Duchenne Muscular Dystrophy:
mutation profiling
in view of the emerging gene-based therapies.

By

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DECLARATION

I, Alina Esterhuizen, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being or is to be submitted for another degree in this or any other university. This study has been approved by the Research Ethics Committee of the Faculty of Health Sciences: reference number 416/2008.

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LIST OF ABBREVIATIONS

ABD - actin-binding domain
ACE - angiotensin converting enzyme
AON - antisense oligonucleotide
BMD – Becker muscular dystrophy
CBC - cap-binding complex
cDNA – complementary DNA
CHG - comparative genomic hybridization
CK - creatine kinase
CNS – central nervous system
CpG – C-phosphate-G dinucleotide
D/BMD - Duchenne and Becker muscular dystrophy
DBS - dystrobrevin-binding domain
DGGE - denaturing gradient gel-electrophoresis
DHPLC - denaturing high performance liquid chromatography
DMD – Duchenne muscular dystrophy
DNA – deoxyribonucleic acid
DOVAM - detection of virtually all mutations
DPC - dystrophin-associated protein complex
DSBs - double stranded breaks
dsDNA - double stranded
ECG – electrocardiogram
EDL - extensor digitorum longus
EJC - exon junction complex
ERG - electroretinogram
ERS - exonic recognition sequences
ESE - exonic splicing enhancer
ESS - exonic splice silencer sites
FSIQ - full scale intelligence quotient
FVC - forced vital capacity
GRMD - golden retriever muscular dystrophy
GSH - Groote Schuur Hospital
HGVS - Human Genome Variation Society
HRM – high resolution melting
hrMCA - high-resolution melting curve analysis
HSF - Human Splicing Finder
ID - intellectual disability
IQ - intelligence quotient
LOVD - Leiden open source variation database
MLPA - multiple ligation-dependant probe amplification
MLPH - multiple ligation-dependant probe hybridisation
mPCR - multiplex PCR
mPCR - multiplex PCR
mRNA – messenger RNA
NHEJ - nonhomologous end joining
NHLS - National Health Laboratory Service
NMD - nonsense mediated decay
nNOS - neuronal-type nitric oxide synthase

ODN - oligodeoxynucleotide
ORF - open reading frame

ABBREVIATIONS cont.

PABP - poly(A)-binding protein
PCR – polymerase chain reaction
PEG - percutaneous endoscopic gastrostomy
PMO - phosphorodiamidate morpholino oligomer
PTC - premature termination codon
PTT - protein truncation test
RE - Restriction Endonuclease
RFLP - restriction-fragment-length polymorphism
RNA – ribonucleic acid
RXH - Red Cross War Memorial Children's Hospital
SCAIP - single condition amplification/internal primer sequencing
SIFT - Sorting of Intolerant From Tolerant
SR proteins - serine/arginine-rich proteins
SSCA - single-strand conformation analysis
SSCP - single strand conformational polymorphism
ssDNA - single stranded
STR - small tandem repeat
TAE - Tris-acetate EDTA
TAP - mRNA nuclear export mediator
TESS - Transcription Element Search System
TFB - transcription factor binding site/s
Tm - melting temperature
UCT - University of Cape Town
UMD–DMD France - Universal Mutation Database for DMD, France
UTR – untranslated region
VIQ - verbal IQ
XLDCM - X-linked dilated cardiomyopathy

ABSTRACT

Duchenne Muscular Dystrophy (DMD) is a lethal, X-linked, recessive muscle-wasting disorder affecting 1 in 3 500 live male births worldwide, for which only palliative care is available to date. Large exonic deletions or duplications are found in approximately 70% of DMD patients, for which diagnostic testing is available. The remaining 30% carry point mutations, which go largely undetected, as no testing is currently offered due to the great size of the *DMD* gene and the logistical challenges involved.

Positive outcomes of research into gene-based therapies necessitate availability of protocols which will extend the scope of testing to detection of point mutations. In this study, the DNA samples of 24 patients previously tested negative for exonic deletions with the mPCR method, were subjected to a complete mutation screen of the coding region of the *DMD* gene using the MLPA and hrMCA. Four deletions and 2 duplications were revealed by the initial screen with the MLPA. The DNA of the remaining 18 patients was then subjected to mutation scanning with hrMCA on the RotorGene™6000, of 96 PCR fragments encompassing the entire coding region of the *DMD* gene, amplified using M13-tailed primers, and subsequent sequencing of variant fragments. The analysis revealed 10 small/point pathogenic changes, 39 polymorphisms and 4 changes of uncertain significance. No pathogenic mutations were found in 8 patients of the cohort.

The 10 disease-causing changes identified, consisted of 3 nonsense, 4 frameshifts, 1 splice-site, 1 compound mutation (a GGTG duplication and a missense), 1 missense, and 1 point substitution in the Dp427promoter/exon1 region of the *DMD* gene. The deleterious nature of the mutations detected was inferred by their nature and by the output of bioinformatic analyses with regard to the effect on splicing, amino acid changes and regulatory sequences. Also, the results of family studies showed that the pathogenic mutations were familial in their origins and that they tracked with the disease within the families. Predictions of future therapeutic options revealed that by virtue of cohort selection none of the patients would benefit from AON-induced skipping of exon 51, which is currently undergoing clinical trials. However, multiexon skipping of exons 6 – 8 could benefit ~30% of the mutation carrying patients in the cohort and skipping exons 45 - 55 could benefit a further 12,5%, emphasizing the potential therapeutic impact of the multiexon skipping approach.

The study findings suggested that hrMCA could be successfully incorporated into the diagnostic protocol for mutation detection in the *DMD* gene, to help find “family mutations”, thus facilitating genetic counselling and ultimate determination of eligibility for mutation-based therapy in the future.

PLAN OF THE THESIS

This thesis is divided into five chapters. The first chapter provides an introductory background to neuromuscular disorders as well as a general overview of the aim of the study and the scientific approach to the investigation. The second chapter details the scientific rationale for each of the molecular investigations undertaken in this project. The third chapter follows with a presentation of the findings of the study. The fourth chapter discusses each finding with respect to the global genetic landscape of the *DMD* gene and patients. Genotype/phenotype correlations are discussed and future prospects for further investigations are addressed. The thesis is concluded in the fifth chapter, where the findings of the molecular studies are summarised and placed in context of the emerging DNA-based therapies currently undergoing clinical trials.

CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION

Muscular dystrophies are a group of inherited disorders, caused by the lack of a specific protein component in the muscle tissue. Starting with identification of the dystrophin gene in the 1980's, a number of muscular dystrophy genes and their proteins have been characterised. The impressive amount of knowledge since gathered on the molecular basis of muscular dystrophies has paved the way to a better understanding of the disease mechanisms and improved diagnosis, both in the clinic and in the laboratory. Availability of more reliable and informative genetic testing is a direct benefit of this knowledge, which allows for more effective patient management and accurate genetic counselling of the family.

Despite the advances in medical technology, muscular dystrophies particularly the allelic Duchenne and Becker muscular dystrophies (D/BMD) remain lethal and devastating to the patients and their families. While standards of care are continually improving worldwide, currently available interventions are limited to the management of symptoms and complications. Progress in terms of therapy has been slow and fraught with pitfalls and setbacks. However, much research has been focused on genetic therapies, and in recent years a number of new experimental approaches appear to hold promise. Of those, read-through therapy and exon skipping are especially relevant to D/BMD and to this study, and will be addressed in more detail later in this review.

Still, the first step remains identification of the disease-causing mutations. Potential availability of mutation-specific gene therapy marks the advent of personalised medicine and emphasizes the need for a rapid and accurate method of detecting small nucleic acid changes in D/BMD and other genetic disorders. Ideally, the technology should be cost-efficient, sensitive yet robust and rapid enough to fit into a diagnostic environment.

1.2 MUSCULAR DYSTROPHIES

Muscular dystrophies fall into the spectrum of neuromuscular diseases and are defined as a group of heterogeneous, inherited disorders, characterised by progressive weakness and wasting of skeletal muscle tissues. The classification scheme devised by Walton and Nattrass in 1954, based on their own clinical observations and still applied today, relies mostly on the distribution of muscle

weakness (Figure1.1), and the mode of inheritance (Walton, 1954). It identifies the following main groups:

- Duchenne/Becker-type (X-linked)
- Facioscapulohumeral (autosomal dominant)
- Limb girdle (genetically heterogeneous but mostly autosomal recessive)
- Emery-Dreifuss (X-linked, autosomal dominant and recessive sub-types)
- Distal (autosomal dominant and recessive clinically and genetically distinct subgroups)
- Oculopharyngeal (autosomal dominant)
- Congenital (autosomal recessive with a more generalised muscle weakness)

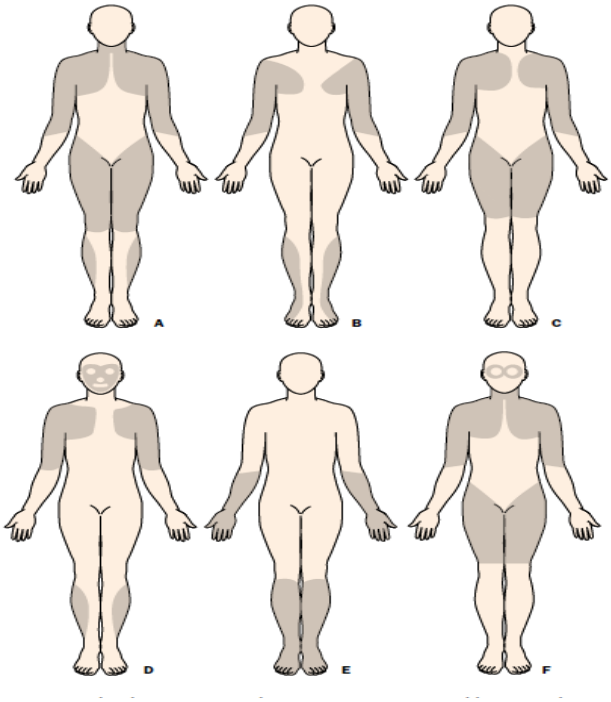


Figure 1.1: Distribution of predominant muscle weakness in different types of dystrophy. A: Duchenne-type and Becker-type, B: Emery-Dreifuss, C: limb-girdle, D: facioscapulohumeral, E: distal, F: oculopharyngeal. Shaded=affected areas. (Emery, 2002)

Muscular dystrophies have since been mapped to 29 different chromosomal loci, extending the above classification into 34 distinct disorders varying in the age of onset, degree of severity, mode of inheritance and the primarily affected muscle groups (Dalkilic & Kunkel, 2003).

1.3 DUCHENNE/BECKER MUSCULAR DYSTROPHY (D/BMD) (OMIM#310200)

Also known as Meryon's disease, DMD is one of the most common and severe of the inherited dystrophies, with an incidence of 1 in 3500 live, male births worldwide (Emery, 2001). Long before the gene and its protein were characterised, early

investigators noted the X-linked recessive heritability of DMD, based upon scrutiny of the affected family pedigrees (Meryon, 1851; Tyler, 2003). Also, early post mortem studies of muscle tissues and the spinal chord suggested the myogenic nature of the disease, with no involvement of the central or peripheral nervous system (Emery & Emery, 1995). The milder, allelic form of DMD, Becker's muscular dystrophy (BMD), has a lower frequency (1 in 30 000 boys), a milder progression and longer life expectancy (Becker & Kiener, 1955; Blake & Kroger, 2000a).

1.4 DISEASE MANIFESTATION

Duchenne muscular dystrophy, usually manifests in boys between the ages of 2 and 5 years, when parents typically notice a delay in motor milestones and symptoms such as frequent falling, difficulty in getting up, gait problems, toe-walking and flat-footedness. About 50% of the affected boys cannot walk independently at 18 months (Bushby et al., 2005).

The natural course of the disease is fairly predictable, although severity varies between patients, depending on the causative mutation (Tuffery-Giraud et al., 2009). Clinical examination generally reveals calf enlargement (**pseudohypertrophy**), lumbar lordosis, which disappears on sitting, and weakness of the neck flexors. Most DMD patients never learn to jump with both feet together. Weakness of the knee and hip extensors causes the typical **Gower's manoeuvre**: in an effort to stand up from lying on his back, the child needs to turn onto his front and push himself erect by moving his hands up his thighs. Muscle weakness is progressive, starting with proximal weakness of the lower limbs, moving onto the distal lower and then upper limbs, ultimately leading to wheelchair dependence (Emery, 2001; Manzur & Muntoni, 2009). **Loss of independent ambulation**, as defined in DMD occurs at 13 (although the mean is 9), in intermediate-type muscular dystrophy (IMD) between 13 and 16 and in BMD, beyond 16 years of age, though the use of steroids has made this data less fixed. Muscle weakness leads to **scoliosis** in 90% of the cases, with ultimate loss of sitting balance, exacerbated by formation of asymmetric **contractures** of the Achilles tendons and hips. In untreated patients, respiratory and cardiac complications are the cause of death at the mean age of 19 (Bushby et al., 2010a). BMD has a later age of onset with a more diverse presentation and progression, in some cases showing only mild myalgia and muscle cramps with no weakness (Beggs et al., 1991).

Cardiac involvement is seen in all D/BMD patients but generally remains subclinical in the early stages. It is likely that the late presentation of cardiac

symptoms is due to the decrease in physical activity, relative to the progressive general muscle weakness. The spread of fibrosis caused by repetitive strain results in left ventricular dysfunction and eventual dilative cardiomyopathy, if left untreated (van Bockel et al., 2009). Cardiomyopathy is seen as the determinant of survival in BMD patients, with an incidence of approximately 72%. It is the cause of death in about 20% of the DMD, and 50% of the BMD patients (Manzur & Muntoni, 2009).

For the most part, **respiratory function** in D/BMD patients is normal before loss of ambulation. As a rule, early loss of ambulation predicts early need for ventilation and respiratory failure. The respiratory function parameter in D/BMD patients is the forced vital capacity (FVC), which peaks shortly before loss of independent ambulation and progressively drops thereafter, with eventual respiratory failure manifesting as lowered energy levels, generalised malaise, weight loss, headaches, sleep disturbance, nocturnal and subsequent daytime hypercapnia. The concurrent increase in the frequency of respiratory infections raises the risk of death from respiratory failure during an infection (Bushby et al., 2005).

Non-progressive **cognitive impairment** in DMD ranges in severity from borderline neuropsychological deficits to severe intellectual disability (ID). Investigators consistently report the full scale intelligence quotient (FSIQ) in DMD patients as approximately 1 standard deviation below the population mean, with FSIQ scores of under 70 points seen in 19–35% of DMD cases, and moderate to severe ID (FISQ<50) noted in 3% of DMD patients (Cotton et al., 2001; Cotton et al., 2005; Taylor et al., 2010). The gross anatomical morphology of DMD-affected brains appears normal, although Yoshioka and colleagues found slight cerebral atrophy in 20 of the 30 DMD cases they examined, the extent of atrophy correlating directly with age and low intelligence quotient (IQ) (Yoshioka et al., 1980). The results of a study by Taylor et al. (2010) correlating FSIQ results with the location of the DMD mutations are highly suggestive of a link between cognitive deficits and cumulative loss of dystrophin isoforms expressed in the central nervous (CNS). Intellectual disability in BMD patients is reported infrequently, and there is speculation that since cognitive disabilities in D/BMD can occur before the onset of muscle weakness, some patients diagnosed with X-chromosome-linked mental retardation might carry mutations in the DMD gene (Blake & Kroger, 2000b).

Dystrophin is also expressed **in the retina** and some DMD patients show impaired scotopic and photopic responses obtained by full-field electroretinogram (ERG). Their visual function does not appear to be seriously compromised, although a

degree of non-progressive, red-green colour-blindness has been documented (Costa et al., 2007).

Long **bone fractures** and fractures of the vertebrae are common in D/BMD, because of low bone mineral density, possibly caused by relative immobility, further exacerbated by the long-term use of corticosteroids. Progressive loss of mobility along with steroid treatment can also lead to excessive **weight gain**, which in turn leads to early immobility. On the other hand, loss of appetite frequently accompanying respiratory failure, results in **weight loss**. **Constipation** is a common complaint in older boys, due to the involvement of smooth muscle. In later stages, **difficulty in swallowing** and frequent aspiration creates further nutritional and even respiratory complications. D/BMD patients can also present with a potentially fatal ‘‘malignant hyperthermia-like’’ reaction with rhabdomyolysis, hyperkalaemia and myoglobinuria, upon exposure to suxamethonium or a halogenated inhaled anaesthetic. This **rhabdomyolytic risk** is a major consideration in their anaesthetic management, and easy access to monitoring aids and intensive care facilities is strongly indicated (Manzur & Muntoni, 2009).

D/BMD along with other muscle disorders ranging in presentation from muscle cramps and myoglobinuria, to DMD-associated dilated cardiomyopathy or X-linked dilated cardiomyopathy (XLDCM), are also referred to as **dystrophinopathies,** since all are caused by production of defective or insufficient levels of dystrophin (Cardamone et al., 2008).

1.5 CLINICAL DIAGNOSIS

The assessments and interventions used in the diagnosis and management of D/BMD have been recently evaluated by the DMD Care Considerations Working Group, in an effort facilitated by the US Centers for Disease Control and Prevention (Bushby et al., 2010a; Bushby et al., 2010b). According to the clinical care recommendations, as set out by the Group, the diagnosis of DMD should be considered irrespective of family history, following one of three triggers:

- observation of **abnormal muscle function** in a male child,
- detection of **increased serum creatine kinase (CK)** levels (in DMD massively elevated by 10 – 100 x normal, since birth), a result of muscle tissue break down,

- discovery of **increased transaminases** (aspartate aminotransferase and alanine aminotransferase, which are produced by muscle as well as liver cells) (Bushby et al., 2010a).

The clinical diagnosis should be confirmed by:

- **Genetic testing**

  Mutation detection provides conclusive evidence and diagnosis. However, a negative result does not negate the diagnosis of D/BMD. A good understanding of the test limitations is therefore required. Testing the mother's carrier status is not strictly part of the diagnosis but facilitates genetic counseling of the family (Abbs et al., 2010).

- **Muscle biopsy**

  Immunohistochemical staining for dystrophin in muscle tissue will reveal absent or reduced dystrophin levels, which can be used to confirm a diagnosis of D/BMD. In most centres however, because of the invasive nature of the procedure, biopsies are taken only if molecular testing is uninformative (Bushby et al., 2010a).

1.6 TREATMENT AND CARE

Ideally, clinical management of a D/BMD patient is a team effort coordinated by the muscle clinic physician and involving a plethora of specialists: orthopedic, cardiac, physiotherapists, dieticians, occupational therapists, psychologists, and family and parent/patient support groups.

- **Glucocorticosteriods** are the mainstay of treatment for muscle weakness in D/BMD, best commenced at the plateau of the child's physical performance but long before loss of ambulation. Careful dosage monitoring and prophylaxis are needed to counteract the negative effects of steroid therapy such as weight gain, behavioural problems, bone density reduction, and compromised immune function after prolonged use.
- **Physiotherapy**: passive or active exercise as well as appropriate orthodesis to prevent and treat contractures, scoliosis and for walking and/or sitting postural support (seating / wheelchairs).
- **Splinting**: as appropriate, depending of the degree of ambulation and ankle dorsiflection.
- **Surgery**: possible elongation of Achilles tendons and correction of scoliosis.
- **Anasthaesia:** careful preoperative assessment of cardiac and respiratory function and consideration of the rhabdomyolytic risk.

- **Respiratory management**: prophylaxis, prompt diagnosis and treatment of lung infections, positive pressure ventilation to treat hypercapnia and respiratory failure.
- **Cardiovascular management:** regular monitoring of cardiac function (echocardiogram and ECG), treatment with ACE (angiotensin converting enzyme) inhibitors. Additional agents after onset of symptoms.
- **Monitoring of bone health:** diet supplementation with vitamin D and calcium, intravenous biphosphonates for vertebral fractures, early mobilization post long bone fracture, to prevent early loss of ambulation.
- **Nutrition:** weight monitoring and diet adjustments, use of mild laxatives to relieve constipation, intubation or percutaneous endoscopic gastrostomy (PEG).
- **Addressing learning and emotional difficulties:** occupational and speech therapy, consultation with psychologists and support groups.
- **Access to adaptive technologies** i.e. electric beds and wheel chairs, computers etc., to aid an independent and functional life (Bushby et al., 2005; Bushby et al., 2010b).

1.7 THE *DMD* GENE AND ITS PRODUCT

Localisation of the gene to the short arm of the X chromosome (Xp) was achieved by extensive work with positional cloning and restriction fragment polymorphisms (Davies et al., 1983; Monaco et al., 1986; Burghes et al., 1987; Koenig et al., 1987) as well as cytogenetic studies of DMD-manifesting females, with balanced X;autosome translocations breaking at Xp21 (Verellen-Dumoulin et al., 1984). The full extent of the gene was eventually revealed by work with deletion-detecting clones on DNA of a patient with four different X-linked disorders, in whom a large contiguous-gene-deletion was confirmed (Francke et al., 1985). Similarly, BMD was shown to map to the same region of the X chromosome (Kingston et al., 1984).

The *DMD* gene encompasses 2.5Mb of genomic sequence, with a full-length protein encoded by a 14kb RNA transcript (Monaco et al., 1992). This is approximately 1.5% of the X chromosome and 0.1% of the entire genome, making the *DMD* gene one of the largest, single protein-encoding genes described in humans to date. It consists of 79 exons with 7 tissue-specific promoters (Figure1.2), which drive the expression of dystrophin isoforms in various tissues (Manole, 1995; Muntoni et al., 2003a):

- **Full-length isoforms** are encoded by promoters with unique first exons, spliced to 78 common exons:

  Dp427M (muscle promoter) drives expression of dystrophin in the skeletal muscle, cardiomyocytes and to a small extent in glial cells of the brain.

  Dp427P (Purkinje promoter), on the other hand, is expressed in the cerebellar Purkinje cells and at low concentrations, in skeletal muscle.

  Dp427B (brain promoter), is expressed primarily in cortical neurons and the hippocampus of the brain (Blake et al., 2002; Muntoni et al., 2003a).

  Dp427L (putative lymphocyte promoter, not included in Figure 1.2), whose status is uncertain but is at this time thought to be physiologically insignificant (Wheway & Roberts, 2003).

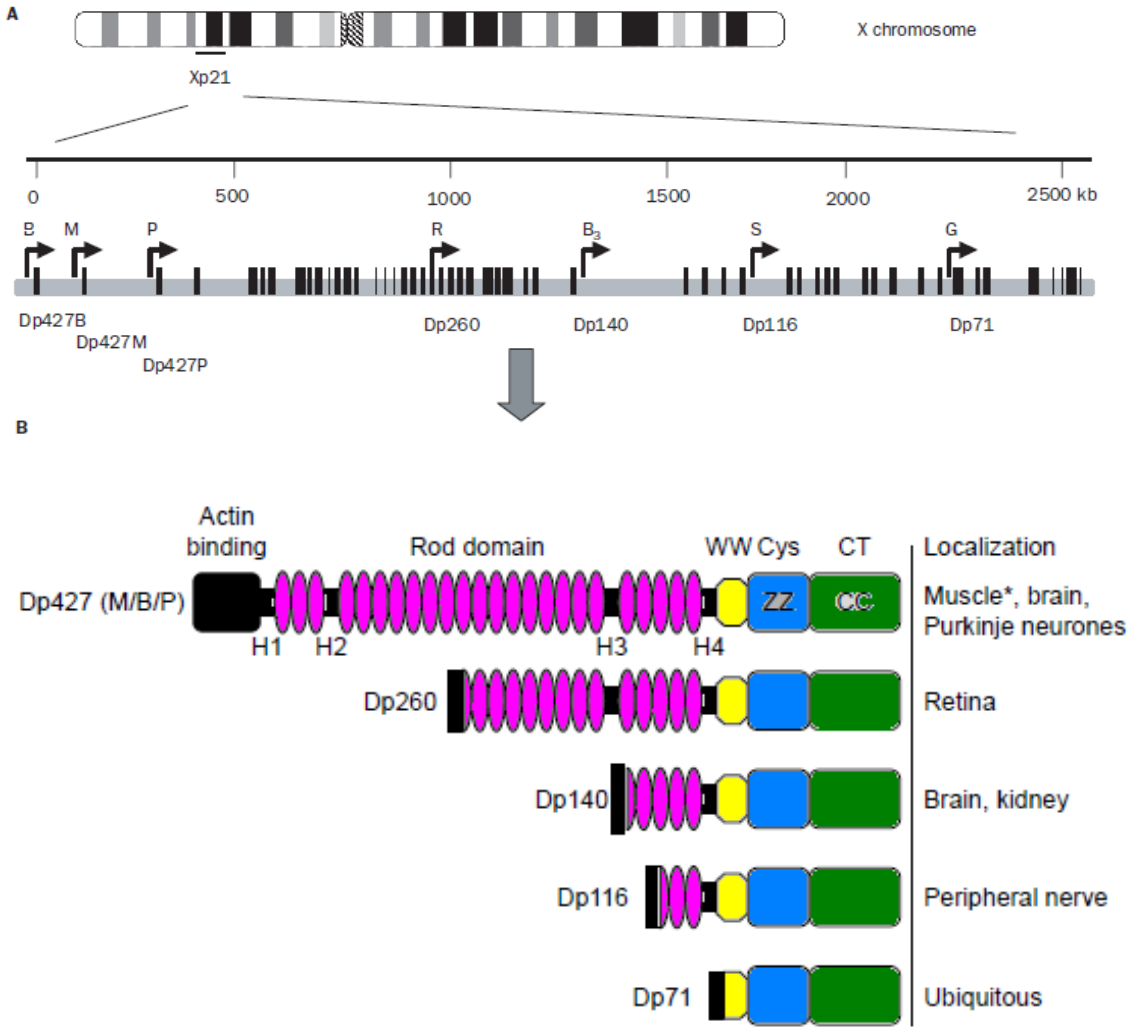


Figure 1.2 Linear representation of the *DMD* gene and its products - adapted from
A: The *DMD* gene position at Xp21 and its linear representation: exons represented as black lines and promoters as arrows). B: Linear representation of the dystrophin isoforms (Blake & Kroger, 2000b; Muntoni et al., 2003b). *skeletal and cardiac muscle

- **Short isoforms** are expressed by at least four internal promoters, which splice to various exons within the gene, generating protein products of 260 kDa (Dp260 to exon 30), 140 kDa (Dp140 to exon 45), 116 kDa (Dp116 to exon 56), and 71 kDa (Dp71 to exon 63) (Blake et al., 2002).

- **Alternative isoforms** of dystrophin are generated through a number of alternative, tissue specific splicing events, such exon skipping and exon scrambling. These events sometimes result in the formation of circular RNA molecules in co-existence with linear RNA, the biological function of which is still unclear (Surono et al., 1999; Gualandi et al., 2003).

1.7.1 Dystrophin

Dystrophin is a large cytoskeletal protein, 427kDa in size, localized to the cytoplasmic face of the muscle cell membrane. It acts as a mechanical link between the cytoskeletal actin and the extracellular matrix (Figure.1.6) (Arahata et al., 1988; Hoffman et al., 1987) and plays a role in intracellular signal trunsduction (Rando, 2001). The dystrophin molecule consists of four, main functional domains (Figure 1.2), each with its own protein-binding capabilities (Blake et al., 2002):

- The **NH_2-terminus,** along with a part of the rod domain binds directly to, but does not cross-link cytoskelatal actin.
- The **central rod domain**, makes up most of the protein and consists of 24 spectrin-like repeats, interrupted by 4 proline-rich hinges. The repeats confer the molecule's structural rigidity and the interspersed hinges allow its flexibility. Spectrin repeats 11 through 17 within the rod region constitute a second actin binding site.
- The **dystroglycan-binding domain** consists of:
    - **WW domain** follows the fourth hinge of the rod region and separates it from the cysteine-rich and the COOH-terminal regions. It is a protein-binding module and is part of a number of signaling and regulatory molecules.
    - The **cysteine-rich domain** consists of two EF-hand motifs, which bind intracellular Ca^{2+} , and a ZZ domain containing a number of cysteine-rich residues predicted to form coordination sites for divalent metal cations e.g. Zn^{2+}. This ZZ-domain is similar to other types zinc finger found in nuclear and cytoplasmic proteins.
- The **COOH-terminal domain** or CC-domain contains two α–helical coiled coils, which mediate its interaction with the syntrophins.

The shorter dystrophin isoforms (Figure 1.2), lack parts of the actin-binding and rod domains but retain the cysteine-rich and the CC-terminal domains, which mediate binding to the dystrophin-associated proteins (dystroglycan, dystrobrevin and syntrophin).

1.8 DYSTROPHIN-ASSOCIATED PROTEIN COMPLEX

Initial biochemical purification of the compound from muscle led to the observation that dystrophin co-purifies with a group of sarcolemmal and sub-sarcolemmal proteins (Ervasti & Campbell, 1991; Ervasti et al., 1991). Together, they have been shown to interact as the dystrophin-associated protein complex (DPC), which consists of dystrophin, sarcoglycans, dystroglycan, dystrobrevins, syntrophins, sarcospan, caveolin-3, and neuronal-type nitric oxide synthase (nNOS) (Blake et al., 2002; Judge et al., 2006). The DPC form a scaffold connecting the actin-based cytoskeleton with the basal lamina, with dystrophin acting as an essential structural link in its assembly (Figure 1.3). Absence or dysfunction of dystrophin therefore, causes instability of the DPC and an abnormally increased susceptibility to damage. The DPC proteins are found in various combinations depending on muscle tissue type and can be subdivided into three distinct protein subcomplexes, according to their location and binding associations within the cell and between one another:

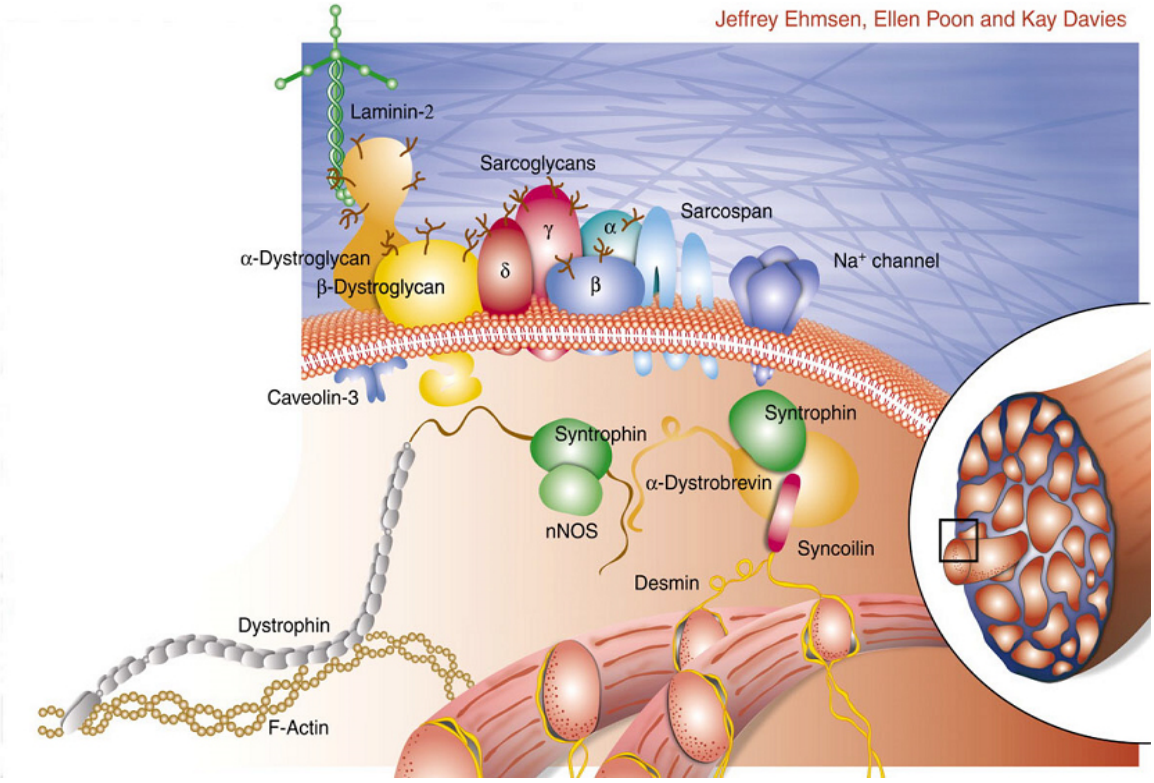


Figure 1.3 Dystrophin and its associations within the cell.
(www.humgen.nl/lab-vdeutekom/introduction.html).

- **The Dystroglycan Complex**

  Dystroglycan forms an essential core of the DPC, as it connects the cytoskeletal components of the DPC to the extracellular matrix (Figure 1.5). Dystroglycan is produced from a single gene and is post-translationally cleaved into α and β subunits (Lapidos et al., 2004). The extreme COOH terminus of the **β-dystroglycan** binds directly to the WW domain and the EF hands of the cystein-rich region of dystrophin, emphasizing the functional importance of both these domains (Crawford et al., 2000). β-dystroglycan also binds to **Grb2**, providing a known signaling pathway for β-dystroglycan. **Caveolin-3,** a transmembrane protein specifically expressed in muscle, also interacts with β-dystroglycan, and may compete for the same binding site as dystrophin. This theory is supported by the reduced levels of dystrophin and β-dystroglycan in autosomal dominant limb-girdle dystrophy type 1C, which is a result of calveolin-3 mutations. On the extracellular side, the **α-dystroglycan** forms an important connection to the α_2-chain of **laminin 2,** forging a link is between the sarcolemma and the extracellular environment (Rando, 2001; Blake et al., 2002; Lapidos et al., 2004).

- **The Sarcoglycan Complex**

  The sarcoglycans are a group of transmembrane-spanning glycoproteins, which co-purify as a complex within the DPC (Figure 1.3). The precise function of the sarcoglycans within the cell membrane remains unclear and although absence of the sarcoglycan-complex in mutant cells does not affect normal distribution of dystrophin, it appears to cause defective cell membrane permeability and subsequent increased fragility and degeneration (Rando, 2001; Blake et al., 2002; Lapidos et al., 2004).

- **Sarcospan** is tightly associated with the sarcoglycans (Figure 1.3) and the DPC and is thought to stabilise the dystroglycan complex in sarcolemma. It is not crucial for normal function of the DPC, nor is it essential for formation of the sarcoglycan complex. Sarcospan may have a signalling function, since it is a member of the tetraspanin protein family, which has been implicated in mediating the integrin-signaling responses in other tissues (Rando, 2001; Blake et al., 2002; Lapidos et al., 2004).

- **The Cytoplasmic (dystrophin containing) Complex**

  Various isoforms of **dystrobrevin** bind directly to dystrophin in the brain, muscle and other tissue. The **syntrophins** bind directly to dystrophin and

dystrobrevin. While the precise role of dystrobrevin in this context is unclear, it is thought to play a role in intracellular signal transduction. The syntrophins possibly function as a link between the membrane-associated proteins and the DPC. Syntrophins also bind to the neuronal-type nitric oxide synthase (**nNOS**), which may play a role in regulation of the vascular tone in skeletal muscle fibres. Binding between dystrobrevin and syntrophin and by extension that between syntrophin and neuronal nitric oxide synthase (nNOS), is mediated by the CC-domain of the dystrophin molecule. In *mdx* mice, nNOS is known to be dislodged from its site in the plasma membrane and becomes cytoplasmic. Vasoconstriction in these mice is impaired, which is an indication of the importance of association between the DPC and nNOS (Blake & Kroger, 2000a; Rando, 2001; Lapidos et al., 2004).

1.9 PATHOLOGY OF DYSTROPHIN-DEFICIENT MUSCLE

It is hypothesised that since dystrophin forms an integral part of a mechanical link between the extracellular matrix and the cytoskeleton, faulty dystrophin causes destabilization of that link and subsequent weakening of the sarcolemma (Judge et al., 2006). Mechanical damage of fragilised membranes, caused by repetitive eccentric contractions, provokes microlesions that are thought to lead to physical sarcolemmal breaks or calcium leak channel openings. This in turn, elevates intracellular free calcium levels triggering calcium-activated proteases and subsequent fibre necrosis. Destabilisation of the sarcolemma is may also be linked to the DPC's involvement in cellular signalling, important for microvascular function, muscle fibre type-determination and general myofiber homeostasis (Judge et al., 2006). The primary manifestations of DMD are therefore thought to result from an imbalance between muscle fiber necrosis and myoblast regeneration, with necrosis as the primary pathologic feature (Deconinck & Dan, 2007).

Histological analysis of muscle samples from affected patients generally reveals changes in the fibre size, fibre necrosis and increased amounts of fat and connective tissue. Degenerating fibers are often observed in clusters confined to segments of the muscle fiber (grouped necrosis). Necrotic or degenerating muscle fibers are characteristically seen in postnatal DMD muscle biopsies even before muscle weakness is clinically observed. In early disease, active regeneration is a sign of fibre necrosis, with the regenerating fibers recognized by their small diameter, basophilic RNA-rich cytoplasm, and large, centrally placed myonuclei. In later stages, this regenerative capacity is exhausted and muscle is replaced by connective and adipose tissue. Necrotic muscle fibers are subject to phagocytosis

and inflammatory cells, predominantly macrophages and CD4 lymphocytes, are seen at perimysial and endomysial sites (Blake et al., 2002).

Immunohistochemical analysis of normal muscle shows dystrophin localised to the sarcolemma with uniform labelling of each fibre. In DMD, dystrophin is mostly absent, whereas in BMD, the fibres show reduced and/or uneven sarcolemmal labeling (Figure 1.4). Some muscle biopsies of DMD, show “revertant fibres”, which manifest low or even near normal expression of dystrophin. Immunolabelling is often uneven on the fibre periphery and longitudinal sections reveal only partial labeling of the fibre. This type of expression arises as a result of restoration of the translational reading frame in some cells but the mechanisms by which this occurs are uncertain (Sewry, 2000). Studies of revertant fibres in the *mdx* mouse suggest various mutation-dependant mechanisms which result in alternative splicing events e.g. ‘‘exon skipping’’ (Winnard et al., 1995; Lu et al., 2000).

An estimated 26% of non-manifesting carriers are reported to exhibit a degree of dystrophin-defective staining (E. M. Hoogerwaard et al., 2005), with occasional dystrophin negative fibres, or uneven labelling. In symptomatic carriers, dystrophin-negative muscle fibres are thought to result from a skewed pattern of X-inactivation in the muscle (Sewry, 2000). The numbers of ‘‘negative’’ fibres are variable and transverse sections in young cases often show mosaic patterns of positive and negative staining (Figure 1.4).

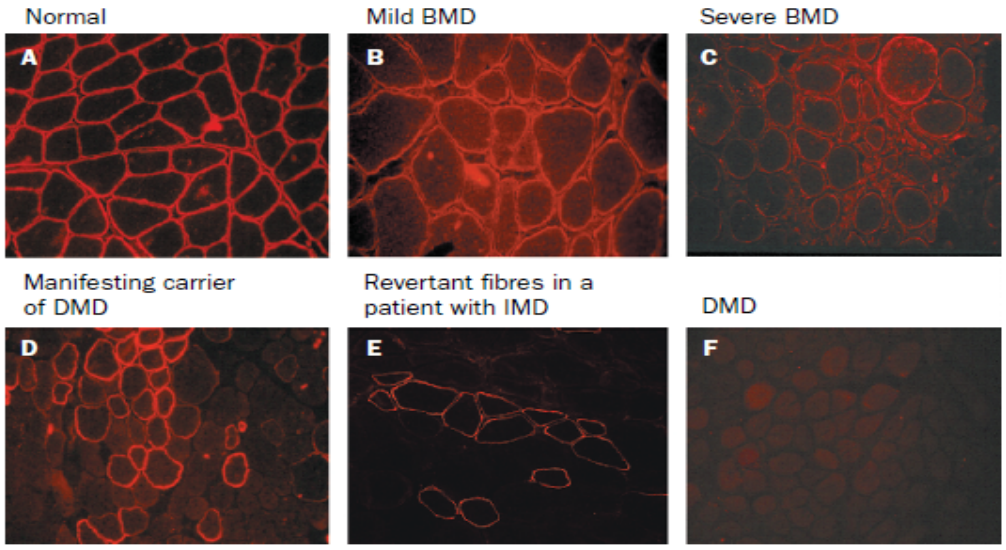


Figure 1.4 Examples of immunocytochemical findings in normal and dystrophin-deficient muscle. Normal control (A): localization to the periphery of each muscle fibre. Patients with BMD (B and C): low level, discontinuous protein expression. Manifesting DMD carrier (D): “mosaic” expression of dystrophin in different fibres. Intermediate phenotype (IMD) (E): relatively abundant revertant fibres. DMD (F): absent protein expression. (Muntoni et al., 2003b)

1.10 MUTATIONS IN THE *DMD* GENE

Because of its size, the *DMD* gene is predisposed to mutations, approximately 65% of which are estimated to be exonic deletions, and 5% to 15% are duplications (Beggs et al., 1991). This broad estimate for the frequency of duplications has been put down to the variable sensitivities of the techniques used, which should change with the current, improved detection methods (Muntoni et al., 2003a). In a recent study by Kesari et al. (2008), duplications were detected in as many as 21% of mutation-positive BMD patients.

While exonic rearrangements can occur at any point within the gene, two hot-spot regions have been identified: one situated towards the central part of the gene and includes **exons 45 to 53** (hot-spot region 1), and the other at the 5' end of the gene, spanning **exons 2 to 20** (hot-spot region 2). Entries in the Leiden Open source Variation Database (*LOVD*) for DMD (www.dmd.nl) record the most deletions in hot-spot region 1, with the deletion of exon 45 being most common (1.7% of all DMD mutations) (Aartsma-Rus et al., 2006). Deletions within the *DMD* gene (and other genes on the X chromosome) are predominantly maternally inherited, as opposed to duplications, which mostly originate in the male germline (Hu et al., 1990). Consequently, duplications are usually seen as familial cases, and the recurrence risk is high. Most duplications occur near hot-spot region 2, with exon 2 being most commonly duplicated (0.4%of all mutations) (Aartsma-Rus et al., 2006; Gualandi et al., 2009). Interestingly, the reciprocal deletion of the most common duplication (exon 2) has never been reported (White et al., 2006; Flanigan et al., 2009).

Analyses of the deletion junctions indicate that they arise from rejoining of broken ends via nonhomologous end joining (NHEJ) of double stranded breaks (DSBs). DSBs arise during DNA replication and other endogenous processes, and are seen to cluster in DSB hot-spot regions. There is evidence to suggest that in DMD, the DSB-prone regions coincide with deletion/duplication hotspots, which also represent major meiotic recombination hot-spots (Sironi et al., 2006; del Gaudio et al., 2008).

The remaining 30% of gene changes in DMD are small/point mutations, mostly resulting in nonsense, frame-shift or splice site mutations (Roberts et al., 1994). Deep intronic mutations have also been described (Béroud et al., 2004), as well as exonic insertions of repetitive sequences and rarely, missense mutations. Small/point mutations in the *DMD* gene do not appear to exhibit the same clustering effect as exonic rearrangements and as mentioned earlier, their detection continues to present a diagnostic challenge. However, compared to the amount of data available on exonic rearrangements, the information on frequencies and distribution

of small/point mutations within the *DMD* gene, is less abundant, mostly due to the technical difficulties in finding them. Therefore, as methods improve and data becomes available, currently undetected trends may be uncovered. For example, Deburgrave et al. (2007), found that of the 124 pathogenic point mutations identified in that study cohort, 20 were in exons 53, 66 and 70 or in their flanking splice sites. Also, the recently published genotype-phenotype analysis of all entries in the UMD-DMD France database (www.umd.be) reported a deficit of point mutations in exons 32 and 48 and conversely, and excess of mutations in exons 4, 6, 7, 35, 66, and 70 (Tuffery-Giraud et al., 2009) . Also, the CpG dinucleotide has been shown to be a hot spot for mutations in humans because it can undergo oxidative deamination of 5-methyl cytosine (Krawczak et al., 1998). Tuffery-Giraud et al. (2009) report that 43.8% (81/185) of substitutions leading to stop codons, involve CpG dinucleotides, with the C-to-T substitution in the CGA codon as the most common event (90%). The highest mutation rate was observed at a CpG site in exon 66. Three CpG sites are located within exon 70 making this exon particularly prone to mutations. Nonsense mutations due to other types of substitutions showed a wider distribution along the gene (Tuffery-Giraud et al., 2009). Data gathered from a large cohort study by Flanigan et al. (2009) supports uniform hypermutability of CGA>TGA mutations but emphasises the absence of hot-spots for point mutations seen to segregate within families as "private mutations".

1.11 GENOTYPE VERSUS PHENOTYPE

The severe DMD phenotype results from mutations, which cause synthesis of a non-functional and unstable protein, as opposed to BMD, where a smaller but semi-functional protein is produced. The disease presentation (DMD as opposed to BMD or IMD) is therefore not relative to the size of the mutation, as much as it is to:

- **Disruption of the DNA translational open reading frame (ORF)**
  The "reading frame hypothesis" can be applied to ~90% of DMD cases (Monaco et al., 1988), and it argues that mutations, which disrupt the ORF result in more severe phenotypes than those, where the reading frame is retained. ORF disruptions cause premature termination of translation which triggers the cellular process of nonsense mediated decay (NMD) with its degradation of the mRNA and the faulty protein (if any).
- **The mutation's position within the gene**
  Disruptions of the actin-binding N-terminus and the carboxy-terminus of dystrophin generally cause a severe phenotype, even if the rearrangement is in-frame. Large, in-frame deletions at the 5' end of the gene, which stretch across

the actin-binding and into the middle of the rod domain (deletions of exons 3 – 31, 3 – 25, 4 – 41, or 4 – 18) result in a DMD phenotype and account for some of the exceptions to the reading frame hypothesis (Nevo et al., 2003). The functional importance of the actin-binding domain is also emphasised by the DMD-causing, smaller deletions at the 5' region, such as those involving exons 3 – 13 (Muntoni et al., 2003b). The central and distal rod domains, on the other hand, appear to be almost dispensable and cases of in-frame deletions of approximately half of the gene have been described, with only a mild effect on the phenotype (England et al., 1990). Conversely, some single exon deletions e.g. exon 44, result in a severe DMD phenotype (Muntoni et al., 2003a). Mutations involving the cysteine-rich domain invariably cause DMD, whereas mutations located in exon 74 or higher are found in both BMD and DMD patients. Phenotypes can also vary between patients carrying identical mutations, even within one family which could be a result of the mutation's epigenetic context (Aartsma-Rus et al., 2006). The schematic overview in Figure 1.5 reflects general findings for in-frame deletions.

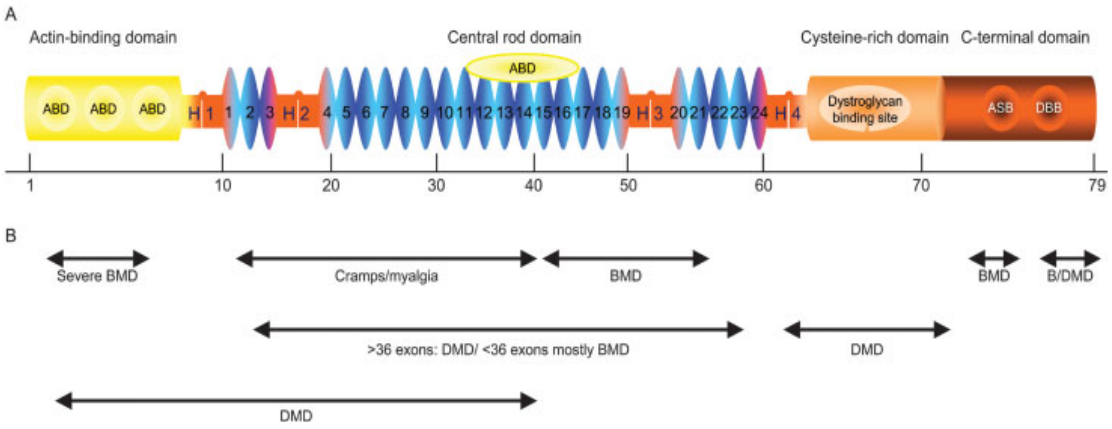


Figure 1.5 Correlation between the location of in-frame deletions and the BMD or DMD phenotype (Aartsma-Rus et al., 2006). Schematic drawing of the dystrophin protein with its fuctional domains. The location of the different exons is shown underneath the protein. (B) Graphic overview of the relation between the location of in-frame deletions and the severity of the phenotype (Beggs et al., 1991).

The effect of an exonic deletion on the ORF and possibly the phenotype can be predicted, as the intron-exon boundaries have been well characterised and show that some exons of the *DMD* gene do not contain an integral number of triplet codons and if deleted, will result in a frame-shift of the mRNA (Baumbach et al., 1989; Koenig et al., 1989). Such predictions are valuable to the clinician, especially in early diagnoses of cases with no family history, as they help to anticipate the disease severity and may influence the approach to treatment. Useful prediction tools such as the Reading-Frame Checker accessible via *The Leiden Muscular Dystrophy Pages* (www.dmd.nl), designed to predict the effect of the rearrangement

on the ORF. Such predictions however, can only be made reliably when based on the mRNA changes. Genomic DNA-based findings may not reflect the true extent of the rearrangements due to events such as altered or inefficient splicing. Reading frame predictions for duplications, based on genomic testing are not recommended, as non-tandem or complex duplications are being reported with increasing frequency (with availability of more efficient testing), and determination of the fragment orientation requires mRNA analysis (Flanigan et al., 2009).

1.12 EXCEPTIONS TO THE READING FRAME RULE

It has been estimated that 8 – 9% of DMD cases violate the reading frame rule, (Aartsma-Rus et al., 2006) and the incidence may be much higher in BMD patient populations, as suggested by results of a study by Kesari et al. (2008), where as many as 30% had out of frame mutations. In-depth studies of such cases have elucidated a number of underlying mechanisms.

1.12.1 Exon skipping and alternative splicing

Alternative splicing events can cause skipping of one or more exons around the deletion/duplication, affecting the ORF and dystrophin production. Rearrangements in certain parts of the gene appear to be more likely to result in exon skipping (5' end, exons 3 – 6; 3 – 7; 5 – 7 and at the 3' end, exons 44, 45, 51, 47 – 52 and 49 – 50) (Beggs et al., 1991; Gualandi et al., 2009). Similar genomic changes, however, have been seen to result in different phenotypes. This could be explained by the unusually large size of some of the introns in the *DMD* gene, ranging in size from 107 to 248 000 bp (average intron size of 27 kb), where the deletion/duplication break-points can occur at different positions, disrupting regulatory sequences in some cases. (Béroud et al., 2004). Gualandi et al. (2003) described a case in point, where deletion of a single exon 5, resulted in DMD in some, and BMD in other patients. Molecular scrutiny of mRNA revealed that the DMD patients produced circular and linear RNA with missing exons 6 and 9. The BMD patients on the other hand, exhibited a linear transcript only, with a probably in-frame deletion of exon 5 (Gualandi et al., 2003).

While the majority of the small/point mutations disrupt the ORF and result in a severe phenotype, a number of BMD patients with nonsense and frameshift mutations have been reported (Franz et al., 2000; Ginjaar et al., 2000; Disset et al., 2006; Deburgrave et al., 2007). Again, alternative splicing and exon skipping events are the mechanisms shown to be active in some of these patients' phenotype rescue. Exon-skipping is also the most common consequence of splice-site

mutations and may result in production of in- or out-of-frame transcripts, depending on whether the particular exon boundry ends with an entire codon (Deburgrave et al., 2007). Splice mutations can also activate cryptic splice sites and alternative splicing events, where intronic sequences become incorporated into the mRNA. Deep intronic mutations can activate entire cryptic exons (Dwi Pramono et al., 2000; Gualandi et al., 2003; Béroud et al., 2004; Z. Zhang et al., 2007).

Studies also suggest that trans-acting splicing factors might play a role. According to this hypothesis, splicing within the *DMD* gene might be affected by individual differences in the levels of expression of CUG-binding proteins, which regulate splicing via direct binding to the gene (Sironi et al., 2003).

1.12.2 Disruption of exonic splicing enhancer (ESE) sites and exonic recognition sequences (ERS's)

Splicing often involves a complex series of interactions between the sarcoplasmic reticulum (SR) proteins, which bind to ESE sites and ERS, which in turn recruit other splicing factors. Regulation of alternative splicing appears to depend on mutation-specific creation of new, or disruption of the existing ESEs and ERS's, or the less common exonic splice silencer sites (ESS) (Cartegni et al., 2002). Reports of BMD patients with nonsense mutations, such as those mentioned by Aartsma-Rus et al. (2006) in their review article, are examples of such events. The two BMD patients each carried a different nonsense mutation in exon 29. This is an apparent paradox, as nonsense mutations generally result in severe phenotypes due to nonsense mediated decay (NMD). In these two patients however, the mutations disrupted exonic ESE sites, causing in-frame splicing out of exon 29, thus effecting restoration of the ORF and production of a truncated dystrophin and a milder phenotype. Once again, the effect is variable and depends on the strength of the particular enhancer or silencer sequence (Aartsma-Rus et al., 2006). ESEs are well documented in the *DMD* gene, and may be present in most exons (Caceres & Kornblihtt, 2002; Cartegni et al., 2002). ESSs on the other hand appear less common (Disset et al., 2006). As in prediction of the frameshift caused by exonic rearrangements, a number of bioinformatic tools such as ESEfinder (Cartegni et al., 2003), RESCU-ESE (Fairbrother et al., 2004) or the Human Splice Finder (Desmet et al., 2009) are available to predict the presence of ESE motifs and to assess the consequence of a single base alteration.

1.12.3 Activation of alternative translation initiation sites

Mutation-specific activation of an alternative translation initiation ATG codon, has been described in a BMD patient with an out-of-frame deletion of exons 3 – 7 and no evidence of exon-skipping in cDNA (Winnard et al., 1995). The authors suggest that the mutation caused activation of an alternative translation initiation site present in exon 8. Activation of an alternative translation initiation site from a methionine in exon 6 has since been directly experimentally demonstrated by Gurvich et al. (2009), in the context of a founder nonsense mutation in exon 1.

1.12.4 Somatic mosaicism

Apparent violation of the frameshift rule can also be seen with somatic mosaicism in the affected tissue. This is considered when immunohistochemical analysis of tissue samples reveals a dual population of dystrophin-positive and dystrophin-negative fibres (Hoffman et al., 1987) and molecular studies reveal two DNA populations (normal and mutant) in blood DNA and in muscle cDNA, in the absence of alternatively spliced transcripts (Deburgrave et al., 2007; Kesari et al., 2009). Since the large *DMD* gene has a high (1/10 000) rate of *de novo* mutations in germ line cells (sperm, eggs) (Caskey et al., 1980; Haldane, 2004), somatic mutations could occur early in embryonic development, leading to production of tissues with both dystrophin-positive and dystrophin-negative muscle fibres.

Despite the gene's high mutation rate, reports of male somaticism are few (Saito et al., 1995; Uchino et al., 1995; Deburgrave et al., 2007; Kesari et al., 2009), possibly due to the low mutation rate in somatic cells relative to germ line cells, or alternatively, poor ascertainment due to mild or unexpected clinical symptoms. An interesting case of somatic mosaicism was reported by Kesari et al (2009). in a patient presenting with acute cardiac failure, and raised CK levels in the absence of overt muscle symptoms. Dystrophin immunostaining of muscle biopsy material revealed a mosaic pattern, with a predominance of dystrophin positive fibres. DNA studies showed no exonic rearrangements but cDNA sequencing presented apparent heterozygosity for a C>T (U) change at position 8713 (r.8713c>u), predicted to cause a nonsense mutation in the *DMD* gene (Arg2905X). The mutation was not identified in the patient's mother, although a number of shared polymorphisms were seen, confirming a case of somatic mosaicism. The frequencies of normal versus mutant genes were determined in blood/DNA (50:50), muscle/DNA (80:20) and muscle/mRNA (90:10) (Kesari et al., 2009). The discordance between muscle and blood, could be explained either as incidental segregation of more dystrophin-positive stem cells into the biopsy region or by

“genetic normalization”, a process whereby the necrotic fibres are replaced with normal stem cells, as seen in the majority of manifesting female carriers (functionally somatic mosaics, but due to X inactivation rather than somatic mutations) who also often present with cardiac symptoms. This process explains the reduction in serum CK levels and improvement in clinical symptoms of some manifesting carriers, with advancing age (Pegoraro et al., 1995; Kesari et al., 2009).

1.12.5 Unusual changes

Non-contiguous duplications, partial triplications and complex rearrangements in the *DMD* gene are being reported with increasing frequency as a result of testing with sophisticated technologies like oligonucleotide array-CGH and shown to have varying effects on the phenotype (White et al., 2002; Kesari et al., 2008; White et al., 2006). Detailed scrutiny of the complex junctions have led investigators to suggest regional genomic instability, aided by the presence of repetitive elements, a stem-loop structure, and possibly preexisting mutations, as the mechanism behind complex rearrangements of the *DMD* gene (del Gaudio et al., 2008; Oshima et al., 2009).

In most instances, the apparent violation of the reading-frame hypothesis is disproved upon in-depth analysis and shown to hold. The importance of understanding the exact genomic context of each mutation with its downstream effect on the transcript and the protein is thus emphasised, not only in predicting its phenotypic consequence, but also as relevant to application of the emerging personalised gene therapy.

An interesting finding of a completely healthy male with a deletion of exon 16 and part of introns 15 and 16 has been published (exon 16 encodes for 60 amino acids situated within the central rod domain of dystrophin), which suggest that even large changes in the dystrophin gene may not always be disease-causing The investigators therefore caution against hasty diagnosis of dystrophinopathy in sporadic cases of single exon in-frame deletions (Schwartz et al., 2007). In an effort to avoid misdiagnosing young patients without positive family history, many neurology centres confirm molecular findings of isolated cases with muscle biopsies.

1.13 MUTATION DETECTION

1.13.1 Detection of exonic rearrangements

The Beggs and Chamberlain **multiplex PCR,** is a simple and reliable method used to amplify selected exons across the hot-spot regions, still used in many diagnostic

laboratories today (Chamberlain et al., 1988; Beggs et al., 1990). In a multiplex reaction, each target is amplified with its own set of primers, which is a limiting factor, as large quantities of primers within a single PCR, result in problems such as dimerisation and false priming. Therefore, the multiplex PCR deletion screen for DMD involves at least three PCR reactions, which together amplify selected exons across the 5' and the 3' mutation hotspots. This allows for detection of ~ 95% of the DMD-causing deletions but all duplications and the remaining 5% of deletions located outside of the hotspots, remain undetected.

The Salsa **Multiple Ligation-dependant Probe Amplification (MLPA)** test, (MRC-Holland) is a sophisticated variant of PCR, which incorporates specific probes for all the exons in the DMD gene (Lalic et al., 2005; Lai et al., 2006). The output is analysed quantitatively and allows for detection of all exonic copy number changes. MLPA currently features in the *The Leiden Muscular Dystrophy Pages* (www.dmd.nl) as the recommended method for deletion/duplication detection in the *DMD* gene. It also used in this study and will be addressed in more detail in Chapter 2 (Materials and Methods). A number of other tests for detection of exonic rearrangements have been developed with varying degrees of success.

The following are listed in the *Leiden Muscular Dystrophy Pages as* the most effective, following the MLPA and the multiplex PCR:

- **MAPH** (Multiple Amplifiable Probe Hybridisation): as informative as the MLPA but more labour intensive and requires more DNA (White et al., 2002).
- **qPCR**: theoretically simple but technically challenging and difficult to optimise in multiplex (Ashton et al., 2008).
- **Southern Blotting**: along with multiplex PCR and qPCR, can be used to determine the deletion/duplication breakpoints (www.dmd.nl).
- recently described high-resolution comparative genomic hybridization **(CGH) microarray**: very expensive but capable of precise detection of intronic breakpoints (Bovolenta et al., 2008; del Gaudio et al., 2008; Hegde et al., 2008). It does not present a major diagnostic advantage at this point but may become a valuable tool with the advent of "personalised gene therapy".
- FISH, CA-repeat marker analysis and exon-specific qPCR: useful for confirmation of known mutations in carriers but not as an initial screen.

1.13.2 Detection of point mutations.

Currently, few laboratories in the world and certainly none in Africa offer detection of point mutations as part of a diagnostic genetic work up for DMD. However, research

into development of personalised genetic and other forms of therapy are beginning to show promise and much effort is directed at formulation of testing protocols and development of methodologies for point mutation detection. *The Leiden Muscular Dystrophy Pages* (www.dmd.nl) and the NCBI *GeneTests* (www.ncbi.nlm.nih.gov) list the following methods as most successful, although others have been described:

- **High-resolution melting curve analysis (hrMCA)**
  HrMCA is simple and very sensitive (>98%). Although it requires specialised equipment with high resolution melting (HRM) capability, it is particularly cost effective as a pre-sequencing screen to resolve variant fragments. HrMCA is the technological platform used to detect point mutation in this study and will be addressed in detail in Chapter 2 (Materials and Methods).
- **Denaturing Gradient Gel-Electrophoresis (DGGE)**
  DGGE approaches 100% detection sensitivity (Hofstra et al., 2004). However, it requires several PCR and electrophoresis conditions, which make it laborious and it difficult to automate.
- **Direct sequencing**
  Direct sequencing (or SCAIP: single condition amplification/internal primer)(), straightforward and powerful method but it is very costly, as over 79 separate exon fragments must be amplified, sequenced and analysed (Flanigan et al., 2003).
- **Single-Strand Conformation Analysis (SSCA)**
  SSCA / DOVAM (SSCA/detection of virtually all mutations)() is simple, cheap and effective but laborious, demanding electrophoresis of over 79 PCR fragments each using several electrophoretic conditions (Mendell et al., 2001; Buzin et al., 2005).
- **Denaturing High Performance Liquid Chromotography (DHPLC)**
  Characteristics for DHPLC are similar to those for SSCA, although it is easier to automate. It does however require specific, specialised equipment (Bennett et al., 2001).
- **Protein Truncation Test (PTT)** is an RNA-based screening method. It is very effective in detecting truncating mutations but requires availability of RNA extracted from a muscle biopsy, which is not always available. PTT lymphocyte RNA is possible, but more difficult to perform (Tuffery-Giraud et al., 2004). The cDNA fragments obtained after reverse transcriptase PCR (RT-PCR) can also be used for sequencing to determine the exact mutation (Hamed & Hoffman, 2006).
- **MyoD-induced in vitro muscle differentiation** can be a source of RNA as well a substrate for protein expression studies in pre and post-natal

diagnoses of cases where DNA analyses are uninformative. This is achieved by transfection and subsequent forced expression of the MyoD gene in a non-muscle cell types to initiate the process of myogenesis. This approach of analysis of muscle protein in non-muscle cells, has been applied to prenatal and postnatal diagnosis of DMD using fibroblasts, amniocytes and chorion villi cells. (Sancho et al., 1993; Davis et al., 1987; Roest et al., 1996; Roest et al., 1999).

For the most part, mutation detection techniques focus on the protein coding regions of the *DMD* gene. Studies analysing other regions (promoters, 5'UTR and 3'UTR) have so far not revealed many changes (e.g. (Tubiello et al., 1995; Flanigan et al., 2003). While DNA-based screening is offered most widely, RNA-based screening is considered most effective, as cDNA analysis can resolve all mutations resulting in protein truncation and those affecting RNA-splicing. The difficulty lies primarily in sample acquisition, as mRNA is best obtained from muscle tissue. Lymphocyte mRNA in this context is not ideal, as certain mutations (e.g. splice mutations) in lymphocyte transcripts may behave differently to those in muscle (Ferlini et al., 1998). This is of particular relevance with the advent of personalised genetic therapy as establishing the downstream effects of the mutation is imperative for therapy design e.g. antisense oligonucleotide (AON) design for exon skipping.

1.13.3 Haplotyping

This is a DNA-based approach to identify the risk chromosome in cases where no mutation could be found with other methods. The original restriction-fragment-length polymorphism (RFLP) assay (Bakker et al., 1985), has been refined to construction of a risk haplotype using informative small tandem repeats (STRs) in and around the *DMD* gene.

1.14 DMD CARRIERS: GENETIC AETIOLOGY AND TESTING

DMD female carriers, although unaffected, have CK levels 2 -10 times the upper level of normal in over 50% of the cases. It is estimated that approximately one fifth of female carriers show some signs of the disease (Hoogerwaard et al., 1999). These symptoms range widely from mild cramps on muscle exertion, calf pseudohypertrophy, varying degrees of muscle weakness (sometimes slowly progressive), to severe weakness and wheelchair dependence in rare cases. Impaired cognitive and/or cardiac function can be a part, or an exclusive manifestation of the disorder. Apart from a few cases associated with chromosomal rearrangements, most girls have been assumed to be affected as a result of skewed X inactivation, though this is not supported by the recent report of Soltanzedeh et al.

(2010), where no definite link could be established between the phenotype in female carriers and the pattern of X-linked inactivation. Muscle weakness, if present, is asymmetric and proximal, involving the pelvic girdle rather than the shoulder girdle in most cases, and can lead to misdiagnosis as the limb-girdle variety of dystrophy. Age of onset is variable, ranging from the first to fourth decade, with early onset (before the age of 15) linked to a more severe involvement. As is the case with male patients, carriers of BMD are less frequently and less severely affected than carriers of DMD (van Essen et al., 1997).

A *DMD* gene mutation in a female can be inherited from a carrier or a somatic/germline mosaic mother, or from a germline/somatic mosaic father. Alternatively, her mutation could have arisen *de novo* in the ovum or sperm from which she arose or in her own germline or somatic cells. Approximately 1/3 of DMD cases result from new mutations and the *a priori* carrier risk for mothers of sporadic cases is estimated at 66%. Absence of other affected family members and low maternal blood CK levels reduce this risk. An affected child of a non-carrier mother can be a result of a new mutation in the ovum from which the child arose or of somatic mosaicism in the affected child. Sporadic cases arising from somatic and/or germline mosaic mother are also seen as new mutations, as are mutations which originated in a germline of a mosaic grandfather, which were passed onto the mother. The recurrence risk of a germline mosaic mother of an affected child lies at an estimated 5 – 10% (Bakker et al., 1989; van Essen et al., 1992). Identification of exonic rearrangements in females is now possible with quantitative analyses such as the MLPH and MLPA. In cases where no mutation is detected in the proband, haplotype analysis is used to track inheritance of the risk chromosome.

Immunohistochemical staining of muscle tissue can be helpful in ascertaining D/BMD carrier status where neither mutation detection nor haplotyping is informative. In absence of a genetic diagnosis, it is essential in differentiating between manifesting DMD female carriers from autosomal forms of muscular dystrophy. A young, dystrophic female with normal muscle dystrophin immunolabelling is unlikely to be a manifesting carrier of DMD. This, however, may not be the case in older females, due to genetic normalization (Webster & Blau, 1990; Hoogerwaard et al., 2005).

1.15 GENE-BASED THERAPY

Much of the research into gene therapy for DMD has been facilitated by studies on two naturally occurring models of DMD: the dystrophic golden retriever dog (GRMD)

mdx mouse (Banks & Chamberlain, 2008). It must be emphasised that that not all of the therapeutic approaches discussed under the broad heading of "gene therapy" can truly be classified as such, as some use conventional pharmacological agents and others target the transcript rather than the underlying gene (Lim & Rando, 2008). In this context, the term is used loosely to describe treatments developed specifically to target genetic disorders.

The approaches to development of genetic therapies can be roughly divided into two main fields: gene replacement and gene modification (Lim & Rando, 2008). **Gene replacement** strategies (viral vectors, utrophin upregulation, myoblast and stem-cell transplantation), aim at delivery of a normal copy of the gene directly to the affected tissue, either as naked DNA in a viral vector (Wolff et al., 2005; Blankinship et al., 2006), or as a gene copy in a myoblast or stem cell (Farini et al., 2009). Proof-of-principle experiments on animal models (the *mdx* mouse), have been conducted and demonstrate that dystrophin expression can be restored to varying degrees to single muscles, muscle groups (limbs) or the entire body. The major advantage of this strategy is that it can be applied to patients regardless of the underlying mutations. Although the research is showing promise, much work still needs to be done to overcome the mechanical and practical difficulties of gene delivery to the muscles, the immunological concerns and the high cost. The other approach to genetic therapy, namely, **gene modification**, also referred to as mutation- or gene-based therapy is currently receiving much attention. Exon skipping and read-through of stop codons, discussed below in more detail, have reached the point of phase II clinical trials. In terms of their application and delivery, these strategies are seen as less difficult and risky as well as significantly cheaper than the gene replacement approaches. Effectively, as identification of the particular mutation is the basis for this approach, **"personalised gene therapy"** is becoming a reality and is of particular relevance to this study.

1.15.1 Exon skipping

The theoretical basis of exon skipping is the naturally occurring phenomenon of restoration of the ORF by mutation-triggered alternative splicing mechanisms, such as those seen in BMD patients with frameshift mutations. Also, characterisation of the *DMD* gene and dystrophin with its functional domains, and experimentation with truncated mini-genes on *mdx* mice has shown that a degree of dystrophin functionality is preserved with parts of the protein missing (Phelps et al., 1995; Harper et al., 2002). Therefore, the aim of exon skipping therapy is restoration of the ORF and amelioration of the phenotype from DMD to BMD, by induced splicing out (or skipping) of one or more exons. Modification of dystrophin mRNA splicing is

achieved with the use of antisense oligonucleotides (**AONs**, also known as "molecular patches"). These small DNA or RNA analogues attach to mRNA sequences crucial to the splicing process and induce alternative splicing out of targeted exons (exon skipping) (Manzur & Muntoni, 2009) (Figure 1.6). The sequences targeted by the AONs include 5' and 3' consensus splice sites and intra-exonic splice regulatory sequences (Lim & Rando, 2008). The feasibility of this approach was tested in the initial in vitro studies on human muscle and the *in vivo* gene model in *mdx* mice (Mann et al., 2001; Lu et al., 2003). Subsequently, much work has been done using in vivo animal models, in search of the optimal oligonuclear conditions and strategies to improve efficiency of delivery, with minimal toxicity and immunogenicity.

Modifications to the chemistry of AONs are still required to increase their half-life and to prevent the cleavage of RNA-RNA hybrids by RNAse H. The most commonly used chemistries for exon-skipping antisense oligonucleotides are 2'*O*-methyl RNA with a negatively charged phosphorothioate backbone (2'*O*MePS) (Lu et al., 2003) and uncharged phosphorodiamidate morpholino oligomers (PMO) (Fletcher et al., 2007), because both are stable and non-toxic.

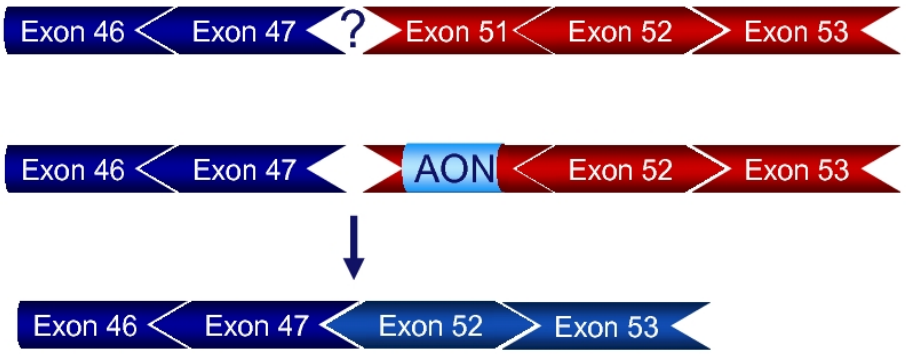


Figure 1.6 Exon skipping to restore the ORF.

Exon 51 is the focus of the first clinical trials, as AONs able to effect efficient skipping of exon 51 have been identified. Also, skipping of this single exon would restore the ORF in patients with deletions of exons 50, 52, 52–63, 45–50, 47–50, 48 – 50 and 49–50, which comprise 13% of the DMD population. Two independent, proof-of-concept and safety trials on DMD patients, both targeting exon 51, have been successfully completed. Intramuscular injections of the 2-*O*-methyl AON PRO051 were administered by the group in Netherlands and the AVI-4658 morpholino approach was adopted by the Muntoni group in the Imperial Collage of London (Kinali et al., 2009; van Deutekom et al., 2007). Both studies yielded good

results without drug-related adverse events. These encouraging results led to the initiation of further trials by both groups. The phase I/II open-label study using subcutaneous administration of PRO051 aimed to evaluate the safety profile of PRO051 and its ability to induce specific exon 51 skipping and novel dystrophin expression, has been recently completed. The preliminary data showed good tolerability and induction of dystrophin expression in a dose related manner. The published results of this study are however still pending. The results of a similar trial in the United Kingdom involving intravenous infusions of AVI-4658 at escalating doses is due to be completed in the first half of 2010 (http://www.clinicaltrials.gov). Systemic delivery of the AONs over an extended period of time will however truly test the promise of this therapy (Aartsma-Rus & van Ommen, 2009; Guglieri & Bushby, 2010).

Successful application of exon 51 skipping, will lead to design, development and testing of AONS for skipping other single exons. However development of AONs specific to each individual mutation, in a gene this size, is a tremendous endeavor, with its risks of variable effect and an immune response to possible newly created epitopes. Since it has been shown that dystrophin can maintain some of its essential function with large parts of the gene (and protein) missing, the feasibility of designing a cocktail of AONs, capable of inducing double- or multi-exon skipping in approximately 60% of all DMD cases, is under investigation. Multi-exon skipping experiments have been conducted with encouraging results on animal models: studies with *mdx4cv* mice, which require skipping of exons 52 and 53 (Mitrpant et al., 2009), and the canine muscular dystrophy dog model (CXMD), where skipping of exons 6 and 8 corrects the splice mutation in exon 7 (Yokota et al., 2009). Also, an AON cocktail targeting all exons between 45 and 55 has already been tested on human cells *in vitro*, however, effective skipping of this number of exons is yet to be achieved (van Vliet et al., 2008).

Theoretically, large duplications in the DMD gene should be an ideal target for the exon skipping therapy, as skipping of the duplicated exons should result in production of the wild type protein. Experiments on cultured muscle cells from duplication-carrying DMD patients, transfected with AONs targeting the duplicated exons, showed some degree of success. Further work is however required, as specific targeting of the duplicated, as opposed to the constitutive exons (or both) has proved problematic in cases (Aartsma-Rus et al., 2007).

An interesting approach towards a more permanent repair has been tested in cell cultures and the *mdx* mouse, where AONs in tandem with a modified U7 small nuclear RNA are expressed from an adeno-associated virus (Goyenvalle et al., 2009). Again, more work is required, as the problems accompanying the gene replacement therapies are also relevant here (Trollet et al., 2009).

1.15.2 Exon editing

Small oligonucleotides are also the basis of another form of therapy, known as oligonucleotide-mediated gene editing or gene repair, which aims at correcting small, in-frame mutations such as nonsense, missense and splice-site mutations (Igoucheva et al., 1998). It is based on the hypothesis that in the presence of an oligonucleotide exactly matching the genomic sequence, except for the single base mismatch, the cellular repair systems can be induced to modify the mutated genomic sequence in a proportion of cells, possibly through mismatch repair enzymes (Gamper et al., 2000; Andersen et al., 2002). This should result in a normal transcript and a full-length protein. The originally used small DNA sequences flanked by 2'-methyl RNA sequences (chimeroplasts) have been superseded by short all-DNA ologonucleotides, which anneal to one strand of the genomic sequence and have been shown to yield more reproducible results and are cheaper and simpler to synthesise. *In vitro* and *in vivo* experiments on the *mdx* mouse model (nonsense mutation in exon 23) and in the golden retriever dog (splice mutation at exon 7) have shown that this **oligodeoxynucleotide (ODN)**-mediated gene repair technology is capable of successful modification of single base changes in genomic DNA and thus correction of dystrophin expression at the sarcolemma, with wild-type dystrophin detectable at the site of the injection for up to 48 weeks. The clear advantage of this therapy is its permanence without the need for ongoing drug administration, although this is yet to be fully established by determining the therapeutic effect of a single dose administration. Also, while the findings are encouraging from the proof-of-concept viewpoint, the efficiency of gene expression achieved in these animal models was low, at only 20% at the injection site. Systemic delivery generally yields even lower levels and therefore much research is focused on optimal ODN design and on improving the efficacy and efficiency of delivery (Bertoni, 2005; Robinson, 2009).

Recently Maguire et al. (2009), took the ODN-mediated gene repair a step further with an experiment on *mdx*^{5cv} mice**,** in which the repair takes place *ex vivo*, in cell cultures enriched with myoblasts. *Mdx*^{5cv} mice have a single point mutation (A to T), which creates a donor splice site within exon 10 and gives rise to a 53-base deletion

in the transcript, which in turn causes a frameshift and a stop codon in exon 11. In normal muscle, the presence of damaged fibres activates the surrounding resting precursor cells into active myoblasts, which divide rapidly and effect regeneration of the injured tissue. Cycles of damage and regeneration are more frequent in DMD-affected tissue, creating an abundance of myoblasts. The authors speculate that isolated myoblasts subjected to ODN-mediated gene repair, could be transplanted back into the muscle, creating long-term restoration of dystrophin expression. To facilitate oligonucleotide binding and higher levels of gene repair, the investigators attempted to further strengthen the reaction by the use of RNAi to knock down the Msh2 protein, a known inhibitor of gene repair. While the results of the experiment with primary myoblast-enriched cell cultures showed significant gene repair and increased dystrophin production, purified myoblasts proved less susceptible to both repair and Msh2 knockdown. The proposed explanation is the difficulty in genetic manipulation of progenitor cells in general, due to natural barriers including lower transfection efficiencies and dosage-dependent toxicity (Maguire et al., 2009). This however, does not negate the possible potential of this approach and reinforces the need for further experiments.

The main challenges of exon skipping and editing therapies lie in overcoming the efficiency of delivery, as according to the best estimates, 30 – 40% of normal levels of dystrophin are needed to effect an improvement in clinical signs and symptoms (Neri et al., 2007). Coupling AONs with carrier molecules such as nanopolymers of polyethylene glycol and polyethyleneimine or polyactide-co-glycolic acid nanospheres and cationic core shell nanoparticles, have shown some promise, although potential toxicities have yet to be established (Williams et al., 2008; Sirsi et al., 2009). The nature and position of the target sequence (e.g. exonic or intronic) also play a significant role. Another current limitation of AON therapy is the inability to target the heart in mdx mouse at doses suggested for clinical applications (Townsend et al., 2008). Recent studies have suggested incorporation of peptide or chemical moieties with the AONs to facilitate cell uptake and prevent disruption of cardiac sarcolemma, cardiac hypertrophy and dysfunction in mdx mice (Townsend et al., 2008; Wu et al., 2008; Yin et al., 2008).

1.15.3 Nonsense read-through

According to the Human Gene Mutation Database, nonsense mutations account for ~12% of all mutations causing human inherited disease (Mort et al., 2008). They arise as a result of single point alterations, which create in-frame UAA, UAG or UGA codons in mRNA coding regions, leading to premature termination of translation and

subsequent **nonsense-mediated decay (NMD).** NMD is a quality-control mechanism, whereby aberrant mRNA molecules containing premature termination codons (PTCs) are degraded to prevent accumulation of incomplete, potentially toxic protein fragments (Figure 1.7). Pre-mRNA splicing in the nucleus, leads to placement of several proteins, including UPF3, on the CBC-bound mRNA, 20–24 nucleotides upstream of exon–exon junctions, to form the exon junction complex (EJC). During translation of normal transcripts, the EJC is remodelled and CBC is replaced by 4E, which promotes mRNA export to the cytoplasm. If a transcript contains a PTC, the ribosome stalls and is converted into a surveillance complex, which is hypothesized to consist of the 40S ribosomal subunit bound to NMD effector molecules (UPF1-3 and SMG1-7). This surveillance complex acts as a link between the premature translation termination factors (eRF1 and eRF3) and the general cellular mRNA decay machinery. Recognition of a stop codon as premature rather than normal appears to depend on its location relative to the downstream sequence elements and associated proteins. Natural stops are typically located within terminal exons, whereas PTCs lie more than 50 - 55 nucleotides upstream of the last exon–exon boundary (Maquat, 2004). While EJCs located in an open reading frame upstream of exon-exon junctions may facilitate ribosomal recruitment prior to displacement by a "pioneer" round of translation, EJCs located downstream of a nonsense codon (and 20–24 nucleotides upstream of splice junctions) (Chang et al., 2007) are not displaced because the ribosome is released from the transcript before reaching it. These remaining EJCs function as tags for recruitment of UPF1, followed by the mRNA's transport out of the nucleus and into the cytosol where the RNA is degraded by, for example, the exosome complex. NMD, however, does not appear to be an invariable consequence of a PTC. Indeed, PTCs located either less than 50–55 nucleotides upstream of the last exon–exon junction or downstream of this junction generally fail to elicit NMD (Behm-Ansmant et al., 2007). Also, even when NMD is triggered, some 5 to 25% of nonsense mutation-containing mRNA routinely escapes NMD (Isken & Maquat, 2007). The pathological effects of these mutations are therefore thought to be due to degradation of the newly synthesized PTC-containing mRNA by NMD, and also in part to the failure of the small amount of the PTC-containing mRNA that escaped NMD, to yield a (semi-) functional protein (Khajavi et al., 2006).

Aminoglycosides are a group of antibiotics, which bind to the 30S or 50S subunits of the bacterial ribosome and can induce misreading of the mRNA codons, as part of their mechanism of action. This capability has been studied as a potential mechanism to read through disease-causing nonsense mutations in humans, as

theoretically, this should lead to generation of a full-length proteins with a single amino acid substitution, thus correcting the primary genetic defect. The initial experiments were conducted on cystic fibrosis, where read-through of known disease-associated nonsense mutations in the transmembrane conductance regulator (*CFTR*) gene, was achieved in cell lines exposed to high doses of aminoglycosides (Howard et al., 1996).

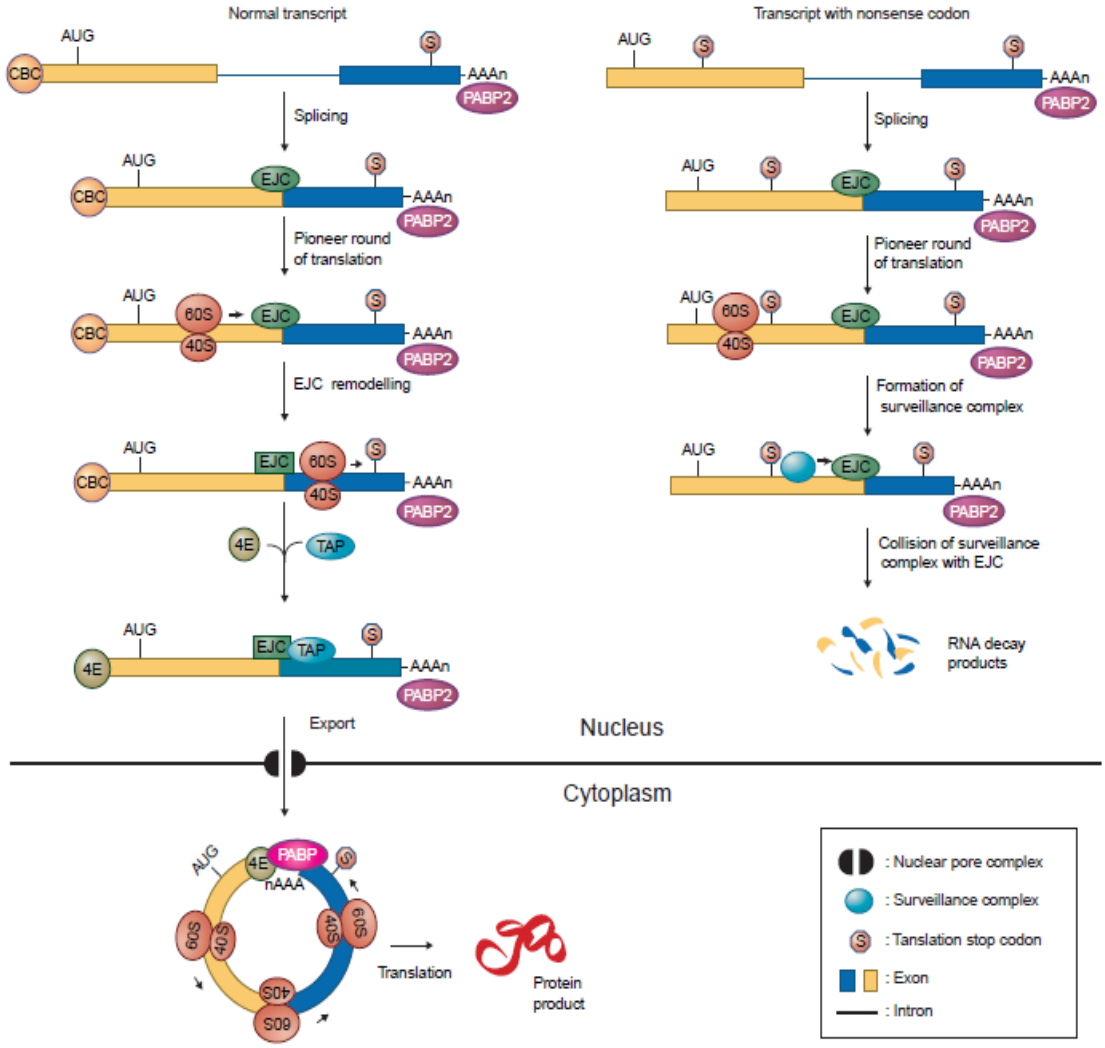


Figure 1.7 Model for nuclear nonsense mediated decay. CBC: cap-binding complex, EJC: exon junction complex, PABP2: nuclear poly(A)-binding protein, PABP: cytoplasmic poly(A)-binding protein, TAP: mRNA nuclear export mediator, 4E: translation initiation factor eIF-4E (Wilkinson & Shyu, 2002).

In 1999, the first *in vivo* evidence of phenotypic suppression of a premature stop codon was obtained by subcutaneous injection of high doses of gentamicin into transgenic *mdx* mice carrying a nonsense mutation in exon 23 of the *DMD* gene (Barton-Davis et al., 1999). Subsequent evaluation of gentamicin as a treatment for cystic fibrosis proved it be of limited clinical use, due to its known oto- and nephrotoxicity in high doses, as well as the need for regular intramuscular and

intravenous administration (Clancy et al., 2001; Politano et al., 2003). Under investigation, is a newly developed aminogylcoside (NB54), with a higher read-through efficacy and an improved toxicity profile in different cell lines, as compared to gentamicin. However, further studies in animal models are required before considering its potential applicability (Nudelman et al., 2009).

The results of the experiments with aminoglycosides did show that read-through of PTCs could be achieved biochemically, which prompted a search for a molecule with a more suitable profile of low toxicity and higher read-through efficiency (Politano et al., 2003). Two high-throughput screens of approximately 8000 000 molecules were conducted against a luciferase reporter that harbors a UGA PTC, identifying PTC124 as a candidate for further development (Welch et al., 2007).

Ataluren (PTC124) is a 284.24Da, achiral, 1,2,4-oxadiazole linked to fluorobenzene and benzoic acid rings. It has no structural similarity to aminoglycosides and is orally bioavailable when prepared in aqueous suspension. Findings of experiments with stable cell lines harbouring *LUC-190* nonsense alleles showed that, as seen in studies with aminoglycosides, the efficiency of read-through in response to Ataluren differed according to the sequence of the stop codon: the UGA codon showed the most efficient read-through, followed by UAG and UAA (Figure1.8). The nature of the nucleotide following the nonsense codon (the +1 position) also appeared to influence its termination efficiency. Similarly to gentamicin, Ataluren was most active with a pyrimidine (especially cytosine, C) in the +1 position. The UGAG termination context was the only efficiently suppressed exception to this trend. Ataluren was shown to be a more powerful nonsense suppressor than gentamicin, exhibiting 4 to 15-fold increase in levels of *in vitro* read-through. Additional experiments with *LUC-190* constructs established that Ataluren did not suppress multiple proximal nonsense codons even at exposure levels far exceeding those achieving maximal activity, and that continuous exposure to Ataluren maximized and maintained suppression activity. Suppression of nonsense alleles was also evident in Ataluren-treated and untreated human and mouse primary muscle cell cultures. Restoration of correctly localized dystrophin expression at the myofibre membrane was noted at all concentrations by immunohistochemistry with the antibody recognizing the carboxy-terminal dystrophin epitope (Welch et al., 2007; Peltz et al., 2009) (Figure1.9).

Protection against contraction-induced injury is seen as the best predictor of long-term therapeutic outcome in DMD. In the same study, Welch et al. (2007) used the

mean percentage drop in force after five eccentric contractions of extensor digitorum longus (EDL) muscles, as an indicator of contraction-induced injury in dystrophic mice. Administration of Ataluren orally, and in the form of intraperitoneal injections into the EDL muscles were assessed respectively at 2, 4 and 8 weeks. Partial protection against contraction-induced injury was seen at only 2 weeks, without significant change at 4 and 8 weeks. A combination of oral and intraperitoneal dosing, resulted in further improvement, where protection against contraction induced injury observed in the treated mice was comparable to that of the wild-type. The combined oral and intraperitoneal dosing also demonstrated significant reductions in serum creatine kinase levels after 2 weeks, which were maintained for up to 8 weeks.

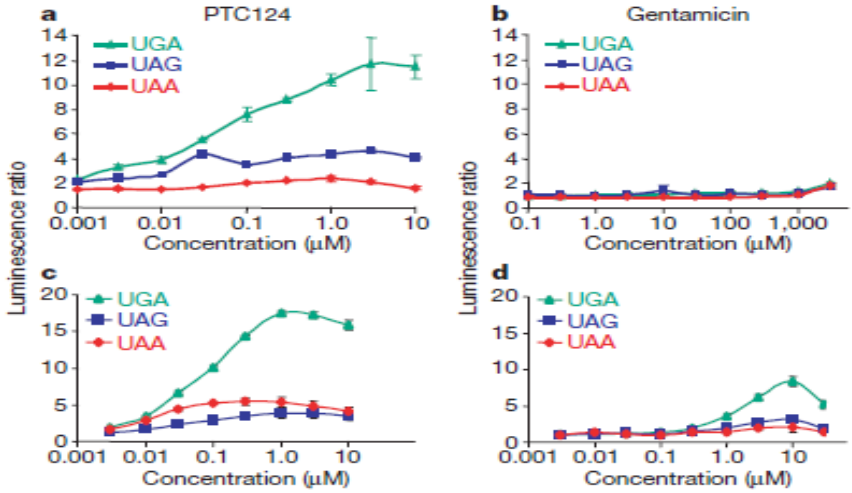


Figure 1.8 PTC124 suppresses premature nonsense codons.
a and b: cultured HEK293 cells harbouring various LUC-190 nonsense alleles, treated with increasing concentrations of PTC124 (a) or gentamicin (b) for 16h and assayed for luciferase activity. c and d: synthetic LUC mRNAs, with different PTCs, subjected to varying concentrations of PTC124 (c) or gentamicin (d), and assayed for luciferase activity after 4h (Welch et al., 2007).

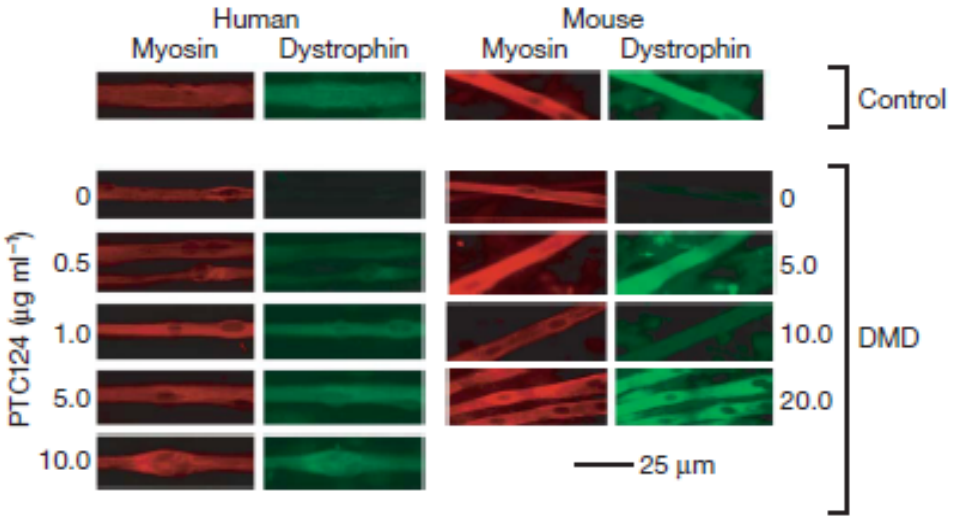


Figure 1.9 Immunohistochemistry of myotubules from primary cell cultures derived from muscle biopsies. The effect of adding varying amounts of PTC124 on levels of detectable dystrophin (green). Myosin (red) levels remain unchanged (Welch et al., 2007).

The authors therefore concluded that treatment with Ataluren leads to decreased muscle fragility in animal models. Recovery of dystrophin production and function was demonstrated by western blotting. Full length dystrophin was detected in muscles of both wild type and ataluren-treated *mdx* mice, with the *mdx* mice exhibiting dystrophin levels 25 – 30% of that in the wild type (Welch et al., 2007).

The exact molecular mechanism of the drug's action is not as yet clear (Welch et al. 2008). However, in an effort to elucidate the compound's mechanism of nonsense suppression, experiments designed to monitor its effects on the translation and stability of nonsense-containing mRNAs were carried out. The results indicate that Ataluren has a negligible if any effect on the synthesis and stability of cellular mRNAs and does not exhibit off-target transcriptional activity. It also does not manifest any antibacterial activity (Welch et al., 2007). It is believed that the drug's selectivity for read-through of premature as opposed to normal stop codons, is a result of the positional and mechanical differences between the two termination processes. Welch et al. (2006) tested this hypothesis by experiments monitoring the accumulation of truncated, full-length and read-through luciferase polypeptides in drug-treated HEK293 cells. They found a truncated product in untreated cells, a full-length product after treatment with Ataluren, and no product of the size expected, after read-through of a normal termination codon. Similar analyses in the same study also failed to detect polypeptides corresponding to putative read-through products in multiple tissues isolated from Ataluren-treated human subjects, rats and dogs. The investigators thus concluded that Ataluren acts on premature and not normal stop codons, even at the drug levels far exceeding the optimal therapeutic dose. Absence of toxic side effects was an important observation, as well as the apparent lack of effect on the NMD pathway, which, considering the importance of NMD as a genomic surveillance mechanism, is seen as a component of its safety (Hirawat et al., 2007).

Recently, a question arose as to Ataluren's actual capability to induce nonsense suppression. In cell-based assays similar to those used by Welch et al. (2006), it was shown that Ataluren is capable of stabilising the firefly (Fluc) luciferase protein, implying that its initial discovery as a nonsense suppressor may have been biased by this effect (Auld et al., 2009). This hypothesis, however, has been rejected (Peltz et al., 2009) due to the differences in the respective assay conditions and most importantly, the fact that the efficacy of Ataluren in the study by Welch et al. (2006), was validated in actual dystrophin deficient muscle *in vitro and in vivo* (Welch et al.,

2007). The therapeutic potential and efficacy of Ataluren is under investigation in clinical trails for DMD, cystic fibrosis and haemophilia A/B (www.ptcbio.com).

The promise and potential availability of Ataluren as therapy for a sub-population of DMD patients, served as the initial trigger for undertaking this project, as eligibility for treatment can only be determined by identification of the putative mutations in each patient. The phase I and II clinical trials of Ataluren for DMD showed good safety and tolerability (Hirawat et al., 2007). However, the preliminary results of the phase IIb clinical trial revealed no statistically significant change in the 6-minute walk distance within the 48-week duration of the study, though the drug was well tolerated and no trial patients discontinued treatment due to an adverse event. Additional efficacy analyses in the subgroup of patients receiving lower doses are currently underway. Ataluren development will also continue for other indications including cystic fibrosis and haemophilia (Allamand et al., 2008; Kerem et al., 2008) (www.ptcbio.com).

1.16 ANIMAL MODELS

1.16.1 The *mdx* mouse

Much of what is known about the molecular biology of dystrophin and its associated proteins along with their interactions and the role of inflammation in regeneration and fibrosis, has come from studies on animal models. A number of mammalian models have been used to this end, some naturally occurring and some engineered, chosen according to the type and purpose of the research at hand. The naturally occurring dystrophin-deficient mouse (C57BL/10ScSn-DMD^{mdx}/J), commonly referred to as the mdx-mouse, with a nonsense mutation in exon 23 has been the most widely utalised model for the study of molecular pathogenesis and the effects of gene manipulation and cell transplantation in DMD. Mdx mice have only a slightly shorter life span than the wild type mice. There is has less muscle fiber degeneration, more regeneration, and less fibrosis than seen in DMD patients. Despite that, it has served extremely well as a model in which to study mechanisms of disease and potential gene, cell, and experimental drug treatments. A number of allelic variants of the *mdx* mouse have been discovered and the specific mutations identified, although the original *mdx* strain remains the most widely chosen DMD rodent model. A number of variant strains have also been created to study the effects of different mutations and phenotypes and effects of experimental drug treatments:

- *mdx* variants (termed *mdx*^{2Cv}, *mdx*^{3Cv}, *mdx*^{4Cv}, and *mdx*^{5Cv}), recovered from a cross of male mice (C3H.X25 _ C57BL/6Ros) treated with mutagen N-ethyl-nitrosourea, and female C57BL/10Sn.*mdx* animals.
- *mdx*52, with a disruption (deletion) of exon 52 to mimic a patients with exonic rearrangements.
- *mdx* double mutants, generated by crossing *mdx* mice with mice deficient in proteins such as utrophin, MyoD, α-dystrobrevin, α7 integrin and parvalbumin. These were created to test the roles of these proteins/factors in the muscle function and disease pathology, and to be used as animal models in experiments for potential therapies (Willmann et al., 2009).

1.16.2 The Golden Retriever Muscular Dystrophy dog (GRMD) has also become a recognized animal model of DMD and is widely used for the experimental evaluation of new treatments prior to human clinical trials. The GRMD carries a point mutation in the 3' consensus splice site of intron 6, leading to skipping of exon 7, consequently disrupting the open reading frame in exon 8 and creating a premature stop codon. Unlike the *mdx* mouse, GRMD dogs suffer from a rapidly progressing, fatal disease similar to DMD in humans. There is, however, a large variation in disease severity as some pups survive only for a few days, while others are ambulant for months or even years. As seen in DMD patients, GRMD dogs display selective muscle involvement although the most affected muscles in dogs (i.e. tongue, masticatory and trunk muscles) are different to those of humans. In addition, myocardial involvement in the GRMD dog very closely matches the cardiac complications observed in DMD patients. Recently, the Golden Retriever mutation was transferred on to the Beagle, which is a more suitable canine model because of its smaller size and extensive prior use in research. Canine dystrophinopathies have been reported in many other purebred and mixed breed dogs (Shelton & Engvall, 2005; Willmann et al., 2009).

1.16.3 The dystrophic cat carries a naturally occurring deletion of the muscle dystrophin promoter and lacks approximately 200 kb of the dystrophin gene, resulting in development of extensive muscle hypertrophy. The cats eventually die due to compression of the esophagus by the hypertrophied diaphragm or because of impaired water intake caused by glossal hypertrophy, which then leads to renal failure. Histological analysis has reveals localized foci of necrosis and regeneration but no signs of fibrosis. Thus, the pathology of the dystrophic cat does not resemble DMD as closely as the GRMD dog because it lacks the hallmarks of generalized muscle wasting (Shelton & Engvall, 2005).

Recently (2008), “TREAT-NMD”, a European Network of Excellence, compared the currently used mammalian animal models for DMD with the aim of selecting and recommending the most appropriate ones for preclinical efficacy testing of new therapeutic strategies. The *mdx* and GRMD models emerged as the best suited animal models for preclinical therapy testing. The particular advantages of using the *mdx* mouse over other mouse models include the good understanding of its muscle, cardiac and respiratory pathology gathered over time from studies and publications, the reproducibility of the phenotype, commercial availability and the relatively low cost. Importantly, the *mdx* model has been used to demonstrate efficacy of several potential treatment strategies including small molecule pharmaceuticals (PTC124) as well as gene correction and cell replacement approaches.

There are a number of significant factors against the use of the GRMD model, such as the high degree of variability in disease severity between breeds and among littermates and a subsequent high number of animals required to substantiate any conclusions about the treatment efficacy. This model is expensive due to the high cost of breeding and the need for a veterinary facility to treat large-sized animals. However, dog models closely resemble the human disease and are becoming appropriately characterized having already been used in tests on treatment efficacy.

Therefore, the recommendation by ‘‘TREAT-NMD” is to use the *mdx* mouse as the model of choice for pre-clinical tests and proof-of-concept studies. The GRMD model is recommended in well controlled experimental settings, when using complementary tests, such as those used in supportive therapy for respiratory and cardiac functions, or as an alternative to *mdx* for specific experimental therapeutic approaches such as the use of stem cells or the testing of viral vectors (Willmann et al., 2009).

1.17 THE LOCAL PERSPECTIVE

The genetic service for DMD was the first of its kind offered nationally by the government health services at the Groote Schuur Hospital (GSH) and the Division of Human Genetics, University of Cape Town (UCT). The service has continued since its inception in 1987 and through the transition to the National Health Laboratory Service (NHLS).

The early testing protocol involved tracking inheritance of the putative chromosome in the affected families using linkage markers and the multiplex PCR (mPCR) deletion screen was introduced later. However, before implementation of the

Multiple Ligation-dependant Probe Amplification (MLPA®) in 2007, studies with linkage markers were the strategy of choice for determining female carrier status and as such, comprised a large part of the referral base.

In total, 1749 individuals from 571 DMD families have been referred for testing to date. Of those, 1264 individuals from 89 families were either referred for linkage analysis only, or were excluded from testing for reasons such as an autosomal recessive inheritance pattern or insufficient or inadequate sample quality. The MLPA® has superseded the mPCR deletion screen as a test for exonic rearrangements in DMD, as it offers a broader mutation detection range and can be used for carrier testing in females, to a large extent removing the need for linkage analysis. Its implementation has significantly improved mutation detection rate, as can be seen from the data in Table 1.1, even though comparatively few individuals have been tested with the MLPA® (40 patients) as opposed to the mPCR (445 patients) thus far. The MLPA® assay itself is described in detail in Chapter 2 and in Appendix B.

PRE-2007 (mPCR): 445 cases		POST-2007 (MLPA®): 40cases		TOTAL TESTED: 485 cases	
FAMILIAL	SOLATED	FAMILIAL	ISOLATED	FAMILIAL	ISOLATED
41/101(41%)	135/344(39%)	4/6 (67%)	16/34(47%)	45/107(41%)	151/378(40%)
Deletion pick up rate: 176/445(40%)		Del/dup pick up rate: 20/40(50%)		Del/dup pick up rate: 196/485(40%)	

Table 1.1 Detection rates of exonic rearrangements with mPCR and MLPA in the diagnostic service at GSH.

The Duchenne service is now offered by two academic centres in South Africa, namely, the NHLS at GSH in Cape Town and in Johannesburg, with their academic links to UCT and the University of Wittwatersrand respectively. Both serve the state and private hospitals and clinics, as well as clinicians in private practice. Many of the patients tested at GSH are referred from the developmental and especially the neuromuscular clinics at the Red Cross War Memorial Children's Hospital (RXH) in Cape Town. The hospital, as South Africa's leading centre for post-graduate specialist paediatric medical and surgical training, offers specialised care facilities and high levels of expertise. "The Guidelines for Medical approach to Care of Children with DMD – Guidelines from the Neuromuscular Clinic", authored by A/Professor Jo Wilmshurst, head of Paediatric Neurology at RXH (and a clinical co-investigator in this study), and approved by PANDA (Paediatric Neurology and Development Association of Southern Africa) offer a clear guide as to the

appropriate and achievable standard of care within the South African context, based on the recommendations set by international bodies (i.e. US Centres for Disease Control and Prevention, DMD Care Considerations Working Group).

It is therefore fitting that a long-standing genetic service for DMD, with links to academic centres with specialist clinics, should offer testing which is comprehensive and as informative as possible to both to the patient and his family. Complementing the MLPA for exonic deletions/duplications, with a screen for small/point mutations in the *DMD* gene, would create such a service. This will be of value to the patients and their families not only from the genetic counselling point of view but also by preparing the groundwork for application of mutation-based therapies in the future.

This said, while the systems and expertise in most tertiary centres (and in the private sector) in South Africa are in keeping with the international standards, easy access to such facilities at a primary care level and in rural areas is still limited to many. Those patients able to take full advantage of the available care can live relatively comfortably and productively well into their thirties. However the social problems of the African context get in the way of every patient receiving timeous intervention. Community health issues such as HIV and especially TB, add a further challenge to the care of a South African DMD patient. The Muscular Dystrophy Foundation SA, who funded this project, is a subsidiary of the International Muscular Dystrophy Association and is actively involved in helping to support and educate all South African families affected with DMD.

1.18 AIMS AND OBJECTIVES

1.18.1 Aims

To test for the presence of **small/point mutations** in the dystrophin gene of DMD patients previously reported as deletion-negative, as determined by mPCR, for the purpose of mutation profiling, family counselling and future determination of eligibility for gene-based therapies.

1.18.2 Objectives

- To re-test the entire cohort of 24 DMD-affected boys for large deletions/duplications using the **MLPA**, as all had been reported deletion-negative, based on the results of the mPCR deletion hotspot screen.
- To **amplify the entire coding region** of the *DMD* gene of each deletion/duplication-negative patient using 96 primer sets designed for subsequent hrMCA.

- To screen for variant fragments with **hrMCA** in a high-throughput, closed-tube format using RotorGene™6000 (Corbett Life Science, Australia).
- To **sequence** all hrMCA variants in search of sequences changes.
- To perform **bioinformatic analyses** of the sequence variants to determine:
  - disease association, by determining possible downstream effects of the mutation on the transcript and the protein
  - mutation-induced alteration of restriction enzyme sites, which may be useful for future testing of family members.
- To perform **family studies** (where possible) of patients in whom pathogenic mutations are detected, for the purpose of authenticating "family mutations" and for genetic counseling of the families.
- To establish a prospective laboratory **testing protocol** for patients such as those in the project cohort, for possible incorporation into the testing repertoire of the diagnostic component of the Division of Human Genetics (NHLS, Groote Schuur Hospital).
- To perform population studies, where required, to aid in determining possible disease-association of *novel* mutations.

CHAPTER 2: MATERIALS AND METHODS

2.1 STUDY COHORT

The full study cohort consisted of 24 unrelated boys, diagnosed with D/BMD on the basis of clinical presentation, elevated CK levels and in some cases, muscle biopsy results. The data presented in Table 2.1 was gathered by communication with clinicians and by perusal of the request forms. All 24 boys had tested negative for exonic deletions with the mPCR hotspot screen, as part of a diagnostic investigation for DMD. Of those, 19 had been referred for testing by the muscle clinic at the RXH, and 5 had been referred via other avenues.

Table 2.1 The complete study cohort

#	DNA code	diagnosis	age/ dob	age first walked	age first symptoms	prominent clinical findings	familial/ isolated	CK	muscle biopsy
1	DMD83.1MTH	?D/BMD	24	?	7	calf hypertrophy	isolated	?	not done
2	DMD306.1GV	DMD	?	?	?	Learning difficulties from early on	isolated	?	not done
3	DMD342.2RIC	DMD	18yrs	18 months	2 years	in-toeing, slight calf hypertrophy	isolated	35950	dystrophin negatiave
4	DMD347.1RIC	?D/BMD	?	?	?	Neuropathy of uncertain type, wheelchair bound	isolated	?	?
5	DMD352.2BRE	DMD	19yrs	12 months	~2years	waddling,falling, calf hypertrophy	familial	5570	dystrophin negative
6	DMD357.1MAS	DMD	?	?	?	?	familial	?	not done
7	DMD372.1DIN	DMD	24	?	?	?	isolated	?	not done
8	DMD378.1TEM	DMD	24yrs	<5yrs	5 yrs	Learning difficulties, calf hypertrophy, waddling gait	isolated	3465	not done
9	DMD386.1ZIP	DMD	16	?	?	Calf hypertrophy	familial	?	?
10	SMS389.1PIE	DMD	?	?	presented for testing at 10yrs	?	familial	2910	?
11	DMD402.1PET	DMD	19yrs	no details	7 yrs	calf hypertrophy, waddling gait	familial	14130	dystrophin negative
12	DMD411.1PET	DMD	Died	?	?	?	familial	?	Dystrophin negative
13	DMD414.1PHI	DMD	21yrs	14 months	10yrs	waddling,toe-walking	isolated	31650	dystrophin negative
14	DMD420.1SIY	DMD	17yrs	1 yr	3 yrs	severe course, posterior cardiac infarction, good intellect	isolated	11712	not done
15	DMD 423.1NIC	DMD	18yrs	14 mo	7yrs	calf hypertrophy, waddling gait	isolated	14000	?
16	DMD428.1AND	Not confirmed	20yrs	?	?	Late presentation, last seen in 2002,	?	1567	not done
17	DMD433.1LUN	DMD	?	?	?	?	familial	?	not done
18	DMD447.1THA	?D/BMD	25	?	?	?	familial	?	?
19	DMD457.1MOG	DMD	16yrs	18months	18months	Poor respiratory function, overweight, stopped walking at 11yrs	Isolated	15624	dystrophin negative
20	DMD501.1NAZ	DMD	12yrs	<2 yr	5 yrs	calf hypertrophy, waddling gait	isolated	6778	dystrophin negative
21	DMD530.1THA	DMD	15yrs	10 mo	7 yrs	calf hypertrophy, waddling, toe-walking	familial	>10000	dystrophin negative
22	DMD548.1THE	DMD	10yrs	11months	5 yrs	ADHD, borderline cardiac function	Isolated	12900	Dystrophin negative
23	DMD550.1SHA	DMD	16yrd	1yr	4yrs	Typical pattern	Isolated	>1000	dystrophin negative
24	DMD552.1RIV	DMD	12yrs	12 mo	3yrs	calf hypertrophy, waddling gait	isolated	23716	absent dystrophin

Blood for DNA extraction was also collected from healthy male volunteers to be used anonymously as wild type test controls. The project was approved by the University of Cape Town Ethics Committee (REF 416/2008).

2.2 SAMPLE COLLECTION AND DNA EXTRACTION

Fresh EDTA-anticoagulated blood samples were collected where possible, and DNA was extracted from buffy coats using the Gentra® PureGene® Blood kit (Qiagen, Germany) or the Maxwell® 16 System (Promega, USA, UK) for automatic nucleic acid extraction, as per the manufacturers' instructions. In cases where fresh blood could not be obtained, previously extracted DNA was retrieved from the DNA Registry at the Division of Human Genetics (Institute of Infectious Diseases and Molecular Medicine, Medical School, UCT, REF 230/2010). The patients' consent for inclusion in the study had been obtained either as part of the NHLS, or the Division of Human Genetics' protocol for informed consent (Appendix A), in keeping with the tenets of the Declaration of Helsinki (2008). Mutation-positive samples for use as controls in the MLPA runs and also for validation of the HRM approach were obtained from the divisional DNA Registry.

2.3 SCREENING FOR EXONIC REARRANGEMENTS

In view of the limitations of the mPCR method, the entire study cohort of 24 DMD patients was re-tested for exonic rearrangements with the SALSA Multiple Ligation-dependant Probe Amplification (MLPA®) test from MRC-Holland (www.mrc-holland.com). The advantages and limitations of the procedure are addressed in Appendix B1 and B2.

2.3.1 MLPA® Principle

MLPA is a sophisticated variety of mPCR, where simultaneous amplification of up to 40 target fragments is achieved with a single primer pair. Each DNA fragment of interest is targeted by an MLPA® probe consisting of two oligonucleotides (**hybridization sequences**), which recognise adjacent target sites on the DNA (Figure 2.1). One of the oligonucleotides contains the sequence recognised by the **forward primer**, and the sequence on the other is recognised by the **reverse primer**. The SALSA MLPA® probes range in size from 130 to 490 nucleotides (nt), and are prepared by restriction enzyme digestion from phage M13 derived clones (www.mrc-holland.com). The length of the amplification product is adjusted with the aid of a "**stuffer sequence**", positioned between the hybridization sequence and the primer sequence in one of the oligonucleotides. This allows for a clear resolution of the subsequent PCR product. It is also possible to prepare cheaper synthetic MLPA® probes for research purposes, the design protocols for which are available online (www.mlpa.com). The lengths of fragments generated by synthetic probes however, have to be limited to 90 -160 nt due to their comparatively inferior quality.

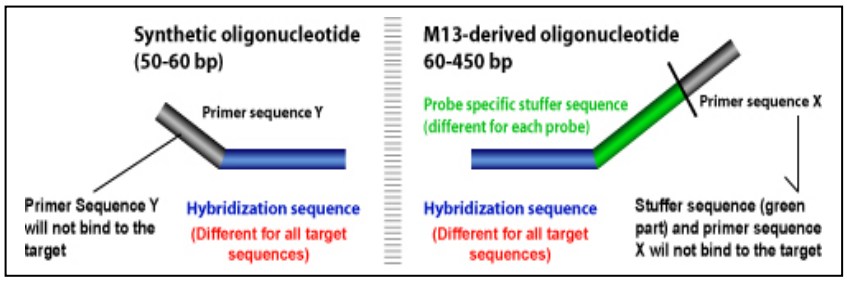


Figure 2.1 Components of a SALSA MLPA® probe (www.mrc-holland.com).

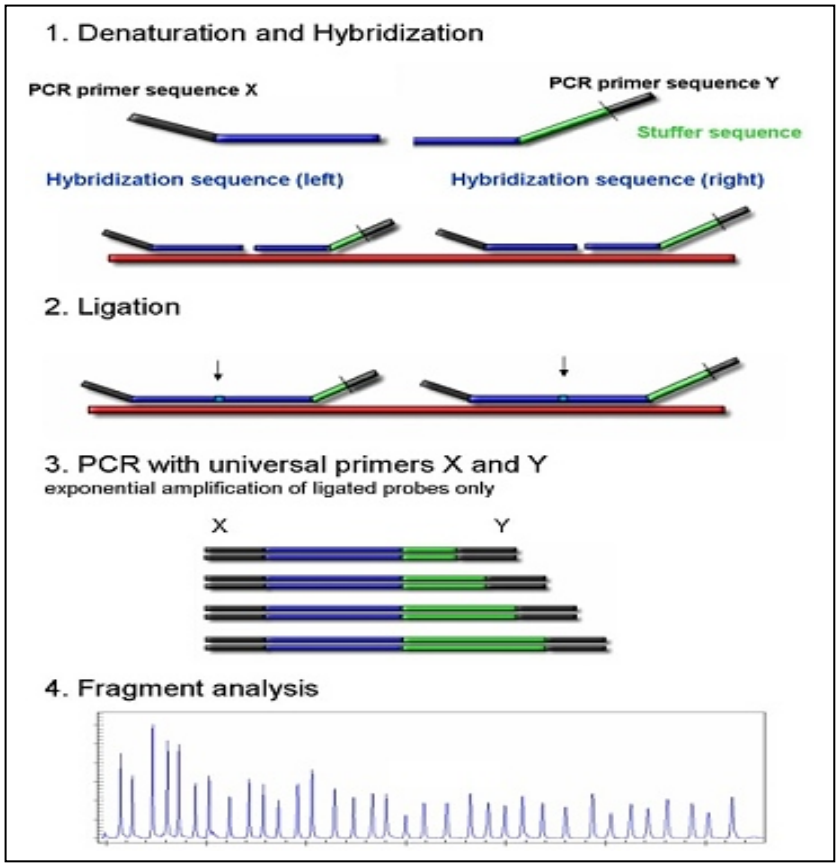


Figure 2.2 The 4 steps of an MLPA® procedure (www.mrc-holland.com).

The first step of a standard MLPA® reaction (Figure 2.2) is **denaturation** of the test DNA and **hybridization** of both probe oligonucleotides to the target sequence. **Ligation** into a complete probe will follow only after the target sequence is found. Next, PCR primers act to **amplify** the fragment. In this way, it is the ligated oligonucleotides, and not the DNA or the unbound probe oligonucleotides, which are amplified. In absence of the DNA target, there will be no ligation of a complete probe and no PCR product formation. The fluorescent labelled primers (FAM) allow for MLPA® product separation by **capillary electrophoresis**. A comparison is made of peak heights (Figure 2.2, step 4) generated from the test sample and a reference (wild type) sample, and the differences reflect copy number changes.

2.3.2 MLPA® quality control

The quality and reliability of MLPA® results is controlled both internally and externally. Internal quality control is achieved by the presence of quantity control fragments (Q-fragments), denaturation control fragments (D-fragments), as well as genomic and sex chromosome-specific reference probes in all MLPA probe mixes. An unusual appearance of the control fragments can be an indication of unreliable results. Each assay is also controlled externally, by inclusion of reference samples. The details of the quality control aspect of an MLPA® assay are available online (www.mrc-holland.com → MLPA® procedure → Interpretation of MLPA® Results). The main aspects are also outlined in Appendix B3.

2.3.3 MLPA® Probes

Two probe mixes were required to screen all 79 exons in the *DMD* gene. The cohort was tested with the MLPA® probe mix version P034-A2 and P035-A2, containing 55 and 54 probes respectively (including control fragments), the details of which are set out in Appendix B4 (also downloadable from www.mrc-holland.com → Products → Complete Product List → DMD). Two MLPA® reactions (one per probe mix) were therefore set up for each patient.

2.3.4 Sample preparation

The concentrations of all test and control DNA samples were measured with the NanoDrop® ND-1000 Spectrophotometer (NanoDrop® Technologies) and brought to a working concentration of 50 – 100 ng/μl in 1 X TE buffer (10mM Tris-HCl pH 8.2 + 0.1 mM EDTA), as recommended in the MLPA® protocol (downloadable from www.mrc-holland.com → MLPA® reaction → MLPA® DNA - Detection/ Quantification protocol). Each MLPA® run included reference DNA from 3 wild type males, and 2 mutation positive samples: a deletion of exons 45 to 53 and a duplication of exons 45 to 53 selected from the DNA Registry.

2.3.5 MLPA® reaction set-up

All MLPA® reactions were set up according to the same basic protocol. This, along with any changes made to the reagents and probe sets (which are regularly updated), as well as additional information and tips on the reaction set up and subsequent product resolution and result interpretation, is clearly set out in the documentation provided with every MLPA® kit (downloadable from www.mrc-holland.com → MLPA® reaction → MLPA® DNA - Detection/ Quantification protocol).

The test was performed according to the manufacturers' instructions, with the exception of using half of the prescribed volumes, throughout the assay. This change features as a possible alternative in the MLPA® protocols online (www.mrc-Holland.com) and has been validated in-house by the Molecular Human Genetics diagnostic laboratory at NHLS GSH. This cost-saving adjustment has also been reported by other laboratories (Lalic et al., 2005).

The brief outline of the MLPA® method is as follows:

- DNA **denaturation**: 98°C for 5 minutes.
- **Hybridization**: DNA + SALSA probemix + MLPA® buffer at 95°C for 1min, followed by 16 hrs at 60°C.
- **Ligation**: addition of ligase mix at 54°C and incubation at 54°C for 15 min, ligase inactivation at 98°C for 5 min.
- **PCR**: ligation reaction mix + primers + dNTPs + polymerase
- **Capillary electrophoresis** and product analysis.

2.3.6 MLPA® product separation

MLPA® products were prepared for capillary electrophoresis by mixing 0.7µl of the product with 9 µl of HiDi formamide and 0.3 µl of the ROX500 size standard (Applied Biosystems, USA), followed by denaturation at 80°C for 2 min and rapid cooling on ice for 5 min. Electrophoresis was carried out on the ABI 3100 Genetic Analyzer (Applied Biosystems, USA) using a 36cm capillary and POP-4 polymer (Applied Biosystems, USA). The specific instrument settings and trouble shooting guidelines are available online at www.mrc-holland.com → MLPA® Procedure → Fragment Separation.

2.3.7 Data analysis and interpretation

The generated data files were analysed using the GeneMapper v3.0 software (Applied Biosystems, USA). The resulting electropherograms were initially scrutinized visually for missing peaks representing exonic deletions in males, since the *DMD* gene is X-linked. Duplications in males and copy number variations in female heterozygotes result in a 35-50% reduction/increase in the relative peak area, making visual recognition possible in most cases. It is however not a reliable way of data interpretation and a statistical analysis of data files for copy number variations was performed using the Coffalyser program recommended by MRC-Holland (www.mrc-holland.com). All data normalization steps are built-in functions of Coffalyser, as is correction for the probe length-dependent decrease in peak area/height (the ski-slope effect). Probe–ratios between 0.7-1.3 were read as

normal, in keeping with the definition by MRC-Holland. Normalization was performed on data which run within the same experiment and tested with the same probe mix. It is also recommended to normalise samples extracted with the same method, although it was not possible in this cohort, as some samples had been retrieved from the DNA Registry's long-term storage facility.

2.4 SCREENING FOR SMALL/POINT MUTATIONS

All 18 patients in the cohort who tested negative for exonic rearrangements with the MLPA®, were further investigated for small/point mutations in the *DMD* gene.

At present, searching for point mutations in this vast gene is considered beyond realistic capabilities of many diagnostic laboratories, though a number of mutation screening technologies have been described in an attempt to reduce the burden of sequencing. This includes single strand conformational polymorphism (SSCP) analysis (Mendell et al., 2001; Buzin et al., 2005), denaturing gradient gel-electrophoresis (DGGE) (Hofstra et al., 2004) and denaturing high performance liquid chromatography (DHPLC) (Bennett et al., 2001). However, in addition to the expense, labour intensity and specialized equipment, all of these methods involve post-PCR sample handling and some require additional processing by enzymatic or chemical reactions and subsequent toxic waste disposal. More recently, assays such as DOVAM (detection of virtually all mutations) and single condition amplification/internal primer (SCAIP) sequencing have successfully achieved sequencing of all the exons and the flanking intronic regions of the *DMD* gene (Buzin et al., 2005; Flanigan et al., 2003). Again, even with an aid of robotics and bioinformatic software, the expense and time required especially during data analysis, is considered too great for full integration into the local routine diagnostic process.

High resolution melting curve analysis (hrMCA) is a powerful new technology currently receiving much attention, capable of high throughput detection of mutations, polymorphisms and epigenetic differences in double stranded DNA. The mutation detection sensitivity of hrMCA is comparable or superior to the prescreening techniques mentioned above and it is inexpensive, rapid and accurate. Also, the technology has already been successfully applied to the *DMD* gene by Almomani et al. (2009). HrMCA was therefore chosen as a pre-sequencing screening tool to be used for detection of small/point mutations in the *DMD* gene, in this study.

2.4.1 HrMCA background and principle

High resolution melting curve analysis (hrMCA) is based on the natural process of DNA denaturation or “melting” upon exposure to a gradual increase in temperature; DNA unwinds and dissociates from a double stranded (dsDNA) to a single stranded (ssDNA) molecule. The reaction takes place in the presence of a DNA intercalating dye, which is included in the preceding PCR reaction set up, and fluoresces only while incorporated into dsDNA. The melting process can be monitored by measuring the gradually diminishing amount of fluorescence during strand dissociation and a melting curve is derived by plotting fluorescence versus temperature (Figure 2.3).

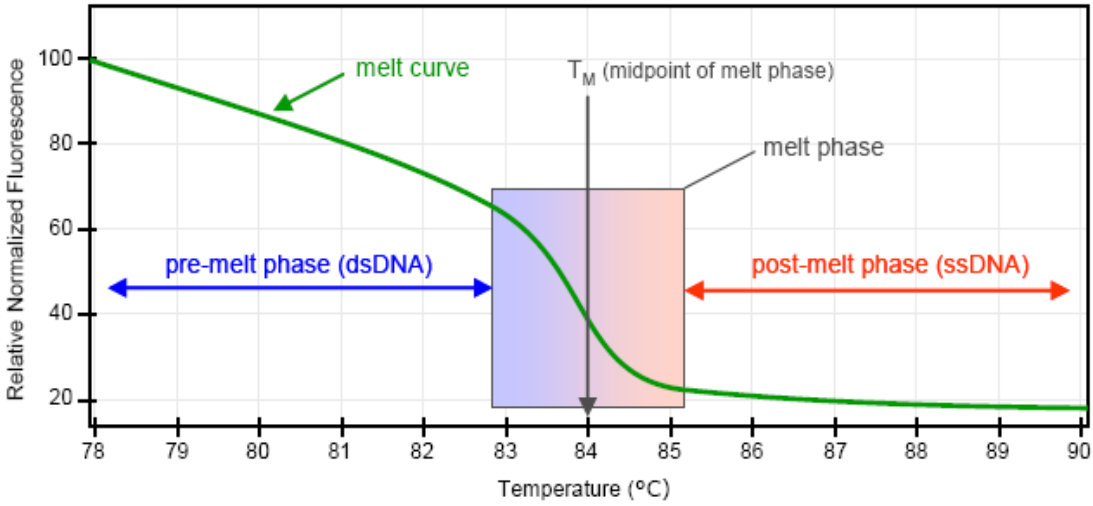


Figure 2.3 Fundamentals of a typical HRM (high resolution melt) plot.
Fluorescence decreases as DNA intercalating dye is released from double-stranded DNA during “melting” into single strands. The midpoint of the melt phase, at which the rate of change in fluorescence is greatest, defines the temperature of melting (TM) of the particular DNA fragment under analysis (CorProtocol™ 6000-1-July06).

Melting curve scrutiny has, until recently, been used mostly to determine the melting temperature (T_m) of amplified dsDNA, which is defined as the temperature at which 50% of dsDNA has dissociated to ssDNA, under standard conditions. It has also been recognized that the precise shape of the melting curve is a function of the DNA sequence. The observed thermal denaturation profile is characteristic of a specific PCR product and is dependent on its sequence length, base and GC content (HRM Assay Design and Analysis Booklet, www.corbettlifescience.com).

2.4.2 HRM curve shape, shift and data normalization

Sample differentiation based on hrMCA, is based on two main characteristics of the melt curve:

- The curve shape, where the actual shape of the curve is examined.
- The curve shift, i.e. the thermal offset of a curve from other curves.

Before the curves are plotted, the HRM raw data is normalized. The fluorescence axis (Y) is normalized onto a 0 to 100% scale, which allows all the curves to be compared with the same starting and ending fluorescent signal level to aid interpretation and analysis. Normalisation of the temperature (X) axis, known as "temperature shifting" is applied by the analysis software of some instruments e.g. Idaho Technology HR-1™ and the Roche LightCycler® 480 (White & Potts, 2006; Herrmann et al., 2007). This has the desired effect of compensating for well-to-well temperature measurement variations between samples. It is particularly useful when analysing heterozygous samples, because their curves have a complex shape and often become more easily discriminated after temperature shifting normalization. Differences between curves of homozygous samples on the other hand, become obscured by temperature normalisation, since the differentiating feature of the curve is its temperature shift and not a change of shape. Temperature normalisation can therefore detrimental for a number of applications such as discrimination of homozygous SNPs or mutation screening in sex-linked disorders (such as DMD). Although homozygous samples have identical HRM curve shapes, they can usually be discriminated by observing a change in their respective T_Ms, provided that temperature shifting normalization is not applied and the HRM temperature data is precise enough (Krypuy et al., 2006; White & Potts, 2006; Herrmann et al., 2007). The RotorGene™6000 (Corbett Research, Australia), used for hrMCA in this study does not use temperature shifting normalization, as the rotor format (as opposed to the heating block on the others), allows for sufficiently precise collection of temperature data due to minimal well-to-well thermal variation (www.corbettlifescience.com). Important considerations for the hrMCA experiment design and the factors affecting the outcome are set out in Appendix C1 and C2.

2.4.3 Validation of the hrMCA approach

Before commencement of the study, the hrMCA approach was validated for this application on the RotorGene™6000, by testing 4 DNA samples from DMD-affected patients with known point mutations in exons 3, 16, 34 and 56. The DNA was sourced from the DNA Registry at the Division of Human Genetics, UCT, and the mutations had been detected during previous research conducted, using SSCP, DHPLC and cycle sequencing.

While successful application of the HRM technology for detection of small changes in the *DMD* gene had already been described by Almomani et al. (2009), a validation run was performed to make sure that a similar approach using the available instrument (RotorGene™6000) as a platform, was a viable undertaking.

The relevant test and wild type DNA samples were amplified with the Sensimix HRM kit (Quantace) and WAVE Leiden DMD primers (Bennett et al., 2001), which were available at the Division of Human Genetics, UCT at the time, and subjected to hrMCA on the Rotor-Gene™6000. PCR and hrMCA was performed on all 4 of the relevant exons in each of the 4 patients, in order to test the specificity as well as sensitivity of the method. The instrument settings were as described below.

2.4.4 PCR primers

Following the validation study, 96 sets of hrMCA-optimised primers with M13 tails were used to amplify all 79 exons of the *DMD* gene, with their respective flanking intronic regions in each of the 18 samples. The primer sequences were obtained through an online enquiry to the Leiden Muscular Dystrophy Pages (www.dmd.nl) and subsequent personal communication with Prof. Egbert Bakker of Leiden University in the Netherlands (Almomani et al., 2009). The primers were manufactured by Integrated DNA Technologies (IDT), Whitehead Scientific (Pty,) Ltd. All primer sequences with their annealing temperatures and the resulting PCR fragment sizes are tabulated in Appendix C3.

2.4.5 PCR and hrMCA experiment set-up

PCR amplification and hrMCA of 96 fragments was performed on the DNA of each of the 18 deletion/duplication negative patients (18 x 96 = 1728). The PCR and hrMCA were performed consecutively in a closed-tube system and in real-time, on the RotorGene™6000. The reactions were set up in 0.1 ml PCR tubes and run on the 72-well rotor. A single run tested 8 exons on 6 patients, 2 wild type male controls and a DNA-free control (blank). Therefore, full hrMCA on 6 patients required 12 test runs. Sample uniformity is essential to the success of this analysis. The test and wild type DNA were brought to the required concentration (100ng/µl) with nuclease-free distilled water (Promega, USA).

A reaction per 0.1 ml PCR tube consisted of 100 ng test DNA, 10pmols of each (forward and reverse) primer, 12.5 µl of the Sensimix HRM (Quantace), I µl of EvaGreen (Biotum Inc., included in the Sensimix HRM™), made up to a total volume of 25.0 µl with nuclease-free distilled water (Promega, USA). The PCR cycling parameters were as follows: 10 minutes at 95°C, 45 cycles of 15 seconds at 95°C, 30 seconds at the annealing temperature for the primer set (Appendix C3) and 40 seconds at 72°C.

The HRM fluorescence data for the amplicon scanning was collected from 65°C to 87°C at a temperature transition rate of 0.1°C with a 2 second hold at each step. Amplification data was set to acquire to the green channel at the end of each extension step, with auto-gain optimization before the first acquisition. After exponential background subtraction, fluorescence data was normalized between 0% and 100%. The HRM data on the RotorGene™6000 automatically acquires to the HRM channel, with the selected option for automatic gain (sensitivity) optimization, at the fluorescence value of less than 70 units.

2.4.6 HRM data analysis

The melting curves generated were analyzed by a combination of the RotorGene™6000 series analysis software set at the default confidence threshold of 90%, and visual scrutiny of the melting profiles. Since the PCR and HRM on the RotorGene™6000 take place in real time and in a closed-tube system, there is no need to check for the presence of PCR product with checking gels or any type of post PCR manipulation. Unsuccessful PCR reactions with no or late amplification were simply terminated or excluded from HRM analysis and repeated. A number of factors can influence the DNA melting profile and must be considered during the analysis. This is addressed in detail in Appendix C2.

2.5 CYCLE SEQUENCING

Sequencing of the variant fragments detected by hrMCA was performed on the GeneAmp®9700 thermocycler (Applied Biosystems, USA) in the sense and antisense direction, using the BigDye®Terminator v3.1 mix (Applied Biosystems, USA) and universal M13 sequencing primers (all primer sequences are listed in Appendix C3). HR-MCA is a non-destructive method, and the EvaGreen™ dye (Biotum Inc.) does not interfere with post HRM fragment manipulation. All amplicons with variant melting profiles could thus be sequenced directly from the original PCR reactions. The pre-sequencing PCR product clean-up was performed using the NucleoSpin® Extract II columns (Macherey-Nagel, Germany), according to the manufacturer's instructions. The presence of all the putative variants detected was confirmed by repeat PCR and cycle sequencing.

2.5.1 Sequencing reaction set-up

A sequencing reaction per 0.2ml PCR tube included 50 – 100 ng of PCR product, 3.2 pmole forward or reverse M13 primer, 1µl of BigDye®, Terminator v3.1, and 2µl of the X5 sequencing buffer (Applied Biosystems, USA), in a total volume of 10µl with nuclease-free distilled water (Promega, USA). The cycling parameters were as

follows: 5 minutes at 95°C, 40 cycles of 15 seconds at 95°C, 15 seconds at 50°C and 4 minutes at 60°C.

2.5.2 Sequence resolution and analysis

The sequencing products were run on the ABI3100 Genetic Analyzer (Applied Biosystems, USA) and the output files analysed with the BioEdit Sequence Alignment Editor and the NCBI Basic Local Alignment Search Tool (BLAST) both freely available online (www.ncbi.nlm.nih.gov). The sequences were compared to the Leiden coding reference sequence (www.dmd.nl), based on the GenBank reference file NM_004006.1 of the Dp427m dystrophin isoform (with one difference: 12505G>A). The *DMD* gene genomic reference sequence used, was generated in collaboration with the NCBI's RefSeqGene project, record NG_012232.1, as the basis for the description of sequence variants in the *DMD* gene. All sequence variants identified in this study were described according to the Human Genome Variation Society's (HGVS) recommendations (den Dunnen & Antonarakis, 2000) .

2.6 BIOINFORMATIC ANALYSES

Over and above the BioEdit and the NCBI BLAST programs, the mutations detected were subjected to select bioinformatic analyses to gain insight into their downstream effects and possible disease association. It must be emphasised that the results of such analyses must be seen as indicators of probable effects rather than definitive proof thereof. The final effect of a given mutation may be influenced by a combination of factors, not necessarily taken into account during a computational search for a specific effect.

2.6.1 Effect on the translational open reading frame (ORF)

The exonic changes detected with the MLPA in 6 patients of the cohort were assessed for their effect on the ORF. An indication of whether or not an exonic rearrangement maintains or disrupts the ORF is intended to help in anticipating the disease severity, which may influence the approach to treatment, especially in isolated cases.

The predictions were made with the aid of the Reading-frame Checker program which is freely accessible online via the Leiden Muscular Dystrophy Pages (www.dmd.nl) (Aartsma-Rus et al., 2006). The UMD-DMD France, which is the Locus Specific Data Base (LSDB) for DMD (www.umd.be), was also used for reading-frame predictions and takes into account the impact of the mutation on the junctional codon, i.e. even if the translational reading frame is maintained and the

two exons are in the same phase, a premature stop codon could be generated at the junction (Tuffery-Giraud et al., 2009). The predictions are based on the exon phasing of the *DMD* gene and direct translation of mRNA, generated by the deletion /duplication of exons, as selected by the user. The program creators caution that the reading frame predictions of changes detected by DNA-based testing cannot be used as evidence for the effect the change will have at the RNA level, as for example, additional exons may be missing in the transcript, due to the disruption or deletion of the splicing signals. Alternatively, intronic sequences may be recognised as exons due to mutation-activated cryptic splicing signals (Dwi Pramono et al., 2000; Caceres & Kornblihtt, 2002; Disset et al., 2006). The Leiden program creators also caution against predicting the effect of duplications detected by genomic DNA testing, as non-contiguous and complex duplications which are not easily detectable at that level, are being reported with increasing frequency (Kesari et al., 2008; Gualandi et al., 2009). More accurate predictions can therefore be achieved with RNA-based results.

2.6.2 ESE-finder and Human Splicing Finder (HSF)

Bioinformatic analyses to search for a possible effect on the splicing elements were carried out with the ESE-finder (Cartegni et al., 2003) and Human Splice Finder v2.4 (HSF) (Desmet et al., 2009) programs, both available online. The programs were used to confirm the deleterious effect of the mutation detected in the consensus splice site of intron 18 in patient DMD372.1DIN (c2293-1G>A), as well as the compound mutation found in exon 55 of patient DMD357.1MAS [c.8028_8031dupGGTG; c.8044G>T]. HSF analysis was also performed on the missense mutation in exon 7 of patient DMD389.1 (c.587T>C) and the silent mutation in exon 26 of patient DMD447.1THA(c.3513A>G). Similarly, the nonsense mutations in exons 7 and 47 of patients DMD552.1RIC and DMD411.1PET respectively, were analysed with HSF for an effect on splice elements and the possibility of exon skipping, which is relevant to eligibility for read-through therapy. The nonsense mutation in exon 34 of patient DMD352.2BRE (c.4729C>T) had been previously analysed for this effect by Nishiyama et al. (2008).

Bioinformatic analyses for a possible effect on splicing can help shed light on the nature and effect of a mutation. It is known that exon-intron junctions are defined by weakly conserved intronic *cis*-elements: the 5' splice site, 3' splice site and branch site, which are important but not necessarily sufficient for accurate splicing. Additional intronic and exonic *cis*-elements required for correct splice-site identification act by either stimulating (enhancers) or repressing (silencers) splicing,

and may be especially relevant for regulating alternative splicing. Exonic splicing enhancers (ESEs), in particular, appear to be prevalent, and may be present in most exons (Cartegni et al., 2002). Nonsense, missense and even translationally silent mutations can disrupt exonic splicing enhancers (ESEs) and cause altered splicing resulting in changes with significant effects on the gene product e.g. exon skipping. Since predictions of the downstream effects of a mutation in most cases, are made on DNA-based sequence information, the prevalence of mutations which cause aberrant splicing may be underestimated. Different classes of ESE consensus motifs have been described, but they are not always easily identified (Cartegni et al., 2002).

ESE-finder (http://exon.cshl.edu/ESE/) is a web-based resource that facilitates rapid analysis of exon sequences to identify putative ESEs responsive to the human SR proteins (serine/arginine-rich proteins) SF2/ASF, SC35, SRp40 and SRp55, and to predict whether exonic mutations disrupt such elements (Cartegni et al., 2003). The HSF software, freely available via the UMD-DMD France website (http://www.umd.be/HSF), includes new algorithms derived from the UMD to allow the evaluation of the strength of the 5' and 3' splice sites and branch points. It includes already published algorithms for identification of *cis*-acting elements, such as those incorporated into RESCUE-ESE and ESE-Finder as well as new algorithms designed to use available or newly created matrices (Desmet et al., 2009). To allow the study of virtually any human sequence, HSF includes all genes and alternative transcripts as well as intronic sequences that were extracted from the Ensembl human genome database (http://www.ensembl.org/).

2.6.3 SIFT (Sorting of Intolerant From Tolerant) analysis

The SIFT program (www.sift.jcvi.org) was used to further investigate the possible pathogenicity of the amino acid substitution caused by the missense mutation in exon 7 (c.587.T>C) of patient DMD389.1PIE. This was done using the SIFTBlink single protein tool option, using the NCBI protein Reference Sequence: NP_003997.1, with the amino acid of interest in position 196, at a 0.05 threshold for intolerance.

The SIFT analysis was performed as a missense mutation does not necessarily lead to an appreciable protein change. An amino acid may be replaced by another amino acid of very similar chemical properties, in which case, the protein may still function normally ("quiet" or "conservative" mutation). Alternatively, the amino acid substitution could occur in a region of the protein which does not significantly affect

the protein secondary structure or function. SIFT is a multi-step algorithm which classifies amino acid substitutions using a sequence homology-based approach. For a given protein sequence, SIFT compiles a dataset of functionally related protein sequences by searching a protein database using the PSI-BLAST algorithm (Altschul et al., 1997) and builds an alignment from the homologous sequences with the query sequence. Each position in the alignement is scanned with a calculation of probabilities for all possible 20 amino acids at that position. These probabilities are normalized by the probability of the most frequent amino acid and are recorded in a scaled probability matrix. SIFT predicts a substitution to affect protein function if the scaled probability, also termed the SIFT score, lies below a certain threshold value. Generally, a highly conserved position is intolerant to most substitutions, whereas a poorly conserved position can tolerate most substitutions (Kumar et al., 2009). The SIFT algorithm was developed at the Fred Hutchinson Cancer Center, and the SIFT server has been transferred to the J. Craig Venter Institute.

2.6.4 The Transcription Element Search System (TESS)

The single base substitution in the Dp427promoter/exon 1 region in patient DMD548.1THE, was analysed with the TESS program (www.cbil.upenn.edu), for possible disruption of transcription factor binding site/s (TFBs), which may negatively affect expression of the DMD gene.

TESS is a web tool for predicting transcription factor binding sites in DNA sequences. It can identify binding sites using site or consensus strings and positional weight matrices from the TRANSFAC, JASPAR, IMD, and our CBIL-GibbsMat database. TESS can be used to search own sequences or user-defined c*is*-regulatory modules (CRMs) genome-wide, near genes throughout genomes of interest. The process of gene regulation includes binding of transcription factors (trans-elements) to short sequences of DNA (cis-elements). The presence of a factor bound to a location in the promoter region of a gene can increase or reduce its expression. Each transcription factor recognizes a family of similar sequences, usually about 4-10 bases long with varying degrees of conservation at each position. The bonds formed with the DNA sequences are stronger with some than with others and identification of a good binding site in the gene promoter suggests the possibility that the corresponding factor may play a role in the regulation of that gene. However, the sequences recognised by transcription factors are typically short and allow for some amount of mismatch. Because of this, binding sites for a factor can typically be found at random every few hundred to a thousand base pairs. TESS has

features to help sort through and evaluate the significance of predicted sites (Schug, 2008).

2.7 POST-PCR MIXING AND HRM

To test if an improved detection rate can be achieved, PCR amplification of all 96 fragments was repeated on DNA from 4 of the 8 patients in the study with no pathogenic mutations detected by the initial hrMCA, as well as on DNA of a wild type male. The PCR products of each test was mixed with that of the wild type and subjected to hrMCA, after a denaturing and cooling step to facilitate heteroduplex formation. The presence of polymorphisms was a specific inclusion criterion for this experiment, in order to compare the degree of variance in mixed and unmixed amplicons.

Theoretically, post-PCR mixing should not be required on the RotorGene 6000, since hrMCA data is not automatically temperature normalized, as is the case with block-based instruments, which is known to obscure homo- or hemizygous changes. However, post-PCR mixing was shown to result in a significantly improved mutation detection rate in the study by Almomani et al. (2009). The melting profiles of variant fragments in heteroduplexes with the wild type were more easily detectable as such, than the same variants previously missed in the unmixed reactions. Although the Almomani et al. (2009) study had been conducted using a block-based instrument (Light Scanner®, Idaho Technology), a similar experiment was carried out to test if an improved detection rate can be achieved with the RotorGene™6000 in this study.

2.7.1 Post-PCR mixing reaction set up

Both the wild type and test samples were amplified in 25µl reactions, as described above. Equal volume aliquots (8.3µl) of the test and the wild type reactions for each exon, were mixed together in a new 0.1µl PCR tube. The mixed PCR product was denatured at 95°C and allowed to cool to 15°C, before hrMCA. In this way, both the original PCR reactions (test and wild type) and the tube of amplicon mix, contained equal volumes of PCR product (16.6µl). All three (the test, the wild type and the mix), were subjected to hrMCA simultaneously at the settings above, for the sake of comparison of their melting profiles in the same run.

2.8 FAMILY STUDIES

DNA for family studies was available from the relatives of 7 mutation-positive probands: DMD352.2BRE, DMD411.PET, DMD83.1MYH, DMD357.1MAS, DMD342.2RIC, DMD372.1DIN and DMD389.1PIE. Therefore, in order to gather

further evidence in support of the deleterious nature of these sequence alterations, DNA of the relatives was tested for the presence of the putative mutations detected in the probands. This was done by PCR, hrMCA and cycle sequencing, as per the protocols described above. To demonstrate an alternative, cheaper approach to the detection of “private” mutations, restriction enzyme (RE) analysis was used instead of sequencing in family DMD 411. In the context of this study, hrMCA was used as a pre-sequencing amplicon screening tool and as such, was not required for mutation detection in the family studies, for which direct sequencing was the better approach. Rather, hrMCA was included for the academic purpose of a complete analysis and comparison of the melting profiles generated by various individuals with their respective genotypes.

2.8.1 Restriction Endonuclease (RE) analysis

RE analysis can be used as an easy, quick and inexpensive way to confirm the presence or absence of a family mutation, which changes a RE recognition site. The Webcutter 2.0 (http://bio.lundberg.gu.se/cutter2) is a free on-line tool for finding restriction map nucleotide sequences and which can be used to determine if a mutation changes a RE recognition site. It operates via a seamless interfaces to NCBI's GenBank, a DNA sequence database, and New England Biolab’s REBase, a restriction enzyme database. This approach was demonstrated on the family of patient DMD 411.1PET, who was found to carry a nonsense mutation in exon 47 of the *DMD* gene (c.6905G>A). Webcutter 2 program (free online), the mutation was shown to destroy a restriction recognition site for TspRI enzyme in the amplicon containing exon 47. The site is situated at position 148 in the 399bp amplicon of wild type DNA, creating two fragments of 148bp and 251bp in length respectively.

PCR amplification of exon 47 of the *DMD* gene was performed on the DNA of patient DMD411.1PET, his mother, his affected brother and a male wild type, using the primers and PCR reaction protocol as described for hrMCA analysis earlier.

A RE reaction per 0.2ml PCR tube contained 100 – 200ng PCR product, 10 units of the TspRI enzyme (New England Biolabs), 10X NEBuffer 4 (New England Biolabs), supplemented with 100x BSA (bovine serum albumin) made up to a total volume of 15 µl with nuclease-free distilled water (Promega, USA). Each reaction mix was incubated in a water bath for 2 hours at 65°C to activate the enzyme. The RE digests were then resolved by gel electrophoresis, where 3µl of each sample was mixed with 3µl of a GelRed™ (Biotum Inc)/Bromothymol Blue loading buffer (1:1000) and loaded into wells of a 2.5% agarose gel, made with 1xTAE buffer (Tris-

acetate EDTA, pH8.0). The loaded gel was run in 1XTAE buffer at a 100V, for 30 minutes and visualized using the UVIPRO gel documentation system (UVITEC Ltd., UK). The restriction pattern of each sample was compared to that of a wild type and an uncut PCR product. A 100bp molecular ladder was also run alongside for size estimation. The results were interpreted accordingly.

2.9 POPULATION STUDIES

In addition to bioinformatic analyses, population studies were carried out on the mutations in patients DMD548.1THE (c.-85T>C) and DMD389.1PIE (c.587T>C) respectively. Both cases were isolated, with no family history of DMD, as per the available information. Neither mutation was found recorded in the Leiden *LOVD*, or found in published material (Aartsma-Rus et al., 2006).

Two anonymised population cohorts were selected from the DNA Registry of the Division of Human Genetic, UCT: a cohort of 17 MLPA-negative DMD-affected patients and a cohort of 75 DMD-unaffected males. Both the cohorts consisted of South African individuals of various ethnicities (Caucasian, African, Cape Malay, Indian and mixed ancestry). The DMD-affected cohort in particular was small in size due to the selection requirements, as only MLPA-tested patients with a definitive clinical diagnosis of DMD were included (also excluded were the patient in the study cohort), considerably reducing the number of eligible subjects. Also, in addition to bioinformatics and family studies the absence of a sequence alteration should be demonstrated in at least 200 wild type individuals, before a confident argument can be made towards its pathogenic nature (Frederic et al., 2009), which extended beyond the constraints of this study.

2.9.1 Experiment setup

Exon 1 and exon 7 were PCR amplified in the DNA of both cohorts as well as the two mutation positive patients, using the HRM primers and the previously described PCR protocol. The presence or absence of the mutations, were then determined by RE analysis as follows:

- Variation c.-85T>C in exon 1 created an additional restriction recognition site for TspRI enzyme at position 221 of the 405bp-long amplicon, resulting in restriction sites at positions 19, 94 and 221 of the amplicon. Four fragments were thus created: 19bp, 75bp, 125bp and 186bp in length. The restriction sites for the TspRI enzyme in the wild type (or variant-negative) amplicon were at positions 19 and 94 of the amplicon, creating 3 fragments: 19bp, 75bp

- Variation c.587T>C in exon 7 created a restriction recognition site for enzyme MspI (Fermentas) at position 184 of the 296bp-long amplicon, creating two fragments: 112pb and 184bp in length. No restriction recognition site for MspI enzyme was noted within the wild type (or variant-negative) amplicon. RE analysis was carried out on both the affected and the wild type cohorts as follows: a reaction per 0.2ml PCR tube contained 100 – 200ng PCR product, 10 units of the MspI enzyme (Fermentas), 10X Buffer Tango (Fermentas) , made up to a total volume of 20 µl with nuclease-free distilled water (Promega, USA). Each reaction mix was incubated in a water bath for 2 hours at 37°C to activate the enzyme. The RE reaction products were resolved by gel electrophoresis as described in Section 2.8.1 above.

C HAPTER 3: RESULTS

3.1 MLPA® analysis

Analysis with the MLPA® revealed 6 exonic changes previously undetected with the mPCR, of which 4 were deletions and 2 were duplications (Table 3.1). The disease-causing nature of large lesions in the *DMD* gene is widely recognized and no further investigations were therefore conducted to prove pathogenicity. It was also noted that the large deletion of exons 61 – 79 in patient DMD306.1GV may extend beyond the *DMD* gene, exceeding the detection range of the DMD MLPA®. Determining the deletion cut off was however not pursued further in this study. Examples of the GeneMapper and Coffalyser output for multiexonic changes found in patients DMD457.1MOG and DMD420.1SIY, are given in Figures 3.1, 3.2 and 3.3.

While further investigations are not required to demonstrate the disease causing nature of large rearrangements, it is necessary to confirm the MLPA results in some instances. Multiexonic deletions of consecutive exons detected with the MLPA are generally accepted as such, however it is necessary to authenticate the presence of single exon deletions with another test method, as possible MLPA® assay artifacts or point mutations in the target sequence can interfere with probe attachment. The deletion of exon 7 in patient DMD414.1PHI, was thus confirmed by single PCRs of exons 6, 7 and 8, using the HRM primers and the PCR protocol for small/point mutation detection described in Chapter 2.

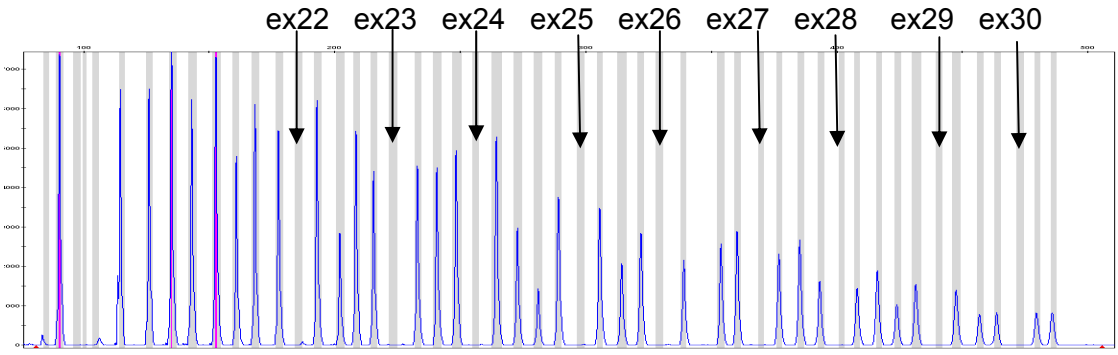


Figure 3.1 MLPA® electropherogram for DMD457.1MOG, probe set PO34–A2: deleted exons 22, 23, 24, 25, 26, 27, 28, 29 and 30.

Both duplications (DMD420.1SIY and DMD530.1THA) were confirmed by repeat MLPA®. In addition, the single duplication of exon 1 in patient DMD530.1THA was further authenticated by testing DNA extracted from a fresh blood sample along with the old DNA, as well as that of an affected sibling, in the same MLPA® run. All three samples manifested the duplication upon analysis with the Coffalyser program.

Table 3.1 Exonic rearrangements detected with the MLPA®.

PATIENT	MLPA®	DNA SEQUENCE CHANGE (HGVS notation)	DYSTROPHIN DOMAIN/S	AFFECTED ISOFORMS	READING FRAME	IMPACT AT THE JUNCTION
DMD378.1TEM	deletion (ex 5 – 7)	c.265-?_649+?del	actin-binding domain	Dp427c Dp427m Dp427p	disrupted	–
DMD414.1PHI	deletion (ex 7)	c.531-?_649+?del	actin-binding domain	Dp427c Dp427m Dp427p	disrupted	–
DMD457.1MOG	deletion (ex22 - 33)	c.2804-?_4674+?del	central rod domain repeats 6 - 11	Dp427c Dp427m Dp427p Dp260	disrupted	–
DMD306.1GV	deletion (ex 61 -79)	c.9085-?_(*2691_?)del	central rod domain repeat 24 and hinge 4, cystein-rich domain, carboxy-terminus	ALL	◊ uncertain	no modification
DMD420.1SIY	duplication (ex 3 -36)	c.94-?_5154+?dup	actin-binding domain and central rod domain ending mid-repeat 13	Dp427c, Dp427m, Dp427p, Dp260	◊◊ maintained	no modification
DMD530.1THA	duplication (ex 1)	c.-244-?_31+?dup	Dp427m-unique N-terminus	Dp427m	◊◊ disrupted	–

◊ The deletion may extend beyond the *DMD* gene and its effect on the ORF or applicability any potential therapy cannot be predicted with the data available here.

◊◊ The reading-frame predictions based on DNA findings may not reflect the actual changes at the mRNA level. Predictions for duplications are particularly risky, and non-tandem or complex duplications are frequently reported and the orientation of the duplicated fragment cannot be determined by DNA analysis (Kesari et al., 2008; Flanigan et al., 2009; Gualandi et al., 2009).

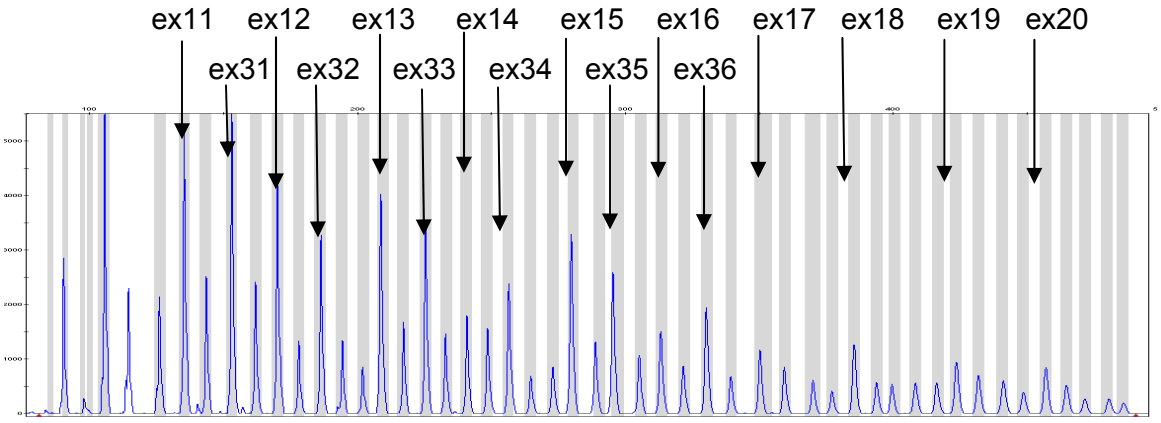


Figure 3.2 MLPA® electropherogram for DMD420.1SIY, probe set PO34- A2: duplicated exons 3, 4, 5, 6, 7, 8, 9, 10

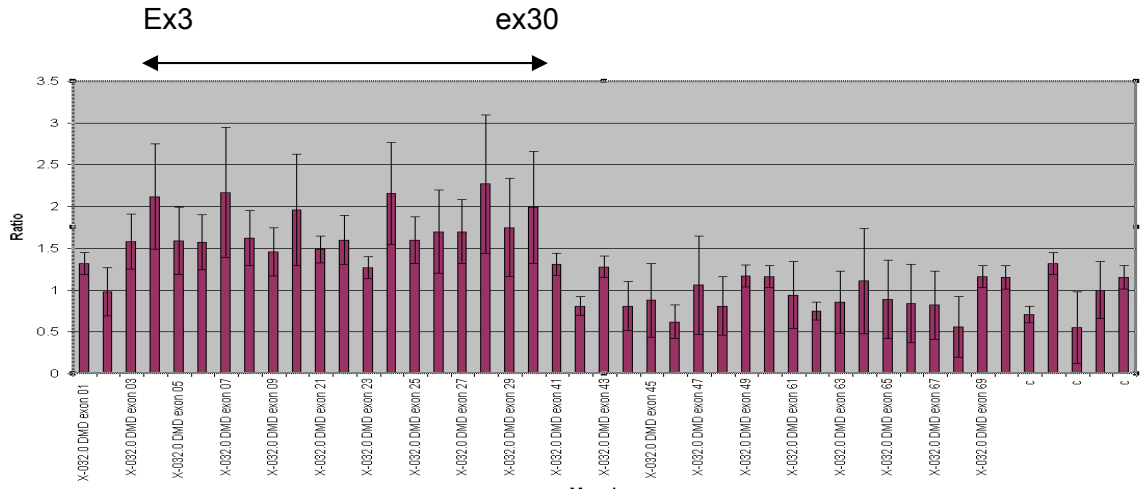


Figure3.3 Coffalyser chart for DMD420.1SIY, probe set P034-A2.

3.2 HrMCA

3.2.1 HrMCA validation run

All 4 of the known mutation-containing fragments manifested shifted melting profiles, as compared to the wild type samples. Also, variations were seen only in the mutated exons and not in the other exons tested per patient, successfully validating the use of the RotorGene™6000 for this application.

3.2.2 HrMCA screen.

HrMCA analysis of 1728 PCR amplicons from 18 deletion/duplication negative boys in the cohort, revealed 240 (14%) variants, as selected by the RotorGene™6000 analysis software and by visual scrutiny of the melting profiles. In total, 53 of these fragments were subsequently confirmed as variant by cycle sequencing. The high number of

fragments falsely selected as variant had been attributed to the variable quality of the available DNA, as fresh blood samples for DNA extraction were not available in every case. Other aspects which influence the outcome of hrMCA are addressed in detail in Appendix C. Also, the criteria applied for variant selection erred on the side of caution, to avoid missing disease-associated mutations.

3.3 CYCLE SEQUENCING AND BIOINFORMATIC ANALYSES

Of the 53 hrMCA variants detected, 10 were identified as disease-causing (Table 3.2), 39 were previously reported polymorphisms (Aartsma-Rus et al., 2006; Tuffery-Giraud et al., 2009) (Table 3.3) and 3 were changes of uncertain clinical significance (Table 3.4).

3.3.1 Disease associated mutations

Among the 10 pathogenic changes identified, 3 were nonsense, 3 frameshift, 1 splice site, 1 compound mutation (splice/frameshift and missense), 1 missense and 1 point substitution in the Dp427 promoter/exon1 region of the *DMD* gene. Of those, only 2 nonsense mutations (c.4729C>T and C.6905G>A), featured in the Leiden *LOVD* and the remaining 8 were *novel* (Aartsma-Rus et al., 2006; Tuffery-Giraud et al., 2009). The changes were shown to cause alterations to RE sites in 8 cases, with the Webcutter 2.0 software (http://bio.lundberg.gu.se/cutter2), offering RE analysis as a tool for easy mutation detection in the relatives who may present for testing in the future (Table 3.2).

Truncating mutations in the *DMD* gene are generally expected to be pathogenic and therefore no further analyses for evidence of disease-association were carried out on the 3 nonsense and 3 frameshift mutations detected in this study (highlighted in yellow in Table 3.2). It is however possible for a nonsense mutation to disrupt the normal splicing process and thus create an alternative rescue transcript (Nishiyama et al., 2008). None of the nonsense mutations detected in this study were shown to have this effect, according to the results of bioinformatic analyses with the Human Splice Finder v2.4 program (HSF) (Desmet et al., 2009).

Only 1 of the 3 nonsense mutations detected in this project involved a CGA codon (c.4729C>T), with the other 2 located at non-CpG sites: an AT:CG transversion at c.620T>G in exon 7 and a GC:AT transition at c.6905G>A in exon 47. It has been

Table 3.2 Disease-associated small mutations detected,

	DNA CODE	SEQUENCE CHANGE	EXON	TRANSLATIONAL EFFECT	RE SITE	AFFECTED DYSTROPHIN ISOFORM	DYSTROPHIN DOMAIN	?NEW
1	DMD552.1RIV	c.620T>G	7	NONSENSE p.(Leu207X)	MnlI +	Dp427c, Dp427m, Dp427p	actin-binding domain	?NEW
2	DMD352.2BRE	c.4729C>T	34	NONSENSE p.(Arg1577X)	SfaNI -	Dp427c, Dp427m, Dp427p, Dp260	central rod domain repeat 12	NO*
3	DMD411.1PET	c.6905G>A	47	NONSENSE p.(Trp2302X)	TspRI -	Dp427c, Dp427m, Dp427p, Dp260, Dp140	central rod domain repeat 18	NO**
4	DMD550.1SHA	c.503delC	6	FRAMESHIFT p.(Ala168fsX2)	MwoI -	Dp427c, Dp427m, Dp427p	actin-binding domain	?NEW
5	DMD83.1MTH	c.836_837delCG	9	FRAMESHIFT p.(Thr278fsX8)	none	Dp427c, Dp427m, Dp427p	central rod domain repeat 1	?NEW
6	DMD342.2RIC	c.8284dupA	56	FRAMESHIFT p.(Iso2762fsX10)	none	Dp427c, Dp427m, Dp427p, Dp260, Dp140, Dp116	central rod domain repeat 22	?NEW
7	DMD372.1DIN	c.2293-1G>A	19	SPLICE	PstI -	Dp427c, Dp427m, Dp427p	central rod domain repeat 4	?NEW
8	DMD357.1MAS	[c.8028_8031dup GGTG; c.8044G>T]	55	SPLICE/FRAMESHIFT and MISSENSE [p.Val2677fsX4; p.Ala2682Ser]	MnlI-	Dp427c, Dp427m, Dp427p, Dp260, Dp140	central rod domain repeat 22	?NEW
9	DMD548.1THE	c.-85T>C	Dp427m/ exon 1	5'UTR	TspRI +	Dp427m	transcription factor binding site	?NEW
10	DMD389.1PIE	c.587T>C	7	MISSENSE p.(Leu196Pro)	MspI +	Dp427c, Dp427m, Dp427p	actin-binding domain	?NEW

* (Aartsma-Rus et al., 2006), **(Sedlackova et al., 2009)

Yellow: nonsense mutations Blue: frameshift mutations Pink: splicing mutations Green: other mutations

shown that the CpG dineucleotide is a hot-spot for mutations in the human genome, because of its vulnerability to spontaneous deamination of 5-methyl cytosine to thymidine (Krawczak et al., 1998). C>T transitions in the CGA codon are reported as the most prevalent stop mutation class in the *DMD* gene and the CGA codon presents 23 out of 28 nonsense mutation targets in the coding region of the gene (Flanigan et al., 2009; Tuffery-Giraud et al., 2009).

Table 3.3 Previously reported polymorphisms.

SEQUENCE CHANGE	EXON	FREQUENCY	DETECTED WITH PATHOGENIC MUTATIONS	REFERENCE
c.94-9dupT	3a	3/18	YES (1pt)	(Aartsma-Rus et al., 2006)
c.960+50delG	9	3/18	YES(1pt)	(Aartsma-Rus et al., 2006)
c.837G>A	9	6/18	YES (2pts)	(Aartsma-Rus et al., 2006)
c.1993-37T>G	17	4/18	YES (3pts)	(Aartsma-Rus et al., 2006)
c.2168+13T>C	17	1/18	NO	(Aartsma-Rus et al., 2006)
c.2645G>A	21	3/18	YES (2pts)	(Aartsma-Rus et al., 2006)
c.5234G>A	37	3/18	YES (2pts)	(Aartsma-Rus et al., 2006)
c.7200+53C>G	49	2/18	YES (1pt)	(Aartsma-Rus et al., 2006)
c.9361+138T>C	64	4/18	YES (2pts)	(Aartsma-Rus et al., 2006)
c.9649+15T>C	66	3/18	YES (2pts)	(Aartsma-Rus et al., 2006)
c.11046+119A>G	78	1/18	NO	(Aartsma-Rus et al., 2006)
c.*38G>A	79	1/18	YES	(Aartsma-Rus et al., 2006)
c.*477_*480dupTACA	79	1/18	NO	(Aartsma-Rus et al., 2006)
c*1562_*1565dupTAAG	79	1/18	YES	(Aartsma-Rus et al., 2006)
c.*2760-*2763delACTT	79	3/18	YES (1pt)	(Aartsma-Rus et al., 2006)

Table3.4 Changes of uncertain significance

PATIENT	SEQUENCE VARIATION	EXON	FREQUENCY	TYPE
DMD447.1THA	c.3513A>G	26	1/18	silent
DMD357.1MAS	c.8668+19A>G	58	1/18	intronic - in a patient with a pathogenic mutation in exon 55
DMD501.1NAZ	c.*1903_*1906delTTAA	79	1/18	3'UTR

Similarly accepted as pathogenic were the mutations affecting splicing (highlighted in pink in Table 3.2). The alteration of the consensus splice acceptor site of intron 18

in patient DMD372.1DIN (c.2993-1G>A), was listed as such based on its location and the fact that a G>T change in the same position was listed as probably pathogenic in the DMD *LOVD* (Dubourg et al., 1999). Furthermore the ESEfinder analysis (Cartegni et al., 2003) for this mutation was strongly indicative of a disrupted splice acceptor site, with a drop in the motif score from 9.37680 in the wild type to 2.1300 in the mutant (Figure 3.4).

The compound mutation identified in exon 55 of patient DMD357.1MAS [c.8028_8031dupGGTG; c.8044G>T], was also listed as a splicing alteration, though the duplication of the GGTG motif could cause either a splicing or a frameshift aberration, possibly exacerbated (or ameliorated) in its effect by the missense mutation 12 bases downstream. Bioinformatic analysis with the HSF (Desmet et al., 2009) revealed possible alterations to the splicing elements by creation of alternative acceptor splice sites, disruption of the SRp40 splice enhancer site and creation of new splice silencer motifs. Figure 3.5 is the graphical representation of the HSF output for the mutant (DMD357.1MAS) as compared to the reference sequence of exon 55 with its flanking intronic regions.

DMD372.1DIN exon 19 (c.2293-1G>A) with flanking intronic regions
Sequence:
gtgaaacatcttaaggcttgaaagggcaagtagaagttataattattgtgtagattcacagtccttgtattgaattactcatctttgctctcatgctgcaa
GCCATAGAGCGAGAAAAAGCTGAGAAGTTCAGAAAACTGCAAGATGCCAGCAGATCAGCTCAGG
CCCTGGTGGAACAGATGGTGAATGtaattacacgagttgatttagataatcttcttagggatttgataa

5SS_U2_human threshold: 6.67	3SS_U2_human threshold: 6.632	BranchSite threshold: 0
Position*/Site/Score	Position*/Site/Score	Position*/Site/Score
168 (-68); CTGGTGGAACAGATGGTGAATGtaattaca; 6.19690	88 (-148); ctctcatgctgcaaGCCATAGAGCGAGAAA; 2.61300	88 (-148); ctctcat; 5.98280

* both positions from 5'end (through 1) and 3'end (through -1) are given

Wild type exon 19 with flanking intronic regions
Sequence:
gtgaaacatcttaaggcttgaaagggcaagtagaagttataattattgtgtagattcacagtccttgtattgaattactcatctttgctctcatgctgcag
GCCATAGAGCGAGAAAAAGCTGAGAAGTTCAGAAAACTGCAAGATGCCAGCAGATCAGCTCAGG
CCCTGGTGGAACAGATGGTGAATGtaattacacgagttgatttagataatcttcttagggatttgataa

5SS_U2_human threshold: 6.67	3SS_U2_human threshold: 6.632	BranchSite threshold: 0
Position*/Site/Score	Position*/Site/Score	Position*/Site/Score
168 (-68); CTGGTGGAACAGATGGTGAATGtaattaca; 6.19690	87 (-149); gctctcatgctgcagGCCATAGAGCGAGAA; 9.37680	88 (-148); ctctcat; 5.98280

* both positions from 5'end (through 1) and 3'end (through -1) are given

Figure 3.4 ESEfinder output for the splice site mutation c.2293-1G>A in patient DMD372.1DIN
The red arrows point to the changed acceptor site motif score.

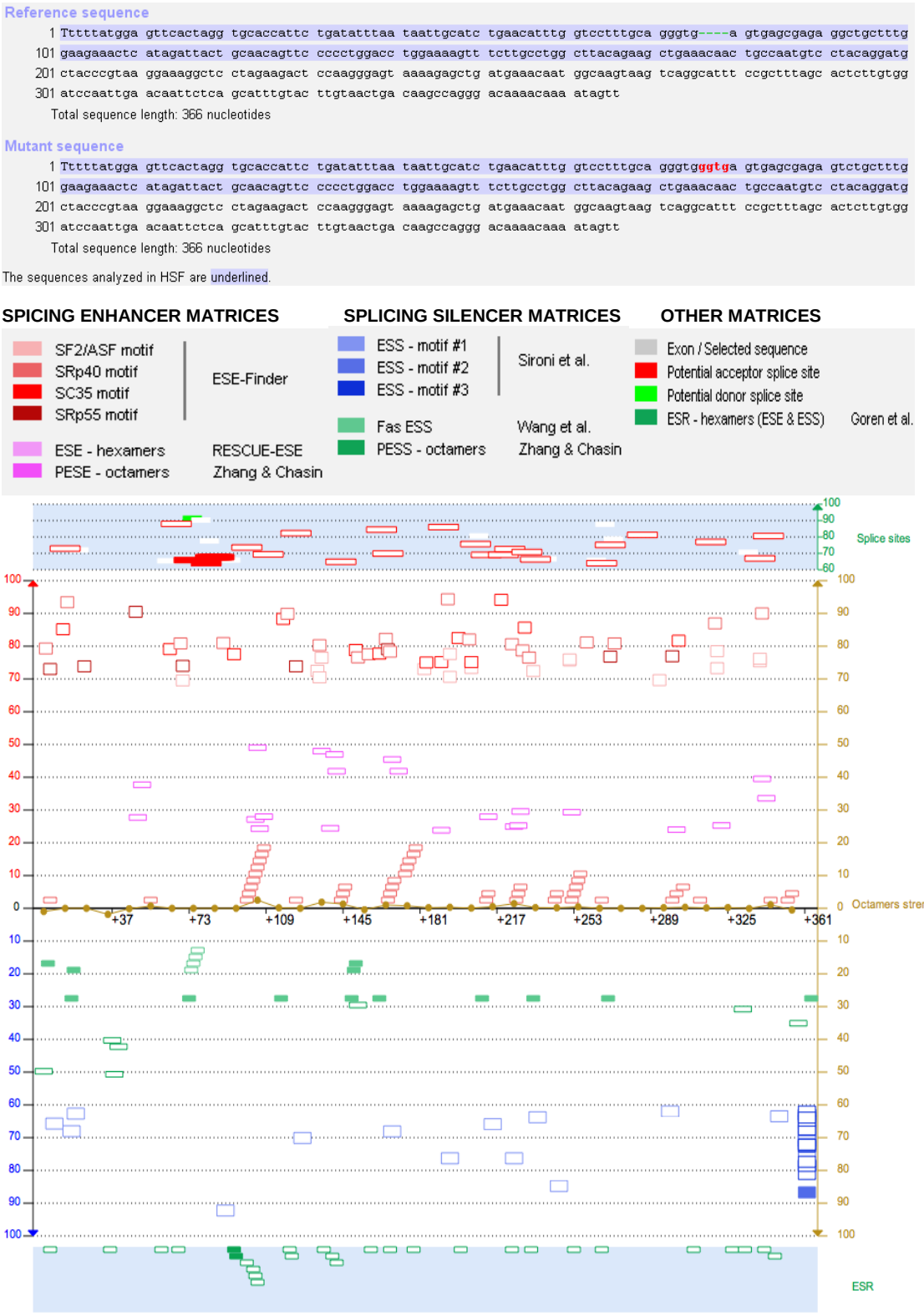
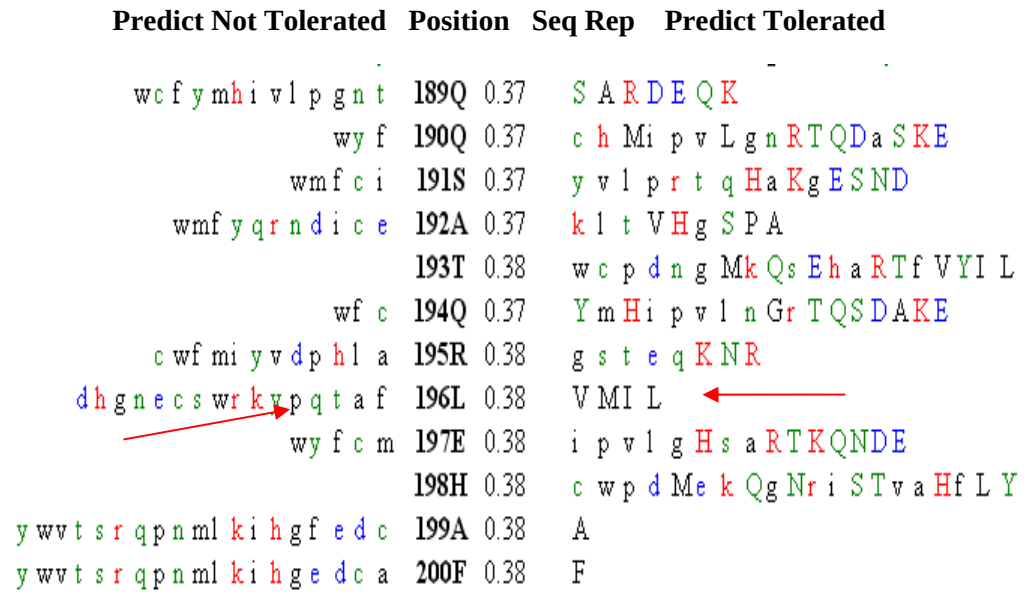


Figure 3.5 Graphical representation of the output from the Human Splicing Finder (HSF) program for the comparison of the reference sequence of exon 55 in the *DMD* gene with that of the mutant in patient DMD372.1PIE (Desmet et al., 2009). **Empty boxes** = this potential splicing element is present in both sequences; **Full boxes** = this potential splicing element is present only in the mutant sequence or with a reduced value in the reference sequence; **Doted boxes** = this potential splicing element is present only in the reference sequences or with a reduced value in the mutant sequence (none present).

A *novel* missense mutation was identified in exon 7 of patient DMD389.1PIE (c.587T>C). Missense mutations are infrequent in the *DMD* gene, comprising approximately 1 – 4% of the small lesions (which in turn account for ~ 30% of all

DMD mutations) (Tuffery-Giraud et al., 2004; Flanigan et al., 2009). Differentiating between benign and deleterious amino acid substitutions is often difficult. In this case, the output of computational analysis with the SIFT program (Sorting the Intolerant From Tolerant), listed the Leucine to Proline amino acid substitution in position 196 of the protein, as “not tolerable” (Figure 3.6).(Kumar et al., 2009). Further suggestion of the pathogenic nature of this mutation is its location in exon 7, as most of the missense mutations reported in the *DMD* gene, cluster in exons 2 – 8 and 62 – 70 (Aartsma-Rus et al., 2006; Tuffery-Giraud et al., 2009). The missense mutation identified as part of the compound mutation in exon 55 in patient DMD357.1MAS (c.8044G>T), was also analysed with the SIFT program. The Alanine to Serine change in this context was found tolerable, though as mentioned earlier, it may be of significance in its co-segregation with the GGTG duplication in exon 55.



Threshold for intolerance is 0.05. Amino acid color code: nonpolar, uncharged polar, basic, acidic.
Capital letters indicate amino acids appearing in the alignment, lower case letters result from prediction.
'Seq Rep' is the fraction of sequences that contain one of the basic amino acids. A low fraction indicates the position is either severely gapped or unalignable and has little information. Expect poor prediction at these positions.

Figure 3.6 The SIFT (Sorting Intolerant From Tolerant) analysis output for the missense mutation c.587T>C in patient DMD389.1PIE. The putative amino acid change from Leucine to Proline in position 196 is predicted as “not tolerated” .

The sequence alteration found in patient DMD548.1THE (c.-85T>C) was listed as pathogenic due to its location in the highly conserved Dp427m promoter/exon 1 region of the *DMD* gene. The conserved nature of the surrounding motif was noted with the aid of the UCSC Genome Browser (http://genome.ucsc.edu) (Figure 3.7), where T and 7 of the adjacent bases appear conserved in all 5 of the mammalian species. It is therefore likely that the mutation disrupts one of the transcription and/or translation factor binding sites, resulting in defective gene expression.

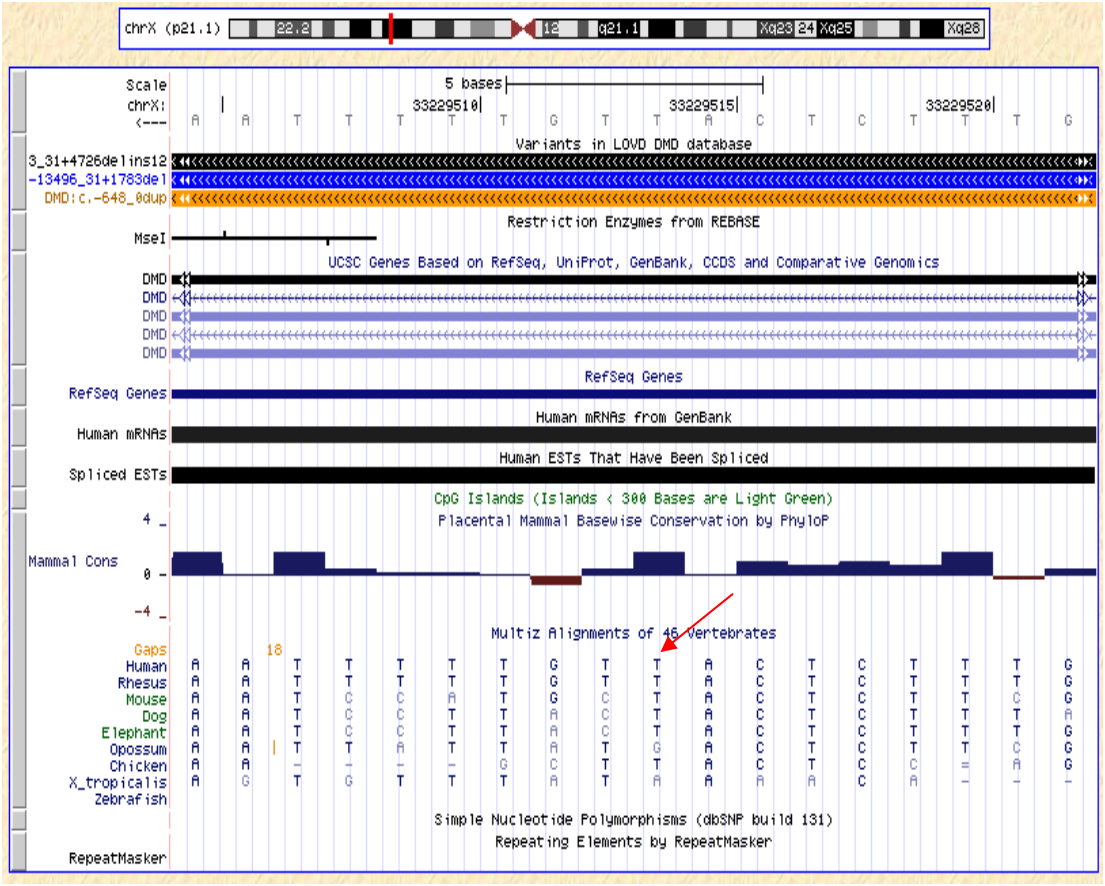


Figure 3.7 Output screen from the UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly. The red arrow points to the position of the T>C change (c.-85T>C) in patient DMD548.1THE (position 33229514).

Analysis with the TESS (www.cbil.upenn.edu) program for identification of transcription factor binding (TBF) sites shows a considerable decrease in the number of possible sites in the mutant as opposed to the wild type sequence (Figure 3.8). The analysis output is strongly suggestive of the change disrupting the MEF-2 (Human Myocyte-specific Enhancer Factor 2) TFB site, specifically the aMEF-2 isoform, (red arrow in Figure 3.7), which has a high likelihood score (L_a) of 18 and is not listed under the mutant sequence. The aMEF-2 is a MyoD-induced transcription activating factor for expression of muscle specific genes in the cardiac, smooth, skeletal muscle; placenta, brain, lung and kidney. Additional bioinformatic tools as well as mRNA expression studies from muscle may be required to elucidate the possible role of this mutation in the disease pathogenesis of this patient.

3.3.2 Polymorphisms

The non-pathogenic nature of 36 of the 39 previously reported polymorphisms (Aartsma-Rus et al., 2006) identified, was confirmed by their presence in multiple patients including those with disease associated mutations (Table 3.3). This confirmation was not possible in 3 cases (c.2168+13T>C, c.11046+119A>G and

c.*477_480dupTACA), which were found in 3 respective patients with no pathogenic mutations.

DMD548.1THE (c-85T>C - mutant sequence)

```
00001  ggtttctcac tgtttttaag  00020
       --------(9.3136) Elf-1/NTF-1 I00239
       ==========(14.00) D1 R03463
        -------(6.8999) Elk-1 I00112
         =====(6.7973) HSF1 M00029
         =====(7.9042) HSTF M00028
           ====== ==(9.8754) NHP-1 I00285
            ----- --(7.6443) GAGA I00244
             ==== ======(18.00) Dof2,MNB1a R08280,R08280
              --- -----(6.6581) fI-fII I00222
                = =========(14.00) MEF-2 (516 AA),MEF-2 R02201,R02201
                  ==========(12.3011) Hb I00248
                   --------(6.0618) Gt I00246
                   ---------(6.5976) TBP Q00171
                   =======(12.00) SEF4 R03650
                   -------(6.5104) GT-1 I00347
                   ========(12.4104) SBF-1 I00353
                    ------(7.6604) TFIID I00338
                    ------(6.4055) TFIID I00217
                    =====(9.7546) Hb Q00091
                      ======(8.2671) MBF-1 I00083
```

DMD wild type sequence

```
00001  ggtttctcat tgtttttaag  00020
       --------(9.3136) Elf-1/NTF-1 I00239
        -------(6.8999) Elk-1 I00112
         =====(6.7973) HSF1 M00029
         =====(7.9042) HSTF M00028
          ------- --(10.7172) Vmw65 I00017
          ------- ------(6.9662) P0U2F1,P0U2F1a M00137
          -------(6.8338) NBF I00323
          ------- -(6.2935) DEF I00073
           ------ --(8.8931) NHP-1 I00285
           ======(12.00) XPF-1 R03499
           ------ ---------(10.1294) BR-C_Z3 M00093
           ====== ========(14.2347) SOX-9 M00410
           ------ -(6.6165) CAP/CRP I00372
            ----- --------(6.0211) LCR-F1 M00285
            ===== =====(13.4966) mat1-Mc M00276
            ----- -------(11.5729) SRY M00160
            ===== =====(14.00) CBF (2),CP1,NF-1 R05057,R05057,R05057
             ==== ======(11.3986) Sox-5 M00042
             ---- ----(7.6319) SRY I00035
              --- ----(7.2929) SEF4 I00355
              --- -----(7.6404) fI-fII I00222
               == ====(12.00) Sox-5 R04109
               -- -----(6.6427) SRY M00148
                = =========(18.00) aMEF-2,MEF-2 (516 AA),MEF-2 R03584,R03584,R03584,R03584
                - --------(6.2935) DEF I00073
                = =========(14.00) MEF-2 (516 AA),MEF-2 R02201,R02201
                = =========(18.00) aMEF-2,D-MEF2,MEF-2 (516 AA),MEF-2 R03587,R09145,R03587,R03587,R03587
                  ==========(12.3011) Hb I00248
                   --------(6.0618) Gt I00246
```

Hit Sense and Strength Coloring Scheme

Color	Strand	Secondary Threshold
=	+	above
=	–	above
–	+	below
–	–	below

Binding site matches

Figure 3.8 The "annotated sequence" output of analysis with TESS.
The red arrows point to the TFB site for aMEF in + and - strand

3.3.3 Variants of uncertain significance

The study findings also revealed 3 changes of uncertain significance: c.3513A>G, c.8668+19A>G and c.*1903_*1906delTTAA. These were initially dismissed as probable polymorphisms as c.3513A>G is a silent mutation, c.8668+19A>G was found in patient DMD537.1MAS along with a disease associated mutation and c.*1903_*1906delTTAA is located in the 3'UTR close to other mutations listed as polymorphisms in the DMD *LOVD* (Aartsma-Rus et al., 2006). This assumption can however not be made with any degree certainty for the following reasons:

- all 3 alterations are *novel*
- 2 of the 3 were detected in patients with no other disease associated changes and the possibility of more than one pathogenic change in a patient cannot be excluded
- intronic mutations as well as silent mutations may disrupt splicing elements and cause altered splicing
- the 3'UTR in the DMD gene is fairly conserved and mutations therein can be disease associated (Greener et al., 2002a).

The silent mutation was shown not to have any significant effect on the splicing elements as per analysis with the HSF programs, which points towards it being a polymorphism. No further analysis was carried out on the remaining 2 changes, as expression studies on mRNA are required to provide a more meaningful and definitive prediction regarding any pathogenic effect.

3.4 POST-PCR MIXING AND HRM

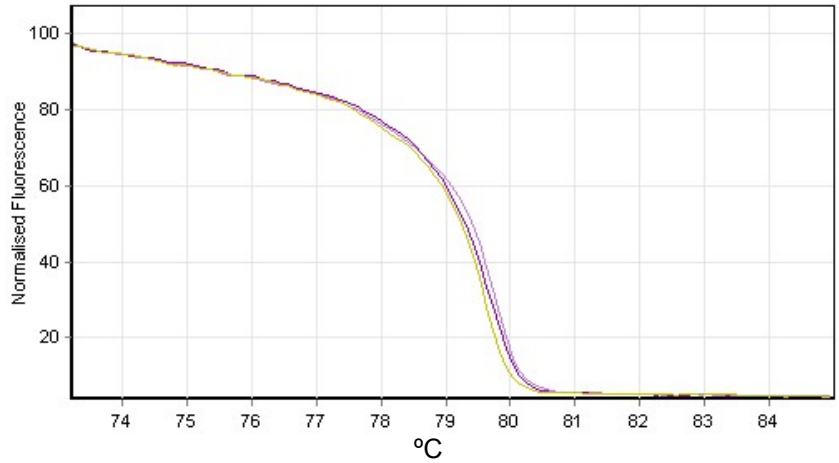
The post–PCR mixing experiment did not result in detection of any additional variants possibly missed with the initial hrMCA. However, the value of this exercise on the RotorGene™6000 is uncertain, as the results were inconsistent (Figure 3.9). Previously detected polymorphisms became more obviously variant after post-PCR mixing in some cases, and equally or less obvious in others. A heteroduplex showed an altered curve shape in most instances, but did not necessarily appear more variant or easily detected than the initial homoduplex. This may be due once again, to the variable sample quality.

3.5 FAMILY STUDIES

The allele segregation analyses within 6 families of probands with putative mutations, revealed that the mutations tracked with the phenotype. The mutation segregation within the relatives was then compared to the individual haplotypes previously constructed as part of the diagnostic work-up for the families using

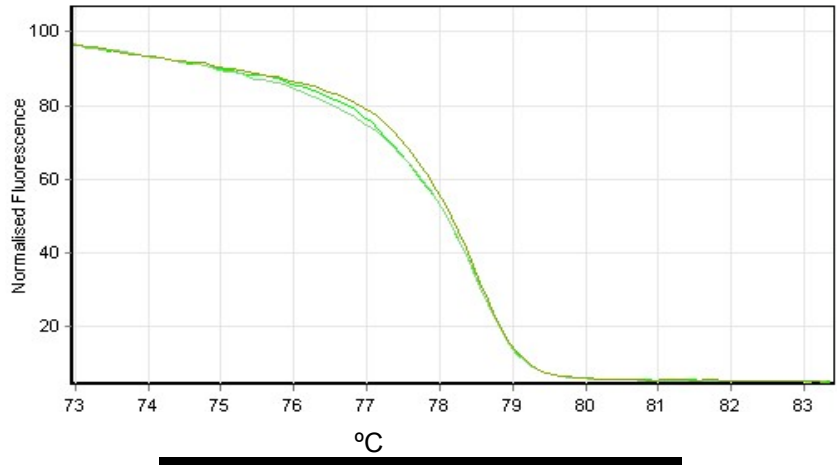
intragenic microsatellite markers. The putative mutations were thus shown to track with the affected haplotypes (Table 3.5).

A



Colour	Name	Genotype	Confidence %
	test exon 49	Variation	46.28
	post mixing exon 9	Variation	76.86
	wild type exon 9	control	100.00

B



Colour	Name	Genotype	Confidence %
	test exon 66	Variation	82.24
	post mixing exon 66	Variation	73.76
	wild type exon 66	control	100.00

Figure 3.9 Examples of the variable results of the post-PCR mixing hrMCA experiment on the RotorGene™ 6000. A: Sequence variant in exon 9 (c.960+50delG) - less prominent after pos-PCR mixing. B: Sequence variant in exon 66 (c.9649+15T>C) - more prominent after post-PCR mixing.

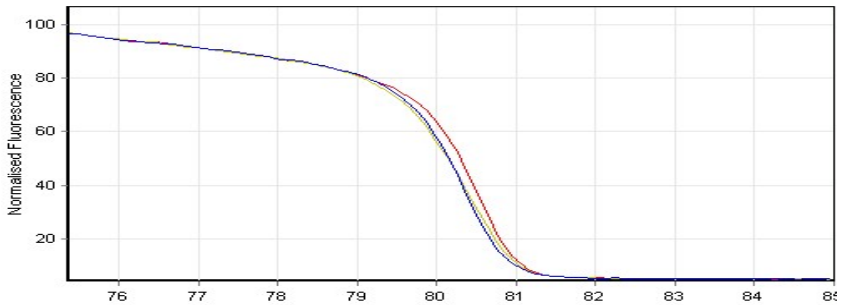
Table 3.5 Family Studies

FAMILY MUTATION	DNA CODE	SEX	RELATIONSHIP TO PROBAND	MICROSAT. ANALYSIS	MUTATION DETECTED Y/N	DETECTION METHODS
exon 34, c.4729C>T, p.(Arg1577X)	DMD352.1LO	F	mother	obligatory carrier	Y(hetero)	hrMCA, sequencing
	DMD352.2BR	M	**proband**	affected haplotype	Y(hemi)	
	DMD352.3BR	F	sister (carrier)	affected haplotype	Y(hetero)	
	DMD352.4AN	F	maternal aunt	uninformative	N	
	DMD352.5SA	M	affected nephew	affected haplotype	Y(hemi)	
exon 47, c.6905G>A, p.(Trp2302X)	DMD411.1PE	M	**proband**	affected haplotype	Y	hrMCA, restriction enzyme digest with TspRI (only proband sequenced)
	DMD411.2MR	F	mother	obligatory carrier	Y(hetero)	
	DMD411.3DIH	M	affected brother	affected haplotype	Y(hemi)	
exon 9, c.836_837delCG p.(Thr278fsX8)	DMD83.1MTH	M	**proband**	affected haplotype	Y(hemi)	hrMCA, sequencing
	DMD83.2NTO	F	mother	/	Y(hetero)	
exon 55, [c.8028_8031dupGGTG; c.8044G>T], [p.Val2677fsX4; p.Ala2682Ser]	DMD357.1MA	M	**proband**	affected haplotype	Y(hemi)	hrMCA, sequencing
	DMD357.2SO	M	brother	affected haplotype	Y(hemi)	
	DMD357.3MO	F	mother	obligatory carrier	Y(hetero)	
	DMD537.4NO	F	sister (carrier)	recombinant haplotype	Y(hetero)	
exon 56, c.8284dupA, p.(Iso2762fsX10)	DMD342.1RE	F	mother (carrier)	**/**	Y(hetero)	hrMCA, sequencing
	DMD342.2RIC	M	**proband**	affected haplotype	Y(hemi)	
	DMD342.3FO	M	foetus (brother)	opposite haplotype	N	
exon 19, c.2293-1G>A splice mutation	DMD372.1DIN	M	**proband**	affected haplotype	Y(hemi)	hrMCA, sequencing
	DMD372.2DE	F	mother	/	Y(hetero)	
	DMD372.3JA	F	maternal aunt	uninformative	N	
exon 7 c.587T>C p.Leu196Pro	DMD389.1PIE	M	**proband**	affected haplotype	Y(hemi)	hrMCA, sequencing
	DMD389.2	F	mother	obligatory carrier	Y(hetero)	
	DMD389.3	M	affected brother	affected haplotype	Y(hemi)	

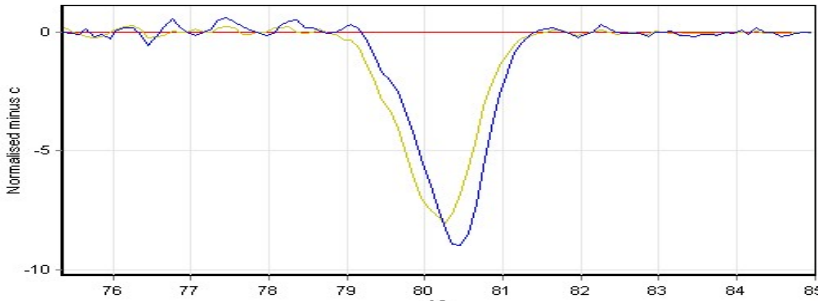
/ = the mother carries the haplotype of an affected child, which does not necessarily make her a carrier in absence of other affected individuals in the family.

RE analysis was included in the family studies for family DMD411 to demonstrate its use and the results concurred with both the hrMCA and the sequencing analysis of the proband. The family pedigree and the RE analysis of family DMD411 includes an affected sibling (DMD411.3DIH) who presented for testing subsequent to the hrMCA analysis on the DNA of his mother and brother (Figures 3.10, 3.11, 3.12).

A



B



Colour	Name	Genotype	Confidence %
	wild type exon 47	control	100.00
	DMD411.2.MRS exon47	Variation	59.82
	DMD411.1.PET exon47	Variation	55.56

Figure 3.10 HrMCA analysis of family DMD411 with a nonsense mutation in exon 47
A: fluorescence normalized curve, B: a difference curve

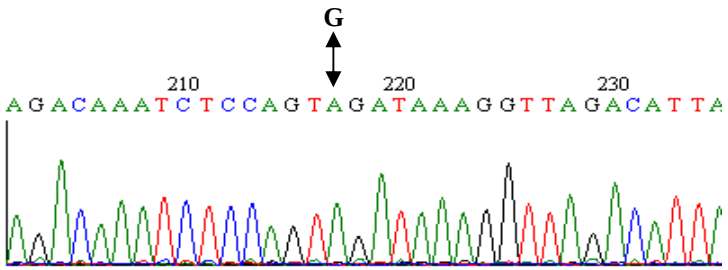


Figure 3.11 A section of the sequencing electropherogram of the affected proband in family DMD411 with a G>A change in exon 47(c.6905G>A)

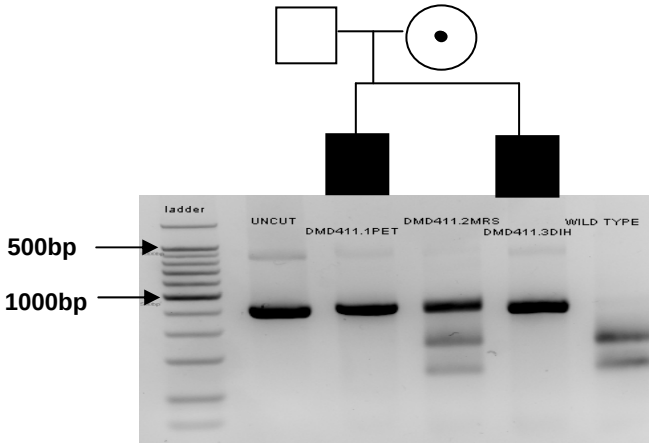


Figure 3.12 RE analysis and pedigree of family DMD411. The mutation c.6905G>A destroys the TspRI RE site in the affected boys and the affected allele of their mother.

3.6 POPULATION STUDIES

The PCR and RE analyses of the population cohorts of affected and wild type samples tested negative for both mutations (c.-85T>C in 5'UTR and c.5878T>C in exon 7 of the *DMD* gene), providing further (though not conclusive) evidence towards the pathogenic and private nature of the mutations.

3.7 POTENTIAL ELIGIBILITY FOR GENE- BASED THERAPY

Based on the mutation type in relation to the exon phasing of the *DMD* gene (Figure 3.13), an attempt was made at determining theoretical eligibility for potential gene modification therapy options for each of the 16 mutation-positive patients in the cohort (Table 3.6). This was a purely academic exercise, as determining eligibility for any mutation-based therapy requires knowledge of the mutation's effect at the transcript level. Also, the selection was made superficially, not taking into account factors other than the mutation type, which must be considered in application of a mutation-based therapy to an individual e.g. feasibility of the specific AON design, efficient delivery to the site of expression, the immunogenic aspects etc. A number of options are given per patient, some theoretically capable of restoring the wild type protein, and some aiming at restoration of the ORF by internal truncation of the *DMD* gene and production of a partially functional protein, leading to amelioration of the phenotype. Consideration was primarily given to the gene-modification strategies, as they appear closest to clinical application at this time (Guglieri & Bushby, 2010).

Theoretically, restoration of the wild type protein is achievable with the gene modification approach is some cases. For example, exonic duplications, such as those in patients DMD530.1THA and DMD420.1SIY in this cohort, are considered an excellent target for exon skipping, as skipping of the duplicated exons (as opposed to the constitutive ones) should restore the original gene structure and function (Aartsma-Rus et al., 2007). Effective readthrough therapy for nonsense mutations should also restore production of full length (or close) dystrophin, with 3 patients (DMD552.1RIV, DMD352.2BRE and DMD411.1PET presenting as potential candidates) (Guglieri & Bushby, 2010). Even more attractive, though at this stage, further from successful application, is long term restoration of the wild type protein at a genomic level achieved by exon editing with short oligodinucleotides (Gamper et al., 2000; Andersen et al., 2002). This has been described in relation to classic splice site mutations such as that in patient DMD372.1DIN, as wells as to point substitutions resulting in nonsense or missense (DMD552.1RIV, DMD352.2BRE, DMD411.1PET and DMD389.1PIE).

Amelioration of the phenotype however, by skipping exons and creating an internally truncated but partially functional protein, is currently seen as the most promising of the gene modification therapies for DMD, with single exon skipping of exon 51 reaching the stage of phase IIb clinical trials (Guglieri & Bushby, 2010; van Deutekom et al., 2007). In this cohort, single exon skipping could benefit 6 patients (Table 3.6), though different AONs are required in each case (DMD378.1TEM, DMD457.1MOG, DMD530.1THA, DMD352.2BRE, DMD411.1PET and DMD83.1MTH).

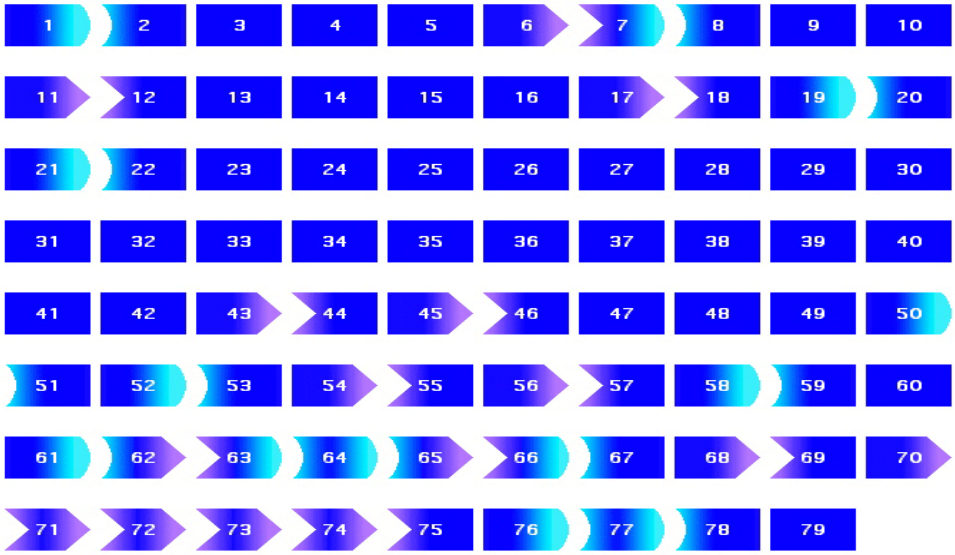


Figure 3.13 Exon phasing of the DMD gene (Beroud et al., 2007). The phase of each Exon is represented by the shape of extremity of the box representing the exon.
Left end of exons: 1) dark blue vertical line: exon starting at the 1st nucleotide of a codon
2) light blue curve: exon starting at the 2nd nucleotide of a codon
3) purple arrow: exon starting at the 3rd nucleotide of a codon.
Right end of exons: 1) dark blue vertical line: exon ends at last nucleotide of a codon
2) light blue curve: exon ends by at the 1st nucleotide of a codon
3) purple arrow: exon ends by the 2nd nucleotide of a codon.

Multiexon skipping of two sets of AONs on the other hand, could present a therapeutic option for a number of the patients in this cohort. Skipping exons 6 – 8 has been successfully achieved in proof of concept experiments with the CXMD dog model (Yokota et al., 2009). In this study, multiexon skipping of exons 6 – 8 could benefit 5 patients. This includes patient DMD83.1MTH, with a frameshift mutation in exon 9, because of the spontaneous skipping of exon 9 achieved along with exons 6, 7 and 8 in the experiment with and without AONs for skipping exon 9. The feasibility of multiexon skipping of exons 45 – 55 has also been assessed (though unsuccessfully, so far) and if achieved, could benefit 3 patients tested in this study cohort (van Vliet et al., 2008). The *DMD* gene modification approach will not be beneficial to all DMD patients. In this cohort, the therapeutic avenues for 2 patients were difficult to predict by virtue of the mutation type and location. Depending on the effect of the point substitution in the Dp427m/exon1 promoter region of patient

DMD548.1THE, one of the gene replacement approaches may be a better therapeutic option (Jorgensen et al., 2009; Mendell et al., 2009; Sonnemann et al., 2009). The deletion of exons 61 – 79 in patient DMD306.1GV possibly extends beyond the *DMD* gene. If that is the case, even the *DMD* gene replacement may be of limited value.

Table 3.6 Eligibility for potential gene modification therapy

PATIENT	MUTATION	POTENTIALLY APPLICABLE GENE-BASED THERAPIES	POSSIBLE THERAPEUTIC EFFECT
DMD306.1GV	deletion (ex 61-79)	–*	
DMD378.1TEM	deletion (ex 5 – 7)	single exon skipping (exon 8), multiexon skipping (e.g. exons 2, 4)	BMD
DMD414.1PHI	deletion (ex 7)	multiexon skipping (e.g. exons 6,8)**	BMD
DMD457.1MOG	deletion (ex22 - 33)	single exon skipping (exon 21)	BMD
DMD420.1SIY	duplication (ex 3 -36)	multiexon skipping of duplicated exons	wild type protein
DMD530.1THA	duplication (ex 1)	single exon skipping of duplicated exon 1	wild type protein
DMD552.1RIV	nonsense codon TAA (exon 7)	nonsense readthrough, multiexon skipping (e.g. exons 6 – 8)**, exon editing.	**** wild type protein ?reduced levels BMD
DMD352.2BRE	nonsense codon TGA (exon 34)	nonsense readthrough, single exon skipping (exon 34), exon editing	**** wild type protein ?reduced levels BMD
DMD411.1PET	nonsense codon TAG (exon 47)	nonsense readthrough, single exon skipping (exon 47), multiexon skipping (45 -55)***, exon editing	*** wild type protein ?reduced levels BMD
DMD550.1SHA	frameshift (delC in exon 6)	multiexon skipping (eg. exon 6 – 8)**	BMD
DMD83.1MTH	frameshift (delCG in exon 9)	single exon skipping (exon 9), exon skipping for exons 6 – 8**	BMD
DMD357.1MAS	frameshift (dup GGTG) and missense in exon 55	multiexon skipping (eg. exon 45 – 55)***	BMD
DMD342.2RIC	frameshift (dupA in exon 56)	multiexon skipping (e.g. exon 55, 56 or 56, 57)	BMD
DMD372.1DIN	G>A substitution (splice acceptor site intron 18)	splice site editing, multiexon skipping (e.g. exons 2 – 19)	wild type protein
DMD548.1THE	T>C substitution in Dp427m promoter/exon1	gene editing or replacement ****	?
DMD389.1PIE	missense in exon 7	multiexon skipping (e.g. 6 – 8)**, exon editing	BMD or wild type protein

* The deletion may extend beyond the *DMD* gene and its effect on the ORF or applicability of any potential therapy cannot be predicted with the data available here.
** The concept of skipping exons 6 – 8 has been successfully proven using the CXMD dog model. The experiment also showed spontaneous skipping of exon 9. (Yokota et al., 2009).
*** The feasibility of multiexon skipping of exons 45 – 55 has been assessed. While not successful at the initial stage, further investigation is under way (van Vliet et al., 2008).
**** In this case, it is possible that the transcription process of the *DMD* gene is inefficient, necessitating gene replacement or repair at the genomic level.

CHAPTER 4: DISCUSSION

Investigation of the entire coding region of the *DMD* gene in 24 affected boys revealed disease-causing mutations in 16 cases, providing genetic confirmation of clinical diagnoses, not achieved with routine diagnostic testing.

Six exonic rearrangements were identified with the DMD MLPA in respective patients; 4 of which were deletions and 2 duplications. These changes had not been detected by diagnostic testing with mPCR, due to the limitations of the test, which screened only selected exons for deletions within the deletion hot-spots. Furthermore mPCR did not have a quantitative component and could not be used to detect duplications (Beggs et al., 1990). However, the deletion of exons 5-7 in patient DMD378.1TEM had been missed with the mPCR despite the presence of exon 6 in the mPCR screen. This was most probably caused by the poor resolution of mPCR products for exons 4 and 6 on agarose gel electrophoresis, as the PCR fragments were of similar size.

The deleterious nature of large lesions in the *DMD* gene is widely accepted and therefore no further investigations were conducted to prove disease association. However, single exon changes detected with the MLPA, require authentication, as assay artifacts or point mutations can interfere with MLPA probe attachment. For this reason, the deletion of exon 7 found in patient DMD414.1PHI, was confirmed with an alternative technique i.e. single exon PCRs of exons 6, 7 and 8.

It was not deemed necessary to authenticate deletions of multiple consecutive exons found in patients DMD378.1TEM (exons 5 – 7), DMD457.1MOG (exons 22 – 33), and DMD306.1GV (exons 61 – 79). It must be noted though, that in the latter case the deletion may extend beyond the *DMD* gene, which exceeds the detection range of the DMD MLPA assay. In a male, involvement of another gene could be demonstrated by testing for the presence or absence of X-linked markers, or possibly by a more sophisticated technology such as CGH-microarray for copy number variations of the *DMD* gene and the surrounding regions (Hegde et al., 2008). Possible involvement of other genes in this patient's disease presentation could not be gleaned from the limited available clinical data. There was however indication of a learning disability from an early age, which was interesting considering the location of this lesion in the distal part of the *DMD* gene (exons 61-79), which was likely to affect all the dystrophin isoforms including the Dp140 and Dp71. This finding was consistent with reports in the literature of a link between

lesions affecting the Dp140 and especially the Dp71 isoform and intellectual disability in DMD patients. This has been hypothetically linked to the abundant levels of Dp140 and Dp71 in the foetal brain, which may be of relevance to cognitive development (Bushby et al., 1995; Bardoni et al., 2000; Felisari et al., 2000; Taylor et al., 2010). Interestingly, learning disability also featured in the clinical data of patient DMD378.1TEM with a deletion of exons 5-7. Mutations located at the 5' end of the gene which affect the full-length dystrophin isoforms (including the Dp427c in the brain) are not generally associated with intellectual disability, which if present, is mild in most such instances (Muntoni et al., 2003b). The overall mean IQ in DMD patients however, is about one standard deviation below the normal mean and in absence of a detailed clinical description, an indication of "learning disability" may not refer to intellectual disability as such.

The exonic duplications in patients DMD420.1SIY (exons 3 – 36) and DMD530.1 THA (exon 1) were found located in the 5' hot-spot region, which is the predominant hot-spot for duplications (Aartsma-Rus et al., 2006). Both were confirmed with repeat MLPA runs. In addition, the duplication of exon 1 in patient DMD530.1THA was confirmed by testing DNA extracted from a fresh blood sample, as well as that from an affected brother, also shown to carry the duplication.

The limited available clinical data of the cohort, allowed for few correlations between the phenotype and the affected dystrophin domain or isoform. However, the reading frame hypothesis held for all but 1 of the large lesions detected. The genotype-phenotype correlation in patient DMD420.1SIY with a duplication of exons 3 – 36, appeared to violate the reading frame hypothesis, as according to the exon phasing of the *DMD* gene (Beroud et al., 2007), this was an in-frame change, which involved part of the actin-binding domain and the adjacent central rod domain. It should therefore theoretically cause a BMD phenotype, when in fact, according to the clinical data available, the patient presented with a particularly severe disease course. This discrepancy was however not surprising, as the reading frame predictions made here, were based on the results of genomic DNA testing, and could not be used as evidence for the effect of the change at the transcriptional level. Furthermore, the frequency of reports of non-tandem duplications, triplications, and complex changes in the *DMD* gene is increasing with the current, improved detection methods (del Gaudio et al., 2008; Oshima et al., 2009). Such changes will in many instances not manifest their true nature on DNA-based analysis and therefore reading frame predictions for duplications detected with DNA-based MLPA

testing is discouraged (White & den Dunnen, 2006; White et al., 2006; Kesari et al., 2008; Flanigan et al., 2009).

The identification of 6 previously undetected large lesions reiterated the superior nature of the MLPA as a mutation detection tool for exonic rearrangements in the *DMD* gene, as compared to the mPCR assay, still used in many diagnostic centres. The MLPA analysis offers a wider detection range and better definition of the deletion/duplication breakpoints, as it features all 79 exons of the *DMD* gene. Very importantly, the quantitative component of the assay allows for improved determination of female carrier status without the need for linkage analysis, which is more expensive and can be less informative. Incorporation of the MLPA into the genetic service at the NHLS in Cape Town has certainly had a positive impact on carrier status determination, as the majority of referrals are isolated, as opposed to familial DMD cases. The MLPA therefore, unlike linkage analysis, allows for determination of carrier status in the mothers of deletion/duplication positive boys with 99% accuracy.

Disease-causing small/point mutations were detected in 10 of the remaining 18 patients of the cohort using hrMCA. These included 3 nonsense, 3 frameshift, 1 splice site, 1 compound mutation (splice/frameshift and missense), 1 missense, and 1 point substitution in the Dp427m promoter/exon1 region of the DMD gene. Only two of the 10 mutations (c.4729C>T and c.6905G>A, both nonsense) were previously reported in the DMD *LOVD* (Leiden Open source Variation Database) (Aartsma-Rus et al., 2006). All were unique to their specific families or in the cases where no family DNA was available for testing, to the probands.

The 3 nonsense mutations were found in patients DMD552.1RIV, DMD352.2BRE and DMD411.1PET in exons 7, 34 and 47 respectively. The expected consequence of a PTC-causing mutation is protein truncation with its associated nonsense mediated decay (NMD), however, nonsense mutations have also been known to cause secondary alterations to splicing, resulting in skipping of the mutated exon and production of a semi-functional protein (provided that the skipped exon does not create a frame-shift) and a milder phenotype in some cases (Zhang et al., 2003; Disset et al., 2006; Deburgrave et al., 2007; Nishiyama et al., 2008). The nonsense mutation c.4729C>T in patient DMD352.1BRE had previously been studied for this effect and found not to cause altered splicing (Nishiyama et al., 2008). Analysis of the remaining two (c.620.T>G and c.6908G>A) with the Human Splice Finder v2.4 software (HSF) (Desmet et al., 2009) did not indicate potential secondary splicing

alterations. Establishing whether a nonsense mutation creates an alternative rescue transcript has an obvious therapy implication for drugs which induce nonsense readthrough, thus emphasising the importance of accurate assessment of eligibility for treatment by analysis of mRNA extracted from muscle tissue.

The 3 frameshift mutations, expected to cause truncation by downstream PTCs and subsequent NMD, were found in patents DMD550.1SHA, DMD83.1MYH and DMD342.2RIC. All 6 of the PTC-associated mutations detected in this study (frameshift or nonsense), were accepted as pathogenic and no further analyses (other than family studies) were carried out to prove disease-association.

A classic splice site mutation was detected in patient DMD372.1DIN, where the G>A change (c.2293-1G>A) was shown to disrupt the consensus splice acceptor site, using the ESEfinder software (Cartegni et al., 2003). The second line of evidence towards the deleterious nature of this mutation, was the fact that a previously detected G>T change at the same site was recorded as pathogenic in the DMD *LOVD* (Aartsma-Rus et al., 2006).

The compound mutation found in exon 55 of patient DMD357.1, on the other hand was not as obvious in its nature. Sequence analysis revealed an alteration involving insertion of a GGTG motif, along with a G>T missense mutation 12 bases downstream. The insertion was annotated according to the HGVS recommendations (den Dunnen & Antonarakis, 2000), as a duplication of a repeat motif at the 3' most position in the query sequence, with GGTG being the first 4 bases of exon 55. Because of the size of the insertion and the surrounding sequence, it was not certain whether the mutation caused a frameshift or altered a splice site. Analysis with the HSF however, was suggestive of an aberration in the splicing process, caused by creation of alternative splice sites, new ESS (exonic splice silencer) motifs and disruption of ESE (exonic splice enhancer) sites. The missense mutation downstream, is a possible exacerbating (or ameliorating) factor which may disrupt splice regulating sequences, though the amino acid change taken on its own, appeared tolerable according to SIFT analysis (Sorting Intolerant From Tolerant) (Kumar et al., 2009). However, determination of the actual effect of both mutations on the transcript, be it a splice variation or a frameshift, requires RNA-based studies on muscle biopsy material.

The missense mutation identified in exon 7 of patient DMD389.1PIE (c.587T>C), resulted in an amino acid substitution of Leucine to Proline. According to

computational analysis with the SIFT program, this was not a tolerable change in the context of the highly conserved residue of the actin-binding domain, which implied pathogenicity. Analysis with the HSF program was not indicative of a significant effect on the splicing elements. Missense mutations are rare in both DMD and BMD patients, reported at a frequency of approximately 1 – 4% of all small lesions (which make up ~ 30% of all mutations in the *DMD* gene) (Aartsma-Rus et al., 2006; Tuffery-Giraud et al., 2009). The missense cases reported in the DMD *LOVD*, cluster in the actin–binding domain of dystrophin encoded by exons 2 – 8, and the cystein-rich domain encoded by exons 62 – 70, defining a possible hot-spot (Aartsma-Rus et al., 2006; Tuffery-Giraud et al., 2009). The location of the missense mutation detected in exon 7 in this study was in keeping with this trend, further pointing towards its pathogenicity. Previously recorded missense mutations in the 5' end of the *DMD* gene were mainly associated with the Becker phenotype, and those at the 3' end, with DMD. According to the limited clinical data available, the patient was diagnosed with DMD but presented for testing at 10 years of age. This is however not necessarily due to late onset of symptoms (as in a BMD phenotype), as in some cases, the socio-economic and cultural aspects of the South African context obstruct access to early diagnosis and intervention.

The single base substitution was identified in the Dp427m promoter/exon1 region of the *DMD* gene (c.-85T>C) in patient DMD548.1THE (Tubiello et al., 1995). In the absence of mRNA studies, the link between this change and the DMD phenotype appeared somewhat tenuous. However, the T>C alteration fell within a highly conserved motif, as noted with the aid of the UCSC Genome Browser (http://genome.ucsc.edu). Also, the analysis output of the TESS (Transcription Element Search System) program, was strongly suggestive of the change disrupting the MEF-2 (Human Myosite-specific Enhancer Factor 2) TFB site, specifically the aMEF-2 isoform, which is a transcription activating factor for expression of muscle specific genes in the cardiac, smooth, skeletal muscle, placenta, brain, lung and kidney. Additional bioinformatic tools as well as mRNA expression studies from muscle may be required to elucidate the possible role of this mutation in the disease pathogenesis of this patient (Navankasattusas et al., 1992; Yu et al., 1992).

DNA was available from relatives of 7 probands and the disease-causing mutations were shown to track with the affected phenotype in all 7 families. Comparison with the diagnostic records of microsatellite analyses within the families showed that the changes also tracked with the affected haplotypes. This in itself is not proof of pathogenesis, as polymorphisms also track with haplotypes. More importantly, the

family studies served to establish the origins of the mutations as familial, by demonstrating their presence in the mothers of the affected probands. Furthermore, finding the deleterious changes in the mothers of isolated cases (families DMD83, DMD342 and DMD372), changed their empirical carrier risk of 70%, to a confirmed positive carrier status. Interestingly, the sister of proband DMD357.1MAS had been shown to carry a recombinant haplotype. She however tested positive for the pathogenic mutation in exon 55 ([c.8028_8031dupGGTG; c.8044G>T]) and closer scrutiny of the haplotype revealed that the recombination breakpoint lay downstream from exon 55, at the 3' end of the *DMD* gene between the STR HI and STR 3'CA microsatellite markers (Beggs & Kunkel, 1990; Tyson et al., 1996).

No DNA was available for testing from healthy male relatives, with the exception of family DMD342, where sample DMD342.3FOE was extracted from amniocytes of the male foetus for a prenatal diagnosis. The foetus, previously found to carry the unaffected haplotype, tested negative for the mutation (c.8284dupA) in this study. No further information regarding the affection status of the subsequently born child was available, as the family were infrequent attendees at the muscle clinic. The child had however not presented at the clinic to date, which is a likely indication of the absence of DMD. This, along with the fact the mutation was a frameshift alteration, supported the conclusion that the c.8284dupA is disease-causing.

Population studies in affected and unaffected cohorts were performed on the missense (c.587T>G) and the Dp427 promoter/exon 1 (c.-85T>C) mutations, in an effort to gain further supporting evidence towards (or against) their possible pathogenicity. Definitive evidence was however not found, as the changes were absent from both cohorts. Detection of a sequence change under investigation in the affected as opposed to the wild type cohort could be indicative of a deleterious mutation (or even a possible founder effect if found in more than one family). On the other hand, its presence in the wild type cohort could point towards non-pathogenicity, bearing in mind, that the population cohort size fell somewhat short of the minimum size required for this type of investigation (at least 200 individuals) (Frederic et al., 2009).

All of the mutations identified, were communicated to the relevant clinicians, for delivery to the probands and their families. The result delivery protocol stipulated that other family members seeking testing for the mutations detected in this research setting should be directed for testing in the diagnostic, accredited environment.

In addition to the pathogenic changes, 39 sequence alterations previously reported as polymorphisms were identified (Aartsma-Rus et al., 2006). The non-pathogenic nature of 36 of these changes was verified by their presence in multiple patients, including those carrying disease associated mutations in other exons. No such verification was possible in 3 cases (c.2168+13T>C, c.11046+119A>G and c.*477_*480dupTACA) as each of the variants was found in one respective patient, in the absence of a co-segregating pathogenic mutation.

Finally, 3 apparently *novel* sequence alterations of uncertain significance were identified, initially dismissed as polymorphisms. The silent mutation c.3513A>G in exon 26 of patient DMD447.1THA was computationally analysed with the HSF program and found to have no significant impact on the splicing elements within the fragment, increasing the likelihood of it being non-pathogenic. The sequence variation located in intron 58 (c.8668 + 19A>G) was also regarded as a polymorphism, since it was found in patient DMD357.1MAS, who was shown to carry a pathogenic mutation in exon 55 (the splice and missense mutation discussed earlier). This may however not be correct, as the possibility of more than one pathogenic change in a patient could not be excluded. Iintronic mutations in the *DMD* gene have been shown to cause disease by activating cryptic splice sites which incorporate intronic sequences into the mRNA thus creating a frameshift. These “pseudoexon mutations” are gaining the status of a newly recognized mutation type in DMD (Béroud et al., 2004; Gurvich et al., 2008). It was therefore dangerous to dismiss an intronic mutation as insignificant, based purely on its location outside of a coding region. Another mutation of an uncertain significance, initially dismissed as a polymorphism, was found in the 3’UTR of the *DMD* gene in patient DMD433.1LUN (c.*1903_*1906delTTAA) in the absence of other disease-causing mutations. However, a growing body of evidence has revealed that dysfunctional translational regulation of gene expression can be linked to disease pathophysiology. The translational ability of mRNA is controlled by sequence sites, signals and motifs residing in the 5’ and 3’ UTRs of eukaryotic genes and involves mechanisms such as alterations in the stability of the mRNA, its accessibility to the ribosomes, its circularization and interaction with the translation machinery (Chatterjee & Pal, 2009). The 3’UTR of the dystrophin gene specifically, exhibits a high degree of conservation, implying that changes in the sequence can be deleterious (Greener et al., 2002b).

Characterization of the downstream effects and clinical significance of ambiguous sequence variants such as the 3 changes described above requires additional

bioinformatic analyses and is most effectively achieved by analysis of mRNA transcripts extracted from muscle tissue, which extended beyond the scope and limitations of this study.

Extrapolation of mutation frequencies in this small cohort, to the DMD-affected population in South Africa cannot be made with any degree of confidence. However, truncating point mutations comprised 60% (6 patients) of the small pathogenic changes found in this study (half of which were nonsense mutations) and 20% (2 patients) involved splicing, which was largely in keeping with the detection frequencies reported in the literature, where over 60% of all reported small lesions in the *DMD* gene are nonsense and frameshifts and approximately 25% are splice mutations. Disease-causing missense mutations are reported at the lowest frequency of 1 – 4% of the small lesions, and 1 was found in this study (Aartsma-Rus et al., 2006; Flanigan et al., 2009; Tuffery-Giraud et al., 2009).

No disease-associated mutations were identified in 8 patients of the cohort (this includes the 4 patients with changes of uncertain significance). The reasons for this may be threefold. Firstly, the clinical diagnosis was not confirmed with muscle biopsies in 4 cases and in the absence of genetic diagnoses, clinical reassessments may be warranted. Secondly, there was the technical consideration of the quality of the DNA used for analysis. Fresh DNA was not available in all cases and long-term stored DNA extracted with a number of methods and reagents, had to be used. This was not ideal, as sample uniformity is important for reliable hrMCA and may therefore have resulted in missed variants. Lastly, some of these patients could carry deep intronic point mutations which create novel splice sites and result in the inclusion of intronic sequences in the mRNA. These “pseudoexon mutations” cannot be picked up with DNA-based testing and the methods used here. Also, the large size of the *DMD* introns still precludes direct intronic sequencing as a practical assay. Consequently, the presence of pseudoexons must be determined by analysis of mRNA from muscle tissue (Gurvich et al., 2008).

At this time, sophisticated high throughput technologies like next generation sequencing are on the horizon as the point mutation detection tool for entire genes, including the introns (Chou et al., 2010). Also, the ultimate tool for detection and elucidation of the full extent of copy number variations in the *DMD* gene is microarray technology with its capability of locating intronic breakpoints, deep intronic mutations and complex rearrangements involving other genes. (del Gaudio et al., 2008; Oshima et al., 2009). The changes detected in studies using these

sophisticated platforms, emphasise the complexity of some mutations underlying the DMD phenotype and help to elucidate the underlying mechanisms. For the moment however, financial limitations place the microarray and next generation sequencing technology out of reach of many diagnostic laboratories. Until such time as these testing platforms become affordable and more widely applied in laboratory medicine (in developing countries), the MLPA, along with an inexpensive and easily performed pre-sequencing screening tool like hrMCA, can become easily incorporated into the diagnostic setting and serve to provide previously unavailable information and a more comprehensive genetic service for DMD.

By and large, the endevour of small/point mutation detection in the *DMD* gene has been confined to research projects, as the expense and logistical challenges of setting up and sustaining a service for small mutation detection in this enormous gene, has been considered beyond realistic capabilities of most diagnostic laboratories. However, this line of thinking is shifting, with the potential availability of mutation-based therapies, the application of which necessitates the knowledge of the genetic basis of the disease in each affected individual. The decision to use hrMCA as a pre-sequencing screening tool for detection of small/point sequence variations in the *DMD* gene in this project was based on its ease, reasonable price, availability of specialized instrumentation and its capability to resolve single base variations with the sensitivity and reproducibility required for a diagnostic application (Krypuy et al., 2006; Krypuy et al., 2007; Takano et al., 2008; Yan et al., 2010).

While relatively quick and easy, the success of hrMCA is wholly dependant on a number of factors, as addressed in detail in Appendix C1 and C2. Besides the importance of availability of a suitable instrument and a sound experiment design, the experience of hrMCA as a technological platform in this project, revealed sample-to-sample uniformity as the prevailing theme at every level of the experiment, and the key to meaningful findings. The important lesson learned, was that troubleshooting of any (optimised) hrMCA experiment must start with assuring sample uniformity, from the DNA extraction method, to the amount of starting DNA, the rate of amplification and the amount of fluorescence at the beginning of the melt. A reliable comparison of DNA melting profiles can only be made between samples extracted and amplified in a similar manner. The importance of comparing uniformly extracted DNA was effectively demonstrated in this study. The wild type DNA from fresh blood samples was extracted manually with the PureGene blood kit (Qiagen) and was initially HRM-tested against test samples extracted with unknown methods, retrieved from the long-term storage facility in the Division of Human Genetics' DNA

Registry. The mediocre results obtained, were thought to be caused by degradation of the old DNA and fresh blood was requested, where possible. Fresh blood samples were obtained from 2 patients and DNA was extracted with the Maxwell16 automatic extractor (Promega). Upon hrMCA, every exon tested appeared significantly variant from the wild type. The problem was solved by repeated DNA extraction from frozen buffy coats using the PureGene kit (Quiagen), and hrMCA comparison of test and wild type DNA extracted with the same method.

Similarly, standardization of the starting amount of DNA in every sample within a run was found to have a significant effect on the resulting hrMCA. Meticulous sample dilution with the same diluent (in this case, nuclease-free distilled water) and careful spectrophotometry was found to be essential. Varying concentrations of DNA will affect the amount of fluorescent signal emitted by the dsDNA intercalating dye, which will in turn affect the shape and shift of the fluorescence-normalized melting curve. Uniform amplification efficiency between samples is also a consideration during hrMCA analysis, as it correlates with the amount of the resulting PCR product and hence, the strength of the fluorescent signal. The amplification log phase beyond cycle 30 is an indication of insufficient or a degraded sample.

It was found that the sample-to-sample uniformity principle should also be applied during data analysis, when adjusting normalization regions. Ideally, the regions should have a default position for each amplicon, relative to its melting temperature. In a high throughput analysis, such as that for the large *DMD* gene, the melting temperature range is set fairly broadly to accommodate a number of different amplicons with varying melting temperatures in one run. The positions of the normalization regions are therefore adjusted for each amplicon, which affects the curve shape and shift and therefore variant recognition. The recommended approach used in this study, was to set the regions over a temperature range of ~ 10°C, with Region 1 set at a point where the lines of the sample and control curves showed uniform melting (parallel), and Region 2 set after complete melting of the samples and controls (no fluorescence). Complicated melting curves such as those for fragments 3a or 23c in this project, were best analysed separately by adjusting the normalization regions for each melting domain.

The advantage of using an instrument like the RotorGene™6000 (Corbett Life Science) is the ability to run real-time PCR amplification and subsequent HRM consecutively in a closed-tube system, without the need for post-PCR manipulation.

Besides reducing labour-intensity, the risk of introducing errors and contamination is also reduced, which is of value in a diagnostic setting.

Theoretically, variant curves should be seen only in fragments with DNA sequence alterations. The experience of this project showed however, that this was not quite the case in practice, as recognition of a variant melting profile was in some instances tricky. Melting curve interpretation is to some extent operator dependant and factors such as sample uniformity, DNA quality, number of melting domains etc. affect the melting profile. In this study, 14% of the amplicons were marked as variant and subsequently sequenced (240 out of 1728). It was however anticipated that this percentage should drop when working with fresh, uniformly extracted test and control DNA. The variant selection criteria also erred on the side of caution, in an effort to avoid missing disease-causing mutations. However, considering the high number of amplicons scanned, the figure of 14% still represented a considerable reduction in the burden of sequencing, as compared to other methods, such as direct sequencing (though it has its own advantages), and a considerable reduction in cost at ~R7.00 per reaction as opposed to ~R100.00 (S.A. currency) per fragment sequenced.

The post-PCR mixing experiment was performed to test if mutation detection could improve with heteroduplex formation, as was found in the study by Almomani et al. (2009). This did not result in the identification of additional sequence changes, although the value of the exercise here is uncertain, due to the inconsistency of the results. Previously detected polymorphisms became more obviously variant after post-PCR mixing in some cases, and equally or less obvious in others. A heteroduplex showed an altered curve shape in most instances, but did not necessarily appear more variant or easily detectable than the initial homoduplex.

No temperature normalization of the data takes place on the RotorGene™6000, due to its rotary format and exquisite thermo-optical accuracy, as claimed by the manufacturers and also as shown by studies comparing the RotorGene™6000 to block-based HRM platforms. In theory, this should remove the need for post-PCR mixing as the melting behaviour of homo- or hemizygous changes usually exhibits a temperature rather than a shape shift, which is obscured by temperature normalisation on block-based instruments (White & Potts, 2006; Herrmann et al., 2007). Also, post-PCR mixing introduces a post-PCR manipulation step, significantly extending the length of the procedure and increasing labour intensity and the chances of introducing errors. However, post-PCR mixing should be considered as

a second step, if no pathogenic change is found with the initial hrMCA, as the assay can be designed in a way which will allow post PCR mixing after the initial PCR and hrMCA. The experience of this study showed that the PCR products with the EvaGreen dsDNA intercalating dye remain stable for a period of time, if frozen or refrigerated in the dark (Mao et al., 2007). Corbett Life Sciences do however suggest in their protocols, that class 4 SNPs may be more easily detectable with post-PCR mixing (http://www.corbettlifescience.com). All but 1 single base alteration detected in this study fell into the class 1 or 2 category, with the single class 3 SNP detected in exon 49 (c.7200+ 53C>G). Class 4 SNPs are the least prevalent in the human genome (Kwok et al., 2007) and none were identified in this cohort.

In light of the long term aim of this study, an attempt was made at scrutiny of each pathogenic alteration found, for therapy eligibility using the exon phasing of the *DMD* gene (Beroud et al., 2007; Tuffery-Giraud et al., 2009) and the mutation type as the selection criteria. In this cohort, skipping of single exons was thought to potentially benefit 6 patients, though different AONs would be required in each case (exons 1, 8, 9, 21, 34 and 47), highlighting one of the difficulties of the exon skipping approach.

The concept of exon skipping as a therapy is based on the natural phenomenon seen in some BMD patients, where mutation-induced, spontaneous skipping of exons affects restoration of the ORF, resulting in a milder phenotype (Kesari et al., 2008). AON-induced skipping of exons and creating an internally truncated but partially functional protein is currently seen as the most promising of the gene modification therapies for DMD (Aartsma-Rus & van Ommen, 2009). Specifically, AON-induced single-exon skipping of exon 51 has been a major research focus. The unpublished results of the independent phase I/II clinical trials in the Netherlands and the United Kingdom, as well as the preliminary outcomes of the phase IIb clinical trials have shown good tolerability and induction of dystrophin expression (van Deutekom et al., 2007; Guglieri & Bushby, 2010). It is estimated that approximately 15% of all DMD patients, could benefit from skipping exon 51, though unfortunately, by virtue of the cohort selection, none of the patients in this study could benefit from this particular therapy, as it targets patients with exonic deletions in the 3' deletion hotspot.

To provide a truly personalised gene-based therapy many different AONs must be designed and tested according to the nature and extent of the primary lesion. This will however result in creation of many types of truncated dystrophin of variable

functional activity and effect on the phenotype. Also, the new exon junctions may create novel epitopes with immunogenic properties (Lu et al., 2003). The design, testing and approval of a range of AONs therefore, carriers with it the obvious issues related to cost and applicability.

Multiexon skipping, on the other hand, where a single cocktail of AONs could be applied to a range of mutations, is an alternative approach to single exon skipping and could hold a significant clinical impact. Applied to the findings in this project, skipping of exons 6 - 8 could theoretically be beneficial to 5 (~30%) of the 16 mutation-positive patients: DMD414.1PHI, DMD552.1RIV, DMD550.1SHA, DMD83.1MTH and DMD389.1PIE). Successful proof of concept experiments for multiexon skipping of exons 6 – 8 have been conducted in the CXDM dog with a spice mutation in exon 7 (exons 6 – 8). The experiment on the CXMD dog, achieved widespread rescue of dystrophin expression with weekly or biweekly systemic intravenous injections of a three-morpholino cocktail over the course of 5 to 22 weeks. Interestingly, in the same study, Yokota et al. (2009) described spontaneous skipping of exon 9 along with exons 6, 7 and 8, consistently achieved with and without AONs for exon 9. If a similar effect is seen in human patients, DMD83.1MTH with a frameshift mutation in exon 9 may also benefit from this therapy (Mitrpant et al., 2009; Yokota et al., 2009).

Another 2 patients (12.5%) in this study cohort could benefit from the already experimentally designed (though not yet unsuccessfully tested), therapy with a cocktail of AONs for multiexon skipping of exons 45 – 55 (DMD411.1PET and DMD357.1MAS (van Vliet et al., 2008).

Exonic duplications, such as those detected in patients DMD530.1THA and DMD420.1SIY in this cohort, are thought to present an excellent target for exon skipping therapy, as AON-induced splicing out of duplicated exons (but not the constitutive exons), should result in restoration of the wild type message and protein (Aartsma-Rus et al., 2007; Gurvich et al., 2008). Also, exon skipping of the duplicated exons and restoration of the wild type does not carry with it the possible functional variability or the immunological risks of the newly created truncated protein. A similar principle applies to pseudoexon mutations (none were detected in this study cohort), as AON-induced exon skipping of intronic sequences should also restore production of the wild type protein.

Besides the importance of designing AONs capable of achieving sufficient levels of exon skipping for a therapeutic effect and are target-specific with good safety and tolerability profiles, a major challenge lies in optimization of systemic AON delivery. Also, in order to optimize AON target sequences in humans, a better understanding is required of the mechanisms through which antisequences interfere with RNA splicing. Another current limitation of AON therapy is its inability to target the heart in the mdx mouse at doses suggested for clinical applications (Townsend et al., 2008). Recent studies have suggested incorporation of peptide or chemical moieties with the AONs, to facilitate cell uptake and prevent disruption of cardiac sarcolemma, cardiac hypertrophy and dysfunction in mdx mice (Townsend et al., 2008; Wu et al., 2008; Yin et al., 2008).

Another, though more distant alternative to exon skipping for some patients, is the oligonucleotide-mediated gene editing or gene repair, which aims at correcting in-frame point mutations such as the 3 nonsense mutations found in this study cohort as well as the splice mutation in intron 18 (c.2293-1G>A), and the missense mutation in exon 7 (c.587T>C). This is an attractive option, as the repair takes place at the genomic level and as such, should permanently restore of the wild type protein. However, much research is still required for optimal ODN design and improved efficacy and efficiency of delivery and the resulting levels of expression (Bertoni, 2005; Robinson, 2009).

The 3 patients in this study cohort shown to carry nonsense mutations, could also be eligible for potential readthrough therapy (DMD552.1RIC, DMD352.2BRE and DMD411.1PET). Recently much hope was placed on the results of the double blind, placebo-controlled phase IIb clinical trial for Ataluren, shown to be an effective read-through agent for nonsense mutations *in vitro* and in animal models (Welch et al., 2007). The trial results were disappointing, with no statistically significant change in the primary endpoint of the 6 minute walking distance, noted within the 48 week duration of the study (Guglieri & Bushby, 2010). However, the data gathered on the effect of the therapy on the subgroup of patients receiving a lower drug dose is under further scrutiny, leaving a glimmer of hope for future studies of similar compounds (www.ptcbio.com). In this vein, a newly developed aminoglycoside (NB54) is under investigation as a readthrough agent, with a higher read-through efficacy and an improved toxicity profile in different cell lines, as compared to gentamicin (which was the initial stimulus for this line of research) (Nudelman et al., 2009). Two of the patients with nonsense mutations detected here (c.620T>G and c.4729C>T), carry TGA stop codons (though neither has a pyrimidine base in the +1

position) which showed the best readthrough response in animal and muscle cell culture models for both gentamicin and Ataluren studies, making them good candidates for possible future application of readthrough therapies (Welch et al., 2007; Peltz et al., 2009).

The therapeutic options for 2 patients in this cohort were difficult to predict. The point substitution in the Dp427 promoter/exon 1 of patient DMD548.1THE presents a dilemma, due to the uncertain downstream effect of this mutation, which could only be elucidated by mRNA from muscle tissue. Gene editing or in particular gene replacement therapy which is currently undergoing intensive research with hopeful results, could present a possible option (Trollet et al., 2009). The large deletion of exons 61 – 79, on the other hand, which may extend beyond the *DMD* gene, makes patient DMD306.1GV ineligible for any of the gene-modification therapies discussed above, since the entire 3' end of the gene is deleted. If the mutation extends beyond the *DMD* gene, even its replacement may be of limited value, as the disease phenotype may be linked to other gene/s.

It must be emphasised that the prediction regarding eligibility for therapy were made superficially, using results of DNA-based analyses which do not necessarily reflect the mutational effect on the transcript. It was a purely academic exercise of interest, in view of the current research and clinical trials. Also, consideration was given mostly to gene-modification approaches closer to clinical application at this time, as opposed to gene replacement or progenitor cell therapies which have shown promising results in animal models but are more distant from clinical application (Harper et al., 2002; Guglieri & Bushby, 2010).

Application of the emerging personalized, genetic therapies for DMD begins with precise mutation detection and knowledge of the downstream effects of this mutation in each patient. This information can be obtained only by studying the mRNA from the primary expression site of the gene i.e. muscle, in each DMD patient, as it has been shown that similar mutations detected in DNA, can have different downstream effects in different patients, even within the same families (Muntoni et al., 2003a; Aartsma-Rus et al., 2006). Sampling a muscle biopsy however, is an invasive procedure, which can be traumatic to a child and which carries its own risks. For this reason clinical protocols in many centres recommend taking biopsy specimens only from patients who require confirmation of diagnosis with immunohistochemistry and dystrophin staining i.e. those with no genetic diagnosis.

It is therefore likely, that until genetic therapy becomes a reality, testing of DNA from blood lymphocytes will remain the mainstay of genetic diagnostics in DMD. There is however every indication that gene therapy is within sight, making the effort of establishing methodologies and protocols for detection of both large and small lesions in the *DMD* gene mandatory, albeit limited to one centre in the country. The information gained with DNA testing in the meanwhile, can be used for improved genetic counselling of the families and will give an edge when the time comes for therapy assessment and additional testing of muscle biopsy material. While the findings of this study may not have a direct therapeutic impact on many of the patients tested in this study, the information gathered here may serve their families in the not-so-distant future.

CHAPTER 5: CONCLUDING REMARKS

Since the initial description of Duchenne and Becker muscular dystrophy in 1851, much progress has been made in establishing intervention strategies for this debilitating childhood disorder. While a cure remains elusive, the currently available treatments focus on early diagnosis and timeous management of symptoms and complications, allowing many D/BMD patients to live productively into their third decade.

The development of genetic therapies for DMD adopting the traditional gene replacement approach has been slow and fraught with setbacks, though it has seen a revival in recent years with mini- and micro-dystrophin or full-length utrophin replacement showing promise. At this time, however, the gene modification avenue appears closest to clinical application, with AON-induced exon skipping and nonsense read-through reaching the stage of clinical trials.

As opposed to gene replacement, the gene modification therapy is mutation-specific and as such, goes hand in hand with mutation detection. Determining eligibility and taylor-making of a particular brand of therapy is wholly reliant on the precise characterization of the disease-causing mutation and its downstream effects, necessitating availability of comprehensive diagnostic testing protocols.

Since the identification and characterization of the *DMD* gene in 1987 (Koenig et al., 1987), technological advancements in molecular science have greatly influenced diagnostic testing protocols. Current methods for detection of exonic rearrangements, which cause the disease in 70% of all B/DMD patients, offer highly accurate diagnostic testing and carrier status assessment for female relatives. Detection of small/point mutations, implicated in 30% of all B/DMD cases, has however been slow in reaching the diagnostic setting, due to the logistical and financial issues involved in interrogation of the vast *DMD* gene. While not yet included in most diagnostic protocols, point mutation detection has been the focus of a considerable amount of research and development in recent years, particularly in view of the prospects for availability of gene-based therapies for DMD.

With that in mind, the main focus of this study lay in detection and characterisation of all pathogenic mutations in the coding region of the *DMD* gene by screening for both exonic rearrangements and small/point mutations in each of the 24 patients of the cohort. Detection of small/point mutations was a particularly significant aspect of

the study, as the methodology and the technological platform employed (hrMCA), if successful, was to result in prompt translation into the local diagnostic service for DMD, complementary to the already offered MLPA® screen for large lesions. Detection of 10 pathogenic mutations with hrMCA was of special significance in this study not only because it provided the patients and their families with previously unavailable genetic diagnoses, but also because it signified successful fulfilment of its main aim. However, it must be emphasised that while research involving point mutation detection in the *DMD* gene has been conducted in this centre before, this is the first study in South Africa, to take an all-encompassing approach of profiling all mutations in the coding region of the *DMD* gene i.e. point mutations, as well as exonic rearrangements.

Availability of genetic diagnosis plays an important part in the clinical protocol, as early testing allows for effective monitoring and timely intervention, prolonging the patient's ambulation and life span. In cases with no family history, knowledge of the mutation type and its location could also help to anticipate the severity of the disorder and aid in adoption of appropriate patient management strategies. Also, availability of effective testing for carrier status is invaluable to female relatives, especially the mothers of isolated cases, whose carrier status could only be established empirically in the past. Implementation of the MLPA® at the NHLS GSH, with its quantitative component, has made a significant impact in carrier testing, as the referral base consists largely of isolated DMD cases. Extending the service further to small/point mutation detection with hrMCA, will offer additional testing options to deletion/duplication negative patients and their families, providing previously unavailable information and a more comprehensive genetic service for DMD.

The experience of this study also brought forth the increasing role of bioinformatic analyses and the need for bioinformatic training in the diagnostic environment, particularly in view of the "private" nature of the small/point mutations found in the *DMD* gene. While certain types of mutations are clearly disease-causing e.g. nonsense and frameshifts, their precise downstream effects as well as the disease association of other types of changes eg. missense, are difficult to determine in the absence of RNA-based studies. This also applies to characterisation of the effect of intronic mutations, the detection of which is becoming more frequent with the availability of ever improving technological platforms, and which have also been shown to play a part in the disease pathogenesis of some *DMD* patients. Analysis of n*ovel*, previously uncharacterised changes requires competency in using

bioinformatic software and ability to interpret the output, which are skills still largely confined to the research environment.

The superior nature of RNA extracted from muscle material, as the basis for definitive determination of the downstream effects of mutations was repeatedly highlighted in this study. Future laboratory protocols for ascertaining eligibility for mutation-based therapies are likely to incorporate RNA analysis as an essential part of the process. This will necessarily lead to changes in clinical protocols regarding biopsy sampling, as at present, muscle biopsies in DMD patients are not routinely performed due to the invasive nature of the procedure. Until such time however, DNA extracted from blood lymphocytes is likely to remain the mainstay of diagnostics testing and the results thus obtained will be of value to the patients and their families now, and at a time of more extensive therapy eligibility testing in the future.

The main findings of this study include:

- 16 pathogenic mutations detected in respective patients:
  - 6 exonic rearrangements (4 deletions and 2 duplications)
  - 10 small/point mutations (8 *novel*):
    - 3 nonsense
    - 3 frameshits
    - 1 splice
    - 1 compound mutation (a GGTG duplication and a missense)
    - 1 missense
    - 1 point substitution in the Dp427m/exon1 promoter region of the *DMD* gene.
- 39 polymorphisms
- 4 changes of uncertain significance

The work presented in this thesis provides the foundation for establishing comprehensive and specific diagnostic tests for each DMD family. It also provides the basis for further research efforts into defining the effects of the changes found at the transcript level. Furthermore, defining mutations underlying the DMD phenotype in the South African population will also facilitate establishing trends within the various population subgroups of South Africa, possibly correlating molecular features to the disease course and facilitating a more strategised approach to the clinical and laboratory diagnostic protocols. The knowledge of the disease-causing mutations detected during this study, provides patients and their families with a

considerable head-start in the future, when mutation-based genetic therapy becomes a veritable therapeutic avenue. Also, the experience and results obtained by employing hrMCA as a mutation scanning tool, demonstrated its aptitude for high throughput mutation detection in a sensitive, yet cost efficient way, thus presenting a possible method of choice for point mutation detection in the *DMD* gene, at this time.

In conclusion, the promising outcomes of research into several strategies of gene-based therapy for DMD show every indication of crossing the divide between the laboratory and the clinic in the not-so-distant future. Availability of a comprehensive testing protocol, capable of determining the genetic basis of the disease in most patients is therefore emphasized as a primary requirement in determining suitability for any mutation-based therapy. The resulting broader scope and range of testing will necessarily increase the detection rate, providing previously unavailable genetic diagnoses, better family counseling and definitive determination of carrier status for female relatives. The information gained as a result, will help fill the hollows in the international DMD mutation databases and increase the body of knowledge on the incidence and prevalence of mutation types and their effects on the gene, the protein and the phenotype. Thus gathered data will add to the general body of knowledge from which future progress and treatments will follow.

References

Aartsma-Rus, A., Janson, A. A., van Ommen, G. J., & van Deutekom, J. C. (2007). Antisense-induced exon skipping for duplications in Duchenne muscular dystrophy. *BMC Medical Genetics, 8*, 43. doi:10.1186/1471-2350-8-43

Aartsma-Rus, A., Van Deutekom, J. C., Fokkema, I. F., Van Ommen, G. J., & Den Dunnen, J. T. (2006). Entries in the Leiden Duchenne muscular dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule. *Muscle & Nerve, 34*(2), 135-144. doi:10.1002/mus.20586

Aartsma-Rus, A., & van Ommen, G. J. (2009). Less is more: therapeutic exon skipping for Duchenne muscular dystrophy. *Lancet Neurology, 8*(10), 873-875. doi:10.1016/S1474-4422(09)70229-7

Abbs, S., Tuffery-Giraud, S., Bakker, E., Ferlini, A., Sejersen, T., & Mueller, C. R. (2010). Best Practice Guidelines on molecular diagnostics in Duchenne/Becker muscular dystrophies. *Neuromuscular Disorders : NMD,* doi:10.1016/j.nmd.2010.04.005

Allamand, V., Bidou, L., Arakawa, M., Floquet, C., Shiozuka, M., Paturneau-Jouas, M., Gartioux, C., Butler-Browne, G. S., Mouly, V., Rousset, J. P., Matsuda, R., Ikeda, D., & Guicheney, P. (2008). Drug-induced readthrough of premature stop codons leads to the stabilization of laminin alpha2 chain mRNA in CMD myotubes. *The Journal of Gene Medicine, 10*(2), 217-224. doi:10.1002/jgm.1140

Almomani, R., van der Stoep, N., Bakker, E., den Dunnen, J. T., Breuning, M. H., & Ginjaar, I. B. (2009). Rapid and cost effective detection of small mutations in the DMD gene by high resolution melting curve analysis. *Neuromuscular Disorders, 19*(6), 383-390. doi:DOI: 10.1016/j.nmd.2009.03.004

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research, 25*(17), 3389-3402.

Andersen, M. S., Sorensen, C. B., Bolund, L., & Jensen, T. G. (2002). Mechanisms underlying targeted gene correction using chimeric RNA/DNA and single-stranded DNA oligonucleotides. *Journal of Molecular Medicine (Berlin, Germany), 80*(12), 770-781. doi:10.1007/s00109-002-0393-8

Arahata, K., Ishiura, S., Ishiguro, T., Tsukahara, T., Suhara, Y., Eguchi, C., Ishihara, T., Nonaka, I., Ozawa, E., & Sugita, H. (1988). Immunostaining of skeletal and cardiac muscle surface membrane with antibody against Duchenne muscular dystrophy peptide. *Nature, 333*(6176), 861-863. doi:10.1038/333861a0

Ashton, E. J., Yau, S. C., Deans, Z. C., & Abbs, S. J. (2008). Simultaneous mutation scanning for gross deletions, duplications and point mutations in the DMD gene. *European Journal of Human Genetics : EJHG, 16*(1), 53-61. doi:10.1038/sj.ejhg.5201916

Auld, D. S., Thorne, N., Maguire, W. F., & Inglese, J. (2009). Mechanism of PTC124 activity in cell-based luciferase assays of nonsense codon suppression. *Proceedings of the National Academy of Sciences of the United States of America, 106*(9), 3585-3590. doi:10.1073/pnas.0813345106

Bakker, E., Hofker, M. H., Goor, N., Mandel, J. L., Wrogemann, K., Davies, K. E., Kunkel, L. M., Willard, H. F., Fenton, W. A., & Sandkuyl, L. (1985). Prenatal diagnosis and

carrier detection of Duchenne muscular dystrophy with closely linked RFLPs. *Lancet, 1*(8430), 655-658.

Bakker, E., Veenema, H., Den Dunnen, J. T., van Broeckhoven, C., Grootscholten, P. M., Bonten, E. J., van Ommen, G. J., & Pearson, P. L. (1989). Germinal mosaicism increases the recurrence risk for 'new' Duchenne muscular dystrophy mutations. *Journal of Medical Genetics, 26*(9), 553-559.

Banks, G. B., & Chamberlain, J. S. (2008). The value of mammalian models for duchenne muscular dystrophy in developing therapeutic strategies. *Current Topics in Developmental Biology, 84*, 431-453. doi:10.1016/S0070-2153(08)00609-1

Bardoni, A., Felisari, G., Sironi, M., Comi, G., Lai, M., Robotti, M., & Bresolin, N. (2000). Loss of Dp140 regulatory sequences is associated with cognitive impairment in dystrophinopathies. *Neuromuscular Disorders : NMD, 10*(3), 194-199.

Barton-Davis, E. R., Cordier, L., Shoturma, D. I., Leland, S. E., & Sweeney, H. L. (1999). Aminoglycoside antibiotics restore dystrophin function to skeletal muscles of mdx mice. *The Journal of Clinical Investigation, 104*(4), 375-381. doi:10.1172/JCI7866

Baumbach, L. L., Chamberlain, J. S., Ward, P. A., Farwell, N. J., & Caskey, C. T. (1989). Molecular and clinical correlations of deletions leading to Duchenne and Becker muscular dystrophies. *Neurology, 39*(4), 465-474.

Becker, P. E., & KIENER, F. (1955). A new x-chromosomal muscular dystrophy. [Eine neue x-chromosomale Muskeldystrophie] *Archiv Fur Psychiatrie Und Nervenkrankheiten, Vereinigt Mit Zeitschrift Fur Die Gesamte Neurologie Und Psychiatrie, 193*(4), 427-448.

Beggs, A. H., Hoffman, E. P., Snyder, J. R., Arahata, K., Specht, L., Shapiro, F., Angelini, C., Sugita, H., & Kunkel, L. M. (1991). Exploring the molecular basis for variability among patients with Becker muscular dystrophy: dystrophin gene and protein studies. *American Journal of Human Genetics, 49*(1), 54-67.

Beggs, A. H., Koenig, M., Boyce, F. M., & Kunkel, L. M. (1990). Detection of 98% of DMD/BMD gene deletions by polymerase chain reaction. *Human Genetics, 86*(1), 45-48.

Beggs, A. H., & Kunkel, L. M. (1990). A polymorphic CACA repeat in the 3' untranslated region of dystrophin. *Nucleic Acids Research, 18*(7), 1931.

Behm-Ansmant, I., Kashima, I., Rehwinkel, J., Sauliere, J., Wittkopp, N., & Izaurralde, E. (2007). mRNA quality control: an ancient machinery recognizes and degrades mRNAs with nonsense codons. *FEBS Letters, 581*(15), 2845-2853. doi:10.1016/j.febslet.2007.05.027

Bennett, R. R., den Dunnen, J., O'Brien, K. F., Darras, B. T., & Kunkel, L. M. (2001). Detection of mutations in the dystrophin gene via automated DHPLC screening and direct sequencing. *BMC Genetics, 2*, 17.

Beroud, C., Tuffery-Giraud, S., Matsuo, M., Hamroun, D., Humbertclaude, V., Monnier, N., Moizard, M. P., Voelckel, M. A., Calemard, L. M., Boisseau, P., Blayau, M., Philippe, C., Cossee, M., Pages, M., Rivier, F., Danos, O., Garcia, L., & Claustres, M. (2007). Multiexon skipping leading to an artificial DMD protein lacking amino acids from exons 45 through 55 could rescue up to 63% of patients with Duchenne muscular dystrophy. *Human Mutation, 28*(2), 196-202. doi:10.1002/humu.20428

Béroud, C., Carrié, A., Beldjord, C., Deburgrave, N., Llense, S., Carelle, N., Peccate, C., Cuisset, J., Pandit, F., Carré-Pigeon, F., Mayer, M., Bellance, R., Récan, D., Chelly, J., Kaplan, J., & Leturcq, F. (2004). Dystrophinopathy caused by mid-intronic substitutions activating cryptic exons in the DMD gene. *Neuromuscular Disorders, 14*(1), 10-18. doi:DOI: 10.1016/S0960-8966(03)00169-X

Bertoni, C. (2005). Oligonucleotide-mediated gene editing for neuromuscular disorders. *ACTA MYOLOGICA, 24*(3), 194.

Blake, D. J., & Kroger, S. (2000a). The neurobiology of duchenne muscular dystrophy: learning lessons from muscle? *Trends in Neurosciences, 23*(3), 92-99.

Blake, D. J., & Kroger, S. (2000b). The neurobiology of duchenne muscular dystrophy: learning lessons from muscle? *Trends in Neurosciences, 23*(3), 92-99.

Blake, D. J., Weir, A., Newey, S. E., & Davies, K. E. (2002). Function and genetics of dystrophin and dystrophin-related proteins in muscle. *Physiological Reviews, 82*(2), 291-329. doi:10.1152/physrev.00028.2001

Blankinship, M. J., Gregorevic, P., & Chamberlain, J. S. (2006). Gene therapy strategies for Duchenne muscular dystrophy utilizing recombinant adeno-associated virus vectors. *Molecular Therapy : The Journal of the American Society of Gene Therapy, 13*(2), 241-249. doi:10.1016/j.ymthe.2005.11.001

Bovolenta, M., Neri, M., Fini, S., Fabris, M., Trabanelli, C., Venturoli, A., Martoni, E., Bassi, E., Spitali, P., Brioschi, S., Falzarano, M. S., Rimessi, P., Ciccone, R., Ashton, E., McCauley, J., Yau, S., Abbs, S., Muntoni, F., Merlini, L., Gualandi, F., & Ferlini, A. (2008). A novel custom high density-comparative genomic hybridization array detects common rearrangements as well as deep intronic mutations in dystrophinopathies. *BMC Genomics, 9*, 572. doi:10.1186/1471-2164-9-572

Burghes, A. H., Logan, C., Hu, X., Belfall, B., Worton, R. G., & Ray, P. N. (1987). A cDNA clone from the Duchenne/Becker muscular dystrophy gene. *Nature, 328*(6129), 434-437. doi:10.1038/328434a0

Bushby, K., Bourke, J., Bullock, R., Eagle, M., Gibson, M., & Quinby, J. (2005). The multidisciplinary management of Duchenne muscular dystrophy. *Current Paediatrics, 15*(4), 292-300. doi:DOI: 10.1016/j.cupe.2005.04.001

Bushby, K., Finkel, R., Birnkrant, D. J., Case, L. E., Clemens, P. R., Cripe, L., Kaul, A., Kinnett, K., McDonald, C., Pandya, S., Poysky, J., Shapiro, F., Tomezsko, J., Constantin, C., & DMD Care Considerations Working Group. (2010a). Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and pharmacological and psychosocial management. *Lancet Neurology, 9*(1), 77-93. doi:10.1016/S1474-4422(09)70271-6

Bushby, K., Finkel, R., Birnkrant, D. J., Case, L. E., Clemens, P. R., Cripe, L., Kaul, A., Kinnett, K., McDonald, C., Pandya, S., Poysky, J., Shapiro, F., Tomezsko, J., Constantin, C., & DMD Care Considerations Working Group. (2010b). Diagnosis and management of Duchenne muscular dystrophy, part 2: implementation of multidisciplinary care. *Lancet Neurology, 9*(2), 177-189. doi:10.1016/S1474-4422(09)70272-8

Bushby, K. M., Appleton, R., Anderson, L. V., Welch, J. L., Kelly, P., & Gardner-Medwin, D. (1995). Deletion status and intellectual impairment in Duchenne muscular dystrophy. *Developmental Medicine and Child Neurology, 37*(3), 260-269.

Buzin, C. H., Feng, J., Yan, J., Scaringe, W., Liu, Q., den Dunnen, J., Mendell, J. R., & Sommer, S. S. (2005). Mutation rates in the dystrophin gene: a hotspot of mutation at a CpG dinucleotide. *Human Mutation, 25*(2), 177-188. doi:10.1002/humu.20132

Caceres, J. F., & Kornblihtt, A. R. (2002). Alternative splicing: multiple control mechanisms and involvement in human disease. *Trends in Genetics : TIG, 18*(4), 186-193.

Cardamone, M., Darras, B. T., & Ryan, M. M. (2008). Inherited myopathies and muscular dystrophies. *Seminars in Neurology, , 28*(2) 250.

Cartegni, L., Chew, S. L., & Krainer, A. R. (2002). Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nature Reviews.Genetics, 3*(4), 285-298. doi:10.1038/nrg775

Cartegni, L., Wang, J., Zhu, Z., Zhang, M. Q., & Krainer, A. R. (2003). ESEfinder: A web resource to identify exonic splicing enhancers. *Nucleic Acids Research, 31*(13), 3568-3571.

Caskey, C. T., Nussbaum, R. L., Cohan, L. C., & Pollack, L. (1980). Sporadic occurrence of Duchenne muscular dystrophy: evidence for new mutation. *Clinical Genetics, 18*(5), 329-341.

Chamberlain, J. S., Gibbs, R. A., Ranier, J. E., Nguyen, P. N., & Caskey, C. T. (1988). Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. *Nucleic Acids Research, 16*(23), 11141-11156.

Chang, Y. F., Imam, J. S., & Wilkinson, M. F. (2007). The nonsense-mediated decay RNA surveillance pathway. *Annual Review of Biochemistry, 76*, 51-74. doi:10.1146/annurev.biochem.76.050106.093909

Chatterjee, S., & Pal, J. K. (2009). Role of 5'- and 3'-untranslated regions of mRNAs in human diseases. *Biology of the Cell / Under the Auspices of the European Cell Biology Organization, 101*(5), 251-262. doi:10.1042/BC20080104

Chou, L. S., Liu, C. S., Boese, B., Zhang, X., & Mao, R. (2010). DNA sequence capture and enrichment by microarray followed by next-generation sequencing for targeted resequencing: neurofibromatosis type 1 gene as a model. *Clinical Chemistry, 56*(1), 62-72. doi:10.1373/clinchem.2009.132639

Clancy, J. P., Bebok, Z., Ruiz, F., King, C., Jones, J., Walker, L., Greer, H., Hong, J., Wing, L., Macaluso, M., Lyrene, R., Sorscher, E. J., & Bedwell, D. M. (2001). Evidence that systemic gentamicin suppresses premature stop mutations in patients with cystic fibrosis. *American Journal of Respiratory and Critical Care Medicine, 163*(7), 1683-1692.

Costa, M. F., Oliveira, A. G., Feitosa-Santana, C., Zatz, M., & Ventura, D. F. (2007). Red-green color vision impairment in Duchenne muscular dystrophy. *American Journal of Human Genetics, 80*(6), 1064-1075. doi:10.1086/518127

Cotton, S., Voudouris, N. J., & Greenwood, K. M. (2001). Intelligence and Duchenne muscular dystrophy: full-scale, verbal, and performance intelligence quotients. *Developmental Medicine and Child Neurology, 43*(7), 497-501.

Cotton, S. M., Voudouris, N. J., & Greenwood, K. M. (2005). Association between intellectual functioning and age in children and young adults with Duchenne

muscular dystrophy: further results from a meta-analysis. *Developmental Medicine and Child Neurology, 47*(4), 257-265.

Crawford, G. E., Faulkner, J. A., Crosbie, R. H., Campbell, K. P., Froehner, S. C., & Chamberlain, J. S. (2000). Assembly of the dystrophin-associated protein complex does not require the dystrophin COOH-terminal domain. *The Journal of Cell Biology, 150*(6), 1399-1410.

Dalkilic, I., & Kunkel, L. M. (2003). Muscular dystrophies: genes to pathogenesis. *Current Opinion in Genetics & Development, 13*(3), 231-238.

Davies, K. E., Pearson, P. L., Harper, P. S., Murray, J. M., O'Brien, T., Sarfarazi, M., & Williamson, R. (1983). Linkage analysis of two cloned DNA sequences flanking the Duchenne muscular dystrophy locus on the short arm of the human X chromosome. *Nucleic Acids Research, 11*(8), 2303-2312.

Davis, R. L., Weintraub, H., & Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell, 51*(6), 987-1000.

Deburgrave, N., Daoud, F., Llense, S., Barbot, J. C., Recan, D., Peccate, C., Burghes, A. H., Beroud, C., Garcia, L., Kaplan, J. C., Chelly, J., & Leturcq, F. (2007). Protein- and mRNA-based phenotype-genotype correlations in DMD/BMD with point mutations and molecular basis for BMD with nonsense and frameshift mutations in the DMD gene. *Human Mutation, 28*(2), 183-195. doi:10.1002/humu.20422

Deconinck, N., & Dan, B. (2007). Pathophysiology of Duchenne Muscular Dystrophy: Current Hypotheses. *Pediatric Neurology, 36*(1), 1-7. doi:DOI: 10.1016/j.pediatrneurol.2006.09.016

del Gaudio, D., Yang, Y., Boggs, B. A., Schmitt, E. S., Lee, J. A., Sahoo, T., Pham, H. T., Wiszniewska, J., Chinault, A. C., Beaudet, A. L., & Eng, C. M. (2008). Molecular diagnosis of Duchenne/Becker muscular dystrophy: enhanced detection of dystrophin gene rearrangements by oligonucleotide array-comparative genomic hybridization. *Human Mutation, 29*(9), 1100-1107. doi:10.1002/humu.20841

den Dunnen, J. T., & Antonarakis, S. E. (2000). Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. *Human Mutation, 15*(1), 7-12. doi:2-N

Desmet, F. O., Hamroun, D., Lalande, M., Collod-Beroud, G., Claustres, M., & Beroud, C. (2009). Human Splicing Finder: an online bioinformatics tool to predict splicing signals. *Nucleic Acids Research, 37*(9), e67. doi:10.1093/nar/gkp215

Disset, A., Bourgeois, C. F., Benmalek, N., Claustres, M., Stevenin, J., & Tuffery-Giraud, S. (2006). An exon skipping-associated nonsense mutation in the dystrophin gene uncovers a complex interplay between multiple antagonistic splicing elements. *Human Molecular Genetics, 15*(6), 999-1013. doi:10.1093/hmg/ddl015

Dubourg, C., Odent, S., Fergelot, P., Le Gall, J. Y., David, V., & Blayau, M. (1999). Identification of three novel mutations in the dystrophin gene detected by the heteroduplex/SSCA screening procedure. Mutations in brief no. 222. Online. *Human Mutation, 13*(2), 173. doi:2-3

Dwi Pramono, Z. A., Takeshima, Y., Surono, A., Ishida, T., & Matsuo, M. (2000). A novel cryptic exon in intron 2 of the human dystrophin gene evolved from an intron by acquiring consensus sequences for splicing at different stages of anthropoid

evolution. *Biochemical and Biophysical Research Communications, 267*(1), 321-328. doi:10.1006/bbrc.1999.1962

Emery, A. (2001). Duchenne muscular dystrophy or Meryon's disease. *Lancet, 357*(9267), 1529. doi:10.1016/S0140-6736(00)04681-X

Emery, A. E. (2002). The muscular dystrophies. *Lancet, 359*(9307), 687-695. doi:10.1016/S0140-6736(02)07815-7

Emery, A. E., & Emery, M. L. (1995). The history of a genetic disease: Duchenne muscular dystrophy or Meryon's disease. *Royal Society of Medicine Press,*

England, S. B., Nicholson, L. V., Johnson, M. A., Forrest, S. M., Love, D. R., Zubrzycka-Gaarn, E. E., Bulman, D. E., Harris, J. B., & Davies, K. E. (1990). Very mild muscular dystrophy associated with the deletion of 46% of dystrophin. *Nature, 343*(6254), 180-182. doi:10.1038/343180a0

Ervasti, J. M., & Campbell, K. P. (1991). Membrane organization of the dystrophin-glycoprotein complex. *Cell, 66*(6), 1121-1131.

Ervasti, J. M., Kahl, S. D., & Campbell, K. P. (1991). Purification of dystrophin from skeletal muscle. *The Journal of Biological Chemistry, 266*(14), 9161-9165.

Fairbrother, W. G., Yeo, G. W., Yeh, R., Goldstein, P., Mawson, M., Sharp, P. A., & Burge, C. B. (2004). RESCUE-ESE identifies candidate exonic splicing enhancers in vertebrate exons. *Nucleic Acids Research, 32*(Web Server issue), W187-90. doi:10.1093/nar/gkh393

Farini, A., Razini, P., Erratico, S., Torrente, Y., & Meregalli, M. (2009). Cell based therapy for Duchenne muscular dystrophy. *Journal of Cellular Physiology, 221*(3), 526-534. doi:10.1002/jcp.21895

Felisari, G., Martinelli Boneschi, F., Bardoni, A., Sironi, M., Comi, G. P., Robotti, M., Turconi, A. C., Lai, M., Corrao, G., & Bresolin, N. (2000). Loss of Dp140 dystrophin isoform and intellectual impairment in Duchenne dystrophy. *Neurology, 55*(4), 559-564.

Ferlini, A., Galie, N., Merlini, L., Sewry, C., Branzi, A., & Muntoni, F. (1998). A novel Alu-like element rearranged in the dystrophin gene causes a splicing mutation in a family with X-linked dilated cardiomyopathy. *American Journal of Human Genetics, 63*(2), 436-446. doi:10.1086/301952

Flanigan, K. M., Dunn, D. M., von Niederhausern, A., Soltanzadeh, P., Gappmaier, E., Howard, M. T., Sampson, J. B., Mendell, J. R., Wall, C., King, W. M., Pestronk, A., Florence, J. M., Connolly, A. M., Mathews, K. D., Stephan, C. M., Laubenthal, K. S., Wong, B. L., Morehart, P. J., Meyer, A., Finkel, R. S., Bonnemann, C. G., Medne, L., Day, J. W., Dalton, J. C., Margolis, M. K., Hinton, V. J., Weiss, R. B., & the United Dystrophinopathy Project ConsortiumOther authors (members of the UDP Consortium) are listed at the end of the article. (2009). Mutational spectrum of DMD mutations in dystrophinopathy patients: application of modern diagnostic techniques to a large cohort. *Human Mutation, 30*(12), 1657-1666. doi:10.1002/humu.21114

Flanigan, K. M., von Niederhausern, A., Dunn, D. M., Alder, J., Mendell, J. R., & Weiss, R. B. (2003). Rapid direct sequence analysis of the dystrophin gene. *American Journal of Human Genetics, 72*(4), 931-939.

Fletcher, S., Honeyman, K., Fall, A. M., Harding, P. L., Johnsen, R. D., Steinhaus, J. P., Moulton, H. M., Iversen, P. L., & Wilton, S. D. (2007). Morpholino oligomer-mediated exon skipping averts the onset of dystrophic pathology in the mdx mouse. *Molecular Therapy : The Journal of the American Society of Gene Therapy, 15*(9), 1587-1592. doi:10.1038/sj.mt.6300245

Francke, U., Ochs, H. D., de Martinville, B., Giacalone, J., Lindgren, V., Disteche, C., Pagon, R. A., Hofker, M. H., van Ommen, G. J., & Pearson, P. L. (1985). Minor Xp21 chromosome deletion in a male associated with expression of Duchenne muscular dystrophy, chronic granulomatous disease, retinitis pigmentosa, and McLeod syndrome. *American Journal of Human Genetics, 37*(2), 250-267.

Franz, W. M., Muller, M., Muller, O. J., Herrmann, R., Rothmann, T., Cremer, M., Cohn, R. D., Voit, T., & Katus, H. A. (2000). Association of nonsense mutation of dystrophin gene with disruption of sarcoglycan complex in X-linked dilated cardiomyopathy. *Lancet, 355*(9217), 1781-1785. doi:10.1016/S0140-6736(00)02266-2

Frederic, M. Y., Lalande, M., Boileau, C., Hamroun, D., Claustres, M., Beroud, C., & Collod-Beroud, G. (2009). UMD-predictor, a new prediction tool for nucleotide substitution pathogenicity -- application to four genes: FBN1, FBN2, TGFBR1, and TGFBR2. *Human Mutation, 30*(6), 952-959. doi:10.1002/humu.20970

Gamper, H. B.,Jr, Cole-Strauss, A., Metz, R., Parekh, H., Kumar, R., & Kmiec, E. B. (2000). A plausible mechanism for gene correction by chimeric oligonucleotides. *Biochemistry, 39*(19), 5808-5816.

Ginjaar, I. B., Kneppers, A. L., v d Meulen, J. D., Anderson, L. V., Bremmer-Bout, M., van Deutekom, J. C., Weegenaar, J., den Dunnen, J. T., & Bakker, E. (2000). Dystrophin nonsense mutation induces different levels of exon 29 skipping and leads to variable phenotypes within one BMD family. *European Journal of Human Genetics : EJHG, 8*(10), 793-796. doi:10.1038/sj.ejhg.5200535

Goyenvalle, A., Babbs, A., van Ommen, G. J., Garcia, L., & Davies, K. E. (2009). Enhanced exon-skipping induced by U7 snRNA carrying a splicing silencer sequence: Promising tool for DMD therapy. *Molecular Therapy : The Journal of the American Society of Gene Therapy, 17*(7), 1234-1240. doi:10.1038/mt.2009.113

Greener, M. J., Sewry, C. A., Muntoni, F., & Roberts, R. G. (2002a). The 3'-untranslated region of the dystrophin gene - conservation and consequences of loss. *European Journal of Human Genetics : EJHG, 10*(7), 413-420. doi:10.1038/sj.ejhg.5200822

Greener, M. J., Sewry, C. A., Muntoni, F., & Roberts, R. G. (2002b). The 3'-untranslated region of the dystrophin gene - conservation and consequences of loss. *European Journal of Human Genetics : EJHG, 10*(7), 413-420. doi:10.1038/sj.ejhg.5200822

Gualandi, F., Neri, M., Bovolenta, M., Martoni, E., Rimessi, P., Fini, S., Spitali, P., Fabris, M., Pane, M., Angelini, C., Mora, M., Morandi, L., Mongini, T., Bertini, E., Ricci, E., Vattemi, G., Mercuri, E., Merlini, L., & Ferlini, A. (2009). Transcriptional behavior of DMD gene duplications in DMD/BMD males. *Human Mutation, 30*(2), E310-9. doi:10.1002/humu.20881

Gualandi, F., Trabanelli, C., Rimessi, P., Calzolari, E., Toffolatti, L., Patarnello, T., Kunz, G., Muntoni, F., & Ferlini, A. (2003). Multiple exon skipping and RNA circularisation contribute to the severe phenotypic expression of exon 5 dystrophin deletion. *Journal of Medical Genetics, 40*(8), e100.

Guglieri, M., & Bushby, K. (2010). Molecular treatments in Duchenne muscular dystrophy. *Current Opinion in Pharmacology, 10*, 331-337.

Gurvich, O. L., Tuohy, T. M., Howard, M. T., Finkel, R. S., Medne, L., Anderson, C. B., Weiss, R. B., Wilton, S. D., & Flanigan, K. M. (2008). DMD pseudoexon mutations: splicing efficiency, phenotype, and potential therapy. *Annals of Neurology, 63*(1), 81-89. doi:10.1002/ana.21290

Haldane, J. B. (2004). The rate of spontaneous mutation of a human gene. 1935. *Journal of Genetics, 83*(3), 235-244.

Hamed, S. A., & Hoffman, E. P. (2006). Automated sequence screening of the entire dystrophin cDNA in Duchenne dystrophy: point mutation detection. *American Journal of Medical Genetics.Part B, Neuropsychiatric Genetics : The Official Publication of the International Society of Psychiatric Genetics, 141B*(1), 44-50. doi:10.1002/ajmg.b.30234

Harper, S. Q., Hauser, M. A., DelloRusso, C., Duan, D., Crawford, R. W., Phelps, S. F., Harper, H. A., Robinson, A. S., Engelhardt, J. F., Brooks, S. V., & Chamberlain, J. S. (2002). Modular flexibility of dystrophin: implications for gene therapy of Duchenne muscular dystrophy. *Nature Medicine, 8*(3), 253-261. doi:10.1038/nm0302-253

Hegde, M. R., Chin, E. L., Mulle, J. G., Okou, D. T., Warren, S. T., & Zwick, M. E. (2008). Microarray-based mutation detection in the dystrophin gene. *Human Mutation, 29*(9), 1091-1099. doi:10.1002/humu.20831

Herrmann, M. G., Durtschi, J. D., Bromley, L. K., Wittwer, C. T., & Voelkerding, K. V. (2006). Amplicon DNA melting analysis for mutation scanning and genotyping: cross-platform comparison of instruments and dyes. *Clinical Chemistry, 52*(3), 494-503. doi:10.1373/clinchem.2005.063438

Herrmann, M. G., Durtschi, J. D., Wittwer, C. T., & Voelkerding, K. V. (2007). Expanded instrument comparison of amplicon DNA melting analysis for mutation scanning and genotyping. *Clinical Chemistry, 53*(8), 1544-1548. doi:10.1373/clinchem.2007.088120

Higuchi, R., Fockler, C., Dollinger, G., & Watson, R. (1993). Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Bio/technology (Nature Publishing Company), 11*(9), 1026-1030.

Hirawat, S., Welch, E. M., Elfring, G. L., Northcutt, V. J., Paushkin, S., Hwang, S., Leonard, E. M., Almstead, N. G., Ju, W., Peltz, S. W., & Miller, L. L. (2007). Safety, tolerability, and pharmacokinetics of PTC124, a nonaminoglycoside nonsense mutation suppressor, following single- and multiple-dose administration to healthy male and female adult volunteers. *Journal of Clinical Pharmacology, 47*(4), 430-444. doi:10.1177/0091270006297140

Hoffman, E. P., Brown, R. H.,Jr, & Kunkel, L. M. (1987). Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell, 51*(6), 919-928.

Hofstra, R. M., Mulder, I. M., Vossen, R., de Koning-Gans, P. A., Kraak, M., Ginjaar, I. B., van der Hout, A. H., Bakker, E., Buys, C. H., van Ommen, G. J., van Essen, A. J., & den Dunnen, J. T. (2004). DGGE-based whole-gene mutation scanning of the dystrophin gene in Duchenne and Becker muscular dystrophy patients. *Human Mutation, 23*(1), 57-66. doi:10.1002/humu.10283

Hoogerwaard, E., Bakker, E., Ippel, P., Oosterwijk, J., Majoor-Krakauer, D., Leschot, N., Van Essen, A., Brunner, H., Van der Wouw, P., & Wilde, A. (1999). Signs and symptoms of Duchenne muscular dystrophy and Becker muscular dystrophy among carriers in The Netherlands: a cohort study. *Lancet, 353*(9170), 2116.

Hoogerwaard, E. M., Ginjaar, I. B., Bakker, E., & de Visser, M. (2005). Dystrophin analysis in carriers of Duchenne and Becker muscular dystrophy. *Neurology, 65*(12), 1984-1986. doi:10.1212/01.wnl.0000188909.89849.59

Howard, M., Frizzell, R. A., & Bedwell, D. M. (1996). Aminoglycoside antibiotics restore CFTR function by overcoming premature stop mutations. *Nature Medicine, 2*(4), 467-469.

Hu, X. Y., Ray, P. N., Murphy, E. G., Thompson, M. W., & Worton, R. G. (1990). Duplicational mutation at the Duchenne muscular dystrophy locus: its frequency, distribution, origin, and phenotypegenotype correlation. *American Journal of Human Genetics, 46*(4), 682-695.

Igoucheva, O., Alexeev, V., & Yoon, K. (2001). Targeted gene correction by small single-stranded oligonucleotides in mammalian cells. *Gene Therapy, 8*(5), 391-399. doi:10.1038/sj.gt.3301414

Isken, O., & Maquat, L. E. (2007). Quality control of eukaryotic mRNA: safeguarding cells from abnormal mRNA function. *Genes & Development, 21*(15), 1833-1856. doi:10.1101/gad.1566807

Jorgensen, L. H., Larochelle, N., Orlopp, K., Dunant, P., Dudley, R. W., Stucka, R., Thirion, C., Walter, M. C., Laval, S. H., & Lochmuller, H. (2009). Efficient and fast functional screening of microdystrophin constructs in vivo and in vitro for therapy of duchenne muscular dystrophy. *Human Gene Therapy, 20*(6), 641-650. doi:10.1089/hum.2008.162

Judge, L. M., Haraguchiln, M., & Chamberlain, J. S. (2006). Dissecting the signaling and mechanical functions of the dystrophin-glycoprotein complex. *Journal of Cell Science, 119*(Pt 8), 1537-1546. doi:10.1242/jcs.02857

Kerem, E., Hirawat, S., Armoni, S., Yaakov, Y., Shoseyov, D., Cohen, M., Nissim-Rafinia, M., Blau, H., Rivlin, J., Aviram, M., Elfring, G. L., Northcutt, V. J., Miller, L. L., Kerem, B., & Wilschanski, M. (2008). Effectiveness of PTC124 treatment of cystic fibrosis caused by nonsense mutations: a prospective phase II trial. *Lancet, 372*(9640), 719-727. doi:10.1016/S0140-6736(08)61168-X

Kesari, A., Neel, R., Wagoner, L., Harmon, B., Spurney, C., & Hoffman, E. P. (2009). Somatic mosaicism for Duchenne dystrophy: evidence for genetic normalization mitigating muscle symptoms. *American Journal of Medical Genetics.Part A, 149A*(7), 1499-1503. doi:10.1002/ajmg.a.32891

Kesari, A., Pirra, L. N., Bremadesam, L., McIntyre, O., Gordon, E., Dubrovsky, A. L., Viswanathan, V., & Hoffman, E. P. (2008). Integrated DNA, cDNA, and protein studies in Becker muscular dystrophy show high exception to the reading frame rule. *Human Mutation, 29*(5), 728-737. doi:10.1002/humu.20722

Khajavi, M., Inoue, K., & Lupski, J. R. (2006). Nonsense-mediated mRNA decay modulates clinical outcome of genetic disease. *European Journal of Human Genetics : EJHG, 14*(10), 1074-1081. doi:10.1038/sj.ejhg.5201649

Kinali, M., Arechavala-Gomeza, V., Feng, L., Cirak, S., Hunt, D., Adkin, C., Guglieri, M., Ashton, E., Abbs, S., Nihoyannopoulos, P., Garralda, M. E., Rutherford, M., McCulley, C., Popplewell, L., Graham, I. R., Dickson, G., Wood, M. J., Wells, D. J., Wilton, S. D., Kole, R., Straub, V., Bushby, K., Sewry, C., Morgan, J. E., & Muntoni, F. (2009). Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study. *Lancet Neurology, 8*(10), 918-928. doi:10.1016/S1474-4422(09)70211-X

Kingston, H. M., Sarfarazi, M., Thomas, N. S., & Harper, P. S. (1984). Localisation of the Becker muscular dystrophy gene on the short arm of the X chromosome by linkage to cloned DNA sequences. *Human Genetics, 67*(1), 6-17.

Koenig, M., Beggs, A. H., Moyer, M., Scherpf, S., Heindrich, K., Bettecken, T., Meng, G., Muller, C. R., Lindlof, M., & Kaariainen, H. (1989). The molecular basis for Duchenne versus Becker muscular dystrophy: correlation of severity with type of deletion. *American Journal of Human Genetics, 45*(4), 498-506.

Koenig, M., Hoffman, E. P., Bertelson, C. J., Monaco, A. P., Feener, C., & Kunkel, L. M. (1987). Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. *Cell, 50*(3), 509-517.

Krawczak, M., Ball, E. V., & Cooper, D. N. (1998). Neighboring-nucleotide effects on the rates of germ-line single-base-pair substitution in human genes. *American Journal of Human Genetics, 63*(2), 474-488. doi:10.1086/301965

Krypuy, M., Ahmed, A. A., Etemadmoghadam, D., Hyland, S. J., Australian Ovarian Cancer Study Group, DeFazio, A., Fox, S. B., Brenton, J. D., Bowtell, D. D., & Dobrovic, A. (2007). High resolution melting for mutation scanning of TP53 exons 5-8. *BMC Cancer, 7*, 168. doi:10.1186/1471-2407-7-168

Krypuy, M., Newnham, G. M., Thomas, D. M., Conron, M., & Dobrovic, A. (2006). High resolution melting analysis for the rapid and sensitive detection of mutations in clinical samples: KRAS codon 12 and 13 mutations in non-small cell lung cancer. *BMC Cancer, 6*, 295. doi:10.1186/1471-2407-6-295

Kumar, P., Henikoff, S., & Ng, P. C. (2009). Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nature Protocols, 4*(7), 1073-1081. doi:10.1038/nprot.2009.86

Kwok, A., Bassam, B., & Reja, V. (2007). *Genotyping a Class 4 SNP by high resolution melt (HRM) using SYBR Green I* (Reasearch reportCorbett Research (Corbett Life Science).

Lai, K. K. S., Lo, I. F. M., Tong, T. M. F., Cheng, L. Y. L., & Lam, S. T. S. (2006). Detecting exon deletions and duplications of the DMD gene using Multiplex Ligation-dependent Probe Amplification (MLPA). *Clinical Biochemistry, 39*(4), 367-372. doi:DOI: 10.1016/j.clinbiochem.2005.11.019

Lalic, T., Vossen, R. H., Coffa, J., Schouten, J. P., Guc-Scekic, M., Radivojevic, D., Djurisic, M., Breuning, M. H., White, S. J., & den Dunnen, J. T. (2005). Deletion and duplication screening in the DMD gene using MLPA. *European Journal of Human Genetics : EJHG, 13*(11), 1231-1234. doi:10.1038/sj.ejhg.5201465

Lapidos, K. A., Kakkar, R., & McNally, E. M. (2004). The dystrophin glycoprotein complex: signaling strength and integrity for the sarcolemma. *Circulation Research, 94*(8), 1023-1031. doi:10.1161/01.RES.0000126574.61061.25

Liew, M., Pryor, R., Palais, R., Meadows, C., Erali, M., Lyon, E., & Wittwer, C. (2004). Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. *Clinical Chemistry, 50*(7), 1156-1164. doi:10.1373/clinchem.2004.032136

Lim, L. E., & Rando, T. A. (2008). Technology insight: therapy for Duchenne muscular dystrophy-an opportunity for personalized medicine? *Nature Clinical Practice.Neurology, 4*(3), 149-158. doi:10.1038/ncpneuro0737

Lu, Q.L., Morris,G.E., Wilton,S.D., Ly,T., Artem'yeva,O.V., Strong,P., Partridge,T.A. (2000). Massive idiosyncratic exon skipping corrects the nonsense mutation in dystrophic mouse muscle and produces functional revertant fibers by clonal expansion. *J.Cell Biol.*, 2000, 148, 5, 985-996.

Lu, Q. L., Mann, C. J., Lou, F., Bou-Gharios, G., Morris, G. E., Xue, S. A., Fletcher, S., Partridge, T. A., & Wilton, S. D. (2003). Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse. *Nature Medicine, 9*(8), 1009-1014. doi:10.1038/nm897

Maguire, K., Suzuki, T., DiMatteo, D., Parekh-Olmedo, H., & Kmiec, E. (2009). Genetic correction of splice site mutation in purified and enriched myoblasts isolated from mdx5cv mice. *BMC Molecular Biology, 10*, 15. doi:10.1186/1471-2199-10-15

Mann, C. J., Honeyman, K., Cheng, A. J., Ly, T., Lloyd, F., Fletcher, S., Morgan, J. E., Partridge, T. A., & Wilton, S. D. (2001). Antisense-induced exon skipping and synthesis of dystrophin in the mdx mouse. *Proceedings of the National Academy of Sciences of the United States of America, 98*(1), 42-47. doi:10.1073/pnas.011408598

Manole, E. (1995). The dystrophin gene and its product--a view. *Romanian Journal of Neurology and Psychiatry = Revue Roumaine De Neurologie Et Psychiatrie, 33*(2), 109-119.

Manzur, A. Y., & Muntoni, F. (2009). Diagnosis and new treatments in muscular dystrophies. *Journal of Neurology, Neurosurgery, and Psychiatry, 80*(7), 706-714. doi:10.1136/jnnp.2008.158329

Mao, F., Leung, W. Y., & Xin, X. (2007). Characterization of EvaGreen and the implication of its physicochemical properties for qPCR applications. *BMC Biotechnology, 7*, 76. doi:10.1186/1472-6750-7-76

Maquat, L. E. (2004). Nonsense-mediated mRNA decay: splicing, translation and mRNP dynamics. *Nature Reviews.Molecular Cell Biology, 5*(2), 89-99. doi:10.1038/nrm1310

Markham, N. R., & Zuker, M. (2005). DINAMelt web server for nucleic acid melting prediction. *Nucleic Acids Research, 33*(Web Server issue), W577-81. doi:10.1093/nar/gki591

Mendell, J. R., Buzin, C. H., Feng, J., Yan, J., Serrano, C., Sangani, D. S., Wall, C., Prior, T. W., & Sommer, S. S. (2001). Diagnosis of Duchenne dystrophy by enhanced detection of small mutations. *Neurology, 57*(4), 645-650.

Mendell, J. R., Rodino-Klapac, L. R., Rosales-Quintero, X., Kota, J., Coley, B. D., Galloway, G., Craenen, J. M., Lewis, S., Malik, V., Shilling, C., Byrne, B. J., Conlon, T., Campbell, K. J., Bremer, W. G., Viollet, L., Walker, C. M., Sahenk, Z., & Clark, K. R. (2009). Limb-girdle muscular dystrophy type 2D gene therapy restores alpha-sarcoglycan and associated proteins. *Annals of Neurology, 66*(3), 290-297. doi:10.1002/ana.21732

Meryon, E. (1851). On fatty degeneration of the voluntary muscles: report of the Royal Medical and Chirurgical Society. *Lancet, 2*, 588-589.

Mitrpant, C., Fletcher, S., Iversen, P. L., & Wilton, S. D. (2009). By-passing the nonsense mutation in the 4 CV mouse model of muscular dystrophy by induced exon skipping. *The Journal of Gene Medicine, 11*(1), 46-56. doi:10.1002/jgm.1265

Monaco, A. P., Bertelson, C. J., Liechti-Gallati, S., Moser, H., & Kunkel, L. M. (1988). An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. *Genomics, 2*(1), 90-95.

Monaco, A. P., Neve, R. L., Colletti-Feener, C., Bertelson, C. J., Kurnit, D. M., & Kunkel, L. M. (1986). Isolation of candidate cDNAs for portions of the Duchenne muscular dystrophy gene. *Nature, 323*(6089), 646-650. doi:10.1038/323646a0

Monaco, A. P., Walker, A. P., Millwood, I., Larin, Z., & Lehrach, H. (1992). A yeast artificial chromosome contig containing the complete Duchenne muscular dystrophy gene. *Genomics, 12*(3), 465-473.

Mort, M., Ivanov, D., Cooper, D. N., & Chuzhanova, N. A. (2008). A meta-analysis of nonsense mutations causing human genetic disease. *Human Mutation, 29*(8), 1037-1047. doi:10.1002/humu.20763

Muntoni, F., Torelli, S., & Ferlini, A. (2003a). Dystrophin and mutations: one gene, several proteins, multiple phenotypes. *Lancet Neurology, 2*(12), 731-740.

Muntoni, F., Torelli, S., & Ferlini, A. (2003b). Dystrophin and mutations: one gene, several proteins, multiple phenotypes. *Lancet Neurology, 2*(12), 731-740.

Navankasattusas, S., Zhu, H., Garcia, A. V., Evans, S. M., & Chien, K. R. (1992). A ubiquitous factor (HF-1a) and a distinct muscle factor (HF-1b/MEF-2) form an E-box-independent pathway for cardiac muscle gene expression. *Molecular and Cellular Biology, 12*(4), 1469-1479.

Neri, M., Torelli, S., Brown, S., Ugo, I., Sabatelli, P., Merlini, L., Spitali, P., Rimessi, P., Gualandi, F., Sewry, C., Ferlini, A., & Muntoni, F. (2007). Dystrophin levels as low as 30% are sufficient to avoid muscular dystrophy in the human. *Neuromuscular Disorders : NMD, 17*(11-12), 913-918. doi:10.1016/j.nmd.2007.07.005

Nevo, Y., Muntoni, F., Sewry, C., Legum, C., Kutai, M., Harel, S., & Dubowitz, V. (2003). Large in-frame deletions of the rod-shaped domain of the dystrophin gene resulting in severe phenotype. *The Israel Medical Association Journal : IMAJ, 5*(2), 94-97.

Nishiyama, A., Takeshima, Y., Zhang, Z., Habara, Y., Tran, T. H., Yagi, M., & Matsuo, M. (2008). Dystrophin nonsense mutations can generate alternative rescue transcripts in lymphocytes. *Annals of Human Genetics, 72*(Pt 6), 717-724. doi:10.1111/j.1469-1809.2008.00468.x

Nudelman, I., Rebibo-Sabbah, A., Cherniavsky, M., Belakhov, V., Hainrichson, M., Chen, F., Schacht, J., Pilch, D. S., Ben-Yosef, T., & Baasov, T. (2009). Development of novel aminoglycoside (NB54) with reduced toxicity and enhanced suppression of disease-causing premature stop mutations. *Journal of Medicinal Chemistry, 52*(9), 2836-2845. doi:10.1021/jm801640k

Ohta, T., Tokishita, S., & Yamagata, H. (2001). Ethidium bromide and SYBR Green I enhance the genotoxicity of UV-irradiation and chemical mutagens in E. coli. *Mutation Research, 492*(1-2), 91-97.

Oshima, J., Magner, D. B., Lee, J. A., Breman, A. M., Schmitt, E. S., White, L. D., Crowe, C. A., Merrill, M., Jayakar, P., Rajadhyaksha, A., Eng, C. M., & del Gaudio, D. (2009). Regional genomic instability predisposes to complex dystrophin gene rearrangements. *Human Genetics, 126*(3), 411-423. doi:10.1007/s00439-009-0679-9

Pegoraro, E., Schimke, R. N., Garcia, C., Stern, H., Cadaldini, M., Angelini, C., Barbosa, E., Carroll, J., Marks, W. A., Neville, H. E., Marks, H., Appleton, S., Toriello, H., Wessel, H. B., Donnelly, J., Bernes, S. M., Taber, J. W., Weiss, L., & Hoffman, E. P. (1995). Genetic and biochemical normalization in female carriers of Duchenne muscular dystrophy: evidence for failure of dystrophin production in dystrophin-competent myonuclei. *Neurology, 45*(4), 677-690.

Peltz, S. W., Welch, E. M., Jacobson, A., Trotta, C. R., Naryshkin, N., Sweeney, H. L., & Bedwell, D. M. (2009). Nonsense suppression activity of PTC124 (ataluren). *Proceedings of the National Academy of Sciences of the United States of America, 106*(25), E64; author reply E65. doi:10.1073/pnas.0901936106

Phelps, S. F., Hauser, M. A., Cole, N. M., Rafael, J. A., Hinkle, R. T., Faulkner, J. A., & Chamberlain, J. S. (1995). Expression of full-length and truncated dystrophin mini-genes in transgenic mdx mice. *Human Molecular Genetics, 4*(8), 1251-1258.

Politano, L., Nigro, G., Nigro, V., Piluso, G., Papparella, S., Paciello, O., & Comi, L. I. (2003). Gentamicin administration in Duchenne patients with premature stop codon. Preliminary results. *Acta Myologica : Myopathies and Cardiomyopathies : Official Journal of the Mediterranean Society of Myology / Edited by the Gaetano Conte Academy for the Study of Striated Muscle Diseases, 22*(1), 15-21.

Rando, T. A. (2001). The dystrophin-glycoprotein complex, cellular signaling, and the regulation of cell survival in the muscular dystrophies. *Muscle & Nerve, 24*(12), 1575-1594.

Reed, G. H., Kent, J. O., & Wittwer, C. T. (2007). High-resolution DNA melting analysis for simple and efficient molecular diagnostics. *Pharmacogenomics, 8*(6), 597-608. doi:10.2217/14622416.8.6.597

Roberts, R. G., Gardner, R. J., & Bobrow, M. (1994). Searching for the 1 in 2,400,000: a review of dystrophin gene point mutations. *Human Mutation, 4*(1), 1-11. doi:10.1002/humu.1380040102

Robinson, R. (2009). Antisense Therapy Restores Function in Dmd Dog. *Neurology Today, 9*(8), 1.

Roest, P. A., Bakker, E., Fallaux, F. J., Verellen-Dumoulin, C., Murry, C. E., & den Dunnen, J. T. (1999). New possibilities for prenatal diagnosis of muscular

dystrophies: forced myogenesis with an adenoviral MyoD-vector. *Lancet, 353*(9154), 727-728. doi:10.1016/S0140-6736(98)05471-3

Roest, P. A., Bout, M., van der Tuijn, A. C., Ginjaar, I. B., Bakker, E., Hogervorst, F. B., van Ommen, G. J., & den Dunnen, J. T. (1996). Splicing mutations in DMD/BMD detected by RT-PCR/PTT: detection of a 19AA insertion in the cysteine rich domain of dystrophin compatible with BMD. *Journal of Medical Genetics, 33*(11), 935-939.

Saito, K., Ikeya, K., Kondo, E., Komine, S., Komine, M., Osawa, M., Aikawa, E., & Fukuyama, Y. (1995). Somatic mosaicism for a DMD gene deletion. *American Journal of Medical Genetics, 56*(1), 80-86. doi:10.1002/ajmg.1320560118

Sancho, S., Mongini, T., Tanji, K., Tapscott, S. J., Walker, W. F., Weintraub, H., Miller, A. D., & Miranda, A. F. (1993). Analysis of dystrophin expression after activation of myogenesis in amniocytes, chorionic-villus cells, and fibroblasts. A new method for diagnosing Duchenne's muscular dystrophy. *The New England Journal of Medicine, 329*(13), 915-920. doi:10.1056/NEJM199309233291303

Schug, J. (2008). Using TESS to predict transcription factor binding sites in DNA sequence. *Current Protocols in Bioinformatics / Editoral Board, Andreas D.Baxevanis ...[Et al.], Chapter 2*, Unit 2.6. doi:10.1002/0471250953.bi0206s21

Schwartz, M., Duno, M., Palle, A. L., Krag, T., & Vissing, J. (2007). Deletion of exon 16 of the dystrophin gene is not associated with disease. *Human Mutation, 28*(2), 205. doi:10.1002/humu.9477

Sedlackova, J., Vondracek, P., Hermanova, M., Zamecnik, J., Hruba, Z., Haberlova, J., Kraus, J., Marikova, T., Hedvicakova, P., Vohanka, S., & Fajkusova, L. (2009). Point mutations in Czech DMD/BMD patients and their phenotypic outcome. *Neuromuscular Disorders : NMD, 19*(11), 749-753. doi:10.1016/j.nmd.2009.08.011

Sewry, C. A. (2000). Immunocytochemical analysis of human muscular dystrophy. *Microscopy Research and Technique, 48*(3-4), 142-154. doi:2-9

Shelton, G. D., & Engvall, E. (2005). Canine and feline models of human inherited muscle diseases. *Neuromuscular Disorders : NMD, 15*(2), 127-138. doi:10.1016/j.nmd.2004.10.019

Sironi, M., Cagliani, R., Comi, G., Pozzoli, U., Bardoni, A., Giorda, R., & Bresolin, N. (2003). Trans-acting factors may cause dystrophin splicing misregulation in BMD skeletal muscles. *FEBS Letters, 537*(1-3), 30-34.

Sironi, M., Pozzoli, U., Comi, G. P., Riva, S., Bordoni, A., Bresolin, N., & Nag, D. K. (2006). A region in the dystrophin gene major hot spot harbors a cluster of deletion breakpoints and generates double-strand breaks in yeast. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 20*(11), 1910-1912. doi:10.1096/fj.05-5635fje

Sirsi, S. R., Schray, R. C., Wheatley, M. A., & Lutz, G. J. (2009). Formulation of polylactide-co-glycolic acid nanospheres for encapsulation and sustained release of poly(ethylene imine)-poly(ethylene glycol) copolymers complexed to oligonucleotides. *Journal of Nanobiotechnology, 7*, 1. doi:10.1186/1477-3155-7-1

Sonnemann, K. J., Heun-Johnson, H., Turner, A. J., Baltgalvis, K. A., Lowe, D. A., & Ervasti, J. M. (2009). Functional substitution by TAT-utrophin in dystrophin-deficient mice. *PLoS Medicine, 6*(5), e1000083. doi:10.1371/journal.pmed.1000083

Soltanzadeh,P., Friez,M.J., Dunn,D., von Niederhausern,A., Gurvich,O.L., Swoboda,K.J., Sampson,J.B., Pestronk,A., Connolly,A.M., Florence,J.M., Finkel,R.S. Bonnemann,C.G., Medne,L., Mendell,J.R., Mathews,K.D., Wong,B.L., Sussman,M.D., Zonana,J., Kovak,K., Gospe,S.M.,Jr, Gappmaier,E., Taylor,L.E., Howard,M.T., Weiss,R.B., Flanigan,K.M. (2010). Clinical and genetic characterization of manifesting carriers of DMD mutations. *Neuromuscul.Disord.*, 20, 8, 499-504, Elsevier B.V.

Surono, A., Takeshima, Y., Wibawa, T., Ikezawa, M., Nonaka, I., & Matsuo, M. (1999). Circular dystrophin RNAs consisting of exons that were skipped by alternative splicing. *Human Molecular Genetics, 8*(3), 493-500.

Takano, E. A., Mitchell, G., Fox, S. B., & Dobrovic, A. (2008). Rapid detection of carriers with BRCA1 and BRCA2 mutations using high resolution melting analysis. *BMC Cancer, 8*, 59. doi:10.1186/1471-2407-8-59

Taylor, P. J., Betts, G. A., Maroulis, S., Gilissen, C., Pedersen, R. L., Mowat, D. R., Johnston, H. M., & Buckley, M. F. (2010). Dystrophin Gene Mutation Location and the Risk of Cognitive Impairment in Duchenne Muscular Dystrophy.

Townsend, D. W., Yasuda, S., Li, S., Chamberlain, J. S., & Metzger, J. M. (2008). Emergent dilated cardiomyopathy caused by targeted repair of dystrophic skeletal muscle. *Molecular Therapy, 16*(5), 832-835.

Trollet, C., Athanasopoulos, T., Popplewell, L., Malerba, A., & Dickson, G. (2009). Gene therapy for muscular dystrophy: current progress and future prospects. *Expert Opinion on Biological Therapy, 9*(7), 849-866. doi:10.1517/14712590903029164

Tubiello, G., Carrera, P., Soriani, N., Morandi, L., & Ferrari, M. (1995). Mutational analysis of muscle and brain specific promoter regions of dystrophin gene in DMD/BMD Italian patients by denaturing gradient gel electrophoresis (DGGE). *Molecular and Cellular Probes, 9*(6), 441-446. doi:10.1006/mcpr.1995.0067

Tuffery-Giraud, S., Beroud, C., Leturcq, F., Yaou, R. B., Hamroun, D., Michel-Calemard, L., Moizard, M. P., Bernard, R., Cossee, M., Boisseau, P., Blayau, M., Creveaux, I., Guiochon-Mantel, A., de Martinville, B., Philippe, C., Monnier, N., Bieth, E., Khau Van Kien, P., Desmet, F. O., Humbertclaude, V., Kaplan, J. C., Chelly, J., & Claustres, M. (2009). Genotype-phenotype analysis in 2,405 patients with a dystrophinopathy using the UMD-DMD database: a model of nationwide knowledgebase. *Human Mutation, 30*(6), 934-945. doi:10.1002/humu.20976

Tuffery-Giraud, S., Saquet, C., Chambert, S., Echenne, B., Marie Cuisset, J., Rivier, F., Cossee, M., Philippe, C., Monnier, N., Bieth, E., Recan, D., Antoinette Voelckel, M., Perelman, S., Lambert, J. C., Malcolm, S., & Claustres, M. (2004). The role of muscle biopsy in analysis of the dystrophin gene in Duchenne muscular dystrophy: experience of a national referral centre. *Neuromuscular Disorders : NMD, 14*(10), 650-658. doi:10.1016/j.nmd.2004.05.002

Tyler, K. L. (2003). William Richard Gowers (1845-1915). *Journal of Neurology, 250*(8), 1012-1013. doi:10.1007/s00415-003-1154-5

Tyson, J., Bellman, S., Newton, V., Simpson, P., Malcolm, S., Pembrey, M. E., & Bitner-Glindzicz, M. (1996). Mapping of DFN2 to Xq22. *Human Molecular Genetics, 5*(12), 2055-2060.

Uchino, M., Tokunaga, M., Mita, S., Uyama, E., Ando, Y., Teramoto, H., Miike, T., & Ando, M. (1995). PCR and immunocytochemical analyses of dystrophin-positive fibers in Duchenne muscular dystrophy. *Journal of the Neurological Sciences, 129*(1), 44-50.

van Bockel, E. A., Lind, J. S., Zijlstra, J. G., Wijkstra, P. J., Meijer, P. M., van den Berg, M. P., Slart, R. H., Aarts, L. P., & Tulleken, J. E. (2009). Cardiac assessment of patients with late stage Duchenne muscular dystrophy. *Netherlands Heart Journal : Monthly Journal of the Netherlands Society of Cardiology and the Netherlands Heart Foundation, 17*(6), 232-237.

van Deutekom, J. C., Janson, A. A., Ginjaar, I. B., Frankhuizen, W. S., Aartsma-Rus, A., Bremmer-Bout, M., den Dunnen, J. T., Koop, K., van der Kooi, A. J., Goemans, N. M., de Kimpe, S. J., Ekhart, P. F., Venneker, E. H., Platenburg, G. J., Verschuuren, J. J., & van Ommen, G. J. (2007). Local dystrophin restoration with antisense oligonucleotide PRO051. *The New England Journal of Medicine, 357*(26), 2677-2686. doi:10.1056/NEJMoa073108

van Essen, A. J., Abbs, S., Baiget, M., Bakker, E., Boileau, C., van Broeckhoven, C., Bushby, K., Clarke, A., Claustres, M., & Covone, A. E. (1992). Parental origin and germline mosaicism of deletions and duplications of the dystrophin gene: a European study. *Human Genetics, 88*(3), 249-257.

van Essen, A. J., Kneppers, A. L., van der Hout, A. H., Scheffer, H., Ginjaar, I. B., ten Kate, L. P., van Ommen, G. J., Buys, C. H., & Bakker, E. (1997). The clinical and molecular genetic approach to Duchenne and Becker muscular dystrophy: an updated protocol. *Journal of Medical Genetics, 34*(10), 805-812.

van Vliet, L., de Winter, C. L., van Deutekom, J. C., van Ommen, G. J., & Aartsma-Rus, A. (2008). Assessment of the feasibility of exon 45-55 multiexon skipping for Duchenne muscular dystrophy. *BMC Medical Genetics, 9*, 105. doi:10.1186/1471-2350-9-105

Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., Gocayne, J. D., Amanatides, P., Ballew, R. M., Huson, D. H., Wortman, J. R., Zhang, Q., Kodira, C. D., Zheng, X. H., Chen, L., Skupski, M., Subramanian, G., Thomas, P. D., Zhang, J., Gabor Miklos, G. L., Nelson, C., Broder, S., Clark, A. G., Nadeau, J., McKusick, V. A., Zinder, N., Levine, A. J., Roberts, R. J., Simon, M., Slayman, C., Hunkapiller, M., Bolanos, R., Delcher, A., Dew, I., Fasulo, D., Flanigan, M., Florea, L., Halpern, A., Hannenhalli, S., Kravitz, S., Levy, S., Mobarry, C., Reinert, K., Remington, K., Abu-Threideh, J., Beasley, E., Biddick, K., Bonazzi, V., Brandon, R., Cargill, M., Chandramouliswaran, I., Charlab, R., Chaturvedi, K., Deng, Z., Di Francesco, V., Dunn, P., Eilbeck, K., Evangelista, C., Gabrielian, A. E., Gan, W., Ge, W., Gong, F., Gu, Z., Guan, P., Heiman, T. J., Higgins, M. E., Ji, R. R., Ke, Z., Ketchum, K. A., Lai, Z., Lei, Y., Li, Z., Li, J., Liang, Y., Lin, X., Lu, F., Merkulov, G. V., Milshina, N., Moore, H. M., Naik, A. K., Narayan, V. A., Neelam, B., Nusskern, D., Rusch, D. B., Salzberg, S., Shao, W., Shue, B., Sun, J., Wang, Z., Wang, A., Wang, X., Wang, J., Wei, M., Wides, R., Xiao, C., Yan, C., Yao, A., Ye, J., Zhan, M., Zhang, W., Zhang, H., Zhao, Q., Zheng, L., Zhong, F., Zhong, W., Zhu, S., Zhao, S., Gilbert, D., Baumhueter, S., Spier, G., Carter, C., Cravchik, A., Woodage, T., Ali, F., An, H., Awe, A., Baldwin, D., Baden, H., Barnstead, M., Barrow, I., Beeson, K., Busam, D., Carver, A., Center, A., Cheng, M. L., Curry, L., Danaher, S., Davenport, L., Desilets, R., Dietz, S., Dodson, K., Doup, L., Ferriera, S., Garg, N., Gluecksmann, A., Hart, B., Haynes, J., Haynes, C., Heiner, C., Hladun, S., Hostin, D., Houck, J., Howland, T., Ibegwam, C., Johnson, J., Kalush, F., Kline, L., Koduru, S., Love, A., Mann, F., May, D., McCawley, S.,

McIntosh, T., McMullen, I., Moy, M., Moy, L., Murphy, B., Nelson, K., Pfannkoch, C., Pratts, E., Puri, V., Qureshi, H., Reardon, M., Rodriguez, R., Rogers, Y. H., Romblad, D., Ruhfel, B., Scott, R., Sitter, C., Smallwood, M., Stewart, E., Strong, R., Suh, E., Thomas, R., Tint, N. N., Tse, S., Vech, C., Wang, G., Wetter, J., Williams, S., Williams, M., Windsor, S., Winn-Deen, E., Wolfe, K., Zaveri, J., Zaveri, K., Abril, J. F., Guigo, R., Campbell, M. J., Sjolander, K. V., Karlak, B., Kejariwal, A., Mi, H., Lazareva, B., Hatton, T., Narechania, A., Diemer, K., Muruganujan, A., Guo, N., Sato, S., Bafna, V., Istrail, S., Lippert, R., Schwartz, R., Walenz, B., Yooseph, S., Allen, D., Basu, A., Baxendale, J., Blick, L., Caminha, M., Carnes-Stine, J., Caulk, P., Chiang, Y. H., Coyne, M., Dahlke, C., Mays, A., Dombroski, M., Donnelly, M., Ely, D., Esparham, S., Fosler, C., Gire, H., Glanowski, S., Glasser, K., Glodek, A., Gorokhov, M., Graham, K., Gropman, B., Harris, M., Heil, J., Henderson, S., Hoover, J., Jennings, D., Jordan, C., Jordan, J., Kasha, J., Kagan, L., Kraft, C., Levitsky, A., Lewis, M., Liu, X., Lopez, J., Ma, D., Majoros, W., McDaniel, J., Murphy, S., Newman, M., Nguyen, T., Nguyen, N., Nodell, M., Pan, S., Peck, J., Peterson, M., Rowe, W., Sanders, R., Scott, J., Simpson, M., Smith, T., Sprague, A., Stockwell, T., Turner, R., Venter, E., Wang, M., Wen, M., Wu, D., Wu, M., Xia, A., Zandieh, A., & Zhu, X. (2001). The sequence of the human genome. *Science (New York, N.Y.), 291*(5507), 1304-1351. doi:10.1126/science.1058040

Verellen-Dumoulin, C., Freund, M., De Meyer, R., Laterre, C., Frederic, J., Thompson, M. W., Markovic, V. D., & Worton, R. G. (1984). Expression of an X-linked muscular dystrophy in a female due to translocation involving Xp21 and non-random inactivation of the normal X chromosome. *Human Genetics, 67*(1), 115-119.

Walton, J. N. (1954). On the classification and natural history of the myopathies. *Transactions of the American Neurological Association, 13*(79th Meeting), 19-21.

Webster, C., & Blau, H. M. (1990). Accelerated age-related decline in replicative life-span of Duchenne muscular dystrophy myoblasts: implications for cell and gene therapy. *Somatic Cell and Molecular Genetics, 16*(6), 557-565.

Welch, E., Goetz, D., & Almstead, N. (2008). Reading through premature stop codons with PTC1 24. Project Catalyst to find more Duchenne drugs. Interview by Guenter Scheuerbrandt. *Acta Myologica : Myopathies and Cardiomyopathies : Official Journal of the Mediterranean Society of Myology / Edited by the Gaetano Conte Academy for the Study of Striated Muscle Diseases, 27*, 63-68.

Welch, E. M., Barton, E. R., Zhuo, J., Tomizawa, Y., Friesen, W. J., Trifillis, P., Paushkin, S., Patel, M., Trotta, C. R., Hwang, S., Wilde, R. G., Karp, G., Takasugi, J., Chen, G., Jones, S., Ren, H., Moon, Y. C., Corson, D., Turpoff, A. A., Campbell, J. A., Conn, M. M., Khan, A., Almstead, N. G., Hedrick, J., Mollin, A., Risher, N., Weetall, M., Yeh, S., Branstrom, A. A., Colacino, J. M., Babiak, J., Ju, W. D., Hirawat, S., Northcutt, V. J., Miller, L. L., Spatrick, P., He, F., Kawana, M., Feng, H., Jacobson, A., Peltz, S. W., & Sweeney, H. L. (2007). PTC124 targets genetic disorders caused by nonsense mutations. *Nature, 447*(7140), 87-91. doi:10.1038/nature05756

Wheway,J.M., Roberts,R.G. (2003).The dystrophin lymphocyte promoter revisited: 4.5-megabase intron, or artifact? *Neuromuscul.Disord.* 13, 1, 17-20.

White, H. E., Hall, V. J., & Cross, N. C. (2007). Methylation-sensitive high-resolution melting-curve analysis of the SNRPN gene as a diagnostic screen for Prader-Willi and Angelman syndromes. *Clinical Chemistry, 53*(11), 1960-1962. doi:10.1373/clinchem.2007.093351

White, H., & Potts, G. (2006). Mutation Scanning by High Resolution Melt Analysis. Evaluation of Rotor-Gene 6000 (Corbett Life Science), HR-1 and 384-well LightScanner (Idaho Technology). Salisbury UK: National Genetics Reference Laboratory (Wessex).

White, S., Kalf, M., Liu, Q., Villerius, M., Engelsma, D., Kriek, M., Vollebregt, E., Bakker, B., van Ommen, G. J., Breuning, M. H., & den Dunnen, J. T. (2002). Comprehensive detection of genomic duplications and deletions in the DMD gene, by use of multiplex amplifiable probe hybridization. *American Journal of Human Genetics, 71*(2), 365-374. doi:10.1086/341942

White, S. J., Aartsma-Rus, A., Flanigan, K. M., Weiss, R. B., Kneppers, A. L., Lalic, T., Janson, A. A., Ginjaar, H. B., Breuning, M. H., & den Dunnen, J. T. (2006). Duplications in the DMD gene. *Human Mutation, 27*(9), 938-945. doi:10.1002/humu.20367

White, S. J., & den Dunnen, J. T. (2006). Copy number variation in the genome; the human DMD gene as an example. *Cytogenetic and Genome Research, 115*(3-4), 240-246. doi:10.1159/000095920

Wilkinson, M. F., & Shyu, A. B. (2002). RNA surveillance by nuclear scanning? *Nature Cell Biology, 4*(6), E144-7. doi:10.1038/ncb0602-e144

Williams, J. H., Schray, R. C., Sirsi, S. R., & Lutz, G. J. (2008). Nanopolymers improve delivery of exon skipping oligonucleotides and concomitant dystrophin expression in skeletal muscle of mdx mice. *BMC Biotechnology, 8*, 35. doi:10.1186/1472-6750-8-35

Willmann, R., Possekel, S., Dubach-Powell, J., Meier, T., & Ruegg, M. A. (2009). Mammalian animal models for Duchenne muscular dystrophy. *Neuromuscular Disorders : NMD, 19*(4), 241-249. doi:10.1016/j.nmd.2008.11.015

Winnard, A. V., Mendell, J. R., Prior, T. W., Florence, J., & Burghes, A. H. (1995). Frameshift deletions of exons 3-7 and revertant fibers in Duchenne muscular dystrophy: mechanisms of dystrophin production. *American Journal of Human Genetics, 56*(1), 158-166.

Wittwer, C. T., Reed, G. H., Gundry, C. N., Vandersteen, J. G., & Pryor, R. J. (2003). High-resolution genotyping by amplicon melting analysis using LCGreen. *Clinical Chemistry, 49*(6 Pt 1), 853-860.

Wolff, J., Lewis, D. L., Herweijer, H., Hegge, J., & Hagstrom, J. (2005). Non-viral approaches for gene transfer. *Acta Myologica : Myopathies and Cardiomyopathies : Official Journal of the Mediterranean Society of Myology / Edited by the Gaetano Conte Academy for the Study of Striated Muscle Diseases, 24*(3), 202-208.

Wu, B., Moulton, H. M., Iversen, P. L., Jiang, J., Li, J., Li, J., Spurney, C. F., Sali, A., Guerron, A. D., Nagaraju, K., Doran, T., Lu, P., Xiao, X., & Lu, Q. L. (2008). Effective rescue of dystrophin improves cardiac function in dystrophin-deficient mice by a modified morpholino oligomer. *Proceedings of the National Academy of Sciences of the United States of America, 105*(39), 14814-14819. doi:10.1073/pnas.0805676105

Yan, J. B., Xu, H. P., Xiong, C., Ren, Z. R., Tian, G. L., Zeng, F., & Huang, S. Z. (2010). Rapid and reliable detection of glucose-6-phosphate dehydrogenase (G6PD) gene

mutations in Han Chinese using high-resolution melting analysis. *The Journal of Molecular Diagnostics : JMD, 12*(3), 305-311. doi:10.2353/jmoldx.2010.090104

Ye, S., Cole-Strauss, A. C., Frank, B., & Kmiec, E. B. (1998). Targeted gene correction: a new strategy for molecular medicine. *Molecular Medicine Today, 4*(10), 431-437.

Yin, H. F., Moulton, H. M., Seow, Y., Boyd, C., Boutilier, J., Iverson, P., & Wood, M. J. A. (2008). Cell-penetrating peptide-conjugated antisense oligonucleotides restore systemic muscle and cardiac dystrophin expression and function. *Human Molecular Genetics, 17*(24), 3909.

Yokota, T., Lu, Q. L., Partridge, T., Kobayashi, M., Nakamura, A., Takeda, S., & Hoffman, E. (2009). Efficacy of systemic morpholino exon-skipping in Duchenne dystrophy dogs. *Annals of Neurology, 65*(6), 667-676. doi:10.1002/ana.21627

Yoshioka, M., Okuno, T., Honda, Y., & Nakano, Y. (1980). Central nervous system involvement in progressive muscular dystrophy. *Archives of Disease in Childhood, 55*(8), 589-594.

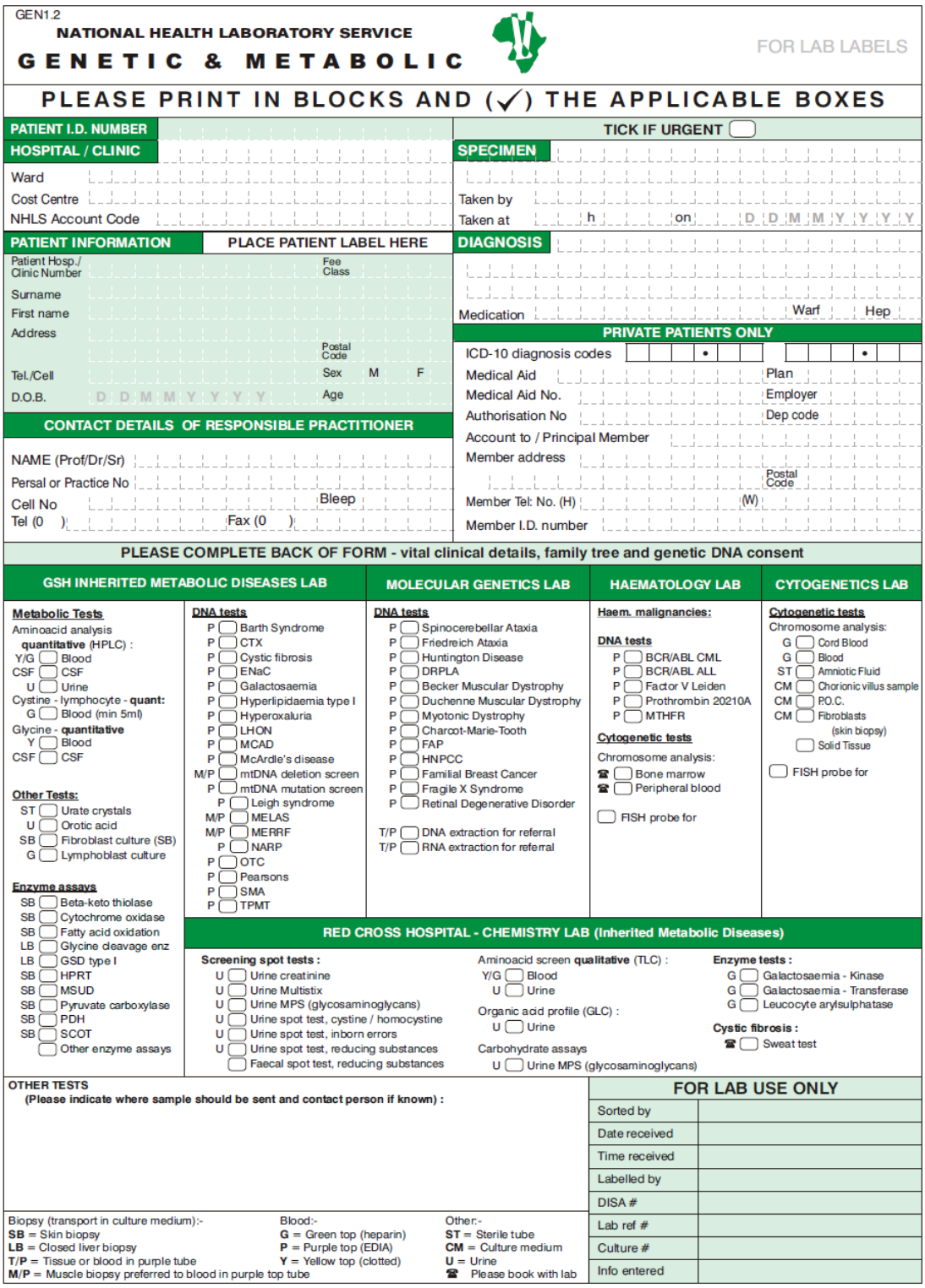
Yu, Y. T., Breitbart, R. E., Smoot, L. B., Lee, Y., Mahdavi, V., & Nadal-Ginard, B. (1992). Human myocyte-specific enhancer factor 2 comprises a group of tissue-restricted MADS box transcription factors. *Genes & Development, 6*(9), 1783-1798.

Zhang, X., Lee, J., & Chasin, L. A. (2003). The effect of nonsense codons on splicing: a genomic analysis. *RNA (New York, N.Y.), 9*(6), 637-639.

Zhang, Z., Habara, Y., Nishiyama, A., Oyazato, Y., Yagi, M., Takeshima, Y., & Matsuo, M. (2007). Identification of seven novel cryptic exons embedded in the dystrophin gene and characterization of 14 cryptic dystrophin exons. *Journal of Human Genetics, 52*(7), 607-617. doi:10.1007/s10038-007-0163-0

APPENDIX A

Request and Consent Forms



GEN1.2

NATIONAL HEALTH LABORATORY SERVICE

GENETIC & METABOLIC

FOR LAB LABELS

PLEASE PRINT IN BLOCKS AND (✓) THE APPLICABLE BOXES

PATIENT I.D. NUMBER

HOSPITAL / CLINIC

Ward

Cost Centre

NHLS Account Code

TICK IF URGENT ☐

SPECIMEN

Taken by

Taken at h on D D M M Y Y Y Y

PATIENT INFORMATION

PLACE PATIENT LABEL HERE

Patient Hosp./ Clinic Number

Fee Class

Surname

First name

Address

Postal Code

Tel./Cell

Sex M F

D.O.B. D D M M Y Y Y Y

Age

DIAGNOSIS

Medication

Warf

Hep

PRIVATE PATIENTS ONLY

ICD-10 diagnosis codes

Medical Aid

Plan

Medical Aid No.

Employer

Authorisation No

Dep code

Account to / Principal Member

Member address

Postal Code

Member Tel: No. (H)

(W)

Member I.D. number

CONTACT DETAILS OF RESPONSIBLE PRACTITIONER

NAME (Prof/Dr/Sr)

Persal or Practice No

Cell No

Bleep

Tel (0)

Fax (0)

PLEASE COMPLETE BACK OF FORM - vital clinical details, family tree and genetic DNA consent

GSH INHERITED METABOLIC DISEASES LAB

Metabolic Tests

Aminoacid analysis quantitative (HPLC) :

Y/G ☐ Blood

CSF ☐ CSF

U ☐ Urine

Cystine - lymphocyte - quant:

G ☐ Blood (min 5ml)

Glycine - quantitative

Y ☐ Blood

CSF ☐ CSF

Other Tests:

ST ☐ Urate crystals

U ☐ Orotic acid

SB ☐ Fibroblast culture (SB)

G ☐ Lymphoblast culture

Enzyme assays

SB ☐ Beta-keto thiolase

SB ☐ Cytochrome oxidase

SB ☐ Fatty acid oxidation

LB ☐ Glycine cleavage enz

LB ☐ GSD type I

SB ☐ HPRT

SB ☐ MSUD

SB ☐ Pyruvate carboxylase

SB ☐ PDH

SB ☐ SCOT

☐ Other enzyme assays

DNA tests

P ☐ Barth Syndrome

P ☐ CTX

P ☐ Cystic fibrosis

P ☐ ENaC

P ☐ Galactosaemia

P ☐ Hyperlipidaemia type I

P ☐ Hyperoxaluria

P ☐ LHON

P ☐ MCAD

P ☐ McArdle's disease

M/P ☐ mtDNA deletion screen

P ☐ mtDNA mutation screen

P ☐ Leigh syndrome

M/P ☐ MELAS

M/P ☐ MERRF

P ☐ NARP

P ☐ OTC

P ☐ Pearsons

P ☐ SMA

P ☐ TPMT

MOLECULAR GENETICS LAB

DNA tests

P ☐ Spinocerebellar Ataxia

P ☐ Friedreich Ataxia

P ☐ Huntington Disease

P ☐ DRPLA

P ☐ Becker Muscular Dystrophy

P ☐ Duchenne Muscular Dystrophy

P ☐ Myotonic Dystrophy

P ☐ Charcot-Marie-Tooth

P ☐ FAP

P ☐ HNPCC

P ☐ Familial Breast Cancer

P ☐ Fragile X Syndrome

P ☐ Retinal Degenerative Disorder

T/P ☐ DNA extraction for referral

T/P ☐ RNA extraction for referral

HAEMATOLOGY LAB

Haem. malignancies:

DNA tests

P ☐ BCR/ABL CML

P ☐ BCR/ABL ALL

P ☐ Factor V Leiden

P ☐ Prothrombin 20210A

P ☐ MTHFR

Cytogenetic tests

Chromosome analysis:

☎ ☐ Bone marrow

☎ ☐ Peripheral blood

☐ FISH probe for

CYTOGENETICS LAB

Cytogenetic tests

Chromosome analysis:

G ☐ Cord Blood

G ☐ Blood

ST ☐ Amniotic Fluid

CM ☐ Chorionic villus sample

CM ☐ P.O.C.

CM ☐ Fibroblasts (skin biopsy)

☐ Solid Tissue

☐ FISH probe for

RED CROSS HOSPITAL - CHEMISTRY LAB (Inherited Metabolic Diseases)

Screening spot tests :

U ☐ Urine creatinine

U ☐ Urine Multistix

U ☐ Urine MPS (glycosaminoglycans)

U ☐ Urine spot test, cystine / homocystine

U ☐ Urine spot test, inborn errors

U ☐ Urine spot test, reducing substances

☐ Faecal spot test, reducing substances

Aminoacid screen qualitative (TLC) :

Y/G ☐ Blood

U ☐ Urine

Organic acid profile (GLC) :

U ☐ Urine

Carbohydrate assays

U ☐ Urine MPS (glycosaminoglycans)

Enzyme tests :

G ☐ Galactosaemia - Kinase

G ☐ Galactosaemia - Transferase

G ☐ Leucocyte arylsulphatase

Cystic fibrosis :

☎ ☐ Sweat test

OTHER TESTS

(Please indicate where sample should be sent and contact person if known) :

FOR LAB USE ONLY

Sorted by	
Date received	
Time received	
Labelled by	
DISA #	
Lab ref #	
Culture #	
Info entered	

Biopsy (transport in culture medium):-

SB = Skin biopsy

LB = Closed liver biopsy

T/P = Tissue or blood in purple tube

M/P = Muscle biopsy preferred to blood in purple top tube

Blood:-

G = Green top (heparin)

P = Purple top (EDIA)

Y = Yellow top (clotted)

Other:-

ST = Sterile tube

CM = Culture medium

U = Urine

☎ Please book with lab

Figure A.1 National Health Laboratory Service (NHLS) request form for genetic testing.

Additional Information needed for cytogenetic and molecular genetic samples

Relevant clinical findings and family history: _____

_____

_____

_____

_____

Indicate ethnic origin of patient's mother _____ **father** _____

If prenatal referral, please give age of mother _____ **gestational age** _____ **sonar #** _____

Abnormalities noted on ultrasound scan _____

_____

Is patient : affected ☐ at risk ☐ carrier ☐ spouse ☐ query ☐ unaffected ☐

Have samples for genetic studies been sent to the lab before? Yes / No / Don't know

Family tree

Consent is mandatory for tests performed in the Molecular Genetics Laboratory, and if DNA storage is required.

Consent for DNA analysis and storage

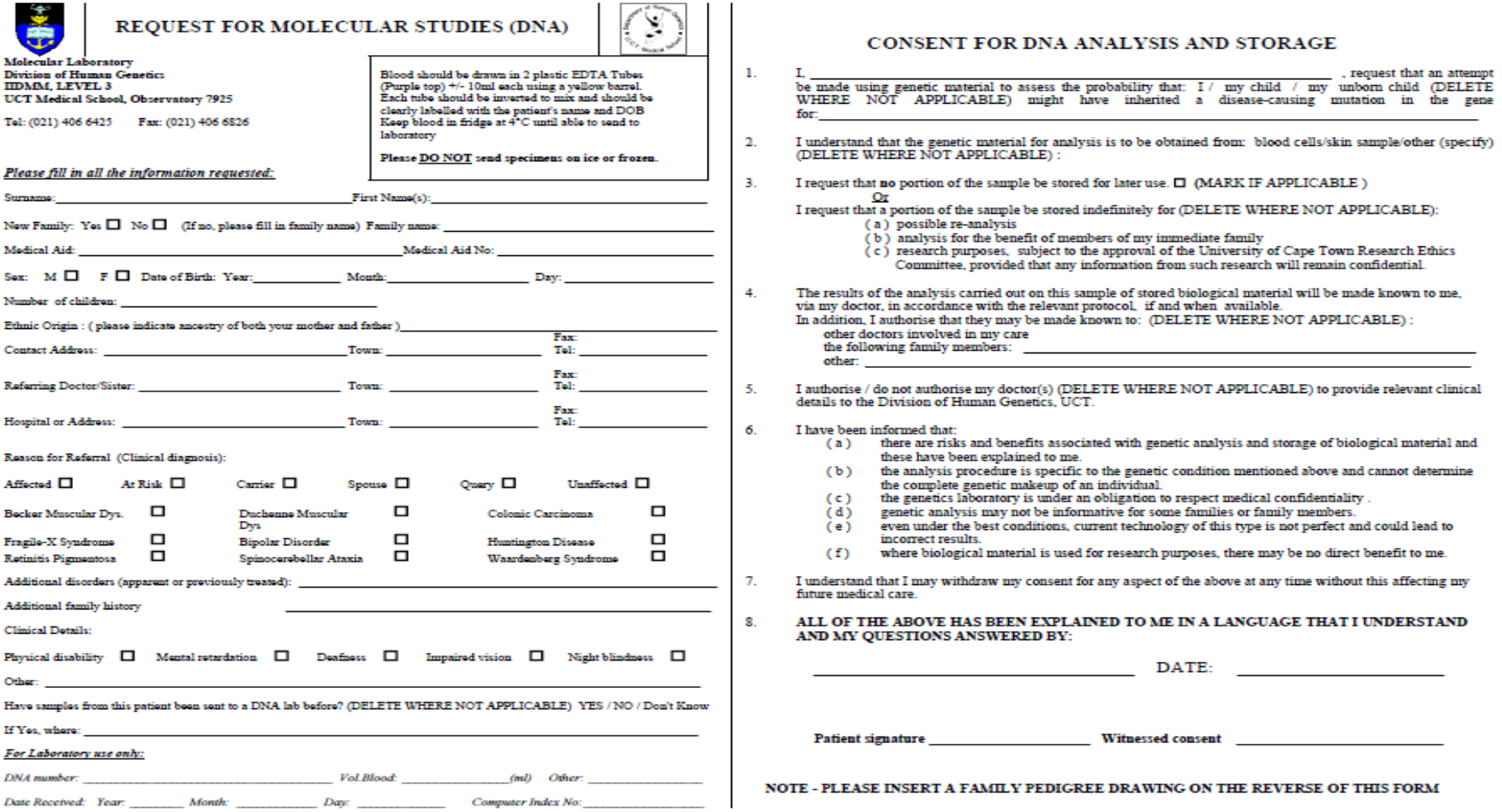
1. I, _____, request that an attempt be made using genetic material to assess the probability that: I / my child / my unborn child (DELETE WHERE NOT APPLICABLE) might have inherited a disease-causing mutation in the gene for: _____
2. I understand that the genetic material for analysis is to be obtained from: blood / skin sample / other (specify) (DELETE WHERE NOT APPLICABLE)
3. I request that no portion of the sample be stored for later use. ☐(MARK IF APPLICABLE)
   OR
   I request that a portion of the sample be stored indefinitely for (DELETE WHERE NOT APPLICABLE):
   (a) possible re-analysis.
   (b) analysis for the benefit of members of my immediate family.
   (c) research purposes, subject to the approval of the Research Ethics Committee, provided that all information will remain confidential.
4. The result of the analysis will be made known to me, via my doctor(s), in accordance with the relevant protocol, if and when available.
5. If clinically relevant, I authorise that the results may be made known to family members.
6. I have been informed that:
   (a) the analysis procedure is specific to the genetic condition and cannot determine the complete genetic makeup of an individual.
   (b) the genetics laboratory is under an obligation to respect medical confidentiality.
   (c) genetic analysis may not be informative for some families or family members.
   (d) even under the best conditions, current technology of this type is not perfect and could lead to incorrect results.
   (e) where biological material is used for research purposes, there may be no direct benefit to me.
7. I understand that I may withdraw my consent for any aspect of the above at any time without this affecting my future medical care.
8. **ALL OF THE ABOVE HAS BEEN EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS ANSWERED BY**

Doctor/Consultant _____ Date _____

Patient / Parent Signature _____ Date _____

CTX = Cerebrotendinous xanthomatosis
DRPLA = Dentatorubral pallidoluysian atrophy
ENaC = Epithelial sodium channel
FAP = Familial adenomatous polyposis coli
GSD = Glycogen storage disease type I
HNPCC = Hereditary non-polyposis colorectal cancer
HPRT = Hypoxanthine phosphoribose tranferase
LHON = Lebers hereditary optic neuropathy
MCAD = Medium chain acyl coenzyme A dehydrogenase
MSUD = Maple syrup urine disease
mtDNA deletion screen = KSS and CPEO
mtDNA mutation screen = Leigh's syndrome, MELAS, MERRF and NARP
MTHFR = Methylene tetrahydrofolate reductase
OTC = Ornithine transcarbamylase
PDH = Pyruvate dehydrogenase
SMA = Spinal muscular atrophy
SCOT = Succinyl coA-acyl transferase
TPMT = Thiopurine methyl transferase

Figure A.2 The NHLS consent form for genetic testing and/or research (printed the reverse of the above request form).



REQUEST FOR MOLECULAR STUDIES (DNA)

Molecular Laboratory
Division of Human Genetics
IIDMM, LEVEL 3
UCT Medical School, Observatory 7925

Tel: (021) 406 6425 Fax: (021) 406 6826

Blood should be drawn in 2 plastic EDTA Tubes (Purple top) +/- 10ml each using a yellow barrel. Each tube should be inverted to mix and should be clearly labelled with the patient's name and DOB
Keep blood in fridge at 4°C until able to send to laboratory

Please DO NOT send specimens on ice or frozen.

Please fill in all the information requested:

Surname: _____ First Name(s): _____

New Family: Yes ☐ No ☐ (If no, please fill in family name) Family name: _____

Medical Aid: _____ Medical Aid No: _____

Sex: M ☐ F ☐ Date of Birth: Year: _____ Month: _____ Day: _____

Number of children: _____

Ethnic Origin : (please indicate ancestry of both your mother and father) _____

Contact Address: _____ Town: _____ Fax: Tel: _____

Referring Doctor/Sister: _____ Town: _____ Fax: Tel: _____

Hospital or Address: _____ Town: _____ Fax: Tel: _____

Reason for Referral (Clinical diagnosis):

Affected ☐ At Risk ☐ Carrier ☐ Spouse ☐ Query ☐ Unaffected ☐

Becker Muscular Dys. ☐	Duchenne Muscular Dys ☐	Colonic Carcinoma ☐
Fragile-X Syndrome ☐	Bipolar Disorder ☐	Huntington Disease ☐
Retinitis Pigmentosa ☐	Spinocerebellar Ataxia ☐	Waardenberg Syndrome ☐

Additional disorders (apparent or previously treated): _____

Additional family history _____

Clinical Details:

Physical disability ☐ Mental retardation ☐ Deafness ☐ Impaired vision ☐ Night blindness ☐

Other: _____

Have samples from this patient been sent to a DNA lab before? (DELETE WHERE NOT APPLICABLE) YES / NO / Don't Know

If Yes, where: _____

For Laboratory use only:

DNA number: _____ *Vol.Blood:* _____ *(ml)* *Other:* _____

Date Received: Year: _____ *Month:* _____ *Day:* _____ *Computer Index No:* _____

CONSENT FOR DNA ANALYSIS AND STORAGE

1. I, _____ , request that an attempt be made using genetic material to assess the probability that: I / my child / my unborn child (DELETE WHERE NOT APPLICABLE) might have inherited a disease-causing mutation in the gene for: _____

2. I understand that the genetic material for analysis is to be obtained from: blood cells/skin sample/other (specify) (DELETE WHERE NOT APPLICABLE) :

3. I request that **no** portion of the sample be stored for later use. ☐ (MARK IF APPLICABLE)
   Or
   I request that a portion of the sample be stored indefinitely for (DELETE WHERE NOT APPLICABLE):
   (a) possible re-analysis
   (b) analysis for the benefit of members of my immediate family
   (c) research purposes, subject to the approval of the University of Cape Town Research Ethics Committee, provided that any information from such research will remain confidential.

4. The results of the analysis carried out on this sample of stored biological material will be made known to me, via my doctor, in accordance with the relevant protocol, if and when available.
   In addition, I authorise that they may be made known to: (DELETE WHERE NOT APPLICABLE) :
   other doctors involved in my care
   the following family members: _____
   other: _____

5. I authorise / do not authorise my doctor(s) (DELETE WHERE NOT APPLICABLE) to provide relevant clinical details to the Division of Human Genetics, UCT.

6. I have been informed that:
   (a) there are risks and benefits associated with genetic analysis and storage of biological material and these have been explained to me.
   (b) the analysis procedure is specific to the genetic condition mentioned above and cannot determine the complete genetic makeup of an individual.
   (c) the genetics laboratory is under an obligation to respect medical confidentiality .
   (d) genetic analysis may not be informative for some families or family members.
   (e) even under the best conditions, current technology of this type is not perfect and could lead to incorrect results.
   (f) where biological material is used for research purposes, there may be no direct benefit to me.

7. I understand that I may withdraw my consent for any aspect of the above at any time without this affecting my future medical care.

8. **ALL OF THE ABOVE HAS BEEN EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS ANSWERED BY:**

_____ DATE: _____

Patient signature _____ **Witnessed consent** _____

NOTE - PLEASE INSERT A FAMILY PEDIGREE DRAWING ON THE REVERSE OF THIS FORM

Figure A.3 Consent for molecular studies (Division Human Genetics, School of Clinical and Laboratory Medicine, University of Cape Town).

INFORMATION SHEET AND CONSENT FORM FOR PARTICIPATION AS A WILD-TYPE CONTROL IN THE PTC124 STUDY

Project title:
DMD: mutation profiling in view of the emerging gene-based therapies.

Project Summary
Duchenne/Becker Muscular Dystrophy (D/BMD) is a lethal, X-linked, recessive muscle wasting disorder, affecting 1 in 3 500 live male births worldwide. It is caused by mutations in the *DMD* gene, which encodes production of dystrophin, a cytoskeletal protein, important for the structure and strength of muscle cells. DMD can result from many different mutations within the gene. Approximately 30% of those mutations remain undetected, owing to the practical difficulties and expense involved. There is no treatment to date and only palliative care is available.

In recent years, much research has been focused on the development of tailor-made (personalized) gene-based therapies, chosen or designed specifically to target different mutations in different patients (private mutations). However, the first step to application of any such future therapies is finding and identifying the disease-causing mutation in each DMD patient. At present, testing for only certain types of mutations (large deletions and duplication) is possible in most diagnostic laboratories, leaving approximately 30% of all DMD patients with no genetic confirmation of the diagnosis and no information relative to possible future eligibility for treatment with a gene-based therapy.

The focus of this project is to set up a comprehensive and cost effective mutation screening strategy for D/BMD patients, to provide genetic confirmation of the clinical diagnosis, facilitate genetic counseling of the patient and his family, and provide information pertaining to possible future application of a gene-based therapy. The methodology will involve multiple ligation-dependant probe amplification (MLPA) for large mutations (exonic rearrangements) and high resolution melting curve analysis for detection of small/point mutations.

Selection and Recruitment
The study cohort consists of patients with clinical diagnoses of D/BMD, currently seen at the Red Cross Children's Hospital Muscle Clinic. DNA of the individuals included in the study was obtained from the DNA Registry storage facility at the Division of Human Genetics, School Clinical and Laboratory Medicine, University of Cape Town. Consent for genetic testing was previously obtained as part of each patient's diagnostic work-up. If required, any additional specimen collection will be conducted in keeping with the existing National Health Laboratory Service (NHLS) protocol for informed consent.

Wild type (normal) controls must be included in all test runs for comparison of profiles and as part of relative quantitation analyses.

Figure A.4 Information sheet for participation in this research project (page 1).

Voluntary Participation and Anonymity

Participation as a wild type control is completely voluntary. Samples from participants will be anonymised and record kept of the sex of the participant only. This is relevant as DMD is an X-linked disorder.

CONSENT FORM

1. I, _____, consent to the use of my genetic material in the study outlined above.

2. I have been informed that:

(a) The genetic material for analysis is to be obtained from a blood sample

(b) The sample I provide will be assigned a unique identification number and that there will be no link between my name and the unique identification number.

(c) Since the sample has been collected anonymously it cannot be withdrawn from the study.

(d) The sample will be stored indefinitely.

(e) The results of the project will be used for theses material and may published in a scientific journal.

(f) The analysis procedure only provides information on variable genetic elements in DNA and cannot determine the complete genetic makeup of an individual.

ALL OF THE ABOVE HAS BEEN EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS ANSWERED BY:

_____ Date: _____

Donor signature: _____

Figure A.5 Consent for participation in this research project (page 2).

APPENDIX B

1. ADVANTAGES OF MLPA®

- A major advantage of this technology is that it is a quantitative scan of the entire *DMD* gene for exonic rearrangements in two multiplex, PCR-based reactions. It can therefore detect duplications in males and determine carrier status in females.
- The use of a single primer pair allows for amplification of up to 45 targets in a single multiplex reaction. It is therefore capable of amplifying all 79 exons of the *DMD* gene (in two reactions) and not just those located in the mutation hotspots.
- MLPA® is superior to other quantitative analyses, such as the qPCR and Southern blot, as the former is difficult to optimise in multiplex, and the latter is expensive, labour intensive and time consuming. The MLPA® reaction is easy to perform, robust and fast, as results are available within 48hrs. It is also relatively inexpensive at ~ EUR15 per reaction (as per price in 2009).
- The MLPA® is internally quality-controlled by inclusion of control fragments and probes in every probe set. Also, consecutive exons are amplified in different probe mixes and are not adjacently lined up on the electropherogram, due to clever fragment size regulation by probe design. This also helps to authenticate any changes found.
- Only 20ng of human genomic DNA is required.

2. LIMITATIONS OF MLPA®

- MLPA for DMD is primarily designed to identify deletions/duplications, *not* point mutations.
- MLPA® is more sensitive to sample impurities than an ordinary PCR.
- Sample uniformity is important. It is advisable to compare DNA extracted with the same extraction method and diluted with the same diluent. Also, DNA sample in a batch should be of similar concentration, which requires pre-test sample preparation.
- A single exon deletion must be confirmed with an independent method, such as a separate PCR with an individual set of primes. The lack of probe signal could be caused by incomplete sample denaturation, failed ligation or another artefact.
- Point mutations and polymorphisms can interfere with probe hybridization and mimic single-exon deletions. For this reason, cycle sequencing should be performed on PCR products of single exons presenting as deleted with

®. This however, can be seen as an advantage, since a disease-causing point mutation can be detected in this way.

3. MLPA® QUALITY CONTROL

The control probes and fragments are part of the test design, acting as an internal quality control of each MLPA® assay. Each assay run is also controlled externally by inclusion of control samples of known genotype.

- **Q-fragments** (quantity controls of 64, 70, 76 and 82 nt) are present in low quantities and are not ligation-dependent. In a reaction with sufficient amount of DNA (Figure B1(C)), the Q-fragments are barely visible, as they are dwarfed by the MLPA® probes. If the amount of sample DNA is below 20ng or if the ligation reaction fails, the Q-fragments co-vary together and become more prominent (higher than half of the D-fragment and the MLPA® probe peaks), rendering the MLPA® results unreliable (Figure B1 (A) and (B)) (www.mlpa.com).
- **D-fragments** (denaturation controls 88, 92 and 96 nt) are DNA- and ligation-dependent with peak sizes comparable to those of the MLPA® probes (Figure B.2(A) and B.3). Invisible or significantly reduced D-fragments indicate insufficient sample denaturation and unsuccessful ligation (Figure B.2 (B) and (C)). The 88 and 96 nt D-fragments target difficult to denature sequences in CpG islands. A peak height 40% lower than that of the 92 nt D-fragment and the other MLPA® probes, is a possible indication of incomplete sample denaturation. Deletions of probes close to CpG islands should always be treated with caution and special attention paid to the signals of the D-fragments. The 92 nt D-fragment, is present in lower quantities and is a measure of hybridization efficiency. A peak size significantly lower than that of the 88 and 96 nt D-fragments, is an indication of incomplete probe-to-target hybridization (www.mlpa.com).
- **The X and Y-specific fragments** can be used for sex determination and can in some cases locate sample switch.
- **Reference probes** consist of chromosomal sequences, which do not show frequent copy number changes. It is unlikely (though not impossible) to see deletions or duplications of reference probes or non-neighbouring target-specific probes and such findings may indicate unreliable MLPA® results.
- **Reference samples** (wild type DNA) are included in every MLPA® run and are essential for data normalization and quantitation analysis. MRC-Holland recommends using at least three reference samples, all extracted by the same method and diluted to a concentration similar to that of the tests samples.

- **Mutation-positive control samples** are useful for testing the integrity of the MLPA® run but are not essential for data analysis.
- **No-DNA control reactions** included as a test for contamination of tests or capillary electrophoresis reagents. These reactions are not completely blank as the Q-fragments, along with some longer aspecific amplification products are sometimes present. However, the results of the no-DNA control reactions should be reproducible.

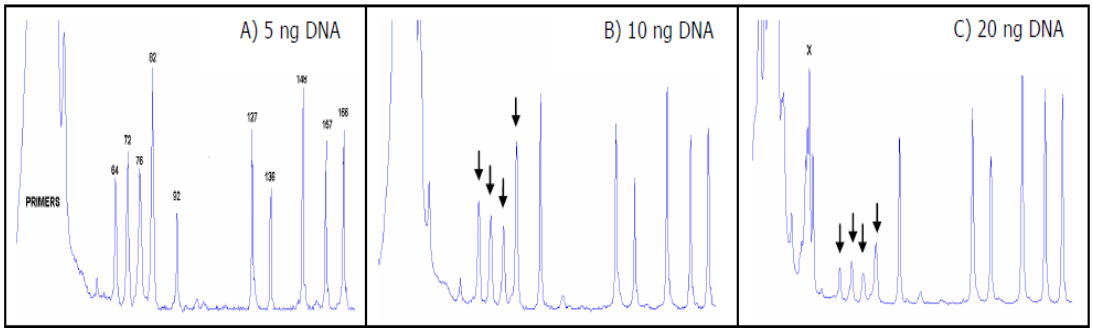


Figure B.1 Effect of DNA quantity on Q-fragments.
MLPA® results with 5 ng (A), 10 ng (B), 20 ng (C). Peaks of the Q-fragments decrease when the amount of sample DNA added is increased. Primer-dimer peaks (x) are often seen at ~55 nt (www.mlpa.com)

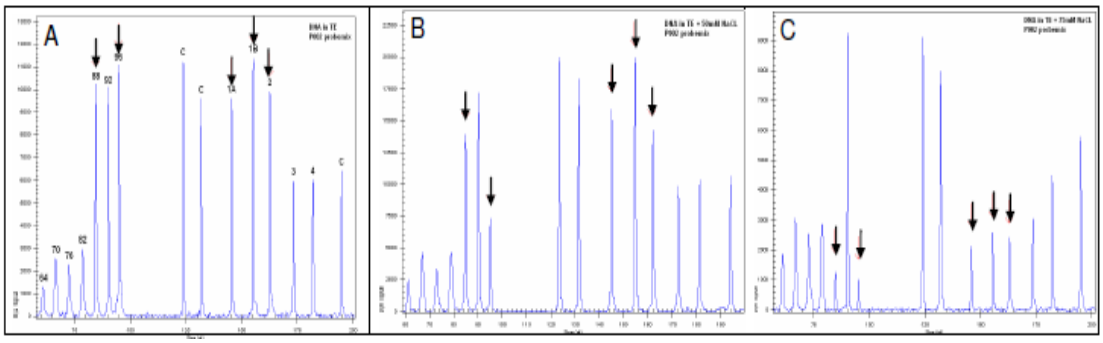


Figure B.2 Effect of a poor denaturation (due to a high salt concentration) on D-fragments.
MLPA® P002 (BRCA1) probes signals from DNA in TE (A), with 50mM NaCl (B), 75 mM NaCl (C). Peak size of control fragments 88, 96 and probes for exon A, B and C are reduced in the presence of a high salt concentration due to incomplete denaturation. 64-96 nt: quality control fragments, c: control probe, 1-5: BRCA1 probes for exons 1-5 (www.mlpa.com).

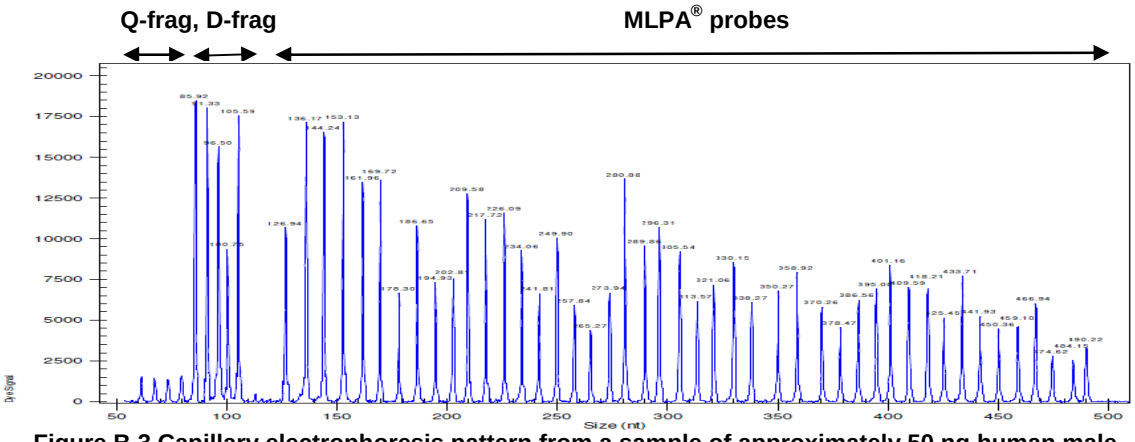


Figure B.3 Capillary electrophoresis pattern from a sample of approximately 50 ng human male control DNA analyzed with SALSA MLPA® kit P035-A2 DMD (lot 0508) (www.mlpa.com).

4. SALSA MLPA® PROBES FOR DMD

Table B.1 SALSA MLPA® P034-A2 DMD probe mix.

Length (nt)	SALSA MLPA® probe	Chromosomal position reference DMD
64-70-76-82	Q-fragments: DNA quantity; only visible with less than 100 ng sample DNA	
88-92-96*	D-fragments: Low signal of 88 or 96 nt fragment indicates incomplete denaturation	
100*	X-fragment: Specific for the X chromosome	
105*	Y-fragment: Specific for the Y chromosome	
118	Y-fragment: Specific for the Y chromosome	
130	Reference probe 1690-L00423	Xq11.2
138	DMD probe 1353-L01001	Exon 1
146	DMD probe 1354-L01002	Exon 41
154	DMD probe 1355-L01615	Exon 21
162	DMD probe 1356-L01004	Exon 61
170	DMD probe 1357-L01005	Exon 2
178	DMD probe 1711-L01279	Exon 42
186	DMD probe 1359-L01007	Exon 22
194	DMD probe 1897-L01008	Exon 62
202	Reference probe 1691-L00465	Xp22
210	DMD probe 1361-L01009	Exon 3
218	DMD probe 1362-L01010	Exon 43
226	DMD probe 1363-L01011	Exon 23
234	DMD probe 1364-L01012	Exon 63
242	DMD probe 1365-L01013	Exon 4
250	DMD probe 1366-L01014	Exon 44
258	DMD probe 1958-L01518	Exon 24
266	DMD probe 1368-L01016	Exon 64
274	Reference probe 1768-L01617	Xq28
282	DMD probe 1954-L01574	Exon 5
290	DMD probe 1370-L01287	Exon 45
298	DMD probe 1371-L01019	Exon 25
306	DMD probe 1372-L01020	Exon 65
314	DMD probe 1373-L01021	Exon 6
322	DMD probe 1374-L01288	Exon 46
330	DMD probe 1375-L01023	Exon 26
338	DMD probe 1376-L01024	Exon 66
354	DMD probe 1713-L01281	Exon 7
362	DMD probe 1378-L01026	Exon 47
370	DMD probe 1379-L01616	Exon 27
378	DMD probe 1960-L01520	Exon 67
386	DMD probe 1715-L01283	Exon 8
394	DMD probe 1382-L01030	Exon 48
402	DMD probe 1716-L01284	Exon 28
410	DMD probe 2482-L02710	Exon 68
418	Reference probe 3766-L03227	Xq13
426	DMD probe 1385-L01033	Exon 9
434	DMD probe 1717-L01285	Exon 49
442	DMD probe 1387-L01035	Exon 29
450	DMD probe 1388-L01036	Exon 69
458	DMD probe 1718-L01286	Exon 10
466	DMD probe 1390-L01038	Exon 50
474	DMD probe 1391-L01039	Exon 30
482	DMD probe 1392-L01040	Exon 70
490	Reference probe 1692-L01531	Xq28

Table B.2 SALSA MLPA® PO35 - A2 DMD probe mix.

Length (nt)	SALSA MLPA® probe	Chromosomal position reference DMD
64-70-76-82	Q-fragments: DNA quantity; only visible with less than 100 ng sample DNA	
88-92-96	D-fragments: Low signal of 88 or 96 nt fragment indicates incomplete denaturation	
100	X-fragment: Specific for the X chromosome	
105	Y-fragment: Specific for the Y chromosome	
130	Reference probe 1690-L00423	Xq11.2
138	DMD probe 1393-L01041	Exon 11
146	DMD probe 1394-L01042	Exon 51
154	DMD probe 1395-L01371	Exon 31
162	DMD probe 1396-L01044	Exon 71
170	DMD probe 1397-L01608	Exon 12
178	DMD probe 2059-L01571	Exon 52
186	DMD probe 1399-L01609	Exon 32
194	DMD probe 1949-L01610	Exon 72
202	Reference probe 1691-L00465	Xp22
210	DMD probe 1899-L01049	Exon 13
218	DMD probe 1892-L01050	Exon 53
226	DMD probe 1900-L01051	Exon 33
234	DMD probe 1893-L01052	Exon 73
242	DMD probe 1950-L01573	Exon 14
250	DMD probe 1894-L01054	Exon 54
258	DMD probe 1901-L01055	Exon 34
266	DMD probe 1902-L01611	Exon 74
274	Reference probe 1768-L01617	Xq28
282	DMD probe 1410-L01057	Exon 15
290	DMD probe 1411-L01058	Exon 55
298	DMD probe 1412-L01059	Exon 35
306	DMD probe 1413-L01060	Exon 75
314	DMD probe 2060-L01572	Exon 16
322	DMD probe 1415-L01062	Exon 56
330	DMD probe 1416-L01063	Exon 36
338	DMD probe 1417-L01612	Exon 76
354	DMD probe 1952-L01065	Exon 17
362	DMD probe 1419-L01066	Exon 57
370	DMD probe 3038-L02508	Exon 37
378	DMD probe 1421-L01068	Exon 77
386	DMD probe 1891-L01069	Exon 18
394	DMD probe 1423-L01070	Exon 58
402	DMD probe 1896-L01071	Exon 38
410	DMD probe 1425-L01072	Exon 78
418	Reference probe 3766-L03227	Xq13
426	DMD probe 1426-L01073	Exon 19
434	DMD probe 1427-L01074	Exon 59
442	DMD probe 1955-L01613	Exon 39
450	DMD probe 1429-L01076	Exon 79
458	DMD probe 1430-L01077	Exon 20
466	DMD probe 1431-L01614	Exon 60
474	DMD probe 1432-L01079	Exon 40
482	DMD probe 1433-L01080	Exon DP427C
490	Reference probe 1692-L01531	Xq28

Table B.3 DMD probes arranged according to chromosomal order.

Length (nt) P034/P035	SALSA MLPA® A probe	DMD exon	Ligation site M18533	Partial sequence (20 nt adjacent to ligation site)	Distance to next exon
P034/P035					
482 *	1433-L01080	Exon DP427c	233-234 in NM_000109.2	CAGTAATAGA-ATGCTTTCAG	127.9 Kb
138 *	1353-L01001	Exon 1	26-27	CCCCCTACAG-GACTCAGATC	191.3 Kb
170 *	1357-L01005	Exon 2	266-267	CAAAAGAAAA-CATTCACAAA	170.4 Kb
210	1361-L01009	Exon 3	335-336	CTCTTCAGTG-ACCTACAGGA	5.0 Kb
242	1365-L01013	Exon 4	435-436	CCCTGAACAA-TGTCAACAAG	21.5 Kb
282	1954-L01574	Exon 5	517-518	AGATGGAAAT-CATAAACTGA	6.7 Kb
314	1373-L01021	Exon 6	618-619	CCAACAGTGA-AAAGATTCTC	7.0 Kb
354	1713-L01281	Exon 7	765-766	GGAATAGTGT-GGTTTGCCAG	110.4 Kb
386	1715-L01283	Exon 8	951-952	TTGAAGCCAT-CCAGGAAGTG	1.2 Kb
426	1385-L01033	Exon 9	1066-1067	ACAGGGATAT-GAGAGAACTT	52.8 Kb
458	1718-L01286	Exon 10	1195-1196	AGACAAGTCA-TTTGGCAGTT	0.8 Kb
138	1393-L01041	Exon 11	1382-1383	TTGACAGCCC-ATCAGGGCCG	29.9 Kb
170	1397-L01608	Exon 12	1647-1648	AGCCTCTTGG-ACCTGATCTT	18.5 Kb
210	1899-L01049	Exon 13	1762-1763	GGTAGTTGAT-GAATCTAGTG	22.0 Kb
242	1950-L01573	Exon 14	1836-1837	GGGCAAACAT-CTGTAGATGG	0.2 Kb
282	1410-L01057	Exon 15	1937-1938	TGGCTTTCAG-AAAAAGAAGA	7.8 Kb
314	2060-L01572	Exon 16	2059-2060	GCAATCCATG-GGCAAACTGT	20.6 Kb
354	1952-L01065	Exon 17	2292-2293	AACAGATCCT-GGTAAAGCAT	27.2 Kb
386	1891-L01069	Exon 18	2433-2434	AAGCTGTGTT-GCAGAGTCCT	16.3 Kb
426	1426-L01073	Exon 19	2526-2527	AAGCTGAGAA-GTTCAGAAAA	10.4 Kb
458	1430-L01077	Exon 20	2650-2651	GTGGATCGAA-TTCTGCCAGT	6.4 Kb
154	1355-L01615	Exon 21	2916-2917	AAGGACAAGG-ACCCATGTTC	12.7 Kb
186	1359-L01007	Exon 22	3051-3052	AGGAGACCAT-GAGTGCCATC	3.6 Kb
226	1363-L01011	Exon 23	3200-3201	GGCCTATACT-ATCTCAGCAC	4.0 Kb
258	1958-L01518	Exon 24	3441-3442	GGCCTGCCCT-TGGGGATTCA	1.1 Kb
298	1371-L01019	Exon 25	3554-3555	CAGAAGATAA-AGAATGAAGC	8.8 Kb
330	1375-L01023	Exon 26	3695-3696	GTAAGCCTCC-AGAAAGATCT	6.2 Kb
370	1379-L01616	Exon 27	3871-3872	TGAGTCTGTA-AATAGTGTCA	7.3 Kb
402	1716-L01284	Exon 28	4020-4021	GGCATGAGTT-ATTGTCATAC	3.0 Kb
442	1387-L01035	Exon 29	4198-4199	ACAGACCCTA-ACAGATGGCG	26.4 Kb

Table B.3 DMD probes arranged according to chromosomal order cont.

Length (nt) P034/P035	SALSA MLPA® probe	DMD exon	Ligation site M18533	Partial sequence (20 nt adjacent to ligation site)	Distance to next exon
474	1391-L01039	Exon 30	4304-4305	AAGTTGCTTG-AACAGAGCAT	21.8 Kb
154	1395-L01371	Exon 31	4519-4520	GGAGGCTGCC-CAAAGAGTCC	0.6 Kb
186	1399-L01609	Exon 32	4674-4675	CTGCATTGGA-AACAAAGAGT	3.1 Kb
226	1900-L01051	Exon 33	4766-4767	TCTGAAGTGG-AAATGGTGAT	5.8 Kb
258	1901-L01055	Exon 34	4947-4948	GAAAGGAAAT-GAATGTCTTG	15.5 Kb
298	1412-L01059	Exon 35	5098-5099	CCTGAAGAGT-ATCACAGAGG	0.5 Kb
330	1416-L01063	Exon 36	5288-5289	ATCACAAAGT-GGATCATTCA	1.8 Kb
370 *	3038-L02508	Exon 37	5498-5497 Rev.	TGGCTGCAAA-TCGATGGTTG	14.4 Kb
402	1896-L01071	Exon 38	5613-5614	CTGAAATTCA-GCAGGGGGTG	2.4 Kb
442	1955-L01613	Exon 39	5700-5701	TGCAAAGAGG-AGACAACTTA	2.8 Kb
474	1432-L01079	Exon 40	5828-5829	AAGGCTCTAG-AAATTTCTCA	1.0 Kb
146	1354-L01002	Exon 41	6019-6020	GGGCTTGTCT-GAGGATGGG	32.0 Kb
178	1711-L01279	Exon 42	6159-6160	CGATGATGGT-GATGACTGAA	22.6 Kb
218	1362-L01010	Exon 43	6403-6404	ATTGCAAAGT-GCAACGCCTG	70.6 Kb
250	1366-L01014	Exon 44	6589-6590	ACAGTTTCTC-AGAAAGACAC	248.6 Kb
290	1370-L01287	Exon 45	6745-6746	AGATGCCAGT-ATTCTACAGG	36.3 Kb
322	1374-L01288	Exon 46	6913-6914	CATTGCTAGT-ATCCCACTTG	2.4 Kb
362	1378-L01026	Exon 47	7017-7018	TCAAACAATT-AAATGAAACT	54.4 Kb
394	1382-L01030	Exon 48	7225-7226	GTTAAATCAT-CTGCTGCTGT	38.5 Kb
434	1717-L01285	Exon 49	7356-7357	AAGAGATTTT-GTCTAAAGGG	16.7 Kb
466	1390-L01038	Exon 50	7455-7456	TAAACCGTTT-ACTTCAAGAG	46.0 Kb
146	1394-L01042	Exon 51	7630-7631	TCTGGCAGAT-TTCAACCGGG	44.4 Kb
178 *	2059-L01571	Exon 52	7837-7836 Rev.	AGCCTCTTGA-TTGCTGGTCT	50.2 Kb
218	1892-L01050	Exon 53	7994-7995	GAGCAGGTCT-TAGGACAGGC	21.3 Kb
250	1894-L01054	Exon 54	8116-8117	GCAGACAAAT-GTAGATGTGG	30.3 Kb
290	1411-L01058	Exon 55	8276-8277	ACTCATAGAT-TACTGCAACA	120.5 Kb
322	1415-L01062	Exon 56	8535-8536	TCCTGTTACA-AAGACGTTTG	10.5 Kb
362	1419-L01066	Exon 57	8686-8687	GAAAGATGAT-GAATTAAGCC	17.8 Kb
394	1423-L01070	Exon 58	8817-8818	AGACTGTACG-AATATTTCTG	0.8 Kb
434	1427-L01074	Exon 59	9005-9006	GATGAGACCC-TTGAAAGACT	33.6 Kb

Table B.3 DMD probes arranged according to chromosomal order cont.

Length (nt) P034/P035	SALSA MLPA® probe	DMD exon	Ligation site M18533	Partial sequence (20 nt adjacent to ligation site)	Distance to next exon
466	1431-L01614	Exon 60	9175-9176	GCCTCTGAAA-GAGAACGTGA	96.0 Kb
162	1356-L01004	Exon 61	9325-9326	GCAGCTGCAT-GAAGCCCAC	25.0 Kb
194	1897-L01008	Exon 62	9394-9395	TCCCTGGGAG-AGAGCCATCT	62.6 Kb
234 *	1364-L01012	Exon 63	9453-9454	AAACAACTTG-CTGGGACCAT	37.9 Kb
266 *	1368-L01016	Exon 64	9547-9546 Rev.	CTGCAGTCTT-CGGAGTTTCA	13.4 Kb
306	1372-L01020	Exon 65	9599-9600	GCTGCATGTG-ATGCCTTGGA	3.0 Kb
338	1376-L01024	Exon 66	9806-9807	CTGTCTTTTA-AAACTGGCAT	2.6 Kb
378 *	1960-L01520	Exon 67	9987-9986 Rev.	ACACTTGGCT-CAATGTTACT	21.1 Kb
410	2482-L02710	Exon 68	10064-10065	GACTGGATGA-GACTGGAACC	2.4 Kb
450	1388-L01036	Exon 69	10238-10239	TTTTTTTCTG-GTCGAGTTGC	1.7 Kb
482	1392-L01040	Exon 70	10385-10386	CCCCGAATGG-GCTACCTGCC	0.8 Kb
162 *	1396-L01044	Exon 71	10450-10451	TCTGATCAAC-TTCTGGCCAG	4.4 Kb
194	1949-L01610	Exon 72	10493-10494	CCTCAGCTTT-CACACGATGA	1.2 Kb
234 *	1893-L01052	Exon 73	10571-10570 Reverse	TATCATTTAG-ATAAGATCCA	2.9 Kb
266	1902-L01611	Exon 74	10693-10694	CCAGATCTTG-ATTTCCTTAG	22.1 Kb
306	1413-L01060	Exon 75	10885-10886	TGAGCTCATT-GCTGAGGCCA	1.0 Kb
338	1417-L01612	Exon 76	11063-11064	ACCTCTCTAC-AGAGGTCCGA	12.2 Kb
378	1421-L01068	Exon 77	11164-11165	CCAGGACACA-AGCACAGGGT	7.5 Kb
410 *	1425-L01072	Exon 78	11245-11246	TGGAAAGCCA-ATGAGAGAGG	4.8 Kb
450	1429-L01076	Exon 79	11286-11287	CCACATGGCA-GATGATTTGG	

*These probes detect the reverse sequence or extend into an intron and are expected to give no signal with cDNA. The two exon 1 sequences are present in alternative transcripts and may also give a weak or no signal on cDNA (Kesari et al., 2008).

Tables of probe mixes were adapted from SALSA MLPA® KIT P034-A2 / P035-A2 DMD / Becker (www.mlpa.com). The identity of the genes detected by the reference probes is available on request from info@mlpa.com.

APPENDIX C

1. IMPORTANT CONSIDERATIONS FOR HR- MCA ASSAY DESIGN

The HRM platform

Successful HRM can only be achieved with specialized instrumentation capable of collecting data with exceptional real-time and thermo-optical precision. A cross-platform comparison by Herrmann et al. (2006) effectively highlighted the limitations of conventional systems and the unique qualities required for accurate HRM analysis, particularly:

- High intensity illumination
- High sensitivity optical detection
- A fast data acquisition rate
- Exquisite sample temperature control
- An absolute minimum of sample-to-sample thermal and optical variation

The first instrument marketed for the sole purpose of exquisitely accurate HRM analysis, was the HR-1™ (Idaho Technology), which remains the performance benchmark (Reed, Kent, & Wittwer, 2007). However, the HR-1™ is not capable of thermal cycling and can only analyze a single sample per run from within a glass capillary, making data analysis time consuming. The multi-well LightScanner® (Idaho Technology) uses a modified block-based design but is also an HRM-only machine, not capable of thermal cycling. Subsequent technological advancements have resulted in introduction of machines with real-time cycling as well as HRM capability i.e. LightCycler®480 (Roche, Germany) and the RotorGene™6000 (Corbett Life Science, Australia) (Figure C.1). A major advantage of this is that the amplification efficiency can be monitored in real time, with the initial PCR cycling followed by HRM in a closed-tube system, without the need for post-PCR manipulation. In addition, the methods used to normalize and display HRM data for intuitive manual analysis or automated genotyping are specific and continue to improve.

The Rotor-Gene™6000, used in this study, stands apart from other analysers by virtue of its unique centrifugal format. Every tube spins quickly in a chamber of moving air. This is especially suitable for HRM, as there is virtually no positional temperature variation such as the recognized “edge effect” observed in block-based instruments. Optically, the Rotor-Gene™6000 is similarly uniform, because every tube moves past identical excitation and detection points (Herrmann et al., 2007; White & Potts, 2006). As a result, the Rotor-Gene™000 HRM performance closely matches the HR-1™ benchmark.

The RotorGene™6000, fulfils the criteria for reliable HRM analysis, with a melting rate which can be adjusted manually to the lowest level of the above mentioned instruments (0.05°C/sec), and a default of 0.1°C increments with a 2 second hold at each step. The Rotor-Gene™6000 has a wide optical range, with 6 channels spanning UV to infra-red wavelengths (www.corbettlifescience.com). It offers the benefits of real-time PCR along with its HRM capability, successfully tested in a number of applications, including:

- Gene expression analysis
- Pathogen detection
- DNA methylation analysis
- Genotyping
- Mutation scanning
- SNP analysis
- High Resolution Melting Analysis

In an evaluation report by Helen White and Gemma Potts of NGRL, the overall HRM sensitivity and specificity of the RotorGene™6000 was found to be 100% and 95% respectively, as compared to HR1™ (Idaho Technology) at 98% and 95%, and the 384-well LightScanner® (Idaho Technology) at 99% and 88% (White & Potts, 2006). Other instrumentation with real-time and HRM capabilities have since been marketed, but at this time, those mentioned above are most often mentioned in the literature and have been subject to cross platform comparisons.

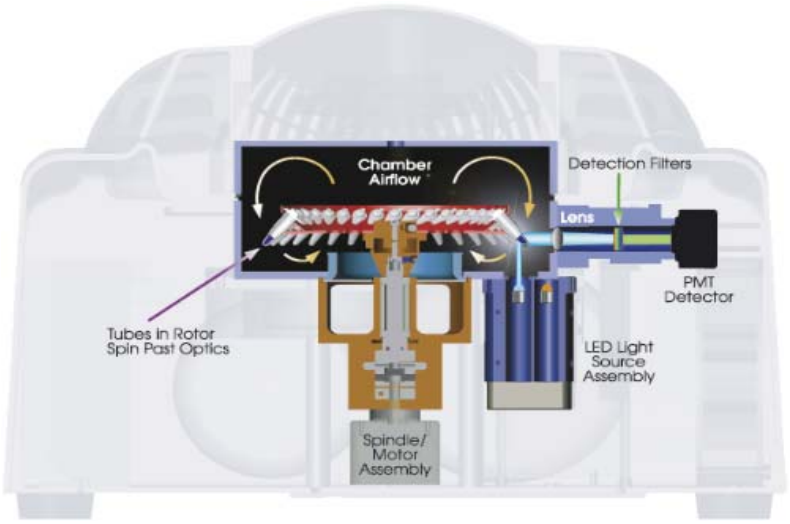


Figure C.1 The RotorGene™6000 with its centrifugal design.

Choice of a dsDNA intercalating dye

The advancements in intercalating dye technology since Higuchi first demonstrated the use of ethidium bromide in qPCR (Higuchi et al., 1993), along with instrumentation that allows highly controlled temperature transitions and acquisition, have played a major role in the emergence of HRM methodology. Different base substitutions produce slight differences in melting behaviour, the resolution of which requires an appropriate intercalating dye. SYBR Green® is the most widely used intercalating dye for monitoring real-time PCR, but is generally regarded unsuitable for use in hrMCA due to its strong tendency to inhibit PCR and promote mispriming at high (saturating) concentrations. According to the proposed "dye saturation hypothesis" (Wittwer et at., 2003; Liew et al., 2004), SYBR Green dissociates from dsDNA during melting, and dynamically re-intercalates into the neighbouring duplexes that melt at higher temperatures (Figure C.2), potentially masking small differences in melting behaviour, which could then go undetected. Low dye concentrations on the other hand, (e.g. < 0.5 µM) can compromise PCR signal strength, and the dye redistribution possibly results in an unreliable DNA melt curve. "Saturating dyes" are thus defined as those that can be used at concentrations sufficiently high to saturate all DNA binding sites without inhibiting the PCR, thereby achieving a greater melting curve resolution (Wittwer et al 2003, Liew et al 2004). New generation dyes such as LC Green®, LC Green®PLUS, SYTO 9®, BEBO, CHROMOFY® and Eva Green™, show minimal PCR toxicity at high levels and allow for accurate genotyping of single-nucleotide polymorphisms and sensitive mutation scanning.

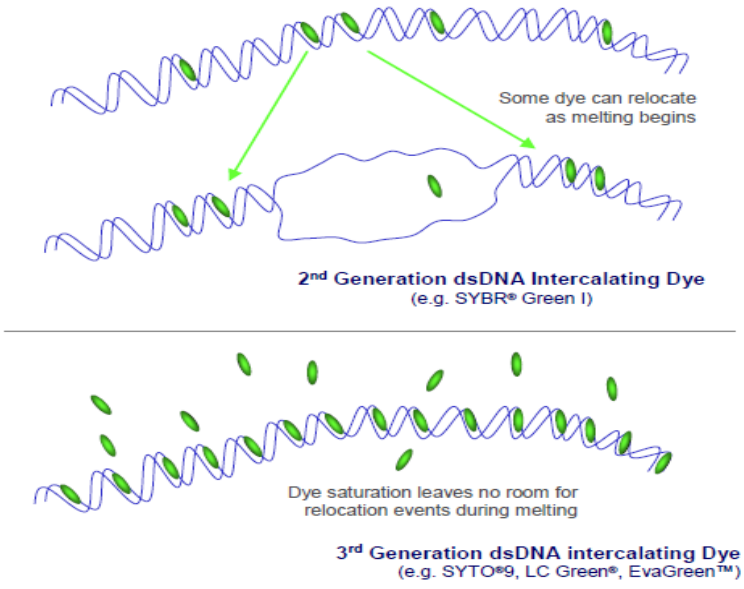


Figure C.2 The dye re-distribution theory and the 2nd and 3rd generation dsDNA intercalating dyes (Cor-Protocol-6000-1-July06)**.**

The dye redistribution theory however, has been contested, as evidence for this theory was collected by a pre-HRM era LightCycler® (Roche Molecular Systems) using templates which included a low mass DNA size ladder (Wittwer et al., 2003; Liew et al., 2004). According to Corbett Research, saturation levels of SYBR Green® are not required when using the Rotor-Gene™6000. The experimental data shows good HRM results and the authors encourage users not to dismiss SYBR Green® as a dye for HRM, provided that access to an appropriate instrument is available. Corbett Research do however concede that while saturating dye levels appear unnecessary for HRM on the Rotor-Gene™6000 (when apparently required on other instrument systems), the reason for this is not clear. They suggest that the explanation may lie in the centrifugal rotary format of the instrument, as that is what sets it apart from other HRM machines.

The dsDNA intercalating utalised in this project, EvaGreen™ (Biotum), is included in the HRM Sensimix kit, optimised by Quantace specifically for use on the RotorGene™6000. EvaGeen is a green fluorescent nucleic acid dye useful in qPCR, hrMCA, real-time monitoring of thermophilic helicase-dependent amplification (tHDA), routine solution DNA quantification and capillary gel electrophoresis. It is also compatible with multiplex PCR, as no dye migration from amplicon to amplicon takes place when used at the recommended concentration. EvaGreen™ is essentially nonfluorescent by itself, but becomes highly fluorescent upon binding to dsDNA. Once bound, it has excitation and emission spectra similar to fluorescein (FAM) or SYBR Green® 1 and is therefore readily compatible with instruments equipped with the 488 nm argon laser or any visible light excitation filter with a wavelength in that region. It is thus compatible with all major brand qPCR instruments and enzyme systems. The extreme thermal, hydrolytic and photo-stability of EvaGreen™ provides convenience during routine handling, as the dye can withstand repeated cycles of freezing and thawing, does not decompose in PCR buffer at 95-100°C for 48 hours and is highly stable under either alkaline or acidic condition. Compared with SYBR Green®, EvaGreen™ is generally less inhibitory toward PCR and less likely to cause non-specific amplification. Unlike SYBR Green®, which enters cells rapidly and is known to be a powerful mutation-enhancer, EvaGreen™ is nonmutagenic and noncytotoxic by being completely impermeable to cell membranes (Ohta et al., 2001; Mao et al., 2007; White et al., 2007). Regardless of the validity of the dye redistribution theory, EvaGreen can be used at a much higher dye concentrations than SYBR Green® I, resulting in a more robust PCR signal.

PCR design and optimisation

Besides the availability of an appropriate HRM platform and choice of a dsDNA intercalating dye, the success of any hrMCA assay is highly dependant on the design and optimisation of the pre-HRM PCR reaction, as the presence of non-specific products and primer dimmers significantly reduces HRM performance. Use of a real-time thermocycler is recommended, particularly when optimizing or troubleshooting an assay, as information on amplicon quality can be monitored in real time.

Primer design

The PCR product should not include sequence variations other than the one investigated e.g. polymorphic regions, as their presence will alter the melting curve. Also, secondary structure formation of the primers and the amplicon will affect the amplification efficiency and should be avoided. Optimal primer design and checking for formation of secondary structures can be achieved with software such as Primer3 and the DINAmelt Servers from the Rensselaer Polytechnic Institute, respectively (Markham & Zuker, 2005). HR-MCA of polymorphic regions however, has been successfully performed but involves inclusion of known, genotype-specific controls (White & Potts, 2006). PCR reaction optimisation for HRM, may require a significant increase in the annealing temperatures (2°C – 10°C), because of the presence of dsDNA intercalating dye.

$MgCl_2$ concentration

The amount of $MgCl_2$ has a strong effect on the melting behaviour of dsDNA and should be optimized carefully. Generally, a higher concentration of $MgCl_2$ is needed in a PCR for HR-MCA, which includes a dsDNA intercalating dye, as opposed to an ordinary PCR, where no dye is present. The general guidelines also indicate that the required $MgCl_2$ concentration varies according to the specific dye chosen e.g. 1.5 mM $MgCl_2$ with SYTO® 9, or 3 mM $MgCl_2$ with LCGreen® or EvaGreen™ (www.corbettlifescience.com)

PCR reagents

It is recommended to use PCR reagents validated or optimized for HRM analysis, when available, although this may be more expensive. Hot start enzymes, generally included in HRM mixes such as the AmpliTaq Gold 360 Master Mix (Applied Biosysems, USA) or the HRM Sensimix (Quantace), are also recommended, as they increase primer specificity.

2. FACTORS AFFECTING THE MELTING CURVE AND RELIABILITY OF hrMCA

DNA and amplicon quality/quantity

The effect of DNA quality on an HRM assay was clearly demonstrated in an experiment by White et al. (2003), where amplifiability of DNA samples of variable quality was assessed by using a multiplexed control gene primer set which amplifies products of 100, 200, 300, 400 and 600bp with equal intensity. Loss of the higher molecular weight products was seen as an indicator of poor DNA quality. It was shown that samples in which products of 200bp or less were amplified, gave false positive results when analysed by HRM.

The use of spectral analysis for determining DNA concentration and purity is recommended, as the amount of starting template should be similar between samples in a run, since it affects the amount of generated PCR product. At 260 nm one absorbance unit is equal to 50 µg/mL of DNA and pure DNA will provide a 260 nm to 280 nm ratio of 2. The amplicon concentration in turn, affects its melting temperature(T_M) and the outcome of HRM analysis. The threshold cycle (C_T) of an efficient amplification plot, should not exceed 30 cycles, as amplification log phase starting beyond that point, is typically due to insufficient amount of starting template or presence of degradation effects. Allowing every reaction to amplify to the plateau phase is important, as a similar extent of amplification for all tests should be achieved irrespective of their starting amount. However, poor reaction might result in a plateau with a different quantity of product, due to factors such as inconsistent assay set-up (e.g. insufficient amount of primer).

Monitoring of the amplification curve in real time is a valuable trouble-shooting tool in hrMCA, as problems and inconsistencies involving the quality and quantity of DNA, the amplicon and the reaction set up can be readily identified. They manifest as late amplification, curves with log-linear phase that is not steep, is jagged, or reaches a low signal plateau as compared to other reactions in the run. HRM data from such samples can be inconclusive and should be excluded from analysis.

Amplicon size

Differentiation of variant melting curves is best achieved in smaller PCR fragments (~100bp). HrMCA analysis of products larger than 300bp can be analyzed successfully but usually with lower resolution. This is simply because a single base variation affects the melting behavior of a 100 bp amplicon more than a 500 bp amplicon and will therefore be easier to detect (www.corbettlifescience.com). Large amplicons are also more likely to have complex melting curves with two or more

melting domains, complicating the analysis and making it less reliable. Ideally, a PCR fragment should have a single melting domain, otherwise each domain must be analysed separately, by manual adjustment of the normalisation regions. Optimal amplicon design and subsequent optimisation are therefore crucial to the success of HRM. For this reason a number of exons in the DMD gene were split into two or more fragments i.e. exons 23, 48, 53, 61, 67, 68, 76 and 79 thus creating 96 sets of PCR primers to amplify 79 exons of the *DMD* gene.

Primer dimers

Like other non-specific products, presence of primer-dimmers may affect the melting curve characteristics and therefore primer concentration should generally not exceed 300nM.

Effect of mutation type or the surrounding sequence context

The position of a mutation within a PCR fragment per se, does not appear to have a significant effect on HRM-based mutation detection. However, an experiment carried out as part of a cross HRM platform comparison by White et al. (2006) showed that certain mutations, such as the 110A>G and 588delA mutations in exons 1 and 7 respectively, are intrinsically difficult to detect by HRM, possibly because they are sited in regions which, when melted, produce only subtle overall changes in fluorescent intensity. Interestingly, it was shown that differences in data handling between the compared platforms (HR-1™, Light Scanner™ and RotorGene™6000), also affect variant detection efficiency of some mutations. Specifically, 1459 C>T, 1451 delA and 1409+14G>A sequence variants in exon13, appear to alter the melting temperature rather than the overall shape of the melting curve. Data from the HR-1 ™ and Light Scanner™ is first normalised and then temperature shifted. When the data are temperature shifted these curves become superimposed on the wild type samples, making detection difficult. Data from the RotorGene™6000 is normalized, but not temperature shifted, and therefore such mutations can be more easily detected as the melt curves do not become overlaid on the wild type curve (White & Potts, 2006).

Homo- and hemizygous changes appear to have similar effect on the melting dynamic of the DNA fragment i.e. they appear to alter the temperature at which the amplicon melts rather than the shape of the melt curve (Kwok et al., 2007; Yan et al., 2010). Temperature-shifting for data analysis, causes the curves of homozygous samples to become superimposed on the wild type masking the change. As mentioned above, this effect does not take place during analysis with the

RotorGene™6000, as the data is normalized but not temperature shifted and homozygous mutations are readily detected.

Sample-to-sample uniformity

Consistency in reaction setup and reagent use is critical to the success hrMCA. Reaction volume and the amount of dye in each tube must be the same. Similarly, identical reaction tubes from the same manufacturer should be used to avoid variations in plastic thickness and autofluorescence properties. DNA melting behavior is affected by salts in the reaction mix, so concentration of buffers, $MgCl_2$ and other salts should be kept uniform in all samples. The various available DNA extraction methods utalise different chemistries and elution buffers, which may affect the DNA melting behavior. It is therefore best to compare samples extracted with the same method, which is an important consideration when attempting analysis of archival samples or samples from other laboratories.

Temperature range during melting

HRM data points over a window of approximately 10°C (or greater), should be captured, centered around the observed T_M of the fragment under analysis This provides enough baseline data points for effective curve normalization and will result in tight replicates and easier data interpretation.

Regions of normalisation

The data analysis software on the RotorGene™6000 allows for both visual and auto-calling of genotypes. Two normalization regions are used to normalize fluorescencece (Y axis), represented by vertical cursors placed pre-melt (region 1) and post-melt (region 2) phases (Figure C.3).

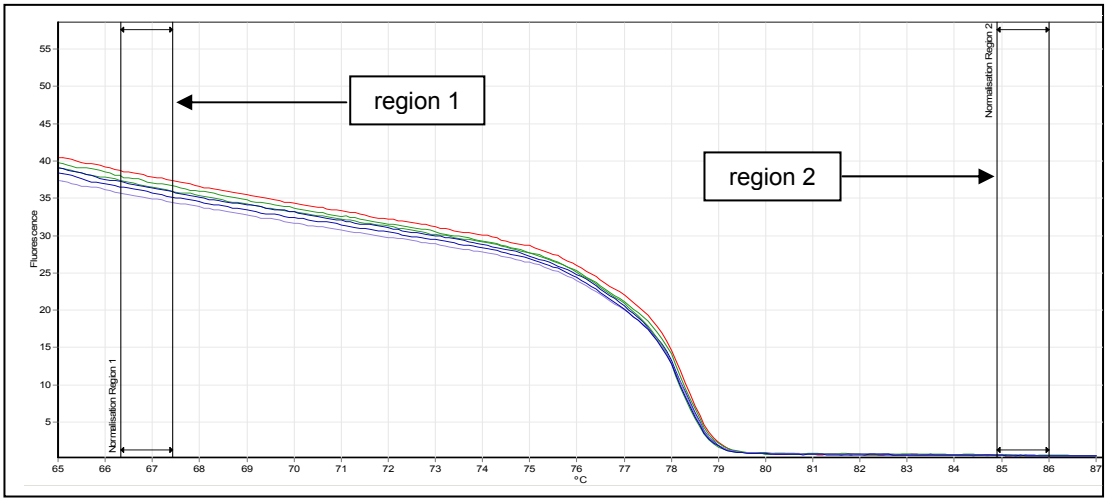


Figure C.3 Adjustable normalisation regions.

The cursors are placed at default positions, which can be manually adjusted. Shifting the cursors can alter the appearance of a normalised curve and cause differences in both qualitative manual calling and automated mutation calling. Therefore, in a diagnostic setting, criteria for positioning the cursors for different amplicons should be incorporated into standard operating procedures to ensure minimal data analysis variability between operators. Corbett Research recommend that these default settings be changed only to avoid certain parts of the curve, such as when analysing complicated curves with multiple melting domains. In such instances, the pre-melt region 1 should be set at a point where all samples in a run show uniform melting (seen as parallel lines), and the post-melt region 2 should be set after complete melting of all samples, so that the normalisation range should stretch across approximately 10°C.

The four classes of base changes

Detection of a single base alteration, as the smallest genetic change underlies the sensitivity of an HRM analysis. Single Nucleotide Polymorphisms (SNPs) often used for genotyping have been divided into 4 classes according to their rarity and difficulty in detection. As presented in Table C.1 below, the A:T to G:C interchange, which is the most common single base variation in the human genome also results in the greatest difference in Tm (>0.5), which is readily detected. Other base changes give rise to smaller differences in Tm, resulting in lower specificity that depends on the exact base change and also on the surrounding sequence.

Table C.1 Classification of SNPs (Venter et al., 2001).

SNP Class	Base Change	Typical T_M Melt Curve Shift	Rarity (in the human genome)
1	C/T and G/A	Large (>0.5°C)	64%
2	C/A and G/T	⇩	30%
3	C/G		9%
4	A/T	Very Small (<0.2°C)	7%

3. PRIMER SEQUENCES

Table C.2 HRM and sequencing primers

#	PRIMER SEQUENCE	FRAGMENT LENGTH	ANNEALING TEMP
EX01-F	TGTAAAACGACGGCCAGTGCAGGTCCTGGAATTTGA	405	61Cº
EX01-R	CAGGAAACAGCTATGACCCAAACTAAACGTTATGCCACA		
EX02-F	TGTAAAACGACGGCCAGTCACTAACACATCATAATGG	269	61Cº
EX02-R	CAGGAAACAGCTATGACCGATACACAGGTACATAGTC		
EX03 A-F	TGTAAAACGACGGCCAGTTCATCCGTCATCTTCGGCAGATTAA	176	61Cº
EX03 A-R	CAGGAAACAGCTATGACCCAGGCGGTAGAGTATGCCAAATGAAAATCA		
EX03B-F	TCTTCAGTGACCTACAGGATGG	184	64Cº
EX03B-R	CGCCCGCCGtgctgtttcaatcagtacctagtca		
EX04-F	TGTAAAACGACGGCCAGTTTGTCGGTCTCTCTGCTGGTCAGTG	233	60Cº
EX04-R	CAGGAAACAGCTATGACCCCAAAGCCCTCACTCAAAC		
EX05-F	TGTAAAACGACGGCCAGTCAACTAGGCATTTGGTCTC	261	61Cº
EX05-R	CAGGAAACAGCTATGACCTTGTTTCACACGTCAAGGG		

Table C.2 HRM and sequencing primers cont.

EX06-F	TGTAAAACGACGGCCAGTTGGTTCTTGCTCAAGGAATG	335	61Cº
EX06-R	CAGGAAACAGCTATGACCTGGGGAAAAATATGTCATCAG		
EX07-F	TGTAAAACGACGGCCAGTCTATGGGCATTGGTTGTC	296	60Cº
EX07-R	CAGGAAACAGCTATGACCAAAAGCAGTGGTAGTCCAG		
EX08-F	TGTAAAACGACGGCCAGTTCGTCTTCCTTTAACTTTG	343	61Cº
EX08-R	CAGGAAACAGCTATGACCTCTTGAATAGTAGCTGTCC		
EX09-F	TGTAAAACGACGGCCAGTTCTATCCACTCCCCCAAACC	318	61Cº
EX09-R	CAGGAAACAGCTATGACCAACAAACCAGCTCTTCAC		
EX10-F	CGACGTTGTAAAACGACGGCCAGTGGAACAATCTGCAAAGAC	350	61Cº
EX10-R	CAGGAAACAGCTATGACCAAAGGATGACTTGCCATTATAAC		
EX11-F	TGTAAAACGACGGCCAGTCAAATAAAACTCAAAACCACACC	337	61Cº
EX11-R	CAGGAAACAGCTATGACCCTTCCAAAACTTGTTAGTCTTC		
EX12-F	TGTAAAACGACGGCCAGTCTTTCAAAGAGGTCATAATAGG	305	61Cº
EX12-R	CAGGAAACAGCTATGACCCATCTGTGTTACTGTGTATAGG		

Table C.2 HRM and sequencing primers cont.

EX13-F	TGTAAAACGACGGCCAGTGCAAATCATTTCAACACAC	387	60Cº
EX13-R	CAGGAAACAGCTATGACCTCTTTAAATCACAGCACTTC		
EX14-15-F	TGTAAAACGACGGCCAGTTGGCAAATTATTCATGCCATT	548	63Cº
EX14-15-R	CAGGAAACAGCTATGACCTGATCCAAGCAAAAATAAACATT		
EX16-F	TGTAAAACGACGGCCAGTATGCAACCCAGGCTTATTC	286	61Cº
EX16-R	CAGGAAACAGCTATGACCCTGTAGCATGATAATTGGTATCAC		
EX17-F	TGTAAAACGACGGCCAGTTTTTCCTTTGCCACTCCAAG	362	61Cº
DEX17-R	CAGGAAACAGCTATGACCCACCACCAACAAAACTGCTG		
EX18-F	CGACGTTGTAAAACGACGGCCAGTTGTCAGGCAGGAGTCTCAGAT	339	63Cº
EX18-R	CAGGAAACAGCTATGACCCGGAGTTTACAAGCAGCACA		
EX19-F	TGTAAAACGACGGCCAGT gattcacgtgataagctgacaga	286	63Cº
EX19-R	CAGGAAACAGCTATGACCCGCCCGCCGCGCttcagctgataaatatgaacctatgtg		
EX20-F	TGTAAAACGACGGCCAGTTGGCTTTCAGATCATTTCTTTC	393	61Cº
EX20-R	CAGGAAACAGCTATGACCAAATACCTATTGATTATGCTCC		

Table C.2 HRM and sequencing primers cont.

EX21-F	TGTAAAACGACGGCCAGTGCAAAATGTAATGTATGCAAAG	355	63Cº
EX21-R	CAGGAAACAGCTATGACCATGTTAGTACCTTCTGGATTTC		
EX22-F	TGTAAAACGACGGCCAGTAGGAAAACATGGCAAAGTGTG	370	63Cº
EX22-R	CAGGAAACAGCTATGACCTGCTCAATGGGCAAACTACC		
EX23 A-F	TGTAAAACGACGGCCAGTACTCATCAATTATTATTCATCAATTAGGGT	126	61Cº
EX23 A-R	CAGGAAACAGCTATGACCCATCTCTTTCACAGTGGTGC		
EX23 B-F	TGTAAAACGACGGCCAGTAGCAACAAAGTGGCCTATAC	135	61Cº
EX23 B-F	CAGGAAACAGCTATGACCGCTGGGAGGAGAGCTTC		
EX23 C-F	TGTAAAACGACGGCCAGTTTGAAGAAATTGAGGGACGC	175	61Cº
EX23C-R	CAGGAAACAGCTATGACCCTTTACAGTTTACAGTGTATCGTTAGG		
EX24-F	TGTAAAACGACGGCCAGTTTGGGCCTGTGTTTAGACATA	327	63Cº
EX24-R	CAGGAAACAGCTATGACCAAATCCACCCCAGCTGTAAAA		
EX25-F	TGTAAAACGACGGCCAGTTGTGGCAGTAATTTTTTTCAG	296	61Cº
EX25-R	CAGGAAACAGCTATGACCAGGAAATCTTAGTTAAGTACG		

Table C.2 HRM and sequencing primers cont.

EX26-F	TGTAAAACGACGGCCAGTTGAGTGTATCTGATCCCCATGA	438	61Cº
EX26-R	CAGGAAACAGCTATGACCTGTTGCATTTCTTTCTTTTTC		
EX27-F	TGTAAAACGACGGCCAGTTGGGATGTTGTGAGAAAGAA	365	63Cº
EX27-R	CAGGAAACAGCTATGACCTGACCATGTATTGACATATCATTGA		
EX28-F	TGTAAAACGACGGCCAGTGAAGTTTTAATAATGAAATGGCAAAA	311	61Cº
EX28-R	CAGGAAACAGCTATGACCTGACCTCTTTTAATACTGCATAT		
EX29-F	TGTAAAACGACGGCCAGTCCAATGTATTTAGAAAAAAAAGGAG	279	63Cº
EX29-R	CAGGAAACAGCTATGACCGCAAATTAGATTAAAGAGATTTTTCAC		
EX30-F	TGTAAAACGACGGCCAGTTACAGAAAAGCTATCAAGAG	297	61Cº
EX30-R	CAGGAAACAGCTATGACCAAAAACAAAAGAATGGAAGC		
EX31-F	TGTAAAACGACGGCCAGTATGGTAGAGGTGGTTGAGGA	296	61Cº
EX31-R	CAGGAAACAGCTATGACCTATAATGCCCAACGAAAACA		
EX32-F	TGTAAAACGACGGCCAGTCAGTTATTGTTTGAAAGGCAAA	322	61Cº
EX32-R	CAGGAAACAGCTATGACCCTTCTTAATGAGGAAAGTCAAGG		

Table C.2 HRM and sequencing primers cont.

EX33-F	CGACGTTGTAAAACGACGGCCAGTTGGAATAGCAATTAAGGG	393	60Cº
EX33-R	CAGGAAACAGCTATGACCGCTAAGACTCTAATCATAC		
EX34-F	TGTAAAACGACGGCCAGTCAGAAATATAAAAGTTCCAAATAAGTG	374	61Cº
EX34-R	CAGGAAACAGCTATGACCCATGTTAATACTTCCTTACAAAATC		
EX35-F	TGTAAAACGACGGCCAGTCCGTTTCATAAGCATTAAATC	307	61Cº
EX35-R	CAGGAAACAGCTATGACCAGCTTCTAGCCTTTTCTC		
EX36-F	CGACGTTGTAAAACGACGGCCAGTTGTCTAACCAATAATGCCATG	257	64Cº
EX36-R	CAGGAAACAGCTATGACCCTGGTGTACAATTTGGACA		
EX37-F	CGACGTTGTAAAACGACGGCCAGTCTTTCTCACTCTTCTCGCTCAC	377	61Cº
EX37-R	CAGGAAACAGCTATGACCTTCGCAAGAGACCATTTAGCAC		
EX38-F	TGTAAAACGACGGCCAGTTTTAGCAACAGGAGGTTGAA	267	64Cº
EX38-R	CAGGAAACAGCTATGACCTTCTTTCCAAATATTTATTTCCACT		
EX39-F	TGTAAAACGACGGCCAGTCTCTGTTAACAATGTACAGCTTTTT	365	64Cº
EX39-R	CAGGAAACAGCTATGACCAAAAACCACAGGCAAGGTAT		

Table C.2 HRM and sequencing primers cont.

EX40-F	TGTAAAACGACGGCCAGTTACAAAAAGATGAGGGAC	387	61Cº
EX40-R	CAGGAAACAGCTATGACCAATAGAAACAAGAACATCAAC		
EX41-F	TGTAAAACGACGGCCAGTGTTAGCTAACTGCCCTGGGCCCTGTATTG	311	61Cº
EX41-R	CAGGAAACAGCTATGACCTAGAGTAGTAGTTGCAAACACATACGTGG		
EX42-F	TGTAAAACGACGGCCAGTATGGAGGAGGTTTCACTGTT	408	61Cº
EX42-R	CAGGAAACAGCTATGACCCCATGTGAAAGTCAAAATGC		
EX43-F	TGTAAAACGACGGCCAGTTTTCTATAGACAGCTAATTCATTTTT	287	63Cº
EX43-R	CAGGAAACAGCTATGACCACAGTTCCCTGAAAACAAATC		
EX44-F	TGTAAAACGACGGCCAGTGTTACTTGAAACTAAACTCTGCAAATG	444	61Cº
EX44-R	CAGGAAACAGCTATGACCACAACAACAGTCAAAAGTAATTTCCATC		
EX45-F	TGTAAAACGACGGCCAGTTTCTTTGCCAGTACAACTGC	357	61Cº
EX45-R	CAGGAAACAGCTATGACCTCTGCTAAAATGTTTTCATTCC		
EX46-F	TGTAAAACGACGGCCAGTCCAGTTTGCATTAACAAATAGTTTGAG	409	64Cº
EX46-R	CAGGAAACAGCTATGACCAGGGTTAAGAAGAAATAAAGTTGTGAG		

Table C.2 HRM and sequencing primers cont.

EX47-F	TGTAAAACGACGGCCAGTTGATAGACTAATCAATAGAAGCAAAGAC	399	61Cº
EX47-R	CAGGAAACAGCTATGACCAACAAAACAAAACAACAATCCACATACC		
EX48 A-F	TGTAAAACGACGGCCAGTTTTGGCTTATGCCTTGAGAAT	175	61Cº
EX48 A-R	CAGGAAACAGCTATGACCATAACCACAGCAGCAGATG		
EX48 B-F	TGTAAAACGACGGCCAGTGCTTGAAGACCTTGAAGAGC	185	61Cº
EX48 B-R	CAGGAAACAGCTATGACCAAATGAGAAAATTCAGTGATATTGCC		
EX49-F	TGTAAAACGACGGCCAGTGTGCCCTTATGTACCAGGCAGAAATTG	475	61Cº
EX49-R	CAGGAAACAGCTATGACCGCAATGACTCGTTAATAGCCTTAAGATC		
EX50-F	TGTAAAACGACGGCCAGTCACCAAATGGATTAAGATGTTCATGAAT	307	64Cº
EX50-R	CAGGAAACAGCTATGACCTCTCTCTCACCCAGTCATCACTTCATAG		
EX51-F	TGTAAAACGACGGCCAGTGAAATTGGCTCTTTAGCTTGTGTTTC	424	64Cº
EX51-R	CAGGAAACAGCTATGACCGGAGAGTAAAGTGATTGGTGGAAAATC		
EX52-F	TGTAAAACGACGGCCAGTGTGTTTTGGCTGGTCTCACA	298	63Cº
EX52-R	CAGGAAACAGCTATGACCCATGCATCTTGCTTTGTGTGT		

Table C.2 HRM and sequencing primers cont.

EX53 A-F	TGTAAAACGACGGCCAGTAAGAATCCTGTTGTTCATCATCCTAGC	252	64Cº
EX53 A-R	CAGGAAACAGCTATGACCCCAGCCATTGTGTTGAATCCTTTAAC		
EX53 B-F	TGTAAAACGACGGCCAGTAGTACAAGAACACCTTCAGAACCG	278	64Cº
EX53 B-R	CAGGAAACAGCTATGACCACTTTACATTAAACATCATTAAATTACAATCTATGG		
EX54-F	CGACGTTGTAAAACGACGGCCAGTGTATTCTGACCTGAGGATTC	378	61Cº
EX54-R	CAGGAAACAGCTATGACCCATGGTCCATCCAGTTTC		
EX55-F	TGTAAAACGACGGCCAGTAATTTAGTTCCTCCATCTTTCTCT	445	61Cº
EX55-R	CAGGAAACAGCTATGACCAAATACATCAGGCTGTATAAAAGC		
EX56-F	TGTAAAACGACGGCCAGTATTCTGCACATATTCTTCTTCCTGC	353	63Cº
EX56-R	CAGGAAACAGCTATGACCGGATGATTTACGTAGACATGTGAG		
EX57-F	TGTAAAACGACGGCCAGTCAATGGAATTGTTAGAATCATCA	320	63Cº
EX57-R	CAGGAAACAGCTATGACCCACTGGATTACTATGTGCTTAACAT		
EX58-F	TGTAAAACGACGGCCAGTTTTTGAGAAGAATGCCACAAGCC	315	63Cº
EX58-R	CAGGAAACAGCTATGACCAAAATATGAGAGCTATCCAGACCC		

Table C.2 HRM and sequencing primers cont.

EX59-F	TGTAAAACGACGGCCAGTAAAGAATGTGGCCTAAAACC	433	64Cº
EX59-R	CAGGAAACAGCTATGACCTTGTGGGAAGATAACACTGC		
EX60-F	TGTAAAACGACGGCCAGTTAAATATTCTCATCTTCCAATTTGC	267	63Cº
EX60-R	CAGGAAACAGCTATGACCTTACTGTAACAAAGGACAACAATG		
EX61A-F	TGTAAAACGACGGCCAGTCGCCCGCCGCttgctttagtgttctcagtcttgg	169	63Cº
EX61A-R	CAGGAAACAGCTATGACCAAAGTCCCTGTGGGCTTCAT		
EX61B-F	TGTAAAACGACGGCCAGTCGTCGAGGACCGAGTCAG	210	63Cº
EX61B-R	CAGGAAACAGCTATGACCCGCCCGCCGCcaggatgatttatgcttctactgc		
EX62-F	TGTAAAACGACGGCCAGTTAATGTTGTCTTTCCTGTTTGCG	221	63Cº
EX62-R	CAGGAAACAGCTATGACCATACAGGTTAGTCACAATAAATGC		
EX63-F	TGTAAAACGACGGCCAGTTACTCATTGTAAATGCTAAAGTC	229	63Cº
EX63-R	CAGGAAACAGCTATGACCTAGCAAGTAACTTTCACACTGC		
EX64-F	TGTAAAACGACGGCCAGTTTCTGATGGAATAACAAATGCT	322	61Cº
EX64-R	CAGGAAACAGCTATGACCCATTCTAGGCAAACTCTAGGC		

Table C.2 HRM and sequencing primers cont.

EX65-F	TGTAAAACGACGGCCAGTagtgtggttcacgtttggt	386	64Cº
EX65-R	CAGGAAACAGCTATGACCtgtacgctaagcctcctgtg		
EX66-F	TGTAAAACGACGGCCAGTGTCAGTAATTGTTTTCTGCTTTG	246	61Cº
EX66-R	CAGGAAACAGCTATGACCATAAGAACAGTCTGTCATTTCCC		
EX67 A-F	TGTAAAACGACGGCCAGTTCAGGTTCTGCTGGCATC	172	60Cº
EX67 A-R	CAGGAAACAGCTATGACCTGCAACTTCACCCAACTGTC		
EX67 B-F	TGTAAAACGACGGCCAGTGCCTCCTTCTGCATGATT	187	61Cº
EX67 B-R	CAGGAAACAGCTATGACCAGAAAACGAAGCTCTGTGG		
EX68 A-F	TGTAAAACGACGGCCAGTCGCCCGCCcagcctagctttgcaaccat	249	61Cº
EX68 A-R	CAGGAAACAGCTATGACCACTGGGGTTCCAGTCTCATC		
EX68 B-F	TGTAAAACGACGGCCAGTAGCGGCCCTCTTCCTAGACT	236	61Cº
EX68 B-R	CAGGAAACAGCTATGACCCGCCCGC taacagcaactggcacagga		
EX69-F	TGTAAAACGACGGCCAGTGAACGTGGTAGAAGGTTTATTAAA	267	61Cº
EX69-R	CAGGAAACAGCTATGACCCTAACTCTCACGTCAGGCTG		

Table C.2 HRM and sequencing primers cont.

EX70-F	TGTAAAACGACGGCCAGTTGGTCATTAGTTTTGAAATCATC	273	63Cº
EX70-R	CAGGAAACAGCTATGACCCATCAAACAAGAGTGTGTTCTG		
EX71-F	TGTAAAACGACGGCCAGTGGCTGAGTTTGCGTGTGTCT	174	61Cº
EX71-R	CAGGAAACAGCTATGACCGAGCGAATGTGTTGGTGGTA		
EX72-F	TGTAAAACGACGGCCAGTAAGCATTCTAGGCCATGTGT	261	61Cº
EX72-R	CAGGAAACAGCTATGACCGGTTAGCTTTCCTTGGTTAGTT		
EX73-F	TGTAAAACGACGGCCAGTACGTCACATAAGTTTTAATGAGC	238	63Cº
EX73-R	CAGGAAACAGCTATGACCATGCTAATTCCTATATCCTGTGC		
EX74-F	TGTAAAACGACGGCCAGTATAAGGGGGGGAAAAAAC	290	63Cº
EX74-R	CAGGAAACAGCTATGACCTGCAAGTGTATGCACTCTG		
EX75-F	TGTAAAACGACGGCCAGTTCTTTTTTACTTTTTTGATGC	380	60Cº
EX75-R	CAGGAAACAGCTATGACCAGTGCTCTCTGAGGTTTAG		
EX76 A-F	TGTAAAACGACGGCCAGTacaatctttgggagggcttc	231	63Cº
EX76 A-R	CAGGAAACAGCTATGACCCTGACTGCTGTCGGACCTCT		

Table C.2 HRM and sequencing primers cont.

EX76 B-F	TGTAAAACGACGGCCAGTCACAACGGTGTCCTCTCCTT	216	63Cº
EX76 B-R	CAGGAAACAGCTATGACCttcagtggctccctgatacc		
EX77-F	TGTAAAACGACGGCCAGTTAATCATGGCCCTTTAATATCTG	306	63Cº
EX77-R	CAGGAAACAGCTATGACCGATACTGCGTGTTGGCTTCC		
EX78-F	TGTAAAACGACGGCCAGTTTCTGATATCTCTGCCTCTTCC	267	61Cº
EX78-R	CAGGAAACAGCTATGACCCATGAGCTGCAAGTGGAGAGG		
EX79 A-F	TGTAAAACGACGGCCAGTAGAGTGATGCTATCTATCTGCAC	385	61Cº
EX79 A-R	CAGGAAACAGCTATGACCTGCATAGACGTGTAAAACCTGCC		
EX79B 1–F	TGTAAAACGACGGCCAGTTTGTGAAGGGTAGTGGTATTATACTG	323	60Cº
EX79B 1–R	CAGGAAACAGCTATGACCTGCCTCAAAGTTTTGTGTGTG		
EX79B 2–F	TGTAAAACGACGGCCAGTCGCCCGCCGAACGCATTTTGGGTTGTTT	284	60Cº
EX79B 2–R	CAGGAAACAGCTATGACCTCAAATGAGCAGTGTGTAGTAGTCA		
EX79C1-F	TGTAAAACGACGGCCAGTCTTCCTCTACCACCACACCAA	242	60Cº
EX79 C1-R	CAGGAAACAGCTATGACCAAGCAGGTAAGCCTGGATGA		

Table C.2 HRM and sequencing primers cont.

EX79 C2-F	TGTAAAACGACGGCCAGTTGTTCATGTCACATCCTAATAGAAA	309	60Cº
EX79 C2-R	CAGGAAACAGCTATGACCCGCCCGCCGTAGCAGCAGGAAGCTGAATG		
EX79D 1-F	TGTAAAACGACGGCCAGTCGCCCGCCGCGAGTAATCGGTTGGTTGGTTGA	265	60Cº
EX79D 1-R	CAGGAAACAGCTATGACC TCCTTCACTTAAAGAGTGGCCTA		
EX79D 2 -F	TGTAAAACGACGGCCAGTGCTGGAGGGCTATGGATTC	280	60Cº
EX79D 2 -R	CAGGAAACAGCTATGACCCGCCCGCCGTCACAAATGTGATGGGGCTA		
EX79E -F	TGTAAAACGACGGCCAGTAATAAACTTTGGGAAAAGGTG	536	64Cº
EX79E -R	CAGGAAACAGCTATGACCGAAGCCGTGTTTGATGTTAAT		
EX79F-F	TGTAAAACGACGGCCAGTGAGAGTGGGCTGACATCAA	532	61Cº
EX79F-R	CAGGAAACAGCTATGACCTCACTCCAGAGCTAATGTGTCT		
EX79G-F	TGTAAAACGACGGCCAGTAGTAAGTTTCATTCTAAAATCAGAGG	531	61Cº
EX79G-R	CAGGAAACAGCTATGACCGTGTTTTCACTGTCTTTCTGGA		
M13 F	TGTAAAACGACGGCCAGT		
M13 R	CAGGAAACAGCTATGACC		