always disclose myotonic discharges, but if these discharges are found this will direct toward DNA analysis of the *PROMM* gene as the next step.

In conclusion, the benefit of EMG for diagnosis in symptomatic and asymptomatic adult patients is probably very limited and mainly aiming at exclusion of the rare disorders SMA and DM2. Nevertheless in asymptomatic patients the EFNS guidelines propose an EMG in the screening process before the indication of muscle biopsy, with the consequence that in case the EMG is normal, a muscle biopsy can be omitted in males > 25 years without exercise related symptoms in whom the serum CK is only mildly elevated (< 3 times normal).

Notably, EMG will not contribute to the diagnosis dystrophinopathy and very mild BMD cases older than 25 years might be missed because following a normal EMG a muscle biopsy is not proposed according to the current European guidelines.

FIGURE 1 Muscle biopsy (anterior tibial muscle) of a 4.5 y old boy with DMD



A: Hematoxylin-Eosin : atrophic and hypertrophic fibers with locally regenerating fibers; endomysial and perimysial fibrosis.

B,C,D: immunohistchemical staining with dystrophin antibodies DYS1 (B), DYS2 (C), DYS3(D): negative, besides one revertant fiber (arrow) E: immunohistochemical staining with spectrin:normal signal (but dystrophic histology). Pictures courtesy dr. S.G. van Duinen

Muscle biopsy

Muscle biopsy in dystrophinopathy has two main goals: to confirm and classify muscular dystrophy in undiagnosed sporadic symptomatic patients and to investigate dystrophin (semi)quantitatively in patients with a phenotype that seems not concordant with the mutation in the *DMD* gene.

Sporadic symptomatic patients

If DMD is suspected in a young boy the diagnosis can be confirmed either with DNA analysis of the *DMD* gene or immunohistopathologically in muscle tissue from a biopsy sample. The advantage of the latter is that the results of histologic and immunological investigations often are achieved faster, and in case the clinical features are not typical for DMD (and BMD is also considered) immunohistochemistry of biopsy tissue will inform about dystrophin expression. The disadvantages is the invasive nature of the procedure for which anesthesia is requested in very young children. If the biopsy shows that dystrophin is absent, DMD is confirmed. Each muscle specimen is routinely examined histopathologically. Typical features in dystrophinopathies are dystrophic features such as abnormal variation in muscle fiber size due to atrophic and hypertrophic fibers, inflammatory features with focal necrosis and regenerating fibers and extensive fibrosis because muscle fibers are replaced by fat and connective tissue (**figure 1**).[91]

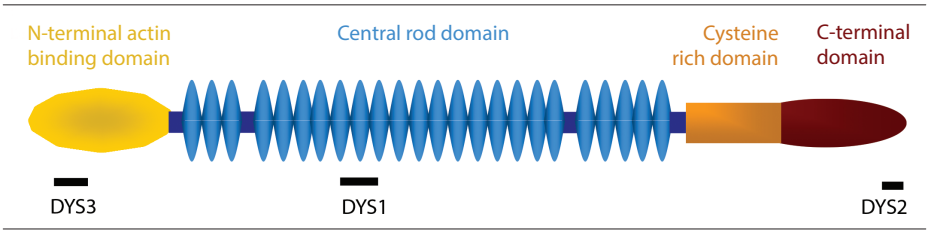
For diagnostic confirmation immunohistochemical examination with antibodies raised against different parts of dystrophin are used. Immunohistochemical analysis of dystrophin is a qualitative measure for dystrophin in muscle tissue. In case of DMD dystrophin will be absent, although sometimes a minority of the fibers will stain positive. These so-called revertant fibers are explained by recovery of the reading frame through alternative splicing or multiple exon skipping.[92]

In Becker muscular dystrophy the pathological changes are similar to those found in DMD. Based on histopathologically features alone BMD cannot be distinguished from DMD.[91] Usually BMD presents at older age compared to DMD and its progression is slower although pathological changes have been found in very young boys with BMD. [91] In contrast to DMD dystrophin is present in muscles of BMD patients but in an aberrant size and/or reduced amounts. Immunohistochemical investigation with different antibodies (e.g. DYS1 against the rod domain, DYS3 against the N-terminus and DYS2 against the Carboxyl terminus of dystrophin; **figure 2**) can show that dystrophin is distributed normally but globally reduced or that the staining is discontinuous with either a normal or reduced intensity. However, often this test is not sufficient to confirm the diagnosis BMD. Western Blot analysis (WB), which is a semi quantitative measurement of dystrophin, can detect abnormal amounts of dystrophin and/or dystrophin with a different molecular weight. A smaller dystrophin may be caused by deletions of one or more exons or by small mutations and a larger size is found in case of duplications of one of more exons. WB is more elaborate and requiring expertise. In the Dutch situation this test is available in one reference laboratory, the Laboratory for Diagnostic Genome Analysis (LDGA) in Leiden. This in contrast to immunohistochemical analysis which is routinely performed in most neu-

romuscular expert centers. Assessment of dystrophin is not only used in diagnostic work up but is also important to evaluate the effect of therapy in clinical DMD trials; especially for quantification of low levels of dystrophin several immunohistochemical methods have been developed.[93-95] Efforts to harmonize dystrophin quantification are ongoing; preliminary results suggest that different methods to assess dystrophin levels result in comparable results both between different protocols for immunofluorescence and when comparing immunofluorescence and Western blotting.[96]

In a female DMD carrier immunohistochemical analysis of muscle tissue may show a mosaic pattern of dystrophin positive and negative muscle fibres. In asymptomatic carriers the sensitivity for immunohistochemistry was only 26%.[97] Moreover, DNA analysis is the first choice to confirm the carrier status in asymptomatic females as this is non-invasive, and genetic diagnosis is necessary if further prenatal testing and counselling is wanted.

Figure 2. Schematic drawing of the dystrophin protein.



Dystrophin consists of four domains: the N-terminal actin-binding domain (yellow), the central rod domain, which contains 24 spectrin like repeats (light blue), interrupted by four hinge regions (dark blue), the cysteine rich domain (orange) and the Carboxy-terminal domain (red). The regions of the dystrophin protein which are stained by tdifferent DYS antibodies on a muscle sample are marked underneath the cartoon: DYS3, Amino acids 67-713; DYs1 amino acids 1181-1388; Dys 2 extreme Carboxy-terminus. Adapted from figure courtesy dr. A. Aartsma-Rus

Discrepancy between mutation and phenotype

In symptomatic patients in whom a mutation in the DMD gene is found the phenotype can be non-conclusive, e.g. a child with milder symptoms than expected for DMD or a youngster with a more progressive course than expected in BMD. Besides this, mutations have been described that can result in phenotype between DMD and BMD and those are associated with outliers.[29, 30, 98] Also, within one family the same mutation can result in different phenotypes.[99] Examination of a muscle specimen can clarify whether dystrophin is absent or present and in the latter case subsequently WB can detect the abnormal size and/or amounts of dystrophin, compatible with BMD or with intermediate dystrophinopathy.

Resuming, from a clinicians viewpoint the role of a muscle biopsy is a diagnostic tool in the following instances: 1) sporadic cases in whom dystrophinopathy is suspected but DNA analysis has not revealed a mutation, 2) in patients in whom the mutation is not conclusive in relation to the phenotype and 3) in adult patients with mild symptoms in whom the differential diagnosis is large and histopathological and/or immunological classification on muscle tissue may guide subsequent DNA requests. In male patients with possible BMD additional Western Blotting could be considered. In female patients sporadic symptomatic carrier status can be detected by immunohistochemical staining with dystrophin antibodies of the muscle biopsy.

DNA analysis

The *DMD* gene is the largest human gene, located on the X-chromosome, and consists of 79 exons. Large re-arrangements in de *DMD* gene account for 70% of the DMD mutations and can be detected by means of several techniques such as Southern blotting, Multiplex-PCR or nowadays Multiple Ligation-dependant Probe Amplification (MLPA) which is more accurate, faster and cheaper.[100, 101] Approximately 60% of the patients has a deletion of one of more exons, and in 5-10 % of the BMD/DMD patients duplications are found.[11] The reading frame hypothesis explains that large deletions can result in mild BMD whereas a deletion of one exon can result the more severe phenotype DMD.[102] In patients with dystrophinopathy in whom no deletion or duplication was found, sequencing techniques can be performed to search for small mutations. Small mutations can result in direct stops (nonsense mutations), truncating frameshifts, amino acid substitutions (missense and neutral mutations), or affect splicing.[101] However, sequencing of the large DMD gene is laborious and mutations in the promoter area and deep intronic mutations that may affect splicing will not be detected. Consequently, in 2-5% of symptomatic cases with DMD no mutation can be found. If no mutation in the *DMD* gene can be found in genomic DNA of the patient or if it is uncertain whether a mutation is pathogenic, RNA studies can be performed to give information about the status of the mutation.[103, 104] Novel mutations and unique variants can be registered at the DMD/BMD database that collects observed mutations from all over the world including the accompanying phenotype DMD or BMD (*www.dmd.nl*). DNA analysis of the *DMD* gene can serve multiple goals: as initial minimally invasive diagnostic tool in sporadic symptomatic patients to confirm the diagnosis dystrophinopathy or to detect the mutation if muscle biopsy analysis has revealed dystrophinopathy. Notably, for future treatment options like exon-skipping it is important to know the exact boundaries of the deletions found.[105] In familial cases DNA analysis will be the first choice and can replace muscle biopsy if the mutation is known.

Furthermore, DNA analysis is used to investigate carrier status in female family members and for prenatal testing. In daily practice results of DNA analysis usually take some time. Screening for deletions or duplications is the standard procedure which regularly takes a few weeks. Sequencing of the whole gene and its interpretation is more time consuming and results may take several weeks. Resuming, molecular-genetic diagnosis is the mainstay in the diagnostic work up of dystrophinopathies, because information about the mutation is important to improve classification for individual patients, to offer genetic counseling of family members and hopefully also to guide future therapies in DMD.

Aim of this thesis

This thesis focuses on heterogeneous clinical aspects of dystrophinopathies. The following topics have been investigated:

Becker muscular dystrophy

Diagnosis and phenotype

The phenotype of BMD may vary from severe muscle weakness with cardiomyopathy and early death to exercise induced myalgia and/or muscle cramps associated with hyperCKemia. Since 1983 genetic analysis was available in the Netherlands, which started with linkage analysis in BMD and DMD families. After the cloning of the DMD gene in 1986 and the identification of dystrophin in 1987 it became clear that dystrophinopathy in the majority (70%) of the patients was caused by large rearrangements like deletions or duplications of one or more exons.[10, 115, 116] Small mutations in the large DMD gene probably cause the disorder in the remaining one-third of the patients and novel techniques to find these mutations became available much later.[117, 118] It is therefore possible that DNA diagnosis in D/BMD patients with small mutations has not been made if they had been referred for DNA analysis in the period before sequencing techniques could be used to screen for small mutations. We were interested whether it was worthy to re-analyse the DNA of patients with potential BMD but without a genetic diagnosis yet. The results of this study are presented in ***chapter 2.***

The phenotype of BMD patients with specific mutations encompassing exon 51 is interesting concerning treatment of boys with DMD with exon-skip therapy.[105] The dystrophin product within the muscles of these BMD patients is the expected end product following successful exon-skip therapy in boys with DMD. ***Chapter 3*** describes the phenotypes of three Dutch BMD patients from two families who have in-frame deletions including exon 51.

Duchenne muscular dystrophy

Imaging of the brain: morphology of the brain and the relation to cognition

Approximately 30% of DMD patients have learning and/or behavioral problems. Full length dystrophin which is essential for normal muscle function is also present in brain tissue although its function in brain tissue is not known. Besides, several dystrophin isoforms are expressed in normal brain (figure 3). Dependent on the location of the mutation in the DMD gene in DMD dystrophin isoforms are expected to be present or absent.[119, 120] Neuropsychological studies in DMD patients have shown a relation between cognitive scores and the location of the mutation; DMD patients with a mutation downstream of exon 44 have a lower IQ and a higher risk of ADHD compared to patients with a mutation upstream of exon 44.[46, 50, 120, 121] ***Chapter 4*** describes quantitative results of an explorative MRI study on brain morphology and microstructure in DMD.

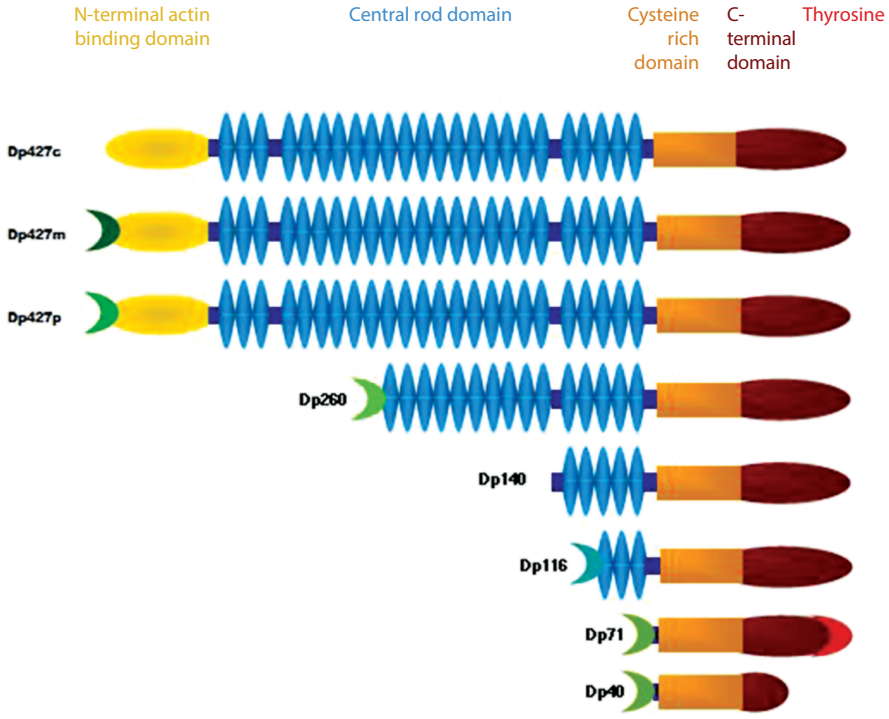
Imaging of the brain: morphology of the skull

Enlargement of the calves in dystrophinopathy is often an early and prominent feature which was already described by Guillaume Duchenne in his observations on DMD in 1868. This enlargement represents muscle hypertrophy and it is known that during progressive clinical stages the muscle tissue is replaced by fat and connective tissue. Also other muscle groups can be hypertrophic like deltoid muscles and the tongue. In a MRI study of the brain, which we performed in DMD boys aged 8-16 years and age-matched healthy controls, we observed on the transverse images of the head that hypertrophy of the temporal muscles was present in all boys with DMD and that the shape of their skulls was rounder. ***Chapter 5*** illustrates these features and presents quantified results.

Early prednisone treatment for Duchenne muscular dystrophy in the Netherlands

Steroid treatment extends the ambulant phase in DMD.[122, 123] In natural cohort studies boys with DMD became wheelchair-bound between 10-12 years.[124] Since the introduction of chronic steroid treatment in the mid 1990's the age of ambulation has improved with at least one year. The benefit depends on the dosage and age at which steroids are started. In the Netherlands guidelines for prednisone treatment were established in 2004.[123] In the rehabilitation center De Trappenberg, however, young boys with DMD have been offered prednisone since 1996 in an intermittent scheme, in which 10 days prednisone 0.75 mg/kg per day was alternated with 10 days without corticosteroids. The study presented in chapter 6 retrospectively evaluated long term follow up of steroid treatment in a cohort of 35 DMD patients and results are compared with the literature..

FIGURE 3 Dystrophin and its isoforms



Isoforms and the corresponding tissues where the isoforms are expressed

Dp427c: Cortical neurons; skeletal and cardiac muscle
Dp427m: Skeletal and cardiac muscle; glial cells
Dp427p: Purkinje cerebellar neurons; (skeletal muscle)
Dp260: Retina; (brain and heart)
Dp140: Brain; kidney
Dp116: Swann cells
Dp71: Brain; liver; cardiac muscle;
Dp40: Ubiquitous

Adapted from figure courtesy dr. A. Aartsma-Rus



PART I

Becker muscular dystrophy



2

Diagnosis of Becker muscular dystrophy:

results of re-analysis of DNA samples

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Abstract

Introduction

The phenotype of Becker muscular dystrophy (BMD) is highly variable, and the disease might be underdiagnosed. We searched for new mutations in the *DMD* gene in a cohort of previously undiagnosed cases who had been referred in the period 1985-1995.

Methods

All requests for DNA analysis of the *DMD* gene in probands with suspected BMD were re-evaluated. If the phenotype was compatible with BMD, and no deletions or duplications were detected, DNA samples were screened for small mutations.

Results

In 79 of 185 referrals no mutation had been found. Analysis could be performed on 31 DNA samples. Eight different mutations, including four novel ones, were found. Long term clinical follow-up is described.

Conclusion

Refining DNA analysis in previously undiagnosed cases can identify mutations in the *DMD* gene and confirm the diagnosis BMD genetically. A delayed diagnosis can still be valuable for the proband or the relatives of BMD patients.

Introduction

The phenotype of Becker Muscular Dystrophy (BMD) was first described in 1955 as a juvenile or benign type of X-linked muscular dystrophy.[8, 129, 130] The symptoms are similar to those of Duchenne Muscular Dystrophy (DMD) but the onset is later and the progression is slower. Moreover, BMD is characterized by a large phenotypical variability, ranging from severe muscle weakness with cardiomyopathy and early death to exercise-induced myalgia and/or muscle cramps associated with hyperCKemia.[13, 80, 131] In addition, severe cardiomyopathy has been found in patients with BMD without skeletal muscle weakness.[132] The diagnosis is supported by an X-linked inheritance pattern as well as by hypertrophy of the calves and increased serum creatine kinase (CK) activity, although neither of the last two features is pathognomonic for dystrophinopathy.[17, 53] The mean age at which muscle symptoms are noticed in BMD patients is 11.2 years (with a range of 10 months to 38 years) whereas the mean age at diagnosis is 17.7 years;[24] however, late onset in the 3rd-7th decade has been reported.[27] This indicates that diagnosis is often delayed. The gold standard for confirming BMD in adult males is molecular-genetically by DNA analysis of the *DMD* gene.[106] For a long time, following the cloning of the *DMD* gene in 1986, DNA analysis of this gene was the first and only test available to patients suffering from a muscular dystrophy.[10, 107] DNA analysis for other forms of muscular dystrophy became available years later, and these are classified as Limb Girdle Muscular Dystrophy (LGMD) with a growing number of subtypes.[133, 134]

In the Netherlands, DNA analysis for dystrophinopathies and limb girdle muscular dystrophies is performed in one referral laboratory, the Laboratory for Diagnostics and Genome Analysis (LDGA) at the Leiden University Medical Center. Seventy percent of the patients with a dystrophinopathy have deletions or duplications in the *DMD* gene, which can be detected by several techniques of which Multiple Ligation-dependant Probe Amplification (MLPA) is currently the most accurate.[100, 101] In the last decade new techniques have been developed for the detection of small mutations in the *DMD* gene; from 2008 until 2012 pre-sequencing techniques such as High Resolution Melting Curve Analysis (HCMCA)[135] were a practical method to screen for small mutations whereas currently direct sequencing is used in regular patient care.

In the present study we have investigated whether BMD had been underdiagnosed in the early decade of DNA analysis and, if so, to see if we could improve the diagnostic yield by applying newer techniques; we have reviewed the genetically undiagnosed cases among patients referred for suspected BMD in the period 1985-1995.

Methods

In 2011, we scrutinized the BMD database of the LDGA for files of patients referred in the period 1985-1995. Besides the initial application form for DNA analysis of the proband, these files may include a family tree, data of family members, results of other investigations performed and information on the clinical follow up. To improve the yield of diagnostic procedures at the LDGA, the stored DNA samples are often re-analysed if a new technique becomes available. One of us (CS) re-examined the files of all male probands in whom no deletions or duplications of one or more exons had been found with Southern blot analysis or the MLPA technique. For our study we included only those patients whose phenotype was considered compatible with BMD: male patients with hyperCKemia and proximal weakness, myalgia, cramps and / or calf hypertrophy. Patients that did not fulfil these criteria were categorised as unlikely BMD and were excluded. Referrals of patients known to be deceased at time of re-evaluation were included if the finding of a new mutation would have consequences for counselling of female family members. Referrals from abroad, which had no follow-up information, were also excluded.

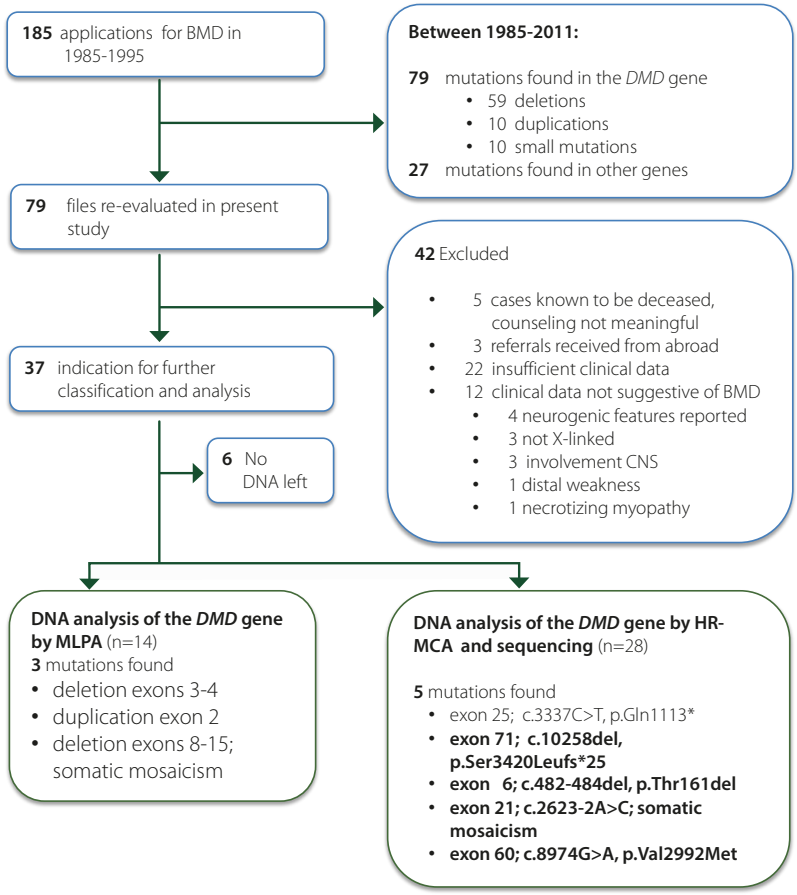
DNA from patients was re-analysed, if still available, by means of MLPA analysis if only Southern blot technique was used in the past, which was the case in around 40% of the patients. Subsequently, in all patients in whom no large deletions or duplications had been found, the DNA was screened for small mutations by means of HR-MCA followed by sequence analysis.[135] All newly identified mutations were reported to the referring physician and to the patient and/or his family. If possible, the patient or family members were approached for a new blood sample to confirm the mutation.

Results

In the period 1985-1995, blood samples from 185 male patients suspected of having BMD were sent to the LDGA for DNA analysis of the *DMD* gene. In the period between 1995 and 2011 a genetic mutation had been found in 106 patients (Figure 1), in 79 cases in the DMD gene and in 27 cases in other genes (Table 1). We reviewed the files of the remaining 79 probands. Of these, 42 cases were excluded because the patients were deceased, the referral had been received from abroad, the clinical information was insufficient or the clinical information was incompatible with the diagnosis of dystrophinopathy (Figure 1).

The remaining 37 probands were considered candidates for renewed diagnostic testing for BMD (Figure 1), but in six probands no DNA was left. Thus, further DNA analysis could

FIGURE 1 Flow diagram of the selection process of patients, referred for suspected BMD between 1985-1995, who were eligible for re-analysis of the DMD gene.



*BMD: Becker muscular dystrophy; HR-MCA: High Resolution Melting Curve Analysis; MLPA: Multiple Ligation-dependant Probe Amplification. Mutation in **bold**: novel mutation*

be performed in 31 probands. In 14 cases we performed MLPA analysis, showing an in-frame deletion of exon 3-4 (no. 1), a duplication of exon 2 (no. 2), and a somatic mosaic frameshift deletion of exon 8-15 corresponding to a previously detected mosaic dystrophin pattern in muscle tissue (no. 3)(Table 2). The *DMD* gene of the remaining 28 probands was analysed by HR-MCA and sequencing. Small mutations in the *DMD* gene were found in five patients (Table 2). Four of these are novel mutations and considered pathogenic

TABLE 1: Diagnoses confirmed by DNA mutation in 185 patients from the diagnostic BMD cohort 1985-1995

GENE	DISORDER	ALL (N)	%
DMD	Dystrophinopathy	87	47,0%
CAPN3	LGMD 2A	7	3,8%
DYSF	LGMD 2B	2	1,1%
SGCA	LGMD 2D	3	1,6%
FKPR	LGMD 2I	2	1,1%
ANO5	LGMD 2L	10	5,4%
SMN1	SMA late onset	2	1,1%
GAA	Pompe's disease late onset	1	0,5%
confirmed	total	114	62%
	from present study	8	7,0%

in three patients: a frameshift mutation in exon 71 (c.10258del, p.ser3420Leufs25*; no. 4), a mosaic splice-site mutation of exon 21 (c.2623-2A>C; no. 5) and a 3-basepair deletion in exon 6 (c.482-484del, p.Thr161del; no. 6) which was not previously reported in the literature although submitted in Leiden Muscular Dystrophy pages database in both one DMD and two BMD patients. The fourth novel mutation is a missense mutation in exon 60 (c.8974G>A, p.Val2992Met; no.7); in silico analysis does not predict to affect splicing of this exon. However, the amino acid valine is evolutionary highly conserved in codon 2992 and previously dystrophin amounts were found to be reduced in muscle tissue of this patient. This variant has not been reported before in literature, the Muscular Dystrophy pages of the Leiden Open Variation Database (LOVD) or the Human Gene Mutation Database.

Finally, a nonsense mutation was found in exon 25 (c.3337C>T, p.Gln1333*) and the severe BMD phenotype of patient no. 8 might be explained by exon skipping of this "in-frame" exon.[99] However, there was no muscle tissue available for RNA analysis. This mutation indeed has been reported in patients with BMD and DMD/BMD phenotypes in the LOVD.

Clinical data of the eight patients with a newly detected mutation are presented in Table 2. All patients with a novel mutation (nos. 4,5,6,7) were alive and three of them (nos.4,6 and 7) agreed to undergo a new clinical assessment. Patient no. 4 with a frameshift mutation in exon 71 had a severe BMD phenotype with normal cognitive development; this mutation has not been reported in the LOVD. Frameshift mutations in exon 71 have been described in

two BMD patients after RNA studies identified two different isoforms of which one was an in-frame transcript lacking exon 71.[136] However, muscle tissue of patient no. 4 was not available for RNA studies. Patient no. 5, who was a somatic mosaic for an acceptor splice-site mutation (c.2623-2A>C) of exon 21, declined clinical follow up. He was interviewed by telephone and said that he had no symptoms at age 23, that he was working full time in the military service and playing sports several times a week. Patient no. 7, with a missense mutation, had very mild symptoms, calf hypertrophy and hyperCKemia. He had suffered from frequent cramps in his teens and as an adult he cannot keep up with his peers in sports. Because his mutation is not classified yet as pathogenic for BMD and in the past the working diagnosis of his referring physician included Miyoshi-type muscular dystrophy (MMD), further DNA analysis was performed; no mutations in the ANO5 gene were found. His phenotype is compatible with mild BMD and therefore we advised long term cardiac monitoring.

Three patients were deceased and counselling could be meaningful for family members. The daughter of patient no. 1 had already requested a test for carrier status in 1985 and re-analysis of her DNA confirmed that she was a carrier of the same deletion; now, her daughter could be counselled for carrier status. The second deceased patient (no. 8) died at the age of 39 from acute cardiac failure and severe cardiomyopathy was confirmed by autopsy. Although he had a phenotype of severe BMD with progressive weakness since the age of six, he had not had recent clinical follow up by a cardiologist. He did not have children and renewed counselling of his sisters was not considered meaningful, as earlier linkage analysis had excluded them as carriers. The third deceased patient (no. 3) had a severe cardiomyopathy and died at age 61 from acute cardiac death. Because he was a somatic mosaic for a frameshift deletion of exon 8-15 his daughters are at risk to transmit DMD to their (grand) sons. Thus genetic counselling and cardiac monitoring is meaningful for his female offspring. Finally, counselling was advised for female family members of patient no. 2 in whom we found a duplication of exon 2, compatible with his severe BMD phenotype.

New mutations were confirmed in new blood samples in four patients and in the daughter of a deceased patient. One patient declined sending a new blood sample and two other patients were deceased.

Resuming, in this study we found a mutation in eight of the 31 patients (26%) whose DNA was re-analysed. Overall a genetic mutation was found in 114 of the 185 patients who were referred in the period 1985-1995 (Table 1). Confirmation of the diagnosis dystrophinopathy in eight patients in the present study has improved the diagnostic yield by about 7%.

Discussion

Our recent re-evaluation of 79 referrals for DNA analysis of the *DMD* gene in cases without a genetic diagnosis enabled more specific genetic testing in 31 patients and revealed mutations in eight patients, at least three of these are novel pathogenic mutations. We have described the clinical characteristics with long term follow up of all eight, including the patient with a missense mutation with very mild symptoms and hyperCKemia, which are compatible with mild BMD. Our findings had relevant clinical implications as we were able to advise the patients to undergo screening for cardiomyopathy and could counsel the family members. It is surprising that none of these eight patients had visited a neuromuscular specialist in all these years, although three of the five patients who are still alive had progressive muscle weakness. Apparently their treating rehabilitation physician, cardiologist or pulmonologist had not realised that the disorder had not been confirmed by a genetic test. A confirmed genetic diagnosis in patients with progressive weakness will enable the treating physicians to employ targeted patient care and to offer genetic counselling to family members. Cardiomyopathy associated with a mutation in the *DMD* gene can present at a later age.[137] Thus confirmation of dystrophinopathy can result in early diagnosis of cardiac manifestation and subsequent treatment.

Renewed DNA analysis based on a requisition form sent in 15-25 years ago has not been described before. Whereas the aim of our study was to investigate undiagnosed cases of suspected BMD, other studies have focused on searching for small mutations after dystrophinopathy had already been proven but genetic classification had not yet been achieved.[138, 139]

The present study has some limitations. Firstly, the retrospective evaluation was based on clinical data that were collected a long time ago. In the period 1985-1995 DNA analysis was the only possibility for confirming a BMD-like phenotype in male adults; little was known about limb girdle muscular dystrophies which, as we know now, encompass a broad clinical spectrum.[134] We therefore confined ourselves to re-evaluating only the *DMD* gene because recalling all unsolved cases for clinical follow up decades after the request for DNA analysis was not practically feasible. One exception was made for the patient who agreed in reassessment after we had found a missense mutation; we thought it was in his interest to exclude MMD type 3. Another limitation is that 22 files were excluded because no clinical data were available. This shows that sufficient and adequate clinical information provided with the request for DNA analysis can help in elucidating diagnoses even after a long time. Finally, screening for deletions/duplications and subsequently sequencing for small mutations with regular methods does not reveal all BMD mutations, in particular those in the promoter area and deep in the introns. RNA analysis on muscle tissue could be helpful in probable BMD

cases with abnormal dystrophin expression in muscle tissue or in BMD patients with frame-shift or nonsense mutations like patients no. 4 and no. 8 respectively in this study. However, muscle tissue was not available (anymore) for most of the cases in this study.

The clinical features of some neuromuscular disorders may show similarities to those of BMD.[19, 21] At the time of our chosen cohort, DNA analysis of the *DMD* gene was the only genetic analysis available to distinguish BMD from sporadic or autosomal recessive LGMD, late onset Pompe's disease or late onset spinal muscular atrophy (SMA). At present, clinicians consider a broad spectrum of muscular dystrophies in the diagnostic work up.[140] Indeed, over time other diagnoses were assigned to 27 patients.

For an efficient diagnostic process it is important that sufficient and standardised clinical information is supplied on the requisition form for DNA analysis. This enables the molecular geneticist to advice on further diagnostic steps to be followed. Next generation sequencing (NGS), which is upcoming in regular patient care, is for now not a first choice diagnostic tool in suspected BMD as the majority of patients with dystrophinopathy has large deletions in the *DMD* gene; therefore sequencing should follow screening the *DMD* gene for large rearrangements. Regarding adult patients with mild muscle symptoms, referring physicians should clarify whether diagnostic work up should start with screening for deletions or duplications in the *DMD* gene or with targeted limb girdle gene panels. A prospective study could help to support a practical diagnostic strategy for daily practice in neuromuscular patient care.

We conclude that extending the analysis by sequencing the *DMD* gene in potential cases of BMD has yielded new mutations in a small but relevant number of patients. The diagnosis of dystrophinopathy was confirmed also in patients with mild symptoms at initial referral. This had clinical implications with regard to cardiac monitoring and genetic counselling.

TABLE 2 **Clinical information on patients with a mutation in the *DMD* gene found in the present study** *p. 44-45 >>*

?: *Information not available; CK: serum creatine kinase;* ***)** *results of Western blot analysis on muscle tissue, as reported in 1992; mutation in* ***bold*****:** *novel*

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The authors thank the patients for undergoing re-evaluation and R. Nooijens for his technical assistance in HR-MCA analysis and the sequencing of the old DNA samples.

Patient		1	2	3	4	5	6	7	8
age	presenting symptoms	10	4	47	5	6	childhood	15	5
	DNA request	56	15	49	43	6	9	26	16
	reassessment	----	40	---	63	23	32	47	----
	deceased	57	----	61	----	----	----	----	39
symptoms	presenting	cramps	walking difficulties in childhood	fatigue after exercise	walking difficulties in childhood	hyperactive behaviour	exercise related cramps	exercise related myalgia in the legs	fatigue after exercise
	calf hypertrophy	yes	yes	yes	?	?	yes	yes	yes
	muscle cramps	yes, in teens	yes, in childhood	yes, only in adolescence, exercise related	?	no	yes	yes, only in adolescence	no
	CK level (U/l)	?	785	1090	638	1600	4500	2430 at age 26; 512 at age 33	1573
muscle weakness	upper limb	?	yes, severe at age 32	not at age 49	yes, severe at age 43	no	yes, severe at age 32	no	yes, in childhood
	lower limb	?	yes, severe at age 13	not at age 49	yes, severe at age 43	no	yes, severe at age 32	no	yes, in childhood
	wheelchairbound	yes, unknown at which age	yes, since age 14	no	yes, at age 36 following trauma	no	yes, since age 25	no	yes, at age 30
	cardiac involvement	?	yes, cardiac failure since age 28	yes, cardiomyopathy and atrial fibrillation; acute cardiac death	no	no	no	atrial fibrillation at age 46	yes, cause of death (autopsy results)
	respiratory support	?	yes, intermittently at age 28, permanently at age 32	no	yes, nocturnal ventilation at age 55	no	no	no	no

muscle biopsy	performed	yes, date not known	yes, at age7	yes	yes, at age 43	not done	yes, at age 9	yes, at age 26	yes, at age 6
	hematoxylin-eosin	dystrophy	dystrophy	mild dystrophic changes	dystrophy		dystrophy	no dystrophic features	dystrophy
	dystrophin immunohisto-chemistry	not done	not done	staining of dystro-phin was positive in some and neg-ative in others	irregular and weak staining of dystrophin		?	no abnormality	not done
	Western blot of dystrophin	not done	not done	not done	no abnormality*)		reduced amount of dystrophin	reduced amount of dystrophin*)	not done
working diagnosis untill 2011		?	Becker muscular dystrophy	possible Becker muscular dystrophy	Becker muscular dystrophy	no neuromus-cular disorder	Becker muscular dystrophy	Miyoshi-type muscular dys-trophy was con-sidered before; no mutations were found in the ANO-5 gene (MMD3)	Becker muscular dystrophy
DNA	*DMD* gene	deletion exons 3-4	duplication exon 2	deletion exons 8-15 somatic mosaic	exon 71	exon 21 somatic mosaic	exon 6	exon 60	exon 25
	mutation				**c.10258del, p.Ser3420Leufs*25**	**c.2623-2A>C**	**c.482-484del, p.Thr161del**	**c.8974G>A, p.Val2992Met**	c.3337C>T, p.Gln1113*
analysis of a new DNA sample		yes in daughter: carrier status confirmed	yes, mutation confirmed	not applicable	yes, mutation confirmed	not done	yes, mutation confirmed	yes, mutation confirmed	not applicable

□



3

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.

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.[105] 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[141]. 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.

Patients and Methods

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 (CS and AH-E).

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*).[142] 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.

Results

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.

Family 1: deletion exons 45-51

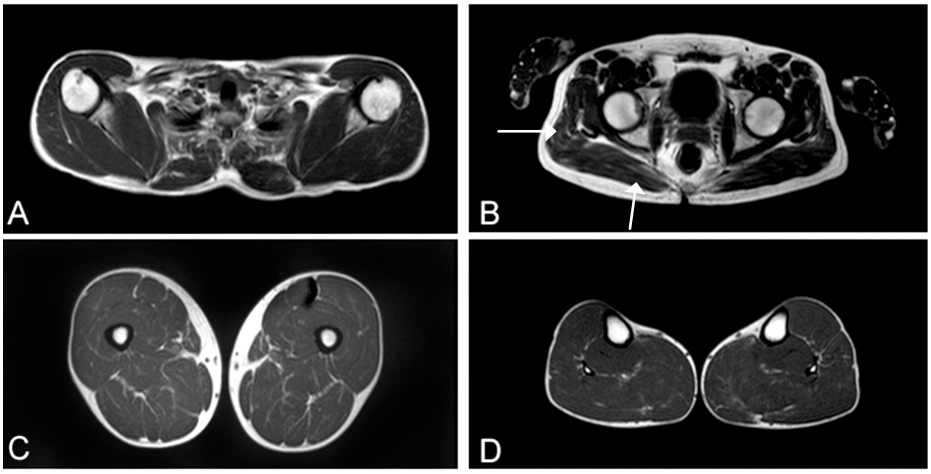
Patient A1, born in 1962, had suffered from painful muscle cramps in his legs since childhood. Cramps were mostly provoked by exercise such as hiking or occurred after he had bumped his leg. Cramps resulted in painful nodules and could last for one and a half hour. Sometimes his father had to carry him 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 (figure 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. H.F.M. Busch, neurologist. The grandfather showed no neurological signs or symptoms of BMD and his CK values were normal.

FIGURE 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.

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 meters was 2.6 sec, (reference: mean time to run 9 meters for healthy boys of 11 years is 2.5 ± 0.28 sec).[143]

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.

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*"[142] is a public repository of variants reported in literature or submitted directly to the database. We used 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, seven 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 local 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 twelve more patients: six with deletions of exons 45-51[144-146] and six with deletion exons 50-51 in the literature.[147, 148]

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.

TABLE 1 BMD patients with a deletion of exons 45-51

Patient number	Reason referral	Age at presentation (y)	Last examination at (y)	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	146
A4	↑CK	8	8	no	2950	normal	M-PCR	dystrophin present	145
A5	cousin of patient A4	7	7	no	896	normal			145
A6	grandfather patient 4	67	67	no	63	normal			145
A7	pain on exercise	8	8	pain, mild calf hypertrophy	1000	normal	M-PCR	dystrophin present	79
A8	↑CK	10	11	no			M-PCR	dystrophin present	144 patient B-19
A9	myoglobinuria	14	14	no			M-PCR	dystrophin present	144 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 database K. Kekou/ E.Kanavakis
A12	↑CK	4	16	no	810	normal	M-PCR	dystrophin present	149

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 (y)	Last examination at (y)	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	147
B3	grandfather patient B2	69	69	no		normal	M-PCR		147
B4	great uncle of patient B2	55	55	no			M-PCR		147
B5	cousin of mother of patient B2	28	28	no			M-PCR		147
B6	maternal cousin of mother of patient B2	29-55	29-55	no			M-PCR		147
B7	maternal cousin DMD patient with another mutation	18	18	no	327		M-PCR	dystrophin present	148

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

Discussion

Our study shows that BMD patients with 45-51 or 50-51 in-frame 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 A1 and 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 problems appear to occur more frequently in boys with Becker muscular dystrophy than in the general population.[51]

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.[145, 147]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[150, 151] and quantitative multiplex PCR. MLPA[152] 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[105] 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 makes the difference between being wheelchair bound at age 9 or climbing trees at age 14. The ultimate goal is to develop specific treatments that 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.[5] 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 may affect the binding of dystrophin to β-dystroglycan.[153] Mice with such a deletion have muscles with severe dystrophic pathology.[154] Furthermore, a deletion of exons 45-55 is associated with a BMD phenotype with a prognosis of a favorable outcome,[155] but can also be associated with X-linked dilated cardiomyopathy depending on the exact location of the breakpoints of the deletion.[156, 157]

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.[158] 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.



PART II

Duchenne muscular dystrophy



4

Reduced grey matter volume and altered white matter in boys with Duchenne muscular dystrophy

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Abstract

Objective

Duchenne muscular dystrophy (DMD) is characterized by progressive muscle weakness caused by DMD gene mutations leading to absence of the full-length dystrophin protein in muscle. Multiple dystrophin isoforms are expressed in brain, but little is known about their function. DMD is associated with specific learning and behavioral disabilities which are more prominent in patients with mutations in the distal part of the DMD gene, predicted to affect expression of shorter protein isoforms. We used quantitative MRI to study brain microstructure in DMD.

Methods

T1-weighted and diffusion tensor images (DTI) were obtained on a 3 tesla MR scanner from 30 patients and 22 age-matched controls (ages 8-18 years). All subjects underwent neuropsychological examination. Group comparisons on tissue volume and DTI parameters were made between DMD and controls, and between two DMD subgroups that were classified according to predicted Dp140 isoform expression (DMD_Dp140^{+} and DMD_Dp140^{-}).

Results

DMD patients had smaller total brain volume, smaller grey matter volume, lower white matter fractional anisotropy, and higher white matter mean and radial diffusivity than healthy controls. DMD patients also performed worse on neuropsychological examination. Subgroup analyses showed that DMD_Dp140^{-} contributed most to the grey matter volume differences and performed worse on information processing.

Interpretation

Grey and white matter is affected in DMD at a whole-brain level. Differences between subgroup DMD_Dp140^{-} and controls indicate an important role for the Dp140 dystrophin isoform in cerebral development.

Introduction

The X-linked disease Duchenne muscular dystrophy (DMD) is characterized by severe and progressive muscle weakness due to mutations in the DMD gene. Mutations cause the absence of dystrophin, a 427 kDa protein.[116] Dystrophin provides structural stability by connecting the contractile filament actin and the dystrophin-associated glycoprotein complex in the sarcolemma. In addition to muscle weakness, DMD is characterized by cognitive impairment which is thought to be non-progressive. In patients, the mean full-scale intelligence quotient (FSIQ) is approximately one standard deviation below normal.[42] DMD patients exhibit problems with verbal short term memory, visuospatial long term memory and verbal fluency.[159]In addition, a higher incidence of autism spectrum disorders, attention deficit and hyperactivity disorders (ADHD) and obsessive-compulsive disorders occurs, as well as specific learning disorders such as dyslexia have been described.[43, 45, 48]

In the central nervous system (CNS), full length dystrophin (Dp427) is expressed mainly in cortical neurons and cerebellar Purkinje cells, but little is known about its function.[160] At least four shorter isoforms of dystrophin are also present in the CNS with molecular weights of 260, 140, 116 and 71 kDa respectively. These isoforms derive from alternative tissue specific promoter sites that use the following exons as the N–terminal domain, exon 30 for Dp260, exon 45 for Dp140, exon 56 for Dp116 and exon 63 for Dp71.[161] Cognitive impairment is more prominent in patients with mutations in the distal part of the DMD gene (downstream of exon 44) that are associated with the loss of Dp140 expression. This suggests a genotype-phenotype correlation between mutation and brain involvement.[121]

Post-mortem studies of DMD patients have not shown consistent abnormalities.[162, 163]. However, on a micro scale, architectural alterations in the cortical-spinal system and the parvalbumin and calbindin-positive cortical circuitry have been found in a mouse model for DMD. [164-166] Radiological studies in DMD patients showed glucose hypometabolism using positron emission tomography (PET) and altered metabolite concentrations with magnetic resonance spectroscopy.[167-169] Magnetic resonance imaging (MRI) studies in DMD are limited to case reports and one quantitative study using voxel-based morphometry analysis and resting state fMRI with a region of interest in the motor cortex.[170] However, quantitative information on in vivo whole brain morphology and microstructure in DMD is not available.

The aim of the present study was to investigate the brain in DMD using quantitative MRI to assess grey and white matter volume and microstructure. Composite scores for cognitive and behavioral functioning were analyzed in relation to MRI data. Finally, MRI data and

composite scores were compared between two DMD subgroups with different mutations predicted to affect the expression of the Dp140 isoform and controls.

Methods

Participants

DMD patients were recruited from the Dutch Dystrophinopathy Database, a nationwide patient registry. Exclusion criteria were the presence of MRI contraindications and the inability to lie supine for at least 30 minutes. DMD diagnosis had previously been confirmed using DNA extracted from whole blood taken from patients by a Gentra Puregene DNA purification kit (Gentra Systems, Minneapolis, USA), following the manufacturer's instructions. Deletion/duplication analysis of the DMD gene was performed using multiplex ligation-dependant probe amplification (MPLA kit Salsa P034/P035 MCR-Holland, Amsterdam, The Netherlands). Small mutations were identified by means of high resolution melting curve analysis (HR-MCA) and subsequent sequence analysis.[135] Within the recruited patients, two patient subgroups were distinguished (Figure 1). Patients carrying mutations upstream of intron 44 (transcription start site) were considered Dp140+. Patients with mutations involving exon 51 (translation start site) or the genomic region downstream of exon 51 were considered Dp140-. Deletions with 3' breakpoints in intron 44 were considered Dp140+ in case the breakpoint was upstream of the Dp140 promoter and unique first exon. A polymerase chain reaction (PCR) based approach assessed whether the Dp140 promoter and first exon were deleted using the forward primer Dp140F 5'-ctttgtgcggaggcattg-3', and the reverse primer Dp140R3 5'-CCAGCTTTTAGGCACACACA-3'. A second PCR was performed for these patients to confirm the presence of exon 51 using the forward primer hEx51F 5'-ggcttggacagaacttaccg-3' and the reverse primer h51r 5'-cttctgcttgatgatcatctc-3'. Patients carrying deletions in the region between intron 44 and exon 51 were excluded due to the unpredictable effect on Dp140 expression. All protocols were approved by the local Medical Ethical Committee. All participants or their legal representatives provided written informed consent.

Procedures

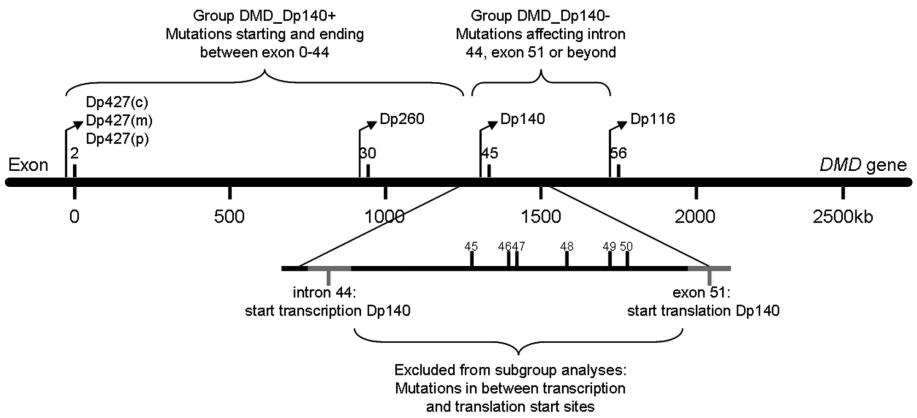
The protocol was designed such that all tests could be performed on the same day. The Brooke scale for upper extremity function and Vignos scale for lower extremity function were used as a measure of muscle function.[171] Participants were taken to a dummy scanner to familiarize themselves with the sounds and layout of an MRI machine. Subsequent neuropsychological evaluation (NPE) lasted a maximum of one hour. MRI scanning was then performed on a 3T scanner (Philips Achieva, Philips Healthcare, Best, The Netherlands) using an 8 channel receive-only headcoil. Participants were placed supine with legs slightly

elevated for comfort. 3D T1-weighted (T1w; echo time (TE) and repetition time (TR) 4.6/9.8 ms; spatial resolution 1.17x0.92x1.2 mm; 4:55 min), T2-weighted (T2w; TE/TR 80/4000 ms; spatial resolution 0.5x0.46x3.6 mm; 2:46 min), fluid attenuated inversion recovery (FLAIR; TE/TR 120/10000 ms; spatial resolution 1x0.8x3.6 mm; 4:00 min), and diffusion tensor (DTI; TE/TR 56/9440 ms; spatial resolution 1.96x2x2 mm; 32 directions, b=0 and 1000 s/mm2; 6:40 min) images were obtained parallel to the corpus callosum. An independent neuroradiologist assessed the scans for gross structural abnormalities and incidental findings.

Neuropsychology

Three composite scores were constructed using predefined parts of standardized and validated instruments reflecting the most important aspects of neuropsychological and behavioral/emotional functioning in DMD [172]. The first composite score represented reading (scores standardized for age with a range of 1-19, mean 10 and standard deviation 3 in healthy controls) and was based on the mono-syllabic word reading test and the one minute reading test derived from CB&WL: "continu benoemen en woorden lezen" (Bos & Lutje Spelberg, Boom test uitgevers, Amsterdam, The Netherlands). The second composite score represented information processing (scores standardized for age with a range or 1-19, mean 10 and standard deviation 3 in healthy controls), and used two subtests from the Kaufman

FIGURE 1. Dp140 transcription and translation sites used for subgroup classification



The prediction of Dp140 expression is based on mutation location in the DMD gene. There is a large gap between the start of transcription and start of translation for Dp140. Patients with a mutation starting and ending exactly in between transcription and translation were excluded from the analyses due to unpredictability of the mutation effect on Dp140 expression.

Assessment battery (number recall for auditory working memory and block counting for conceptual thinking) and one subtest from the Wechsler Intelligence Scale for Children (symbol search). The third composite score for emotional and behavioral problems (range 0-40) was constructed on the basis of the four problem based subscales from the Dutch version of the Strengths and Difficulties Questionnaire for parents. [173] The Personal Adjustment and Role Skills scale (PARS) –III, a parent report measure studied in 287 DMD patients, was used to assess psychosocial adjustment.[174] General intellectual level was assessed by the Peabody Picture Vocabulary test (PPVT-III-NL). This test measures receptive vocabulary, is normalized for age, and requires no motor response. It was previously used in 130 Duchenne boys.[175]

MRI

T1w and DTI scans were analyzed with FSL software v5.0.[176] Intracranial volumes were obtained using the Brain Extraction Tool with BET2 + betsurf (BET v2.1)[177] with a fractional intensity threshold (f) of 0.35. Grey matter, white matter and cerebrospinal fluid (CSF) were segmented using the automated segmentation tool FAST.[178] Quantification of the volumes was performed with fslstats -V using the partial volume corrected output from FAST. Diffusion tensor imaging scans were corrected for motion and distortion using ExploreDTI.[179] ExploreDTI descriptive statistics was used to calculate mean whole brain FA and MD. Maps of axial diffusivity (AxD), radial diffusivity (RD), fractional anisotropy (FA) and mean diffusivity (MD) were also created. These maps were then exported for tract-based spatial statistics (TBSS) in FSL.[180]

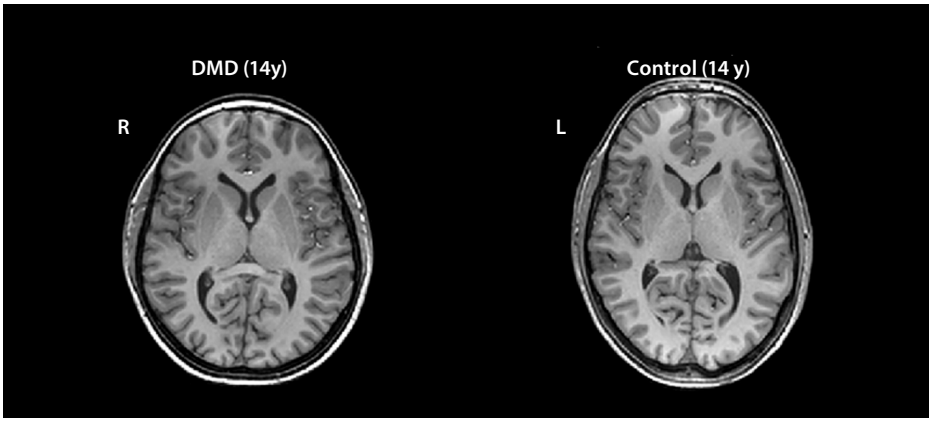
Statistical analysis

PARS and PPVT-III-NL scores of the DMD group were compared to known DMD populations using one sample t-tests. One sample t-tests were also used to test volumetric differences in whole brain intracranial, white matter, grey matter, and CSF volumes, as well as NPE scores between patients and controls. To correct for multiple comparisons, we used the method of Benjamini and Hochberg which limits the false discovery rate (FDR) to 5%.[181] One-way ANOVA was used to assess differences in these same parameters between the subgroups DMD_Dp140+, DMD_Dp140- and controls.

A univariate general linear model with Duchenne status yes/no was used to assess the relation between three whole brain MR measures decided upon before the analysis to reduce multiple comparisons (grey matter volume, mean FA and mean MD) and the neuropsychological composite scores for each group, and to assess the relation between the whole brain MR measures and age. All analyses were performed using SPSS v20.0 (IBM Corp., NY, USA) and considered significant at $p<0.05$.

To localize MRI findings in specific areas of the brain, voxelwise statistical analyses were performed. For grey matter, voxel-based morphometry analysis (VBM) was performed with FSL-VBM.[182] For white matter, FA, MD, RD and AxD, data were analyzed with age as covariate using Tract-Based Spatial Statistics (TBSS).[180] An additional TBSS analysis was performed with ICV and TBV as covariates to assess the relationship between volume and white matter microstucture. The aligned maps from each subject were projected onto the FA skeleton, and the resulting data used for voxel-wise cross-subject statistics using RANDOMISE ttest_2 with multiple comparison correction tcfe_corrp.[183]

FIGURE 2. Representative T1w-images



A 14 year old boy with DMD is depicted on the left and an age-matched healthy control on the right. Images are displayed in the radiological convention with the left hemisphere on the right of the image. Note the absence of gross structural differences between the patient and the age-matched healthy control, which explains why routine visual assessment of the MR images did not show abnormalities.

Results

Participants

Thirty-three DMD boys (ages 8-18 years) and twenty-two healthy age-matched control boys (ages 8-16 years) participated in the study. Three DMD boys were excluded because they withdrew informed consent prior to the MRI scan (n=2), or the scans could not be made due to technical problems (n=1). In five DMD boys, one or more of the MRI scans could not be evaluated due to motion artifacts (one T1w and five DTI), leaving 29 DMD boys for T1w analysis and 25 for DTI analysis. In the controls, one DTI scan was also excluded

due to motion artifacts. Some NPE tests could not be completed within one hour prior to the MRI, resulting in the missing composite scores. Clinical characteristics and an overview of the available MRI and NPE assessments are summarized in Table 1. Representative T1w images are shown in Figure 2. DMD general intellectual level was not different from data reported in the literature using the PPVT-III (97.56 vs 98.15 respectively).[159] Psychosocial adjustment, reflected by the PARS-III score, was significantly better than in historical controls (91.62 vs 84.43, p<0.001).[174] From the 29 DMD patients, 12 were classified as DMD_Dp140+ and 12 as DMD_Dp140-. Five DMD patients were excluded from the subgroup analysis because of the unpredictability of the mutation effect on Dp140 expression. No mutations downstream of exon 56 containing promoter sites of Dp116 and Dp71 were found.

TABLE 1. Patient characteristics and available assessments

	Control	DMD	DMD _ Dp140+	DMD _ Dp140-
Participants (***n***)	22	29	12	12
Age (years)*	13.1 (2.0)	12.3 (2.8)	13.1 (3.3)	12.0 (2.7)
Range (years)	8-16	8-18	9-18	8-16
Steroid treatment (***n***)	-	24	10	10
On/off 10 cycle (***n***)	-	22	10	9
Brooke scale*	1	2.1 (1.6)	2.0 (1.6)	2.5 (1.7)
Vignos scale*	1	5.9 (3.1)	6.2 (3.1)	6.1 (3.2)
Wheelchair bound (***n***)	-	15	7	6
Age of loss of ambulation (years)*	-	10.2 (2.1)	11.0 (2.6)	9.9 (1.3)
Regular education (***n***)	22	17	8	6
Reading composite score (***n***)	21	25	11	12
Information composite score (***n***)	21	27	11	12
SDQ composite score (***n***)	22	28	11	12
3D T1 scans (***n***)	22	29	12	12
DTI scans (***n***)	21	25	10	10

** Data are presented as mean with standard deviation. n: number of participants; SDQ: Strengths and Difficulties Questionnaire (for emotional and behavior problems); 3D: 3 dimensional; DTI: diffusion tensor imaging*

MRI

Visual assessment of the T1w, T2w and FLAIR images did not reveal any gross structural brain abnormalities. Quantitative analysis of the T1w images showed a significantly smaller total brain and grey matter volume in DMD patients compared to controls (Table 2). Intracranial volume was also smaller in DMD, although this difference failed to reach statistical significance (p=0.056). Adding intracranial volume as a covariate in the analysis of grey matter and total brain volume showed that these structures were not smaller compared to total intracranial volume. VBM analysis showed small regions within the occipital cortex and the left insula that were significantly smaller in patients compared to controls (Figure 3a).

White matter FA was significantly lower in DMD in the occipital lobe (Figure 4). Significantly higher MD in DMD was found mainly in the corpus callosum. RD was significantly higher in DMD throughout the white matter. Taking ICV and TBV as covariates in the TBSS analysis revealed a relationship between these parameters and all three DTI measures, while part of the occipital lobe showed changes in FA and RD independent of volumetric parameters (data not shown).

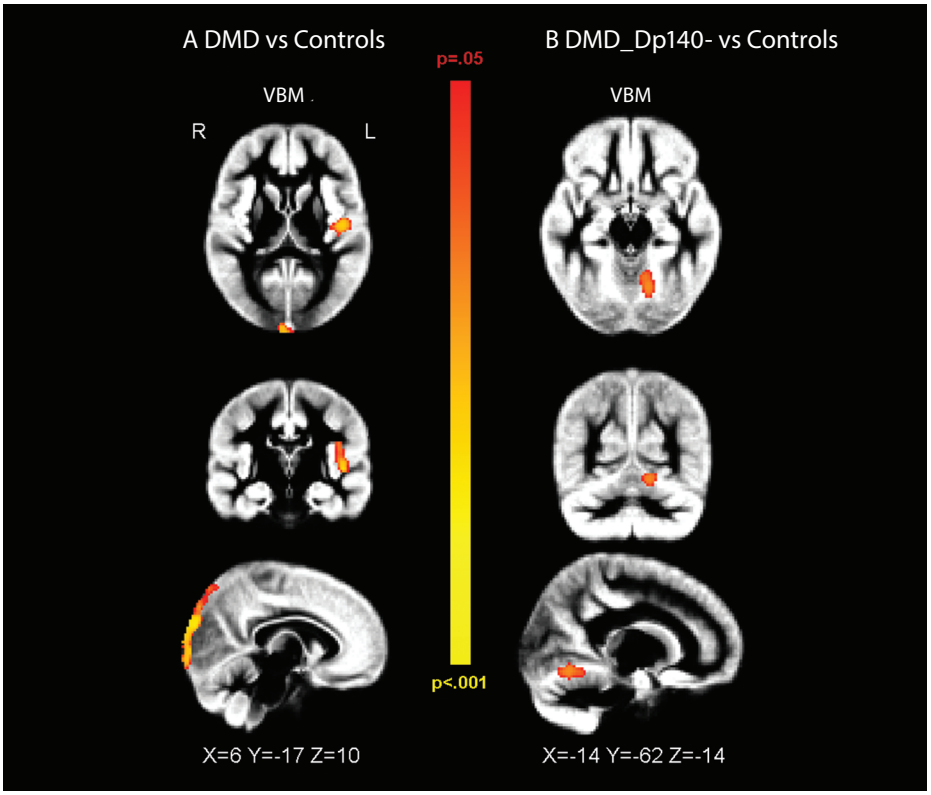
DMD_Dp140- patients had the smallest grey matter, total brain and intracranial volumes (Figure 5a-c). VBM analysis showed differences between DMD_Dp140- and controls in a different but similarly small area as found in the whole DMD group versus control analysis (Figure 3b). TBSS showed similar results for DMD_Dp140+ and DMD_Dp140- versus control with significantly lower FA and higher RD, but no differences in MD. There was no effect of age on grey matter, white matter or total brain volumes. However, age did correlate with white matter microstructure, with higher FA and lower MD and RD with increasing age.

TABLE 2. Brain structure volumes in DMD patients and age-matched controls

Volumes	Control	DMD	DMD _ Dp140+	DMD _ Dp140-
Intracranial (cc)	1651 ± 95	1584 ± 137	1633 ± 149	1550 ± 123
Total brain (cc)	1430 ± 78	1355 ± 118 *	1401 ± 121	1324 ± 114 *
Grey matter (cc)	811 ± 42	757 ± 56 ***	770 ± 53	749 ± 63 ***
White matter (cc)	619 ± 45	598 ± 70	631 ± 75	576 ± 61
CSF (cc)	222 ± 30	229 ± 32	232 ± 39	226 ± 29

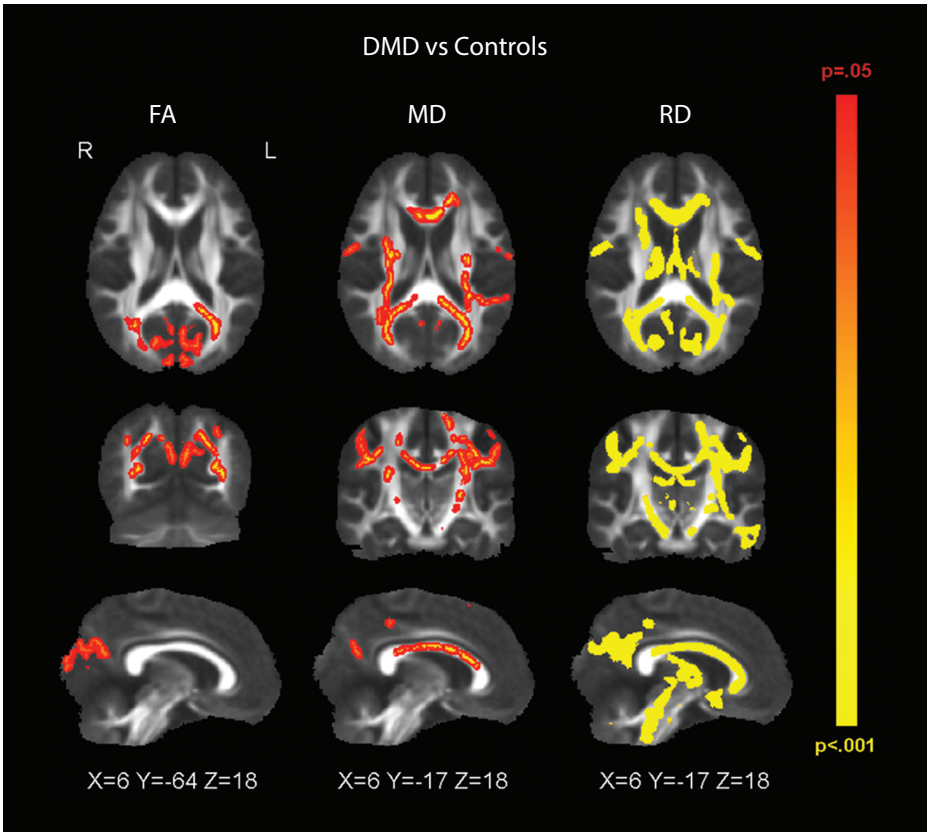
*Significant differences between patient groups and controls are indicated by * for p<0.05 and *** for p<0.001 after multiple comparison correction. CSF: cerebrospinal fluid*

FIGURE 3. Grey matter voxel-based morphometry results



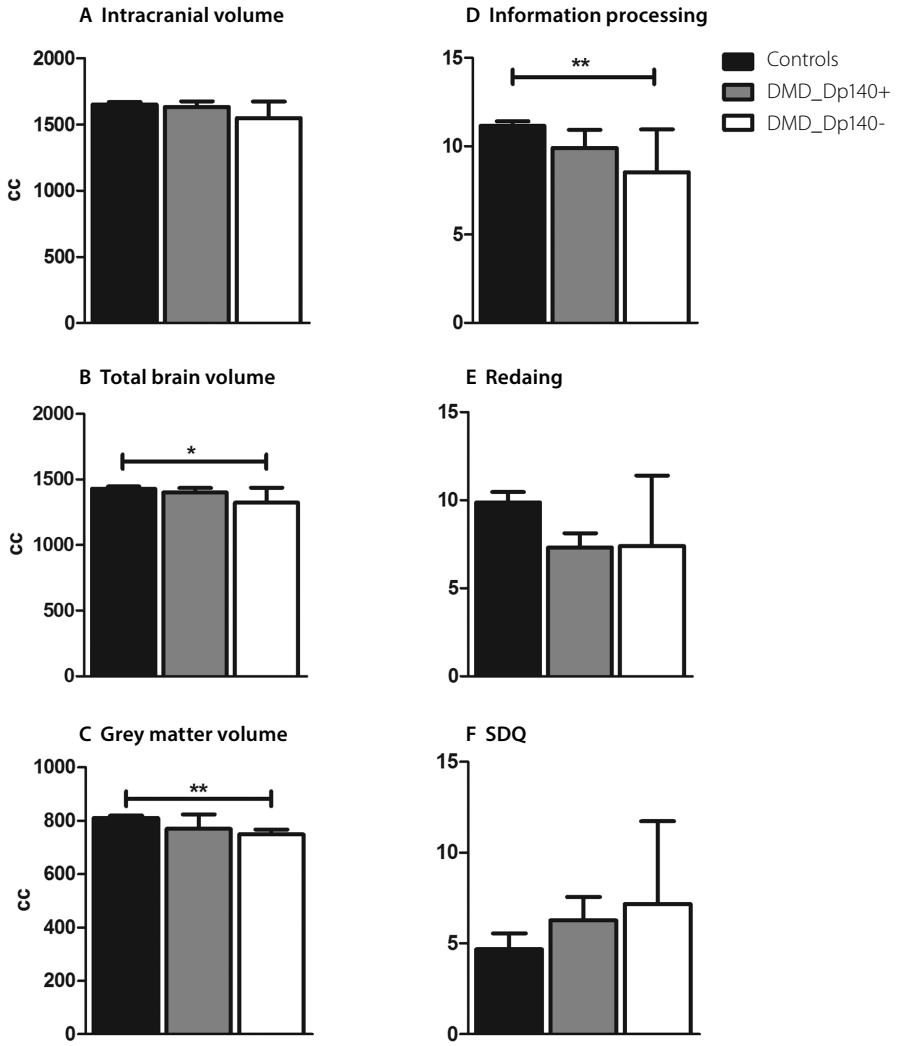
Local grey matter reductions in part of the left insula and occipital lobe are demonstrated by VBM when comparing the whole patient group to controls (a). Local grey matter reductions in part of the lingual gyrus and left cerebellar cortex are demonstrated by VBM when comparing the DMD_Dp140- subgroup to controls (b). No differences were found between DMD_Dp140+ and controls ***(data not shown).*** *Images are displayed in the radiological convention with the left hemisphere on the right side.*

FIGURE 4. DTI analyses of the white matter



Widespread alterations in white matter with reduced fractional anisotropy (FA) in the occipital lobe, and increased mean diffusivity (MD) and radial diffusivity (RD) throughout as demonstrated by tract-based spatial statistics (TBSS).

FIGURE 5. T1w volumes and composite scores



*Subgroup analyses for controls (black) DMD_Dp140^{+} (grey) and DMD_Dp140^{-} (white). Volumes (cc) of the intracranial (a), total brain (b) and grey matter (c) are depicted on the left. Composite scores for information processing (d), reading (e) and emotional/behavioral problems derived from Strengths and Difficulties Questionnaire (SDQ) (f) are shown on the right. Significant differences between groups are indicated by * for p<0.05, ** for p<0.01 after multiple comparison correction.*

Neuropsychological parameters

All three NPE composite scores were significantly different between DMD patients and controls, with DMD patients performing worse. Subgroup analysis showed that DMD_Dp140- patients performed worst on all composite scores, although this difference was statistically significant only for information processing. Scores of DMD_Dp140+ patients were between DMD_Dp140- and controls (Figure 5d-f). Within each subgroup, there were no significant correlations between the three composite scores and whole brain grey matter volume, whole brain FA or whole brain MD using the univariate general linear model.

Discussion

In DMD patients, grey matter and total brain volume are smaller, and white matter microstructural integrity is altered compared to healthy age-matched controls. Strikingly, comparison of DMD patients with and without Dp140 expression with controls shows that DMD_Dp140- patients, in whom the expression of the Dp140 isoform is predicted to be impaired, were more severely affected in terms of total brain and grey matter volumes as well as neuropsychological scores.

The smaller grey matter volume in DMD is global, as the regions in the occipital lobe and left insula identified by VBM are too small to account for the difference. This is in contrast to a previous quantitative MRI study in DMD brain, in which VBM identified a smaller volume in the left primary sensorimotor cortex, although no analysis of absolute grey matter volumes was performed.[170] One CT study reported smaller grey matter volume in 20 out of 30 DMD patients and contributed this difference to cortical atrophy. In the present study, skull size was proportional to total brain volume. There is a dynamic interaction between the developing brain and skull growth,[184, 185] with 25-27% increase in whole brain and intracranial volume between early childhood and adolescence. Therefore, correcting total brain volume with respect to intracranial volume enables the detection of atrophy. This implies that, smaller brains in DMD are due to differences in maturation rather than atrophy.

In contrast to grey matter, white matter showed no difference in volume, but was significantly affected on a microstructural level as shown by DTI. The smaller FA and higher MD found in DMD indicate compromised structural complexity, and suggest reduced fiber density, increased membrane permeability, and/or decreased structural organization.[186] The higher RD in DMD indicates more fiber branching, decreased structural organization, or demyelination which can lead to increased membrane permeability.[187, 188] The white matter microstructural differences between DMD and controls were associated with intracranial and total brain volume, although these cross-sectional data do not allow any con-

clusions regarding a causal relationship between the two. The global nature of the reduction in grey matter volume and microscopic nature of the white matter alterations could explain why these changes have not been described in previous post mortem studies and conventional MRI studies, as they can only be observed with whole brain quantitative MRI.

The global reduction in grey matter volume of patients could be related to the fact that full length Dp427, affected in all patients, is normally located in cortical and hippocampal pyramidal cells, the fetal cerebral cortex and cerebellar purkinje cells.160.[160] In our DMD_Dp140- subgroup, no mutations downstream of exon 56 were found. This implies that differences in MRI and NPE assessments in this study are mainly due to a lack of expression of the Dp140 isoform and do not include Dp116 and Dp71. The involvement of the Dp140 isoform in cognitive impairment was previously identified.[121] Dp140 is predominantly active during fetal development in the astroglial processes.[160] DMD patients with mutations disturbing Dp71 expression were even more severely affected mentally.[189] Therefore, the loss of more dystrophin isoforms may well have a cumulative effect on altered brain maturation. The correlation analyses did not reveal a significant association between mean whole brain grey matter volume, FA or MD and the neuropsychological composite scores within the groups. A modest, yet significant association between grey matter volume and total IQ was previously reported by Reiss et al[185] in 69 healthy children. Our study was not powered to find such function to morphology associations within the group of DMD patients or controls, but aimed at finding differences between these groups.

There are two potentially confounding issues in this study, which need to be specifically addressed. First, the brain still undergoes crucial developments in the age range of our subjects. Maximum global grey matter volume is reached between 6-9 years of age, after which it linearly declines approximately 5% per decade.[184] White matter volume increases substantially from infancy through early adulthood with changes in myelination and axonal packing.[190] Age had a significant effect on FA, MD and RD which is in line with recent results.[190] This made a correction for age essential in the TBSS analysis despite matching for this parameter upon recruitment.

Secondly, the steroid treatment could have affected brain morphology. Volume reductions of hippocampus and amygdala have been observed in patients with asthma or rheumatic diseases receiving steroid treatment.[191] Children with excess endogenous glucocorticoids due to Cushing syndrome showed a significantly smaller total brain volume, larger ventricles, and smaller amygdala volumes than controls.[192] Five DMD patients in our study were steroid naïve or had only been on steroid treatment for a short period of time at least ten years before participating. Two of these patients were in DMD_Dp140+ and

two in DMD_Dp140-. Given that DMD_Dp140- showed the most extensive differences with controls in all tests, this strongly suggests that steroid use cannot be the main contributor to the morphological findings. However, a secondary contributing effect of the steroids on the morphological MRI findings cannot be excluded.

In conclusion, we observed significant global morphological and microstructural differences in the brain between DMD boys and controls. Results suggest that these differences arise from altered brain maturation rather than atrophy. Differences between DMD_Dp140+ and DMD_Dp140- patients indicate an important role for the Dp140 dystrophin isoform in cerebral development. Future studies could include longitudinal data to confirm the lack of atrophy or focus on assessment of the structural and functional connectivity between different brain networks (the connectome) and hopefully further dissect the role of each of the dystrophin brain isoforms.



5

Temporal muscle hypertrophy and skull eccentricity in Duchenne muscular dystrophy

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Abstract

Muscle hypertrophy and muscle weakness are well known in Duchenne Muscular dystrophy (DMD). Decreased muscle force can have secondary effects on skeletal growth and development such as facial and dental morphology changes. In this study we quantified temporal muscle thickness, circumference and eccentricity of the skull and the head on T1-weighted MRI scans of the head of 15 Duchenne muscular dystrophy patients and 15 controls. Average temporal muscle thickness was significantly increased in patients (12.9 ± 5.2 mm) compared to controls (6.8 ± 1.4 mm) ($p<0.0001$), whereas the shape of the skull was significantly rounder compared to controls. Temporal muscle thickness and skull eccentricity were significantly negatively correlated in patients, and positively in controls. Hypertrophy of the temporal muscles and changes in skull eccentricity appear to occur early in the course of Duchenne muscular dystrophy. Further studies in younger patients are needed to confirm a causal relationship.

Introduction

Duchenne muscular dystrophy (DMD) is characterized by progressive weakness of skeletal, respiratory and cardiac muscles due to the absence of dystrophin. Medical and supportive care have improved life expectancy from 20 up to 25-30 years. Muscle weakness in the legs usually manifests itself when a boy begins to walk on his toes and falls frequently. A prominent feature is calf hypertrophy, which in combination with a disturbed walking pattern and increased serum creatine kinase level suggests the clinical diagnosis of DMD.

Muscle hypertrophy in DMD is not restricted to the calf muscles, but has also been reported in other muscle groups. Specifically, the gracilis and sartorius muscles in the upper legs, deltoid and infraspinatus muscles in the shoulder region and the tongue can be hypertrophic[193,194] One case report has described temporal muscle hypertrophy in DMD.[195]

It is likely that muscle hypertrophy in DMD is a response to dysfunction of weak skeletal muscles. It is currently not known if hypertrophy in DMD leads to an increase in muscle strength.[196,197] Mechanical forces exerted by skeletal muscles can influence bone structure[198] and, therefore, secondary effects of muscle weakness or hypertrophy could lead to differences in bone development. For example, decreased bite force has been suggested as related to skeletal changes in the oral-facial region.[199,200] Furthermore, dental malocclusion in DMD has been described as a result of imbalance of muscle strength in the orofacial region and developmental changes in jaw bones.[200] During an MRI study of the brain in DMD patients we consistently observed temporal muscle hypertrophy and a different skull shape compared to healthy age-matched controls. The difference was so pronounced that, given the knowledge of the impact that skeletal muscle force can have on bone, we choose to quantify and report our observations.

Methods

Subjects

15 Caucasian DMD boys (8-16 years) and 15 age-matched healthy Caucasian (9-15 years) boys participated in the study. Patients were recruited from the Dutch dystrophinopathy database. Only patients with a genetically confirmed diagnosis were included. Controls were recruited from schools or sports clubs. In patients using steroids (intermittent scheme, 10 days on, 10 days off) the scans were performed during the off-period. The study was approved by the institutional Ethics Committee. All participants and their parents gave informed consent.

MR imaging

Three-dimensional T1-weighted images (echotime (TE) 4.6ms, repetition time (TR) 9.8ms, voxel size 1.17 x 1.17 x 1.2 mm, acquisition time 4:56min) of the head were obtained at 3T (Achieva, Philips Healthcare, Best, The Netherlands) using an 8-channel head coil. Quantitative determination of eccentricity and circumference of the skull (excluding muscles, fat and skin) and the head (including muscles, fat and skin) was performed by two independent observers. Transversal images from each boy were assessed from the anterior and posterior commissure (AC/PC) upwards (reconstructed to 10 slices, 1.5 mm thick, 2.0 mm slice gap). Regions of interest (ROIs) of skull and head were manually drawn in MIPAV (http://mipav.cit.nih.gov) and averaged for the 10 slices. MIPAV was also used to determine the largest cross-section of left and right temporal muscles.

Statistics

Differences in skull eccentricity, head eccentricity, temporal muscle thickness, skull circumference and head circumference between DMD and controls were assessed. This was done using the values averaged for two independent observers by means of a Students t test with Welch's correction were the variances where significantly different. Interobserver variability was computed using the Intraclass Correlation Coefficient (ICC). Pearson correlation was computed between skull eccentricity and temporal muscle thickness, age and temporal muscle thickness, and between age and eccentricity. Statistical significance was set at $p<0.05$. Statistical analyses were performed by using SPSS (version 17.0, SPSS Inc, Chicago, IL, USA)

Results

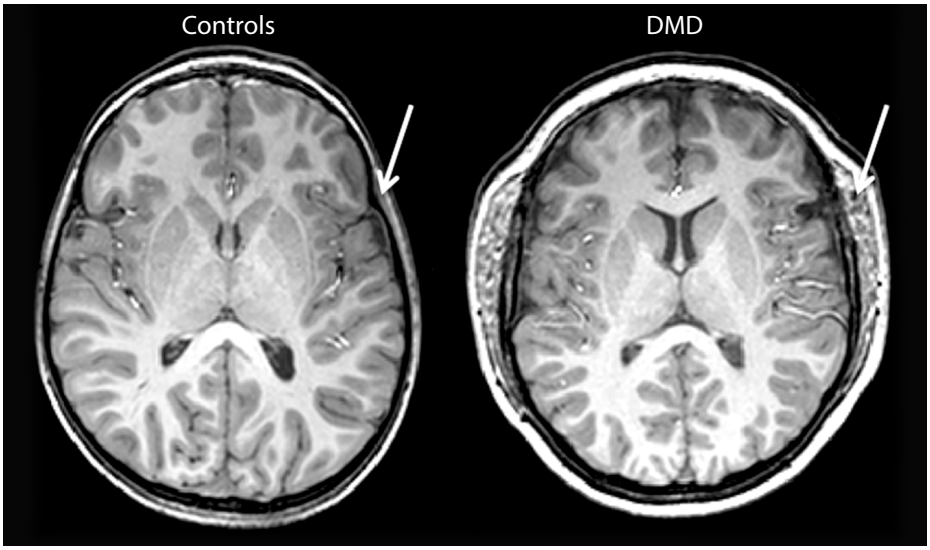
Descriptive data of the participants are shown in Table 1. On visual inspection of the images, hypertrophy of the temporal muscles was observed in all DMD patients as well as a rounder shape of the head compared to more ovoid shaped in healthy controls (Figure 1).

The average cross-sectional temporal muscle size was 12.9 ± 5.2 mm in DMD compared to only 6.8 ± 1.4 mm in controls ($p<0.001$; Figure 2A). In controls, a positive correlation between temporal muscle size and age was observed, while in DMD no such relation was observed (Figure 2B). The ICC for measurements of eccentricity and circumference of the skull and head was 0.994 ± 0.001. The circumference of skull and head showed no significant difference between DMD and controls. As a parameter for shape, eccentricity was calculated from ROIs drawn on head circumference and on skull circumference (Figure 1). Eccentricity ranges between 0 and 1, in which "0" represents a perfect circle and "1" a parabola.

TABLE 1 Descriptive data of the participants

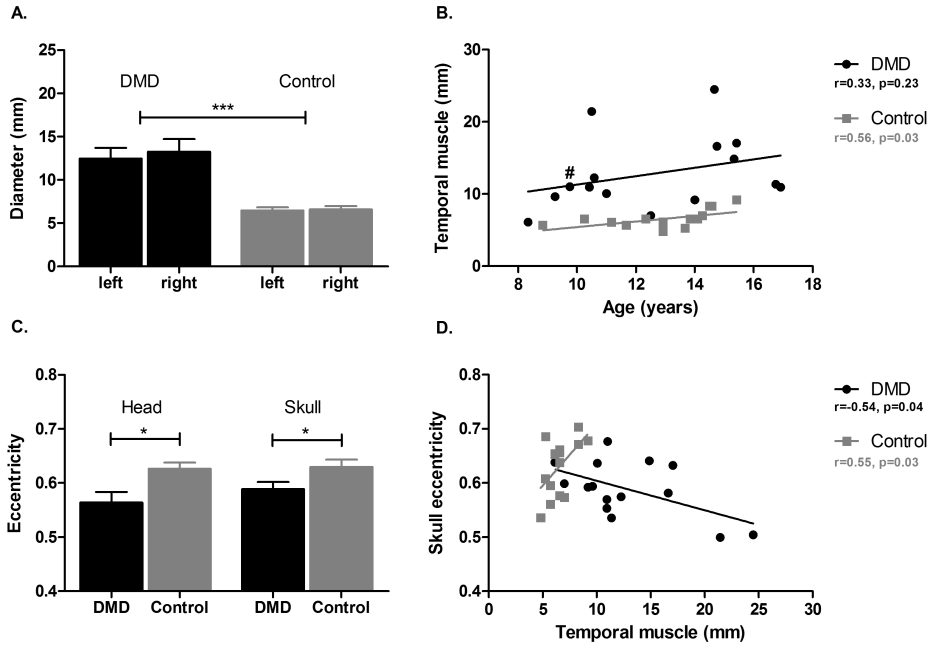
	DMD N=15	Controls N=15
Age		
Mean ± *SD*	12.4 ± 2.7	12.4 ± 1.7
Median (range)	14.0 (8-16)	12.9 (9-15)
Use of Steriods (n)		
Current use	12	0
Stopped >1year	2	
Never used	1	
Wheelchair bound (n)	**9**	**0**
Head circumference (cm)		
Mean ± *SD*	56.0 ± 2.8	55.5 ±1.4
Range	52.5-60.0	53.0-58.5

FIGURE 1 T1-w MRI scans of the head



Transverse sections taken parallel to the anterior and posterior commissure (AC/PC) of a healthy 10-year-old boy (left) and 10-year-old boy with DMD. The DMD boy shows a rounder head and skull shape and bilateral temporal muscle hypertrophy (indicated by arrows).

FIGURE 2. Temporal muscle thickness and skull morphology in DMD boys and healthy age matched controls



In all images DMD is depicted in black and control in grey.

A: average thickness of left and right temporal muscles with SEM in DMD and in controls.

B: correlation between temporal muscle thickness (averaged values for left and right) and age in DMD and in controls. Indicated by # is a DMD boy that has never used steroids.

C: eccentricity (0=perfect circle, 1= parabola) of the head and the skull with SEM in DMD and in controls.

D: correlation between temporal muscle thickness and skull eccentricity in DMD and controls; in DMD larger temporal muscle size correlates to a rounder skull shape, but in controls larger temporal muscles correlate to a more ovoid shaped skull.

** = p<0.05*

**** = p<0.0001*

SEM: standard error of the mean

The average skull eccentricity in DMD (0.59 ± 0.05) was significantly different from skull eccentricity in controls (0.63 ± 0.05) ($p<0.05$; figure 2C). DMD boys had a rounder skull shape and controls had a more ovoid shaped skull on transversal images. Skull eccentricity was not correlated with age.

Finally, a significant correlation between temporal muscle size and skull eccentricity was observed (figure 2D). In DMD, thicker (hypertrophic) temporal muscles were related to a rounder shaped skull ($r=-0.54$; $p=0.04$). In controls, thicker temporal muscles were correlated with an ovoid skull shape ($r=0.55$; $p=0.03$).

Discussion

Our results showed systematic temporal muscle hypertrophy in DMD boys which is clearly apparent on transverse MR images of the head. Additionally, in the transverse plane, the shape of the skull in DMD patients is rounder compared to healthy age-matched controls. Temporal muscle hypertrophy and skull eccentricity are significantly correlated in DMD boys, whereby a thicker muscle is associated with a rounder skull. On clinical examination temporal hypertrophy is visible and palpable and should not be confused with a steroid-induced "moon face".

Hypertrophy of skeletal muscles is commonly observed after training and is associated with an increase in muscle strength. In DMD, however, hypertrophy of the leg muscles is not accompanied by an increase in strength but rather by a decrease in strength, indicating a lower quality of the increased muscle mass.[196,197] It is unclear if the increased muscle mass of the temporalis muscles we observed results in an increased or decreased force, as we did not assess muscle strength of the temporalis muscles. Previous literature suggests that muscle force of the temporalis muscles decreases despite the hypertrophy, as bite force is known to be impaired in DMD patients above the age of eight.[199,201]

In addition, our results show that thicker temporal muscles are associated with a more ovoid skull shape in controls. Taken together, this could suggest that the weaker, hypertrophic cranial muscles, among which the temporal muscles, influences the development of the skull, resulting in a rounder shape. We did not observe a correlation of skull shape with age. Therefore, it is likely that the changes in skull shape are present at a young age. In support of this suggestion are data from 7-week-old dystrophic (dy/dyC57BL/6J) mice where the anatomical shape of the skull and mandible was different compared to control mice. This would suggest that bone development is affected early in the muscular

dystrophy.[202,203] Furthermore, histopathological dystrophic features were already present in the temporal muscles in 7-week-old mdx mice.[204] An alternative hypothesis is that the observed skull shape is independent from temporal muscle hypertrophy, but generally related to bone differences such as short stature [205] and different bone mineral density[206] as recently described in DMD.

As the majority of the DMD boys included in this study used chronic steroid treatment this could have influenced the changes we observed. Though hypertrophic muscles due to anabolic steroids have been reported [207], current literature does not describe any relation between corticosteroid use and muscle hypertrophy. On the contrary, corticosteroid therapy is known to result in muscle wasting and type II muscle fibre atrophy[208, 209] In addition, Guillaume Duchenne described muscle hypertrophy in the 19th century[210] when corticosteroid therapy was not yet present. As corticosteroid therapy is usually not initiated in DMD before the age of 6 years, it is highly unlikely that steroid therapy is solely responsible for such a profound effect on skull morphology. Further studies in younger patients, preferably before they commence steroid treatment, could possibly clarify the pathophysiological mechanism of skull eccentricity and the relation to hypertrophy of the temporal muscles.

Improvement of muscle strength and function is the main goal for new treatment strategies in DMD. Future introduction of new therapies aimed at improving muscle strength, will it is hoped improve long-term outcome and life expectancy in DMD. Irreversible skeletal changes may limit recovery of muscle function. Therefore, it is important to pay attention to both the evaluation of muscle function as to skeletal deformation, such as we have described for the skull in this report.



6

Prednisone 10 days on/ 10 days off in patients with Duchenne muscular dystrophy

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Abstract

Corticosteroids are effective in improving motor function in patients with Duchenne muscular dystrophy within 6 month-2 years of treatment initiation, but there is as yet no consensus on which treatment scheme is the best. We retrospectively analyzed data of 35 DMD patients who were treated with prednisone 0.75 mg/kg/day intermittently 10 days on/10 days off. Prednisone was started during ambulant phase at age 3.5-9.7 years (median 6.5 years). The median period of treatment was 27 months (range 3-123 months). The median age at which ambulation was lost was 10.8 years (mean 10.9 years; 95% confidence interval 10.0-11.8 years). Nine patients (26%) had excessive weight gain. Eight boys (21%) had a bone fracture, which was when four of these eight children lost ability to walk. Treatment was stopped in two obese patients, in two hyperactive boys and in one patient following a fracture. Our data suggest that prednisone 10 on/10 off has relatively few side effects and extends the ambulant phase by 1 year compared to historical controls.

Introduction

In 2004, Manzur et al. reported that corticosteroid treatment in Duchenne Muscular Dystrophy (DMD) effectively improves muscle strength for 6 months-2 years and improves respiratory muscle strength for 6 months.[211] In 2005, published guidelines in the Netherlands advocated treatment of DMD patients with 0.75 mg/kg prednisone daily for at least the ambulant years.[212] This conclusion was based on trials using daily dose corticosteroids, which proved to be superior to placebo[213, 214] and to an alternate-day scheme (one day on/one day off).[215] Different doses as well as different intermittent schemes of administration have been tried in clinical trials with the aim of reducing major side effects.[216-220] However, there is as yet no internationally accepted consensus on which corticosteroid treatment scheme is the best for patients with DMD. In addition, there has yet to be a randomized study involving a head-to-head comparison.

Until 2005 clinical experience with corticosteroids treatment in DMD patients had been scarce in the Netherlands and mainly restricted to a few rehabilitation physicians who prescribed prednisone. In rehabilitation center De Trappenberg, however, young boys with DMD have been offered prednisone since 1996. The treatment was given in an alternating scheme, 10 on/10 off, in which 10 days prednisone 0.75 mg/kg/day was alternated with 10 days without corticosteroids; this was in accordance with the dosage scheme of the Hammersmith Hospital in London.[221, 222] This particular therapeutic drug regimen was chosen on the assumption that a sustained clinical effect can be achieved during ten days corticosteroids, while side effects are decreased due to an off phase in which the pituitary-adrenal axis can restore its function.

Here, we describe a follow up study of these young DMD patients who were administered this alternating prednisone therapy, with focus on motor skills and the side effects of prednisone.

Patients and Methods

Patients and clinical data

Between 1996 and 2005, 43 patients with DMD, accompanied by their parents, consulted the rehabilitation centre De Trappenberg for treatment with corticosteroids. We have studied the files of these patients retrospectively and evaluated the data. All patients showed progressive muscle weakness typical of DMD, and diagnosis was confirmed by DNA analysis. The rehabilitation physician (IG) and the pediatrician (GJB) performed the clinical monitoring.

The prednisone starting dosage was 0.75 mg/kg/day in an alternating schema of 10 days on and 10 days off. The treating physician adjusted the dose during growth. In the post-ambulant phase dose was lowered to 0.3-0.5 mg/kg/day 10 days on/ 10 days off. The following assessments were performed prior to the initiation of prednisone treatment and every six months thereafter: (1) time to run or walk 10 meters as fast possible; (2) time to stand up from a sitting position on the floor with crossed legs, with assistance of the hands, but no support from the wall or furniture. The time tests were performed independently of the phase of prednisone therapy. During the visits body weight, height and blood pressure were measured. Weight gain was interpreted with the Standard Deviation Score (SDS) on a weight-to-height growth chart. On this chart +2.0 SDS refers to the P^{98} percentile on the reference chart for Dutch children, whereas 0 SDS refers to the P^{50} percentile. Blood pressure was correlated to height and age and interpreted with a reference table.[223]

Radiological examinations of the spine in anteroposterior and lateral direction were performed yearly in order to follow any development of scoliosis and to register subclinical vertebral fractures. The morning serum cortisone level was measured twice yearly during the off period. In the period 1996-2001 cortisone was measured on days 11, 16 and 20 at 9 am and 4 pm. From 2002 onwards, only the morning cortisone level was measured on day 20, which is the last day of the off period. All patients received a standard advice to increase prednisone dose during periods of stress, like high fever or before surgery. The incidence of bone fractures and the age at which ambulation was lost were registered. Loss of ambulation was defined as being unable to walk unsupported indoors.

Statistical Analysis

Descriptive statistics are shown as median or mean ± standard deviation (SD).
The Kaplan Meier survival curve of ambulation was calculated with the help of SPSS ver. 12.0 (SPSS, Chicago, IL).

Results

Clinical data

The median age at which DMD was diagnosed was 4 years (range 9 months-10.4 years) while the median age at time of evaluation was 10.5 years (range 5- 18 years)

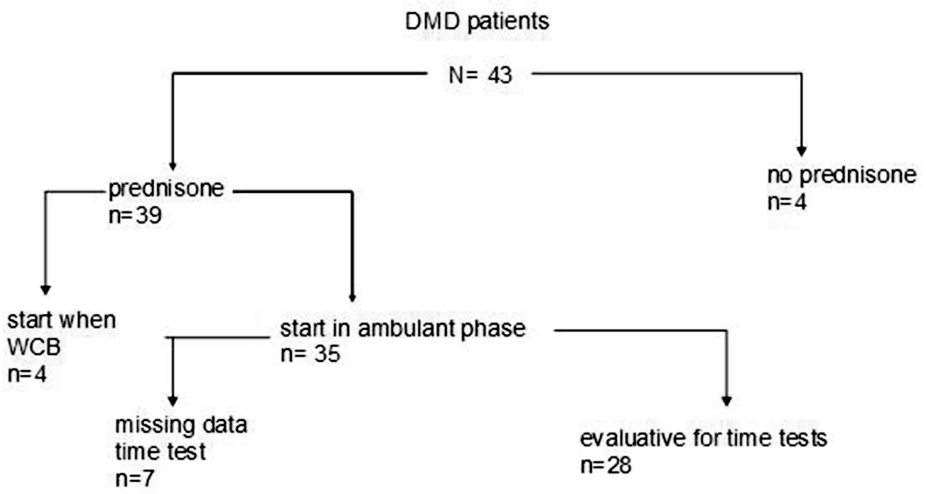
Eighteen patients (42 %) were living in the region of the rehabilitation center and the other 25 patients were seen on tertiary referral base. Twenty-five boys (58%) were attending a regular school, and 18 were attending a special school for children with impaired motor

and/or cognitive skills. The parents of four patients decided, after receiving details of the treatment, not to start treatment with prednisone because of behavioral problems (three patients: autism/retardation) or a mild clinical pattern (one patient). Two of the untreated patients became wheelchair bound (WCB) following a leg fracture due to a trauma at age 11.0 years and 12.6 years, respectively. The remaining 39 patients received prednisone for a period of 3-123 months (median 27 months, average 38 months). In four patients prednisone was started during the non-ambulant phase (Figure 1).

The median age at which prednisone was started was 6.5 years (range 3.5-9.7 years). The dosage of 0.75 mg/kg was adjusted to body weight during follow up, except in four boys because of obesity. After the loss of ambulation, the dosage of prednisone was lowered to 0.3-0.5 mg/kg.

Scoliosis surgery was performed on two patients aged 13.5 years and 8 years, respectively. The former had used prednisone for 9 months until he became WCB at age 9.9 years. The second had already lost ambulation at the time of the operation- at age 6.5 years due to a femur fracture. No patient needed respiratory support during follow up. Three patients received cardiac medication from their cardiologist.

FIGURE 1 Schematic overview of the cohort of patients with Duchenne muscular dystrophy that were followed in De Trappenberg between 1996-2005



WCB: wheelchair bound

FIGURE 2 Cumulative prolongation of ambulation plotted against age

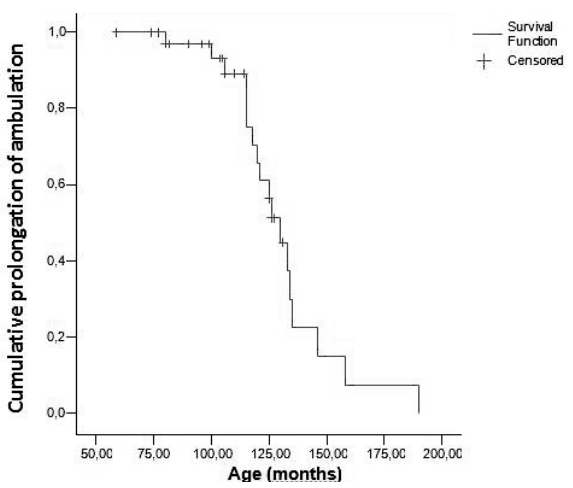
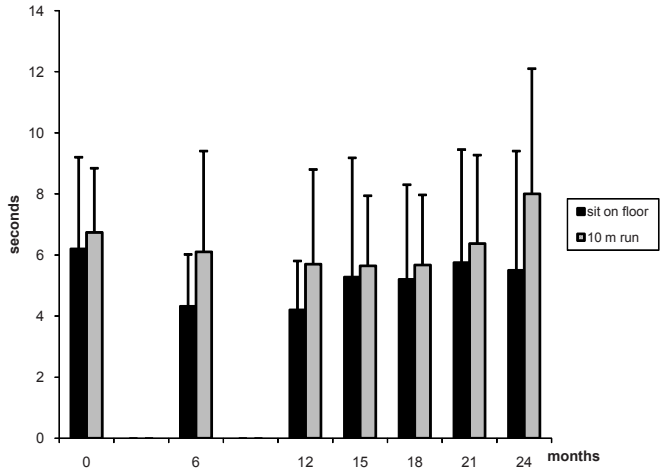


FIGURE 3 Results of the motor-time tests during the follow-up of the DMD patient cohort, from start treatment with prednisone onwards



Motor skills

Ambulation

The median age at which ambulation was lost in the 35 prednisone treated patients was 10.8 years (130 months; with a mean age of 10.9 years, 95% confidence interval (CI) 10.0-11.8 years; Figure 2).

Functional scores

The data obtained in the motor-time tests for 28 treated patients was evaluated (Figure 1). The follow-up was less than 6 months in four patients and in two other patients the treatment was stopped due to hyperactivity. During the first months of treatment the time needed to perform the running or standing up test decreased or remained stable, subsequently increasing when disease progressed (Figure 3).

Side effects

Side effects and complications of prednisone are shown in Table 1**.** Five patients stopped treatment due to side effects of whom three had lost ambulation at that time. Eighteen patients (46%) showed a weight gain on the weight-to-height growth chart during prednisone (nine patients ≤ 1SDS weight gain, nine patients > 1 SDS weight gain). None of the ambulant boys had a weight gain of more than 1.0 SDS. The body weight in seven DMD patients was already at 1.0 SDS before the start of prednisone.

Eight boys (21%) had a fracture during prednisone treatment, with trauma (falling) being the cause of the fracture in six boys; the cause was unknown in the other two boys. All four patients with a femur fracture became WCB due to the fracture. Two boys had a fore arm fracture and two others a lower leg fracture. Of the latter, one boy was only temporarily immobilized while the other had lost ambulation 6 months prior to the fracture.

On one occasion, three boys had a systolic blood pressure (SBP) or diastolic blood pressure (DBP) level that was >95% percentile after correction for age and height. In two of them (one boy at age 10 following 5 years prednisone and the other at age 13 following 7.5 years prednisone respectively) the cardiologist also found a diminished ejection fraction on echocardiography; treatment with an angiotensin converting enzyme inhibitor was commenced. In the third boy, high blood pressure was found three years after he had stopped prednisone; he was given digoxin but data on cardiac function were lacking. The blood pressure in all other patients remained < 95% percentile during follow up.

In the first group of 16 patients that received prednisone in the period 1996-2001, serum cortisone levels were very low to nearly zero on the first day without prednisone (day 11),

while in all patients the morning level restored to normal before restart of prednisone. From 2002 onwards, morning cortisone levels on day 20 were normal, except in two patients who had a low level of 0.08 µmol/l (reference 0.18-0.72 µmol/l) on one occasion. None of the patients suffered from adrenal insufficiency during the follow-up period.

TABLE 1: Reported side effects and complications in the cohort of 35 DMD patients treated with prednisone 10 days on/10 days off.

Side effects/ complications	Yes	No	Reason to stop steroids
Weight gain (n=32)		14	
≤1 SDS	9		
> 1 SDS	9		2
Body Height (n=28)			
Average (0 SDS)	14		
≤- 1 SDS	14		
Behavior problems (n=20)	4 hyperactive	12	2
	4 nycturia		0
Adrenal insufficiency day 20 (n=35)	0	35	
Fracture (n=35)	8	27	1

Discussion

Among our pediatric patient group with DMD, the mean age at which patients treated with the 10 on/10 off prednisone scheme became WCB was 10.9 years. Comparable studies in which daily doses of steroids were used have reported a mean age of 11.5[224] and 12.5 years[225] and those in which alternate-day high dose prednisone (1.3-2.0 mg/kg) were used have reported 14 [226] or 12.2 years.[227] However, a drop-out percentage of 55% was reported during daily prednisone 0.75 mg/kg/day due to obesity or infection-related complications.[226]

Control patients who were not treated in these non-randomized studies and DMD patients reported in historical series from 1967-2002 became WCB at mean age of 8.5-12 years.[228-230] The age at which ambulation is lost is a clinical meaningful endpoint to evaluate efficacy of treatment in DMD patients although it has not been a primary endpoint in randomized studies with steroids in DMD patients up until now.[231] There is no clear consensus on the

definition of loss of ambulation. We used a rather practical definition that ambulation was lost if the patient could no longer walk in a household setting.[232] Although the median treatment period was nearly two years, the patients in our series had a long-term follow up which allowed the construction of a Kaplan-Meier survival curve of the prolongation of walking. In four patients of our series, the follow up of ambulation during steroids was complicated by a leg fracture that resulted in the loss of ambulation. Our study reflects the daily practice of treatment with steroids in DMD. It provides important information when long-term benefits and side effects of steroid treatment are being evaluated.

In a study in which the inclusion criterion was a minimum of 4 years of treatment with deflazacort in an alternating 20/10 scheme, Biggar et al. [218] reported that 35% of the patients were still ambulant at age 12. In young healthy children, functional scores like running or rising from the floor, improve as the child ages from 4 to 10 years due to the growth-related physiological increase in strength.[143] In contrast, DMD patients in the same age group not treated with prednisone showed decreasing mean functional scores during childhood, due to progressive muscle weakness.[143] Although we lack a control group, our patients showed an improvement or stabilization on their functional scores for at least 12 months while on the prednisone 10/10 on/off therapeutic regimen (Figure 3). Most randomized studies have evaluated corticosteroid treatment using functional scores, with a surprisingly large variation in outcome depending whether a metric or non-metric measure was chosen; for example time to run has been evaluated in 10 meters,[217, 218] 9 meters (30 feet)[171, 214, 215, 219, 220, 224] or 8.54 m (28 feet).[221, 233]

Time to stand up from the floor has also been evaluated starting from a lying position,[219, 220] from sitting position with outstretched legs,[217] sitting position with crossed legs, or not well defined. This diversity in evaluation endpoints makes it more difficult to compare studies using different schemes or dosages of corticosteroids.

The main advantage of treatment with steroids in an alternating scheme instead of daily dose would be a similar efficacy with fewer side effects. In most studies evaluating steroids in DMD, weight gain is an important problem. Studies in which the drug regimen consisted of daily dose 0.75 mg/kg/day have reported weight gain > 10% in about 75% of the patients,[213, 214] while we found this in only 25% of our patients and, more remarkably, only in boys after they got WCB. Since seven of the eleven patients that reached a body weight of +2SDS had a body weight of +1SD before start of prednisone, the latter seems to be a risk factor for excessive weight gain. In our group, we found that 40% of the patients did not experience a weight gain, which is comparable to what others found in a small series of six ambulant DMD patients less than five years.[222]

The prevalence of bone fractures in our patients is in accordance with results of a previous study that found 21% fractures in treated as well as not treated DMD boys.[234] Daily steroid treatment has been reported to increase the risk of long bone fractures by 2.6 fold.[225] The majority of the patients was WCB, which is a risk factor for long bone fractures in untreated neuromuscular patients.[234] Remarkably we did not find any vertebral fractures in intermittently treated patients, which is in contrast to the reported 32% compression fractures in the daily steroid treated group.[225] In our DMD cohort hypertension was not a primary problem of intermittent prednisone therapy, although in two patients did have a blood pressure >95% percentile concomitant with insidious cardiomyopathy.

In conclusion, our data show the results of long-term follow up in a large cohort of patients; these data reflect the outcomes observed in daily practice. The retrospective nature of the analysis and the lack of control group limit the interpretation of our results on functional scores. However, controlled studies on the effect of steroids often lack data on long-term endpoints, such as the development of scoliosis and cardiomyopathy.

Until better evidence is available on long-term effects, the reporting of observational data can be helpful in terms of making future decisions on the continuation of steroids for DMD patients who have lost ambulation.

Compared to the daily prednisone therapeutic regimens used in other studies, the 10 on /10 off does seem to be effective in extending ambulant phase in DMD boys; it is also well tolerated. Weight gain is an issue of concern, especially when ambulation is lost, but it seems to occur less often on the 10 on/10 off steroid regimen than when prednisone is administered daily. A prospectively randomized study, with internationally standardized endpoints would still be very useful in providing data for determining the best dose and the best scheme for steroid administration in DMD patients.



7

Summary and future perspectives

Samenvatting in het Nederlands

Summary

Duchenne muscular dystrophy(DMD) and Becker muscular dystrophy (BMD) are like mirror images: alike but different. The two disorders share clinical features such as calf hypertrophy, proximal weakness, hyperCKemia and cardiomyopathy, but they are different concerning clinical course, prognosis and therapeutic perspective. Both disorders are X-linked and allelic on the *DMD* gene, but whereas the protein dystrophin is virtually absent in muscle tissue of DMD patients, in BMD a partly functional dystrophin of different size can be produced. The lack of dystrophin in muscles of DMD patients results in progressive weakness which generally starts in childhood. If dystrophin is not lacking but expressed in reduced amounts or in an aberrant size, the clinical presentation of muscle symptoms is more variable, not only in severity but also in age of onset.[131] Indeed clinical symptoms in BMD are highly variable ranging from mild symptoms, or even asymptomatic with only hyperCKemia, to severe muscle weakness in a limb girdle pattern. This heterogeneity may be the reason why diagnosis in BMD is often delayed.[15]

Diagnosis can be challenging in dystrophinopathies. In general, diagnosis in muscular dystrophies is the resultant of a good interpretation of clinical symptoms and signs in combination with the results of appropriate ancillary investigations ultimately leading to a molecular-genetic classification.

In **chapter 1** the different clinical phenotypes of dystrophinopathies are described and diagnostic steps are discussed. A rule of the thumb is that dystrophinopathy goes hand in hand with an elevated serum CK level. On the other hand the list of differential diagnoses in patients with hyperCKemia is long. Therefore diagnostic algorithms for asymptomatic and paucisymptomatic hyperCKemia are also discussed extensively in the first chapter and a schematic overview is given of neuromuscular disorders that may present with hyperCKemia. Nevertheless, molecular- genetic diagnosis is the mainstay in the diagnostic work up of dystrophinopathies. Information on the DNA mutation is important for several reasons: first to improve classification for individual patients, secondly for genetic counseling of family members and finally to guide future therapies in DMD.

Part 1 of this thesis concerns BMD and two studies are presented.

The study in **chapter 2** deals with delayed diagnosis in BMD. We recently re-evaluated a cohort of 185 patients for whom DNA analysis was requested between 1985-1995. The *DMD* gene was the first gene for which DNA analysis became available for routine diagnostic testing in patients suspected to have a muscular dystrophy; the standard analysis method in the early period of DNA testing was screening for deletions and duplications in

the *DMD* gene. These large rearrangements account for 70% of the mutations in dystrophinopathies. More recently, newer techniques have been developed to screen for small mutations in the gene and currently DNA analysis of the *DMD* gene is a multiple step procedure. If no deletions or duplications have been found using conventional techniques like MLPA, sequencing of the *DMD* gene can be performed to detect small mutations.
It was not known whether mutations had been missed and BMD was underdiagnosed in those patients that were referred for DNA analysis in the early period when sequencing to screen for small mutations was not yet available. In this study we were interested if extending the standard analysis by sequencing the *DMD* gene would improve the yield of diagnosis in patients in whom DNA analysis was requested in the early decade following cloning of the *DMD* gene. All requisition forms of undiagnosed patients with suspected BMD were re-evaluated; if the available clinical information was compatible with BMD, stored DNA was re-analyzed and screened for small mutations. In this cohort re-analysis of DNA could be performed in 31 of 79 patients without a diagnosis. Eight different mutations were found, including four novel mutations. Long term clinical follow up 15-25 years after initial referral was possible in all four patients with a novel mutation. The mutation is pathogenic and leading to a frame shift in at least three of them, including two patients with progressive limb girdle weakness and one patient with a somatic mosaic who was asymptomatic. In addition, in one patient a novel missense mutation was found and he had mild symptoms and hyperCKemia. The clinical relevance of a delayed, genetically confirmed, diagnosis of BMD is that screening for cardiomyopathy can be advised and that family members can be counseled and tested for carrier status. Late diagnosis was achieved because new techniques were applied. The clinical information provided by the referring physician at initial request for DNA analysis was helpful to clarify the diagnosis years later. In contrast, 22 of the undiagnosed 79 cases of the aforementioned cohort were excluded because the requisition forms did not include clinical data. We concluded that collaboration between clinician, molecular geneticist and clinical geneticist can improve the outcome in unsolved cases. One step in this collaboration is that clinical information and, if possible, data of follow up are shared with the geneticist.

The clinical phenotype BMD is very interesting not only because its heterogeneity, but also because the phenotype may serve as a model for treatment of DMD. Currently treatment strategies for Duchenne dystrophy are being developed with the potential to enable production of dystrophin in DMD patients. If the genetic defect in the muscle tissue is changed from out-of-frame to in-frame, resulting in restoration of the reading frame, dystrophin can be produced in the muscle fibers.[105] Since a large spectrum of mutations in the *DMD* gene can cause DMD, customized treatment is required to restore the reading frame. Thus, depending on the location of the mutation, different specific agents will

have to be developed. Exon skipping of exon 51 was the first treatment for DMD that was put on clinical trial.[235] Skipping specific exons in DMD patients to enable dystrophin production will result in an expected end product that is similar to the partly functional dystrophin in patients with BMD who have a deletion including the skipped exon. **Chapter 3** describes the phenotypes and clinical follow up of the two families with BMD in the Netherlands that were known to have such a mutation. In all three patients the course of the disease was very mild. In the first family the index patient had come to medical attention at age 14 years because he was suffering from painful muscle cramps since childhood. His CK level was increased 50 times above normal and an in-frame deletion of exons 45-51 of the *DMD* gene was found. His maternal grandfather, who also suffered from cramps in childhood and adolescence, carried the same mutation. Both males were examined at adult age and none of them had muscle weakness. The grandfather was asymptomatic at age 78 with a normal CK and the grandson had calf hypertrophy at age 46 with minimal exertional related muscle symptoms and a marginally increased serum CK level. In the second family the index patient was eight years old when he was referred to a pediatrician for hyperactive behavior. High serum CK levels were found at routine laboratory investigation. DNA analysis of the *DMD* gene showed in-frame deletions of exons 50-51. He was re-examined at age 14 and had no muscle weakness. Sporadically he experienced exertional muscle cramps. He received medical treatment for his hyperactivity. The very mild clinical course in both BMD families could be promising for DMD patients who are eligible for exon 51 skipping.

Part 2 of this thesis concerns Duchenne muscular dystrophy.

It is generally accepted that the central nervous system is involved in DMD but the pathophysiologic mechanism has not been elucidated yet. Dystrophin, which has an important function in muscles, is also expressed in other tissues, including the brain. Besides the full-length dystrophin protein Dp427 several shorter isoforms are transcribed, which arise from promotors located more distally on the DMD gene. They are known as Dp 260, Dp140, Dp116 and Dp71, each with a preferential distribution in different tissues. (see also figure 3 on page 31). More specifically, Dp140 and Dp71 are present in fetal brain and it is suggested that these isoforms are important for cognitive function.[120] However, the precise role of dystrophin and its isoforms in the brain is not known. DMD patients with severe cognitive impairment often have a mutation in the distal part of the DMD gene.[136, 236] A relation between cognitive impairment and mutations that result in absence of isoforms Dp140 and Dp71 has been suggested.[120, 236] Several studies investigated cognitive function in DMD.[42, 237, 238] Most of them are descriptive, and generally the mean IQ is one standard deviation below the mean in the normal population.[238, 239] Moreover the IQ can range from below 50 till 130 between DMD patients. Although longitudinal follow up

studies are lacking, the cognitive deficits do not seem to be progressive. The literature is not very consistent whether specific domains are affected, but executive function and verbal working memory are both involved.[172] Besides, reading skills seems to be impaired in DMD.[43, 44] Finally, behavioral problems like Attention Deficit and Hyperactivity Disorder (ADHD), Obsessive Compulsive Disorder (OCD) and autism spectrum disorders are more prevalent in DMD.[45, 46, 48]

To investigate the role of dystrophin in the brain a non-invasive tool is necessary. MRI is a safe and suitable method to investigate the brain in detail. Imaging of the brain in DMD is a subject of growing interest, but so far limited imaging studies of the brain in DMD have been performed. Different techniques have been used such as positron emission tomography (PET), which showed region specific glucose hypometabolism,[168] MRI showed focal changes in gray matter concentration[170] and Magnetic resonance spectroscopy (MRS) showed metabolic changes in specific regions like the cerebellum and the temporo-parieto region.[167, 169] We decided to perform an explorative quantitative MRI study of the brain in DMD. In a large series of 30 DMD patients and 22 age-matched boys (aged 8-18 years) MRI images of the brain (T1-weighted and diffusion tensor images) were obtained. Also cognitive function and behavior were examined in all participants. **Chapter 4** describes that the total brain volume and grey matter volume are smaller in DMD patients than in healthy controls. We also found that within the white matter parameters for microstructure were different compared to controls; this suggests that fiber density may be reduced, the permeability of cell membranes may be increased and/or the structure of the white matter may be less organized in DMD. The mean scores on neuropsychological tests were lower in DMD patients. Subgroup analyses showed that DMD patients with a mutation that predicts the lack of isoform Dp140, had significantly smaller brain volumes and grey matter volumes than healthy controls, while in DMD patients with a mutation that predicts presence of Dp 140 we did not find these differences compared to controls. This indicates that the dystrophin isoforms are important for cognitive functioning but also for development of the brain in infancy and early childhood.

Chapter 5 describes and illustrates a serendipity we have found during the study on the brain in DMD. It concerns the cranium and the extracranial muscles. We observed that the skull of boys with DMD has a different shape compared to healthy age-matched controls. Whereas in controls the skull was ovoid shaped in DMD the circumference of the skull was rounder in the transverse plane, which could be determined quantitatively by eccentricity; the mean eccentricity was reduced in DMD.

In muscular dystrophies affected muscles may be hypertrophic. On visual inspection of the MRI images we observed that the temporal muscles in the DMD patients were clearly hypertrophic; the quantified thickness was indeed significantly increased compared to controls.

One could speculate whether a different morphology of the skull has clinical consequences. We found a positive correlation between thickness of the temporal muscles and a rounder shape of the skull in DMD. We hypothesized that the different skull shape was secondary to weakness of the temporal muscles which are involved in masticatory function. The masticatory muscle group include masseter muscles, temporal muscles, medial and lateral pterygoid muscles. Impaired function of masticatory muscles seem to start at a young age as decreased bite force has been observed in DMD patients of 6 years and older[199, 201] as well as dental malocclusion.[240] Both can lead to impaired chewing. The morphology of the skull may be related to malocclusion as individuals with specific morphology such as short lower face show a lower incidence of malocclusion and people with long-faced morphology have weak facial muscles and are predisposed to skeletal open bite.[241, 242] In humans few intervention studies have been done and these lack randomized controls; in young healthy adults bite force increased following training with systematic exercises using chewing gum.[243] In a small study of 13 children (age 7-12 years) with long-faced morphology who had been practicing exercises with chewing gum for at least two hours a day during a year, bite force had increased compared to reference values of untrained age-matched children.[241] More interesting, during this study the morphology of the orofacial structures (anterior mandibular rotation) had changed more than was observed in an historical control group of untreated children of the same age. This suggests that changes in muscle force indeed can influence bony structures. Our study is the first to report a rounder skull shape in patients with DMD who are known to develop weakness of the masticatory muscles. Muscle weakness might be related not only to impaired masticatory function and orofacial morphology but also to skull shape; moreover, it is not known whether the observed rounder skull shape is reversible or may become fixed in older children. In the latter case this may limit the restoration of orofacial function in DMD if new treatments become available that improve muscle strength. For clinical follow up it is important to anticipate that masticatory muscles could be involved early in the disease.

In **chapter 6** we investigated steroid treatment in Duchenne muscular dystrophy in a Dutch cohort. Steroid treatment extends the ambulant phase in DMD.[123, 213, 244] In natural cohort studies boys with DMD became wheelchair-bound between 8.5-12 years.[124] The introduction of chronic steroid treatment in the mid 1990's has prolonged ambulation for at least one year. The benefits are related to the dosage regimen of the steroids and should be weighed against the side effects.

In the Netherlands guidelines for prednisone treatment were established in 2004.[123] In the rehabilitation center *De Trappenberg*, however, young boys with DMD have been offered prednisone since 1996 in an intermittent scheme, in which 10 days prednisone 0.75 mg/kg per day was alternated with 10 days without corticosteroids. In our study we retrospectively analyzed data of a series of 35 DMD patients who have been treated with prednisone 0.75 mg/kg/day intermittently 10 days on/10 days off. Prednisone was started during the ambulant phase at age 3.5-9.7 years (median 6.5 years). The median period of treatment was 27 months (range 3-123). The median age at which ambulation was lost was 10.8 years (mean 10.9 years; 95% confidence interval 10.0-11.8).

Possible side effects of chronic steroid treatment are weight gain, increased risk of osteoporosis and bone fractures. When treatment starts in childhood decreased body height and behaviour problems like hyperactivity have been reported, which all seem to be more pronounced in daily steroid regimes.[225, 244, 245] In most studies evaluating steroids in DMD, weight gain is an important issue. During daily dose of 0.75 mg/kg/day weight gain > 10% was reported in three quarter of the patients.[213, 214] In our study weight gain was found in one quarter of the patients, and more remarkably, only in boys after they got wheelchair bound. Seven of the eleven patients that a reached body weight of +2SDS already had a body weight of +1SDS before start of prednisone. Therefore, this is probably a risk factor for excessive weight gain during steroid treatment. Moreover, we found that 40% of the patients in our study group did not have weight gain. Our data indicate that prednisone 10 on/10 off has relatively few side effects and extends the ambulant phase with one year compared to historical controls. In contrast to the 10 day on/10 day off scheme that is custom in the Netherlands, elsewhere daily dosage of steroids is being used, which may be more successful in sustaining ambulant phase. In a recent observational study the median age for loss of ambulation was 12 years in boys on an intermittent steroid scheme and 14.5 years in boys using daily steroid treatment.[245] In clinical practice, usually treating physicians will discuss with the parents the expected advantages and disadvantages of daily versus intermittent treatment, which will result in shared decision making. Of note, a multicentre randomised controlled trial to search for the optimum steroid regimen is ongoing: FOR-DMD (*www.clinicaltrials.gov*).

In addition, it is reported that continuing steroids treatment in the non-ambulatory phase prevents scoliosis, reduces the need for spinal surgery and delays the progression of respiratory function [225, 244, 246] Resuming, steroid treatment plays an important role in the standard treatment of boys with Duchenne and is recommended in the standards of care for DMD.[16] Moreover, care for DMD will benefit from a multidisciplinary approach in which the different caregivers cooperate and interact as a team.[16]

Future perspectives

During the last decade the organisation of care for patients with DMD has improved a lot in the Netherlands. For young boys with DMD and their parents multidisciplinary teams have been formed in different expert centres. More recently the process of transition for boys with DMD emerging adulthood is getting more attention.[247] In contrast, the connection between the care for DMD and BMD is not always clear. This is somewhat surprising because clinical signs and symptoms of BMD resemble those of DMD. Besides, treatment strategies for DMD aim to improve muscle weakness by restoring the reading frame.[105, 235, 248] This would enable dystrophin expression and chronic treatment might result in a milder phenotype. Theoretically, a milder phenotype will be similar to a BMD phenotype.

For a group (13%) of DMD patients, who have a specific (nonsense) mutation that results in a prematurely stop in the production of normal dystrophin, a drug has been developed that repairs the effect of premature stop codons but does not affect normal stop codons; this enables dystrophin expression in DMD boys with nonsense mutations. [249] Recently this drug (called Translarna or ataluren) has got a positive opinion on a conditional marketing authorisation by the European Medicines Agency (www.ema.europa.eu).

Recently reported but unpublished yet, is that the extended studies of the clinical trials with exon51 skipping show that long term, non-controlled, treatment longer than one year resulted in stable motor function in ambulant boys with DMD.

Moreover, new therapies that are being developed for DMD might also apply for BMD. Several studies now underway aim to improve muscle strength using drugs whose action is not-related to the mutation in the *DMD* gene. For example treatment with an analogue of co-enzyme Q_{10} idebenone, which aims to stabilize respiratory function, seems promising for patients with DMD who are not on steroid treatment; results are not published yet. Results of clinical studies in DMD with phosphodiestersase-5 inhibitors tadalafil/sildenafil are awaited; recently was found that sildenafil does not improve cardiac function in adult patients with dystrophinopathy neither was short term treatment with sildenafil in adult BMD patients effective on skeletal muscle performance.[250, 251] Drug studies on metformine & L-citrulline, aminoglycoside arbekacin-sulfate and pure tea analogue epicatechin are in the recruiting stage (*www.clinicaltrials.gov)*.

Also early detection of cardiac abnormalities and subsequent treatment with ACE inhibitors and/or β-blockers may improve long term outcome in BMD and DMD. Clinicians could anticipate on upcoming new treatments for DMD and get expertise in the management

of care for the whole clinical spectrum of dystrophinopathies. Therefore if the care offered by multidisciplinary expert teams would cover all patients with a dystrophinopathy, care for both DMD and BMD may improve. In addition, multidisciplinary follow up for all dystrophinopathy patients would also facilitate that standardised clinical data on natural history of both disorders are registered prospectively.

The study described in **chapter 4** is the first to report quantitative measures of brain volumes in a large controlled series. Total brain and gray matter volumes are smaller in DMD, and this is more likely congenital or developmental than acquired, theoretically due to genetic nature of the disease and the fact that the cognitive deficit is apparently stable. We also observed that the intracranial volume and the skull size were proportional to the brain volume. In case of to acquired (cortical) atrophy a larger intracranial volume in relation to brain volume would have been expected.

Furthermore, we found a relation between brain volumes and the predicted absence of isoform Dp140 which is in agreement with previous studies on neuropsychological profiles in DMD patients with different mutations.[50, 120] Nevertheless, the smaller gray matter volume and the less organized white matter structure might also be a consequence of having cognitive deficits and/or learning problems. If a young boy lacks dystrophin in the brain and has a learning problem, this might influence and delay brain maturation which might be expressed in a less organized white matter structure and in a smaller gray mater volume. Longitudinal analysis that starts at a very young age and includes measurements at multiple time points might clarify the evolution of brain volumes and of white matter structure during early development in DMD boys, compared to healthy age-matched controls. However, logistically it will be very problematic to perform MRI scans in very young children, as they usually need sedation to lay still in a scanner. Since verbal and language skills seem to improve in older DMD boys, particularly those of 14 years and older,[42] longitudinal follow up with renewed MR imaging and neuropsychological testing of the participants of our study may also be informative. A follow up study has started.

Furthermore, imaging techniques such as MRI and functional MRI (fMRI) are excellent tools to observe and quantify brain structures parameters longitudinally. The neuropsychological profile is a heterogeneous aspect of DMD and its relation to dystrophin in the brain is probably complex. It would be very informative if brain activity could be visualized during execution of specific tasks like reading or information processing.

The diagnostic work up in dystrophinopathies is sometimes simple but can be challenging, especially if the phenotype is not typical. Especially in BMD the clinical symptoms are known to be variable and the differential diagnosis may be large in adult patients; moreover the prevalence of BMD is significantly lower than of DMD. Accordingly, it is possible that in BMD patients with mild symptoms the diagnosis is missed. It is not likely that extensive work up including muscle biopsy, immunoblotting and/or DNA analysis is performed in all cases with mild symptoms such as exertional induced muscle cramps and myalgia or with asymptomatic hyperCKemia. Myalgia and cramps are frequently occurring and non-specific symptoms for which many patients are referred to outpatient clinics.[252, 253] HyperCKemia can occur in neuromuscular as well as non-neuromuscular disorders, and is also known as an idiopathic condition.[53, 84] Besides, mild BMD has been described with normal CK[56, 254, 255] and the prevalence of BMD with mild symptoms is probably higher than is yet known.[22, 256] Immunohistochemical studies on dystrophin may be normal in BMD.[144, 257-259] Western blotting is more sensitive to detect disturbed dystrophin production in BMD but is technically more difficult, costly and reserved to specialized laboratories to which fresh or frozen muscle tissue has to be sent.

Indeed the extent of the diagnostic work up in cases with mild symptoms or only hyperCKemia should be personalised. In daily practice there will be a trade-off between the benefits and the discomfort of a muscle biopsy, the costs of diagnostic procedures and the consequences of the expected results. A prospective study is needed to find out which cases with mild symptoms and/or hyperCKemia will benefit from extensive work up including Western blot analysis of muscle tissue and DNA analysis including sequencing.

Samenvatting in het Nederlands

Duchenne spierdystrofie (DMD) en Becker spierdystrofie (BMD) zijn als spiegelbeelden: gelijkend maar toch verschillend. Gemeenschappelijke kenmerken zijn: kuithypertrofie, spierzwakte in de spieren van bovenbenen en later ook in de onderbenen en bovenarmen, verhoogde spiegel van creatinekinase (CK) in het bloed en cardiomyopathie. De aandoeningen zijn verschillend van elkaar betreft het beloop, prognose en therapeutische mogelijkheden. In genetisch opzicht worden beide aandoeningen veroorzaakt door een mutatie op het DMD gen, dat zich op het X-chromosoom bevindt. De leesraamhypothese houdt in dat als een mutatie het leesraam verstoort, dystrofine ontbreekt. Dit leidt tot een ernstig verlopend ziektebeeld, Duchenne spierdystrofie. Blijft ondanks de mutatie het leesraam intact (in-frame) dan leidt dit tot een milder fenotype, genaamd Becker spierdystrofie. Op het eerste gezicht lijkt heterogeniteit meer van toepassing op BMD dan op DMD, omdat bij BMD de aandoening ernstig maar ook zeer mild kan verlopen.

In ***hoofdstuk 1*** worden de verschillende klinische fenotypen van dystrofinopathieën beschreven en diagnostische overwegingen besproken. Vuistregel is dat bij een dystrofinopathie het serum CK is verhoogd. Omgekeerd is er bij patiënten met een verhoogd CK een uitgebreide lijst van differentiële diagnoses. Daarom worden diagnostische algoritmes voor asymptomatische en weinig symptomatische hyperCKemie uitgebreid besproken en wordt in een schematisch overzicht weergegeven welke neuromusculaire aandoeningen zich kunnen presenteren met hyperCKemie. Het diagnostische proces bij patiënten met een spierdystrofie wordt idealiter voltooid met een moleculair-genetische diagnose, dat wil zeggen dat ook de DNA mutatie bekend is. Informatie over de DNA mutatie is belangrijk om de volgende drie redenen: het helpt om patiënten beter te classificeren, waarop de zorg ook kan worden toegespitst, ten tweede om erfelijkheidsadvisering aan familieleden te kunnen aanbieden en tot slot om toekomstige behandelingen voor patiënten met een dystrofinopathie goed te kunnen implementeren.

Deel 1 van dit proefschrift gaat over BMD en twee studies worden gepresenteerd.
De studie uit ***hoofdstuk 2*** beschrijft dat bij BMD de mutatie soms veel later gevonden wordt dan het moment waarop verwijzing plaatsvindt. Voor deze studie werd een cohort van 185 patiënten opnieuw bekeken; het betrof patiënten voor wie DNA-onderzoek naar mogelijke BMD was aangevraagd in de periode 1985–1995. Het gen voor dystrofinopathie (DMD gen) was het eerste gen dat ontdekt werd en beschikbaar kwam voor diagnostische bepaling bij patiënten bij wie de clinicus dacht aan een spierdystrofie. In deze eerste periode van DNA-onderzoek van het DMD gen was de standaardanalysemethode screenen naar deleties en duplicaties. In 70% van de patiënten met een dystrofinopathie is er sprake

van een deletie of een duplicatie. Voorts heeft 10-15% van de patiënten met de dystrofinopathie dat bij patiënten een puntmutatie in het DMD gen; nieuwe technieken om deze kleine mutaties op te sporen zijn het laatste decennium ontwikkeld. Als geen deleties of duplicaties worden gevonden bij de standaardanalyse kan het onderzoek tegenwoordig worden uitgebreid met sequentie-analyse om kleine mutaties te vinden. Terugkijkend is het dus mogelijk dat bij patiënten die lang geleden verwezen zijn voor diagnostiek naar dystrofinopathie en bij wie destijds geen mutatie was gevonden de diagnose BMD is gemist omdat zoeken naar kleine mutaties nog niet mogelijk was.

De onderzoeksvraag was of diagnostische opbrengst kan worden verbeterd door alsnog sequentie-analyse toe te passen bij de groep patiënten voor wie in deze vroege periode DNA-onderzoek was aangevraagd. Alle dossiers van de patiënten, die in de periode 1985–1995 waren verwezen voor diagnostiek naar mogelijke BMD en bij wie nog geen diagnose was gesteld, werden opnieuw bekeken; als de klinische informatie op het aanvraagformulier van destijds nog steeds paste bij mogelijk Becker spierdystrofie en er nog DNA bewaard was, werd dit opnieuw geanalyseerd.

Bij 31 van de 79 patiënten zonder diagnose kon DNA opnieuw worden geanalyseerd. Er zijn acht verschillende mutaties gevonden in deze groep, waarvan vier niet eerder gepubliceerd (novel). Van alle patiënten met een novelmutatie konden we nieuwe klinische informatie krijgen, 15 – 25 jaar na initiële verwijzing. In drie van de patiënten is de mutatie pathogeen en leidt tot een frameshift; twee van hen hadden progressieve limb girdle spierzwakte en bij een patiënt was er sprake van een somatische mozaïek, hij blijkt asymptomatisch. Ook was er een patiënt met een novel missense mutatie; hiervan is niet zeker of deze pathogeen is. Deze patiënt had milde spierklachten, forse kuiten en hyperCKemie, wat ook kan passen bij een milde vorm van BMD.

Een late diagnose kan ook na jaren nog relevant zijn omdat dit duidelijkheid geeft aan de betrokkenen, en zij verwezen kunnen worden naar de cardioloog voor onderzoek naar betrokkenheid van de hartspier; in dat geval is vaak medicatie gewenst. Daarnaast kunnen vrouwelijke familieleden worden getest op draagsterschap als de mutatie bekend is.

Het vinden van mutaties en daarmee genetisch bevestigen van de diagnose BMD werd bereikt omdat nieuwere technieken werden toegepast op bewaard materiaal. Essentieel hiervoor is dat bij de aanvraag voor diagnostiek klinische informatie wordt gegeven opdat de moleculair- geneticus kan beoordelen bij welke patiënten vervolgdiagnostiek zinvol is indien er nieuwe diagnostische inzichten zijn. Helaas moesten we 22 patiënten van dit retrospectief bekeken cohort uitsluiten omdat er geen klinische informatie beschikbaar was

op het aanvraagformulier. Een aanbeveling uit deze studie is dat samenwerking tussen clinicus, moleculaire geneticus en klinisch geneticus de diagnostische uitkomst kan verbeteren bij patiënten zonder diagnose. Hiervoor is het dus belangrijk dat klinische gegevens van patiënten worden gedeeld.

Het klinische fenotype Becker spierdystrofie is niet alleen interessant vanwege de heterogeniteit maar ook omdat dit fenotype als model kan dienen wanneer nieuwe therapieën voor Duchenne spierdystrofie worden ontwikkeld waarbij dystrofine wordt aangemaakt in de spieren bij DMD patiënten. Bij patiënten met spierdystrofie en een deletie van een of meer exonen die het leesraam verstoort, kan door een exon te skippen de deletie veranderen van out-of-frame (DMD fenotype) naar in-frame (BMD fenotype); Bij in-frame is het leesraam, dat de DNA code afleest, hersteld waardoor in de spiercel een verkorte vorm van het eiwit dystrofine geproduceerd kan worden. Exonskipping geschiedt via antisense oligonucleotiden (AONs); aangezien er bij DMD veel verschillende mutaties voorkomen zullen er dus verschillende AONs moeten worden ontwikkeld die ieder gericht zijn op een specifieke plaats op het dystrofine-gen. Na chronische toediening van een AON, dat een specifiek exon skipt, kunnen de spiercellen dystrofine gaan produceren. Dit dystrofine is vergelijkbaar met het gedeeltelijk functionele dystrofine van patiënten met Becker spierdystrofie die van nature een deletie hebben die ook het target exon van de AON therapie omvat. De eerste behandeling die in klinische trials werd getest was exonskipping van exon 51.

In ***hoofdstuk 3*** beschrijven we de fenotypes en de klinische follow-up van de twee families met Becker spierdystrofie die in Nederland bekend zijn met een mutatie inclusief exon 51. Dit fenotype zou model kunnen zijn voor patiënten met Duchenne spierdystrofie die behandeld worden met een AON die exon 51 skipt, hetgeen tot nu toe alleen in studieverband is geschied.

Het beloop van de aandoening van de drie patiënten uit deze families was zeer mild. De index patiënt uit de eerste familie werd gezien door een neuroloog op de leeftijd van 14 jaar omdat hij veel last had van krampen tijdens inspanning sinds zijn kinderleeftijd. Zijn CK spiegel in het bloed was 50 maal verhoogd en hij had een in-frame mutatie deletie van exonen 45–51 van het DMD gen. Zijn grootvader van moederszijde had ook krampen van kinderleeftijd tot jong volwassen leeftijd en hij had dezelfde mutatie. Beide mannen zijn onderzocht op volwassen leeftijd. Geen van beiden had spierzwakte. De grootvader was asymptomatisch op 78-jarige leeftijd en had een normaal CK in het bloed. Kleinzoon had forse kuiten op de leeftijd van 46 jaar en zeer geringe inspanningsgebonden spierklachten en daarbij een marginaal verhoogde CK spiegel in het bloed.

In de tweede familie was de indexpatiënt acht jaar toen hij verwezen werd naar een kinderarts vanwege hyperactief gedrag. Bij routine laboratoriumonderzoek werden hoge CK spiegels gevonden. DNA-onderzoek toonde een in-frame deletie van de exonen 50–51. Toen hij opnieuw werd onderzocht op de leeftijd van veertien jaar had hij geen spierzwakte. Sporadisch had hij inspanningsgebonden spierkrampen.

Het zeer milde beloop in beide BMD families, met een mutatie die exon 51 omvat, kan veelbelovend zijn voor de patiënten met Duchenne spierdystrofie die in aanmerking komen voor behandeling met exon 51 skipping.

Deel 2 van het proefschrift gaat over Duchenne spierdystrofie.
Hierbij zijn cognitie en gedrag heterogene aspecten. Naast de bekende spierzwakte die bij jongens vaak al vanaf de kleuterleeftijd optreedt, heeft een deel van de patiënten met DMD een lager IQ en/of leerproblemen, wat in zeldzame gevallen ook de eerste uiting van de aandoening kan zijn. Deze cognitieve stoornissen duiden erop dat ook het centraal zenuwstelsel is betrokken bij DMD hoewel de oorzaak en het mechanisme nog niet zijn opgehelderd. Het is bekend dat dystrofine, dat zeer belangrijk is voor stabiliteit van de spiercelmembraan en daardoor voor de functie van spieren, ook expressie heeft in andere weefsels waaronder de hersenen. Naast transcriptie van het volledige dystrofine eiwit Dp427 zijn er ook verschillende kortere isoforms, waarvan de promotor meer distaal is gelokaliseerd op het DMD gen. Deze isoforms, Dp260, Dp 140, Dp 116 en Dp 71 komen in diverse weefsels tot expressie (zie ook figuur 3 op pagina 31). Bijvoorbeeld, van Dp140 en Dp 71 is bekend dat deze isoforms in foetaal hersenweefsel tot expressie komen; waarschijnlijk zijn deze belangrijk voor cognitieve functies. Echter, wat de specifieke functie is van deze isoforms in de hersenen, is niet bekend. Patiënten met DMD en een ernstige cognitieve beperking hebben vaker een mutatie in het distale deel van het DMD gen waardoor Dp 140 en Dp 71 ontbreken.

In eerdere studies is cognitieve functie bij DMD meestal onderzocht door middel van descriptief neuropsychologisch onderzoek; op groepsniveau is het gemiddelde IQ een standaarddeviatie onder het gemiddelde IQ van de normale populatie. De variatie van het gemeten IQ bij DMD is echter breed met een range van 50-130. De cognitieve problemen bij DMD lijken niet progressief, echter longitudinaal onderzoek hiernaar is niet verricht. Uit de literatuur blijkt dat meerdere domeinen betrokken zijn waaronder executieve functies en het verbale werkgeheugen. Ook is de leesvaardigheid vaak minder bij jongens met DMD. Gedragsstoornissen zoals attention deficit en hyperactivity disorder (ADHD), obsessief–compulsieve stoornis (OCS) en stoornis in het autismespectrum worden vaker gezien bij jongens met DMD. Om de rol van het dystrofine in het brein

te onderzoeken is een niet-invasieve onderzoeksmethode nodig. MRI is een veilige en goede methode om de hersenen in detail te onderzoeken. Eerdere studies, meestal met kleine series patiënten, toonden aanwijzing voor een veranderd metabolisme bij DMD. Verschillende technieken zijn gebruikt zoals positron emission tomografie (PET), waarbij scans een veranderd glucose metabolisme aantoonden in specifieke regio's. Bij MRI studies werden op scans lokale veranderingen in de grijze stof gevonden en bij magnetic resonance spectroscopie (MRS) werden metabole veranderingen in het cerebellum en de tempero–pariëtale gebieden gevonden.

Onze onderzoeksgroep is gestart met een exploratieve kwantitatieve MRI studie naar de rol van dystrofine in het brein bij DMD. Een grote groep van 30 patiënten met DMD en een groep van 22 leeftijd gematchte controles (leeftijd 8-18 jaar) werden geïncludeerd. MRI scans (T 1-gewogen opnames en diffusion tensor image (DTI) opnames) werden gemaakt en alle deelnemers kregen neuropsychologisch onderzoek. Tevens werden vragenlijsten voor gedrag ingevuld.

In ***hoofdstuk 4*** beschrijven we dat het totale breinvolume en het volume van de grijze stof kleiner is in DMD patiënten dan in gezonde controles. Ook vonden we dat microstructuur in de witte stof verschillend was vergeleken bij controles. Dit suggereert ten eerste dat de vezeldichtheid in de witte stof bij DMD verminderd is en ten tweede -mogelijk- dat de permeabiliteit van de celmembranen verhoogd is of dat de organisatie van de witte stof minder goed ontwikkeld is. De gemiddelde score op de neuropsychologische testen was lager in de groep patiënten DMD. In een subgroepanalyse werd gevonden dat bij patiënten bij wie de mutatie voorspelt dat isoform Dp 140 ontbreekt, het volume van de hersenen en van de grijze stof significant kleiner was ten opzichte van gezonde controles. In DMD patiënten met een mutatie die aanwezigheid van Dp 140 voorspelt vonden we dit verschil ten opzichte van controles echter niet. Dit kan betekenen dat dystrofine isoforms inderdaad belangrijk zijn voor het cognitief functioneren en ook voor de vroege ontwikkeling van het brein.

Hoofdstuk 5 beschrijft een serendipiteit die wij tegenkwamen bij de MRI breinstudie in DMD. Het betreft de schedel en de spieren die aan de buitenzijde van de schedel aanhechten. Wij deden de observatie dat de vorm van de schedel van jongens met Duchenne anders is vergeleken met gezonde leeftijdsgenoten. In jongens zonder spierziekte is de schedel ovaalvormig en in DMD blijkt dat de omtrek van de schedel ronder is in het transversale vlak. Een maat voor ronding is eccentriciteit en bij jongens met DMD was de gemiddelde eccentriciteit verlaagd. Daarnaast zagen we bij visuele beoordeling van de MRI-plaatjes dat de musculus temporalis die ter hoogte van de slapen aanhecht, aan beide zijden hypertrofisch is, dat wil zeggen dikker. Bij kwantificeren was de dikte van deze spier

inderdaad groter bij DMD patiënten vergeleken met gezonde controles.

Het is niet zeker of een andere schedelvorm klinisch relevant is. We vonden een positieve correlatie tussen dikte van de temporalis spieren en een rondere schedelvorm bij jongens met DMD. Een hypothese is dat de verandering van de schedelvorm secundair is aan zwakte van de temporalis spieren, die deel uitmaken van de kauwspieren. De groep kauwspieren omvat de musculus masseter, de musculus temporalis en de mediale en laterale musculus pterygoideus, alle zowel links als rechts aangelegd. Waarschijnlijk zijn de kauwspieren al vroeg aangedaan bij DMD. Eerder werd gevonden dat de bijtkracht verminderd is en dat de boven- en onderkaak niet goed op elkaar sluiten (dentale malocclusie) bij patiënten met DMD van de leeftijd van zes jaar. Beide zijn van belang om goed te kunnen kauwen. Eerder is gevonden dat er een relatie lijkt te bestaan tussen de schedelvorm en dentale malocclusie ook zonder onderliggende spierziekte; mensen met een langwerpige gezichtsvorm hebben vaker zwakkere aangezichtspieren en hebben een groter risico op een open beet, terwijl mensen met een kort breder gezicht minder kans hebben op deze malocclusie. Interventiestudies zijn echter beperkt beschikbaar en hierbij ontbreken controlegroepen. In een studie werd gevonden dat bij jonge gezonde volwassenen de bijtkracht verbeterde na systematisch dagelijks oefenen met harde kauwgom. In een andere serie met 13 kinderen (leeftijd 7-12 jaar) met langwerpig gezichtsvorm, die trainden met kauwgom tenminste twee uur per dag gedurende een jaar, was de bijtkracht groter vergeleken met de referentiewaarden bij ongetrainde leeftijdsgenoten; opmerkelijk veranderden de vorm en de positie de kaak en gerelateerde structuren (anterior mandibular rotation) ten opzichte van een historische controlegroep. Dit suggereert dat wanneer spierkracht verandert, de stand van botstructuren beïnvloed kan worden.

De studie uit ***hoofdstuk 5*** is de eerste publicatie van een rondere schedelvorm in DMD patiënten, van wie bekend is dat zij op enig moment ook zwakte van de kauwspieren krijgen. Een mogelijke verklaring zou kunnen zijn dat spierzwakte niet alleen gerelateerd is aan minder kauwfunctie en veranderde kaakvorm maar ook verandering geeft van de schedelvorm. Uit onze observationele studie die op één moment een meting heeft verricht en waar meten van kracht (functie) niet tot de studiedoelen hoorde, kan niet de conclusie worden getrokken dat zwakte de oorzaak is van een rondere schedelvorm. Verder is niet bekend of een rondere schedelvorm reversibel is ofwel dat deze gefixeerd is bij kinderen die uitgegroeid zijn. Als het laatste het geval is, zou een veranderde schedelvorm, die niet reversibel is, verbetering van kauwfunctie bij DMD kunnen belemmeren als nieuwe behandelingen beschikbaar komen die spierkracht kunnen verbeteren. Voor clinici is het belangrijk in de begeleiding en behandeling van patiënten met DMD erop te anticiperen

dat kauwfunctie mogelijk vroeg in de ziekte is aangedaan.

In ***hoofdstuk 6*** gaat over corticosteroïdbehandeling bij DMD patiënten. Behandeling met steroïden kan de loopfase (ambulante fase) bij jongens met DMD verlengen. In historische cohortstudies was gevonden dat gemiddelde leeftijd waarop jongens met DMD rolstoelgebonden werden lag tussen de 8.5-12 jaar. Na de introductie van chronische steroïdbehandeling werd de ambulante fase met tenminste een jaar verlengd. Het gunstige effect van steroïden moet worden afgewogen tegen de bijwerkingen. In Nederland zijn richtlijnen voor steroïden geïmplementeerd in 2004. In het revalidatie centrum De Trappenberg in Huizen werden jonge kinderen met DMD reeds behandeld met prednison sinds 1996; het behandelschema was intermitterend: 10 dagen prednison 0.75 mg/kilogram/dag gevolgd door 10 dagen zonder steroïden. In de studie uit ***hoofdstuk 6*** hebben we retrospectief van serie van 35 DMD patiënten beschreven die met prednison zijn behandeld. Prednison was gestart gedurende de ambulante fase op de leeftijd van 3.5-9.7 jaar (mediaan 6.5 jaar). De mediane periode van behandeling was 27 maanden (range 3-123). De mediane leeftijd waarop loopfunctie stopte pas 10.8 jaar (gemiddeld 10.9 jaar; 95% betrouwbaarheidsinterval 10.0-11.8)

Mogelijke bijwerkingen van chronische behandeling met steroïden zijn gewichtstoename, verhoogd risico op osteoporose en botfracturen. Ook is beschreven dat chronische steroïdbehandeling, gestart in de kinderleeftijd, kan leiden tot korte lichaamslengte en tot gedragsproblemen zoals hyperactiviteit; beide lijken met name gerelateerd aan doseringsschema's waarbij steroïden dagelijks worden gebruikt. Eerder is gerapporteerd dat dagelijks gebruik van prednison 0.75 mg/kilogram dag kan leiden tot gewichtstoename van meer dan 10% bij driekwart van de patiënten. Echter in onze studie werd gevonden dat gewichtstoename optrad bij een kwart van de patiënten, en opmerkelijk, alleen in patiënten die rolstoelgebonden waren. Bovendien trad een lichaamsgewicht van +2 SDS op bij zeven van de 11 patiënten die reeds vóór start prednison een lichaamsgewicht hadden van +1 SDS. Waarschijnlijk is een risico voor het ontwikkelen van excessief overgewicht de aanwezigheid van mild overgewicht vóór de start van prednison. Daarentegen had 40% van de patiënten in onze studie geen overgewicht. Intermitterend prednison, 10 op/10 af, lijkt gepaard te gaan met relatief weinig bijwerkingen. Momenteel wordt het intermitterende schema veel gebruikt in Nederland bij patiënten met DMD. Elders wordt vaak dagelijkse dosering steroïden voorschreven; hiermee wordt ambulante fase waarschijnlijk meer verlengd. In een recente observationele studie uit 2013 werd gevonden dat de mediane leeftijd voor verlies van ambulantie 12 jaar was bij intermitterend steroïdengebruik en 14.5 jaar bij jongens die het dagelijks gebruikten. In de dagelijkse klinische praktijk zal de behandelend arts met de ouders de te verwachten voordelen en nadelen van da-

gelijks prednison gebruik bespreken en kunnen zij gezamenlijk een keuze maken voor intermitterend of dagelijks corticosteroïdengebruik. Overigens loopt er op dit moment een multicenter gerandomiseerd onderzoek waarin dagelijks en intermitterend gebruik wordt vergeleken: FOR-DMD (www.clinicaltrials.gov).

Tevens zijn er aanwijzingen dat het voortzetten van steroïdbehandeling in de non-ambulante fase ernstige scoliose kan voorkomen waardoor minder vaak een grote rugoperatie noodzakelijk is; waarschijnlijk vertraagt prednison ook de achteruitgang van de longfunctie en van de hartfunctie. Samenvattend spelen steroïden een belangrijke rol in de standaardbehandeling van jongens met DMD en wordt behandeling aanbevolen in de behandelrichtlijnen voor DMD. Daarnaast dient de zorg bij DMD worden gegeven door een multidisciplinair werkend team.

Reflecties op de toekomst

In de laatste decade is een organisatie van zorg voor patiënten met DMD in Nederland sterk verbeterd. Voor kinderen met DMD en hun ouders zijn er multidisciplinaire behandelteams in verschillende expertisecentra. Meer recentelijk gaat de aandacht naar verbetering van het proces van transitie voor jongens met DMD op weg naar volwassenheid. Verrassend is dat de zorg voor DMD en BMD weinig overlap heeft. Immers beide aandoeningen hebben vergelijkbare klinische symptomen hoewel beloop van de ziekte en de prognose verschillend is. Er wordt toegewerkt naar een gerichte behandeling voor DMD om de spierzwakte te verbeteren door productie van dystrofine in de spier mogelijk te maken. Het beoogd effect is minder snelle achteruitgang van spierzwakte. Als dit lukt, en het ziekteproces milder verloopt, zullen de symptomen en problemen lijken op het beloop van de aandoening bij BMD.

Voor een deel van de patiënten (13%) met DMD die een specifieke mutatie heeft, waarbij de mutatie leidt tot een voortijdig stopcodon, dat zorgt dat productie van dystrofine voortijdig gestopt wordt, is er recente een doorbraak; een medicijn dat het effect van een voortijdig stopcodon teniet doet, genaamd Translarna of ataluren, is na fase 3 klinische trial door de autoriteiten voorwaardelijk goedgekeurd om toe te passen bij ambulante patiënten met DMD en een specifieke stopcodonmutatie. Ook de (nog niet gepubliceerde) resultaten van lange termijnbehandeling (langer dan 1 jaar) met AONs die exon 51 skippen zijn veelbelovend aangezien een groot deel van de behandelde ambulante patiënten stabiele scores heeft voor motorische functies, met name loopfunctie.

Daarnaast worden er therapieën ontwikkeld voor DMD die ook van toepassing kunnen zijn bij BMD. Diverse middelen worden onderzocht waarvan de werking niet is gerelateerd aan de mutatie in het DMD gen. Bijvoorbeeld het middel idebenone, een analoog van co-enzym Q10, lijkt een gunstig effect te hebben op de longfunctie van jongens met DMD die geen prednison gebruiken; de resultaten van deze studie moeten nog gepubliceerd worden. Klinische studies in DMD patienten met fosfodiesterase-5remmers, tadalafil en sildenafil, zullen de komende tijd hun resultaten bekend maken. Andere studies werven nu patiënten voor deelname: studies naar het effect van metformine en L-citrulline, aminoglycoside Arbekan-sulfaat en naar puur thee analoog epicatechin.

Tenslotte is het voor de lange termijn behandeling van belang dat hartproblemen vroegtijdig worden opgespoord en zonodig behandeld worden met ACE-remmers en/of bètablokkers die de lange termijn uitkomst van hartfunctie kunnen verbeteren zowel in BMD als DMD. Door de zorg voor beide aandoeningen gecombineerd en multidisciplinair te organiseren kunnen behandelaars expertise krijgen in het hele klinische spectrum van dystrofinopathie en kan men anticiperen op toekomstige therapieën voor DMD. Uiteindelijk zullen zowel patiënten met BMD als DMD hiervan profiteren. Ook is het zinvol om het beloop van de aandoening bij patiënten met DMD en BMD prospectief en in gestandaardiseerde vorm vast te leggen gedurende de multidisciplinaire follow-up.

De studie beschreven in ***hoofdstuk 4*** is de eerste die in een grote serie patiënten met DMD kwantitatieve metingen heeft gedaan naar breinvolumes en deze heeft vergeleken met controles. De hypothese is dat de gevonden kleinere volumes van totale hersenen en van grijze stof waarschijnlijk al in aanleg aanwezig zijn of ontstaan tijdens de ontwikkeling van het jonge kind; dit vanwege het feit dat DMD een genetische aandoening betreft waarbij dystrofine in aanleg ontbreekt en het feit dat de cognitieve problemen stabiel blijken. Bovendien vonden wij een relatie tussen het volume van de hersenen en de voorspelde afwezigheid van isoform Dp 140 hetgeen in overeenstemming is met voorgaande studies omtrent neuropsychologische profielen in DMD patiënten met verschillende mutaties. Overigens is niet uitgesloten dat een kleiner volume van de grijze stof en minder georganiseerde witte stofstructuur gevolgen zijn van het hebben van cognitieve stoornissen en/of leerproblemen. Om de evolutie van het volume van de hersenen en van de structuur van de witte stof van jongens met DMD goed te bestuderen zijn meerdere meetpunten gewenst inclusief een eerste meting op zeer jonge leeftijd tijdens de vroegste ontwikkeling. In praktische zin is dit echter logistiek lastig omdat zeer jonge kinderen voor het uitvoeren van een MRI scan gesedeerd moeten worden om stil te blijven liggen. Er zijn aanwijzingen dat de verbale en de taalvaardigheden juist verbeteren bij oudere jongens met DMD, in het bijzonder jongens boven 14 jaar en ouder; een tweede meetpunt door middel van

herhalen van de MRI scans en van het neuropsychologisch onderzoek bij dezelfde groep deelnemers uit onze studie kan informatief zijn. Een follow-upstudie is gestart. Daarnaast kan het visualiseren van hersenactiviteit tijdens uitvoerende en/of leertaken zoals lezen en informatieprocessing zinvol zijn. Andere MRI technieken zoals functionele MRI (f MRI) kunnen een geschikte meetinstrument zijn om verschillende structuren in de hersenen verder te observeren en metingen te kwantificeren.

Tot slot, het diagnostisch proces bij dystrofinopathie is soms eenvoudig maar kan ook moeilijk zijn, met name als het fenotype niet typisch is. Juist bij volwassen patiënten met BMD variëren de klinische symptomen van mild tot ernstig en de lijst van differentiële diagnoses kan lang zijn; bovendien is de prevalentie van BMD significant lager dan van DMD. Mogelijk wordt de diagnose BMD ook gemist bij patiënten met milde symptomen. Sporadische patiënten met milde symptomen zoals inspanningsgebonden krampen, spierpijn of asymptomatisch hyperCKemie zullen niet allemaal een uitgebreide diagnostische work-up krijgen naar een mogelijke dystrofinopathie inclusief spierbiopsie, met Western blotting en/of DNA-onderzoek. Spierpijn en spierkrampen zijn veel voorkomende symptomen en bovendien niet specifiek voor dystrofinopathie; hoewel hyperCKemie kan optreden als enige uiting van een milde dystrofinopathie kan dit ook optreden bij infecties of als bijwerking van medicatie en tevens komt hyperCKemie idiopathisch voor. Omgekeerd zijn er patienten beschreven die een normaal CK hadden en toch een milde BMD; waarschijnlijk is de prevalentie van BMD met milde symptomen dan ook hoger dan tot nu toe bekend. Aanvullend onderzoek hoeft niet afwijkend zijn, inclusief immunohistochemisch onderzoek (dystrofine-kleuring op coupes van spierweefsel). Western blotting op spierweefsel is meer sensitief om een gestoorde dystrofine productie bij BMD op te sporen. Technisch gezien is dit moeilijker dan immunohistochemisch onderzoek en ook kostbaarder, en wordt over het algemeen alleen verricht in gespecialiseerde laboratoria. In hoeverre de diagnostische work-up bij patiënten met milde klachten en/of alleen hyperCKemie uitgebreid moet zijn zou per patiënt moeten worden bekeken. In de dagelijks praktijk zal de behandelaar een afweging maken tussen de opbrengst van aanvullend onderzoek en de ongemakken of complicaties van een spierbiopsie; maar ook de kosten van de procedure en de consequentie van gevonden resultaat zouden moeten worden meegenomen. Een prospectieve studie kan behulpzaam zijn om duidelijk te krijgen welke patiënten met milde symptomen en/of hyperCKemie baat hebben van uitgebreide work-up inclusief Western blot analyse van spierweefsel en DNA-onderzoek inclusief sequensen.



A

Appendices

Supplementary Tables

References

Curriculum vitae

List of publications

Dankwoord

List of abbreviations

Supplementary Tables

Neuromuscular diagnoses that can present with hyperCKemia and mild symptoms [53, 84, 87]

TABLE A CK level and symptoms
page 126-129 >>

TABLE B Signs and diagnostic tools
page 130-133 >>

AD: autosomal dominant;
AR: autosomal recessive;
APR: Achilles tendon reflex;
BMD: Becker muscular dystrophy;
CK: creatine kinase;
DMD: Duchenne muscular dystrophy;
EMG: electromyography;
ER: exercise related;
ESR: erythrocyte sedimentation rate;
IHC: immunohistochemical staining of muscle tissue;
LGMD: limb girdle muscular dystrophy;
MMD: Miyoshi-type muscular dystrophy;
PA: histopathological analysis;
RRF: ragged red fibers;
WB: Western Blot analysis of muscle tissue; y: year

TABLE A Disorder	Gene	Inheritance	CK (U/L)	Asymptomatic hyperCKemia	CK in asymptomatic cases (U/L)	Myo-globinuria	Age presenting	Cramps	Exercise-intolerance	Calf hypertrophy
Musculardystrophies										
Dystrophinopathy	DMD	X-linked	very high to 100x	yes in carriers and sometimes in BMD	high	yes in BMD; in DMD in ambulant boys on steroid treatment	DMD childhood; BMD childhood-adult	yes, exercise related	possible	yes
LGMD 2B/MMD1	DYSF	AR	10-72x normal; up to to 27,000	yes	343-3000	occassional	generally 10 to 39 y			some
LGMD 1C	CAV3	AD	3-40x normal	yes; ♂>♀	478-2600		5- adulthood	yes, exercise related		yes
LGMD 2A	CAPN3	AR	nomal-80x normal; 190-11,000	yes			childhood-adult	no		no
Alpha-Sarco-glycanopathy	SGCA	AR	5x nomal, often>5000	yes	1000	yes	2-15 y			some
LGMD 2I	FKPR	AR	1000-8000	yes	8-26x normal	yes	variable; 61% less than 5 y	yes, late onset	yes	yes
LGMD 2L/MMD3	ANO5	AR	very high to 35,000	yes	3000-4000	yes	2nd-3rd decade		yes	yes
Myofibrillary myopathy with Desmin mutation	MFM1	AD/AR	338-1023; ♂>♀	yes	<1000		> 2nd decade			some

Limb girdle syndrome with Central cores	RYR1	AD	15x normal, may be normal	yes	may be normal	yes, following trigger	teens	yes	no	no
LGMD unspecified			elevated							
Facioscapulohumeral dystrophy	D4Z4 repeat retraction	AD	normal (25%) to<5 x normal		may be normal		congenital to late age; mean 3rd decade	no	no	no
Metabolic myopathies										
Carnitine palmitoyltransferase II (CPT2) deficiency	CPT2	AR	high at rhabomyolysis normal/ mildly elevated between episodes		<900	yes	adolescence-adult (mean 13 y; range 1-40 y)	yes	yes	
Myophosphorylase deficiency (McArdle)	PYGM	AR ♂predominance	to 12,000		5500	yes	usual<15		yes	yes
Phophofructokinase-deficiency	PFKM	AR ♂predominance	high			yes	2nd-4th decade	yes, proximal legs	yes	
Alpha-glucosidase deficiency (M. Pompe)	GAA	AR	< 10x normal, normal in 5% adults	yes			usual<15	yes, proximal		yes
Phosphorylase-b deficiency	PHKA1	X linked/AR	2-10x		<900	yes, occassionally	childhood-5th decade	yes	yes	
Adenylate-deaminase-deficiency	AMPD1	AR	normal to slightly elevated						yes	

Table A continued

Disorder	Gene	Inheritance	CK (U/L)	Asymptomatic HyperCKemia	CK in asymptomatic cases (U/L)	Myo-globinuria	Age presenting	Cramps	Exercise-intolerance	Calf hypertrophy
Mitochondrial myopathies										
with episodic HyperCKemia	MTTK	mitochondrial	200-11,000 (episodic) 67-320 (usual)				15-69 y			
with exercise intolerance and encephalopathy	MTCO3	mitochondrial, complex IV	high or normal			yes	4-20 y			
with myoglobinuria and exercise intolerance	MTCO1 MTCYB	mitochondrial; sporadic mitochondrial, complex III	CK normal at rest			recurrent yes	childhood-adult childhood	yes yes	yes yes	
Inflammatory myopathies										
Polymyositis		sporadic	up to 100x normal				>20 y			
Inclusion body myositis		sporadic; ♂predominance	2-5x normal				>50 y			
Macrophagic myofaciitis		sporadic	40% increased mildly to high				3th-5th decade			
Sarcoid myoparthy		sporadic; ♀predominance	moderately high				middle aged females			

Congenital myopathies										
Centronuclear	DNM2	AD/sporadic	rarely mildly high				variable: neonatal to adult			
Multicore	SEPN1	AR /sporadic	normal to slightly elevated				eearly delayed motor milestones			
Miscellaneaous										
Proximal myopathy with tubular aggregates		AD	normal-mildly high				5th-6th decade	yes	yes	
Proximal myopathy with tubular aggregates		sporadic /AR	normal-mildly high				teens	yes		
Thyroid disorder		sporadic; ♀predominance	high in hypothyroidism normal or low in hyperthyroidism				adult	yes		
Myotonia flucuans[125]	SCN4A	AD	2-4x normal				teens			
Myotonic dystrophy type 2 (PROMM)[126,127]	ZNF9	AD	mild< 10x normal	yes	up to 1400	yes	8-60y		yes	
Malignant hyperthermia[90,128]	RYR1	AD	in rest normal to mildly elevated; CK during hyperthermia very high up to 100,000			yes	related to exposure to volatile and depolarizing anesthetics			

TABLE B	Signs				Diagnostic tools			
Disorder	WEAKNESS PRESENTING	SPECIFIC	PRO-GRESS	CARDIAC INVOLVEMENT	LABORATORY	MUSCLE BIOPSY	IMMUNOLOGICAL STUDIES	GENE
Muscular dystrophies								
Dystrophino-pathy	proximal. first legs, later arms	presentation may be atypical with developmental delay, cognitive deficit, behavioral problems or cardiac failure	fast in DMD slower in BMD	yes,cardio-myopathy		dystrophy	DMD: IHC dys1,2,3 absent BMD: IHC dys1,2,3 reduced WB : in BMD different size or amount of dystrophin	DMD
LGMD 2B MMD1	limb girdle or distal gastrocnemius	posterior muscles; legs> arms asym-metry common later onset: varying phenotypes, also in same family	leg	no		dystrophy	IHC: dysferlin reduced or absent. WB: dysferlin absent or reduced	DYSF
LGMD 1C	proximal leg	also mutation specific distal myopathy has been described onset age 3rd-5th decade	usual-ly mild	mutation related		mild myopa-thic; fibrosis	WB: caveolin-3 reduced	CAV3
LGMD 2A	<12y shoulder; > 30y pelvic	weakness mild to se-vere in early onset	slow	no		myopathic with necrosis and regeneration	WB: Calpain-3 reduced	CAPN3
Alpha -Sarcogly-canopathy	proximal, progressive	late onset: less severe phenotype heterozygotes may have myalgia and scapular winging but normal CK	yes	occasionally		dystrophy	IHC: alpha sarco-glycan absent	SGCA
LGMD 2I	proximal> distal	exertional pain may be early symptom mild facial weakness in some	yes	cardiomy-opathy is common		dystrophy type 1 predominance	WB: reduced alpha dystroglycan	FKPR
LGMD 2L MMD3	proximal quadriceps; atrophy posterior lower leg	asymmetric weakness	slow	usually not		dystrophy	WB: reduced dystrophin and/or calpain-3	ANO5

Myofibrillary myopathy with Desmin mutation	posterior lower leg	cardiac failure may be presenting symptom; even sudden death	yes	frequently arrhythmia and heart failure		myopatic; subsarcolemmal & sarcoplasmic aggregates		MFM1
Limb girdle syndrome with Central cores	proximal symmetric	at risk for malignant hyperthermia following anaesthesia. During episode CK may be up to 100,000	slow			central cores		RYR1
LGMD unspecified	proximal symmetric		yes			dystrophy		
Facioscapulohumeral dystrophy	face (eye and/or lip closure); scapular muscles and biceps	often asymmetric rarely presentation with foot drop or lower limb proximal weakness	yes	arrhytmia and conduction block in 2-5%		varied fiber size, small angular fibers and hypertrophic type 2 fibers		D4Z4 repeat retraction
Metabolic myopathies								
CPT2 deficiency	no, in late stage weakness in some				acylcarnitineprofile			CPT2
Myophosphorylase deficiency	mild> 40 y. proximal > distal; arm >leg	second wind ; rhabdomyolysis; renal failure 10%; muscle wasting and hypertrophy are described			exercise test: reduced lactate		IHC: myophosphorylase absent	PYGM
Phophofructokinase deficiency	late onset fixed myopathy in some	second wind			exercise test: reduced lactate			PFKM
Phosphorylase-b deficiency	distal > proximal				exercise test: nl or reduced rise lactate			PHKA1
Adenylate deaminase deficiency	no weakness	no second wind			exercise test: reduced ammonia rise, normal lactate	normal morphology	IHC: AMPDA absent	AMPD1

Table B continued	Signs				Diagnostic tools			
Disorder	Weakness presenting	Specific	Pro-gress	Cardiac involvement	Laboratory	Muscle biopsy	Immunological studies	Gene
Mitochondrial myopathies								
with episodic HyperCKemia	proximal symmetric	episodic myalgia				SDH+ & COX- muscle fibers		MTTK
with exercise intolerance & encephalopathy	proximal in some patients	recurrent encephalopathy; migraine, myalgia and fatigue	improve-ment in teens		lactate acidemia	COX reduced	childhood: COX deficieny 20y RRF and COX+ fibers	MTCO3
with myoglobin-uria and exercise intolerance	no weakness (MTCO1 mutation)				serum lactate normal; low venous oxygen use at exercise test.	COX I&II reduced	COX deficieny; mild defects complex 1&III	MTCO1
	mild proximal (MTCYB mutation)				serum lactate high	RRF; SDH+/COX +	deficient com-plex III activity	MTCYB
Inflammatory myopathies								
Polymyositis	proximal >distal	dysphagia 40% arthritis/arthralgia 40%	slow	heart can be involved myocarditis		PA: inflammation		
Inclusion body myositis	proximal leg, distal wrist/finger flexor	in course often dysphagia	slow			PA: rimmed vacuoles/ inclusion bodies		
Macrophagic myofaciitis	mild proximal 43%	ESR elevated in 55%				PA: infiltration macrophages		
Sarcoid myopathy	proximal ± distal					PA: granuloma-tous myopathy		

Congenital myopathies							
Centronuclear	distal>prox	often dysphagia or ptosis/strabismus	slow			central nuclei	DNM2
Multicore		often rigid spine and scoliosis >10 y				multiple small cores	SEPN1
Miscellaneaous							
Proximal myopathy tubular aggregates AD	proximal	AD disorder; also known with younger onset with opthalmoplegia (limited upgaze) and high CK		no		tubular aggregates in type 2 fibres	
Proximal myopathy tubular aggregates sporadic /AR	proximal					tubular aggregates	
Thyroid disorder	mild proximal	hypothyroidsm: myxedema; APR absent; hyperthyroidism: brisk reflexes	resolving after treatment		TSH/Ft4	non-specific	
Myotonia fluctuans	no weakness	myotonia often delayed after exercise			needle EMG		SCN4A
Myotonic dystrophy type 2 (PROMM)	proximal leg> arm distal hand: flexors of thumb and fingers	often muscle pain/ stiffness	yes slow	cardiac arrhythmia		abnormal type 2 fibers	ZNF9
Malignant hyperthermia		Malignant hyperthermia can also be related to mutations in ion channel genes with transient weakness and normal or marginally elevated CK		yes, arrhythmia during episodes			RYR1

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Curriculum Vitae

Chiara Straathof was born on 6 December 1963 in Eindhoven. She grew up in Valkenswaard and in 1973 she moved to The Hague where she graduated from high school (Gymnasium β) at the Aloysius College in 1982. After this, she started her medical training at the Rijksuniversiteit Leiden. In 1987 she worked for six months as a research student at the Department of Neurology at the Johns Hopkins University, Baltimore, U.S.A. (prof. D.S. Zee) and studied the Optokinetic After-nystagmus in healthy subjects. The report was awarded a "Hippocrates studiefonds-prijs" in 1988. In 1990 she followed a clinical fellowship Neurology at the National Hospital for nervous diseases, Queen Square, London (dr. A.E. Harding) for three months. After obtaining her medical degree in May 1990 she worked as a house officer in Internal Medicine at "Ziekenhuis de Lichtenberg", at present Meander MC, in Amersfoort (dr. H.Ch. Hart).

From 1992 to 1998 she was resident in Neurology at the Dijkzigt Hospital, at present Erasmus MC, in Rotterdam (Prof. dr. F.G.A. van der Meché). In 1994 she received a research fellowship from the Dutch Cancer Society (de Nederlandse Kankerbestrijding) and did a research project on chemotherapy for malignant glioma in a rat model at the Daniel den Hoedkliniek in Rotterdam (Prof. dr J.H.M. Schellens, Prof. dr M.J. van den Bent), followed by an internship Neuro-oncology (dr. Ch.J. Vecht) in the same clinic.

As a neurologist she worked for six months at the Erasmus MC, at the departments of Neurology and Clinical Neurophysiology. From 1999 to 2002 she worked as a neurologist in the Bronovo Ziekenhuis in The Hague and since 2002 she is member of the clinical staff Neurology at the Leiden University Medical Center (LUMC). She received a clinical fellowship Neuro-myology from the Prinses Beatrix Spierfonds and was trained at the LUMC (Prof. dr. J.J.G.M. Verschuuren), the Academic Medical Center (Prof. dr. M. de Visser) and the University Medical Center Utrecht (Prof. dr. J. Wokke), followed by registration as neuromyologist in 2006.

In 2007 she started the studies for her PhD (Prof. dr. J.J.G.M. Verschuuren).

At present she is involved in the care of adult patients with neuromuscular disorders in the LUMC and participates in the multidisciplinary clinic for young adults with muscular dystrophies. Concurrently she is head of the Outpatient Department of Neurology since 2011.

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Dit proefschrift was niet tot stand gekomen zonder de bereidheid van patiënten om mee te werken aan de studies. Ik wil hierbij de jongens met Duchenne spierdystrofie en hun ouders bedanken voor hun deelname aan de MRI brein studie, hun geduld en hun altijd goede humeur.

Voor de retrospectieve studies naar Becker spierdystrofie waren patiënten bereid, soms jaren nadat zij voor het eerst een neuroloog bezocht hadden, opnieuw hun verhaal te vertellen en zich te laten onderzoeken. Mijn waardering hiervoor is groot.

Veel respect verdient de patiëntenvereniging Duchenne Parent Project en haar voorzitter Elizabeth Vroom om de niet aflatende inzet voor de patiënten met Duchenne spierdystrofie en de ondersteuning van wetenschappelijke studies naar dystrofinopathie.

Een van de leukste kanten van het doen van onderzoek is voor mij het contact en de samenwerking met collega's van andere afdelingen. Ik was vanaf het begin betrokken bij het MRI breinonderzoek bij Duchenne spierdystrofie. Het is fantastisch om te zien hoe dit project zich ontwikkeld heeft met fulltime inzet van Nathalie Doorenweerd en onder constructieve leiding van met name dr. Hermien Kan. Inmiddels is er een grote MRI onderzoeksgroep die zich bezighoudt met onderzoek van brein en spieren bij dystrofinopathie. Van die groep wil ik met name bedanken voor de samenwerking Janneke van den Bergen, Nathalie Doorenweerd, dr. Eve Dumas, dr. Jos Hendriksen, dr. Hermien Kan, dr. Erik Niks en Beatrijs Wokke.

Het idee voor de studie uit hoofdstuk 6 ontstond toen ik samen met dr. Truus Plandsoen tijdens de startfase van ons multidisciplinair spreekuur voor kinderen met chronische spierziekten meeliep met het Duchenne spreekuur van dr. Imelda de Groot destijds in revalidatiecentrum De Trappenberg. Ik kijk terug op een fijne samenwerking met beiden.

Daarnaast hebben ook vele anderen geholpen om bij de retrospectieve studies patiënten te traceren en oude, papieren, statussen na te slaan; zonder hun inzet waren de studies uit de hoofdstukken 2 en 3 niet mogelijk geweest. In het bijzonder wil ik de collega's werkzaam in andere centra noemen: dr. Esther Brusse, prof. dr. Pieter van Doorn, Elly Ippel, dr. Anneke van der Kooi, Jan Post, dr. Aad Verrips, prof. dr. Marianne de Visser en dr. Nicol Voermans.

De ideeën voor de studies van de hoofdstukken 2 en 3 zijn gegroeid bij de gezamenlijke besprekingen met de afdeling klinische genetica. Dr. Ieke Ginjaar heeft mij wegwijs ge-

maakt in de genetische onderwerpen en geholpen met het interpreteren van data die zeer lang geleden zijn ingestuurd. Ik denk ook met plezier terug aan de samenwerking met dr. Paula Helderman bij ons gezamenlijke artikel uit hoofdstuk 3.

Mijn belangstelling voor de neuromusculaire ziekten werd gewekt in Leiden, mede door het enthousiasme van prof. dr. Jan Verschuuren, die nu mijn promotor is. Het fellowship neuromusculaire ziekten was de kers op de taart, waarbij ik de gelegenheid kreeg om in drie centra klinische ervaring op te doen. Het was inspirerend en leerzaam de verschillen in stijl van werken te zien.

De status aparte als staflid met promotieopdracht was niet altijd gemakkelijk. "*It always seems impossible until it's done" (Nelson Mandela)* kan ik beamen. Ik heb het gewaardeerd dat mijn collega's niet steeds vroegen hoe mijn proefschrift vorderde.

Hierbij wil ik gebruik maken van de gelegenheid om twee clinici waarvan ik veel geleerd heb postuum te noemen: dr. Peter Kreek, in leven internist in Amersfoort, en mijn eerste supervisor. Zijn stijl van opleiden had iets onconventioneels, en hij nam het altijd op voor de (jonge) assistenten. Hij kwam, ook als hij geen dienst had, op zaterdag visite lopen bij zijn eigen patiënten. Van zijn klinische blik heb ik veel geleerd en zijn lijfspreuk "*Il faut cultiver notre jardin" (Voltaire)* in herinnering gehouden.
Prof. dr. Herman Busch, in leven neuroloog in Rotterdam. Hij had een schat aan klinische kennis en ervaring en als assistent kon je altijd bij hem terecht met een vraag. Hij liet je overigens vooral zelf het antwoord te bedenken.

Zonder een stabiel thuisfront was de eindfase niet bereikt. Lisette Visser is al ruim 10 jaar het rustpunt thuis na schooltijd voor de kinderen en zij zorgt dat wij als gezin in elk geval de helft van de week 's-avonds op tijd warm eten. Ronald, rots in de branding, is er altijd wanneer nodig en ook bereid de flexibiliteit op te brengen die nodig is in een gezin met twee werkende ouders en schoolgaande kinderen. Tot slot, Laurine en Reinier, jullie zijn een bron van heel veel vreugde, en dankzij jullie is het gemakkelijker een hoop zaken relatief te zien.

List of abbreviations

AD	Autosomal dominant
ADHD	Attention-deficit and hyperactivity disorder
AON	Antisense oligonucleotide
AR	Autosomal recessive
AxD	Axial diffusivity
BMD	Becker muscular dystrophy
CK	Creatine kinase
CPT2	Carnitine palmitoyltransferase II
DM1	Myotonic dystrophy type 1
DM2	Myotonic dystrophy type 2
DMD	Duchenne muscular dystrophy
DTI	Diffusion tensor imaging
ECG	Electrocardiography
EFNS	European federation of neurological societies
EMG	Electromyography
FA	Fractional anisotropy
FSIQ	Full scale intelligence quotient
HR- MCA	High resolution melting curve analysis
ICV	Intracranial volume
LDGA	Laboratory for Diagnostic Genome Analysis
LGMD	Limb girdle muscular dystrophy
MD	Mean diffusivity
MH	Malignant hyperthermia
MLPA	Multiplex ligation-dependant probe amplification
MMD	Miyoshi-type muscular dystrophy
MND	Motor neuron disease
M-PCR	Multiplex-Polymerase chain reaction
MRI	Magnetic resonance imaging
NPE	Neuropsychological examination
OCD	Obsessive-compulsive disorder
PROMM	Proximal myotonic myopathy
RD	Radial diffusivity
SD	Standard deviation
SDS	Standard deviation score
SDQ	Strengths and Difficulties Questionnaire
SEM	Standard error of the mean
SMA	Spinal muscular atrophy
SMN1	Survival motor neuron 1
TBV	Total brain volume
U/I	Units/liter
VBM	Voxel based morphometry
WB	Western Blot analysis
WCB	Wheelchair bound