Chapter 1

Introduction

Muscular dystrophy (MD) is a class of muscular disorders, classified by its specific mode of inheritance, gene mutation, and muscle groups affected by the pathological progression. First identified in the 17th century, this class of disorders remains a subject in need of continuous research as no effective treatment is available to date.

Dysferlinopathy is sub-classified as a limb girdle type of muscular dystrophy, inherited in an autosomal recessive way. It is affecting the musculature of the pelvic and shoulder girdles. Onset of this muscle deteriorating genetic disease varies from as early as the 2nd decade of life to as late as the 8th decade. Identification of the gene mutated in the dysferlinopathies (Bashir *et al*., 1998; Liu *et al*., 1998), just over a decade ago, created high expectations for an intervention in the limitations inevitably suffered by those affected. Yet, the answer to a full life for dysferlinopathy patients remains to be unravelled. One of the goals of MD research is underscored by the understanding of how sarcolemmal damage is initiated. How it is repaired, and how the sarcolemma can be protected, or the damage minimized by pharmacologic or therapeutic intervention (Lovering *et al*., 2005). The present study follows an in-line approach by employing a neutraceutical treatment strategy.

The mutation in the dysferlin gene results in a deficiency of the protein dysferlin. Dysferlin has been shown to be involved in membrane repair machinery (Bansal *et al*., 2003), and has lately also been implicated in the process of chemotaxis (Chiu *et al*., 2009). From this point of view, and considering its interactions with numerous other proteins as reviewed in chapter 2 of this thesis, it seems likely that dysferlin may also be involved in other cellular processes, and responses, yet to be determined. The deficiency in this protein eventually leads to muscle cell necrosis, muscle deterioration, and in due course, loss of ambulation.

A major focus of biological research, as it is known today, is to elucidate the molecular and cellular mechanisms that underlie human health and disease (Doran *et al*., 2007), and to aim at manipulating the alterations in disease towards a normal phenotype. The possible diversity in the function of dysferlin and the even more diverse cellular effects brought about by its deficiency; render it viable to explore alternative treatment strategies that focus on the cellular effects, rather than the mechanism of the pathology itself. To address the cellular effects caused by dysferlin deficiency at microscopic level will comprise amongst others, the effective inflection of the cellular environment

to maintain cell stability for longer and reduction of the rate of dystrophic progression. Rather than repairing the membrane defect or the defective chemotactic response. The present study follows an alternative approach in applying antioxidant supplementation to provide relief to a cellular environment possibly subjected to oxidative stress.

Professor Karl Folkers was one of the pioneers who realized the body's need for optimal levels of the antioxidant, Coenzyme Q10 (CoQ10). In two successful double-blind trials, Folkers and Simonsen, 1995, were able to show that CoQ10 administration results in definite improved physical performance in a variety of muscular dystrophies. Folkers' team demonstrated CoQ10 to be therapeutically beneficial to a range of dystrophies and atrophies, probably attributed to its essentiality in the bioenergetics of life (Folkers and Simonsen, 1995). Evidence of its deficiency in the muscular dystrophies (Folkers and Simonsen, 1995) renders CoQ10 ideal for an alternative therapeutic approach in the dysferlinopathies.

Deficiency of CoQ10 was recently demonstrated to be an autosomal recessive disorder with heterogenous phenotypic manifestations and genetic background (Gempel *et al*., 2007). Primary CoQ10 deficiency is considered the only treatable mitochondrial disorder, since patients have a response to oral CoQ10 supplementation (Montini *et al*., 2008). In order for CoQ10 to maintain its physiological properties in biological systems, the need exists for optimum levels of the coenzyme in order to meet the metabolic demand. Literature is expanding with reports focusing on the deficiency of CoQ10 in the body.

Folkers and Simonsen, 1995, reported that the muscular dystrophies may have one or more significant vitamin deficiencies of those vitamins required in the biosynthesis of CoQ10 and that these additional diverse deficiencies may be major for the dystrophies. In 1985, Folkers and co-workers showed a deficiency of CoQ10 in muscle mitochondria of muscular dystrophy patients (Folkers *et al*., 1985). Folkers and Simonsen, 1995, suggested that patients suffering from muscular dystrophies should be treated with CoQ10, indefinitely. Available evidence indicates that, owing to its metabolic roles, CoQ10 could positively influence the natural course of disease and potentially enhance the effect of other treatments by correcting some metabolic derangements (Littarru and Tiano, 2005). Metabolic derangements are often not causative; they are known to be the consequences of secondary aspects of diseases (Littarru and Tiano, 2005). Because of its pivotal role in bioenergetics in all cells of the body, numerous systems are affected when the availability of CoQ10 becomes limited. It might therefore be imperative to explore the effect of CoQ10 supplementation in the MDs.

The statement by Bermúdez-Crespo and López in 2007 that it takes a broad, comprehensive and systematic approach to understand biology that is generally unbiased and not dependent on existing knowledge, encouraged the approach followed in the present study. A novel approach in investigating the cellular effects brought about by the supplementation of the SJL/J mouse model for dysferlinopathy with the antioxidants CoQ10 and resveratrol was followed. The main focus of the study was to determine, on a cellular level, the histopathology and ultrastructural changes in the SJL/J mouse model following a 90 day trial with antioxidant supplementation. In addition to studying the morphology, the study also paid attention to parameters that are non- or less-specific to the dysferlinopathies. The SJL/J mouse model was decided upon as the animal model of choice in the present study since a reduction in dysferlin protein in SJL/J mice, consistent with the dysferlin reduction in dysferlinopathy patients renders this model ideal for dysferlinopathy research (Bittner *et al*., 1999).

Non-specific parameters in the present context are regarded as factors that are not directly related to muscle cell structure and not directly affecting histopathology in dysferlin-deficient muscular dystrophy. These parameters include body weight, physical strength, the levels of inflammatory leukocytes at the haematopoietic level, and creatine kinase (CK) and lactic dehydrogenase (LDH) levels in the blood.

The body weight of the mouse is a measure of muscle mass as well as of overall well-being (Li *et al*., 2005), and was measured twice weekly in accordance with the quality assurance of the study, to maintain the animals in optimal condition. Force transduction strength testing is a non-invasive procedure that allows extremely quantitative assessment of treatment (Weller *et al*., 1997). In addition, the tensile strength test is a useful method for assessing disease progression in the SJL/J mouse model, and the effect of different treatments on physical strength.

Massively elevated serum levels of muscle enzymes, including CK, LDH, and aldolase, have been reported in early stage dysferlinopathy (reviewed by Urtizberea *et al*., 2008). In addition, serum CK levels of SJL/J mice have been found to increase along with exacerbation of the inflammatory changes (Nemoto *et al*., 2007).

Leucocytes travel out of the circulatory system in order to elicit an immune response in the tissues where needed. It follows that increased demand for particular leucocytes in various sites is reflected by increased numbers in the circulation. It was therefore decided to carry out a quantitative analysis of white blood cell counts, blood CK and LDH levels, as well as the weight change and tensile

strength of animals in present study. This was done to establish how these parameters were affected by antioxidant supplementation.

Both histo- and ultrastructural pathology have been well established in dysferlinopathy patients (Selcen *et al*., 2001; Fanin and Angelini, 2002; Cenacchi *et al*., 2005) and dysferlin-deficient animal models (Weller *et al*., 1997; Bittner *et al*., 1999; Bansal *et al*., 2003). Nevertheless, the effect of antioxidant supplementation on this level has not been described previously. Therefore, this thesis includes a chapter on the histopathology where the morphology of all groups was compared to reveal the effect of antioxidants. Chapter 5 also investigated the variation that occurred in the muscle fiber size of SJL/J quadriceps and gastrocnemius muscles and the alteration in the variation pattern afforded by antioxidant supplementation.

This thesis elaborated on the histopathological findings by including a chapter on the ultrastructure of the affected quadriceps muscles. The ultrastructural study included the ultrastructural hallmarks of the disease that has previously been described (Selcen *et al*., 2001; Cenacchi *et al*., 2005), but also paid attention to non-specific alterations at the ultrastructural level of the SJL/J mouse. Non-specific alterations were expected to provide insight on possible alternative ongoing pathological processes, or metabolic disturbances, as an effect of the disease. The strong qualitative focus of these two chapters forms the core of the present thesis and provides a detailed outline of muscle morphology, and how it is affected by dysferlin-deficiency in the SJL/J mouse model. It furthermore provided evidence that justified the investigation of the last experimental chapter of this thesis which includes the analysis of oxidative stress levels in the animals.

It has previously been reported that oxidative stress is primarily involved in the patho-meganistic muscle deterioration in the mdx mouse model for dystrophin-deficient MD (Disatnik *et al*., 1998). The lack of previous findings to support this notion in the SJL/J mouse model supported the initiative to determine the levels of oxidative stress in this model. The final experimental chapter of this thesis, therefore, provides an investigation into the level of oxidative stress in the quadriceps muscle of SJL/J mice and how these levels were affected by antioxidant supplementation.

The aim of the study was to determine whether the supplementation of two potent antioxidants, namely resveratrol and CoQ10, could render any benefit to the SJL/J mouse model for dysferlinopathy, when administered as a single daily dose. The study investigated this objective by studying the cellular effects afforded by the antioxidant supplementation in the SJL/J mouse model.

In order to reach this aim the study objectives that underlined the above methodological approach can be summarized as follows: The implementation and execution of a 90 day antioxidant supplementation trial with the SJL/J mouse model to study:

a. Non-specific parameters, including:
    i. Animal weight as a parameter of animal well-being during the course of the trial
    ii. Tensile strength as a parameter of disease progression and physical strength of SJL/J mice
    iii. Enzyme levels of creatine kinase and lactate dehydrogenase in the blood
    iv. White blood cell counts as indicating factor for ongoing inflammatory events

b. Histopathology
    i. As a qualitative parameter for morphological characteristics in the SJL/J mouse on light microscopic level
    ii. As a quantitative parameter for morphometric assessment of fiber size variation

c. Ultrastructure, to study hallmarks of the disease in the SJL/J mouse, and to pay attention to non-specific alterations, and what they might imply

d. Oxidative stress levels in quadriceps muscles by measuring the levels of malondialdehyde (MDA) and the total antioxidant status (TAS). Calculation of the oxidative stress index (OSI) from these two parameters gives a quantitative indication of the degree of oxidative stress.

Also, to establish how these parameters are affected by the antioxidant supplementation with CoQ10 and resveratrol, alone and in combination, at various concentrations.

Two different concentrations of CoQ10, one concentration of resveratrol, and a combination of CoQ10 and resveratrol were utilized. The combination preparation consisted of the same concentration CoQ10 used as the lower dose tested, and the same concentration of resveratrol, as the single supplementation dose administered. It is expected that beneficial effects afforded by the supplementation with antioxidants will be more pronounced with supplementation of the high dose CoQ10, and the combination of resveratrol/CoQ10.

The following hypothesis was formulated upon execution of the present study:

The supplementation of SJL/J mice with the antioxidants CoQ10 and resveratrol will be beneficial to the animals, and the beneficial effect will be evident at cellular level.

This thesis concludes with a summary of the outcome of the present study and how these findings might be implicated in future development of therapeutic strategies.

Chapter 2

Literature Review

2.1 INTRODUCTION

The muscular dystrophies (MDs) are a genetic group of disorders of muscle degeneration due to mutations in genes that encode a wide variety of proteins. These proteins include extracellular matrix proteins, transmembrane and membrane-associated proteins, cytoplasmic enzymes, and nuclear matrix proteins (Cohn and Campbell, 2000). The diseases vary enormously in terms of severity, age of onset, selective muscle involvement, and inheritance pattern. Even different mutations of single genes may cause dystrophies that range from the mildest to the most severe (Rando, 2002). Despite these variabilities, the most significant common feature among all of these disorders is necrotic degeneration of skeletal muscle cells, leading to, sooner or later, a loss of ambulation in the affected. Intensive investigation of the literature in this chapter will provide a comprehensive overview of the muscular dystrophies. It will more specifically shed light on the specific molecular events and mechanisms involved in the course of the dysferlinopathies.

Evidence of oxidative stress as the primary pathogenic factor contributing to the dystrophic process, and the progression of the disease condition, has been reported in dystrophin-deficient MD (Murphy and Kehrer, 1989; Disatnik *et al*., 1998). Although this has not yet been proved in the dysferlinopathies, it seems likely to be relevant. Considering its less aggressive pathologic course, compared to DMD, such an observation would strengthen the possibility that the application of antioxidant supplementation in dysferlin-deficient subjects may render significant value in the dysferlinopathies.

It therefore the aim of the present study to determine, on a cellular level, how the morphology and ultrastructure in dysferlin-deficient SJL/J mouse muscle, will be affected by antioxidant supplementation. In order to proceed, it is of the essence to have an in depth understanding of the disease and its pathologic progression. A detailed review of the specific mechanisms, unravelled to date, in the dysferlinopathies, as well as their involvement in the pathogenesis and disease progression, is presented.

2.2 MUSCULAR DYSTROPHY

The muscular dystrophies are a heterogeneous group of inherited disorders characterized by progressive weakness and degeneration of skeletal muscles. Although known for the selective involvement of skeletal muscles, this selectivity in the MDs is not restricted only to weakness, but also extends to muscle wasting and enlargement (Pradhan, 2006). They have traditionally been classified by clinical presentation, mode of inheritance, age of onset, and overall progression. The development of molecular genetic mapping techniques has shown that a number of clinically similar conditions are linked to a variety of distinct single-gene disorders. So far, the MDs have been mapped to at least 29 different genetic loci, that give rise to at least 34 different clinical disorders (Dalkilic and Kunkel, 2003), and additional information is accumulating rapidly (Lovering *et al*., 2005). Muscular dystrophy includes a spectrum of disorders caused by loss of the linkage between the extracellular matrix and the actin cytoskeleton (Laval and Bushby, 2004).

Duchenne muscular dystrophy (DMD), is the most common form of MD, affecting approximately one in 3 500 newborn males worldwide (Biggar *et al*., 2001; Metules, 2002; Wagner, 2002). It is an X-linked disorder that was first discovered more than a century ago (Duchenne, 1861). Duchenne MD is characterized by progressive wasting of skeletal muscles with the first signs of weakness detectable in the limb girdle muscles. The first symptoms of the disease manifest as early as 5 years of age, followed by an inability to walk by the age of 8 to 12 years (Brooke *et al*., 1989; McDonald *et al*., 1995).

Duchenne muscular dystrophy is caused by the absence of the protein dystrophin, a 427 kilodalton (kDa) protein localised to the cytoplasmic surface of the plasma membrane of muscle fibers in skeletal and cardiac muscle (Lovering *et al*., 2005). Becker muscular dystrophy (BMD) is an allelic variant of DMD. Whereas DMD is caused by the essential absence of dystrophin, BMD is caused by abnormalities in the quality or quantity of dystrophin (Lovering *et al*., 2005). It is generally believed that a greater amount of dystrophin results in a less severe form of the myopathy (Kakulas, 1999). The onset of BMD is usually between the ages of 5 and 15 years, but can occur as late as the fourth decade of life (Lovering *et al*., 2005). The phenotypic presentation of BMD is similar to that of DMD, but is clinically milder and with more variability and a much slower progression. Patients with BMD do not have contractures or severe scoliosis, and many live well into adulthood, sometimes to a normal life span (Lovering *et al*., 2005).

The dystrophin protein is responsible for mechanical stability of the sarcolemma. It is likely involved in force transmission between the intracellular contractile apparatus and the extracellular matrix (ECM), which envelops the fiber and is connected to the tendon (Petrof *et al*., 1993). Without dystrophin the structural stability of the sarcolemma is compromised, leading to fragility and inability to withstand the stresses of normal muscle contractions (Petrof *et al*., 1993). The resulting membrane damage leads to increased intracellular Ca^{2+}, which activates proteases that ultimately result in fiber death or necrosis.

The regeneration of myofibers that normally occurs after damage to healthy skeletal muscle, also in the first few years of life in muscle of DMD patients, does not persist as these patients mature. The regenerative capacity of dystrophin-deficient muscle becomes inadequate to effectively replace lost muscle fibers (Blau *et al*., 1983; Webster and Blau, 1990; Bockhold *et al*., 1998). Necrotic fibers are replaced by fat and connective tissue, leading not only to muscle wasting and weakness, but also to an apparent 'pseudohypertrophy' (Lovering *et al*., 2005). This phenomenon is mostly observed in calves of boys suffering from Duchenne's. This muscle wasting and weakness is responsible for a clinical hallmark of the disease termed Gowers' sign that was first described in 1887. It is also referred to as Gowers' manoeuvre.

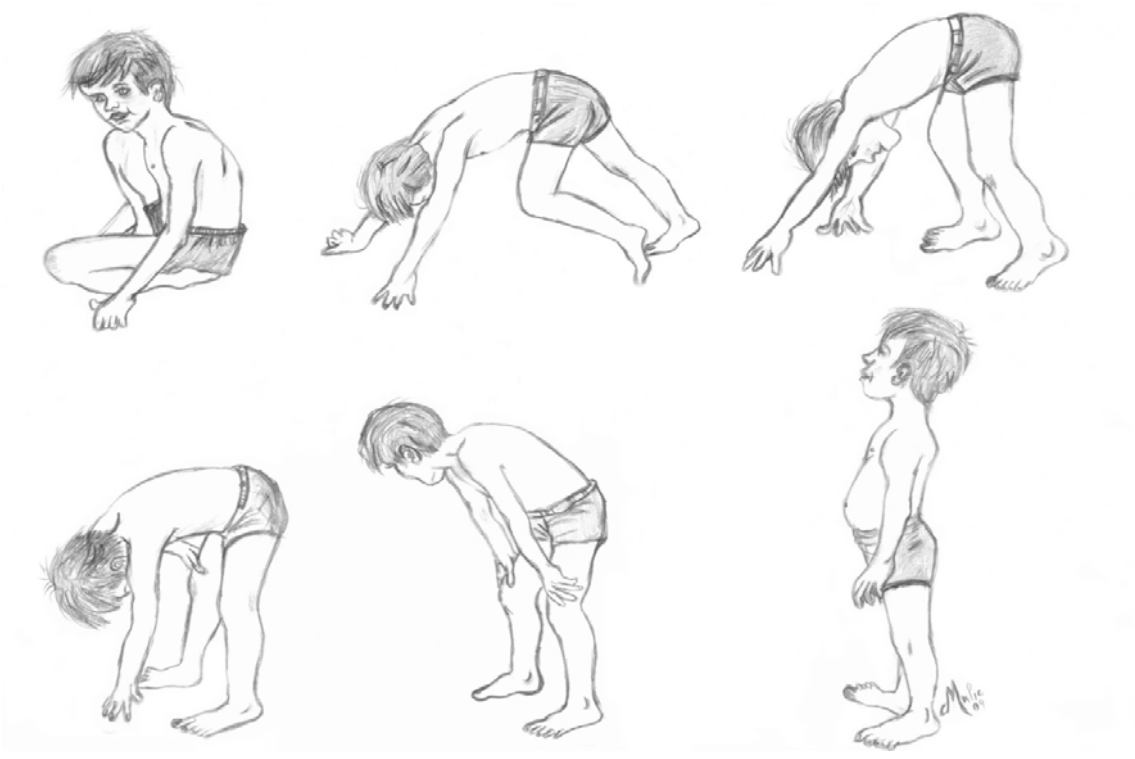

Figure 2.1: Schematic representation of Gower's sign in the muscular dystrophies.

Gowers' sign (Figure 2.1) was first illustrated by Sir William Richard Gowers (1845-1915). He described the characteristic peculiarity in which children with Duchenne dystrophy rise from a seated position. These children use their upper body strength by placing their hands on their knees, climbing up their thighs by pushing with their hands and arms in order to overcome weakness of the pelvic girdle muscles when attempting to rise from the floor (Figure 2.1)(Pearce, 2000; Hartman *et al*., 2007).

The clinical hallmark of a condition, then termed pseudo-hypertrophic muscular paralysis, known as Gowers' sign, was described with exceptional detail by Gowers (Gowers, 1886 in Pearce, 2000). As this detailed clinical illustration still holds diagnostic value in modern medicine, Sir Gowers' exact description is sketched in his own words in text box 2.1.

Text box 2.1

Gowers' sign

"The difficulty in going upstairs is especially due to the weakness of the extensors of the knee and hip. The defect of the extensors of the hip causes the gait to have a peculiar oscillating characters. The greatest defect, however, is in the power of rising from the floor, and the most characteristic peculiarity is the mode in which this is achieved, if it be still possible, and no objects near, by which the patient can aid himself. He commonly has not sufficient power to extend the knees when the weight of the trunk is on the upper extremity of the femur, which is then a lever in which power, applied between the fulcrum and the weight, acts at least advantage. He therefore places his hands on his knees, his arms thus bring much of the weight of the upper part of the trunk on the femur close to the fulcrum, between this and the power, which can then act at greater advantage. When the knees are extended, the power of the extensors of the hip may be sufficient to raise the body into the upright position, or the patient may aid them by an upward push with the hand as he takes it off. If, however, these extensors are weak, the hands are often moved higher and higher up the thighs, grasping alternately, and thus pushing up the trunk. To get the requisite support, the knees must not be quite extended, and if their extensors have no power, the device cannot be employed, and the patient is altogether unable to rise. In many cases, especially when extension of the hip is easy, the patient achieves the extension of the knees in another way; he puts the hands on the ground, stretches out the legs behind him far apart, and then, the chief weight of the trunk resting on the hands, by keeping the toes on the ground and pushing the body backwards, so as to bring a larger portion of the weight of the trunk over the legs. Then one hand is placed upon the knee, and a push with this, and with the other hand on the ground, is sufficient to enable the extensors of the hip to bring the trunk into the upright position." (Sir William R Gowers, 1887)

A great deal about the genetic basis of the dystrophin-deficient disease has been unravelled (Lovering *et al*., 2005), since the cloning of the mutated gene in the 1980s (Murray *et al*., 1982; Kunkel *et al*., 1985). The identification of its protein product, dystrophin, (Hoffman *et al*., 1987) the complex it forms in muscle, (Ehmsen *et al*., 2002) and the mapping of mutations linked several MDs and their associated proteins to dystrophin.

In skeletal muscle injuries, particularly those resulting from lengthening, or eccentric contractions, the membrane is damaged and the cytoskeleton is disrupted (Lovering and De Deyne, 2004), leading to a compromise in the structural stability of the muscle cell. The initial injury is followed by pain, inflammation, and weakness that usually results in necrosis, which is then followed by regeneration of muscle fibers (Lovering *et al*., 2005). As an effect of the disease, continuous cycles of active regeneration and degeneration follows until this sequence of events eventually falls out of equilibrium, inevitably resulting in muscle wasting.

Dystrophin binds indirectly or directly to a group of proteins at the sarcolemma, collectively known as the dystrophin-associated protein complex (DAPC or DPC) or the dystrophin-glycoprotein complex (DGC) (Straub *et al*., 1997; Allamand and Campbell, 2000; Ehmsen *et al*., 2002; Lovering *et al*., 2005). Dystrophin binds to this complex through the interaction of its WW domain. The W represents the letter code of tryptophan. The WW domain is a protein-binding module composed of 35 to 40 amino acids (aa) that include two conserved moieties of tryptophan (Bork and Sudol, 1994) and nearby sequences with the carboxyterminal (C-terminal) cytoplasmic sequence of β-dystroglycan (Jung *et al*., 1995), a transmembrane protein (Petrucci *et al*., 2001). The N-terminal and extracellular portion of β-dystroglycan associates with α-dystroglycan, which in turn connect the DGC to the ECM (Petrucci *et al*., 2001). The major ligand of α-dystroglycan in the ECM is likely laminin-2, a major component of the basement membrane surrounding the muscle fibers. Neurexin and agrin may also be significant binding partners (Michele *et al*., 2002).

Additional components of the DGC include several sarcoglycans, which also span the sarcolemmal membrane, and peripheral membrane proteins, including syntrophins and dystrobrevins that bind to the C-terminal region of dystrophin. Inside the muscle fiber, the N-terminus of dystrophin binds to F-actin (Norwood *et al*., 2000), connecting the DGC to the actin cytoskeleton and ultimately to the contractile apparatus. Therefore, dystrophin is the central component of the molecular link that connects the contractile apparatus inside the muscle fiber to the ECM outside the muscle fiber (Lovering *et al*., 2005). Dystrophin plays a crucial structural role in stabilizing the sarcolemma during

muscle contraction (Petrof *et al*., 1993), and is thought to transmit force laterally across the sarcolemma to the ECM (Tidball, 1991; Patel and Lieber, 1997). In contrast to DGC-linked muscular dystrophies, dysferlin-linked muscular dystrophies introduce a new class of the disease where the repair, and not the structure of the plasma membrane, is disrupted (Bansal and Campbell, 2004). This is a novel mechanism of muscle degeneration (Bansal and Campbell, 2004).

Table 2.1 The Muscular Dystrophies

Disease	*Protein Missing/Deficient*	*Age of onset*	*Muscles Affected*	*Complications*
DUCHENNE (DMD)	Dystrophin	Early childhood	Muscles of hips, legs, shoulders, and spine, and the heart	Severe muscle weakness and wasting, scoliosis, contractures, respiratory failure, pneumonia, and dilated cardiomyopathy. Death early 20s.
BECKER (BMD)	Dystrophin	Adolescence or adulthood	Similar to DMD	Muscle weakness as in DMD, but slower progress and much less severe. Cardiomyopathy
LIMB-GIRDLE (LGMD)				
LGMD 1A	Myotilin	Adolescence to early adulthood	Proximal shoulder/pelvic girdle musculature	Walking may not be possible within 20 years of onset
LGMD 1B	Lamin			
LGMD 1C	Caveolin			
LGMD 1D	Not identified			
LGMD 1E	Not identified			
LGMD 1F	Not identified			
LGMD 2A	Calpain-3	Infancy to early adulthood	Proximal shoulder/pelvic girdle musculature	Type 2 LGMD is much more severe than type 1 LGMD and some result in a DMD-like phenotype. Cardiac complications, sometimes occurring in later stages.
LGMD 2B	Dysferlin			
LGMD 2C	γ-sarcoglycan			
LGMD 2D	α-sarcoglycan			
LGMD 2E	β-sarcoglycan			
LGMD 2F	δ-sarcoglycan			
LGMD 2G	TCAP			
LGMD 2H	TRIM 32			
LGMD2I	FKRP			
LGMD 2J	Titin			
DISTAL MUSCULAR DYSTROPHIES				
Miyoshi myopathy	Dysferlin	Late adolescence	Posterior compartment of legs	Can eventually affect anterior compartment and distal arm muscles. Slow progression and able to maintain independent ambulation throughout life.
Tibial muscular dystrophy	Titin	Late adulthood	Anterior compartment of legs	Slowly progressive. Eventually can affect upper extremities and heart.
Welander myopathy	Unknown	Fifth decade	Distal muscles of upper limbs	Slowly progressive. Eventually affects lower limbs.
Nonakel myopathy (also known as distal myopathy with rimmed vacuoles) and inclusion body myopathy	Acetylglucosamine epimerase	Late adulthood	Anterior compartment of legs	Display red-rimmed autophagocytic vacuoles. Weakness can progress to proximal muscles, but quadriceps femoris muscles are spared.
Laing myopathy	Unknown	Ranges from infancy to adulthood	Anterior compartment of legs and neck flexors	Weakness can progress to proximal muscles.
Myofibrillary / desmin-related myopathy	Desmin		Initially distal lower extremities, but eventually proximally	Aggregation of desmin and other proteins inclusion bodies. Slow progress, eventual cardiomyopathy.

Disease	*Protein Missing/Deficien*	*Age of onset*	*Muscles Affected*	*Complications*
CONGENITAL MUSCULAR DYSTROPHIES (CMD)				
MDC 1A	Laminin α2 (merosin)	At birth	Proximal limb muscles	Joint contractures, cognitive and speech problems, seizures. Most do not learn to walk. White matter changes and structural abnormalities on magnetic resonance image.
MDC 1B	Not identified	At birth		
MDC 1C	FKRP	At birth		
MDC 1D	LARGE	At birth		
Fukuyama CMD	Fukutin	At birth	Proximal upper limbs, distal lower limbs, face and neck.	Cortex malformation and brainstem hypoplasia. Primarily in Japanese population.
α7 integrin congenital myopathy	α7 integrin	At birth	Mild myopathy	Generalized weakness.
Rigid spine CMD	Selenoprotein N1	At birth	Contractures of spinal extensors	Spine rigidity, early restrictive lung disease.
Muscle-eye-brain disease	POMGnT1 glycotransferase	At birth	Generalized	Retinal abnormality, myopia, cataracts, optic nerve atrophy, seizures.
Bethlem myopathy / Ullrich syndrome	Type VI collagen	At birth	Proximal limb muscles	Early contractures, flat feet. Rare and mild.
OTHER TYPES				
Emery-Dreifuss (EDMD)	Emerin/lamin	Childhood to early teens	Proximal upper extremity and distal lower extremities	Early contractures, cardiomyopathy.
Epidermolysis bullosa	Plectin	Childhood	Generalized	Skin blistering with range of severity, joint contractures, dysphagia.
Oculopharyngeal (OPMD)	Poly-A-binding protein 2	Age 40-60 years	Eyelids, throat	Ptosis of eyelids, dysphagia, aspiration pneumonia.
Facioscapulohumeral (FSHD)	Not identified	Childhood to early adolescence	Face, shoulders, proximal upper extremities	Cardiac conduction defects, mild hearing loss, retinal abnormalities.
Myotonic(DM)	Myotinin protein kinase, ZNF 9	Infancy (more severe) to adulthood	Distal extremity muscles first, then proximal as well	Myotonia, cataracts, hypogonadism, cardiac arrhythmias. Adult form is mild compared to early onset.

Key

TCAP	Telethonin, a protein that interacts with, or 'caps', another protein in muscle called titin
TRIM 32	One of 37 TRIM proteins containing a tripartite motif (TRIM)
FKRP	Fukutin-related protein
LARGE	The LARGE gene was so named because it covers over 660 kb of genomic DNA; the protein it encodes is a putative glycosyltransferase
POMT1	Protein O-linked mannose β-1,2-N-acetylglucosaminyltransferase
ZNF9	Zinc finger protein 9

Reproduced with permission of the authors (Lovering *et al.*, 2005)

2.3 LIMB-GIRDLE MUSCULAR DYSTROPHIES

Limb girdle muscular dystrophy is the generic name for a heterogenous group of neuromuscular disorders inherited either dominantly or recessively. They are characterized by muscle wasting and atrophy mostly in the shoulder and pelvic girdle but with a quite variable degree of severity and disease course (Urtizberea *et al*., 2008).

The diagnostic criteria for this subclass of MDs include raised serum creatine kinase (CK) activity, myopathic electromyography, muscle biopsy with features ranging from mildly myopathic to overt dystrophic changes and absence or reduction of a protein involved in the specific form of LGMD, on a Western blot (Borsato *et al*., 2006). Differential diagnosis of these disorders requires the careful application of a broad range of disciplines including, clinical assessment, immunohistochemistry and immunoblotting, using a panel of antibodies and extensive molecular genetic analysis (Laval and Bushby, 2004).

Within this subclass of MDs are the forms of limb-girdle muscular dystrophy caused by deficiencies of the sarcoglycan complex and by aberrant glycosylation of α-dystroglycan caused by mutations in the fukutin-related protein gene. However, other forms of this disease have distinct pathophysiological mechanisms (Laval and Bushby, 2004). Deficiency of dysferlin disrupts sarcolemmal membrane repair, whilst loss of calpain-3 may exert its pathological influence either by perturbation of the IκBα/NF-κB pathway or through calpain-dependent cytoskeletal remodelling (reviewed in Laval and Bushby, 2004). Implicated in numerous cell-signalling pathways (Shaul and Anderson, 1998), caveolin-3 is also involved in the biogenesis of the transverse tubule (T-tubule) system (Parton *et al*., 1997). Alterations to the nuclear lamina caused by mutations in lamin A or C, sarcomeric changes in titin, telethonin or myotilin at the Z-disc, and subtle changes in the extracellular matrix proteins laminin-α2 or collagen VI can all lead to limb-girdle muscular dystrophy phenotypes. However, the specific pathological mechanisms remain obscure (Laval and Bushby, 2004).

The muscle pathology is characterized by necrotic and regenerating fibers, increase in fiber size variation, fiber splitting and centrally located myonuclei. Chronic cycles of degeneration and regeneration of muscle fibers result in replacement of muscle with fatty and fibrous tissue (Borsato *et al*., 2006). The quest for order in the group of diseases designated 'limb-girdle' muscular dystrophy (LGMD) in the classifications of the 1950's has led in various directions over the past 50 years (Bushby, 1999 a). For most of this time, LGMD has been an unpopular and poorly defined

diagnostic term conveying little information for either the clinician or the patient. In the initial classifications, the description of LGMD emphasized heterogeneity. This include the potential for either autosomal recessive (a defect or mutation on the gene from the chromosome of each parent is involved) or autosomal dominant (single gene defect on a chromosome from either parent or one copy of a mutant gene and one normal gene) inheritance, onset of disease in either the pelvic girdle or the shoulder girdle musculature, and slow or more rapid progression. Even sporadic cases of LGMD have been reported (Stübgen and Stipp, 1997; Stübgen, 2008), emphasising the heterogeneity in this group of disorders.

Earlier on, exclusion of alternative diagnoses was the only diagnostic tool, and for a while the existence of a separate 'LGMD' category at all, was questioned (Bushby, 1999 a). When it became clear that the level of heterogeneity was likely to be great, with several independent loci identified, a locus-based classification was proposed by a consortium meeting under the auspices of the European Neuromuscular Centre. It was agreed to name the different LGMD loci based on chronological order and mode of inheritance; with the dominant LGMD loci designated LGMD 1A, B, C, *etc*., and the recessive forms as 2A, B, C, *etc*., in the order of their identification. The suffix '1' points out the dominant form, and the suffix '2', the recessive form (Bushby, 1999 a; Urtizberea *et al*., 2008).

The starting point for investigation of these patients is clinical examination, as many clues can be gained from the history and examination as to what type of LGMD might be present (Bushby, 2000 a). Just more than a decade ago, the protein involved in chromosome 2-linked LGMD, limb girdle muscular dystrophy type 2B (LGMD 2B), was identified (Bashir *et al*., 1998; Liu *et al*., 1998). Dysferlin is the product of a novel mammalian gene which shows homology to a nematode protein, ferlin-1 (FER-1), involved in spermatogenesis (Achanzar and Ward, 1997; Bashir *et al*., 1998). In 1999, Yasunaga and co-workers identified a second human gene, otoferlin, which shows high homology to both dysferlin and FER-1. Otoferlin was implicated in an autosomal recessive form of non-syndromic deafness. All three proteins were predicted to share a number of common characteristics: a C-terminal transmembrane domain and at least three C2 domains (Bushby, 1999, b). Davis and co-workers, 2000, described the identification and characterization of another member of the ferlin family, which they called myoferlin, a protein nearly identical in size to dysferlin. Myoferlin was found to associate with the plasma membrane fraction of skeletal muscle, but was also found in nuclei, and enriched in nuclear membrane fractions (Davis *et al*., 2000).

Dysferlin localizes to the muscle fiber membrane and is expressed from very early in human development (Anderson *et al*., 1999). Dysferlin was simultaneously shown to be involved in two forms MD; LGMD 2B and the predominantly distal muscular dystrophy, Miyoshi myopathy (MM) (Bashir *et al*., 1998; Liu *et al*., 1998), now collectively termed dysferlinopathy. The underlying means by which mutations in this gene are responsible for these different phenotypes is yet to be revealed. One possible factor is environmental influences, as genes are not sealed in a vacuum (Bermúdez-Crespo and López, 2007) they are susceptible to multi-factorial alteration. The environment plays a determining role in gene expression and, in many genes the expression is regulated by multiple promoters, which reflects the complexity behind gene expression (Bermúdez-Crespo and López, 2007).

Although the MDs are classified traditionally by their patterns of muscle involvement, this mode of classification is challenged by the involvement of a single gene in two phenotypically different forms of muscular dystrophy (Bushby, 1999 a). The same homozygous mutation has been described in association with a variable presentation in large chromosome 2-linked families. Different family members were found to present with either proximal (LGMD 2B-like) or distal (MM-like) phenotypes in the presence of a shared haplotype. This is suggestive of another factor, genetic or environmental, that may act to modify the phenotype (Bushby, 1999 b).

2.4 DYSFERLINOPATHY

Dysferlinopathy encompasses a large variety of neuromuscular diseases characterized by the absence of dysferlin in skeletal muscle and an autosomal recessive mode of inheritance. The term ‘dysferlinopathy’ was coined in 1999 by Bushby after MM and LGMD 2B were found to be allelic disorders. Nowadays, it corresponds to the various clinical phenotypes related to a complete or partial absence of dysferlin (Urtizberea *et al*., 2008).

Historically, the first phenotype of dysferlinopathy was described by a Japanese physician, Miyoshi, in 1967 and subsequently in 1986 (Miyoshi *et al*., 1967; Miyoshi *et al*., 1986). In Miyoshi’s original publication, four patients from two consanguineous families presented with recessively inherited late-onset distal myopathy, associated with clear-cut muscular dystrophy and significantly elevated CK levels. This phenotype was called Miyoshi myopathy. Long thought to be confined to Japan, the disease was later reported in Europe and elsewhere thereafter (Urtizberea *et al*., 2008).

To date, three main phenotypes have been reported: MM, LGMD 2B, and distal myopathy with anterior tibial onset (DMAT) (Illa *et al*., 2001). Although rare, dysferlinopathies can account for up to

30% of progressive recessive muscular dystrophies in certain geographical areas, notably in the Middle East and the Indian subcontinent (Urtizberea *et al*., 2008). Guglieri and Bushby, 2008, reported the population frequency of the LGMDs to be highly variable and the prevalence of different forms of LGMD to differ between populations, depending on geographical and ethnic factors. For many areas, including South Africa, there is a lack of demographic data, on the dysferlinopathy prevalence. This lack of demographic data may possibly be attributed to the lack of accurate diagnosis of the disease in clinical practice. Alternatively, since there is no treatment, and the disease is not life threatening, and does not result in early mortality; following diagnosis, many patients do not return to the clinic.

Shared features between the dysferlinopathies so far, are the autosomal recessive form of inheritance, age at onset, most commonly in the late teens or early 20's, high serum CK levels, and transient calf myalgia (Ueyama *et al*., 2002). The myopathological diagnosis of a primary dysferlinopathy is currently based on the presence of degenerative muscle alterations in conjunction with a marked reduction or absence of sarcolemmal anti-dysferlin immunoreactivity (Kesper *et al*., 2008). A variety of other muscular dystrophies such as BMD, LGMD 1C and LGMD 2A may also display altered dysferlin immunostaining (Tagawa *et al*., 2003). However, the final diagnosis of a primary dysferlinopathy can, to date, only be established by the absence of dysferlin staining on Western blotting and/or the presence of two mutated alleles in dysferlin gene analysis (Kesper *et al*., 2008).

Misdiagnosis is commonplace in patients with primary dysferlinopathy. Worse, it can lead to unnecessary and potential hazardous therapeutic interventions such as long-term oral administration of corticosteroids or immunosuppressors (Urtizberea *et al*., 2008). This group of patients are, in particular, likely to be misdiagnosed as myositis, which appears to be non-responsive to steroids (Guglieri and Bushby, 2008). In a study by Brunn and co-workers, 2006, clear differences between the cellular composition of inflammatory infiltrates and their cytokine expression pattern in dysferlinopathies and idiopathic inflammatory myopathies were revealed. The findings were indicative of a pathogenically relevant mechanism of muscle fiber injury in dysferlinopathies, distinct from idiopathic inflammatory myopathies. A pro-inflammatory milieu with expression of interferon-γ (IFN-γ) in the absence of interleukin-10 (IL-10) expression and membrane attack complex deposits were reasoned as possible contributors to progressive muscle damage in primary dysferlinopathies (Brunn *et al*., 2006). Bansal and co-workers, 2003, reported a functional DGC in dysferlin-null mice

and confirmed that the mechanism in LGMD 2B and MM patients is different from the DGC-linked muscular dystrophies.

2.4.1 Clinical Phenotypes

Limb girdle muscular dystrophy type 2B (LGMD 2B)

In LGMD 2B, the clinical phenotype shares a great deal of similarities with MM, making exact initial diagnosis very difficult. The age of onset is also in the late teens and progression is usually slow. Distribution of muscle weakness, although selective, is predominant in the proximal pelvic girdle muscles while the shoulder girdle is relatively spared until late in the disease. Distal involvement in the lower legs can occur, after years of progression, and can result in foot drop (Mahjneh *et al*., 2001). In some instances the initial presentation is a proximo-distal muscle weakness. This distal involvement happens to be a key clue in diagnosis (Urtizberea *et al*., 2008).

Miyoshi Myopathy (MM)

Miyoshi myopathy is the most common form of autosomal recessive distal myopathy. It has also been reported to be the most recognizable subtype of dysferlinopathies, at least in theory (Urtizberea *et al*., 2008). Muscle weakness, initially affecting the gastrocnemius muscle from late teens or early adulthood, characterizes MM. One of the most striking features on examination is the wasting of both calves. Muscle weakness can extend to the pelvic girdle muscles and to the upper limbs, more distally, over time.

Cardiac and respiratory complications are not part of the mainstream form of dysferlinopathy (Urtizberea *et al*., 2008). Serum levels of muscle enzymes (CK, lactic dehydrogenase (LDH), and aldolase) are massively elevated in the early stage of the disease, and the pattern of myopathic changes in muscle is clearly dystrophic with numerous inflammatory foci (Urtizberea *et al*., 2008). At first, patients complain of an inability to stand on tip-toe, therefore, corroborating the primary involvement of the gastrocnemius muscle. Other distal symptoms include difficulties such as going down stairs, Gowers' sign, episodes of ankle subluxation and even foot drop when the leg anterior compartment of the lower extremities becomes affected. Patients occasionally complain of painful legs, sometimes unilaterally, with concomitant calf swelling but without any myoglobinuria. Disease progression is generally slow, over decades, but 10-20% of MM patients become wheelchair dependent (Urtizberea *et al*., 2008).

Distal Myopathy with onset in Tibialis anterior

Distal myopathy with onset in tibialis anterior (DMAT), also referred to as distal anterior compartment myopathy (DACM), comprises a new entity of the dysferlinopathy phenotype found by Illa and co-workers, in 2001. An extended Spanish consanguineous family was shown to carry one homozygous mutation in the dysferlin gene (*DYSF)* but with an atypical clinical presentation. If age of onset, CK levels and histological changes were indeed comparable to MM, the muscle weakness distribution was found to be significantly different. The anterior tibial muscles being the first muscle group involved. As disease progresses, muscle weakness also extend to the posterior compartment (Illa *et al*., 2001).

Other Clinical Variants and novel cases

Other clinical variants with mutations in the dysferlin gene have been described lately (Nagashima *et al*., 2004; Diers *et al*., 2007; Nguyen *et al*., 2007; Klinge *et al*., 2008; Okahashi *et al*., 2008; Paradas *et al*., 2009). These cases included an intermediate form with proximal and distal weakness from onset, patients with rigid spine and leg muscle stiffness, onset after the seventh decade of life, and a novel case resembling congenital muscular dystrophies with onset in the first decade of life. While the clinical symptoms in dysferlinopathies may be distinct at onset, the patterns of muscle involvement often become less specific as the disease progresses with the consequence that phenotypes may overlap in the later stages of the disease.

Recently, an intermediate 'proximodistal phenotype' (PD) has been described with weakness of the proximal lower limbs and calf atrophy as presenting symptoms (Nguyen *et al*., 2007). It appears that there is no genotype-phenotype correlation as mutations can lead to different phenotypes even within the same family (Illarioshkin *et al*., 2000).

Nagashima and co-workers, 2004, reported dysferlinopathy and rigid spine syndrome occurring in a 50-year-old male patient. The patient presented with not only the LGMD type of muscle involvement, but also generalized joint contractures, and rigid spine syndrome. Muscular weakness and wasting of the lower extremities had developed since age 40-years, accompanied by a limitation of anterior bending of the spine. Gene analysis of the dysferlin gene disclosed compound heterozygotes for frameshift and missense mutations. The authors concluded that although it might be by chance that the patient has both dysferlinopathy and rigid spine syndrome. A possibility remains that some mutations in the dysferlin gene might be related to rigid spine syndrome (Nagashima *et al*., 2004).

Diers and co-workers, 2007, reported on an adolescent female with a severe and rapidly progressing clinical course of LGMD 2B which has been suggested by the muscle histopathology and western blot and proven by mutation analysis in the dysferlin gene. The Caucasian girl presented with painful enlargement and mild contractures of calf muscles. A novel compound heterozygous mutation of which one affects the extracellular part of the protein was detected. This report was the first on a mutation in this region of dysferlin and might explain the unusual phenotype of the patient (Diers *et al*., 2007).

Klinge and co-workers, 2008, reported on a patient with disease onset at 73 years, the eldest age of onset of dysferlinopathy reported to date. The presenting symptom was exercise-induced stiffness of the trunk and proximal leg muscles without major progression over a period of 12 years. Gastrocnemius muscle biopsy revealed dystrophic morphology and biochemical depletion of dysferlin. Sequence analysis revealed two novel compound heterozygous splicing mutations of the dysferlin gene. Both mutations were located outside the coding region for C2 domains but the functional significance of this remains to be elucidated (Klinge *et al*., 2007).

Spuler and co-workers, 2008, reported a novel finding that mutations in the dysferlin gene can cause amyloidosis. Amyloid is the abnormal deposition of autologous proteins and peptides in fibrillar aggregates. It was found that mutations located between exons 7 and 16, corresponding to the predicted second and third C2 domain of dysferlin, were amyoloidogenic. In this study, Hamlet antibodies (against dysferlin protein) failed to stain the amyloid deposits, and in contrast, a peptide antibody directed against the second C2 domain of dysferlin, readily stained the amyloid deposits. These findings suggested that the amyloid protein is a proteolytic cleavage product of dysferlin (Spuler *et al*., 2008).

In 2009, Paradas and co-workers, described a new phenotype of dysferlinopathy resembling congenital muscular dystrophies (CMD) caused by a mutation in the dysferlin gene. The two patients, aged 2 years and 5 years, showed weakness in proximal lower limbs and neck flexor muscles at birth. The presence of normal CK levels during the first years of life was noted. Initial magnetic resonance imaging (MRI) showed no abnormalities, but short-time-inversion-recovery (STIR) sequences revealed myoedema in gastrocnemius and hamstring muscles at the age of 5 years. No inflammation and no specific structural muscle alterations were observed except for mild dystrophic changes. The group suggested that these features probably reflect that muscle degeneration in dysferlinopathies become significant several years after birth with increasing

muscular activity, especially after walk acquisition. This explains the normal CK level at birth in the reported case (Paradas *et al*., 2009). The dysferlin mutation reported, was in a homozygous state and has previously been described and associated with different phenotypes, including asymptomatic hyperCKemia at second decade. This report was the first on congenital onset in dysferlinopathies. The authors suggested that it could be attributed to genetic modifier factors that may be responsible for the phenotype variability of this muscular dystrophy (Paradas *et al*., 2009).

2.4.2 Clinical Features

Many patients usually participated actively in sports during adolescence, before disease onset. The first symptoms usually appear during the second or third decade of life as 'clumsiness when running', 'fatigue when walking long distances' and 'difficulty in climbing stairs' (Borsato *et al*., 2006). All of these difficulties are related to proximal lower limb weakness, and are characteristic of patients with the LGMD phenotype whereas the 'incapacity to walk on tiptoes' is characteristic of the distal phenotype. Calf hypertrophy and tendon contractures are rare. Hyperlordosis and waddling gait are also present, and patients can remain asymptomatic for many years with only hyperCKaemia before showing the first clinical signs of disease (Laval and Bushby, 2004; Borsato *et al*., 2006).

Probably the most insightful and astute research study on the disease progression in the field of dysferlinopathy is the study by Mahjneh and co-workers, in 2001. The study reported on a 23-year follow-up of a family where 10 members, age 17 to 76 years, homozygous for the same frameshift dysferlin mutation, were evaluated. A 23 base pair (bp) insertion which has resulted from tandem duplication, presumably as a result of replication slippage, occurred. The mutation was predicted to introduce premature termination of the protein 41 aa downstream.

The detailed clinical results of the study represent a comprehensive review of the clinical features of dysferlinopathy in the family, and allow comparison with other forms of LGMD. Progression of muscle weakness in patients with LGMD 2B on the lower limbs were measured with the functional scale according to the method by Walton (1981), and modified by the group of Mahjneh, 2001. This detailed evaluation not only provides insight to the clinical manifestation and progression of the disease, but also offers a standard that can be used to assess the stage of dysferlinopathy in clinical practice. The different stages of progression in dysferlinopathies, clinically present as the loss of ambulation are summarized in text box 2.2.

Text box 2.2

Different stages of progression in dysferlinopathy

Grade 1	A patient walks normally, but is unable to run freely.
Grade 2	A detectable defect in posture or gait occurs, and the patient is almost unable to run. Climbing stairs take place with slight difficulty. A patient can rise from the floor with a slight Gowers' sign.
Grade 3	The patient is unable to run. Dysferlin gait is observed, and the patient is able to walk at least 2 km. When climbing stairs the patient needs support of one hand on the thigh. A patient can rise from floor with slight Gowers' sign.
Grade 4	Slight dysferlin gait is observed, and the patient is able to walk more than 500 m. Climbing stairs require support of both hands on the thigh. The patient rises from a chair supporting on both thighs, and rises from the floor with moderate Gowers' sign.
Grade 5	Slight dysferlin gait is observed while the patient is able to walk more than 500 m. Climbing stairs requires support with both hands the on thigh. The patient rises from the floor with a severe Gowers' sign.
Grade 6	Moderate dysferlin gait is observed while the patient can only walk more than 200 m. Climbing stairs take place in an upright position with one hand supporting on the railing. The patient rises from a chair supporting on the arms of the chair, and rising from floor requires support on objects.
Grade 7	Severe dysferlin gait is observed, while the patient can only walk more than 100 m. Climbing stairs requires clinging to the railings with both hands. The patient needs to support on a table to rise from a chair, and supporting on objects to rise from the floor.
Grade 8	Severe dysferlin gait is observed, and the patient walks with aid of a cane. Only a few steps can be climbed, while clinging to the railings with both hands. The patient needs to support on a table to rise from a chair, and supporting on objects to rise from the floor.
Grade 9	The patient needs to use a wheelchair outside. The patient has lost the ability to climb stairs or rise from the floor, and can rise from a chair only with assistance from a person.
Grade 10	A patient becomes wheelchair-confined.

(Mahjneh *et al*., 2001)

It was reported on the standing posture of dysferlinopathy patients that signs of abnormality in begin to be apparent only about seven years after disease onset, when a slight degree of hyperlordosis begins to show (Mahjneh *et al*., 2001). "Lower limbs are abducted and externally rotated, usually with a wide base support, due to weakness in the hip muscles. Hyperextension of the lower limbs is present due to the weakness of the quadriceps and gastrocnemius muscles. This results in the phenomenon of back-kneeing in order to avoid sudden flexion of the knee and consequent falls. Bilateral gastrocnemius weakness produces ankle plantar flexion.

The upper limbs are limp and hang along the sides with internal rotated arms and hands facing backwards. This posture abnormality becomes more evident after 10 years, when patients start presenting with balance problems. A patient has difficulty in keeping his/her position for a prolonged period, as there is a prevalence of inversion rather than eversion of the feet. The patient tries to compensate for this with toe flexion in order to keep the feet fixed firmly on the ground. Some degree of rotation of the lower edge of the scapulae may be evident at this stage, although scapular winging never sets in. The deltoid has a characteristic appearance in the intermediate stages as a step at the scapulohumeral level, due to the wasting of the proximal part and hypertrophy of the distal part of the muscle."

In gait analysis Mahjneh and co-workers found that in the early stages (clinical grades/stages 1-2, described above), a patient may have some minor gait abnormalities. "Due to weakness of the quadriceps muscle, the acceleration of the lower limbs forward, during rapid walking, is deficient and the knee extension during the heel strike phase is weak. Patients tend to compensate for weakness of the quadriceps muscle by externally or internally rotating the thigh, leg and foot during the gait phases. This allows a decreased effect of gravity. Furthermore, the calf muscle weakness leads to an inefficient push off movement phase during the gait. Step length is normal but stride width is wider due to hip adductor and quadriceps weakness.

Marked and evident gait abnormalities are present after 10 years of disease (around clinical stage 4). The abnormalities are due to diffuse pelvic girdle- and lower limb muscle weakness. The gait becomes less complex and the stance phase is constituted by only the 'foot flat' period. There is a loss of heel strike and push off thereafter the foot leaves the ground with a slight double flexion of the knee and hip. With the knee in a bent position that falls heavily to the ground with a flat foot. At this stage there is a double support phase which is not stable due to deficient knee extension, and the other leg leaves the ground. The swing phase is greatly reduced due to the marked knee flexion deficiency. There is slight hip tilting due to the weakness in hip and back extensor muscles. Severe deficiency of accelerating the lower limbs forward during rapid walking is noted. At this stage the patients have mild hyperlordosis. Upper limb swing is deficient; the arms are thrown backwards, internally rotated and the forearms and hands externally rotated. There is a wide base of support, and step length becomes shorter with a wide asymmetrical stride width." (Mahjneh *et al*., 2001)

Careful clinical analysis of the distribution of the muscle weakness combined with gait analysis in LGMD 2B patients allowed the characterization of a distinct type of gait which have been termed

'dysferlin gait' by the group of Mahjneh, 2001. Dysferlin gait is clearly different from the gait observed in dystrophinopathies and sarcoglycanopathies (Ben Hamida *et al*., 1983; Eymard *et al*., 1997), who show a typical waddling gait. Mahjneh and co-workers stated that dysferlin gait is pathognomonic for dysferlinopathy patients. Making use of the selective involvement of specific muscles in the muscular dystrophies, researchers has described particular appearances in different muscular dystrophies, such as the 'valley sign' in DMD and BMD, the 'poly-hill sign' in facioscapulohumeral dystrophy, and the 'shank sign' in myotonic dystrophy (Pradhan, 2006). These signs were found to make use of the fact that some muscles show relative enlargement of preserved bulk, whereas others show wasting in the same region (Pradhan, 2006).

The involvement of the shoulder girdle is believed to be a much later event in the dysferlinopathies (Straub and Bushby, 2008). Pradhan, 2006 has observed a particular appearance in muscles of the shoulder girdle in MM-type dysferlinopathy that appeared to be specific to this phenotype. When patients were asked to raise their arms with shoulders abducted and elbows flexed to 90°, an appearance, described as 'calf heads on a trophy sign', was perceived. The sign was marked by six specific features.

- First, a prominent deltoid muscle looking like a calf's head with the mouth at the muscle's insertion and one of the several linear depressions appearing like a half-closed eye was observed. The prominence of the calf's head was found to be further enhanced at its lower border by slight wasting of the long head of the triceps muscle laterally, and the lateral part of the infraspinatus muscle medially.
- The second specific feature was found to be a cordlike upper border of the trapezius muscle appearing like a horn on the calf's head. The horn appeared more prominent because of wasting of the supraspinatus muscle and the overlying trapezius muscle below it.
- The third feature that marked the sign was found to be a prominent upper medial part of the infraspinatus muscle. The sharp demarcated wasting of the lower and lateral part looked like a downward directed ear of a calf.
- The fourth feature was the spinous process of the scapula. This was found to become prominent because of the wasted supraspinatus above it, appearing like the upper border of a calf's neck. A medial continuation as a subnuchal dorsal hump caused by the levator scapulae and rhomboid minor muscles shwoed a lump-like prominence at the insertion over the upper medial border of the scapula.

- The fifth feature that marked the sign was a ventral frill of a calf's neck formed by enlarged latissimus dorsi and teres major muscles.
- The last feature was the overall appearance of the upper back that created the impression of a trophy with superolateral concave arches formed by 'calf heads', and lower lateral convex arches formed by the ventral frill of a calf's neck. The prominent upper borders of the latissimus dorsi muscles were found to form the inferior border of the trophy on either side. The prominent lower borders of the trapezius muscles, which meet in the middle, formed the medial border. The hollowing of the interscapular region, owing to wasting of the rhomboid major muscle and the overlying trapezius muscle were found to be responsible for the prominent appearance of the sign (Pradhan, 2006).

In addition to the calf head sign, Pradhan, 2008, reported the 'diamonds on quadriceps sign' in MM and LGMD 2B phenotypes, which indicated selectivity of the dystrophic process not only among different muscles but also within a muscle. Patients showed asymmetric diamond shaped bulges with wasting of muscles above and below. The sign was suggested to be specific to the dysferlinopathies (Pradhan, 2008). Collectively these findings provide a platform for studying affected muscles in the dysferlinopathies. Together with the clinical picture described by the team of Mahjneh, 2001, this information is not only valuable for diagnostic purposes but may be very valuable in the application of cell-based therapies, as they stipulated the exact muscle involvement of the shoulder girdle, and the extent to which muscles are affected.

2.4.3 Muscle Pathology

Fanin and Angelini, 2002, assessed several parameters in muscle biopsies from dysferlinopathy patients, in order to evaluate their role in the development and progression of the disease. The assessed parameters included the extent and localization of inflammatory response, the amount and type of cellular infiltrate, the extent of muscle fiber degeneration and regeneration, the fiber type composition and the severity of histopathological changes. The series of muscle biopsies were scored in four different categories, according to the characteristics and severity of the pathology picture. Mild myopathy, moderate dystrophy, active dystrophy and advanced-stage dystrophy, were described. The latter categories were characterized by increased fiber size variability, fibro-fatty replacement, and advanced-stage dystrophy that showed many lobulated fibers.

Most biopsies that showed active dystrophy were found in patients younger than 30 years, and no definite correlation between the severity of the pathological picture and the duration of disease or

type of muscle biopsied, was found. An evident increase in the percentage of type 1 fibers in patients with an advanced stage of dystrophic process was observed. The highest rate of degeneration and regeneration in muscle biopsies showing acute dystrophy was observed, mostly from young patients.

An increased inflammatory response was observed in the majority of muscle biopsies, where macrophages were the most abundant cell types, either surrounding or invading muscle fibers or localized in the endomysium. T lymphocytes were localized both in the perimysium and in the perivascular region, and in the endomysium or invading fibers in phagocytosis. The authors reported an increased myosin heavy chain class I (MHC-1) positive reaction in association with the presence of macrophages that was localized to the cytoplasm of regenerating fibers. This reaction was occasionally found on the surface of non-necrotic and non-regenerating fibers suggesting that the inflammatory response might precede necrosis. Occasional MHC-1 positive fibers that were in different planes of sectioning were surrounded or invaded by phagocytes, and showed disruption of cellular integrity. The finding of expression of MHC class I molecules on muscle fibers is indicative of muscle fibers that were immunologically activated.

The extent of muscle fiber degeneration and regeneration was related to the stage of the dystrophic process. Severe muscle changes were observed either in the active phase of a necrotizing myopathy or in the advanced stage of a long-term dystrophy. Fanin and Angelini further observed that the normal fiber type distribution was shifted towards a predominance of type 1 fibers in an advanced stage of dysferlinopathy. The percentage of type 1 fibers was inversely correlated with the amount of degenerating and regenerating fibers, supporting their notion that type 1 fiber predominance, is a feature of advanced-stage dysferlinopathy. This observation was possibly due to a preferential type 2 fiber loss or a fiber type conversion. De Palma and co-workers, 2006, reported a remodelling of fiber type in dysferlinopathy quadriceps muscles. An increased proportion of slow fibers, in addition to a shift towards oxidative metabolism were observed.

Fanin and Angelini found inflammatory reactions to occur in the majority of biopsies, where it appeared secondary to necrosis, as demonstrated by the predominance of macrophage infiltrates. Positive labelling for the membrane attack complex (MAC) was observed on the surface of isolated non-necrotic fibers. This observation suggested that both muscle inflammation and complement cascade activation precede necrosis, and might be triggered by the presence of a structurally altered membrane (Fanin and Angelini, 2002).

2.4.4 Muscle Involvement

Computed tomography (CT) has established that in the early stages of dysferlinopathy, weakness is detected only in the posterior compartment muscles of the lower limbs; hamstrings, adductors, gastrocnemius and soleus and to a lesser degree in the quadriceps and gluteus (Mahjneh *et al*., 2001).

The anterior tibial (TA) and posterior tibial (PT) muscles are involved later, around clinical stage 4. However, long flexor and extensor muscles of the toes (extensor digitorum longus (EDL), and brevis (EDB) and extensor hallucis longus (EHL)), and intrinsic foot muscles are seen to be involved only at clinical stage 9, with mild degree (Weiler *et al*., 1996; Mahjneh *et al*., 2001).

Later on, weakness becomes detectable in the upper limbs and is most pronounced in the biceps, less so in the triceps, pectoralis, rhomboid, infraspinatus and supraspinatus muscles (Bejaoui *et al*., 1995; Weiler *et al*., 1996; Mahjneh *et al*., 2001). The deltoid as well as the trapezius muscles becomes affected very late (Weiler *et al*., 1996). The latissimus dorsi, and small muscles of the hand are relatively spared. Early contractures due to iliotibial band tightness may be present to a slight degree. Slight contractures become more widespread following loss of ambulation. Winging of the scapulae has not been reported in early stages, but Mahjneh and co-workers found that in the late stages it was observed as deficient abduction of the lower edge of the scapulae in six of their patients. The involvement of the proximal muscles of the thigh is much more prominent in LGMD 2B patients than in MM (Mahjneh *et al*., 2001).

The muscle CT in the LGMD 2B patients provided evidence of the presence of some degree of hypodensity on spinal muscles, whereas these aspects were not present in the muscle CT of the MM patients (Linssen *et al*., 1997; Mahjneh *et al*., 2001). Patients with the MM-type show early involvement of gluteus minimus in muscle CT scans, while this muscle is almost normal in LGMD 2B patients. In upper limbs, distal involvement of the forearm muscles is present in patients with MM but not in LGMD 2B patients (Miyoshi *et al*., 1986).

Beyond diagnostic implications, MRI techniques help to detect selectivity of skeletal muscle affection in inherited muscular dystrophies (Kesper *et al*., 2008). The MRI technique has been described as a sensitive method for detecting the earliest changes in dysferlinopathy (Okahashi *et al*., 2008). Whole-body high-field MRI is a non-invasive method to demonstrate various degrees of skeletal muscle alterations and disease progression in MDs (Kesper *et al*., 2008).

High-field MRI is becoming increasingly available in the clinical setting (Schick, 2005), and it shows a high diagnostic accuracy in the detection of early fatty replacement in muscles (Kesper *et al*., 2008). Fat suppressed T2-weighted images also allow the detection of edema as another possible early feature of muscle involvement (Mercuri *et al*, 2007).

In the first detailed whole-body high-field MRI analysis in patients with primary dysferlinopathies, Kesper and co-workers, 2008, found a characteristic pattern of skeletal muscle abnormalities. Evaluation with MRI was found to be sensitive in detecting subclinical muscle involvement in symptomatic patients. In addition, this evaluation gave normal results in LGMD 2B patients when studying muscles of the shoulder girdle. Patients with MM showed moderate to severe degeneration of the subscapularis and teres major as well as mild to moderate involvement of latissimus dorsi muscles. Moderate fatty degeneration of the erector spinae muscle mainly affecting its caudal and lateral parts was observed in both LGMD 2B and MM patients. Pectoralis muscles showed atrophy in all MM patients, while obliquus externus muscles showed atrophy or at least moderate degeneration in all MM patients.

All MM patients and one LGMD 2B patient showed a mild degree of fatty degeneration of the gluteus maximus muscles following investigation of the pelvic musculature Signs of focal edema of gluteus maximus muscles were additionally observed in all MM patients. One LGMD 2B patient and all MM patients in Kesper's study presented with a severe degeneration of the gluteus minimus. All MM patients displayed mild fatty degeneration of the gluteus medius muscles.

All symptomatic patients showed an involvement of thigh muscles with a characteristic sparing of sartorius and gracilis muscles. The anterior thigh compartment (quadriceps femoris), the internal hamstring (semi-membranosus) and external hamstring (biceps femoris) muscles were found to be severely affected. The adductor magnus showed a moderate fatty degeneration in all symptomatic patients. All MM patients further showed a moderate involvement of adductor longus and brevis muscles. Evaluation of lower legs, revealed an involvement mainly affecting the gastrocnemius in all symptomatic patients. When compared with the lateral heads, the medial heads of gastrocnemius muscles were found to be relatively mildly affected. The TA muscles and the peroneal group in the anterior compartment of the lower legs were affected in four and five patients, respectively (Kesper *et al*., 2008).

Okahashi and co-workers, 2008, reported a case where typical distribution of muscle involvement was demonstrated by MRI but not by CT, which contributed to facilitating diagnoses in the earliest

stage of preclinical dysferlinopathy, presenting with asymptomatic elevation of serum CK (Okahashi *et al*., 2008). The work of Kesper's group provides new diagnostic potential that may have value superior to current invasive diagnostic techniques. It also provides a viable technique for identification of muscle involvement and the extent to which muscles are affected in a patient-specific manner. This application will become increasingly useful in cell-based therapeutic strategies for direct identification of affected muscles.

2.4.5 Mutations

In the late 1990's, the gene mutation responsible for the pathology in dysferlinopathy has been identified (Bashir *et al*., 1998; Liu *et al*., 1998). Dysferlinopathies can manifest either as LGMD 2B, MM or DMAT. Moreover, the same mutation in the dysferlin gene has been shown to lead to both LGMD 2B and MM phenotypes within a single family (Weiler *et al*., 1999; Illarioshkin *et al*., 2000) or lead to overlapping in phenotypes in a single patient (Nguyen K *et al*., 2005). Such clinical heterogeneity has previously been reported in other human genetic disorder and is of particular importance in muscular dystrophies (Nguyen K *et al*., 2005). It is expected that additional factors such as genetic-, functional-, or environmental-, might play a role in the phenotypic expression of the clinical and morphological heterogeneity.

The dysferlin gene (*DYSF)* is located on chromosome 2p13, contains 55 coding exons and spans 150 kb of genomic DNA. The transcript is 6.3 kb large and is mainly expressed in skeletal muscle. The dysferlin gene is large, and the 55 exons spanning at least 150 kb of genomic DNA, predict a cDNA of around 7 kb and a protein of 2 088 amino acids (Aoki *et al*., 2001). All mutations in *DYSF* found to date include point mutations or small deletions or insertions distributed all over the entire coding sequence (Nguyen K *et al*., 2005). No hotspot has been identified (Nguyen K *et al*., 2005). Missense as well as nonsense or frameshift mutations have been reported (Aoki *et al*., 2001; Takahashi *et al*., 2003). Nguyen K and co-workers, 2005, reported a series of 54 sequence variations in *DYSF,* 34 of which were novel, in a large group of patients with various ethnic origins. The study highlighted the large number of non-pathogenic polymorphisms disseminated along *DYSF*, which could, in some instances, lead to misdiagnosis in patients in whom the levels of expression of dysferlin have not been evaluated (Nguyen K *et al*., 2005).

2.4.6 Pathogenic Mechanism of Dysferlinopathy

The muscle fiber plasma membrane (sarcolemma), is a highly structured and very specialized cellular structure. It is the physical boundary and limit to the cell, acting as the surface through which the

cell interacts with its environment. The sarcolemma has a very close and highly organized association with the ECM and requires a highly efficient means of self-repair. These specialized features are necessitated by the stresses involved in repeated rounds of muscle contraction and relaxation (Laval and Bushby, 2004).

In normal muscle, sarcolemmal injuries lead to accumulation of dysferlin-enriched membrane patches and resealing of the membrane in the presence of Ca^{2+} (Wenzel *et al*., 2005). Disruption of the plasma membrane is a common event in various normal mammalian cells, and membrane-repair machinery is essential to prevent disruption-induced cell death. In skeletal muscle membrane disruptions are most often observed under physiological conditions, because muscle fibers contract repeatedly and are often susceptible to varying degrees of mechanical stress. The fragility of the sarcolemma makes it unable to withstand these mechanical stresses, and a membrane-repair defect will more easily results in necrosis of the muscle fibers (Hayashi, 2003).

Vesicles must accumulate and fuse with the plasma membrane to afford rapid repair of a disrupted membrane (Hayashi, 2003). Membrane-repair machinery is thought to be involved in Ca^{2+}-dependent exocytosis of lysosomes, which are mainly predocked at the plasma membrane in non-secretory cells (Jaiswal *et al*., 2002). Due to its homology to a nematode gene that mediates vesicle fusion to the plasma membrane in spermatids, dysferlin has been suggested to be involved in membrane fusion (Hayashi, 2003). Further studies reported abnormal accumulation of subsarcolemmal vesicles in dysferlin-deficient muscle to be indicative of a defective membrane-fusion process (Selcen *et al*., 2001; Bansal *et al*., 2003; Cenacchi *et al*., 2005).

In 2003, Bansal and co-workers investigated dysfunction in the membrane-repair machinery in dysferlinopathy. They were able to show that dysferlin accumulates at sites of membrane disruption in normal muscle, although other sarcolemmal proteins, including caveolin-3, and δ-sarcoglycan were lost at the site of injury. At direct assessment of resealing efficiency, laser-damaged membrane of wild-type mouse muscle resealed within a minute in the presence of Ca^{2+}. This Ca^{2+}-dependent resealing of injured sarcolemma was defective in dysferlin-null muscle fibers, suggesting a direct role for dysferlin in the Ca^{2+}-dependent membrane-repair process (Bansal *et al*., 2003). These findings lead to the hypothesis that failure to repair sarcolemmal damage following injury causes loss of cell homeostasis, resulting in cell death (Chiu *et al*., 2009).

2.5 DYSFERLIN

Dysferlin is an ubiquitously expressed 230 kDa molecule that is localized to the periphery of muscle fibers (Anderson *et al*., 1999). The protein is found in a variety of tissues including skeletal and cardiac muscle, kidney, placenta, lung, and brain, and it is most highly expressed in skeletal and cardiac muscle (Bashir *et al*., 1998; Han and Campbell, 2007). Dysferlin was identified through a positional cloning strategy aimed at determining the gene involved in LGMD 2B and MM (Bashir *et al*., 1998; Liu *et al*., 1998). It was named according to the only sequence homology detected at the time of its cloning, to the *Caenorhabditis elegans* (*C.elegans*) protein FER-1 (Bushby, 2000 b).

The predicted cytoplasmic components of dysferlin contains Ca^{2+}-binding motifs homologous to C2 domains (Liu *et al*., 1998), which are believed to trigger Ca^{2+}-signalled membrane fusion (Rizo and Sudhof, 1998). It was later reported that dysferlin contains seven C2 domains (some authors still report only six C2 domains) and a single transmembrane domain located at its C-terminus (Han and Campell, 2007; Glover and Brown, 2007). A single missense mutation in any of five dysferlin C2 domains (C2A, B, C, D, E and G) has been reported to cause muscular dystrophy (Therrien *et al*., 2006), again suggesting non-redundancy among individual C2 domains (Han and Campbell, 2007). Mutations in these C2 domains may also lead to dysferlin misfolding and thus degradation (Therrien *et al*., 2006; Fujita *et al*., 2007). The dysferlin C2A domain binds phospholipids in a Ca^{2+}-dependent manner (Davis *et al*., 2002), consistent with its role in skeletal muscle membrane repair (Lennon *et al*., 2003; Bansal *et al*., 2003; Han and Campbell, 2007).

Also supported by this novel function is the ultrastructural observation of prominent subsarcolemmal small vesicle aggregations of unknown origin (Selcen *et al*., 2001; Bansal *et al*., 2003; Cenacchi *et al*., 2005) in dysferlin-deficient skeletal muscle. Furthermore, the sarcolemma shows many gaps and microvilli-like projections rather than being continuous and smooth (Bansal *et al*., 2003; Cenacchi *et al*., 2005), and the basal lamina is multilayered in some regions (Cenacchi *et al*., 2005; Han and Campbell, 2007). Dysferlin is a type II transmembrane protein with a membrane topology suggesting that it anchors to the plasma membrane by its C-terminal transmembrane domain, whereas the N-terminal part of the protein resides in the cytoplasm of the muscle fiber (Bansal and Campbell, 2004).

Dysferlin can be detected at Carnegie stage 15 or 16 (embryonic age 5-6 weeks) and is therefore present at a stage of development when the limbs start to show regional differentiation (Anderson *et al*., 1999). Lack of dysferlin at this critical time may contribute to the pattern of muscle

involvement that develops later with the onset of muscular dystrophy primarily affecting proximal or distal muscles (Anderson *et al*., 1999). Studies performed using the C2C12 mouse cell line showed that dysferlin was expressed at low levels in myoblasts and at high levels in mature myotubes (Davis *et al*., 2002). In normal primary human muscle cultures, both dysferlin mRNA and protein expression were found to be higher in multinucleated myotubes than at the myoblast stage. This is suggesting a role for dysferlin in muscle differentiation (De Luna *et al*., 2004). Dysferlin deficiency delays myoblast fusion/maturation *in vitro* (De Luna *et al*., 2006), suggesting that dysferlin may also participate in muscle differentiation and/or regeneration (Han and Campbell, 2007).

As discussed above, Bansal and co-workers, 2003, have identified a role for dysferlin during the membrane fusion step of the repair process in muscle fibers. Such a role is consistent with the presence of several C2 domains in dysferlin and with its homology to several known proteins with a role in vesicle fusion. It has been proposed that dysferlin, present on the vesicles, facilitate vesicle docking and fusion with the plasma membrane either by interacting with the other dysferlin molecules or with some other unknown protein-binding partner(s) at the plasma membrane (Bansal *et al*., 2003).

Chiu and co-workers, 2009, have identified a role of dysferlin in chemotactic signalling from internal vesicles in order to facilitate the attraction of neutrophils to a site of injury. The team suggested an extension to the muscle membrane repair model as they revealed a novel molecular pathomechanism affecting muscle regeneration and maintenance in dysferlinopathy. Emphasis was also laid on the content of vesicles which fuse with the cell membrane after muscle injury, which include neutrophil chemotactic factors. Together with the function of dysferlin in vesicle fusion (Bansal *et al*., 2003; Lennon *et al*., 2003), the observation of reduced cytokine release by dysferlin deficient cells provides an alternative explanation, that part of the function of dysferlin is to release cytokines from internal vesicles in order to attract neutrophils to the site of injury (Chiu *et al*., 2009).

2.5.1 Other Members of the Ferlin-Protein Family

After the identification of dysferlin, several novel genes showing protein structure and sequence homology to dysferlin were identified (Yasunaga *et al*., 1999; Britton *et al*., 2000). Consequently, a new family of mammalian proteins named ‘ferlin-1 like proteins’ was predicted on the basis of structural similarity and sequence homology (Han and Campbell, 2007). These include dysferlin or FER1L1 (Bashir *et al*., 1998; Liu *et al*., 1998), otoferlin or Fer1L2 (Yasunaga *et al*., 1999; Yasunaga *et al*., 2000), myoferlin or Fer1L3 (Britton *et al*., 2000; Davis *et al*., 2000), FER1L4, FER1L5 and FER1L6.

Each protein contains multiple C2 domains, which anchors to the membrane via a single C-terminal transmembrane domain, and shows sequence homology to the *C. elegans* ferlin-1 (*FER-1*) gene (Han and Campbell, 2007).

High conservation between homologous C2 domains of dysferlin and myoferlin has been reported (an average of 74% homology), which implicate that the C2A of dysferlin is more related to C2A of myoferlin than it is to the other C2 domains in dysferlin (an average of 15%) (Davis *et al*., 2002). A phylogenetic tree constructed from the alignment of individual mammalian ferlin C2 domain sequences showed that in all ferlins, a given C2 domain is more similar to other C2 domains at similar positions in the other proteins than to C2 domains in different positions of the same protein (Washington and Ward, 2006). This observation suggests that the multiple C2 domains may be required for protein activity (Han and Campbell, 2007), as was shown by Klinge and co-workers, 2007, where all the C2 domains were required to function in membrane fusion and repair.

FER-1

The spermatogenesis factor, FER-1, is specifically expressed in primary spermatocytes of *C.elegans*. In spermatids, mutations in FER-1 cause infertility by impairing the fusion of large vesicle with the plasma membrane (Ward *et al*., 1981; Achanzar and Ward, 1997). The fusion of these vesicles with the plasma membrane adds extra membrane to the plasma membrane at the fusion site. Mutations in FER-1 have been found to lead to immobile spermatids and consequently sterility in *C.elegans* (Bansal and Campbell, 2004).

Otoferlin

Mutations in otoferlin are responsible for DFNB9, a specific type of autosomal recessive deafness in humans (Yasunaga *et al*., 1999). Sequence and northern blot analysis on the RNA isolated from human tissues suggests that otoferlin is smaller than dysferlin. It shows 64% similarity and 31% identity to dysferlin at the level of amino acid sequence (Bansal and Campbell, 2004). Otoferlin encodes a 1230 aa protein with a molecular mass of 140 kDa, and has three predicted C2 domains and a transmembrane domain at its C terminus (Yasunaga *et al*., 1999). Dysferlin shows homology to otoferlin mainly at its C-terminal region, and the C2 domains of otoferlin correspond to the last three C2 domains of dysferlin.

In addition to the 140 kDa isoform, a longer isoform of otoferlin was detected in human and mouse tissues which encode a protein composed of 1997 amino acid residues, with a molecular mass of

≈227 kDa (Yasunaga *et al*., 2000). Similar to dysferlin, this longer isoform possesses seven C2 domains and a single transmembrane domain (Bansal and Campbell, 2004; Han and Campbell, 2007). The transcripts for the long isoform of otoferlin could be detected in both humans and in mice while the transcripts for the shorter isoforms (three C2 domains) could only be detected in human tissues (Yusanaga *et al*., 2000).

Mouse otoferlin shows expression in the cochlea, vestibule and brain. A basal level of expression could also be detected in several other tissues, including lung, kidney, skeletal muscle and heart (Yusanaga *et al*., 1999). Otoferlin has been shown to be essential for a late step of synaptic vesicle exocytosis and may act as the major Ca^{2+} sensor, triggering synaptic vesicle-plasma membrane fusion at the inner hair cell ribbon synapse (Roux *et al*., 2006).

Myoferlin

Following the identification and characterization of myoferlin it was named according to its high homology to dysferlin and its high expression in cardiac and skeletal muscle. Myoferlin was found to be nearly identical in size to dysferlin; ≈230 kDa in molecular weight (Davis *et al*., 2000). The sequence of myoferlin has been reported to be highly homologous to that of dysferlin, whereas Fer1L4 has been found most homologous to that of otoferlin (Bansal and Campbell, 2004).

Myoferlin is predicted to be a type II transmembrane protein, with a large cytoplasmic domain containing seven C2 domains and a C-terminal membrane spanning domain. Databases do not agree on the number of C2 domains. There are probably seven C2 domains in both myoferlin and dysferlin, but both the fifth and the seventh domains are only weakly predicted by two databases with expect values (E values) > 0.001 (Patel *et al*., 2008), and therefore, many authors only report six C2 domains.

Immunohistochemical analysis suggested that, similar to dysferlin, myoferlin is present on the plasma membrane but, in contrast to dysferlin, it is also present in the nucleus of the muscle fibers (Bansal and Campbell, 2004; Han and Campbell, 2007). This localization suggests a function unique to that of dysferlin and may explain the need for two highly homologous proteins in the same tissues (Davis *et al*., 2000). In contrast to dysferlin, myoferlin has been shown to function in myoblast fusion during muscle differentiation/maturation and myoferlin-null mice show muscle atrophy (Doherty *et al*., 2005). Immunoelectron microscopic analysis using an anti-myoferlin antibody revealed that most of the signals of myoferlin were found localized to the inside surface of the normal skeletal myofiber (Inoue *et al*., 2006).

Unlike dysferlin, myoferlin has an SH3 domain that may mediate interactions with other proteins. Myoferlin is upregulated at the plasma membrane of muscle fibers in dystrophin-deficient mdx mice, the murine model for DMD (Sicinski *et al*., 1989; Durbeej and Campbell, 2002), and is hypothesized to play a role in muscle regeneration and, potentially, muscle repair (Davis *et al*., 2000). Because of its high homology to dysferlin, myoferlin has been suggested as a possible compensator for the absence of dysferlin (Inoue *et al*., 2006). Although the myoferlin gene seems to be a candidate gene for the modifier, the compensatory overexpression of myoferlin was not detected in dysferlin-deficient muscles (Inoue *et al*., 2006). The gene for myoferlin (*MYOF*) mapped to chromosome 10q24, and is reported to be a novel candidate gene for muscular dystrophy or cardiomyopathy (Davis *et al*., 2000).

Fer1L4, 5, and 6

The Fer1L4, Fer1L5, and Fer1L6 proteins are predicted from the human and mouse genomic sequences but have not yet been characterized (Han and Campbell, 2007). The open reading frame of Fer1L4 predicts a 1952 aa protein with five C2 domains (Doherty and McNally, 2003). Research is in progress investigating a possible link between mutations in the genes encoding these proteins and human disease (Bansal and Campbell, 2004).

2.5.2 C2 Domains

Crystallographic studies have shown that the C2 domain is an independently folding domain composed of eight β strands forming a β sandwich structure (Sutton *et al*., 1995; Shao *et al*., 1996). At one end of the β sandwich structure, Ca^{2+}-binding loops reside, and Ca^{2+} binding is mediated through a conserved group of aspartic acid residues (Davis *et al*., 2002). The Ca^{2+}-binding sites of topology II C2 domains, similar to dysferlin and myoferlin, have been noted to often lack one or more of the five conserved aspartic acid or glutamic acid residues and to contain alternative residues capable of coordination (Nalefski and Falke, 1996).

Proteins directly implicated in membrane fusion such as the synaptotagmins contain two C2 domains (Davis *et al*., 2002). The first C2 domain of synaptotagmin binds Ca^{2+} and anionic phospholipids (Davletov and Sudhof, 1993; Chapman and Jahn, 1994). The second synaptotagmin C2 domain aids in protein-protein interactions and homo-oligomerization (Chapman *et al*., 1995) and was shown to bind phospholipids (Fernandez *et al*., 2001; Bai *et al*., 2002). Data has emerged supporting the role of synaptotagmins as Ca^{2+} sensor regulating the process of fast exocytosis (Sugita

et al., 2001; Fernandez-Chacon *et al*., 2001). In synaptotagmins, up to three Ca^{2+} ions are known to bind at three loops at the end of the β sandwich structure (Davis *et al*., 2002).

Davis and co-workers, 2002, tested the suggested six C2 domains of moyferlin and found that only C2A demonstrated the binding to phospholipids under the experimental conditions. They reported the role of the remaining ferlin C2 domains to be unknown, but suggested that, similar to other C2 domain-containing proteins, these domains may be involved in protein-protein interactions. The study also found the first C2 domain of dysferlin to demonstrate similar Ca^{2+} and phopholipid binding properties to myoferlin C2A. The cooperative binding of these two C2 domains is consistent with binding multiple Ca^{2+} ions. The C2A domains are found at the extreme amino termini of dysferlin and myoferlin, most remote from the transmembrane domain at the C-terminus. It is speculated that this placement may indicate a role for the C2A domain in localizing the amino terminus to the plasma membrane or possibly in the attraction of ferlin-containing vesicles to the plasma membrane (Davis *et al*., 2002). To function in membrane fusion and repair, all the C2 domains of dysferlin are required (Klinge *et al*., 2007), most likely mediating interactions with other proteins, known to interact with dysferlin, as described in section 2.4.5 below.

It is worth mentioning that the two novel compound heterozygous splicing mutations of the dysferlin gene, found in a 73 year-old patient, were located outside the coding region for C2 domains (Klinge *et al*., 2007). The authors stated that the implication of the finding is unknown. However, it is tempting to suggest that the patient-specific mutation in dysferlinopathy plays a particular role in the disease onset, and progression. Klinge and co-workers, reported a case only affected at a very late age, in conjunction with unaffected C2 domains. This observation may provide the first evidence that the severity, onset and progression of the dysferlinopathies might be directly attributable to the coding region affected by the mutation. More specific, the unaffected region coding for C2 domains, might result in a milder phenotype.

2.5.3 Dysferlin-Mediated Membrane Repair: Response to Membrane Wounding

Progress towards elucidating the mechanisms involved in Ca^{2+}-dependent membrane repair has led to the proposal of two mechanisms: the lipid flow promotion hypothesis and the patch hypothesis (Han and Campbell, 2007). It has recently been shown that a minor portion of dysferlin is also associated with the T-tubule system in skeletal muscle (Ampong *et al*., 2005; Huang *et al*., 2007), whereas the sarcolemmal labelling is not attributed to peripheral T-tubule profiles or caveolae (Anderson *et al*., 1999; Ampong *et al*., 2005). In numerous types of MD, a secondary displacement of

dysferlin to the cytoplasm has been observed (Piccolo *et al*., 2000). Subsarcolemmal accumulation of vesicles is uniquely found in dysferlin deficient skeletal muscle (Selcen *et al*., 2001).

Klinge and co-workers, 2007, examined the subcellular localization of dysferlin in untreated and wounded C2C12 myotubes to study its role in early myogenesis. It was found that although dysferlin is mainly expressed in the T-tubular network in developing muscle, it is able to translocate to the site of injury at the plasma membrane as an immediate and specific response to membrane wounding. The results demonstrated that in the early stages of C2C12 myotube maturation dysferlin does not localize to the sarcolemma but is expressed at the T-tubule system and at sites of cell fusion. The C2C12 myotubes were found to be capable of resealing membrane ruptures in a Ca^{2+}-dependent fashion involving translocation of dysferlin to the site of membrane injury (Klinge *et al*., 2007). The study by Klinge's group showed that in differentiated C2C12 myotubes dysferlin accumulates at the site of myoblasts fusing to myotubes. At this stage of muscle development, dysferlin is predominantly expressed in the T-tubule system of multinucleated myotubes, and the full-length protein is required for its correct localization (Klinge *et al*., 2007).

T-tubules form an intracellular membrane system that penetrates the myofiber allowing the action potential to reach the muscle fiber interior and to facilitate excitation contraction coupling (Flucher *et al*., 1993). T-tubules develop from beaded tubular invaginations of the plasma membrane and partly from longitudinal, cytoplasmic profiles, also termed beaded tubules, which are formed of strings of caveolae (Ishikawa, 1968; Schiaffino *et al*., 1977; Flucher *et al*., 1991). The results of Klinge's group strengthen the hypothesis of synaptic vesicle fusion machinery, a process analogous to the patch hypothesis for membrane repair. They have shown for the first time the focal accumulation of dysferlin positive structures in a Ca^{2+}-dependent manner, at and around the sites of injury as a consequence of membrane wounding. While in mature skeletal muscle the source of dysferlin accumulation at the wounding site is unknown, the group of Klinge have shown that in early myogenesis, dysferlin accumulation at the wounding sites is derived from a T-tubule localization. This response requires the full-length dysferlin protein, and this mechanism is in place in an early stage of development, as T-tubules have not yet orientated into the typical transverse pattern (Takekura *et al*., 2001).

2.5.4 Proteins that Interact with Dysferlin

2.5.4.1 Caveolin-3

Caveolae are vesicular invaginations of the plasma membrane, measuring 50-100nm in diameter (Engelman *et al*., 1998), and function as 'message centres' for regulating signal transduction events (Galbiati *et al*., 2001 a). Caveolin, a 21 to 24 kDa integral membrane protein, has been shown to be a principal component of caveolar membranes *in vivo* (Glenny, 1989; Glenny and Soppet, 1992; Rothberg *et al*., 1992). It has been proposed that caveolin family members participate in vesicular trafficking events, and signal transduction processes, by acting as scaffolding proteins to organize and concentrate specific lipids and lipid-modified signalling molecules within caveolar membranes (Galbiati *et al*., 2001 b). The expression of Caveolin-3 is muscle-specific, and it is the principal structural protein of caveolar membrane domains in striated and smooth muscle cells (Galbiati *et al*., 2001 a). Its expression is induced during the differentiation of skeletal myoblasts, and caveolin-3 is localized to the sarcolemma where it forms a complex with dystrophin and its associated glycoproteins (Song *et al*., 1996).

The importance of caveolin-3 is emphasized by the observation that mutations in the *CAV3* gene cause a dominantly inherited LGMD (LGMD 1C) (Minetti *et al*., 1998). These mutations apparently behave in a dominant negative manner, forcing aggregation of both mutant and wild-type caveolin-3 proteins in the golgi apparatus (Galbiati *et al*., 1999). Matsuda and co-workers, 2001, proposed the first description of a possible dysferlin interacting protein. The team suggested that one function of dysferlin may be to interact with caveolin-3 to subserve signalling functions of caveolae. In addition, caveolin-3 has been implicated in T-tubule system biogenesis (Parton *et al*., 1997), and when caveolin-3 expression is defective, dysferlin localization has been reported to be abnormal (Matsuda *et al*., 2001; Tateyama *et al*., 2002; Fisher *et al*., 2003; Yabe *et al*., 2003).

Ampong and co-workers, 2005, have found Dysferlin and caveolin-3 to coprecipitate with the dihydropyridine receptor (DHPR), a protein complex localized to T-tubules. Dysferlin and DHPR were observed to partially co-localize by double immunofluorescent staining in skeletal muscle fibers. The findings suggested that dysferlin might be involved in the fusion of caveolin-3-containing vesicles with T-tubules (Ampong *et al*., 2005). The team of Hernández-Daviez, 2006, described the cellular distribution of dysferlin with respect to caveolin-3 and showed that muscle disease-associated caveolin-3 mutants affects dysferlin traffic to the plasma membrane. It was demonstrated that co-expression of epitope-tagged dysferlin and mutated caveolin-3 or caveolin-1 caused a dramatic accumulation of dysferlin in the golgi complex in both muscle and non-muscle cells. Dysferlin traffic

to the cell surface was found to be impaired in the absence of caveolin-1 and caveolin-3, suggesting that caveolins are necessary for plasma membrane localization of dysferlin (Hernández-Daviez *et al.*, 2006).

2.5.4.2 CALPAIN 3

Calpain 3 is a skeletal muscle-specific member of the calpain superfamily of non-lysosomal, Ca^{2+}-dependent cysteine proteases (Goll *et al.*, 2003). Several cytoskeletal components have been identified as partners and substrates for calpain 3, linking its function to the regulation of cytoskeleton structure and cytoskeleton-membrane interactions (Guyon *et al.*, 2003; Taveau *et al.*, 2003). This protein has been shown to predominantly localize in several regions of the sarcomere, associated with the protein, titin (Sorimachi *et al.*, 1995).

Duguez and co-workers, 2006, found a deregulation of sarcomere remodelling due to a lack of proteolysis of substrates by calpain 3, to be a possible mechanism of LGMD 2A pathogenesis. A secondary reduction for calpain 3 was reported for a subset of patients with dysferlinopathy (Anderson *et al.*, 2000). The reciprocal has been observed by the team of Chrobáková, 2004, in a subset of LGMD 2A calpain-deficient patients. In co-immunoprecipitation experiments, using llama-derived antibody fragments, Huang and co-workers, 2005, have shown that calpain interacts with dysferlin. Inhibition of calpain activity has been shown to prevent the Ca^{2+}-dependent plasmalemmal resealing of crayfish giant axons (Godell *et al.*, 1997).

Like dysferlin, calpain 3 was suggested to be implicated in the patch fusion repair of the muscle membrane through its interaction with the annexins A1 and A2, as calpain 3 cleavage of annexins A1 and A2 might be critical for patch formation and/or membrane insertion (Lennon *et al.*, 2003). Glover and Brown, 2007 reported annexins A1 and A2 to be likely targets for calpain cleavage in the context of the putative muscle repair protein complex, because both proteins harbour a putative recognition sequence for calpain in their amino termini (Barnes and Gomes, 2002). In addition, annexin A1 has been shown to undergo proteolysis by calpain *in vitro*, a process that enhances the Ca^{2+} sensitivity of annexin A1 for phospholipid binding (Ando *et al.*, 1989).

Calpain 3 and AHNAK were found to be in complex with dysferlin (Huang *et al.*, 2007). Huang and co-workers, 2007, demonstrated that AHNAK can interact with calpain 3 and serves as a direct substrate of calpain 3 in cell culture. The interaction of both these proteins and the cleavage of AHNAK by calpain 3 were supported by their colocalization in skeletal muscle (Huang *et al.*, 2007).

Calpain 3 cleavage caused a loss of AHNAK in cell culture, and AHNAK was found to be increased at the sarcolemma in patients with calpainopathy (Huang *et al*., 2007). Calpain 3-mediated proteolysis of AHNAK prevents interaction of AHNAK with dysferlin and myoferlin. This provided new mechanistic insight for the physiological function of calpain 3 in the dysferlin protein complex in skeletal muscle (Huang *et al*., 2008). The regulatory role of calpain 3 in the dysferlin protein complex may implicate an intimate relationship between muscle membrane repair and remodelling of the sarcomere and subsarcolemmal cytoskeleton architecture (Huang *et al*., 2008).

2.5.4.3 Annexins A1 and A2

Annexins are widely expressed Ca^{2+}- and phospholipid-binding proteins that are implicated in membrane trafficking, transmembrane channel activity, inhibition of phopholipase A2 and cell-matrix interactions (Raynal and Pollard, 1994). The functions of many of the annexins are not clear (Lennon *et al*., 2003). However, annexins A1 and A2 have been shown to aggregate intracellular vesicles and lipid rafts in a Ca^{2+}-dependent manner at the cytosolic surface of plasma membranes in many cells (Babiychuk and Draeger, 2000; Lambert *et al*., 1997). Annexin A1 mediates this aggregation by forming a heterotetramer with the protein S100A11, and annexin A2 has been postulated to have a similar relationship with the protein S100A10 (Gerke and Moss, 2002).

A Ca^{2+}-dependant interaction between dysferlin and annexins A1 and A2, expected to play a role in the aggregation and fusion of intracellular vesicles in response to membrane injury, were described by the team of Lennon, 2003. After membrane injury, a disruption of dysferlin binding to annexin A1, Ca^{2+}-dependent vesicle aggregation, and fusion with the surface membrane were observed (Lennon *et al.*, 2003). It was shown that this membrane repair process is severely upset in dysferlinopathic myotubes (Lennon *et al*., 2003). In addition, microarray analysis of expression patterns in dysferlinopathic biopsies indicated an over-expression of annexin A2, compared to normal subjects (Campanaro, 2002).

Cagliani and co-workers, 2005, investigated annexins A1 and A2 expression levels in dysferlinopathic patients and in subjects suffering from different muscle pathologies. All patients displayed increased annexin levels and a significant positive correlation was evidenced between protein expression and clinical severity. Annexin levels were also found to parallel dystrophic alterations as assessed by histological examination of muscle sections. The findings indicated that increased annexin levels are not a specific marker for dysferlinopathies. They rather represent a secondary phenomenon in the

development of muscle disease, which is largely independent from the underlying pathogenic mechanism (Cagliani *et al*., 2005).

McNeil and co-workers, 2006, demonstrated a requirement for annexin A1 in resealing responses, as the inactivity of annexin A2 were found to inhibit resealing. It was proposed that Ca^{2+} entering through a plasma membrane disruption causes cytosolic annexin A1 to bind to membranes surrounding the disruption site. It thereby initiates the homotypic and exocytotic membrane fusion events of resealing. This event initiates emergency fusion events wherever and whenever required (McNeil *et al*., 2006). Due to the correlation of annexin expression levels with disease severity in dysferlinopathy, irrespective of the clinical phenotype variability, annexins cannot be regarded as good candidates for modifier factors in this disease (Cagliani *et al*., 2005).

2.5.4.4 AHNAK

AHNAK, also called desmoyokin and is located on human chromosome 11q12-13 (Kudoh *et al*., 1995). This protein contains three main structural regions; the N-terminal, 498 aa; a large central region of ≈4300 aa with multiple repeated units, most of which are 128 amino acids in length, and the C-terminal of 1002 aa (Shtivelman *et al*., 1992). A second AHNAK nucleoprotein-like protein, AHNAK nucleoprotein 2, located on chromosome 14q32, was recently identified in a search for homologous sequences in the human genome (Komuro *et al*., 2004).

AHNAK is a family of two proteins of exceptionally large size, ≈600-700 kDa, characterized by common amino acid sequences and structural features (Komuro *et al*., 2004). Like dysferlin, high expression levels of AHNAK are observed in all muscle cells, including cardiomyocytes and skeletal muscle cells (Gentil *et al*., 2003). The exact biological function of AHNAK is largely unknown (Huang *et al*., 2008). However, the C-terminal region of AHNAK binds to G-actin and cosediments with F-actin *in vitro*, suggesting a role for AHNAK in the maintenance of the structural and functional organization of the subsarcolemmal cytoarchitecture in cardiomyocytes (Hohaus *et al*., 2002).

Huang and co-workers, 2007, described a novel interaction of AHNAK with dysferlin. The interaction sites have been identified as the C-terminal, 500 aa of both AHNAK variants and the C2A domain of dsyferlin and its homologue myoferlin (Huang *et al*., 2007). The functional significance of the interaction between dysferlin and AHNAK comes from the observation that AHNAK is, like dysferlin, primarily localized at the sarcolemma. AHNAK is also secondarily reduced in muscle from patients

with genetically confirmed dysferlinopathy, generally comparable to the loss of dysferlin (Huang *et al*., 2007).

In LGMD 1C, dysferlin and AHNAK showed a secondary overall reduction to the primary loss of caveolin-3. In contrast, in unrelated muscular dystrophies, dysferlin and AHNAK showed normal muscle staining (Huang *et al*., 2007). Evidence has been provided for a functional cooperation between dysferlin and AHNAK during muscle regeneration. Dysferlin and AHNAK show a marked increase and cytoplasmic localization during regeneration. This is consistent with the direct interaction between them and mobilization of the AHNAK-dysferlin complex during repair and regeneration (Huang *et al*., 2007).

In 2008, Huang and co-workers have demonstrated that AHNAK can interact with, calpain 3, and that AHNAK serves as a direct substrate of calpain 3 in cell culture. The interaction of both proteins and the cleavage of AHNAK by calpain 3 were supported by their colocalization in skeletal muscle (Huang *et al*., 2008). Calpain 3-mediated proteolysis of AHNAK prevents the interaction of AHNAK with dysferlin and myoferlin (Huang *et al*., 2008). In agreement with the proposed membrane repair function for AHNAK, it was demonstrated that AHNAK is also an integral component of a newly discovered Ca^{2+}-regulated vesicle capable of rapid exocytosis, called enlargosome. Enlargosome exocytosis is induced by plasma membrane disruption and is thought to be involved in both plasma membrane differentiation and repair (Borgonovo *et al*., 2002).

2.5.4.5 Affixin (β-Parvin)

Affixin is a novel focal adhesion (FA) protein that contains two tandem calponin homology (CH) domains (Matsuda *et al*., 2005). While ubiquitously expressed, the expression levels of affixin were found to be highest in the heart and skeletal muscles of humans (Matsuda *et al*., 2005). Affixin co-localizes with integrin-linked kinase (ILK) at focal adhesions of well-spread cultured cells and is suggested to be involved in integrin-ILK signalling (Matsuda *et al*., 2005). In human skeletal muscle, affixin and integrin-linked kinase co-localize at the sarcolemma (Yamaji *et al*., 2001). The biological significance of this interaction is not yet fully understood (Matsuda *et al*., 2005).

Affixin was found to be a dysferlin-binding protein by Matsuda and co-workers, 2005. The team showed that a deficiency of dysferlin caused a secondary reduction of affixin at the sarcolemma of MM and LGMD 2B muscles even though the total protein content did not change. The results suggested that affixin is closely associated with dysferlin at the sarcolemma of normal human muscle. The affixin-binding domain identified in dysferlin was the C-terminal intracellular region,

which does not show homology to any known protein motifs, while the CH1 domain of affixin was identified as a dysferlin-binding region (Matsuda *et al*., 2005).

Affixin plays an essential role in the formation of FA and actin stress fibers (Yamaji *et al*., 2001). It has also been shown to interact with α-actinin and participate in reorganization of F-actin (Yamaji *et al*., 2004). F-actin is depolymerised in response to membrane disruption and plays an important role in wound healing (McNeil, 2002). Although the physiological function of the dysferlin-affixin protein interaction is not yet fully understood, there is a possibility that dysferlin and affixin play some role in membrane repair through the organization of F-actin (Matsuda *et al*., 2005).

2.5.4.6 MYOGENIN

Myogenin or myogenic factor 4 is a member of the basic helix-loop-helix gene family which is essential for muscle development. In adult skeletal muscle myogenin expression is concurrent with satellite cell differentiation and fusion (Cooper *et al*., 1999). It is essential for terminal muscle differentiation downstream of myognesis and is upregulated in differentiating myoblasts in a pattern, similar to dysferlin (Glover and Brown, 2007).

Null mutations in the myogenin gene cause a severe reduction of skeletal muscle mass, showing that myogenin is essential for muscle development i*n vivo* (Hasty *et al*., 1993). In a study by De Luna and co-workers, 2006, human skeletal muscle primary cultures from dysferlinopathy patients showed that the absence of dysferlin remarkably alters the process of muscle differentiation *in vitro*. This finding indicated a signalling pathway that would involve dysferlin and myogenin. Severe reduction of myogenin in dysferlin-null myotubes suggested that myogenin could be involved in the defective muscle differentiation observed in dysferlin-null cell cultures (De Luna *et al*., 2006). The study supported the role of dysferlin in human muscle fusion and differentiation *in vitro*. Furthermore, it provided evidence of a link between dysferlin and myogenin and suggested they share a signalling pathway involved in differentiation of skeletal muscle *in vitro* (De Luna *et al*., 2006).

2.5.4.7 BIN 1

Bin 1 is a conserved member of the BAR family of genes and has been implicated in myoblast differentiation and membrane deformation (Lee *et al*., 2002). Caveolin-3 and Bin 1 have been shown to be involved in T-tubulogenesis (Parton *et al*., 1997; McNally *et al*., 1998; Lee *et al*., 2002). In mice lacking either caveolin-3 or Bin 1, T-tubules are abnormal but not absent (Galbiati *et al*., 2001 a;

Muller *et al*., 2003), suggesting that additional factors are likely to contribute to T-tubule development (Lee *et al*., 2002).

Klinge and co-workers, 2007, found that dysferlin co-localizes with Bin 1 in C2C12 myotubes. Bin 1 was found to be highly abundant at fusion sites of myotubes, co-localizing with full-length dysferlin. The authors suggested that this finding supports a possible joint role of Bin 1 and dysferlin in membrane fusion processes in developing muscle. Since dysferlin and Bin 1 expression both start at similar time points and fusion already takes place before this point, their role in fusion appears to be important in the later stages of differentiation (Klinge *et al*., 2007). This is reflected by the fact that Bin 1 and dysferlin deficient myotubes display impairment but not absence of fusion and differentiation (Lee *et al*., 2002; De Luna *et al*., 2006). The T-tubule localization of dysferlin and Bin 1 has important implications in the view of the fact that the T-tubule system is involved in the generation of membrane for the elongation and repair purposes (Engel and Franzini-Armstrong, 2004). Furthermore, it serves as a membrane source for membrane bound vacuoles. Subsarcolemmal vacuoles contiguous with the T-tubules are one of the ultrastructural hallmarks in dysferlin-deficient skeletal muscle (Selcen *et al*., 2001; Klinge *et al*., 2007). This could be explained by an abnormal membrane fusion/budding mechanism in dysferlin deficient muscle. In normal muscle the T-tubule system initiates the formation of, and serves as a membrane source for autophagic vacuoles and vesicles (Engel and Franzini-Armstrong, 2004).

2.6 DIAGNOSIS AND THERAPY

Reduced or absent expression of dysferlin are the most relevant features leading to the diagnosis of dysferlinopathy and prompt molecular geneticists to search for mutations at the genomic or transcriptional level in *DYSF* (Nguyen K *et al*., 2005). This genetic screening is of particular interest for the accuracy of diagnoses and therefore for more reliable genetic counselling and appropriate medical care (Nguyen K *et al*., 2005). Kesper and coowrkers, 2008, reported that the final diagnosis of a primary dysferlinopathy can to date only be established by the absence of dysferlin staining on Western blotting and/or the presence of two mutated alleles in dysferlin gene analysis.

Ho and co-workers, 2002, developed a new protein-based diagnosis using non-muscle tissues and less invasive sampling techniques in order to avoid the painful and invasive procedure of muscle biopsy for dysferlinopathy screening. The group reported a novel finding that dysferlin is expressed in blood monocytes, and more importantly, it was shown that dysferlin expression in monocytes

correlates with its expression in muscle. The findings led to the development of a new protein-based diagnostic assay for LGMD 2B and MM, without the need for a muscle biopsy (Ho *et al*., 2002).

Before employment of this procedure as a routine diagnostic screening assay for the dysferlinopathies, it should be kept in mind that the expression of dysferlin in monocytes is restricted to the type II (non classical) monocyte population (Chiu *et al*., 2009), that is distinct from the monocyte population which initially invade muscle following injury (Arnold *et al*., 2007). However, Chiu and co-workers, found the monocyte expression of dysferlin unlikely to be involved in the defective regeneration of dysferlin-deficient muscle. The reason being that type II monocytes are not involved until after the resolution of the inflammatory phase. At this point the regeneration of dysferlinopathic muscle is already abnormal (Chiu *et al*., 2009).

In dysferlinopathy, muscle CT and MRI clearly show characteristic muscle involvement of the posterior compartment of the legs (Bruss *et al*., 2004). The hallmark can be seen in the earliest stages or even in the preclinical stage of disease (Argov *et al*., 2000; Bruss *et al*., 2004). Brummer and coworkers showed that MRI change precedes the CT change. The CT change was found to be replacement of muscle by fat tissues, while changes like edema, which was the most sensitive parameter to detect dystrophic alteration in skeletal muscle, were detected with MRI (Brummer *et al*., 2005). MRI appears to be the far superior diagnostic tool, to date, due to its non-invasive mode of operation, in accurate diagnosis of the dysferlinopathies.

It is expected that diagnosis based on clinical presentation specifically in the pelvic girdle, can effectively be evaluated following the criteria described by Mahjneh and co-workers, 2001, which was modified from the functional scale described by Walton (1981). The different stages (text box 2.2) of progression in dysferlinopathies may offer a useful tool in clinical practice.

There is currently no effective treatment for patients suffering from dysferlinopathy. The care of patients with LGMD2 mainly consists of symptomatic treatment strategies and supportive measures. These include physical therapy, assistive devices and monitoring of respiratory function and heart health (Bushby and Straub, 2005), as well as treatment of disease complications (Straub and Bushby, 2008). These strategies mainly aim at prolonging survival and improving quality of life (Bushby and Straub, 2005).

Of all treatment strategy approaches in the muscular dystrophies, the most viable seems to be cell-based therapy. Murphy and Kehrer, 1989, stated that the underlying biochemical defect of muscular

dystrophy, as with nearly all inherited diseases, can only be cured if techniques are developed to repair or replace the defective genetic material.

Case by case approaches are necessary (Danièle *et al*., 2007), as the diversity of proteins involved in the muscular dystrophies, and their diverse functions within the muscle cell, render it almost impossible to develop a universal treatment. Cell grafting offers the possibility of gene delivery into dystrophic muscle (Danièle *et al*., 2007), while reimplantation of genetically modified host myoblasts or healthy donor cells not only has the potential to correct the gene deficiency but also has the capability to remodel the dystrophic tissue (Danièle *et al*., 2007). This could be of particular importance when addressing progressed cases of muscular dystrophy.

When considering the diverse functions of dysferlin, discussed in the present chapter, and the possibility of even more unrevealed functions and interactions of this protein, dysferlinopathy appears to be a candidate for cell-based therapies. Such therapeutic strategies should aim at restoring the protein defect by grafting of normal healthy cells into the dystrophic host tissue.

The group of Kong, 2004, has shown some success in a cell-based approach. Human umbilical cord blood cells were injected into dysferlin-deficient SJL mice. Fibers expressing human dysferlin, although in very small numbers, were detected. The study found that muscle precursor cells are present in human umbilical cord blood and that they can fuse with host muscle fibers after systemic delivery (Kong *et al*., 2004). Vieira and co-workers, 2008, achieved even more success in the field with systemic delivery of human adipose stromal cells into SJL mice, without immunosuppression. The team showed for the first time that human adipose stromal cells were able to fuse with the host muscle, express significant amounts of human muscle proteins, and to improve motor ability of injected animals (Vieira *et al*., 2008).

2.7 Oxidative Stress

Free radicals are highly reactive molecules or chemical species containing unpaired electrons that cause oxidative stress. This state can be defined as an imbalance between antioxidants in favour of the oxidants, potentially leading to damage (Sies, 1997). They are formed during normal physiological metabolism or caused by toxins in the environment. Oxidative stress can damage lipids, proteins, enzymes, carbohydrates and DNA in cells and tissue. The result: damage to membranes, fragmentation or random cross linking of molecules like DNA, enzymes and structural proteins, and ultimately cell death induced by DNA fragmentation and lipid peroxidation (Beckman and Ames, 1998).

The basis of our life on earth is the oxygen present in the atmosphere. However, it can under a number of conditions, be a very toxic substance (Bentinger *et al.*, 2007). Derivatives such as hydroxyl (OH^-) and superoxide (O_2^-) radicals, hydrogen peroxide (H_2O_2) and singlet oxygen (O_3) may be formed and are called reactive oxygen species (ROS). ROS appear not only in diseases but also under normal physiological conditions and interact with basic tissue components with consequences of disturbed function (Bentinger *et al*., 2007).

Various types of antioxidant systems are available in all organisms for limitation and elimination of these unwanted species (Bentinger *et al*., 2007). The functional activity of the mitochondria greatly influences the extent of ROS formation. Low intracellular levels of adenosine diphosphate (ADP) and high mitochondrial membrane potential gives high levels of ROS, whereas high ADP levels and low membrane potential result in low production of ROS (Bentinger *et al*., 2007). Mitochondrial electron transport accounts for two-thirds of the cellular oxygen consumption, and the observed limited leakage of electrons, 1 to 2%, is the largest contribution to the cellular O_2^- and H_2O_2 production in healthy cells (Papa and Skulachev, 1997).

The imbalance between the generation of ROS and their removal by enzymatic and nonenzymatic cellular defence systems could arise from its overproduction. This event occurs under certain pathological conditions and in association with inflammation, or from an impairment of the defence mechanisms, as seen in certain genetic loss-of-function disorders and deficiency states (Halliwell and Gutteridge, 1989). Implicit in this definition is the notion that such an imbalance is sufficient to lead to the oxidation of various cellular constituents and to cause cellular dysfunction and injury (Rando, 2002). As such, oxidative stress may also be viewed as a condition in which the production of oxidative products exceeds their removal by cellular repair mechanisms, which may lead to acute cellular metabolic disturbances and even cell death, if such changes accumulate (Rando, 2002).

During normal cellular metabolism, the primary generation of ROS comes from the leakage of the superoxide anion, O_2^-, from the electron transport chain (Halliwell and Gutteridge, 1989). A series of linked enzymatic reactions are responsible for the detoxification of O_2^-, where it is converted to H_2O_2 by the action of superoxide dismutase (SOD). Most animal cells contain two forms of SOD, the cytoplasmic Cu,Zn-SOD and a mitochondrial Mn-SOD (Theate *et al*., 1985). The selenium-containing enzyme glutathione peroxidase (GPx), is responsible for metabolizing H_2O_2 to oxygen and water, a reaction in which glutathione (GSH) is used as a cofactor (Murphy and Kehrer, 1989). Glutathione peroxidase converts most of the H_2O_2 in the cytoplasm. At sites of relatively high concentrations of

H_2O_2, such as peroxisomes, catalase (CAT) is an important antioxidant enzyme that also converts H_2O_2 to water (Murphy and Kehrer, 1989).

Inside a cell, H_2O_2 can react with metal ions to produce the highly reactive hydroxyl radical, OH^- (Halliwell and Gutteridge, 1990). Superoxide can react with nitric oxide ($NO^\bullet$) to produce peroxynitrite ($ONOO^-$) (Beckman and Koppenol, 1996). The OH^- and $ONOO^-$ are among the most reactive species present in biological systems and are capable of oxidizing nucleic acids, proteins, lipids, and carbohydrate moieties in the cell (Rando, 2002).

2.7.1 Susceptibility of Skeletal Muscle to Oxidative Stress

There is a very high concentration of myoglobin in muscle, and such heme-containing proteins are known to confer greater sensitivity to free radical induced damage by conversion of H_2O_2 to more reactive species (Ostdal *et al*., 1997). Free radicals preferentially attack polyunsaturated fatty acids within membranes Therefore; requirement of skeletal muscle membrane for phospholipids containing large proportions of polyunsaturated fatty acids may render those membranes particularly susceptible to oxidative damage (Murphy and Kehrer, 1989). The specific patterns of cell and tissue injury produced by hereditary or induced GSH depletion, suggest specific susceptibilities to oxidative stress that depend on both age and species (Rando, 2002). The findings of necrotic degeneration in adult mice rendered deficient of GSH, highlights the importance of antioxidant defences in skeletal muscle during normal activity (Martensson and Meister, 1989).

2.7.2 Evidence of Oxidative Stress in Muscular Dystrophy

Disatnik and co-workers, 1998, presented evidence that is consistent with the hypothesis, that free radical mediated injury contributes to the pathogenesis of muscle necrosis in the muscular dystrophies (Murphy and Kehrer, 1989). The hypothesis was initially based on the similarities between the pathology in the dystrophies and the pathology of muscle exposed to oxidative stress in vitamin E deficiency (Murphy and Kehrer, 1989).

Lipid peroxidation is a common index of free radical mediated injury (Halliwell and Gutteridge, 1989). Previous measurements of lipid peroxidation in dystrophin-deficient muscles have indicated elevated levels in both humans and mice (Kar and Pearson, 1979; Jackson *et al*., 1984; Mechler *et al*., 1995; Ragusa *et al*., 1997). The team of Disatnik, 1998, investigated the role of oxidative injury in muscle necrosis in mdx mice. In order to avoid secondary consequences of muscle necrosis, all experiments were executed in the pre-necrotic phase. An induction of expression of genes encoding antioxidant enzymes, indicative of a cellular response to oxidative stress, was recorded. Greater

levels of lipid peroxidation were recorded in mdx muscle than in control muscles (Disatnik *et al*., 1998). No relationship with $NO^{\bullet}$-mediated toxicity was found, but evidence of increased oxidative stress preceding the onset of muscle cell death in dystrophin-deficient mice was detected (Disatnik *et al*., 1998).

The imbalance between free radical synthesis and the antioxidant capacity has been reported, almost four decades ago, to be a possible contributor to the necrotic process (Mendell *et al*., 1971). Although the significance and precise extent of the oxidative stress contribution in the degenerative process of dystrophic muscle is poorly understood, there is increasing evidence that the degenerative process may indeed be due to oxidative stress (Niebrój-Dobosz and Hausmanowa-Petrusewicz, 2005).

2.8 IMPLICATION FOR ANTIOXIDANTS

Antioxidants are substances, which counteract free radicals and prevent the damage caused by them (Venkat Ratnam *et al*., 2006). Antioxidants are enzymes and nonenzymatic agents that can provide a high stability to lipids (Marinova *et al*., 2002) and prevent the formation of, or remove ROS (Turunen *et al*., 2004). There are four major groups of naturally occurring lipid soluble antioxidants, carotenoids, tocopherols, estrogens and coenzyme Q/ubiquinol/CoQ10 (Betinger *et al*., 2007). Antioxidant enzymes include SOD and various peroxidases such as GPx, CAT, thioredoxin reductase and peroxiredoxin. Nonenzymatic agents include vitamins C and E, cartenoids, glutathione, α-lipoic acid, flavinoids and the reduced form of CoQ10, $CoQH_2$. All these agents all rely on a mechanism of regeneration in the cell (Turunen *et al*., 2004).

A new approach for using highly specific anti-cytokine therapies to treat DMD was reported by Grounds and Torrisi, 2004. The study tested the novel hypothesis that the initial sarcolemmal breakdown resulting from dystrophin deficiency is exacerbated by inflammatory cells and that cytokines, specifically tumor necrosis factor-α (TNFα), contribute to muscle necrosis. The antibody Remicade® was used to neutralize activity of the TNFα protein in mdx mice to assess whether blockade of TNFα in patients with DMD has therapeutic value. Remicade® does not cause generalized suppression of the immune system and has been used clinically to treat inflammatory disorders (Gabay, 2002). Remicade® was administered weekly from before the onset of necrosis and dystro-pathology, which set in around 21 days of age in the mdx model. Pharmacological blockade of TNFα activity with Remicade® were found to clearly delay and greatly reduce the breakdown of

dystrophic muscle, in marked contrast to the situation in mdx and mdx/TNFα(-/-) mice (Grounds and Torrisi, 2004).

Antioxidants were found to exert a beneficial effect in experimental models of chronic injury in diabetic animals (Aragno *et al*., 1999; Eriksson and Simán, 1996). Aragno and co-workers, 2004, showed that dehydroepiandosterone (DHEA) or vitamin E treatment in diabetic rats restored the oxidative balance and improved muscle gene transcription (Aragno *et al*., 2004). It has been shown that DHEA, a multifunctional steroid secreted by the adrenal gland and brain (Baulieu *et al*., 2000), possesses a multitargeted antioxidant effect and prevents tissue damage induced by acute and chronic hyperglycaemia (Aragno *et al*., 2002). Treatment with DHEA prevented the reduction of antioxidant compounds and the increase of oxidant species induced in other tissues by chronic hyperglycaemia (Aragno *et al*., 1999).

A study by Buck and Chojkier, 1996 showed that the decreased body weight, muscle wasting and skeletal muscle molecular abnormalities of cachexia were prevented by treatment of TNFα mice with the anitoxidants D-α-tocopherol or BW755c, or the nitric oxide synthase (NOS) inhibitor nitro-L-arginine. TNFα induces oxidative stress and NOS in skeletal muscle of these mice, leading to decreased myosin creatinine phosphokinase (MCK) expression and binding activities.

Kaczor and co-workers, 2007 determined the effect of low intensity training on markers of lipid and protein damage by ROS as well as antioxidant and mitochondrial enzyme activities in skeletal muscle of mdx mice. Significantly higher levels of lipid and protein peroxidation markers were detected in the muscle of mdx as compared to wild-type mice. The activities of antioxidant enzymes, SOD, MnSOD, and CAT were found to be higher in white and red gastrocnemius muscles of mdx mice than in wild-type mice. Elevated levels of the protein content for GPx in EDL muscle of mdx were reported to be higher than that in wild-type mice. Mitochondrial enzymes, oxoglutarate dehydrogenase (OGDH), and cyclooxygenase (COX) activity were only significantly elevated in white gastrocnemius fibers of mdx as compared to wild-type mice. The study found that low intensity exercise had a beneficial effect on skeletal muscle of mdx mice, resulting in decreasing lipid and protein oxidative damage (Kaczor *et al*., 2007). Low intensity exercise was found to induce 'pathophysiological adaptation' in skeletal muscle of mdx mice. The authors believed that in the first few weeks of low intensity exercise, ROS generation increased and markers of oxidative stress were elevated. However, adaptation were found to occur over the duration of training, resulting in a lower

generation of free radicals and decreased level of malondialdehyde (MDA) and protein carbonyls in mdx mice (Kaczor *et al*., 2007).

The generation of ROS which appears to play an important role in DMD motivated the study of Buetler and co-workers, 2002, who tested whether the antioxidant effect of green tea extract could diminish muscle necrosis in mdx mice. The study provided evidence that dietary supplementation with green tea extract preferentially protected the EDL muscle of mdx mice from necrosis. The mechanism for green tea extract protection is not clear but was expected to be mediated by its antioxidant activity. In addition, green tea extract dose-dependently protected C2C12 myotubes from tert-butylhydroperoxide- (BHP) induced oxidative stress (Buetler *et al*., 2002). Furthermore, the levels of protection afforded by green tea extract supplementation were found to be similar to results described for creatine (Pulido *et al*., 1998; Passaquin *et al*., 2002).

Together, these studies suggest that the reduction of oxidative stress, by different methodologies, whether inhibitory- (Grounds and Torrisi, 2004; Buck and Chojkier, 1996), inductive-(Aragno *et al*., 2002; Kaczor *et al*., 2007), or other (Buetler *et al*., 2002), lead to a reduction in the adverse events in the concerned pathologies. These findings motivated the present application of antioxidant supplementation in dysferlin-deficient mice. The present investigation followed a similar approach but employed a different strategy. It was argued that if elevated levels of oxidative stress, responsible for the adverse effects in numerous pathologies, are also present in dysferlin-deficient mouse muscle; these adverse changes will be detectable at the cellular level by morphological analysis. If these changes were found to be present, what will the effect of a direct free radical scavenging approach be?

Further review of the literature provided informative guidelines for the selection of an appropriate single antioxidant, and a combination of antioxidants. The selection criteria were simple: ideally there should be evidence for potent antioxidant potential of a supplement to be applied in a study like the present. In addition, evidence of beneficial application in other disease conditions, preferably in animals and man, with a high dose tolerance and low side effect profile in subjects is desirable. Two antioxidant substances that complied with the criteria, in addition to its numerous other beneficial effects, were CoQ10 and resveratrol.

2.9 PROSPECTIVE ANTIOXIDANTS

2.9.1 COENZYME Q10

Coenzyme Q (CoQ) is a naturally occuring compound with properties similar to those of vitamins. Because of its ubiquitous distribution in nature, it is also known as ubiquinone (Bhakgavan and Chopra, 2006). CoQ belongs to a homologous series of compounds that share a common benzoquinone ring structure but differ in length of the isoprenoid side chain (Bhakgavan and Chopra, 2006). In humans and a few other mammalian species, the side chain is comprised of ten isoprenoid units; hence it is called Coenzyme Q10 (CoQ10) (Bhakgavan and Chopra, 2006).

CoQ10 is similar in chemical structure to vitamin K, but it is not considered a vitamin because of its *de novo* synthesis in the body (Bhakgavan and Chopra, 2006). It is a lipid-soluble component of virtually all cell membranes, and is located in the hydrophobic domain of the phospholipid bilayer of cellular membranes (Quinzii *et al*., 2007; Sohal and Forster, 2007). CoQ10 is the only endogenously synthesized lipid with a redox function in mammals and exhibits a broad tissue as well as intracellular distribution (Dallner and Sindelar, 2000). It is also the only known lipid-soluble antioxidant that animal cells can synthesize *de novo*, and for which there exists enzymatic mechanisms which can regenerate it from its oxidized product formed in the course of its antioxidant function (Ernster and Dallner, 1995). It is a hydrophobic molecule and its properties are given by the 10-isoprenoid side chain (Crane, 2001).

In humans, CoQ10 is biosynthesized from tyrosine through a cascade of eight aromatic precursors, via the mevalonate pathway (Folkers, 1996) where cells rely on *de novo* synthesis for their supply of CoQ10. The levels of CoQ10 are subjected to regulation by physiological factors that are related to the oxidative activity of the organism (Ernster and Dallner, 1995; Tran and Clarke, 2007). CoQ10 is distributed among cellular membranes and it has a significant concentration in the plasma membrane (Gómez-Díaz *et al*., 2000). It is also present in the endomembranes of cells as well as in the mitochondria, where it serves as a central component of the transmembrane electron transport system (Sun *et al*., 1992).

The finding of CoQ10 in all membranes brought on a concept of CoQ10 as an important antioxidant (Kagan *et al*., 1990). Even greater significance became apparent when it was shown that reduced CoQ10 could restore antioxidant function to oxidized tocopherol (Crane, 2007). The first successful application of CoQ to a medical condition was Yamamura's treatment of congestive heart failure

(Crane, 2007). In addition to its central role in the mitochondrial respiratory chain, CoQ10 is now involved in a number of aspects of cellular metabolism (Turunen *et al*., 2004).

A number of medical conditions have shown to respond to supplementation with CoQ10: immune deficiency (Bliznakov, 1987), congestive heart failure (Langsjoen and Langsjoen, 1998), indication of therapeutic effects in diabetes(Hodgson *et al*., 2002), Parkinson's and Huntingtons disease (Shults, 2003; Beal, 2004; Ryu and Ferrante, 2005), relief of statin side effects (Littarru and Tiano, 2005), ataxia or encephalomyopathy (Quinzii *et al*., 2006; Gempel *et al*., 2007; Le Ber *et al*., 2007), and Cancer (Hodges *et al*., 1999; Brea-Calvo *et al*., 2006). CoQ10 deficiency has recently been shown to be an autosomal recessive disorder with heterogenous phenotypic manifestations and genetic background (Gempel *et al*., 2007).

In order for CoQ10 to maintain its physiological properties in biological systems, there is a constant need for optimum levels of the coenzyme in order to meet the metabolic demand. Literature is expanding with reports focusing on the deficiency of CoQ10 in the body. The focus of CoQ10 deficiency states in pathological conditions might further be attributable to a reduced mechanistic efficiency of one or more of the biochemical and/or physiological pathways where CoQ10 is directly or indirectly involved. This phenomenon raises the notion of whether supplementation of pathological conditions with CoQ10, might address the level of severity and progression of the symptoms of disease states where CoQ10 deficiency has been diagnosed.

Primary CoQ10 deficiency is considered to be the only treatable mitochondrial disorder, since patients have a response to oral CoQ10 supplementation (Montini *et al*., 2008). The disease usually manifests with nephropathy and endocephalomyopathy (Salviati *et al*., 2005). It has been shown that oral CoQ10 may stop the progression of encephalopathy, but no benefit has been noted with respect to the evolution of renal disease associated with this deficiency (Salviati *et al*., 2005; Rötig *et al*., 2007). This observation emphasizes the superior value of pre-symptomatic supplementation. Montini and co-workers recently described the outcome of long-term CoQ10 supplementation on CoQ10 deficiency caused by a homozygous missense mutation in the *COQ2* gene. The study found that early administration of CoQ10, before any symptoms have developed, was important for the resolution of renal symptoms and for preventing neurologic damage in a patient with CoQ10 deficiency (Montini *et al*., 2008).

There is no evidence of a CoQ10 deficiency state in the dysferlinopathies, only a few earlier reports on deficiency of CoQ in mice with hereditary muscular dystrophy (Littaru *et al*., 1970), and on dystrophic mice responding to Coenzyme Q4 therapy (Scholler *et al*., 1968) (CoQ10 was not readily available in those days). Furthermore, definitely improved physical performance was reported after two double-blind clinical trials, supplementing patients suffering from different muscular dystrophies, with CoQ10 (Folkers and Simonsen, 1995). Folkers and co-workers, 1985, reported that CoQ10 does not alter genetic defects but can benefit the sequelae of mitochondrial impairment from such defects.

Rötig and co-workers reported that CoQ10 administration to a large number of individuals with or without ubiquinone deficiency were found to be safe. This was attributed to the fact that CoQ10 is not taken up by the cells with normal ubiquinone content as there is no possibility to place more lipids into the limited space in the mitochondrial membrane (Rötig *et al*., 2000). When the lipid is missing, as is the case in ubiquinone synthesis defects, the inner mitochondrial membrane has the capacity to accept exogenous CoQ10 that can restore the electron flow (Turunen *et al*., 2004).

2.9.2 CoQ10 as an Antioxidant

Mitochondrial $CoQH_2$ is efficiently regenerated by the respiratory chain and is normally kept in a highly reduced state (Aberg *et al*., 1992). Early studies had shown that the CoQ hydroquinone is an excellent free radical scavenging antioxidant but its role was restricted to mitochondria until a general membrane distribution was shown by Ernster and Dallner, 1995 (Crane, 2007). Experiments on liposomes, mitochondria, microsomes, beef heart submitochondrial particles and lipoproteins of the blood, demonstrated that CoQ10 in reduced form, ubiquinol ($CoQH_2$), is an effective antioxidant and inhibits lipid peroxidation (Bentinger *et al*., 2007). Bentinger and co-workers reported that CoQ10 is our only endogenously synthesized lipid soluble antioxidant, and is mainly present in the activated (reduced) form.

CoQ10's effectiveness as lipid peroxidation inhibitor is based on its complex interaction during the process of peroxidation. The primary action is the prevention of lipid peroxyl radical ($LOO^{\bullet}$) production during the initiation process. This is the first phase of the process, where an abstraction of a hydrogen atom from a methylene group of a fatty acid occurs, presupposing that it has several double bonds. $CoQH_2$ reduces the initiating perferryl radical with the formation of ubisemiquinone and H_2O_2. It is also possible that $CoQH_2$ eliminates $LOO^{\bullet}$ directly.

The reduced lipid effectively regenerates vitamin E from the α-tocopheroxyl radical (Bentinger *al*., 2007). There are several mechanisms in protein oxidation and it appears that the most common is the direct oxidation of amino acid residues (Stadtman ER, Levine RL, 2000). Protein oxidation may also occur by lipid-derived free radicals and by breakdown products of phospholipid hyperperoxides. These compounds link covalently to basic amino acid residues and in the latter case also to sulfhydryl groups, causing intra- and intermolecular cross-linking (Bentinger *et al*., 2007). CoQ10 is effective in preventing protein oxidation by quenching the initiating perferryl radical and functioning as a chain-breaking antioxidant, thus preventing the process of propagation. This is the second phase in lipid peroxidation, where $LOO^{\bullet}$ abstracts a hydrogen atom from an additional unsaturated fatty acid, leading to formation of a carbon-centered radical ($L^{\bullet}$) and lipid hyperoxide (LOOH), which can be reoxidized to $LOO^{\bullet}$. This will result in reinitiation of lipid peroxidation (Bentinger *et al*., 2007).

The sensitivity of proteins to oxidative stress depends on their structure, composition and localization. The close spatial relationship of CoQ10 to the neighbouring membrane proteins is the main factor for its protective effect against protein oxidation (Bentinger *et al*., 2007). CoQ10 also protects DNA against oxidative damage, which is of particular interest for mitochondrial DNA, since damage is not easily reparable (Bentinger *et al*., 2007). Oxidative stress may damage DNA by initiating a series of metabolic reactions in the cell leading to activation of nuclease enzymes that cleave the DNA backbone. A more common event is the interaction of H_2O_2 with metal ions bound to DNA, which leads to the generation of hydroxyl radicals. DNA oxidation in isolated mitochondria takes place in the presence of ADP-Fe^{3+} and ascorbate, resulting in elevated content of 8-hydroxy-deoxyguanosine (8-OH-dG) (Ernster and Dallner, 1995). Incubation in the presence of succinate and antimycin, which maximize the endogenous ubiquinol pool, eliminated the oxidative damage and decrease the increased strand breaks caused by ADP-Fe^{3+} (Bentinger *et al*., 2007).

In mitochondria, ubisemiquinone radical is formed during respiration which is effectively reduced to ubiquinol by the 'protonmotive Q cycle' described by Mitchell in 1975 (Mitchell, 1975). The large reducing capacity of the cell, which is able to regenerate CoQ10 by reduction at all locations of the cell, is a very important property, contributing to the effectiveness of CoQ10 as an antioxidant (Bentinger *et al*., 2007). Available evidence indicates that, owing to its metabolic roles, CoQ10 could positively influence the natural course of disease and potentially enhance the effect of other treatments by correcting some metabolic derangements (Littarru and Tiano, 2005). Metabolic derangements are often not causative; they are known to be the consequences of secondary aspects of diseases (Littarru and Tiano, 2005). Emily Bliznakov stated that: 'Treatment of patients with

various forms of cardiovascular diseases by a combination of CoQ10 and other antioxidants, concomitant with standard drug treatment, is justified and recommended" (Bliznakov, 1999).

2.9.3 RESVERATROL

Resveratrol (3,5,4'-trihydroxystilbene) was first isolated from the roots of *Veratrum grandiflorum O. Loes* (white hellebore) in 1940 (Takaoka, 1940), and later in 1963, from the roots of *Polygonum cuspidatum* (Japanese knotweed) (Baur and Sinclair, 2006). It is a polyphenolic phytoalexin found in many plants, nuts, and fruits and is abundant in grapes and red wine, and in the traditional Japanese medicine, Kojokon. Its function in nature is to protect the plant from injury, UV irradiation and fungal attack (Birell *et al*., 2005; Matsuoka *et al*., 2008).

Resveratrol has been reported to be along with flavonoids, at least partially, responsible for the health benefits of red wine (Frankel *et al*., 1993 a; Soleas *et al*., 1997). It has previously been reported that resveratrol is a rather weak antioxidant (Hu *et al*., 2007), and that its antioxidant effects may be due to its direct interaction with biomolecules that confer cellular stress resistance (Robb *et al*., 2008). Robb and co-workers have identified the endogenous antioxidant enzyme MnSOD as a specific target for resveratrol in human MRC-5 cells. A progressive and dramatic induction of MnSOD expression and activity elicited by the treatment of an untransformed human cell line with micromolar concentrations were reported (Robb *et al*., 2008). The overexpression of MnSOD has previously proven its sole capability of reducing intracellular oxidative stress and conferring stress resistance, in mouse cortical neurons subjected to chronic intermittent hypoxia-mediated oxidative damage (Shan *et al*., 2007).

Resveratrol attracted little interest until 1992 when this naturally occurring plant antibiotic, initially characterized as a phytoalexin, was postulated to explain some of the cardioprotective effects of red wine (Siemann and Creasy, 1992). This theory provides the basis for the observation that the incidence of cardiovascular diseases in France is lower than that expected for the French diet, which is rich in saturated fat. The so-called 'French Paradox' (Kopp, 1998; Cho *et al*., 2008). Resveratrol has shown to prevent or slow down the progression of cancer (Jang *et al*., 1997), cardiovascular disease (Bradamante *et al*., 2004), and ischaemic injuries (Wang *et al*., 2002 and Sinha *et al*., 2002), as well as enhancing stress resistance and extending the lifespan of various organisms, from yeasts to vertebrates (Howitz *et al*., 2003 and Valenzano *et al*., 2006).

Its free radical scavenging effects has been expanded by the finding that resveratrol decreases the traumatic effects of partial hepatectomy in rats, by reducing the levels of MDA, while increasing the

levels of the cofactor GSH. The authors recommended supplementation with resveratrol for the same application in donors and recipients undergoing living donor liver transplantations (Kirimlioglu *et al*., 2008). Resveratrol has shown the ability to reduce serum cholesterol in hamsters by down-regulating hepatic HMG-CoA reductase mRNA expression, following a high-fat diet (Cho *et al*., 2008). The cardiovascular benefits derived from resveratrol, have also been attributed to its estrogenic action, where resveratrol has been shown to be a phytoestrogen that exhibits variable degrees of estrogen receptor agonism in different test systems (Gehm *et al*., 1997).

Pharmokinetic studies have cast doubt on the physiological relevance of high concentrations of resveratrol, typically used in *in vitro* studies, since it is rapidly metabolized. It is unclear whether the benefits of resveratrol are merely the results of fortuitous interactions with dozens of mammalian proteins or is it acting through a specific genetic pathway that has evolved to increase disease- and stress resistance (Baur and Sinclair, 2006). Resveratrol has been suggested to be cardioprotective via various mechanisms such as its antioxidant activity (Fauconneau *et al*., 1997), inhibition of platelet aggregation (Pace-Asciak *et al*., 1996), induction of NO production (Hsieh *et al*., 1999), and modulation of the synthesis of hepatic apolipoprotein and lipids (Frankel *et al*., 1993 b).

Jang and co-workers, 1997, proposed a chemo-preventive property of resveratrol, as it was proposed to inhibit all three phases of tumour development: initiation, promotion, and propagation. Birell and co-workers, 2005, reported the ability of resveratrol to reduce lung tissue neutrophilia in an asthmatic rat model, to a similar magnitude as that achieved by treatment with budesonide. The results were associated with a reduction in the inflammatory mediators, by an NF-κB-independent mechanism. It was therefore suggested that resveratrol may possess anti-inflammatory properties via a novel mechanism. Resveratrol has been considered to be a caloric restriction mimetic in lower organisms, primarily on the basis of its activation of sirtuin proteins and its capacity to extend lifespan (Baur and Sinclair, 2006).

The maximum tolerated dose of resveratrol has not been thoroughly determined, but 300 mg/kg body weight showed no detrimental effects in rats (Crowell *et al*., 2004). Although the exact mechanism of resveratrol's action in mediating health benefits, is not yet fully understood, accumulating evidence suggest that resveratrol shows great potential in the treatment of the leading causes of morbidity and mortality. So far, little evidence suggests that these health benefits are coupled with deleterious side effects (Baur and Sinclair, 2006).

2.10 STUDY OBJECTIVES

It was the aim of the present study to determine, on a cellular level, how the morphology and ultrastructure in dysferlin-deficient SJL/J mouse muscle, whether subjected to oxidative stress or not, will be affected by antioxidant supplementation. CoQ10 and resveratrol, singly and in combination were used as the antioxidants of choice. The present study was directed by the following research objectives:

1. Implementation of the SJL/J mouse model for investigation of the cellular response to the administration of the antioxidants CoQ10 and resveratrol over a 90-day period, as articulated through the muscle morphology. Distribution of animals into different experimental groups, and calculation of antioxidant doses, comparable to human physiological levels, by a body surface area (BSA) normalization formula.

2. Assessment of non-specific parameters in SJL/J mice:
   a. Animal weight as a parameter of animal well-being during the course of the trial.

   b. Tensile strength as a parameter of disease progression as well as the effect of antioxidant supplementation on the physical strength of SJL/J mice, during week 7 to 11 of the 90 day trial.B

   c. Blood levels of the enzymes creatine kinase (CK) and lactate dehydrogenase (LDH).

   d. Quantitative analysis of white blood cell counts.

3. Assessment of histopathology in the quadriceps muscle as a qualitative parameter for the disease characteristics and the effect of antioxidant supplementation thereof.
   Evaluation will be done at:

   a. light microscopic level; the qualitative state of histopathological characteristics, as well as quantitative morphometric significance of fiber diameter variation in control and treated groups, as determined by the minimal Feret's diameter. In addition, the quantification of fibers with nuclei in the central position will be done.

   b. ultrastructural level, as determined by TEM and SEM, to study the ultrastructural hallmarks of the disease and any variation thereof, as a result of antioxidant supplementation. The ultrastructural analysis will also pay attention to non-specific

alterations and how they could be related to the disease processes in dysferlin-deficient muscle.

4. Assessment of the oxidative stress index (OSI) in the quadriceps muscles as a parameter of the degree of oxidative stress in the muscle tissue of the SJL/J mouse.

    a. Assessment of the malondialdehyde (MDA) levels and the effect of antioxidant supplementation over a 90 day period.

    b. Assessment of the antioxidant activity in quadriceps muscle, expressed as total antioxidant status (TAS), and the variation afforded by antioxidant supplementation over a 90 day period.

    c. Calculation of the OSI as a measure of the relationship between lipid peroxidation and antioxidant activity in quadriceps muscle.