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CHAPTER ONE

INTRODUCTION

The muscular dystrophies are defined as a group of genetic disorders with progressive muscle wasting and weakness. At least 1 in 3,000 individuals are affected by an inherited neuromuscular disorder, of which the muscular dystrophies make up a considerable proportion (Emery, 1998). The exact pathogenesis of many of the muscular dystrophies is, however, still unknown.

Facioscapulohumeral muscular dystrophy (FSHD) is the third most common inherited disorder of muscle after Duchenne and Myotonic dystrophy, with a prevalence of at least 1 in 20,000 (Padberg, 1982). FSHD is characterised on a clinical level by progressive weakening and atrophy of the face, shoulder-girdle and upper arm, but other skeletal muscles may also become involved with progression of the disorder.

FSHD is also known as Landouzy-Déjérine disorder after the two physicians, who described it in 1884 (Landouzy and Déjérine, 1884). Duchenne de Boulogne was, however, the first to give a classical description of FSHD in the late 1800's, stating its myopathic nature and inheritance pattern (Kazakov *et al.*, 1974). A historical discussion therefore arose toward the end of the last century between Erb and Landouzy-Déjérine regarding the first report of "facio-scapulo-humeral muscular dystrophy" (FSHD), as FSHD was for the first time described by Duchenne de Boulogne under the name 'progressive muscular atrophy of childhood' and Erb described a 'juvenile shoulder-girdle' type. Landouzy and Déjérine described patients who differed from those described by Duchenne with regard to the progression of the muscles affected from the upper to the lower part of the body. The patients described by Landouzy and Déjérine corresponded to the cases described by Erb, but a new name (FSH type) was used by these two authors. The forms described by Duchenne and Erb were therefore not distinct from the FSH type, but were rather included into this type. Eventually the first report of FSHD was attributed to the report by Landouzy and Déjérine in 1884.

More than a century after the first report of FSHD, the FSHD locus was assigned to chromosome 4q35 through linkage analysis (Wijmenga *et al.*, 1990). This region consists of 3.3 kb repeat arrays (D4Z4) and a deletion of an integral number of these repeats has been observed in individuals affected with FSHD (van Deutekom *et al.*, 1993). To date no gene has been identified for FSHD although the molecular defect of FSHD has been defined. A detailed history of FSHD is presented in chapter two.

This is the first extensive molecular study to characterise individuals affected with FSHD in the South African population. Haplotype analysis conducted with nine short tandem repeat polymorphism (STRP) markers located on chromosome 4q35 and Southern blot analysis were performed utilising probe p13E-11 to detect DNA rearrangements of the D4Z4 repeat arrays. In chapter three and four the protocols and results obtained via these analyses are described.

Conclusions drawn from the results obtained in this study are presented in chapter five. The results obtained in this study to date excluded the possibility of the existence of genetic heterogeneity in the South African FSHD population, but suggested a Dual Founder Effect for FSHD in the South African population. Some aspects of the genetic aetiology of FSHD in this population could therefore be determined in this study.

CHAPTER TWO

THE AETIOLOGY AND PATHOGENESIS OF FACIOSCAPULOHUMERAL MUSCULAR DYSTROPHY

Muscular dystrophy is not a single disorder, but various types have been defined over the years. The muscular dystrophies are a large and heterogeneous group of inherited disorders and the one feature that the muscular dystrophies as a group share is progressive muscle wasting and weakness.

2.0 THE MUSCULAR DYSTROPHIES

Walton and Nattrass (1954) originally classified the muscular dystrophies into three main groups based on the mode of inheritance (Appendix B) and the distribution of the muscle groups that are predominantly affected:

- Duchenne (X-linked recessive type),
- Facioscapulohumeral (autosomal dominant), and
- Limb girdle (autosomal recessive and sporadic limb-girdle type).

These authors also distinguished three relatively uncommon, but clinically and genetically distinctive forms of muscular dystrophy, namely: Distal, Oculopharyngeal, and Congenital muscular dystrophy. The distribution of muscles that are predominantly affected in six of the muscular dystrophies are illustrated in Figure 2.1. These muscular dystrophies can be distinguished on both the clinical and molecular levels. The six muscular dystrophies presented in Figure 2.1 are discussed in the subsequent paragraphs.

Duchenne muscular dystrophy (DMD) [MIM 310200] and Becker muscular dystrophy (BMD) [MIM 300376] are the dystrophin associated muscular dystrophies. DMD is a more severe form than BMD with DMD being the most common form of muscular dystrophy. Both are inherited as X-linked recessive disorders and therefore predominantly affect boys. DMD and BMD are caused by deletions of one, or many, exons in the dystophin gene that was mapped to Xp21.2. The most distinctive feature of DMD is proximal muscle

weakness with characteristic pseudohypertrophy of the calves. Disease onset is typically before the age of three and individuals become wheel-chair dependent by 12 and are generally deceased by the age of 20. Individuals affected by BMD are usually affected in their twenties or thirties and generally have a normal life span. (Emery 1998; OMIM 2001a)

Dreifuss and Emery described Emery-Dreifuss muscular dystrophy (EDMD) [MIM 310300, 181350] in the mid-nineteen sixties. Significant features are cardiomyopathy, generally presenting as atrioventricular block, weakness with a humeroperoneal distribution in the early stages and early contractures of the elbows and spine. Muscles of the lower extremities are usually first affected by the age of four or five. By the early teens individuals develop a waddling gait with increased lumbar lordosis, and weakness of the shoulder girdle muscles appears later. EDMD is inherited as an X-linked recessive disorder and is caused by a mutation in the emerin gene located on Xq28. An autosomal dominant form of EDMD also exists which results from mutations in the lamin A/C (LMNA) gene, encoding two components of the nuclear lamina (lamins A and C), located on chromosome 1. (Emery 1998; OMIM 2001b)

Limb-girdle muscular dystrophy (LGMD) [MIM 159000, 159001, 253600, 253601, 253700, 254110, 600119, 601287, 601173, 601954, 603511, 604286] is a clinically and genetically heterogeneous group of disorders. Onset is generally in childhood, but it can also present later in life. Muscle involvement is generally first noted in either the pelvic, or the shoulder girdle areas. Asymmetry is often observed when the upper limbs are first involved. LGMD is inherited in either an autosomal dominant or autosomal recessive form. At least six dominant and nine recessive sub-types have been identified. LGMD2A is caused by a muscle specific protease (calpain 3) deficiency, and four other recessive types have been found to be caused by deficiencies of sarcoglycans, which form part of the dystrophin associated protein complex of the muscle membrane (Figure 2.2). (Emery 1998; OMIM 2001c)

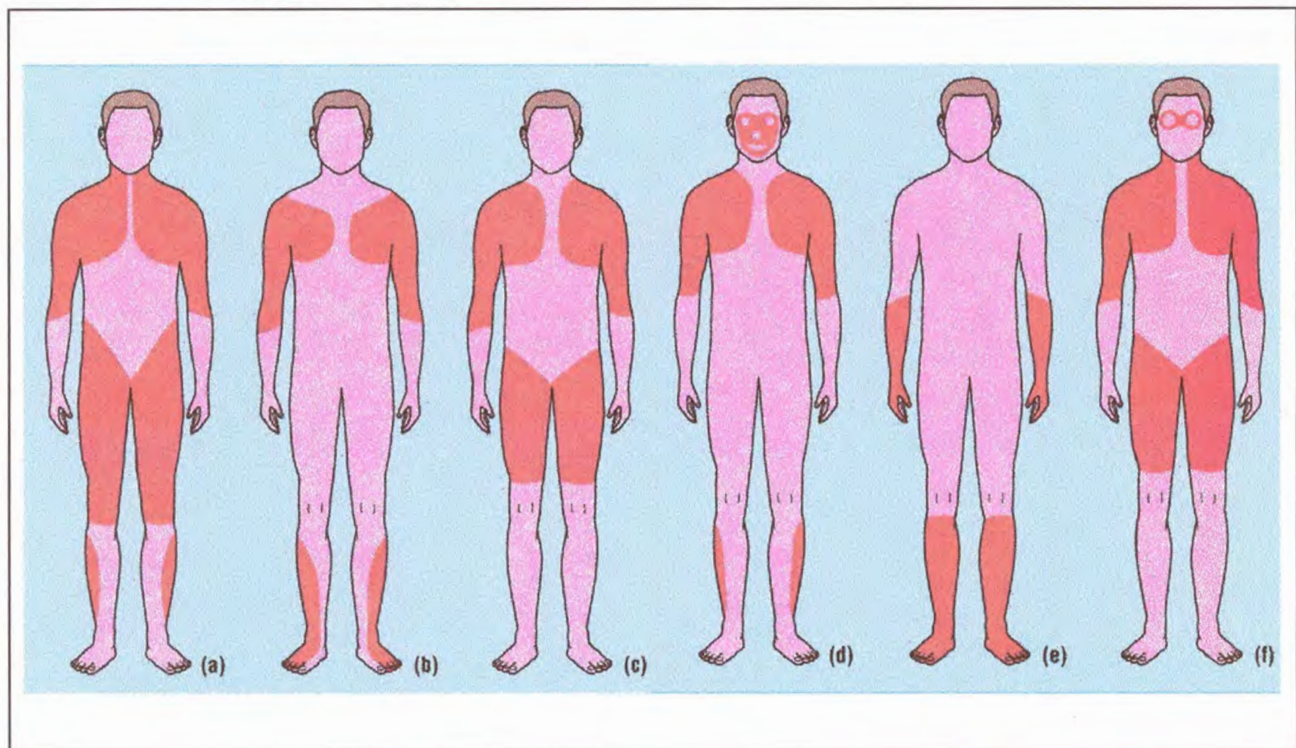
Distal myopathies [MIM 254130] are relatively rare and are associated with wasting and weakness of the distal muscles. Individuals affected with a distal myopathy are generally only mildly affected, however, some may develop problems in walking. (Emery 1998; OMIM 2001d)

Oculopharyngeal muscular dystrophy (OPMD) [MIM 164300] presents late in life and is characterised by dysphagia and progressive ptosis of the eyelids. Weakness of the

muscles of the neck and proximal upper limbs can also be present. OPMD is caused by an expansion of a $(GCG)_6$ repeat encoding a polyalanine tract located at the N terminus of the poly(A)-binding protein 2 located on chromosome 14. (Emery 1998; OMIM 2001e)

Facioscapulohumeral muscular dystrophy (FSHD) [MIM 158900] is characterised by progressive muscle wasting of the facial, shoulder, and upper arm muscles, however, other muscles such as the abdominal, foot extensor and pelvic girdle muscles may also be involved. FSHD is an autosomal dominant disorder caused by a DNA rearrangement on chromosome 4q35. The gene involved in FSHD has not been determined yet. The study presented here focuses on facioscapulohumeral muscular dystrophy (FSHD), which is discussed in section 2.0 (Emery, 1998; OMIM 2001f).

Figure 2.1: Distribution of muscle groups predominantly affected in various muscular dystrophies

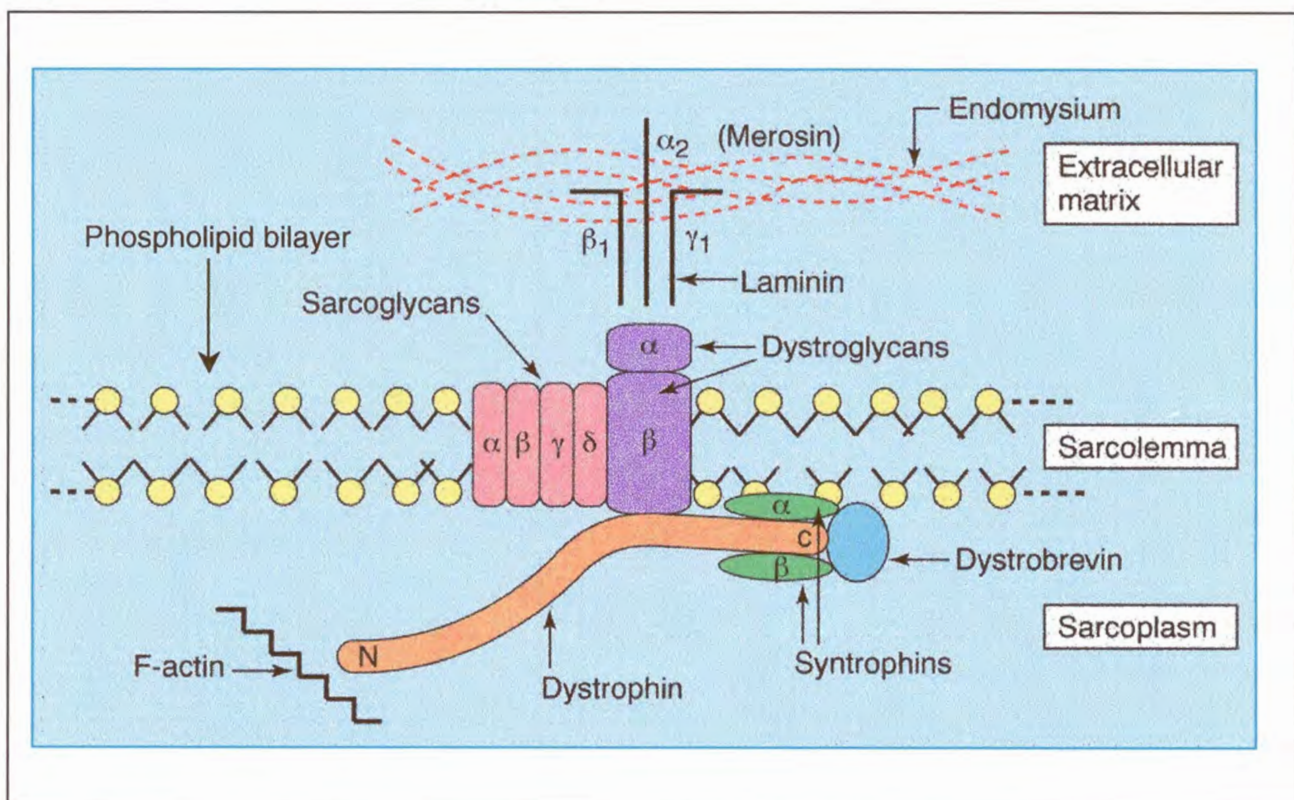


Different types of muscular dystrophies: a = Duchenne and Becker; b = Emery-Dreifuss; c = Limb girdle; d = Facioscapulohumeral; e = Distal; f = Oculopharyngeal. Adapted from Emery *et al.* (1998).

Immunohistochemical techniques enabled the identification of specific deficiencies of various membrane proteins, including dystrophin and sarcoglycan referred to as dystrophin associated glycoproteins. This identification resulted in the classification of some of the dystrophies on a histological level into dystrophinopathy which included Duchenne and Becker muscular dystrophy and sarcoglycanopathy consisting of some of the Limb girdle type muscular dystrophies. The sarcoglycan complex (SGC) consists of five units: α-, β-, γ-, δ-, ε-sarcoglycans, with the first four being associated with muscular

dystrophies (Figure 2.2). The SGC comprises the dystrophin-glycoprotein complex (DGC) together with dystrophin and the dystroglycan complex which are membrane-associated muscle proteins that span the muscle sarcolemma and form the link between the extracellular matrix (endomysium) and intracellular F-actin as illustrated in Figure 2.2. The dystrophin-glycoprotein complex is suggested to have a role in the maintenance of the stability, integrity and strength of the muscle membrane. Disruption of the complex could therefore cause a cascade of events resulting in muscle weakness (Emery 1998; Mak *et al.,* 2001). However, the precise mechanism as to how the absence of these proteins results in muscle weakness remains unresolved.

Figure 2.2: Muscle membrane proteins



Adapted from Emery *et al.* (1998)

2.1 FACIOSCAPULOHUMERAL MUSCULAR DYSTROPHY

Facioscapulohumeral muscular dystrophy (FSHD) is part of a group of inherited disorders characterised by progressive muscle wasting and weakness. FSHD is the third most common inherited disorder of muscle after Duchenne and Myotonic dystrophy, with a prevalence of at least 1:20,000 in Caucasians, including familial and sporadic cases (Padberg, 1982).

2.1.1 CLINICAL ASPECTS OF FSHD

Landouzy and Déjérine, after whom FSHD was initially named, published their first article in the "Revue de Medicine" (Review of Medicine) entitled "La myopathie atrophique progressive; myopathie sans neuropathie debutant d'ordinaire dan l'enfance, par la face", (Progressive atrophic myopathy, myopathy without neuropathy starting usually in childhood, by the face) in 1884 (Landouzy and Déjérine, 1884). In this paper the term 'facioscapulohumeral' was mentioned for the first time. They described an autopsy on a man who died of tuberculosis when he was 24 years old. Atrophy of the facial muscles was the only symptom noted at the age of three while atrophy of the shoulder girdle and upper arm muscles developed at the age of 17. The weakness progressed slowly to the muscles of the trunk and pelvic girdle. The muscles of the tongue, pharynx, larynx masseter, temporal and pterygoid muscles were not affected. No abnormalities on the brain, spinal cord, peripheral nerves and intramuscular nerve endings were present. The younger brother and sister of the patient were similarly affected and an autosomal dominant pattern of inheritance was observed. The father of the patient developed muscle weakness in the shoulder girdle at the age of 26 and facial weakness was observed when he was 32 years old (Padberg, 1982). The complex and difficult diagnosis of FSHD on the clinical level was thus already observed in the first reported case, more than a century ago. Unfortunately the difficulties associated with the clinical diagnosis of FSHD still complicate diagnosis today and are mainly due to the complex clinical phenotype of this disorder.

2.1.1.1 Presenting symptoms

This disorder is characterised at onset by asymmetrical weakness and atrophy of the facial and shoulder girdle muscles (Padberg 1982). The following muscles may also be affected: abdominal, foot extensor, upper arm, pelvic girdle and lower arm muscles. However, the degree to which the muscles can be affected varies dramatically, which forms the basis of the extremely heterogeneous nature of the FSHD phenotype (Padberg 1982).

Diagnostic criteria for FSHD have been defined by the International FSHD Consortium and are provided in Appendix C. In summary the following criteria define FSHD (Padberg *et al.*, 1991):

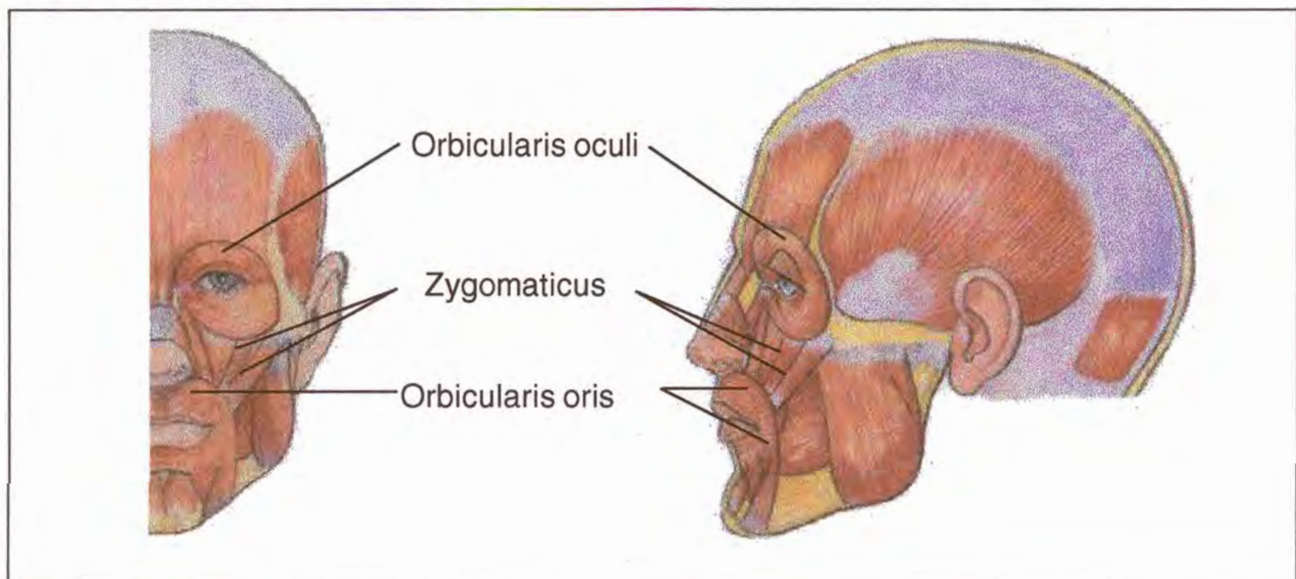
1. weakness of the facial or shoulder girdle muscles, but sparing of the extra-ocular, pharyngeal and lingual muscles and the myocardium,

2. facial weakness in more than 50% of the affected family members,
3. autosomal dominant inheritance in familial cases, and
4. presence of a myopathic disorder in both the electromyography (EMG) and muscle biopsy in at least one affected family member.

2.1.1.1.1 The facial muscles

Facial weakness is generally present in the early stages of the disorder and the zygomaticus, orbicularis oculi and orbicularis oris muscles are generally affected (Figure 2.3). Weakness of the zygomaticus muscles results in the inability to raise the corners of the mouth and patients express a grin instead of a smile. The orbicularis oris allows pursing of the lips, whistling and retaining of air under pressure. Patients find it difficult to bury the eyelashes completely when forced closed due to the weakness of the orbicularis oculi muscles. If the weakness progresses, a small piece of the sclera may be visible when attempting to close the eyes. Individuals are often seen sleeping with open eyes, and blinking is slowed and incomplete (Padberg, 1982). Other facial muscles may also become involved, resulting in an unlined forehead and a smooth, expressionless face, resulting in the term "facies myopathica" or myopathic face (Landouzy and Déjérine, 1885).

Figure 2.3: Facial muscles affected in FSHD



Adapted from Dorland's Pocket Medical Dictionary, (1995).

Facial weakness is, however, not obligatory in the diagnosis of FSHD. Felice *et al.* (2000) examined seventeen unrelated patients with facial-sparing scapular myopathy. Fourteen of the seventeen patients agreed to DNA testing and ten of the fourteen (71%) had a *Bln* I

resistant deletion fragment, which defines FSHD on the molecular level (see paragraph 2.1.2.2). Felice *et al.* (2001) also reported three individuals with no visible facial weakness. The first individual was at first diagnosed with facial-sparing scapular myopathy with symptoms of scapular winging, humeral and quadriceps atrophy together with a waddling-type gait. The second individual had difficulty climbing stairs during high school and had limited movement of wrist and finger flexors and extensors, as well as foot and toe flexors. There were no facial, scapular winging, or other characteristic features of FSHD. This individual was eventually diagnosed with limb-girdle muscular dystrophy. The last individual displayed asymmetric scapular winging and mild right trapezius atrophy. His limb-muscle strength was normal, and there was no facial weakness. FSHD was, however, confirmed on a molecular level in all three of these individuals.

Muscle disorders, clinically similar to FSHD include scapuloperoneal and scapulohumeral dystrophies, which are associated with minimal or complete absence of facial weakness, and may represent milder forms of the disorder since there is evidence for involvement of the same genetic locus (Jardine *et al.,* 1994a). The scapuloperoneal syndrome is difficult and sometimes even impossible to distinguish from FSHD on the clinical level. Identification of the FSHD gene might elucidate whether the disorders are indeed different on the genetic and molecular levels.

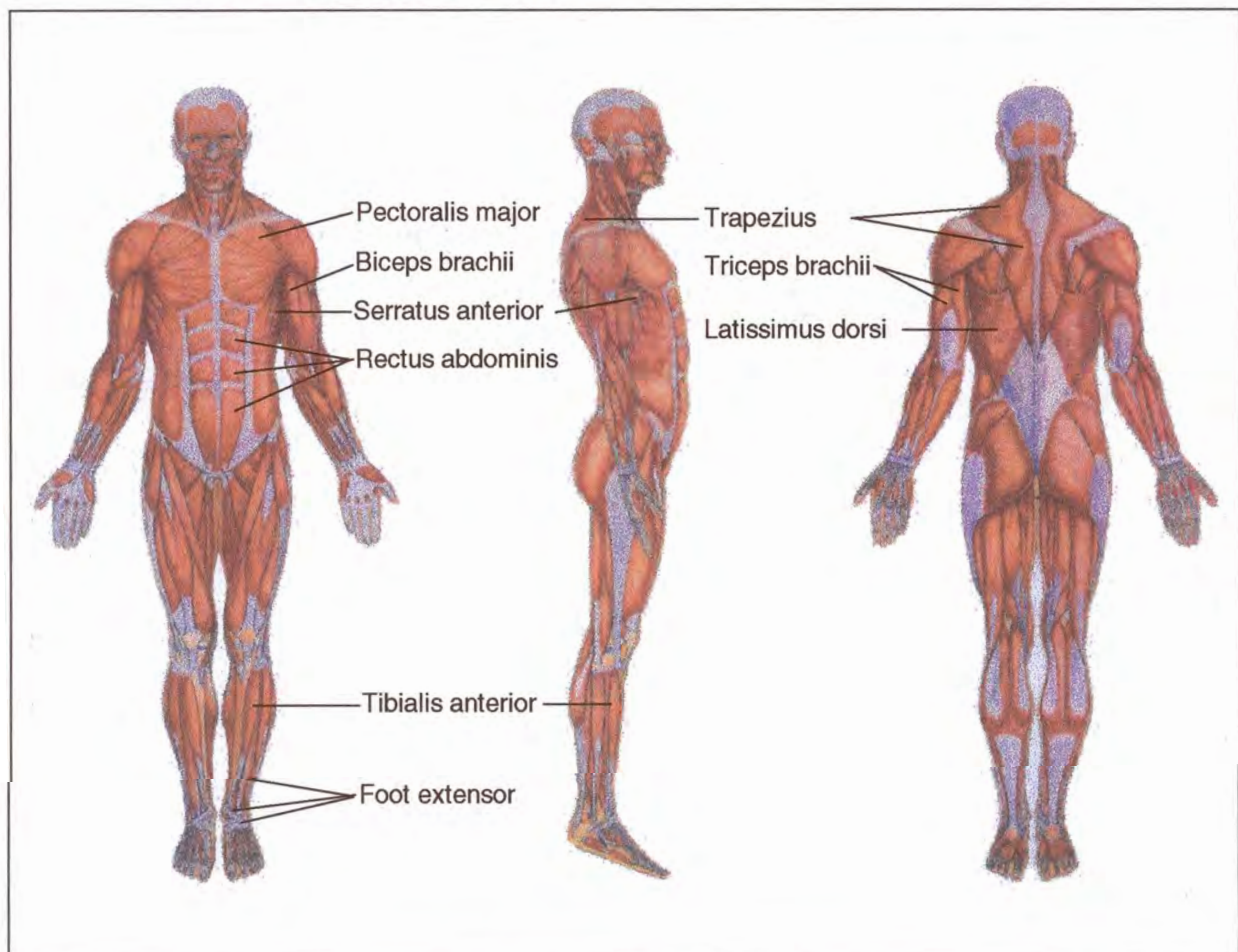
2.1.1.1.2 The upper extremities, shoulder girdle and neck muscles

Apart from the weakness of the facial muscles, one of the earliest symptoms in FSHD is the gradual loss of fixation of the scapula. The rhomboids, the lower part of the trapezius and the serratus anterior muscles, which stabilise the scapula to the torso, are all involved (Padberg, 1982). Figure 2.4 displays the muscles involved, except for the rhomboid muscle, which is part of the deep muscles and is therefore not visible on this figure. Weakness of these muscles results in allowing the scapulae to rotate slightly laterally, and to move upwardly, laterally and anteriorly over the thorax. If the rhomboid and serratus weakness progresses, scapulae alatae appear. The positional change of the scapulae contributes to the development of drooping shoulders. The clavicles lose their normal upward slope, rotate anteriorly and may ultimately even slope downwards. If the scapula fixation becomes weaker, the arms cannot be raised completely and can, for instance, be swung upwards to catch an object that is above shoulder height. When the scapular fixation, and especially the serratus function worsens further, elevation of the arm above shoulder level becomes impossible. When attempting abduction of the arms, the scapulae

ride upward over the back and their upper borders rise high up into the normal location of the trapezius muscles.

Weakness of the latissimus dorsi and the sternocostal part of the pectoralis muscles may also occur (Figure 2.4). Wasting of the latter will result in a flattened outline of the anterior thoracic wall, with a change in the direction of the axillary crease, running more horizontally instead of vertically, and pointing to the sternoclavicular joint (Padberg, 1982). The time between the onset of shoulder girdle weakness and the onset of upper arm weakness may be quite variable. Atrophy of the biceps and triceps in the upper arms may become quite severe even early in the course of the disorder resulting in the upper arm appearing thinner than the lower arm (often referred to as "Popeye" arms), due to the relative sparing of the lower arm muscles.

Figure 2.4: Upper extremities, lower extremities, shoulder girdle and truncal muscles affected in FSHD



Adapted from Dorland's Pocket Medical Dictionary, (1995).

2.1.1.1.3 The truncal muscles

Padberg (1982) reported abdominal muscle weakness in 58% of the patients who were investigated. Weakness of the abdominal muscles (Figure 2.4) was observed to add to the pelvic tilt and the increased lumbar lordosis caused by the anterior convexity of the lumbar spine. The lumbar lordosis was also observed to become more severe when an individual was wheelchair dependent.

2.1.1.1.4 The lower extremities and the pelvic girdle muscles

Landouzy and Déjérine (1885) and Chyatte *et al.* (1966) observed the early weakness of the anterior tibial muscles in FSHD patients. Weakness of the foot extensors (Figure 2.4) interferes with walking, resulting in a steppage gait, referring to the pattern of how an individual walks, and an inability to run (Padberg, 1982). Steppage gait is characterised by the dropping of the foot, where the foot hangs with the toes pointing down, causing the toes to touch the ground while walking. Patients tend to trip easily over small objects, falling forward onto their knees. If pelvic girdle weakness develops, a waddling gait characterised by a distinctive duck-like walk will be visible and gradual rising from a chair or climbing stairs becomes increasingly difficult.

2.1.1.1.5 Asymmetry of muscle involvement

Distinct asymmetry of muscle involvement is an important and common feature of FSHD (Padberg, 1982). The asymmetry can be present in the facial as well as in the shoulder girdle muscles, and the extremities. No correlation was observed between right or left handed individuals, the side of muscle involvement, or the severity of affected muscles (Tawil *et al.*, 1994).

2.1.1.1.6 Extramuscular involvement

Sensorineural deafness and retinal vascular abnormalities have been described by many authors to be associated with FSHD (Gieron *et al.*, 1985; Gurwin *et al.*, 1985; Korf *et al.*, 1985; Matsuzaka *et al.*, 1986; Voit *et al.*, 1986; Fitzsimons *et al.*, 1987; Yasukohchi *et al.*, 1988; Brouwer *et al.*, 1991; Pauleikhoff *et al.*, 1992; Padberg *et al.*, 1995a). High-frequency hearing loss has been observed in 50 to 64% of FSHD patients (Brouwer *et al.*, 1991; Padberg *et al.*, 1995a). Brouwer *et al.* (1991) observed a significant difference in

hearing level between 4,000 Hertz (Hz) and 6,000 Hz in FSHD patients when compared to controls. The hearing loss was also observed to be progressive and, with time, tends to involve lower frequencies of the spectrum. These authors observed that the severity of the hearing loss varied between individuals and that it was not age dependent.

Originally, retinal vascular abnormalities were mainly reported in severe cases with early-onset FSHD (Gurwin *et al.*, 1985). Fitzsimons *et al.* (1987) and Brouwer *et al.* (1993) observed exudative retinal vasculopathy, with capillary telangiectasis, microaneurysms, and capillary closure to be present in 50 to 75% of FSHD patients. This is characterised by damage or breakdown of the retina due to subretinal fluid accumulation and the capillaries being longer, wider and fewer in number than normal. No correlation between the severity of the muscular weakness, and the severity of the hearing loss or the retinal vasculopathy was observed. The pathogenic mechanism of hearing loss and retinal vasculopathy in FSHD is still unknown.

Cardiac and respiratory muscles are generally not affected in FSHD patients (Padberg 1982; Tawil *et al.*, 1998). Emery-Dreifuss muscular dystrophy, a phenotypically similar but genetically distinct disorder, is distinguished from FSHD on the clinical level by the presence of cardiac muscle involvement. Stevenson *et al.* (1990) provided the first evidence of cardiac involvement in FSHD, unfortunately, no molecular data was available to confirm the clinical diagnosis of FSHD. Laforet *et al.* (1998) reported the presence of cardiac involvement in genetically confirmed FSHD patients. This study included 100 patients of whom five had conduction defects or arrhythmia. It is therefore evident that patients with FSHD may have cardiac involvement, although to date it has only been observed in a small number of cases. Finsterer *et al.* (2000) investigated the presence of cardiac involvement (CI) in several myopathies, such as DMD, BMD, EDMD, FSHD, sarcoglycanopathies, myotonic dystrophies type 1 and 2 and mitochondrial myopathies, and observed CI to be present in all the disorders. CI can lead to symptoms such as: impulse generation defects, impulse conduction defects, thickened myocardium, left ventricular hypertrabeculation, dilatation of the cardiac cavities, intracardial thrombus formation, and heart failure with systolic and diastolic dysfunction. The above findings argue strongly in favour of FSHD patients being investigated for possible cardiac involvement.

Inflammatory cellular infiltrates are frequently (40 to 80%) observed in the skeletal muscle of FSHD patients. General histological changes in FSHD muscle are rather non-specific

with variations in fibre size and scattered small angulated fibres being the most commonly observed anomalies (Arahata *et al.*, 1995).

Involvement of the lingual muscle is considered to be one of the exclusion criteria of FSHD (Appendix C). However, Korf *et al.* (1985) reported four patients with tongue abnormalities, such as atrophic changes and movement disturbances. No findings on the genetic studies of these patients were, however, reported. Tongue atrophy was also reported by Yamanaka *et al.* (2001) in seven Japanese patients, all belonging to a group of early-onset FSHD patients with small *Eco* RI deletion fragments (10 to 17 kb). Miura *et al.* (1998) also reported a female sporadic case with early-onset FSHD with tongue atrophy with a 10 kb *Eco* RI deletion fragment. These studies therefore suggest that individuals with FSHD can have involvement of the lingual muscles and that this clinical aspect should not be used as an exclusion criterium of FSHD. The occurrence of tongue abnormalities in FSHD, especially in early onset FSHD, should, however, be investigated in larger populations.

2.1.1.2 Clinical heterogeneity in the FSHD phenotype

Clinical diagnosis of the FSHD phenotype is complex due to extreme variability in various aspects of the phenotype, including its penetrance. The phenotypical expression varies in severity, rate of progression and the age of onset, ranging from almost asymptomatic individuals, to almost 20% of patients who will be wheelchair dependent by the age of 40 years or older (Lunt *et al.,* 1991). Variability is observed both between and within families, thus complicating genetic counselling. The penetrance of the FSHD gene was estimated to be different for various age groups as listed in Table 2.1. The life expectancy of individuals affected with FSHD is not shortened since cardiac and respiratory functions are generally unaffected (Padberg 1982).

Table 2.1: Penetrance of FSHD

<5%	0 to 4 years
21%	5 to 9 years
58%	10 to 14 years
86%	15 to 19 years
95%	20 years.

Adapted from Lunt *et al.* (1989a).

Tawil *et al.* (1993a) studied monozygotic twins with extreme clinical variability. One of the twins had progressive shoulder girdle weakness from the age of 10. Upon examination, at the age of 27, severe facial, scapular, peroneal and abdominal muscle weakness was observed. The asymptomatic

twin brother showed only mild weakness of the orbicularis oculi muscles. Zygosity was first determined through red cell phenotyping as well as human leukocyte antigen (HLA) typing. High-resolution cytogenetic analysis was performed to exclude any cytogenetic abnormalities. Five markers (D4S139, D4S163, D4S171, D4S130 and D4F35S1), closely linked to FSHD, were also studied to provide further evidence of monozygosity and to exclude the possibility that submicroscopic rearrangements of distal 4q had occurred. One possible explanation for the observed variability is the presence of a somatic mutation in one of the twins. Another explanation is that both twins have FSHD and that their phenotypes represent an extreme case of variability of expression within the family. The second was the most likely explanation and, therefore, further molecular studies were necessary to confirm the presence of a deletion fragment in both individuals. Tawil *et al.* (1993b) performed the additional studies and reported the presence of a unique 4q35 DNA rearrangement in the affected individual. Results from the second study confirmed that in this particular case, the discordance was probably due to a *de novo* postzygotic mutation after the twinning process had occurred.

Griggs *et al.* (1995) re-examined the monozygotic twins discordant for FSHD, first examined by Tawil in 1993, as well as two additional sets of monozygotic twins concordant for FSHD. The twins concordant for FSHD were similarly affected in terms of age of onset, overall degree of disability, and quantitative tests of muscle, but extreme differences in the symmetry of the muscle involved were observed. The inheritance pattern of the discordant twins could not be established, since sufficient symptomatic family members were not available for examination. Upon molecular analysis, deletion fragments were observed in the two twin pairs concordant for FSHD. A deletion fragment was also present in the affected individual of the other twin pair. No deletion fragment was, however, present in the twin with the mild facial weakness. These authors also concluded that the *de novo* postzygotic mutation or mitotic crossover probably occurred during the twinning process. Based on the results from the concordant twin pairs the authors suggested that the deletion fragment determines the age of onset and severity of the disorder, and that the asymmetries observed in FSHD patients are due to other factors, which may be for instance, environmental.

Tupler *et al.* (1998) described two monozygotic male twins affected by FSHD, carrying an identical *de novo Eco* RI deletion fragment. Neurological examination of their parents was normal, with no sign of muscular dystrophy. Haplotype analysis identified that the *de novo* rearrangement on chromosome 4q had occurred in paternal gametogenesis or

postzygotically in the paternal chromosome 4 before twinning. The genetic identity of the two twins did however not concur with their respective clinical phenotypes. One twin was severely affected and the other one was almost asymptomatic. The medical history was the same for the two brothers, except for an antirabies vaccination performed at the age of five in the more severely affected twin. Tupler *et al.* (1998) hypothesised that the vaccination might have triggered an inflammatory immune reaction, which contributed to the more severe phenotype. The pathogenesis of this external factor with relation to the severe FSHD phenotype has, however, not been elucidated.

Miura *et al.* (1998) reported two unrelated, severely affected female sporadic cases diagnosed with early-onset scapulohumeral muscular dystrophy with mental impairment (intelligence quotients (IQs) ranging from 33 to 45) and epilepsy. One patient suffered from epilepsy since her second birthday and muscle weakness of the face, shoulder girdle, and upper arms was observed from the age of four years. Lack of facial expression was noticed in the second patient from the age of one. She developed epilepsy at the age of nine years and weakness of her lower limbs progressed from the age of ten, eventually becoming wheelchair dependent by the age of fourteen. She also had moderate sensorineural hearing loss and tongue atrophy. Genetic analysis confirmed the presence of a 10 kb *Eco* RI deletion fragment on chromosome 4q35 in both individuals.

Van der Kooi *et al.* (2000) reported six sporadic patients with symptoms and signs that initially caused confusion upon clinical examination. FSHD was subsequently confirmed via genetic analysis. Three patients presented with foot extensor weakness, one with thigh weakness, one with calf muscle weakness resulting in an inability to walk on his toes, and the other with mild shoulder symptoms, such as tiredness and muscle pain. None of the patients had visible facial weakness upon initial examination. An expert physical examination, however, indicated the presence of an abnormality in the facial expressions of the patients and abnormal shoulder posture or scapular winging when lifting the arms. This study highlights the need for expert clinical examination of FSHD patients. Moreover it is preferable if one clinician can investigate all patients included in a study to ensure consistency in diagnosis of this complex clinical phenotype.

A patient reported by Felice *et al.* (2001) was diagnosed with late-onset autosomal dominant distal myopathy, and presented with progressive bilateral foot drop, and developed difficulties when climbing stairs. Other problems included mild, late-onset

sensorineural hearing loss, occasional heart palpitations, cataracts and arthritis. Neurological examination revealed mild eye closure weakness, mild hearing loss, anterior foreleg muscle atrophy and a steppage gait pattern. There were no visible signs of scapular winging or humeral atrophy. FSHD was, however, confirmed on a molecular level.

The studies of Miura *et al.* (1998), Van der Kooi *et al.* (2000) and Felice *et al.* (2001) confirm the clinical variability of FSHD, and strengthen the need for DNA analysis, as well as examination by a clinician with experience in the clinical diagnosis of this clinically heterogeneous disorder.

2.1.1.2.1 Infantile FSHD

Infantile FSHD, also known as early-onset FSHD, is the most severe form of this disorder (McGarry *et al.,* 1983). Individuals affected by this form of FSHD are generally symptomatic before the age of five, display signs and symptoms of shoulder girdle weakness before the age of ten and are wheelchair dependent by the age of nine or ten (McGarry *et al.,* 1983; Brouwer *et al.*, 1993). Brooke was the first to describe infantile FSHD as a special form of the disorder and even suggested a specific clinical course and mode of inheritance (Brouwer *et al.*, 1994).

This form of FSHD accounts for less than 5% of affected cases (Brouwer *et al.*, 1993). Early facial weakness is visible during the first two years of life, and progressive weakness of the shoulder causing scapular winging, upper-arm and foot-extensor muscle weakness, characterised by foot drop, is observed, followed later by development of pelvic girdle weakness presenting as lumbar lordosis (Korf *et al.,* 1985; Bailey *et al.,* 1986). The presence of high frequency hearing loss, retinal vascular abnormalities and mental retardation have also been reported in many of the infantile FSHD patients (Korf *et al.,* 1985; Fitzsimons *et al.,* 1987; Brouwer *et al.*, 1995).

Death as a result of FSHD was reported by McGarry *et al.* in 1983. This child died at the age of five after progressive weakness and recurrent pneumonias. Her creatine phosphokinase, lactate dehydrogenase and aldolase were all elevated four to ten fold on different occasions. There was no family history of any neurological or muscle disorders. Bailey *et al.* (1986) also reported the death of four individuals, all with onset in infancy,

before the age of 20. Sporadic and familial cases occur, but on average the sporadic cases seem to be more severely affected than the familial cases (Brouwer *et al.*, 1994). Brouwer *et al.* (1995) concluded that infantile FSHD does not differ clinically, or genetically, from adult onset FSHD.

Funakoshi *et al.* (1998) examined 140 Japanese FSHD patients from 91 unrelated families, of whom twenty patients were classified as early onset FSHD. Nine of the twenty patients had small (10-11 kb) *Eco* RI fragments, and a high frequency of epilepsy (44%) and mental retardation (89%) was observed. These authors concluded that FSHD patients with a large deletion in the FSHD region tend to have a higher chance of clinical phenotypes being associated with central nervous system (CNS) abnormalities. Okinaga *et al.* (1997) also reported the presence of two early-onset FSHD cases in the Japanese population. Both children were observed to sleep with their eyes slightly open and demonstrated an inability to smile before the age of one. FSHD was confirmed on the molecular level in these two children by the presence of deletion fragments (13 kb and a 15 kb).

2.1.1.3 Current treatments for FSHD

Individuals affected with any form of muscular dystrophy should consider a few general principles, which could assist them in adapting to the muscle weakness and to continue their everyday activities. Physical therapy, including light exercise, helps preserve flexibility and prevents contractures that result from immobility. Swimming is the preferred form of light exercise as it puts little strain on the muscles. A good diet is necessary to avoid unnecessary weight gain and reduces stress on the already weakened muscles.

Currently there is no treatment or cure for FSHD, but there are aids that can provide symptomatic relief. Individuals affected with FSHD have a relatively normal life expectancy and therefore sometimes require procedures that will provide prolonged relief.

In a procedure known as scapulothoracic arthrodesis, the scapula of some FSHD individuals has been fixed to the thoracic wall, to improve stability for activities involving the upper limbs. Bunch *et al.* (1993) fixed the scapula to the ribs in twelve FSHD patients and did re-examinations from three to twenty one years after the procedure. Instability of the scapula due to weakness of the muscles that stabilise the scapula results in an inability

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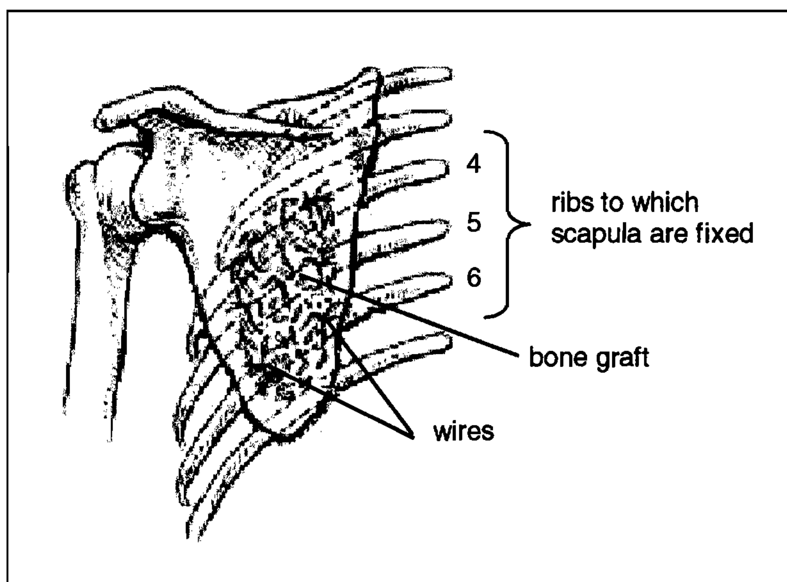
to raise the arms forward or above the head. This group of FSHD patients did, however, have sufficient strength in the supraspinatus and deltoid muscle to abduct the arm if the scapula had been stabilised. Approximately 50% of the individuals examined by Bunch *et al.* (1993) had preserved deltoid function. The scapula is positioned over the seventh rib and fixation to ribs four, five and six is generally adequate, but as many as five ribs have been used as depicted in Figure 2.5. Holes are drilled through the scapula, and two one-millimetre-diameter stainless-steel wires are placed under each rib and through the drill-holes in the scapula. The wires are pulled tightly to compress the scapula against the graft. The resistance of the tightened wires secures the position of the scapula. The wire-ends are bent and buried within the infraspinatus muscles.

Figure 2.5: Scapular fixation in FSHD



Adapted from Bunch *et al.* (1993).

A solid fusion between the ribs and scapula is necessary, as the wire loops do not provide a rigid enough fixation, as they can slide on the ribs. Some form of external support is therefore required to ensure solid fusion. For example, a shoulder spica, which holds the arm abducted in a salute position for two months. Subsequently the arm should be placed in a figure-of-eight dressing until radiographs show a solid fusion. The twelve patients described by Bunch *et al.* (1993) all obtained solid fusion, and all of them experienced a more stable shoulder while they were carrying and lifting objects. All but one patient was capable of forward flexion and abduction to 90 degrees or more. These patients had an average of 30 degree flexion preoperatively, which increased to 65-125 degrees postoperatively. Bunch *et al.* (1993) favour this method as it is technically relatively easy and is associated with few complications.

Andrews *et al.* (1998) also performed scapulothoracic arthrodesis in six FSHD patients. In general the range of abduction and flexion increased and all the patients could continue their work after recovery. The scapula was clinically and radiologically fused to the chest wall in all the patients. Scapulothoracic arthrodesis was also performed by Letournel *et al.*

(1990) in fifteen patients. Flexion increased on average by 33 degrees and abduction by 25 degrees and, upon a sixty-nine month follow-up, the results had not deteriorated. Scapulothoracic arthrodesis offers a good long-term benefit to FSHD patients and also contributes to an increase in quality of life.

Steroids and other anti-inflammatory drugs are generally reserved for the treatment of the inflammatory myopathies, eg. polymyositis. There is, however, not a large amount of literature available on the use of steroids in FSHD. Munsat *et al.* (1972) found that creatine kinase (CK) levels dropped and symptoms improved in a small number of FSHD patients. In a more recent study by Tawil *et al.* (1997), eight FSHD patients were treated with prednisone for 12 weeks. There were, however, no significant changes in the muscle strength or mass. It was therefore concluded that treatment with prednisone had no benefit for FSHD patients, however, the effect on disease progression could not be evaluated over this short period of time.

Kissel *et al.* (1998) investigated the effect of albuterol, a β_2-agonist, in FSHD patients. β_2-agonists have been shown to induce satellite cell proliferation, increase muscle protein production, inhibit muscle proteolysis and retard the loss of muscle mass due to muscle injury, denervation, disuse, steroid atrophy, malnutrition, tumor, sepsis, and surgery. Kissel *et al.* (1998) treated fifteen FSHD patients for three months and reported improved muscle mass and overall improvement of 12% in strength. The results were encouraging and the effects of albuterol were further evaluated in a larger, randomised, double-blind, placebo-controlled trial by these authors. From this study, Kissel *et al.* (2001) reported that although treatment with albuterol for one year did not improve global strength or function in patients affected with FSHD, a significant increase in muscle mass and grip strength was observed. This indicated that albuterol does have some anabolic effect in the treated patients. The authors are, however, not sure as to why the increase in muscle mass did not translate into increased strength.

Bushby *et al.* (1998) reported four patients with FSHD in whom pain was the most distinctive symptom. Treatment of the pain was difficult, since there was a poor response to conventional anti-inflammatory therapy and eventually morphine was prescribed for one of the patients. The other three patients experienced improvement from swimming. All patients complained of a feeling of frustration together with depression and irritability. Two of the patients were treated with antidepressants and an improvement in their mood and partial improvement in their pain control was reported.

2.1.2 GENETIC ASPECTS OF FSHD

FSHD is an autosomal dominant, neuromuscular disorder and has a prevalence of 1 in 20,000 individuals in the European population and a prevalence of 1 in 14,763 individuals in Utah, U.S.A. (Walton, 1955; 1956; Lunt 1989a; Flanigan *et al.,* 2001). A higher prevalence was reported for the Utah population, as the entire geographical region contains only one muscular dystrophy referral centre, which examines almost all the reported cases within this region. It is also known that the Utah population consists of very large families. A relatively high proportion of new mutations (10 to 30%) have also been reported in FSHD (Padberg 1982; Padberg *et al.,* 1995b; Zatz *et al.,* 1995; Tawil *et al.,* 1996; Lunt 1998; Zatz *et al.,* 1998).

2.1.2.1 Linkage of FSHD to chromosome 4q35

Possible linkage was observed for the Gm locus encoding the constant region of the heavy chains of the IgG immunoglobulins (Padberg *et al.,* 1984). Gm was known to be located on the long arm of chromosome 14 (near 14q32). A subsequent study by Padberg in 1988, utilising DNA-probe D14S1, confirmed the linkage between Gm and D14S1, but no linkage was observed between FSHD and the D14S1 loci. This excluded the FSHD locus from the distal part of chromosome 14.

An international collaboration of research groups involved in the linkage analysis of FSHD was organised to reduce unnecessary duplication through the pooling of linkage data and to construct an exclusion map. Four groups consisting of 20 participants contributed linkage data: Padberg and Frants (Leiden); Upadhyaya, Sarfarazi, Lunt and Noades (Cardiff, Manchester, and London); Lucotte (Paris), and Pericak-Vance, Siddique and Shaw (Durham, NC and Manchester). A total of 57 markers on various autosomes were tested for possible linkage to FSHD, but no significant linkage between any marker and the disorder was detected (Lucotte *et al.,* 1989; Lunt 1989b; Sarfarazi *et al.,* 1989; Siddique *et al.,* 1989; Upadhyaya *et al.,* 1989). The overall data indicated that the likelihood for the location of the FSHD gene to be on chromosome 11 was 6.47 times more than being on any other chromosome. Other possible chromosomes were 19 (likelihood of 2.67) and 5 (likelihood of 2.22). Insufficient data was available for chromosomes 3, 10 and 15 and therefore remained unexcluded. Eventually participants of the international consortium

excluded almost 80% of the genome (Sarfarazi *et al.,* 1989). Jacobsen *et al.* (1990) excluded chromosomes 1, 2, 5, 7, 10 and 16 by using a panel of restriction fragment length polymorphism (RFLP) markers that were evenly spaced at approximately 20 centimorgan (cM) intervals.

Linkage was subsequently established in 1990 between the FSHD phenotype and the Mfd22 short tandem repeat polymorphism (STRP) marker at locus D4S171 in the sub-telomeric region of chromosome 4q35 (Wijmenga *et al.*, 1990). Ten multigeneration Dutch families consisting of 69 affected, 58 unaffected sibs and 25 spouses were investigated. Sixty microsatellite loci were analysed for linkage to FSHD. A maximum lod score of 6.34, at a theta value of 0.13, was observed for marker Mfd22. Only one of the ten families was uninformative for this marker. The microsatellite marker Mfd22 had previously been assigned to chromosome 4, with the use of a somatic cell hybrid panel (Weber and May, 1990).

Upadhyaya *et al.* (1990) confirmed the location of the FSHD locus to the subtelomeric region of chromosome 4, through the identification of a flanking marker, more closely linked to the FSHD locus. This variable number of tandem repeat (VNTR) marker pH30, at the D4S139 locus, was demonstrated to be tightly linked to the FSHD locus. The D4S139 locus was mapped to the distal portion of the long arm of chromosome 4 by *in situ* hybridisation (Milner *et al.*, 1989). Wijmenga *et al.* (1991) also mapped D4S139 to chromosome 4q35-qter by *in situ* hybridisation. It was thus possible to construct a primary map of the area surrounding the FSHD locus with D4S171 and D4S139, to estimate the most likely position of the gene. Several recombination events in ten families favoured the following locus order: D4S171-D4S139-FSHD-TEL. This paved the way for presymptomatic and prenatal diagnosis in an independent linkage panel of 24 families with FSHD from Great Britain that were also reported to be closely linked to the D4S139 locus (Upadhyaya *et al.,* 1991).

Members of the International FSHD Consortium for linkage analysis of the FSHD gene have pooled data in an attempt to determine the precise location of the FSHD gene as well as the order of four DNA markers on 4q35. Two additional markers (F11 and D4S163) to those used by Wijmenga in 1991 were utilised to form a linkage group consisting of four polymorphic loci in the area of the FSHD locus (D4S171, F11, D4S163 and D4S139) and covering a total distance of ca. 20 cM. Recombinants have been identified with these four

i 16609 539X
b15465366

markers, which allowed the order of the FSHD locus and marker loci to be determined. The most likely locus order and the relative position of the FSHD gene was thus: CEN-D4S171-F11-D4S187-D4S163-D4S139-FSHD-TEL (Gilbert *et al.,* 1992; Mathews *et al.,* 1992; Mills *et al.,* 1992; Sarfarazi *et al.,* 1992; Upadhyaya *et al.,* 1992; Weiffenbach *et al.,* 1992a; Wijmenga *et al.,* 1992a, Winokur *et al.,* 1993). Identification of flanking markers in the 4q35 region contributed towards establishing a reliable diagnostic test for FSHD and will eventually facilitate in achieving the ultimate goal of cloning the FSHD gene.

2.1.2.2 The FSHD locus on chromosome 4q35

Wijmenga *et al.* (1992b) isolated cosmid clone 13E in a search for homeobox genes. The clone was mapped to 4q35, distal to the D4S139 locus through *in situ* hybridisation. Cosmid 13E was subcloned and a 0.8 kb probe, designated p13E-11, was obtained. Probe p13E-11, at locus D4F104S1 (also known as D4S810), was observed to detect a DNA rearrangement in individuals affected with FSHD. Fragment sizes between 14 to 28 kb were observed to segregate in ten Dutch FSHD families upon *Eco* RI digestion. The DNA rearrangements detected by probe p13E-11 were thus identified to play a role in the aetiology of FSHD.

Restriction mapping of cosmid 13E indicated that the *Eco* RI fragment detected by p13E-11 contained 3.3 kb tandem repeats with each 3.3 kb repeat flanked by *Kpn* I sites as illustrated in Figure 2.6 and Table D.1 (Wijmenga *et al.*, 1992b). The human genome contains hundreds of copies of this 3.3 kb family of tandem repeats in regions associated with heterochromatin. Different members of this long interspersed nuclear element (LINE) repeat family have been found on the short arms of all the acrocentric chromosomes, in the heterochromatic regions adjacent to the ribosomal DNA gene clusters, and on chromosomes 1, 2, 3, 4, 10, 18 and Y (Lyle *et al.,* 1995; Winokur *et al.,* 1994; Winokur *et al.,* 1996).

Hewitt *et al.* (1994) determined the sequence of the 3.3 kb repeat units and reported the presence of a double homeobox, and two repetitive sequences, namely *Lsau* and *hhspm3* within each repeat, as illustrated in Figure 2.6 and Table D.1. *Lsau* is a middle repetitive 68% GC rich element associated with β satellite DNA and found in heterochromatic regions of the genome while *hhspm3* is a low copy GC rich repeat element. Lee *et al.*

2.1.1.1.3 The truncal muscles

Padberg (1982) reported abdominal muscle weakness in 58% of the patients who were investigated. Weakness of the abdominal muscles (Figure 2.4) was observed to add to the pelvic tilt and the increased lumbar lordosis caused by the anterior convexity of the lumbar spine. The lumbar lordosis was also observed to become more severe when an individual was wheelchair dependent.

2.1.1.1.4 The lower extremities and the pelvic girdle muscles

Landouzy and Déjérine (1885) and Chyatte *et al.* (1966) observed the early weakness of the anterior tibial muscles in FSHD patients. Weakness of the foot extensors (Figure 2.4) interferes with walking, resulting in a steppage gait, referring to the pattern of how an individual walks, and an inability to run (Padberg, 1982). Steppage gait is characterised by the dropping of the foot, where the foot hangs with the toes pointing down, causing the toes to touch the ground while walking. Patients tend to trip easily over small objects, falling forward onto their knees. If pelvic girdle weakness develops, a waddling gait characterised by a distinctive duck-like walk will be visible and gradual rising from a chair or climbing stairs becomes increasingly difficult.

2.1.1.1.5 Asymmetry of muscle involvement

Distinct asymmetry of muscle involvement is an important and common feature of FSHD (Padberg, 1982). The asymmetry can be present in the facial as well as in the shoulder girdle muscles, and the extremities. No correlation was observed between right or left handed individuals, the side of muscle involvement, or the severity of affected muscles (Tawil *et al.*, 1994).

2.1.1.1.6 Extramuscular involvement

Sensorineural deafness and retinal vascular abnormalities have been described by many authors to be associated with FSHD (Gieron *et al.*, 1985; Gurwin *et al.*, 1985; Korf *et al.*, 1985; Matsuzaka *et al.*, 1986; Voit *et al.*, 1986; Fitzsimons *et al.*, 1987; Yasukohchi *et al.*, 1988; Brouwer *et al.*, 1991; Pauleikhoff *et al.*, 1992; Padberg *et al.*, 1995a). High-frequency hearing loss has been observed in 50 to 64% of FSHD patients (Brouwer *et al.*, 1991; Padberg *et al.*, 1995a). Brouwer *et al.* (1991) observed a significant difference in

hearing level between 4,000 Hertz (Hz) and 6,000 Hz in FSHD patients when compared to controls. The hearing loss was also observed to be progressive and, with time, tends to involve lower frequencies of the spectrum. These authors observed that the severity of the hearing loss varied between individuals and that it was not age dependent.

Originally, retinal vascular abnormalities were mainly reported in severe cases with early-onset FSHD (Gurwin *et al.*, 1985). Fitzsimons *et al.* (1987) and Brouwer *et al.* (1993) observed exudative retinal vasculopathy, with capillary telangiectasis, microaneurysms, and capillary closure to be present in 50 to 75% of FSHD patients. This is characterised by damage or breakdown of the retina due to subretinal fluid accumulation and the capillaries being longer, wider and fewer in number than normal. No correlation between the severity of the muscular weakness, and the severity of the hearing loss or the retinal vasculopathy was observed. The pathogenic mechanism of hearing loss and retinal vasculopathy in FSHD is still unknown.

Cardiac and respiratory muscles are generally not affected in FSHD patients (Padberg 1982; Tawil *et al.*, 1998). Emery-Dreifuss muscular dystrophy, a phenotypically similar but genetically distinct disorder, is distinguished from FSHD on the clinical level by the presence of cardiac muscle involvement. Stevenson *et al.* (1990) provided the first evidence of cardiac involvement in FSHD, unfortunately, no molecular data was available to confirm the clinical diagnosis of FSHD. Laforet *et al.* (1998) reported the presence of cardiac involvement in genetically confirmed FSHD patients. This study included 100 patients of whom five had conduction defects or arrhythmia. It is therefore evident that patients with FSHD may have cardiac involvement, although to date it has only been observed in a small number of cases. Finsterer *et al.* (2000) investigated the presence of cardiac involvement (CI) in several myopathies, such as DMD, BMD, EDMD, FSHD, sarcoglycanopathies, myotonic dystrophies type 1 and 2 and mitochondrial myopathies, and observed CI to be present in all the disorders. CI can lead to symptoms such as: impulse generation defects, impulse conduction defects, thickened myocardium, left ventricular hypertrabeculation, dilatation of the cardiac cavities, intracardial thrombus formation, and heart failure with systolic and diastolic dysfunction. The above findings argue strongly in favour of FSHD patients being investigated for possible cardiac involvement.

Inflammatory cellular infiltrates are frequently (40 to 80%) observed in the skeletal muscle of FSHD patients. General histological changes in FSHD muscle are rather non-specific

with variations in fibre size and scattered small angulated fibres being the most commonly observed anomalies (Arahata *et al.*, 1995).

Involvement of the lingual muscle is considered to be one of the exclusion criteria of FSHD (Appendix C). However, Korf *et al.* (1985) reported four patients with tongue abnormalities, such as atrophic changes and movement disturbances. No findings on the genetic studies of these patients were, however, reported. Tongue atrophy was also reported by Yamanaka *et al.* (2001) in seven Japanese patients, all belonging to a group of early-onset FSHD patients with small *Eco* RI deletion fragments (10 to 17 kb). Miura *et al.* (1998) also reported a female sporadic case with early-onset FSHD with tongue atrophy with a 10 kb *Eco* RI deletion fragment. These studies therefore suggest that individuals with FSHD can have involvement of the lingual muscles and that this clinical aspect should not be used as an exclusion criterium of FSHD. The occurrence of tongue abnormalities in FSHD, especially in early onset FSHD, should, however, be investigated in larger populations.

2.1.1.2 Clinical heterogeneity in the FSHD phenotype

Clinical diagnosis of the FSHD phenotype is complex due to extreme variability in various aspects of the phenotype, including its penetrance. The phenotypical expression varies in severity, rate of progression and the age of onset, ranging from almost asymptomatic individuals, to almost 20% of patients who will be wheelchair dependent by the age of 40 years or older (Lunt *et al.,* 1991). Variability is observed both between and within families, thus complicating genetic counselling. The penetrance of the FSHD gene was estimated to be different for various age groups as listed in Table 2.1. The life expectancy of individuals affected with FSHD is not shortened since cardiac and respiratory functions are generally unaffected (Padberg 1982).

Table 2.1: Penetrance of FSHD

<5%	0 to 4 years
21%	5 to 9 years
58%	10 to 14 years
86%	15 to 19 years
95%	20 years.

Adapted from Lunt *et al.* (1989a).

Tawil *et al.* (1993a) studied monozygotic twins with extreme clinical variability. One of the twins had progressive shoulder girdle weakness from the age of 10. Upon examination, at the age of 27, severe facial, scapular, peroneal and abdominal muscle weakness was observed. The asymptomatic

twin brother showed only mild weakness of the orbicularis oculi muscles. Zygosity was first determined through red cell phenotyping as well as human leukocyte antigen (HLA) typing. High-resolution cytogenetic analysis was performed to exclude any cytogenetic abnormalities. Five markers (D4S139, D4S163, D4S171, D4S130 and D4F35S1), closely linked to FSHD, were also studied to provide further evidence of monozygosity and to exclude the possibility that submicroscopic rearrangements of distal 4q had occurred. One possible explanation for the observed variability is the presence of a somatic mutation in one of the twins. Another explanation is that both twins have FSHD and that their phenotypes represent an extreme case of variability of expression within the family. The second was the most likely explanation and, therefore, further molecular studies were necessary to confirm the presence of a deletion fragment in both individuals. Tawil *et al.* (1993b) performed the additional studies and reported the presence of a unique 4q35 DNA rearrangement in the affected individual. Results from the second study confirmed that in this particular case, the discordance was probably due to a *de novo* postzygotic mutation after the twinning process had occurred.

Griggs *et al.* (1995) re-examined the monozygotic twins discordant for FSHD, first examined by Tawil in 1993, as well as two additional sets of monozygotic twins concordant for FSHD. The twins concordant for FSHD were similarly affected in terms of age of onset, overall degree of disability, and quantitative tests of muscle, but extreme differences in the symmetry of the muscle involved were observed. The inheritance pattern of the discordant twins could not be established, since sufficient symptomatic family members were not available for examination. Upon molecular analysis, deletion fragments were observed in the two twin pairs concordant for FSHD. A deletion fragment was also present in the affected individual of the other twin pair. No deletion fragment was, however, present in the twin with the mild facial weakness. These authors also concluded that the *de novo* postzygotic mutation or mitotic crossover probably occurred during the twinning process. Based on the results from the concordant twin pairs the authors suggested that the deletion fragment determines the age of onset and severity of the disorder, and that the asymmetries observed in FSHD patients are due to other factors, which may be for instance, environmental.

Tupler *et al.* (1998) described two monozygotic male twins affected by FSHD, carrying an identical *de novo Eco* RI deletion fragment. Neurological examination of their parents was normal, with no sign of muscular dystrophy. Haplotype analysis identified that the *de novo* rearrangement on chromosome 4q had occurred in paternal gametogenesis or

postzygotically in the paternal chromosome 4 before twinning. The genetic identity of the two twins did however not concur with their respective clinical phenotypes. One twin was severely affected and the other one was almost asymptomatic. The medical history was the same for the two brothers, except for an antirabies vaccination performed at the age of five in the more severely affected twin. Tupler *et al.* (1998) hypothesised that the vaccination might have triggered an inflammatory immune reaction, which contributed to the more severe phenotype. The pathogenesis of this external factor with relation to the severe FSHD phenotype has, however, not been elucidated.

Miura *et al.* (1998) reported two unrelated, severely affected female sporadic cases diagnosed with early-onset scapulohumeral muscular dystrophy with mental impairment (intelligence quotients (IQs) ranging from 33 to 45) and epilepsy. One patient suffered from epilepsy since her second birthday and muscle weakness of the face, shoulder girdle, and upper arms was observed from the age of four years. Lack of facial expression was noticed in the second patient from the age of one. She developed epilepsy at the age of nine years and weakness of her lower limbs progressed from the age of ten, eventually becoming wheelchair dependent by the age of fourteen. She also had moderate sensorineural hearing loss and tongue atrophy. Genetic analysis confirmed the presence of a 10 kb *Eco* RI deletion fragment on chromosome 4q35 in both individuals.

Van der Kooi *et al.* (2000) reported six sporadic patients with symptoms and signs that initially caused confusion upon clinical examination. FSHD was subsequently confirmed via genetic analysis. Three patients presented with foot extensor weakness, one with thigh weakness, one with calf muscle weakness resulting in an inability to walk on his toes, and the other with mild shoulder symptoms, such as tiredness and muscle pain. None of the patients had visible facial weakness upon initial examination. An expert physical examination, however, indicated the presence of an abnormality in the facial expressions of the patients and abnormal shoulder posture or scapular winging when lifting the arms. This study highlights the need for expert clinical examination of FSHD patients. Moreover it is preferable if one clinician can investigate all patients included in a study to ensure consistency in diagnosis of this complex clinical phenotype.

A patient reported by Felice *et al.* (2001) was diagnosed with late-onset autosomal dominant distal myopathy, and presented with progressive bilateral foot drop, and developed difficulties when climbing stairs. Other problems included mild, late-onset

sensorineural hearing loss, occasional heart palpitations, cataracts and arthritis. Neurological examination revealed mild eye closure weakness, mild hearing loss, anterior foreleg muscle atrophy and a steppage gait pattern. There were no visible signs of scapular winging or humeral atrophy. FSHD was, however, confirmed on a molecular level.

The studies of Miura *et al.* (1998), Van der Kooi *et al.* (2000) and Felice *et al.* (2001) confirm the clinical variability of FSHD, and strengthen the need for DNA analysis, as well as examination by a clinician with experience in the clinical diagnosis of this clinically heterogeneous disorder.

2.1.1.2.1 Infantile FSHD

Infantile FSHD, also known as early-onset FSHD, is the most severe form of this disorder (McGarry *et al.,* 1983). Individuals affected by this form of FSHD are generally symptomatic before the age of five, display signs and symptoms of shoulder girdle weakness before the age of ten and are wheelchair dependent by the age of nine or ten (McGarry *et al.,* 1983; Brouwer *et al.*, 1993). Brooke was the first to describe infantile FSHD as a special form of the disorder and even suggested a specific clinical course and mode of inheritance (Brouwer *et al.*, 1994).

This form of FSHD accounts for less than 5% of affected cases (Brouwer *et al.*, 1993). Early facial weakness is visible during the first two years of life, and progressive weakness of the shoulder causing scapular winging, upper-arm and foot-extensor muscle weakness, characterised by foot drop, is observed, followed later by development of pelvic girdle weakness presenting as lumbar lordosis (Korf *et al.,* 1985; Bailey *et al.,* 1986). The presence of high frequency hearing loss, retinal vascular abnormalities and mental retardation have also been reported in many of the infantile FSHD patients (Korf *et al.,* 1985; Fitzsimons *et al.,* 1987; Brouwer *et al.*, 1995).

Death as a result of FSHD was reported by McGarry *et al.* in 1983. This child died at the age of five after progressive weakness and recurrent pneumonias. Her creatine phosphokinase, lactate dehydrogenase and aldolase were all elevated four to ten fold on different occasions. There was no family history of any neurological or muscle disorders. Bailey *et al.* (1986) also reported the death of four individuals, all with onset in infancy,

before the age of 20. Sporadic and familial cases occur, but on average the sporadic cases seem to be more severely affected than the familial cases (Brouwer *et al.*, 1994). Brouwer *et al.* (1995) concluded that infantile FSHD does not differ clinically, or genetically, from adult onset FSHD.

Funakoshi *et al.* (1998) examined 140 Japanese FSHD patients from 91 unrelated families, of whom twenty patients were classified as early onset FSHD. Nine of the twenty patients had small (10-11 kb) *Eco* RI fragments, and a high frequency of epilepsy (44%) and mental retardation (89%) was observed. These authors concluded that FSHD patients with a large deletion in the FSHD region tend to have a higher chance of clinical phenotypes being associated with central nervous system (CNS) abnormalities. Okinaga *et al.* (1997) also reported the presence of two early-onset FSHD cases in the Japanese population. Both children were observed to sleep with their eyes slightly open and demonstrated an inability to smile before the age of one. FSHD was confirmed on the molecular level in these two children by the presence of deletion fragments (13 kb and a 15 kb).

2.1.1.3 Current treatments for FSHD

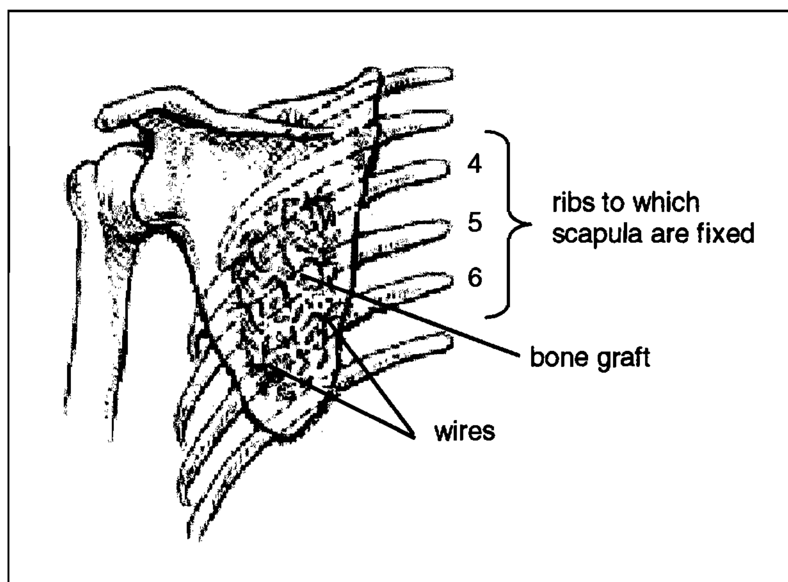
Individuals affected with any form of muscular dystrophy should consider a few general principles, which could assist them in adapting to the muscle weakness and to continue their everyday activities. Physical therapy, including light exercise, helps preserve flexibility and prevents contractures that result from immobility. Swimming is the preferred form of light exercise as it puts little strain on the muscles. A good diet is necessary to avoid unnecessary weight gain and reduces stress on the already weakened muscles.

Currently there is no treatment or cure for FSHD, but there are aids that can provide symptomatic relief. Individuals affected with FSHD have a relatively normal life expectancy and therefore sometimes require procedures that will provide prolonged relief.

In a procedure known as scapulothoracic arthrodesis, the scapula of some FSHD individuals has been fixed to the thoracic wall, to improve stability for activities involving the upper limbs. Bunch *et al.* (1993) fixed the scapula to the ribs in twelve FSHD patients and did re-examinations from three to twenty one years after the procedure. Instability of the scapula due to weakness of the muscles that stabilise the scapula results in an inability

to raise the arms forward or above the head. This group of FSHD patients did, however, have sufficient strength in the supraspinatus and deltoid muscle to abduct the arm if the scapula had been stabilised. Approximately 50% of the individuals examined by Bunch *et al.* (1993) had preserved deltoid function. The scapula is positioned over the seventh rib and fixation to ribs four, five and six is generally adequate, but as many as five ribs have been used as depicted in Figure 2.5. Holes are drilled through the scapula, and two one-millimetre-diameter stainless-steel wires are placed under each rib and through the drill-holes in the scapula. The wires are pulled tightly to compress the scapula against the graft. The resistance of the tightened wires secures the position of the scapula. The wire-ends are bent and buried within the infraspinatus muscles.

Figure 2.5: Scapular fixation in FSHD



Adapted from Bunch *et al.* (1993).

A solid fusion between the ribs and scapula is necessary, as the wire loops do not provide a rigid enough fixation, as they can slide on the ribs. Some form of external support is therefore required to ensure solid fusion. For example, a shoulder spica, which holds the arm abducted in a salute position for two months. Subsequently the arm should be placed in a figure-of-eight dressing until radiographs show a solid fusion. The twelve patients described by Bunch *et al.* (1993) all obtained solid fusion, and all of them experienced a more stable shoulder while they were carrying and lifting objects. All but one patient was capable of forward flexion and abduction to 90 degrees or more. These patients had an average of 30 degree flexion preoperatively, which increased to 65-125 degrees postoperatively. Bunch *et al.* (1993) favour this method as it is technically relatively easy and is associated with few complications.

Andrews *et al.* (1998) also performed scapulothoracic arthrodesis in six FSHD patients. In general the range of abduction and flexion increased and all the patients could continue their work after recovery. The scapula was clinically and radiologically fused to the chest wall in all the patients. Scapulothoracic arthrodesis was also performed by Letournel *et al.*

(1990) in fifteen patients. Flexion increased on average by 33 degrees and abduction by 25 degrees and, upon a sixty-nine month follow-up, the results had not deteriorated. Scapulothoracic arthrodesis offers a good long-term benefit to FSHD patients and also contributes to an increase in quality of life.

Steroids and other anti-inflammatory drugs are generally reserved for the treatment of the inflammatory myopathies, eg. polymyositis. There is, however, not a large amount of literature available on the use of steroids in FSHD. Munsat *et al.* (1972) found that creatine kinase (CK) levels dropped and symptoms improved in a small number of FSHD patients. In a more recent study by Tawil *et al.* (1997), eight FSHD patients were treated with prednisone for 12 weeks. There were, however, no significant changes in the muscle strength or mass. It was therefore concluded that treatment with prednisone had no benefit for FSHD patients, however, the effect on disease progression could not be evaluated over this short period of time.

Kissel *et al.* (1998) investigated the effect of albuterol, a β_2-agonist, in FSHD patients. β_2-agonists have been shown to induce satellite cell proliferation, increase muscle protein production, inhibit muscle proteolysis and retard the loss of muscle mass due to muscle injury, denervation, disuse, steroid atrophy, malnutrition, tumor, sepsis, and surgery. Kissel *et al.* (1998) treated fifteen FSHD patients for three months and reported improved muscle mass and overall improvement of 12% in strength. The results were encouraging and the effects of albuterol were further evaluated in a larger, randomised, double-blind, placebo-controlled trial by these authors. From this study, Kissel *et al.* (2001) reported that although treatment with albuterol for one year did not improve global strength or function in patients affected with FSHD, a significant increase in muscle mass and grip strength was observed. This indicated that albuterol does have some anabolic effect in the treated patients. The authors are, however, not sure as to why the increase in muscle mass did not translate into increased strength.

Bushby *et al.* (1998) reported four patients with FSHD in whom pain was the most distinctive symptom. Treatment of the pain was difficult, since there was a poor response to conventional anti-inflammatory therapy and eventually morphine was prescribed for one of the patients. The other three patients experienced improvement from swimming. All patients complained of a feeling of frustration together with depression and irritability. Two of the patients were treated with antidepressants and an improvement in their mood and partial improvement in their pain control was reported.

2.1.2 GENETIC ASPECTS OF FSHD

FSHD is an autosomal dominant, neuromuscular disorder and has a prevalence of 1 in 20,000 individuals in the European population and a prevalence of 1 in 14,763 individuals in Utah, U.S.A. (Walton, 1955; 1956; Lunt 1989a; Flanigan *et al.,* 2001). A higher prevalence was reported for the Utah population, as the entire geographical region contains only one muscular dystrophy referral centre, which examines almost all the reported cases within this region. It is also known that the Utah population consists of very large families. A relatively high proportion of new mutations (10 to 30%) have also been reported in FSHD (Padberg 1982; Padberg *et al.,* 1995b; Zatz *et al.,* 1995; Tawil *et al.,* 1996; Lunt 1998; Zatz *et al.,* 1998).

2.1.2.1 Linkage of FSHD to chromosome 4q35

Possible linkage was observed for the Gm locus encoding the constant region of the heavy chains of the IgG immunoglobulins (Padberg *et al.,* 1984). Gm was known to be located on the long arm of chromosome 14 (near 14q32). A subsequent study by Padberg in 1988, utilising DNA-probe D14S1, confirmed the linkage between Gm and D14S1, but no linkage was observed between FSHD and the D14S1 loci. This excluded the FSHD locus from the distal part of chromosome 14.

An international collaboration of research groups involved in the linkage analysis of FSHD was organised to reduce unnecessary duplication through the pooling of linkage data and to construct an exclusion map. Four groups consisting of 20 participants contributed linkage data: Padberg and Frants (Leiden); Upadhyaya, Sarfarazi, Lunt and Noades (Cardiff, Manchester, and London); Lucotte (Paris), and Pericak-Vance, Siddique and Shaw (Durham, NC and Manchester). A total of 57 markers on various autosomes were tested for possible linkage to FSHD, but no significant linkage between any marker and the disorder was detected (Lucotte *et al.,* 1989; Lunt 1989b; Sarfarazi *et al.,* 1989; Siddique *et al.,* 1989; Upadhyaya *et al.,* 1989). The overall data indicated that the likelihood for the location of the FSHD gene to be on chromosome 11 was 6.47 times more than being on any other chromosome. Other possible chromosomes were 19 (likelihood of 2.67) and 5 (likelihood of 2.22). Insufficient data was available for chromosomes 3, 10 and 15 and therefore remained unexcluded. Eventually participants of the international consortium

excluded almost 80% of the genome (Sarfarazi *et al.,* 1989). Jacobsen *et al.* (1990) excluded chromosomes 1, 2, 5, 7, 10 and 16 by using a panel of restriction fragment length polymorphism (RFLP) markers that were evenly spaced at approximately 20 centimorgan (cM) intervals.

Linkage was subsequently established in 1990 between the FSHD phenotype and the Mfd22 short tandem repeat polymorphism (STRP) marker at locus D4S171 in the sub-telomeric region of chromosome 4q35 (Wijmenga *et al.*, 1990). Ten multigeneration Dutch families consisting of 69 affected, 58 unaffected sibs and 25 spouses were investigated. Sixty microsatellite loci were analysed for linkage to FSHD. A maximum lod score of 6.34, at a theta value of 0.13, was observed for marker Mfd22. Only one of the ten families was uninformative for this marker. The microsatellite marker Mfd22 had previously been assigned to chromosome 4, with the use of a somatic cell hybrid panel (Weber and May, 1990).

Upadhyaya *et al.* (1990) confirmed the location of the FSHD locus to the subtelomeric region of chromosome 4, through the identification of a flanking marker, more closely linked to the FSHD locus. This variable number of tandem repeat (VNTR) marker pH30, at the D4S139 locus, was demonstrated to be tightly linked to the FSHD locus. The D4S139 locus was mapped to the distal portion of the long arm of chromosome 4 by *in situ* hybridisation (Milner *et al.*, 1989). Wijmenga *et al.* (1991) also mapped D4S139 to chromosome 4q35-qter by *in situ* hybridisation. It was thus possible to construct a primary map of the area surrounding the FSHD locus with D4S171 and D4S139, to estimate the most likely position of the gene. Several recombination events in ten families favoured the following locus order: D4S171-D4S139-FSHD-TEL. This paved the way for presymptomatic and prenatal diagnosis in an independent linkage panel of 24 families with FSHD from Great Britain that were also reported to be closely linked to the D4S139 locus (Upadhyaya *et al.,* 1991).

Members of the International FSHD Consortium for linkage analysis of the FSHD gene have pooled data in an attempt to determine the precise location of the FSHD gene as well as the order of four DNA markers on 4q35. Two additional markers (F11 and D4S163) to those used by Wijmenga in 1991 were utilised to form a linkage group consisting of four polymorphic loci in the area of the FSHD locus (D4S171, F11, D4S163 and D4S139) and covering a total distance of ca. 20 cM. Recombinants have been identified with these four

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markers, which allowed the order of the FSHD locus and marker loci to be determined. The most likely locus order and the relative position of the FSHD gene was thus: CEN-D4S171-F11-D4S187-D4S163-D4S139-FSHD-TEL (Gilbert *et al.,* 1992; Mathews *et al.,* 1992; Mills *et al.,* 1992; Sarfarazi *et al.,* 1992; Upadhyaya *et al.,* 1992; Weiffenbach *et al.,* 1992a; Wijmenga *et al.,* 1992a, Winokur *et al.,* 1993). Identification of flanking markers in the 4q35 region contributed towards establishing a reliable diagnostic test for FSHD and will eventually facilitate in achieving the ultimate goal of cloning the FSHD gene.

2.1.2.2 The FSHD locus on chromosome 4q35

Wijmenga *et al.* (1992b) isolated cosmid clone 13E in a search for homeobox genes. The clone was mapped to 4q35, distal to the D4S139 locus through *in situ* hybridisation. Cosmid 13E was subcloned and a 0.8 kb probe, designated p13E-11, was obtained. Probe p13E-11, at locus D4F104S1 (also known as D4S810), was observed to detect a DNA rearrangement in individuals affected with FSHD. Fragment sizes between 14 to 28 kb were observed to segregate in ten Dutch FSHD families upon *Eco* RI digestion. The DNA rearrangements detected by probe p13E-11 were thus identified to play a role in the aetiology of FSHD.

Restriction mapping of cosmid 13E indicated that the *Eco* RI fragment detected by p13E-11 contained 3.3 kb tandem repeats with each 3.3 kb repeat flanked by *Kpn* I sites as illustrated in Figure 2.6 and Table D.1 (Wijmenga *et al.*, 1992b). The human genome contains hundreds of copies of this 3.3 kb family of tandem repeats in regions associated with heterochromatin. Different members of this long interspersed nuclear element (LINE) repeat family have been found on the short arms of all the acrocentric chromosomes, in the heterochromatic regions adjacent to the ribosomal DNA gene clusters, and on chromosomes 1, 2, 3, 4, 10, 18 and Y (Lyle *et al.,* 1995; Winokur *et al.,* 1994; Winokur *et al.,* 1996).

Hewitt *et al.* (1994) determined the sequence of the 3.3 kb repeat units and reported the presence of a double homeobox, and two repetitive sequences, namely *Lsau* and *hhspm3* within each repeat, as illustrated in Figure 2.6 and Table D.1. *Lsau* is a middle repetitive 68% GC rich element associated with β satellite DNA and found in heterochromatic regions of the genome while *hhspm3* is a low copy GC rich repeat element. Lee *et al.*

YACs were digested with several common and rare cutter restriction enzymes for the production of a fine restriction map around the locus D4S104S1, orientating the rearranged fragment detected by the probe p13E-11. Wijmenga *et al.* (1993a) and Weiffenbach *et al.* (1994) determined the order of the loci around the D4S104S1 locus as: CEN-D4F35S1-D4S1101-D4S104S1-D4Z4-TEL. The homeobox probe 9B6A was identified to map to each copy of the tandem repeat, therefore confirming the presence of a homeobox sequence in each copy of the repeat motif.

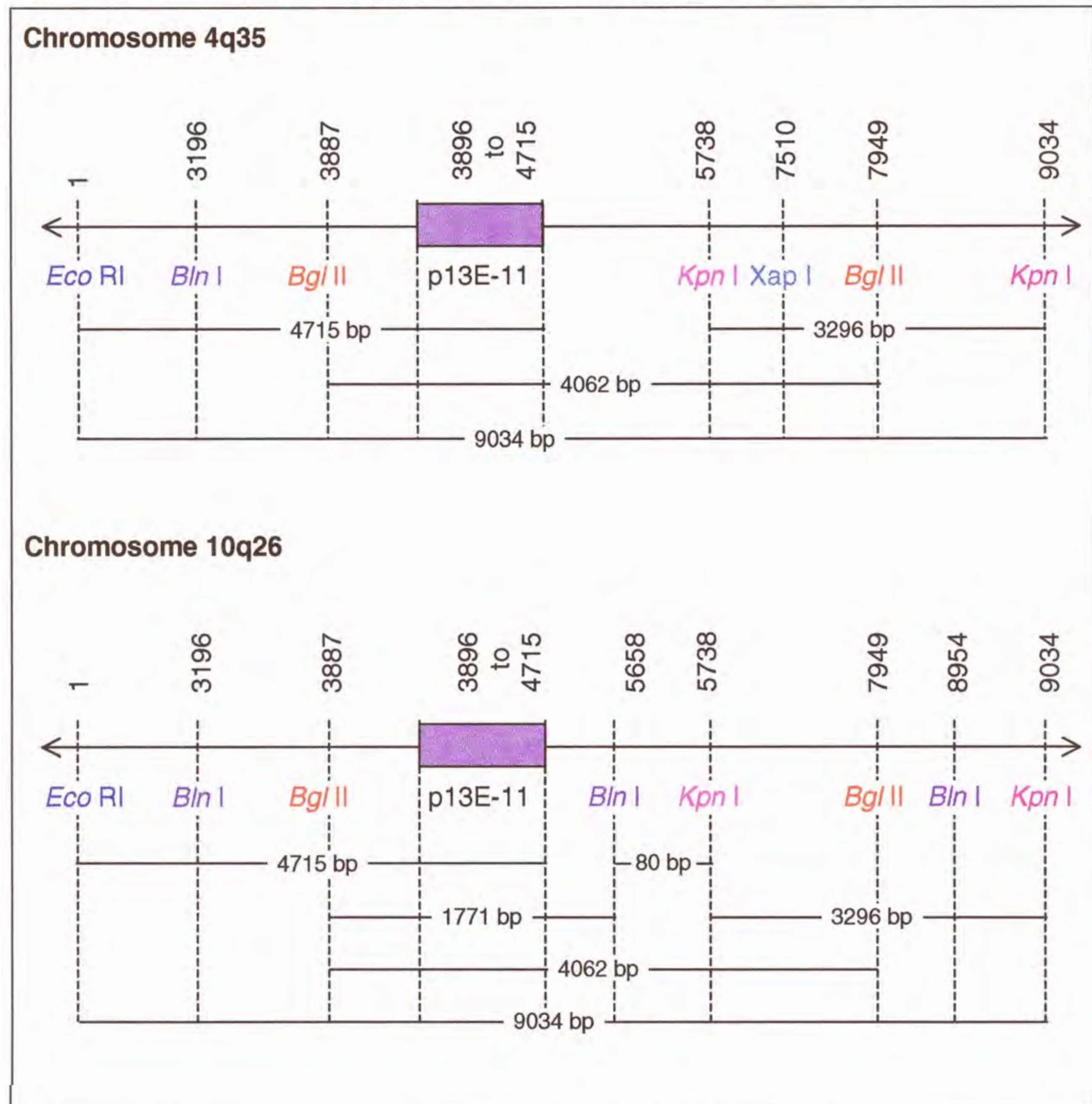
A decrease in the number of 3.3 kb tandem repeats was detected in individuals with FSHD (Van Deutekom *et al.*, 1993). The variability in fragment size was caused by the deletion of an integral number of the repeats. This was in contrast to three other muscular dystrophies, X-linked spinal and bulbar muscular atrophy, oculopharyngeal muscular dystrophy and myotonic dystrophy, in which an expansion of unstable repeats are the cause of the disorder (Lieberman and Fischbeck, 2000). FSHD negative individuals have 10-100 copies of the chromosome 4 repeat elements, corresponding to 35-300 kb fragments and patients with FSHD have less than 10 copies, thus resulting in deletion fragments which are less than 35 kb in size (Van Deutekom *et al.*, 1993).

Probe p13E-11 (D4F104S1) detects the rearranged *Eco* RI fragments. The interpretation of Southern blot analyses were, however, complicated since probe p13E-11 revealed two polymorphic loci as well as a 10 kb Y-specific fragment due to cross-hybridisation (Wijmenga *et al.*, 1993b and Weiffenbach *et al.*, 1993). Haplotype analysis could assign one of the loci to chromosome 4q35, but the location of the second locus remained unknown. Potential candidate regions were identified upon cross hybridisation of cosmid 13E using FISH, and included chromosomes 1q and 10q26, in addition to satellites of all the acrocentric chromosomes. The non-4q35 polymorphic fragment was subsequently shown to segregate with 10q telomeric microsatellite markers (Bakker *et al.*, 1995).

Deidda *et al.* (1995) also cloned a 13 kb non-4q35 fragment segregating in an FSHD Italian family and confirmed the localisation of the fragment to 10q26 via haplotype and *in situ* hybridisation. Restriction mapping of this region indicated that the 10q26 region contains a similar arrangement of *Kpn* I tandemly repeated units and flanking sequences than the FSHD region on 4q35. Comparison of sequences between the 4q35 and 10q26 fragments (Figure 2.7 and Table E.1), identified the presence of a unique chromosome 10 specific *Bln* I site, 80 nucleotides upstream of the *Kpn* I site (Deidda *et al.*, 1996).

Chromosome 10 derived repeat units are sensitive to *Bln* I, thus allowing *Bln* I to discriminate between chromosome 4 and chromosome 10 alleles. Cacurri *et al.* (1998) compared a total of 4 kb of the 4q35 sequences with the 10qter sequences and found the degree of sequence homology to be 98 to 100% between these two regions.

Figure 2.7: Restriction endonuclease maps of the 4q35 and 10q26 regions



This figure is not drawn to scale. Colours and nucleotide numbers correspond with those used to identify specific sequences in Table D.1 and Table E.1.

Upadhyaya *et al.* (1997) screened 200 control and 130 FSHD patients and indicated that double digestion with *Eco* RI and *Bln* I has a sensitivity of 95% and is therefore a valuable diagnostic test for FSHD. Orrell *et al.* (1999) also investigated 82 unrelated FSHD cases

to determine whether the *Eco* RI and *Bln* I double digestion would improve the molecular diagnosis of FSHD. Utilising the double digestion method a definitive molecular diagnosis could be made in all the affected individuals investigated. Orrell *et al.* (1999) therefore concluded that the combination of double digestion with *Eco* RI and *Bln* I, followed by pulsed field gel electrophoresis (PFGE) is the most reliable protocol for the molecular diagnosis of FSHD individuals.

Lemmers *et al.* (2000) identified a *Xap* I restriction endonuclease site on chromosome 4q35 within each 3.3 kb repeat unit (Figure 2.7; Table D.1 and Table E.1). *Xap* I displayed the opposite characteristic of *Bln* I, by uniquely digesting 4-type repeat units, leaving the 10-type units undigested. The combination of *Eco* RI, *Eco* RI/*Bln* I and *Eco* RI/*Xap* I digestions therefore allows the characterisation of each allele, even after translocation events (paragraph 2.1.2.2.1) between 4-type and 10-type repeats has occurred. The combination of these restriction endonucleases thus represents an optimal strategy for diagnosis of FSHD on the molecular level.

2.1.2.2.1 Translocation events between chromosomes 4q and 10q

Translocation events between the repeats on chromosome 4 and chromosome 10 could be demonstrated via the presence of *Bln* I sites within the *Kpn* I repeats from 10q26. The presence of 10-type repeats on chromosome 4, and *vice versa*, was observed in 160 independent Dutch familial or isolated cases (Van Deutekom *et al.*, 1996a). The frequency of these exchanges was determined via the screening of 50 unrelated control samples. A relatively high translocation frequency of 20% was observed, suggesting that it is unlikely that the FSHD gene resides within the repeated units at chromosome 4q35. Only short repeat arrays on chromosome 4 are pathogenic, irrespective of the origin of the repeat unit. A short array of 4-type or 10-type repeats on chromosome 4 therefore causes FSHD. Short repeat arrays located on chromosome 10 are, however, nonpathogenic (Cacurri *et al.*, 1998, Lemmers *et al.*, 1998).

Van der Maarel *et al.* (2000) indicated a numerical excess of 4-type repeats on chromosome 10. It was postulated that this excess is a significant, if not the major predisposing factor that gives rise to the FSHD-type deletion. A further hypothesis was formulated which stated that the FSHD deletion occurs mainly via somatic

interchromosomal gene conversion, in which the presence of a fully homologous repeat array on a non-homologous chromosome is a predisposing factor.

Van Overveld *et al.* (2000) examined the 3.3 kb D4Z4 repeat array configurations on chromosome 4 and its homologue on chromosome 10 to study the behaviour of these subtelomeric domains. PFGE was performed in 208 Dutch blood donors, and the alleles were assigned to their chromosomal origin based on their *Bln* I sensitivity and/or resistance. A standard pattern (4-type arrays on chromosome 4 and 10-type arrays on chromosome 10) was observed in 76% of the individuals. A non-standard configuration due to translocated repeat arrays has been observed in 21% of the individuals. Four-type repeat arrays on chromosome 10 occur as frequently (12%) as the reverse configuration (9%) of 10-type arrays on chromosome 4, but the composition of these translocated arrays differs significantly (Table 2.2).

Table 2.2: Configuration and composition of translocated arrays

Configuration of arrays			
76%	Standard configuration: 4-type arrays on chromosome 4 and 10-type arrays on chromosome 10		
21%	Non-standard configuration		
3%	Unpredictable configuration		
Composition of arrays			
10-type repeat arrays on chromosome 4		**4-type repeat arrays on chromosome 10**	
Homogeneous	Heterogeneous (hybrid arrays)	Homogeneous	Heterogeneous (hybrid arrays)
17%	33%	44%	10%

Adapted from Van Overveld *et al.* (2000).

The 10-type repeat arrays on chromosome 4 were more heterogeneous and the 4-type arrays on chromosome 10 were more homogeneous. This suggested a biological difference between these homologous repeat arrays, resulting in a preference of 4-type repeat arrays on chromosome 4 (Van Overveld *et al.*, 2000). It is known that repetitive sequences in the genome undergo concerted evolution. This process homogenises repetitive sequences and is thought to be important for the maintenance and integrity of each repeat unit. However, the high level of heterogeneity observed for the repeat units at the D4Z4 locus suggests that these loci escaped concerted evolution and evolved by inter- and intrachromosomal recombination. This may imply that the open reading frame,

present in each repeat unit, may have lost its function due to the rapid expansion of this repeat array (refer to paragraph 2.1.2.10.3).

An unexpected high frequency (3%) of 4-type repeat arrays shorter than 38 kb was also observed in this control population. These individuals were not included with the previously described 76% that harboured a classical standard configuration. If these short fragments reside on chromosome 4, they should be associated with FSHD. The authors proposed several possibilities for this unexpected finding:

- It is possible that the short array does not reside on chromosome 4. As reported by Van Deutekom *et al.* (1996a) translocations occur between chromosomes 4 and 10 in 20% of the Dutch population. The presence of 'double exchanged' alleles is therefore predicted to be ca. 1%.
- One of the individuals carried three 4-type repeat arrays. It is therefore possible that the short array may reside on chromosome 10.
- 30% of gene carriers are asymptomatic, and a correlation has been established between the residual repeat size and the age of onset and severity of FSHD. The array in three of the individuals was larger than 30 kb, and these individuals could therefore have subclinical characteristics or display non-penetrance.

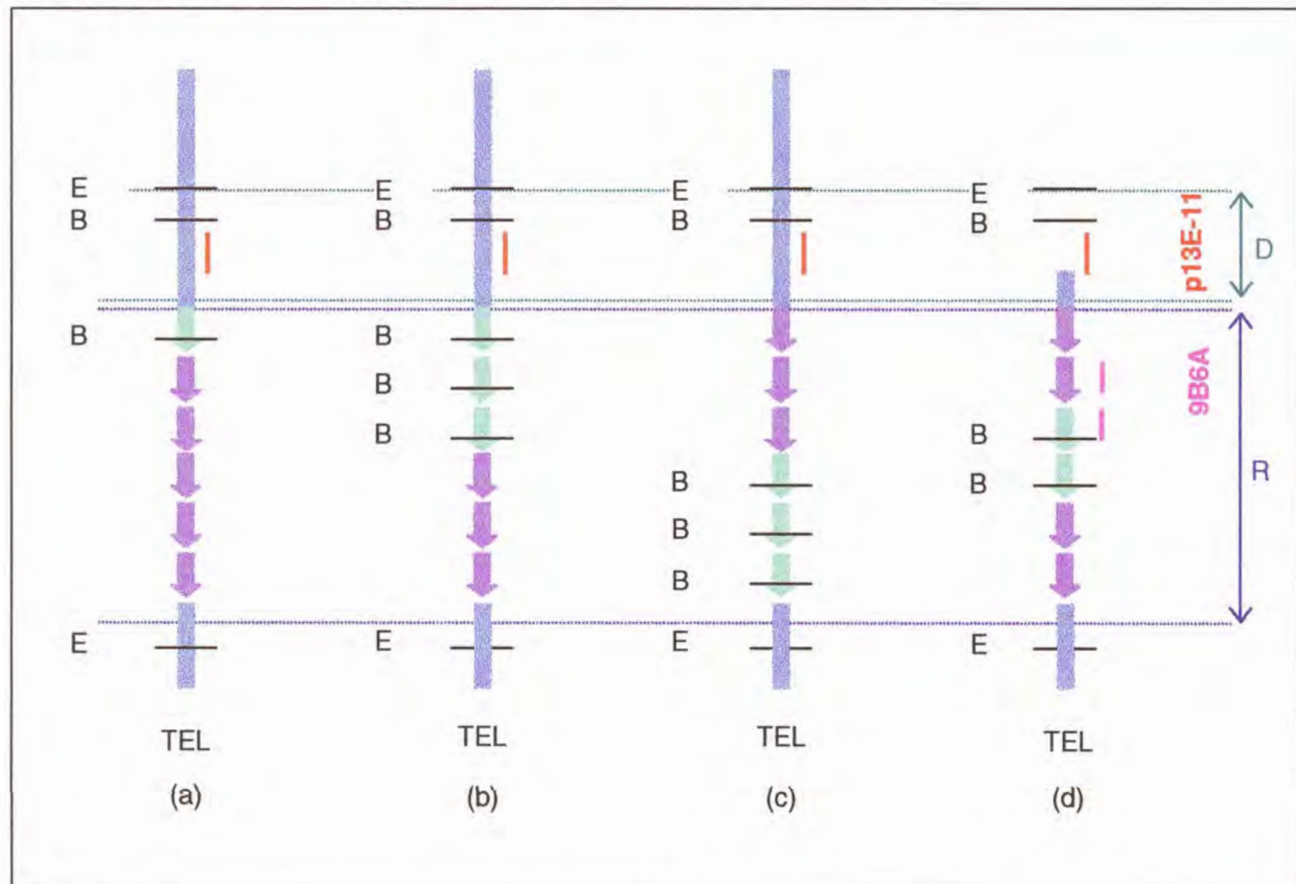
The presence of interchromosomal exchanges between the repeats on chromosome 4 and chromosome 10 has implications for both the specificity and sensitivity of diagnostic DNA testing of FSHD. The high frequency of 20% observed in the Dutch population therefore suggested the need to determine the percentage of interchromosomal exchanges in other populations as well.

<u>2.1.2.2.2 Hybrid repeat arrays and deletion of p13E-11 hybridisation site</u>

The molecular diagnosis of FSHD was further complicated by the identification of a deletion of the p13E-11 hybridisation site and hybrid fragments, comprising repeats of both chromosome 4 and 10 (Van Deutekom *et al.*, 1996a; Lemmers *et al.*, 1998). This was observed after hybridisation with probe 9B6A, which is complimentary to the repeat unit (D4Z4) itself. Individuals who have a deletion of the p13E-11 site will appear to have no small fragment on 4q35 when hybridised with p13E-11 as illustrated in Figure 2.8(d). Upon PFGE only 3 fragments will be observed, the fourth fragment will be visible after hybridisation with marker 9B6A, since marker 9B6A hybridises to the homeobox sequences within each repeat unit.

Hybrid repeat arrays, consisting of 10-type repeats attached to the distal end of a 4-type repeat array as depicted in Figure 2.8(c), could lead to a false positive diagnosis. The *Eco* RI/*Bln* I double digest will yield a small *Bln* I resistant fragment, but this will only correspond to the length of the 4-type repeats. The 10-type repeats will be digested with *Bln* I. On the other hand, hybrid repeat arrays, consisting of 4-type repeats attached to the distal end of a 10-type repeat array as displayed in Figure 2.8(a, b), could lead to false negative diagnosis. The *Eco* RI/*Bln* I double digest will show no deletion fragment, since the *Bln* I site within each chromosome 10-type repeat will lead to the digestion of these repeats. PFGE and the hybridisation with probe 9B6A will be able to detect the 4-type repeats on the distal end. To minimise the risk of false negative diagnosis due to hybrid repeat arrays or deletion of the p13E-11 recognition site the utilisation of PFGE, probe p13E-11 and probe 9B6A is essential in the molecular diagnosis of FSHD.

Figure 2.8: Schematic representation of hybrid chromosomes



Purple arrows represent 4-type repeat units, green arrows represent 10-type repeat units, a = Hybrid repeat arrays consisting of 4-type repeats attached to the distal end of a 10-type repeat array, b = Hybrid repeat arrays consisting of 4-type repeats attached to the distal end of 10-type repeat arrays, c = hybrid repeat arrays consisting of 10-type repeats attached to the distal end of a 4-type repeat arrays, d = hybrid repeat arrays with a deletion of the p13E-11 hybridisation site, B = *Bln* I restriction sites, D = region distal to repeat arrays, E = *Eco* RI restriction site, R = repeat units, TEL = telomere. The probe recognition sites for probe p13E-11 and probe 9B6A are indicated. Adapted from Lemmers *et al.* (1998).

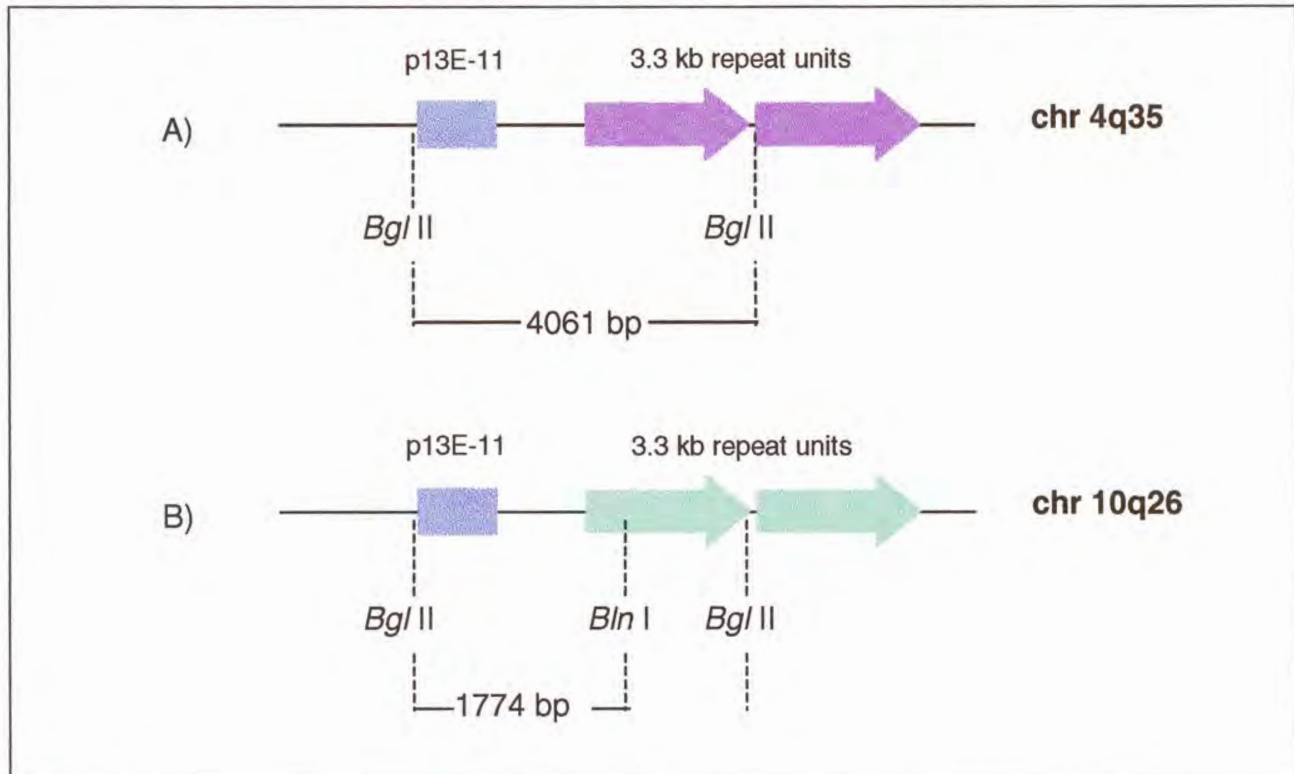
2.1.2.2.3 The *Bgl* II – *Bln* I dosage test

The diagnosis of FSHD with PFGE therefore has the advantage that all four alleles, as well as the presence of translocations, are visualised. The success of PFGE relies, however, on the quality of the aqueous DNA used and it is often difficult to detect alleles exceeding 200 kb. Also, many diagnostic laboratories do not have the facilities, or the expertise, to successfully carry out the PFGE analyses. Van der Maarel *et al.* (1999) therefore developed the *Bgl* II-*Bln* I dosage test (Figure 2.9) to provide a protocol to determine the ratio of 4-type to 10-type repeats. Deletions of the p13E-11 site (as described in paragraph 2.1.2.2.2) are also detected via the dosage test.

The *Bgl* II – *Bln* I dosage test utilises the *Bln* I polymorphism in the first D4Z4 repeat, and *Bgl* II instead of *Eco* RI to obtain a smaller fragment, to discriminate between chromosome 4 and chromosome 10 repeat units. A double digestion with *Bgl* II and *Bln* I will result in a 4,061 bp chromosome 4 specific fragment and a 1,774 bp chromosome 10 specific fragment, due to the extra *Bln* I site on chromosome 10. After hybridisation with p13E-11 the ratios between the signal intensities from chromosome 4 and 10 fragments should be equal (2:2) in the absence of translocation events. Individuals who carry one or three 4-type repeat arrays due to a translocation event should have ratios of 1:3 and 3:1 respectively. Individuals with only 4-type repeat arrays will show no hybridisation for the chromosome 10 fragment.

The dosage test will, however, fall short if the translocation between chromosome 4 and 10 occurred distal to the first polymorphic *Bln* I site or if an individual carries a complex rearrangement resulting in a hybrid repeat array (consisting of both chromosome 4 and 10 repeat units). Diagnosis of FSHD with PFGE using *Bln* I has a sensitivity of 96.5% and a specificity of >99%. In contrast, the sensitivity of the diagnosis utilising the conventional linear gel electrophoresis is 92% with a specificity of 99% (Van der Maarel *et al.*, 1999). If the dosage test is added to the conventional diagnosis the sensitivity and specificity will be improved to that of the PFGE diagnosis. The *Bgl* II – *Bln* I dosage test can also be utilised to study translocation events in large populations. Moreover it can be used in addition to the PFGE diagnosis to prevent false identification of deletions of the p13E-11 region due to the difficult visualisation of large fragment sizes (>200 kb) as a result of poor genomic DNA (gDNA) quality brought about by shearing of the gDNA prior to PFGE.

Figure 2.9: Schematic representation of the *Bgl* II – *Bln* I dosage test



A = Repeat array on chromosome 4, B = Repeat array on chromosome 10. Adapted from Van der Maarel *et al.* (1999).

Van der Maarel *et al.* (1999) observed via PFGE that the entire repeat array was translocated to the non-homologous chromosome in most of their cases. Only a small group of the translocations resulted in hybrid repeat units. The utilisation of the *Bgl* II-*Bln* I dosage test led to the conclusion that the translocations between chromosome 4 and 10 must have occurred proximal to the polymorphic *Bln* I site within the first repeat unit, in a recombination hotspot. The exact localisation of this translocation breakpoint proximal to the repeat arrays has implications for the localisation of the putative FSHD gene by narrowing down the candidate gene region. These authors also mentioned that recombination hotspots co-localise with open chromatin domains in yeast, such as promoter or coding sequences. A similar mechanism may play a role in recombination in vertebrates and the putative recombination hotspot proximal to the D4Z4 array may indeed indicate a new FSHD candidate gene locus.

2.1.2.3 Somatic and germline mosaicism

Somatic mosaicism is indicated by a fifth fragment upon PFGE analysis and hybridisation with probe p13E-11. This phenomenon has been reported by many authors (Griggs *et al.*, 1993; Weiffenbach *et al.*, 1993; Bakker *et al.*, 1995; Upadhyaya *et al.*, 1995; Zatz *et al.*,

1995; Köhler *et al.*, 1996; Bakker *et al.*, 1996;). Van der Maarel *et al.* (2000) investigated 35 sporadic FSHD families for which the chromosomal origin and size of each of the four repeat arrays (chromosomes 4 and 10) in both patients and parents could be determined for 23 families. The remaining 12 families had incomplete information and the authors stated that the "DNA quality was not sufficient" to assign all the alleles for one of the individuals. All the patients had a *Bln* I resistant deletion fragment of <35 kb (9 repeats), confirming their FSHD diagnosis. The deletion fragments ranged from 8 kb (1 repeat unit and flanking sequences) to 25 kb (6 repeats). One patient, however, inherited a 25 kb *Bln* I resistant deletion fragment from his clinically asymptomatic father and this is therefore a familial and not a sporadic case.

Van der Maarel *et al.* (2000) observed 14 cases of somatic mosaicism. Mosaicism for the disease allele was observed in three of the unaffected parents (two mothers and one father) from the 23 fully informative families. Five patients of the 23 families (4 males and 1 female) were mosaic. In the 12 families that had incomplete information, mosaicism was observed in one father, one mother, and four patients (3 males and 1 female). In 73% of mosaic individuals, the smallest D4Z4 allele was reduced to an FSHD-sized deletion fragment. Somatic mosaicism was therefore observed in 40% of cases, either in the patient or in an asymptomatic parent. The degree of mosaicism could, however, be much more, since complete allele information of only 23 of the 35 *de novo* families was available. It was therefore postulated that this high degree of somatic mosaicism implies that the deletion event is mainly mitotic.

As described in paragraph 2.1.2.2.1, interchromosomal repeat translocations occur between the homologous repeats from chromosomes 4 and 10. Van der Maarel *et al.* (2000) observed one or more 4-type repeat arrays on chromosome 10 in 46% of the mosaic individuals. This type of repeat array is, however, only present in 10% of the Dutch population (Van Deutekom *et al.*, 1996a). The reverse configuration was also present in 10% of the population but was not observed in the mosaic individuals. In mosaic individuals, the presence of 4-type arrays on chromosome 10, is therefore increased by almost five times. Somatic mosaicism was also observed in 3% of normal control individuals from the Dutch population (4 males and 2 females). One of the mosaic individuals carried an extra 4-type repeat array on chromosome 10 (Van Overveld *et al.*, 2000).

The presence of somatic mosaicism can only be observed upon PFGE, which emphasises the need to utilise PFGE in the diagnosis of FSHD. The presence of somatic mosaicism in FSHD patients might facilitate the elucidation of some aspects of the clinical variability of this disorder.

2.1.2.4 Anticipation

Myotonic dystrophy was the first disorder identified to display anticipation, which is characterised by an earlier onset with increase in the severity of clinical symptoms in subsequent generations. Here, the disease causing mutation is an unstable expansion of the CTG repeat in the myotonin-protein kinase (DMPK) gene on chromosome 19. Anticipation is caused by an increase in the number of trinucleotide repeats. The age of onset and clinical severity correlate directly with the size of the trinucleotide expansion. It has been observed that the size of the expansion can also increase with time resulting in the progression of the disorder from generation to generation (Mak *et al.*, 2001).

Anticipation for FSHD was reported by a few authors (Lunt *et al.*, 1995a; Zatz *et al.*, 1995; Tawil *et al.*, 1996). One shortcoming of all these papers reporting anticipation in their populations was, however, that affected parent-offspring pairs, thus only two generations, from multiple families were studied. Lunt *et al.* (1995a) studied 15 families over two to four generations, while Zatz *et al.* (1995) reported anticipation in onset among 28 parent-offspring pairs in 17 families and Tawil *et al.* (1996) studied 23 parent-offspring pairs from multiple families. Lunt *et al.* (2000) did mention that it was not clear how anticipation would be able to occur with a fixed mutation in each family.

Flanigan *et al.* (2001) investigated the presence of anticipation in 66 parent-offspring pairs and 21 grandparent-parent-child sets from a single family (homogeneous population) originally described by Tyler and Stephens in 1950. Tyler and Stephens (1950) described 1,249 descendants of a man who was originally born in England in 1775, but emigrated to Utah in 1840. Tyler and Stephens (1950) identified 159 affected individuals, either by history or examination, and reported on the examination results of 58 people. Twenty four of the 58 individuals (41%) who were asymptomatic by self-report were affected upon examination, and 13 of 18 individuals (72%) who were 20 years of age were also affected upon examination. This family provides one of the earliest descriptions of the frequency of individuals who are asymptomatic by self-report, but affected by examination. Flanigan *et al.* (2001) re-examined the large kindred reported in 1950 by Tyler and Stephens and

extended the pedigree to include 2,220 individuals. Genetic characterisation of the affected individuals was performed and a 20 kb disease-associated deletion fragment was observed to segregate over 12 meioses, in seven different branches of this extended family. No compelling evidence for anticipation, in either reported age of onset or in disease severity, was, however, observed in this extended family. Additional studies, utilising quantitative muscle testing, historical data, and genotyping will help to elucidate the aspects of anticipation, gender effects, and parent-origin effects in FSHD.

2.1.2.5 Female and male transmission effects

Padberg *et al.* (1995b) observed that a higher proportion of females are generally asymptomatic. Zatz *et al.* (1998) investigated 52 families, consisting of 172 patients (60% males and 40% females). An excess of affected males was observed in the patients examined. This might be explained by a greater proportion of asymptomatic females and a significantly greater number of affected sons than daughters of asymptomatic mothers. The penetrance at the age of 30 was 95% for males but only 69% for females. It was also observed that new mutations occurred more frequently in females than in males among somatic and/or germline mosaic cases. Severely affected cases were more commonly the result of sporadic mutations or mutations transmitted through maternal lines, including mosaic mothers. Males were on average more severely affected than females, and more clinically affected sons were observed in the offspring of asymptomatic mothers.

Van der Maarel *et al.* (2000) observed that mosaic males were typically affected, although mosaic females were more often the unaffected parent of a nonmosaic *de novo* patient. A difference in the clinical presentation of females and males was also observed by Busse *et al.* (2000). A 35 kb *Bln* I resistant deletion fragment was confirmed in an unaffected mother, two unaffected daughters and an affected son. This would imply that the two unaffected sisters have a 50% chance of transmitting the 35 kb disease causing deletion fragment to their offspring and that a son would have a higher risk of being affected than a daughter.

2.1.2.6 Sporadic FSHD

The frequency of sporadic cases was reported to be 0.33 (Zatz *et al.*, 1995). Wijmenga *et al.* (1992c; 1993b) identified the presence of *de novo* DNA rearrangements in FSHD. Within each FSHD family the size of the deletion fragment was constant and displayed

stable inheritance in future generations. The parental origin of the mutation was identified via PFGE and haplotype analysis. Griggs *et al.* (1993) observed eight sporadic patients from seven families displaying novel *Eco* RI rearrangements ranging between 15 kb and 23 kb.

Weiffenbach *et al.* (1993) and Griggs *et al.* (1993) examined affected sibling pairs with clinically unaffected parents. In these families, a small *Eco* RI fragment was observed in both of the affected children but not in the parents. It is highly unlikely that the FSHD-associated fragments of identical size in both of the affected offspring was a result of two independent chromosomal rearrangements. It was concluded that one of the parents should have been mosaic harbouring the FSHD-associated rearrangement in the sperm or ovarian cell lines. Jardine *et al.* (1993) also identified a *de novo* DNA rearrangement in a proband and his two affected children. A 15 kb DNA fragment was detected after *Eco* RI digestion and hybridisation with probe p13E-11 in the proband and his affected children, but was absent in both parents of the proband.

The presence of *de novo* DNA rearrangements was also confirmed by Jardine *et al.* (1994b), Padberg *et al.* (1995b) and Brouwer *et al.* (1994, 1995). As mentioned earlier in paragraph 2.1.2.6, the sporadic early-onset FSHD cases reported by Brouwer *et al.* (1995) were on average more severely affected than the familial cases. This can, however, be due to ascertainment bias as mildly affected sporadic cases might often go undiagnosed. Sporadic cases should, however, only be classified as such if both parents have been examined on a molecular level.

2.1.2.7 Phenotype-Genotype correlation

A direct correlation between the residual repeat length, the age of onset and the severity of FSHD was observed by several authors (Lunt *et al.*, 1995a, Lunt *et al.*, 1995b, Tawil *et al.*, 1996, Lunt, 1998, Ricci *et al.*, 1999). FSHD patients who harbour the smallest alleles of only 10 kb (thus one 3.3 kb repeat) present at a younger age and with a more severe form of the disorder. The phenotype of these patients is often accompanied by mental retardation and epilepsy, as discussed in paragraph 2.1.1.2.1 (Funakoshi *et al.*, 1998; Muira *et al.*, 1998). The presence of anticipation was also reported, as mentioned in paragraph 2.1.2.4. It was observed that either the age of onset or the degree of weakness, in consecutive generations in familial FSHD, becomes more severe. If

anticipation exists in FSHD, its molecular mechanism has not yet been elucidated, and can therefore not be utilised in phenotypic-genotypic correlation studies.

Ricci *et al.* (1999) studied the correlation between the clinical severity and the residual repeat length in 122 FSHD families, including 253 affected and 200 unaffected individuals. The authors utilised the 10-grade clinical severity (CS) scale, ranging from 0.5 to 5.0, to determine the severity of muscle weakness (Table 2.3). Higher scores (3 to 5) were assigned to individuals with pelvic and proximal lower limb muscle involvement, since the weakness of these muscles generally follows after weakness of the facial and shoulder muscles. Individuals with no proximal lower limb involvement will therefore have a CS score of less than three, whereas a CS score of 4.0 to 5.0 will imply that the individuals have severe lower limb involvement.

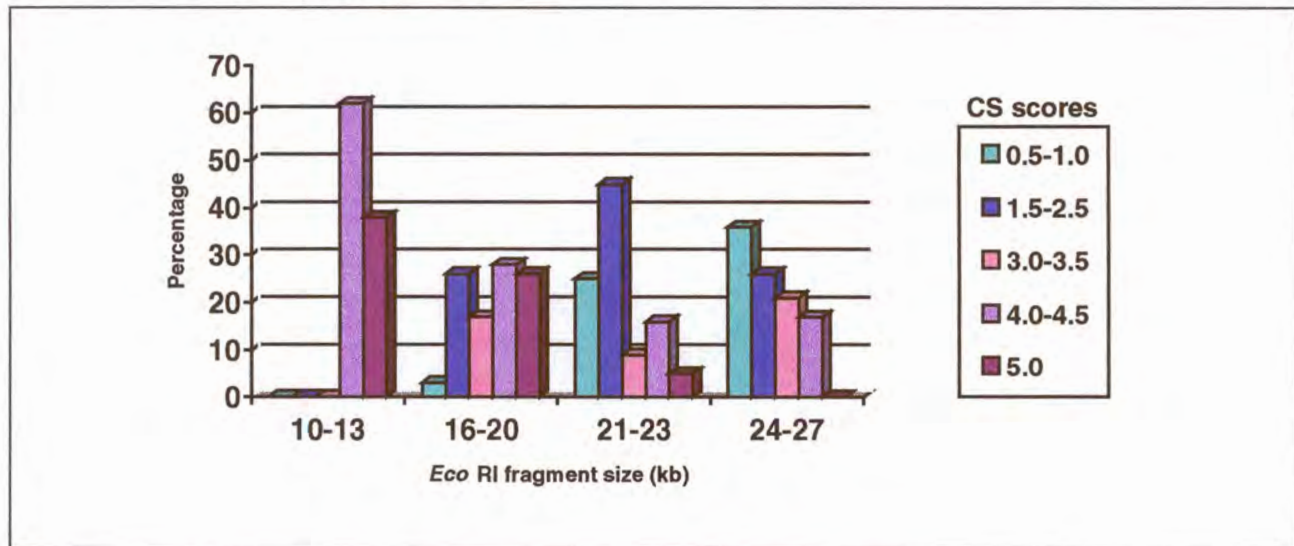
Table 2.3: Clinical Severity Scale for FSHD

Score	Clinical symptom
0.5	Facial weakness
1.0	Mild scapular involvement without limitation of arm abduction; no awareness of disorder symptoms is possible
1.5	Moderate involvement of scapular and arm muscles or both (arm abduction >60° and strength ≥3 in arm muscles); no involvement of pelvic and leg muscles
2.0	Severe scapular involvement (arm abduction <60° on at least one side); strength <3 in at least one muscular district of the arms; no involvement of pelvic and leg muscles
2.5	Tibioperoneal weakness; no weakness of pelvic and proximal leg muscles
3.0	Mild weakness of pelvic and proximal leg muscles or both (strength ≥4 in all these muscles); able to stand up from a chair without support
3.5	Moderate weakness of pelvic and proximal leg muscles or both (strength ≥3 in all these muscles); able to stand up from a chair with monolateral support
4.0	Severe weakness of pelvic and proximal leg muscles or both (strength <3 in at least one of these muscles); able to stand up from a chair with double support; able to walk unaided
4.5	Unable to stand up from a chair; walking limited to several steps with support; may use wheelchair for most activities
5.0	Wheelchair bound

Adapted from Ricci *et al.* (1999).

Graph 2.1 displays the proportion of patients with different CS scores and *Eco* RI deletion fragments. It was observed that the probability of developing a more severe form of the disorder, with a CS score of 4.0 - 5.0, is 100% in the presence of 1 - 2 repeats (10 kb), it decreased to 54% in patients harbouring 3 - 4 repeats (16 to 20 kb) and was less than 21% if the fragments were larger that 20 kb (>4 repeats). The size of the deletion fragment was therefore observed to be a major factor in determining the severity of the FSHD phenotype, thereby having an impact on the clinical prognosis, as well as genetic counselling of FSHD individuals.

Graph 2.1: Proportion of patients with different clinical severity scores and *Eco* RI deletion fragment sizes



Adapted from Ricci *et al.* (1999).

It is interesting to note from the data presented in the above graph that the percentage of severely affected individuals, with CS scores of 4.0 – 5.0, decreases significantly as the *Eco* RI fragment size increases. The percentage of individuals with CS scores of 0.5 to 3.0 increases with the increase of the *Eco* RI fragment size.

2.1.2.8 Prenatal diagnosis

Even though the gene for FSHD has not yet been identified, the detection of rearrangements at the D4Z4 locus with probe p13E-11 provides a reliable indirect method for diagnostic purposes. This therefore enables the prenatal diagnosis of FSHD.

Eggers *et al.* (1993) compiled a questionnaire about the interest in and demand for prenatal and presymptomatic diagnosis for FSHD. The questionnaire was sent to 46 patients and most of the patients indicated that they would have liked to know their diagnosis earlier to enable them to seek more efficient help, to prepare them emotionally, to avoid strenuous activities, or to choose an appropriate profession. Most of the patients also favoured prenatal diagnosis although only two indicated that they would terminate a pregnancy in the case of an affected fetus.

Upadhyaya *et al.* (1999) reported that their laboratory has been involved in the prenatal diagnosis of FSHD since 1993. Several difficulties were encountered during their study

including quality and quantity of DNA needed for the molecular diagnoses. At least 10 µg of high quality, high molecular weight DNA was required to allow good resolution, visualisation, sizing of DNA fragments up to at least 50 kb, and to avoid long autoradiographic exposure times. To obtain the quantity of DNA needed, a sufficient amount of chorion villus tissue had to be biopsied. Upadhyaya *et al.* (1999) also suggested that by completing the molecular diagnosis of an FSHD family prior to the pregnancy can improve the prenatal diagnosis as the molecular defect of the family will already be known and this will decrease the time needed for the prenatal diagnosis.

Galluzzi *et al.* (1999) reported the prenatal diagnosis of FSHD in 15 Italian families. The analysis was performed on DNA isolated from chorionic villi samples, collected at 10-11 weeks of gestation. A deletion fragment associated with the disorder was observed in nine of the fetuses. Parents chose to terminate the pregnancies in eight of the nine cases.

2.1.2.9 Genetic heterogeneity

Evidence for genetic heterogeneity has been reported by several authors (Gilbert *et al.*, 1993, Cacurri *et al.*, 1994, Deidda *et al.*, 1994, Bakker *et al.*, 1996). Gilbert *et al.* (1993) observed two FSHD families from the United States with facial weakness, scapular winging, proximal muscle weakness, and myopathic changes on muscle biopsy without inflammatory or mitochondrial pathology. The phenotype observed in these individuals therefore complied with the clinical requirements for the diagnosis of FSHD, but did not display linkage to the 4q35 region. Cacurri *et al.* (1994) investigated 19 Italian families with FSHD of whom two did not link to 4q35.

It is currently estimated that at least 5% of FSHD families do not display linkage to chromosome 4q35, implying genetic heterogeneity. The finding of heterogeneity in FSHD has important implications for genetic counselling, clinical investigations, prenatal diagnosis, and eventually the cloning of the FSHD gene. Investigation of genetic heterogeneity is therefore included as one of the top ten priorities in international FSHD research. Despite the report of genetic heterogeneity in FSHD almost a decade ago, a second locus has yet to be identified.

2.1.2.10 Candidate genes

The identification of genes associated with the chromosome 4q35 region linked to FSHD has been a difficult undertaking, largely due to the homologous regions in the genome, the high density of repeat units and the gene-poor nature of the region. Despite several attempts the gene, or gene(s) whose modification of expression culminates in the FSHD phenotype still eludes identification. The next section will describe the genes so far identified in the FSHD region.

2.1.2.10.1 Actinin-associated LIM protein gene (ALP)

Piétu *et al.* (1996) cloned a novel human specific complementary DNA (cDNA) by differential screening of a human muscle cDNA array with the aim of identifying muscle specific transcripts. The gene was mapped in the 4q34-qter region. A rat homologue was cloned, and the encoded protein was named actinin-associated LIM protein (ALP), because ALP contains a C-terminal LIM (Lin-11/Isl-1/Mec-3) domain and an N-terminal PDZ (Postsynaptic density protein, Disc-large tumor suppressor and the Zonula occludens protein) domain, which interacts with α-actinin-2 in myofiber Z-lines (Xia *et al.*, 1997). The LIM domain is a specific double-zinc finger motif and the PDZ domain is a protein module composed of β-strands and α-helices. The LIM and PDZ domains are both found in a growing variety of proteins and mediate direct protein-protein interactions. The rat sequence was used to assemble the corresponding human expressed sequence tags (ESTs) and mapped the human gene between D4S171 and the Factor XI gene (Xia *et al.*, 1997). No difference was, however, observed in the expression of ALP in the deltoid muscle from FSHD patients and controls. Bouju *et al.* (1999) therefore concluded that ALP is not a candidate gene for FSHD.

2.1.2.10.2 Adenine nucleotide translocator gene (ANT)

The adenine nucleotide translocator (ANT), also called adenine nucleotide translocase or adenosine diphosphate (ADP)/ATP translocator, is the most abundant mitochondrial protein. This protein facilitates the exchange of adenine nucleotides across the mitochondrial inner membrane. It is a 30 kD homodimer and is embedded asymmetrically in the inner membrane of the mitochondria. The dimer forms pores through which ADP can move from the matrix into the cytoplasm (Neckelman *et al.*, 1987). Three isoforms of

ANT cDNA have been isolated: human skeletal muscle (ANT1), human fibroblast cells (ANT2) and human liver (ANT3) [Neckelmann *et al.*, 1987, Houldsworth and Attardi, 1988].

Minoshima *et al.* (1989) and Li *et al.* (1989) originally mapped ANT1 to chromosome 4 and Fan *et al.* (1992) subsequently assigned ANT1 to 4q35 by fluorescent *in situ* hybridisation. Wijmenga *et al.* (1993c) and Haraguchi *et al.* (1993) mapped the ANT1 gene to 4q35 and more specifically to a site proximal to the FSHD gene. No abnormality was, however, observed after analysing the transcripts of ANT1 from several FSHD patients (Haraguchi *et al.*, 1993).

2.1.2.10.3 Double homeobox gene 4 (DUX4)

Ding *et al.* (1998) cloned a 170 amino acid protein (DUX1) containing a double homeodomain and showed that it is expressed in human rhabdomyosarcoma TE671 cells. Subsequently, two new genes containing double homeodomains and 3.3 kb repeats (DUX2 and DUX3) with similar promoters and ORFs were isolated (Gabriëls *et al.*, 1999). These genes were shown to map to the acrocentric chromosomes and are therefore not involved in FSHD (Beckers *et al.*, 2001). Gabriels *et al.* (1999) aligned the sequences of the 3.3 kb repeat units derived from D4Z4 with those derived from DUX1, DUX2 and DUX3 and identified a promoter and open reading frame encompassing the double homeobox in each 3.3 kb repeat unit and termed the putative gene DUX4 (Figure 2.6 and Table D.1). DUX4 encodes a 391 amino acid protein containing 2 homeodomains. *In vitro* transcription and translation of the ORF in a rabbit reticulocyte lysate yielded two products, corresponding to the DUX4 monomer and dimer. It was proposed that each of the 3.3 kb repeat units could therefore harbour a DUX4 gene, which in turn encodes a double homeodomain protein.

The following hypothesis for the role of the DUX4 gene in FSHD was put forward by Gabriels *et al.* (1999): The deletions of 3.3 kb repeat elements at the D4Z4 locus destabilises the heterochromatin allowing expression of the gene in some repeats in some cells. The DUX4 protein is, however, toxic to muscle cells, possibly because of its strong dimerisation potential. This hypothesis would also explain the dominant character of FSHD and the fact that the severity correlates inversely with the residual number of repeats.

Leclercq *et al.* (2001) reported the presence of a 3.3 kb repeat 40 kb centromeric of the D4Z4 locus. This repeat was, however, observed to be inverted and the authors hypothesised that this repeat might function as an enhancer.

DUX4 might therefore play a role in the pathogenesis of FSHD as partial deletion of the number of 3.3 kb repeat units may alter DUX4 expression in FSHD patients. Confirmation of this hypothesis would, however, be challenging, due to expression of the large number of homologous 3.3 kb repeats scattered all over the human genome.

2.1.2.10.4 Fibroblast growth factor (FGF) and FGF receptors

Growth factors have been indicated to play an important role in muscle regeneration or degeneration. For this reason Saito *et al.* (2000) investigated the expression of several growth factors and their receptors in FSHD patients to determine whether there is any association with FSHD pathogenesis. Saito *et al.* (2000) reported one severely affected FSHD patient with an overexpression of fibroblast growth factor (FGF) and FGF receptor 4. The other patients with FSHD, DMD, BMD, and LGMD did not show any overexpression. No significant difference in the expression levels of the platelet-derived growth factor (PDGF) and receptors, fibroblast growth factor receptor 1 (FGF-R1), FGF receptor 3 (FGF-R3), platelet-derived growth factor receptor α (PDGF-Rα), PDGF receptor β (PDGF-Rβ), and heparan sulfate proteoglycan (HSPG) were observed in the entire group of patients examined. Saito *et al.* (2000) hypothesised that the severe clinical phenotype of the FSHD patient investigated might have been due to the overexpression of FGF, which caused excessive fibrosis of the skeletal muscle. The overexpression of FGF and FGF receptor 4 in the severely affected FSHD patient therefore suggests that the above should be investigated in other FSHD patients to determine whether it is a common feature in severely affected FSHD patients.

2.1.2.10.5 The FSHD region gene 1 (FRG1)

Van Deutekom *et al.* (1996b) identified the first functional gene (FRG1) that mapped 100 kb centromeric of the repeat units on chromosome 4q35. A 1.1 kb FRG1 transcript was observed upon Northern blot analysis of adult muscle, lymphocytes, fetal brain, muscle and placenta. No homology to other known genes could be demonstrated. A polymorphism in exon one of this gene was observed and reverse transcriptase

polymerase chain reaction (RT-PCR) from lymphocytes and muscle biopsies from patients and controls indicated that both alleles were transcribed and that there was no evidence for transcription suppression. No differences in FRG1 messenger ribonucleic acid (mRNA) levels in FSHD patients compared to controls were observed. FRG1 can however not be excluded before the disease mechanism has been determined.

Grewal *et al.* (1998) investigated FRG1 on an evolutionary bases through the comparison of the genomic organisation of this gene in two species, the mouse and the Japanese puffer fish (*Fugu rubripes*). Although the puffer fish gene was found to be five times smaller than that of the mouse, it was observed that the intron and exon structure of FRG1 was identical throughout the protein coding region. The authors also reported that FRG1 was homologous in the two nematodes, *Caenorhabditis elegans* and *Brugia malayi*. It was therefore observed that the human FRG1 gene is highly conserved in both vertebrates as well as invertebrates. Furthermore, the comparison of the vertebrate homologues revealed that all the proteins contained a lipocalin sequence motif near the N-terminal. FRG1 may therefore play a role in protein transport as lipocalins are known to be a large family of extracellular proteins, which transport small hydrophobic molecules such as steroids, retinoids and lipids. The FRG1 protein (FRG1P) was also observed to localise in the nucleolus, Cajal bodies and speckles, and this could imply a fundamental role in RNA processing (Van Koningsbruggen *et al.*, 2000; Van Geel, 2001).

2.1.2.10.6 The FSHD region gene 2 (FRG2)

The FRG2 gene was identified by means of *in silico* exon prediction and confirmed in subsequent expression studies found it to map only 37 kb proximal to the D4Z4 repeat array (Van Geel, 2001). The gene consists of four exons and a strong promoter including a 5' TATA and CCAAT box was predicted. It was observed that FRG2 expression is absent in all tissues tested, however, low, but distinct levels of FRG2 expression was observed in differentiating myoblasts from FSHD patients. No expression of FRG2 could be detected in control myoblasts. FRG2 is therefore the first gene on 4qter that was found to be specifically expressed in FSHD cells. It was further observed that FRG2 is expressed in patient and control fibroblasts undergoing forced myogenesis by adenoviral *MyoD* expression, suggesting a muscle-specific role for this gene. *MyoD* plays a unique role in satellite cell activation and differentiation in myofibers. The exact function of FRG2 could not be determined but results indicated that the FRG2 protein is a nuclear protein

involved in myogenesis and that its transcriptional deregulation is related to FSHD. FRG2 therefore remains an important candidate gene for FSHD. (Van Geel, 2001)

2.1.2.10.7 Human beta-tubulin gene (TUBB4Q)

The TUBB4Q gene maps 80 kb proximal to the FSHD associated D4Z4 repeats on chromosome 4q35 (Van Geel *et al.*, 2000). The gene contains four exons, encoding a protein of 434 amino acids. Although the genomic structure shows all functional aspects of a gene, no transcripts could be detected upon RT-PCR analysis on RNA samples from a wide variety of human adult and fetal tissues. No evidence has therefore been generated to prove that expression of TUBB4Q is involved in FSHD (Van Geel, 2001).

2.1.2.11 Molecular models proposed for the aetiology of FSHD

The molecular mechanisms underlying FSHD are still unclear. It was proposed that the tandem repeats at the D4Z4 locus promote non-homologous unequal recombination between regions within the genome showing homology to it, such as the repeats within the telomeric region of 10qter, thus causing the deletions associated with FSHD. Two hypotheses are postulated in paragraphs 2.1.2.11.1 and 2.1.2.11.2 that might explain the unique pathogenesis of FSHD.

2.1.2.11.1 Homeodomain

Homeobox genes play a crucial role in establishing the anterior-posterior axis during embryogenesis in vertebrates and invertebrates. These genes are therefore candidates to determine the differences observed in specific muscle groups in FSHD (Fischbeck and Garbern, 1992). The two homeobox sequences observed in each 3.3 kb repeat are 67 to 68% homologous on a nucleotide level and were 50 to 52% identical in amino acid sequences (Hewitt *et al.*, 1994; Lee *et al.*, 1995b). Open reading frames have been observed within the homeobox sequences, but not through the whole repeat. The two homeodomain sequences were similar to that of the human paired-type homeodomains, (Paired box gene [Pax] 3, Pax 6, and Orthodenticle [Otx] 1), *Xenopus* mesoderm induced homeobox [Hmix], *Drosophila* paired (prd) [HmprD] and the muscle specific homeodomain protein [Mhox]. Proteins coded by homeodomains display sequence-specific DNA binding

and play an important role in the process of transcription as transcription regulation factors. (Lee *et al.*, 1995b)

The human Pax 3 gene is important in muscle differentiation and mutations in this gene cause Waardenburg syndrome types I and III. Mhox is only expressed in muscle tissue and regulated the expression of muscle-specific creatine kinase and other factors, by binding to the upstream regulatory sequences of the muscle creatine kinase gene (Lee *et al.*, 1995a).

The homeobox sequences within the repeat units provided evidence for a possible gene within the repeat unit. The homeodomain proteins bind DNA and regulate other genes. If the FSHD gene is located at the D4Z4 locus within the repeat units, a minimum number of the repeats may be necessary for normal gene function and deletions of the repeats could result in the loss of the FSHD gene, thus resulting in the disorder. However, no transcripts have been identified from these homeodomains, making it less likely that the FSHD gene is located within these repeat units.

2.1.2.11.2 Position effect variegation

A position effect is displayed when a change in the level of gene expression is due to a change in the position of the gene relative to its normal chromosomal environment (Kleinjan and Van Heyningen, 1998). The expression of genes is influenced by their position in the genome, thus whether located in heterochromatic or euchromatic regions. Chromosomal rearrangements can lead to the alteration of the gene's environment and may therefore change the expression of the gene which is referred to as a position effect.

Winokur *et al.* (1993) and Hewitt *et al.* (1994) postulated that the deletion of the D4Z4 repeats results in a position effect, by disrupting the local chromosomal structure. Deletions of the repeats could result in the expansion of telomeric heterochromatin into adjacent euchromatin altering the expression of the gene. Position effect variegation (PEV) has been observed in *Drosophila* and yeast and it was shown that physical proximity to the centromere, to the telomere or to other heterochromatic regions may actively suppress gene expression (Fisher and Upadhyaya 1997). The down regulation is presumably as a result of an alteration of the structure of euchromatic domains by the

heterochromatic regions. A gradient effect can be observed as genes that are closer to the rearrangement breakpoint are more severely affected than those further away.

Alternatively, the appropriate expression of the gene could require a heterochromatic environment showing a position effect if moved to an euchromatic region. It has been observed that several *Drosophila* genes require a heterochromatic environment for normal function showing position effects if placed within euchromatin. Locus D4Z4 contains *Lsau* repeats that are usually present within heterochromatic regions. The deletions at this locus could therefore result in a position effect on euchromatic gene(s) being moved into heterochromatic regions or alternatively heterochromatic genes being placed in a euchromatic environment as the deletion of GC-rich repeats could result in the loosening of the heterochromatin structure causing a position effect (Winokur *et al.*, 1994).

Position effects occur over several hundred kilobases, therefore, the FSHD gene could be located at quite a distance from D4Z4. The disease causing mutation might be the deletion of the 3.3 kb repeats which lead to FSHD due to the altered transcriptional activity of a gene(s), rather than a mutation within the gene itself. The median repeat array on chromosome 4 was found to be 21 kb larger than that on chromosome 10. This might reflect the requirement for a larger subtelomeric domain on chromosome 4 to prevent gene silencing, or expression, of critical genes in the region proximal to the telomere (Van Overveld *et al.*, 2000).

Genotype-phenotype correlation studies indicated an inverse relationship between the residual repeat length and the clinical severity (paragraph 2.1.2.7). PEV could explain this since the FSHD candidate region is moved further into the 4q heterochromatic telomeric region as the deletion increases, resulting in an increased disease severity. Furthermore the effect on the chromosomal structure from heterochromatic to euchromatic increases as the deletion increases, influencing gene expression correspondingly.

This is the first study to investigate FSHD on both the clinical and molecular levels. FSHD is clinical extremely heterogeneous which results in a complex clinical diagnosis. It is, however, possible to detect the presence of the disease causing DNA rearrangement on chromosome 4q35. This therefore highlighted the need and importance of a molecular study in the South African FSHD population.

2.1.2.12 Objectives of this study

This is the first study to investigate the molecular basis of FSHD in the South African population. Five extended Caucasian FSHD families, consisting of 100 individuals, from the South African population were selected for this study. Haplotype analyses will be performed to study the segregation of STRP markers in the 4q35 region with the FSHD phenotype; and the DNA rearrangements at the D4Z4 locus, associated with FSHD, will be detected via Southern blot analyses utilising probe p13E-11.