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**THE ROLE OF EMERIN IN THE PATHOGENESIS OF
SPORADIC INCLUSION BODY MYOSITIS**

PhD Thesis submitted by

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Declaration of originality

I hereby declare that this thesis and the work reported here represents my original work and that any additional sources have been duly cited.

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ABSTRACT

Idiopathic Inflammatory Myopathies (IIMs) are a heterogeneous group of acquired diseases characterised by inflammation of the skeletal muscle. The most common forms are sporadic inclusion body myositis (sIBM), dermatomyositis, polymyositis, overlap syndromes and necrotising myopathy. Sporadic IBM is the most common acquired myopathy over the age of 50 years, however its pathogenesis remains largely unsolved and there is still debate on whether it is a primary inflammatory or a degenerative condition with secondary inflammatory component. I used an *in silico* approach to identify molecules of interest that might shed light on the pathogenesis of sIBM. Emerin, a nuclear envelope protein, resulted as one of the candidate molecules.

To verify *in silico* analysis results I reviewed a cohort of patients with sIBM phenotype identified at Charing Cross Hospital. This review allowed me to select 10 sIBM patients and identify 3 further patients with sIBM-like phenotype and good response to steroids. The selected patients have been then tested through immunohistochemical, immunoblotting and sequencing studies. Immunohistochemistry studies using anti-emerin antibodies showed abnormal distribution of emerin in the sIBM patients but not in the 3 patients with sIBM-like phenotype. Interestingly, sequencing of the emerin gene identified missense mutations in the 5'-untranslated region.

The three IBM-like patients were extensively studied and serum analysis allowed me to identify a novel autoantibody that proved to target the sarcoglycans. Sequencing of the sarcoglycans genes in the three patients identified a missense mutation in the β -sarcoglycan affecting the 3D-structure of the extracellular component. The identification of this novel autoantibody prompted a further pilot study to develop a diagnostic test to identify novel autoantibodies to sarcolemmal proteins. Variable positive reactivity against sarcolemmal proteins, suggestive of presence of autoantibodies against sarcolemmal proteins was identified through immunoblotting in the sera of 10 further IIM patients, but not in 9 controls.

This study highlights the complexity behind the pathogenesis of IIM and importance of unified classification criteria. Although further studies on a larger scale will be needed I suggest that emerin immunolabeling could be used as diagnostic biomarker in patients with sIBM. In addition, a pilot study using of skeletal muscle protein

extract revealed novel autoantibodies in IIM whose pathogenicity remains to be established.

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INTRODUCTION

Idiopathic Inflammatory Myopathies (IIMs) are a heterogeneous group of acquired diseases characterised by inflammation of the skeletal muscle. They are part of a major class of muscle diseases called Inflammatory Myopathies (IM). IMs are divided in two main categories: infective and idiopathic (or autoimmune) (Mastaglia, 2008). The infective forms can be caused by viral, bacterial, fungal, protozoal or helminthic infection; the autoimmune IM are further divided in generalized and focal forms (Mastaglia, 2008). The generalized forms include following conditions: sporadic inclusion body myositis (sIBM), dermatomyositis (DM), polymyositis (PM), overlap syndromes, necrotising myopathy, eosinophilic myositis and granulomatous myositis (Mastaglia, 2008). The focal forms include the localized nodular myositis, monomelic myositis, angiopathic myositis, eosinophilic myositis, macrophagic myofascitis, inflammatory pseudotumour and orbital myositis. IIMs are a heterogeneous group of acquired conditions and are characterised by inflammatory infiltrate in muscle fibres and variable upregulation of major histocompatibility complex-I (MHC-I) and degenerative changes in the muscle fibres (Dubowitz & Sewry, 2006). They involve mainly the skeletal muscle, and patient usually shows predominant proximal muscle weakness but other organs may be involved during the acute or chronic phase of the disease. Creatine kinase (CK) is usually elevated. The most common forms are PM, DM and sIBM (Cox et al, 2010). Although the different forms of inflammatory myopathies have some common features, they have distinct clinical and pathological features and a correct diagnosis is crucial for prognosis and treatment.

Wagner first described myositis in 1863; the first case of DM was documented in 1875 by Potain and in 1886 Unvericht described the first case of PM (Oddis & Medsger, 1995; Bohan, 1988) while sIBM was recognised only in late 1967 by Chou (Chou 1967).

Over the past 40 years authors have suggested various diagnostic criteria and classifications (Medsger 1970; DeVere & Bradley 1975; Bohan & Peter 1975; Targoff 1997; Dalakas & Hohlfeld, 2003; Pestronk 2011) with the most widely used criteria being those proposed by Bohan & Peter in 1975. However, the scientific community has not yet agreed and approved a worldwide classification for IIM. Over the years classification criteria were variably based on clinical and histological features,

laboratory findings; these include muscle weakness, elevated muscle enzymes [CK, lactate dehydrogenase (LDH), aldolase], myopathic features on electromyography (EMG) and muscle biopsy (Cox et al, 2010). The classification proposed by Bohan and Peter in 1975 divides inflammatory myopathies into 5 groups (PM, idiopathic DM, PM-DM associated to tumour, juvenile PM-DM and PM-DM associated to other connective tissue disease or overlap syndrome). Although widely used, this classification has never been validated and has recently been reviewed by Dalakas and Hohlfeld (2003) with the inclusion of sIBM. The new criteria include immunopathological features and point out the role of the MHC-I antigen and of cytotoxic CD8 positive T-cells as specific markers that help differentiating antigen-mediated inflammatory response, typical of PM and sIBM, from secondary non-specific inflammatory response of other muscular diseases like muscular dystrophies and genetic determined myopathies (Briani et al., 2006). Although the classification proposed by Bohan and Peter criteria (Bohan & Peter, 1975) is the most widely used, recent studies demonstrated that its specificity is very low (29%) compared to new ones, and the classification criteria proposed by Dalakas (Dalakas & Hohlfeld, 2003) have the highest specificity (99%) (Linklater et al, 2013). Furthermore, the Bohan and Peter criteria often can lead to misdiagnose PM from sIBM or some types of muscular dystrophies (Dalakas & Hohlfeld, 2003).

Over the years, thanks to the identification of myositis specific antibodies (MSA) and myositis associated antibodies (MAA) new classifications have been proposed based on the serological status of the patient (Love et al 1991, Troyanov et al, 2005). MSA are autoantibodies that target either nuclear or cytoplasmic cell components and are highly selective and usually characteristic of a particular IIM clinical phenotype (Targoff, 2008). Based on this new criteria suggested by Love in 1991 patients were classified according Bohan and Peter's classification with the inclusion of sIBM and taking into account their serological status (Love et al 1991). Troyanov, years later, suggested a similar classification based on revised Bohan and Peter criteria with the inclusion of overlap myopathies (Troyanov et al, 2005). This classification is thought to have high sensitivity (87%) when used to identify overlap syndrome (Troyanov et al, 2005). In the last few years Pestronk proposed another classification using myopathologic features as guidelines for IIM classification (Pestronk, 2011).

Although Bohan and Peter's classification criteria are nowadays considered obsolete due to low sensitivity and specificity compared to all the new classifications that have been proposed in more recent years (Linklater et al, 2013), an agreement has yet to be reached by the scientific community and classification criteria have to be validated.

As a result of variability and non-unanimity of classification criteria a precise estimation of epidemiology of IIM and of its subtypes is very difficult. The approximate reported annual incidence ranges from 2 – 8 cases per million people per year (Oddis et al 1990, Benbassat et al 1980, Weitoft et al 1997, Koh et al 1993). Overall, it appears that DM is the most common with an incidence of 8 cases/million population/year, PM less frequent with 4 cases/million population/year and sIBM the most frequent in the older population. The prevalence of sIBM is estimated to be 4.3-9.3 per 1 000 000 before the age of 50 years but 35.3 per 1 000 000 in the population over the age of 50 years (Mastaglia & Phillips, 2002; Engel & Askanas, 2006; Needham et al, 2008). PM and DM affect females more commonly with a ratio male/female of [1:2], while sIBM is more common in male with a male/female ratio of [3:1] (Prieto & Grau, 2010; Dimachkie et al, 2014). A geographic variability of the incidence of PM and DM has been observed throughout the years which is mainly to be attributed to differences in diagnostic criteria that have been applied to reach or define the diagnosis (Cox et al, 2010).

IIMs share some clinical and pathological features such as progressive muscular weakness, raised CK, dysphagia, inflammatory infiltrate, various degenerative changes in the muscle fibres and sarcolemmal upregulation of MHC-I. However, they also have distinct characteristics, which help to distinguish the various forms and possibly achieve a final diagnosis; these will be described in the next section.

Since this study focuses on sIBM and PM/DM as controls, these three conditions will be described into more detail.

Dermatomyositis and juvenile Dermatomyositis

Dermatomyositis is a microangiopathy and is characterised by a disease onset in adult life with sub-acute, symmetric proximal muscle weakness and extra-muscular manifestations, typically there is skin rash. CK is usually raised at up to 50 times the normal values (Dimachkie & Barohn, 2012). Electromyography shows polyphasic

motor unit action potentials of short duration and low amplitude on voluntary contraction with increased insertional and spontaneous activity, fibrillation potentials, sharp waves and/or repetitive discharges (Ernste & Reed, 2013).

DM can occur in association with skin changes, or skin changes without myositis (DM sine myositis or amyopathic DM), and in the absence of skin changes a diagnosis of possible DM sine dermatitis can be raised based on histopathological findings and other clinical criteria with exception of skin rash. Skin changes are most commonly characterised by erythematous and photosensitive heliotrope rash which involves face, neck, anterior chest (V-neck sign) or upper back (shawl sign) and extensor surface of arms; violaceous papules (Gotton's papules) or macules (Gotton's sign) can be found at metacarpophalangeal and interphalangeal joints. In severe cases skin goes under dramatic changes becoming tight, shiny and reddened (Dubowitz & Sewry, 2006). Apart from skin changes there can be involvement of other organs; pulmonary, gastrointestinal and cardiovascular systems may be affected. Typically patients can present with interstitial lung disease (ILD), dysphagia and myocarditis or arrhythmias (Briani et al 2006).

DM can occur in association with malignancies or other autoimmune conditions and mixed connective tissue disorders. The risk of malignancies appears to be in the five years preceding the presentation of the disease and in the first five years following the diagnosis (Mastaglia, 2008). Malignancy seems to occur in at least 25% of adult patients within the five years preceding diagnosis. Tumours that are most commonly found in association with DM are breast and ovarian cancer in women and prostate and lungs cancer in men (Ernste & Reed, 2013). Presence of autoantibodies anti-p155/140 in DM patients is associated with malignancy (Betteridge et al 2009, Gunawardena et al, 2009).

Histopathological findings are characterised by variation in fibres size, internal nuclei, necrotic fibres, perifascicular atrophy, perimysial and perivascular infiltration of CD4 positive T-cells (Dalakas & Hohlfeld, 2003). Deposits of membrane attack complex (MAC) are found on capillaries and sarcolemma as well as up-regulation of MHC-I on capillaries and sarcolemma in the perimysium; notably there is a reduction of the number of capillaries (Dalakas, 2011).

Juvenile DM is the paediatric counterpart of DM and has an onset between 5 and 15 years old (Boccaletti et al, 2014). In addition to the skin rash and weakness, some children develop as well calcinosis and contractures. Disease severity can vary from milder forms to severe and life threatening disease manifestations. In contrast to DM, the association with malignancy is uncommon in children (Ernste & Reed, 2013).

Polymyositis usually affects the adult population after the second decade of life and is very rarely seen in the paediatric population. PM is a subacute inflammatory myopathy that progresses over weeks to months with proximal upper and lower limb muscle involvement; distal weakness, dysphagia and facial weakness can also occur (Dimachkie et al, 2014). Compared to DM and sIBM, it is the least frequent and often misdiagnosed as sIBM. The diagnosis of PM is based on exclusion of skin rash, family history for neuromuscular disorders, exposure to myotoxic drugs, endocrine disease, neurogenic disease, muscular dystrophies and metabolic myopathies and sIBM. CK serum level is raised up to 50x the normal upper limit (van der Meulen et al, 2003; Dimachkie et al, 2014).

PM frequently occurs in association with other autoimmune and connective tissue disorders, and sometimes with malignancies with an approximate frequency of 10-15%, however the latter one is less compared to DM (Ernste & Reed, 2013). The muscle biopsy shows variation in fibres size, internal nuclei, necrotic fibres, regenerating fibres, and endomysial inflammatory infiltrate with invasion of non-necrotic fibres. The inflammatory infiltrate is mainly composed by CD8-positive cytotoxic T-cells invading MHC-I expressing muscle fibres (Dalakas, 2011). No deposits of complement, MAC or immunoglobulins are found in the microvasculature.

Pestronk described a novel form of PM, which they called PM-mito, a subtype of IM with vacuoles, aggregates and mitochondrial pathology (IM-VAMP) (Temiz et al, 2008; Pestronk, 2011). PM-mito is a slowly progressive inflammatory myopathy with onset typically above the age of 60 years, affecting predominantly quadriceps, wrist, finger flexors and knee extensors (Temiz et al, 2008). Histopathological features are endomysial and perivascular inflammatory infiltration composed mainly of CD4-positive and CD8-positive T-cells. Other histopathological features include irregular-

shaped vacuoles with basophilic granular debris, cytoplasmic myofibre aggregates which may contain phosphorylated proteins and amyloid, abnormal myonuclei and mitochondrial abnormalities such as mtDNA deletions. In PM-mito mtDNA deletions are about 90% (Pestronk, 2011) and fibres are frequently COX-negative (Chahin and Engel, 2008). Patients with less than 10% of COX negative fibres respond better to methotrexate treatment while usually there is poor response to corticosteroid treatment (Temiz et al, 2008).

Sporadic IBM is the most common acquired myopathy over the age of 50 years and affects men more commonly than women with a ratio [3:1].

In the past 20 years various studies have analysed previously published criteria and in order to achieve consensus on diagnostic criteria for sIBM (Rose et al 2013; Lloyd et al, 2014) and revised diagnostic criteria have been proposed: ENMC IBM Research Diagnostic Criteria 2011 (Rose et al, 2013). Based on these criteria sIBM can be defined as clinico-pathological IBM, clinical defined IBM or probable IBM depending on number of clinical and laboratory features present (Rose et al, 2013).

Clinical features include slowly progressive weakness and wasting of the distal and proximal muscles and early involvement of quadriceps and finger flexors; CK is usually normal or slightly increased. Typically patients with sIBM do not respond to treatment (Dalakas, 2006; Needham & Mastaglia, 2008). In contrast, PM and DM cause symmetrical non-selective proximal myopathy and are generally steroid responsive. Muscle biopsy is essential for the diagnosis and is characterised by multifocal endomysial inflammatory infiltrates composed of CD8-positive cells but also of B-cells and plasma cells, rimmed vacuoles (RVs) in fibres not invaded by lymphocytes, sarcoplasmic inclusions, ragged red-fibres, COX-negative fibres and ubiquitous expression MHC class I antigen (Dalakas 2006; Needham & Mastaglia 2008). Electron microscopy (EM) reveals prominent myonuclear changes including irregular nuclear profiles and intranuclear filamentous 7 nm inclusions. Furthermore EM reveals accumulation of tubulo-filamentous inclusions (15-21 nm) and cytoplasmic amyloid-like filaments (6-10 nm) (Askanas & Engel, 2003).

The association between sIBM and human leukocyte antigen genes, DR β 1*0301 and DQ β 1*0201 alleles, confers susceptibility to sIBM. The B8-DR3-DR52-DQ2 haplotype is found in 67% of sIBM patients and is associated with earlier disease

onset (Koffman et al., 1998; Badrising et al., 2004; O'Hanlon et al., 2005; Mastaglia et al., 2009).

The pathogenesis of sIBM remains still unknown. In the past 40 years several theories regarding the pathogenesis of sIBM have been raised, but it is still unclear whether sIBM is primarily a T-cell mediated inflammatory myopathy like PM, or a myodegenerative disorder with abnormal protein aggregations responsible for a secondary inflammatory response (Finch 2006; Needham & Mastaglia 2008). Several hypotheses have been suggested to explain its pathogenesis ranging from viral infection to autoimmune or degenerative origin (Benveniste et al 2015). Many studies have suggested pathological similarities between sIBM and neurodegenerative diseases such as Alzheimer's disease (Finch, 2006; Needham & Mastaglia, 2008).

Several studies have suggested that a viral infection may act as a trigger to develop sIBM, although molecular techniques have failed to confirm presence of any viruses in muscle. This hypothesis is supported by several reports of patients with retroviral infection (HIV or HTLV-1) and associated sIBM (Cupler et al., 1996; Ozden et al., 2004; Saito et al., 2002; Dalakas, 2006; Dalakas et al., 2007). Clonally in situ expanded CD8-positive T-cells that invade MHC-I positive muscle fibres were found to be retrovirus specific as amino acid residues of the CDR3 region were specific for viral peptides bound to HLA. It was hypothesised that the viral infection followed by immune response might be responsible for the chronic inflammatory process leading IBM clinico-pathological features (Dalakas, 2011).

Various authors support the hypothesis of a primary autoimmune origin in sIBM, suggesting that the inflammatory component is more prominent in the early stage rather than late stage of the disease when the degenerative component predominates. The endomysial inflammatory cells are predominantly CD8-positive T-cells (Dalakas, 1998), which are clonally expanded with a restricted T-cell receptor (Müntzing et al., 2003). Plasma cells have also been described as part of the inflammatory infiltrate and immunoglobulin transcripts have been demonstrated with microarray studies (Greenberg et al., 2005; Raju & Dalakas, 2005).

In the last years the role of B cells in the pathogenesis of sIBM has gained importance. Their role in the inflammatory component of other IIM is well known and

in recent years researchers demonstrated overexpression of a number of immunoglobulin genes together with CD138 positive plasma cells in muscle tissue (Greenberg et al, 2002; Bradshaw et al, 2007). The typical maturation environment of B cells is the lymphoid germinal centre in the lymphoid tissue, which is composed of abundant follicular dendritic cells and B cell follicles (Salajegheh et al, 2010). Due to the instability of RNA transcripts, the presence of immunoglobulin transcripts in the muscle could be due only to local cellular production (and not to deposition from circulation) (Greenberg et al, 2005); it has been suggested that this could be attributed to a permissive environment for local B cell maturation in muscle. Regions of dense B cells infiltrates have been described both in perimysial areas around or near large blood vessels and in endomysial regions. These infiltrates called “nodular infiltrates”, contained also T cells (CD3 positive), myeloid dendritic cells (BDCA1 positive) and CD138- positive plasma cells (Salajegheh et al, 2010). The presence of myeloid dendritic cells has been confirmed thanks to further analyses by Greenberg who has demonstrated that they surround and sometimes invade non-necrotic myofibre regions (Greenberg et al, 2007). There, naïve B cells mature into antibody-producing plasma cells thus transforming the muscle in an immune cell host where plasma cells and myeloid dendritic cells activate T-helper cells (Greenberg, 2007; Salajegheh et al, 2010). Moreover, the abundance of immunoglobulin gene transcripts in the muscle correlates well with the presence of visible nodular infiltrates (Salajegheh et al, 2010). Microarray mRNA expression studies on sIBM documented up-regulation of genes associated with immunoregulatory pathways, cell adhesion molecules and the cytoskeletal network in sIBM, but failed to identify an expression profile that is characteristic of sIBM (Raju & Dalakas, 2005; Greenberg et al, 2005; Greenberg 2007; Parker et al 2009).

Further studies of clonal expansion, immunoglobulin isotype usage and somatic mutations in sIBM and PM muscle tissue have demonstrated the presence of antibodies in sIBM and PM patients’ sera (Targoff, 1993) supporting the hypothesis of an antibody-mediated autoimmunity as a response to an unknown antigen. B cells in the muscle tissue are clonally expanded and it has been observed that most of them had switched from IgM to either IgG or IgA. This is peculiar because usually naïve resting B cells do not class switch till the encounter with an antigen, which means that these cells are mature and have become plasma cells. These cells had

also undergone somatic mutations which supported the antigen-driven response hypothesis. Another finding in support of this theory is the presence of common amino acid mutations in unrelated B cells clones, probably due to the recognition of a common antigen (Bradshaw et al, 2007).

Recently, Salajegheh et al. (2011) identified autoantibodies against a 43 kDa antigen in 52% of the patients with sIBM. This finding was then confirmed in two other separate studies, which showed autoantibodies to cytosolic 5'-nucleotidase 1A (anti-cN1A) in sIBM (Larman et al, 2013, Pluk et al 2013). However, so far this newly identified antibody does not seem to be solely present in sIBM but was identified at a lower frequency also in other subtypes of IIM (Pluk et al, 2013). In sIBM, MHC-I antigen is almost ubiquitously expressed on sarcolemma and sarcoplasm, which is in normal conditions not expressed on muscle fibres. In some other conditions, even in muscular dystrophies MHC-I can be up regulated in some degenerating or regenerating fibres. The exact mechanism of MHC-I up-regulation is still unknown and it is thought that its up-regulation could be induced by cytokines. Chronic up-regulation of MHC-I might in turn induce endoplasmic reticulum stress (Nagaraju et al., 2005; Dalakas, 2006).

A degenerative process with secondary inflammatory response has also been hypothesised as being the cause for sIBM. Evidence for a degenerative process in sIBM is provided by the presence of RVs and the intracellular deposition of proteins such as tau, β -amyloid, α B-crystallin and TDP43 and oxidative stress proteins. In addition, proteasomal abnormalities and dysfunction has been documented and reported in sIBM leading to accumulation of unfolded and misfolded proteins (Fratta et al 2005). Disrupted myonuclear structure, intranuclear filamentous inclusions and the presence of emerin and histone1 in RVs are also features of sIBM that suggest a role of nuclear proteins in this condition but little emphasis has been given to these findings (Karpati and Carpenter 1993; Askanas & Engel, 2003; Greenberg et al, 2006; Fidzianska et al, 2008; Nakano et al, 2008; Nishii et al, 2011). Greenberg et al (2006) showed that RVs were lined with emerin and lamin A/C, further to this they also showed with the use of immunohistochemistry and EM focal rupture of myonuclear membranes. In particular Nakano et al (2008) demonstrated presence of histones H1, emerin and DNA in sIBM RVs and Nishii et al (2011) demonstrated increased percentage of DNA double strand breaks in sIBM myonuclei and defects

of nuclear translocation of Ku70 protein that is responsible for DNA double strand break repair. However the etiopathogenesis of sIBM still remains largely object of controversy.

Serology of Myositis

In recent years there is increasing evidence of a relationship between genotype, serotype and clinical phenotype in IIM (Love et al, 1991; Targoff, 2008; O'Hanlon et al, 2005).

Autoantibodies detected in patients with IIM are classified in MSA and MAA and a high-titre autoantibody is found in 50% - 80% of myositis patients (Betteridge et al 2009; Gunawardena et al, 2009). MAA are mainly found in patients with other connective tissue disorders and can be found in overlap syndromes while MSA are usually highly selective, mutually exclusive and are characteristic of a particular IIM clinical phenotype; in general they are not found in other types of connective tissue disorder.

MSA have been reported to be associated with particular genotypes (Chinoy et al, 2009; 2011). MSA target either nuclear or cytoplasmic cell components, which may have a role in gene transcription, protein translocation and even in anti-viral responses.

Serological status of patients with IIM has gained increasing role over years and autoantibodies have now been included in the classification criteria (Trojanov et al, 2005) and are being used as diagnostic biomarkers to achieve a diagnosis and guide pharmacological treatment (Targoff, 2006).

Myositis Specific Autoantibodies (MSA)

- Anti-aminoacyl-tRNA synthetase (anti-ARSs):
 - ❖ anti-Jo1 (anti-histidyl synthetase)
 - ❖ anti-PL7 (anti-threonyl synthetase)
 - ❖ anti-PL12 (anti-alanyl synthetase)
 - ❖ anti-EJ (anti-glycyl synthetase)

- ❖ anti-OJ (anti-isoleucyl synthetase)
- ❖ anti-KS (anti-asparaginyl synthetase)
- ❖ anti-Ha (anti-tyrosyl synthetase)
- ❖ anti-Zo (anti-phenylalanyl synthetase)

- Anti-SRP (signal recognition particle)
- Anti-Mi-2
- Anti-CADM-140/MDA5
- Anti-p155/140
- Anti-p140

Anti-ARs are directed against a group of cytoplasmic enzymes that catalyses the binding of specific amino acids to their respective tRNA (Gunawardena et al, 2009). Anti-ARS are the most common MSA in both PM and DM and are predominantly found in the adult myositis patients (Targoff, 2006; Gunawardena et al, 2009). Patients typically have a unique clinical picture known as antisynthetase syndrome which is characterised by muscle weakness and pain, ILD, fever, mechanic hands and non-erosive arthritis. These symptoms can present individually or associated depending on which anti-ARS has been identified in the patient.

The reason why ARS are targeted as autoantigens is still unknown. However, it has been demonstrated that the epitope recognized by the autoantibodies is located at the N-terminus of the proteins (Park et al, 2008). These autoantigens tend to cluster on the surface of apoptotic cells when cell death is induced by granzyme B which then cleaves the autoantigens into fragments (Casciola-Rosen et al, 1994). They have chemoattractant properties through CCR3 and CCR5 receptors expressed by leukocytes. The continuous production of these autoantigens associated with chemoattractant activity may lead to the spreading of inflammation in muscle (Betteridge, Gunawardena et al, 2009).

Association with some forms of cancer has also been described, particularly for mutations in isoleucyl-tRNA synthetase that are associated with hereditary non-polyposis colon cancer (HNPCC) and for mutations in phenylalanyl-tRNA synthetase which have been reported in lung solid tumours and acute phase chronic leukaemia

(Park et al, 2008).

- ❖ **Anti-Jo1 antibodies** were first described in 1980 and are the most common of the anti-ARS antibodies; they are detected in about 25-30% of the patients with myositis and in 60-70% of patients with ILD.

Increased expression of anti-Jo1 autoantibody has been demonstrated in regenerating fibres, supporting the hypothesis that production of these autoantigens during regeneration attempt may induce a propagation of the autoimmune response (Betteridge, Gunawardena et al, 2009). However, it is unlikely that these are disease causative since they are normally not expressed in adult muscle apart during regeneration.

- ❖ **Anti-PL7, anti-PL12, anti-EJ, anti-OJ, anti-KS, anti-Ha and anti-Zo antibodies** collectively may be detected in about 20% of PM and DM patients and individual frequency ranges from 1 to 5 % (Betteridge et al, 2007; Gunawardena et al, 2008).

Anti-Signal Recognition Particle (anti-SRP) antibodies target a highly conserved RNP complex (proteins - 7SLRNA), which is responsible for regulation of protein translocation through the endoplasmic reticulum (Ghirardello et al, 2013). Anti-SRP autoantibodies are detected in approximately 5% of IIM patients. Usually patients can have a severe myopathy with an acute onset and high CK level; other features include dysphagia which can be also refractory to treatment, severe cardiac involvement can also occur and is associated to poor survival (Targoff, 2006; Gunawardena et al, 2009).

Anti-Mi-2 antibodies target a nuclear helicase protein that is part of the nucleosome-remodelling deacetylase (NuRD) complex which is involved in gene transcription (Gunawardena et al, 2009). Mi-2 acts together with other component of the NuRD complex as a transcriptional suppressor; however it is also involved through its N-terminus domain in transactivating proteins. Anti-Mi-2 antibodies are detected in approximately 9% of patients with myositis and in approximately 20% of

patients with DM. It was found that Mi-2 protein expression in keratinocytes increases following UV exposure and it was suggested that this triggers anti-Mi-2 production in UV-induced dermatitis (Betteridge et al, 2009; Gunawardena et al, 2009). Although patients with anti-Mi-2 appear to have more severe cutaneous symptoms, prognosis usually is favourable and there is a good response to corticosteroid treatment. In addition, myositis associated with anti-Mi-2 has a lower incidence of malignancy compared to forms of IIM (Trojanov et al, 2005; Mammen, 2010).

Anti-CADM-140/MDA5 antibodies target a cytoplasmic 140 kD protein called melanoma-differentiation associated gene 5 (MDA5) which is involved in innate immune-responses against viral infections (Sato et al, 2013; Silveira et al, 2015). Anti-CADM-140/MDA5 antibodies have been identified in patients with clinically amyopathic DM and are predominantly found in the Japanese and US DM population. Usually these patients do not present any or little muscle involvement, however are at increased risk of developing rapidly progressive ILD (Sato et al, 2013; Labrador-Horillo et al, 2014; Silveira et al, 2015).

Anti-p155/140 antibodies target a 155 kD and 140 kD proteins which are believed to be the same protein. Anti-155/140 is a DM-specific antibody and patients are at increased risk of malignancy; it was shown that patients with anti-155 kD antibodies have a 89% specificity and 78% sensitivity of developing cancer-associated DM (Trallero-Araguas et al, 2010). Recently, it was shown that anti-p155/140 target a family of proteins called TIF1 (transcriptional intermediary factor-1), which are involved in several pathways including carcinogenesis (Targoff, 2008, Betteridge et al, 2009; Gunawardena et al, 2009). Anti-p155 specifically targets a TIF1- γ , a nuclear protein involved in cell differentiation (Gunawardena et al, 2009).

Anti-NXP-2 (formally anti-MJ) antibodies target another 140 kD protein that was subsequently identified as nuclear matrix protein 2 (NXP2). NXP2 is a nuclear protein involved in gene transcription. Anti-NXP2 is associated with severe muscle

weakness, contractures, calcinosis, intestinal vasculitis and polyarthrititis. These antibodies are found in JDM, DM and PM and are usually associated with a younger age of disease onset, however patients generally have a good response to treatment (Gunawardena et al, 2008, 2009).

Anti-SAE antibodies target a 40 kD and 90 kD proteins that was subsequently identified as being a small ubiquitin-like modifier activating enzyme 1 and 2 (SAE) which are involved in post-translational modification of various targets (Betteridge et al, 2009, Gunawardena et al, 2009). Patients with anti-SAE present with DM and a specific clinical phenotype characterised by of widespread skin involvement, muscle involvement, dysphagia and other systemic features. Anti-SAE antibodies were found to have a strong association with HLA-DRB1*04/DQA1*03/DQB1*03 haplotype (Betteridge et al, 2009; Gunawardena et al, 2009; Chinoy et al, 2009).

Myositis Associated Autoantibodies (MAA)

- ❖ Anti-URNPs family
- ❖ Anti-PM-Scl
- ❖ Anti-Ro/SSA
- ❖ Anti-Ku

MAA have been mainly described in scleroderma and overlap syndromes, and have been described also in association with some cases of anti-ARS negative IIMs with ILD (anti-PM-Scl, anti-U1-RNP, anti-U2-RNP and anti-U5-RNP) (Betteridge et al, 2009; Gunawardena et al, 2009; Troyanov et al, 2014).

Anti-U-RNP antibodies are a family of antibodies that target small nuclear ribonucleoproteins (snRNP) that are important in processing pre-mRNA. Anti-U1-RNP is generally found in patients with mixed connective tissue disorder,

scleroderma or systemic lupus erythematosus (Ihn et al, 1999; Targoff, 2002).

Anti-PM-Scl antibodies target a nucleolar macromolecular complex of peptides of which play a role in RNA processing; the main PM-Scl antigens are a 100 kD protein and a 75 kD protein (Targoff, 2002). These antibodies are detected in patients with IIM, isolated systemic sclerosis or overlap scleroderma/myositis. Patients generally present with prominent arthritis and other systemic involvement such as Raynaud's phenomenon or ILD (Targoff, 2002; Troyanov et al, 2014).

Anti-Ro/SSA antibodies are among the most frequently detected antibodies targeting extractable nuclear antigens (ENA). They are mainly detected in patients with systemic lupus erythematosus and Sjögren syndrome, and can be found also in other autoimmune diseases like systemic sclerosis, PM, DM, mixed connective tissue disease and rheumatoid arthritis (Franceschini & Cavazzana, 2005).

Anti-Ku antibodies target a DNA binding protein (Ku70/Ku80) involved in DNA repair, gene transcription, gene recombination and telomere protection. Anti-Ku antibodies have been detected in various autoimmune conditions which include systemic lupus erythematosus, systemic sclerosis, mixed connective tissue disorder, idiopathic lung fibrosis and scleroderma/myositis (Targoff, 1993; Belizna et al, 2010). Patients usually present with muscular and joint symptoms and Raynaud's phenomenon. Unlike ILD in patients with anti-Ku antibodies, musculoskeletal symptoms generally respond well to treatment (Targoff, 1993; Belizna et al, 2010).

Novel Myositis Autoantibodies

Anti-cN1A or NT5C1a antibodies (previously identified as anti-Mup44) target the cytosolic 5'-nucleotidase 1A, which is part of a family of enzymes that is involved in the regulation of intracellular nucleotide pools, energy balance, cell metabolism and cell replication (Careddu et al 2008, Kulkarni et al 2011).

Recently these antibodies were found to be prevalent in sIBM patients (Larman et al, 2013; Pluk et al 2013), however, a more recent study detected the same antibodies also in 4-21% of DM patients and in patients with connective tissue disorders and no

myositis, like systemic lupus erythematosus and Sjögren syndrome. These recent findings reduce the initial excitement since anti-NT5C1a is not only specific to sIBM and cannot be used as a biomarker (Lloyd et al, 2015).

Anti-3-hydroxy-3-methylglutaryl-coenzyme A reductase (anti-HMGCR) - Statins are drugs usually prescribed to lower blood cholesterol levels and they are commercially available since 1987. Necrotising myopathy is amongst the side effects of high doses administration of statins that have been reported, and is characterised by muscle pain, weakness and tenderness of proximal limbs and axial muscles, moderated to markedly elevated serum CK levels, EMG changes, MHC-I expression in necrotic muscle fibres and MAC deposits on scattered non-necrotic muscle fibres (Le Quintrec & Le Quintrec, 1991; Gaist et al, 2001; Lahaye et al, 2014). Unlike other IIM, necrotising myopathy is characterised by little to no inflammatory infiltrate (Hamann et al, 2013). A mean incidence of 2.3 per 10000 persons/years has been reported in a large UK cohort between 1991 and 1997 (Gaist et al, 2001).

In 2010, the presence of antibodies directed against 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), which is the target enzyme of statins, has been described (Christopher-Stine et al, 2010). This immune-mediated necrotising myopathy is responsive to immunosuppressive treatment. The antibody targets a 100/200 kD protein complex and is highly specific for this type of myopathy, though rare (Mammen et al, 2011). Its presence is also associated with myalgia, arthralgia and dysphagia (Mammen et al, 2011).

HMGCR is a ~ 97 kD membrane protein composed of a small extracellular domain (N-terminus), seven membrane spanning domains and an intracellular catalytic domain (C-terminus). The antibody specifically recognizes the C-terminus of the enzyme (Mammen et al, 2011). The 100 kD protein has been demonstrated to be the HMGCR antigen while additional analyses have hypothesized that the 200 kD could be an HMGCR dimer (Mammen et al, 2011). Further studies suggested that statins up-regulate HMGCR expression thus triggering the autoimmune response. High levels of HMGCR in regenerating muscle fibres may perpetuate the immune response even after discontinuation of the therapy (Mammen et al, 2011).

HMGCR antibody was also described in patients that had previously no exposure to statins, suggesting genetic predispositions to develop these antibodies.

HMGCR antibody was further described in a small cohort of white and African-American patients, and a strong correlation between anti-HMGCR antibody and HLA-DRB1*1101 was reported as a risk factor. The same study proved that DQA1 and DQB16 were protective factors (Mammen et al, 2011; Gaudet et al, 2012). A genome-wide association study has shown a strong association of necrotising myopathy with the single nucleotide polymorphism rs4149056 C allele of the SLCO1B1 gene on chromosome 12. This gene encodes OATP1B1, an organic anion-transporting polypeptide, which regulates the hepatic uptake of drugs; this association increases the risk of statin-induced myopathy (SEARCH Collaborative group et al, 2008). However, the prevalence of this SLCO1B1 polymorphisms in patients with HMGCR antibodies is similar to the general population (Mammen et al 2011).

Immunogenetics of IIM

The cause of IIM remains still unsolved and various hypotheses have been raised over the years. It has been suggested that disease expression is triggered by a combination of environmental and/or genetic causes in genetically predisposed individuals (Prieto & Grau, 2010). Furthermore a correlation between higher prevalence of IIM and African-American and Caucasian populations has been reported as previously discussed, so studies have been conducted to analyse the possible role of genetic factors in the diseases.

A recent study performed low to high-resolution genotyping to define the possible role of HLA (class I: HLA-A, -B, -Cw; class II: HLA-DRB1 and -DQA1) alleles as predisposing or protective factor for IIM (O'Hanlon et al, 2005).

TNF α -308A polymorphism is also predisposing factor which has been reported in DM and PM patients (Dalakas & Hohlfeld, 2003; O'Hanlon et al, 2005). Among all the pro-inflammatory cytokines that have been described, TNF α performs quiet a remarkable role in protein catabolic process: it may induce protein loss via oxidative activation of NF- κ B (myogenic transcription factor Nuclear Factor Kappa B).

Furthermore, a link between TNF α -308A and HLA-B*08 has been previously reported by Chinoy and co-workers who demonstrated that HLAB*08/TNF α -308A/DRB1*03/DQA1*05/DQB1*02 haplotype is a risk factor for all subgroups of IIMs (Chinoy et al, 2007). This ancestral haplotype has been further analysed for possible association with anti-Jo1 or anti-PM-Scl autoantibodies: a strong association between DPB1*0101 and anti-Jo1 antibody has been observed while no statistical significance has been reported for the association between the allele and anti-PM-Scl antibody. DPB1*0101 is associated with DRB1*0301 and only when both alleles are present, DPB1*0101 is considered a risk factor for IIM, in contrast DRB1*0301 remains an important risk factor even in the absence of DPB1*0101 for anti-Jo1 positive patients.

Another haplotype that confers risk for developing both anti-Jo1 and anti-PM-Scl antibodies is DRB1*0301/DPB1*0401 (Chinoy et al, 2009), while DRB1*0302 has been demonstrated to be a risk factor for developing anti-Mi-2 autoantibody in African American patients (Chinoy et al, 2011).

In a further more recent study, Chinoy and co-workers analysed NF-kB polymorphisms that could be involved in the pathogenesis of IIM but no significant data has been reported (Chinoy et al, 2012). Other cytokines that have been investigated for having a role in IIM susceptibility are INF γ and IL-4. INF γ is a Th1 cytokine that strongly induces MHC-I expression in IIM patients, as previously discussed while IL-4 is a Th2 cytokine which functions as B-cell growth factor.

A genetic association analysis has been performed in a UK Caucasian cohort where significant data has been reported only for INF γ : the main INF γ associations with the disease have been observed in the microsatellite marker where C(14) repeat represents a risk factor specifically for anti-U1-RNP positive patients. These associations are independent from HLA-II alleles (Chinoy et al, 2007).

The presented data thus seems to demonstrate that there is a strong relationship between HLA haplotype and IIM even though it has been hypothesized that a combination of more than 25 genes, epigenetic and stochastic events associated with sufficiently environmental exposure are required for developing an autoimmune disease (O'Hanlon et al, 2005). To unravel the complexity of IIM immune-pathogenic mechanisms many efforts and years of study are still necessary.

Therapeutic options for IIM

Treatment of IIM aims to modulate or suppress the inflammatory reaction, restore muscular activity and prevent extramuscular organs involvement.

DM and PM generally are responsive to immunosuppressive therapy while sIBM is known to be resistant, although an initial mild response may be observed in some cases. Ideally, the immunotherapy should be a targeted choice of drug focus on the inflammatory component driving the disease (Gordon et al, 2012). Several new drugs are being trialled or released on the market, however at present the first choice relies on older drugs.

Steroids, namely prednisone or prednisolone, are the first choice to be started at high dose of 1mg/kg/day (maximum 60-80 mg/day). The dosage is reduced after 4 weeks down to the lowest dose necessary to control symptoms. Patients that do

not respond after the third month of treatment are likely to be non-responsive to steroids and treatment should be discontinued by tapering down the drug.

Steroid sparing agents such as methotrexate, azathioprine, mycophenolate mofetil and ciclosporin are used in addition to low dose steroids in order to achieve greater immunosuppression minimizing steroid induced side effects (Dalakas, 2011).

Methotrexate is an immunosuppressant agent which inhibits DNA and RNA synthesis by antagonizing folate metabolism. Folate is required for the synthesis of thymidine which itself is required for DNA synthesis. Methotrexate is started at 7.5mg per week and increased gradually after 3 weeks up to 15 – 20 mg per week. Compared to other steroid sparing agents its action is faster and therefore preferred, but side effects can be more severe. Hepatotoxicity, ulcerative stomatitis, low white blood cells, gastrointestinal symptoms, fatigue and fever are the most common side effects (Dalakas, 2011; Marie & Mouthon, 2011; Brandao & Marinho, 2011).

Azathioprine is an antipurine antimetabolite and acts by inhibiting an enzyme, amidophosphoribosyltransferase, required for DNA synthesis. Azathioprine is used at a dosage up to 3mg/kg/day and usually it takes at least 6 months to kick in. Most common side effects are gastrointestinal side effects, skin rash and fatigue (Dalakas, 2011; Marie & Mouthon, 2011; Brandao & Marinho, 2011).

Mycophenolate mofetil is like azathioprine an antipurine antimetabolite, however compared to azathioprine it works faster though is more expensive. Mycophenolate mofetil inhibits the production of new B- and T-cells, hence when used in combination with prednisone both auto-reactive existing and new lymphocytes are targeted. Mycophenolate mofetil is used at a dose of 2-3 g per day. The most common side effects include gastrointestinal symptoms, joint pain, fatigue and headache (Dalakas, 2011; Marie & Mouthon, 2011; Brandao & Marinho, 2011).

Ciclosporin lowers the T-cell activity and compared to the above-mentioned immunosuppressants it has a higher toxicity and requires close monitoring of the full blood count, kidney and liver function. Ciclosporin works faster than azathioprine and mycophenolate mofetil and is used at a dose of 150 mg bi-daily. Side effects encountered with ciclosporin are gastrointestinal symptoms, hypertension, fever, confusion, sensorineural symptoms, dyspnoea and convulsions (Dalakas, 2011; Marie & Mouthon, 2011; Brandao & Marinho, 2011).

In severe cases where steroid treatment has failed to induce remission of symptoms, intravenous immunoglobulins (IVIg) are used alone or in addition to steroids. IVIg are used at 2g/kg over 2-5 days every 4-6 weeks depending on individual clinical response (Dalakas, 2011).

For refractory patients further drugs should be considered, however diagnosis should be revisited before administering more aggressive drugs or new biologicals. New biologicals are drugs that target B cells, T cells, T cell migration, complement, TNF-alpha, IL1 receptor and cellular adhesion molecules. Several of these new biologicals have been trialled already and approved in other immune driven conditions like multiple sclerosis. The muscle biopsy can help in directing the choice of which monoclonal antibody to use depending on the predominant inflammatory component (Dalakas, 2011; Marie & Mouthon, 2011; Brandao & Marinho, 2011).

IBM represents a challenge in terms of treatment, as it is known to be poorly if not responsive at all to steroids and other immunosuppressant drugs. This could be attributed to the nature of the pathology found in sIBM which is both an inflammatory T cell-mediated component alongside a degenerative component. An initial response to steroids can be observed however the disease continues to slowly progress and so far none of the drugs has proven to be effective in treating this insidious disease (Dimachkie & Barohn, 2012).

AIM OF THE STUDY

Idiopathic Inflammatory Myopathies are a heterogeneous group of acquired diseases characterised by inflammation of the skeletal muscle with the most common forms being sIBM, DM, PM, overlap syndromes and necrotising myopathy. Sporadic IBM is the most common acquired myopathy over the age of 50 years, however despite huge efforts in identifying its cause, the pathogenesis of sIBM remains largely unsolved and there is still debate on whether it is a primary inflammatory or a degenerative condition with secondary inflammatory component.

Aim of this study was to identify molecules of interest that may shed light on the pathogenesis of sIBM by applying a recently developed mathematical model to process and analyse expression microarrays, called Chromowave (Turkheimer et al., 2006), to analyse already published mRNA expression datasets of sIBM, DM and PM available in GEO (www.ncbi.nlm.nih.gov/GEO.com).

During the first phase of the study, the revision of patients diagnosed with IIM between 2001 and 2011 at Charing Cross Hospital allowed me to identify three patients with an sIBM-like phenotype which have been further investigated for their peculiar phenotype. Analysis of their sera allowed identification of a novel autoantibody and its target. This finding prompted me to develop a diagnostic test to identify novel autoantibodies in patients with IIM which led to the filing of a patent.

STRUCTURE OF THE STUDY

Chapter 1 - Chromowave analysis of already published expression datasets allowed identification of molecules of interest, which was then further investigated on selected cases following a revision of patients diagnosed with IIM between 2001 and 2011 at Charing Cross Hospital.

Chapter 2 - A study on three patients, identified through the audit of IIM diagnosed between 2001 and 2011, is reported. The three patients have a very similar phenotype and had muscle biopsy features of sIBM, however responded well to steroid treatment. Analysis of their sera allowed me to identify a novel autoantibody and its target. This finding led to further modelling studies of the antigen.

Chapter 3 - A pilot study for the identification of novel autoantibodies is reported. This last part of the project is a result of outcomes reported in chapter 2 and led to the filing of a patent to develop a diagnostic method for the identification of novel autoantibodies in IIM.

CHAPTER 1

CHROMOWAVE ANALYSIS OF EXPRESSION MICROARRAY DATASET OF MUSCLE SAMPLES OF sIBM, DM, PM PATIENTS AND CONTROLS

INTRODUCTION TO CHAPTER 1

Previous studies described disrupted myonuclear structure in sIBM, suggesting that RVs might originate from myonuclear degradation. Greenberg et al. (2006) showed the presence of emerin in RVs and Nakano et al. (2008) identified histone 1 and emerin in RVs suggesting that nuclear membrane damage could lead to transfer of nuclear material to the cytoplasm (Karpati & Carpenter, 1993; Fidzianska et al., 2008; Nishii et al., 2011). Microarray mRNA expression studies documented up-regulation of several genes associated with immunoregulatory pathways, cell adhesion molecules and the cytoskeletal network in sIBM (Raju & Dalakas, 2005; Greenberg et al., 2005; Greenberg, 2007; Parker et al., 2009), but failed to identify significant differences between sIBM, PM and DM that could define an expression profile characteristic of sIBM.

Turkheimer et al. (2006) have developed a mathematical model called Chromowave, to process and analyse expression microarrays. This is a powerful method for investigating expression datasets and has recently been applied successfully to the analysis of expression changes in human gliomas and Huntington's disease (Anderson et al., 2008).

I applied Chromowave analysis to expression microarray of sIBM compared to DM, PM and normal subjects with the aim to identify candidate genes that may shed light on the possible pathogenesis of sIBM. I performed immunohistochemistry, immunofluorescence, PCR and sequencing for the identified candidate genes.

MATERIAL AND METHODS

Chromowave

Chromowave is a newly developed mathematical model (Turkheimer et al., 2006) (written in MATLAB 7, the Mathworks Inc., Natick MA, USA) used to analyse expression microarrays.

Expression microarrays are a standard technique used to quantify gene expression in a set of tissues for thousands of genes simultaneously.

After normalising the microarray to the background, expression values were transformed on a log₂ scale and mapped to their corresponding chromosomal location using the genome alignment information contained in the manifest of each platform (http://www.affymetrix.com/support/technical/manual/taf_manual.affx).

Chromowave uses single value decomposition and Haar wavelets to detect variations in expression of spatially related genes and to represent such changes on physical maps of chromosomes. A wavelet is a wave-like oscillation and as a mathematical function can be used to extract information from different type of data. A wavelet transform (WT) is the representation of a function by wavelets. Wavelets were introduced for the analysis and encoding of genomic data by Lio (Lio, 2003). On the contrary of conventional statistical analysis based on variation in single gene expression, Chromowave applies wavelets to explore the variation in expression of gene clusters and to identify variation in expression of large regions of chromosomes. Chromowave applies the WT to the spatial distribution of the probes and converts the original expression values to wavelet coefficients that are functions of the expression of adjacent genes. The noise level is identical on the original raw data and at all WT levels. This is advantageous because a cluster of genes with similar expression is transformed into a WT coefficient that is greater the larger the number of genes in the cluster. Therefore the net effect of the application of the WT is that genes with individual expression below the noise level, that would otherwise go undetected, are identified when clustered together because their combined energy condenses into a greater wavelet coefficient that arises over the noise.

The microarray dataset that analysed global gene expression in muscle samples of sIBM, DM, PM patients and controls were taken from published studies that used Affymetrix oligonucleotide microarrays (Greenberg et al., 2005; 2007) and is

available in the GEO database. The dataset contain n = 21 healthy controls and n = 23 sIBM samples, n = 6 PM samples and n = 18 DM samples.

The analysis was performed comparing sIBM to healthy controls, PM to healthy controls and DM to healthy controls.

Standard gene by gene analysis has been performed to confirm data.

Case selection

The patients reported in this study provided written informed consent for the genetic tests and functional and immunological analyses. The Ethics Committee of Imperial College London (UK) approved the study.

Ten sIBM cases and 10 PM/DM samples, available at the Imperial College Healthcare Tissue Bank London, were selected following an audit of patients with diagnosis of IIM between 2001 and 2011. Seventy-five control muscle samples (available at Imperial College Healthcare Tissue Bank London) from patients who had a muscle biopsy with suspicion of vasculitis and appeared normal at pathological examination. All cases have been reviewed by Dr F. Roncaroli to confirm the diagnosis.

Inclusion criteria for selection of sIBM cases: age onset > 50 years; sIBM clinical phenotype; normal or CK level lower than 1,000 IU/L; no response to steroids.

Exclusion criteria: high CK level >1000 IU/L; steroid responsiveness; infectious disease; other autoimmune disorder.

Immunohistochemistry

I tested anti-emerin antibody (mouse monoclonal, Novocastra, UK) on frozen muscle tissue of 10 patients with sIBM, 10 PM/DM and 75 control muscle tissues supplied by the Tissue Bank at the Charing Cross Hospital. Ten-micron sections of the frozen tissue were incubated with normal horse serum (Invitrogen, USA) (1:10) for 30 minutes to suppress nonspecific staining and incubated for 1 hour at room temperature with anti-emerin antibody (1:200). After washing with 0.1% PBS/Triton,

sections were developed with the Super Sensitive™ IHC Detection System (BioGenex, CA).

RNA and DNA extraction from muscle tissue

RNA and DNA were isolated from frozen muscle tissue. Ten sIBM and ten control samples were processed.

RNA was isolated from tissue using Trizol reagent (Invitrogen, USA). Trizol reagent 800µl and glycogen (20µg/µl; Invitrogen, USA) 200µl were added to 1 mg of muscle tissue and then homogenized using an electronic homogeniser (Wolflabs, UK).

Following homogenization, samples were centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant containing RNA was transferred to a fresh tube and 0.2 mL of chloroform per 1 mL of Trizol reagent and DNase (Ambion, UK) were added. Tubes were shaken by hand for 15 seconds and incubated at 15-30°C for 3 minutes. Following centrifugation at 12,000 g for 15 minutes at 4°C, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. The aqueous phase, containing RNA, was transferred to a new tube and butanol 500 µl was added, mixed and incubated at room temperature for 10 minutes, then centrifuged at maximum speed for 15 minutes at 4°C. The supernatant was removed and ethanol 75% 200 µl was added. Samples were centrifuged at maximum speed at 4°C for 10 minutes, ethanol was removed and samples were air dried at room temperature. RNA was eluted in 50 µl of distilled molecular water.

DNA was isolated according to the following protocol: Tissue was incubated at 50°C in TNE (10mM Tris, 100mM NaCl, 1mM EDTA) 1x/0.5% sodium dodecyl sulphate and proteinase K (Invitrogen, USA) (100µg/ml) for one week. Fresh proteinase K 2-5 µl was added every other day to speed up the digestion. When all tissue was gone, phenol 500 µl was added and rotated on the shaking plate for 15 minutes. Samples were centrifuged at maximum speed for 20 minutes at room temperature. The top aqueous layer was placed in a new tube; RNase A (2mg/ml) 2µl (Qiagen, Netherlands) was added and stored at room temperature for 30 minutes. Phenol 500 µl was added and mixed as above. Samples were centrifuged at maximum speed for 15 minutes, the top aqueous layer was placed in a new tube and ethanol 100% was added. Samples were stored overnight at -20°C and then centrifuged at maximum speed for 30 minutes at 4°C. Ethanol was removed and ethanol 70% added.

Samples were centrifuged for 30 minutes, ethanol was removed and samples were air-dried. DNA was eluted in 50 µl of distilled molecular water.

DNA and RNA quantity and quality were assessed using a spectrophotometer (NanoVue Plus, GE Healthcare Life Sciences, UK).

Polymerase Chain Reaction (PCR)

To amplify each individual emerin exon (1-6) including the intron/exon boundaries, primers were designed from genomic sequence of the emerin gene using the human genome browser gateway (<http://genome.ucsc.edu/cgi-bin/hgGateway>) (Fig.1.1, Table 1.1) (Bione et al., 1995).

PCR was performed in a total volume of 20µl using 100 ng of genomic DNA as template and 0.1µl each of forward and reverse primers at a concentration of 100µmole. Final reagent concentrations in the PCR reaction mixture (1.1x ReddyMix PCR Master Mix 1.5mM MgCl₂- Thermo Scientific, USA) were ThermoPrime Taq DNA Polymerase 0.625 units, Tris-HCl 75mM, (NH₄)₂SO₄ 20 mM, MgCl₂ 1.5mM, 0.01% Tween 20, dATP, sCTP, dGTP, and dTTP 0.2mM.

After denaturation at 95°C for 3 minutes, 35 cycles followed each consisting of 95°C for 1 minute, 60°C for 1 minute, and 72°C for 90 seconds. Elongation followed at 72°C for 10 minutes. The PCR products were run in a 2% agarose gel for size analysis and in a 0.8% agarose gel for purification. PCR products were purified from the gel using NucleoSpin Extract II kit (Macherey-Nagel, Germany). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer has been used as control. All 6 exons have been amplified for all patients and controls.

Specific primers to cover the entire first exon, including the 5'-untranslated region (5'-UTR) have been designed that is otherwise not covered by primers used also for diagnosed purpose as this is a CG-rich region and difficult to sequence. AccuPrime CG-rich DNA polymerase (Thermo Scientific, USA) was used to amplify the 5'-UTR CG-rich region.

Figure 1.1

Chromosome X graphic illustration from Human genome browser. Red vertical bar shows position of emerin gene in Xq28. Below chromosome X, emerin genes exons and introns are illustrated. Graphic illustration of emerin gene and region of coverage of primers (GEMD1-6).

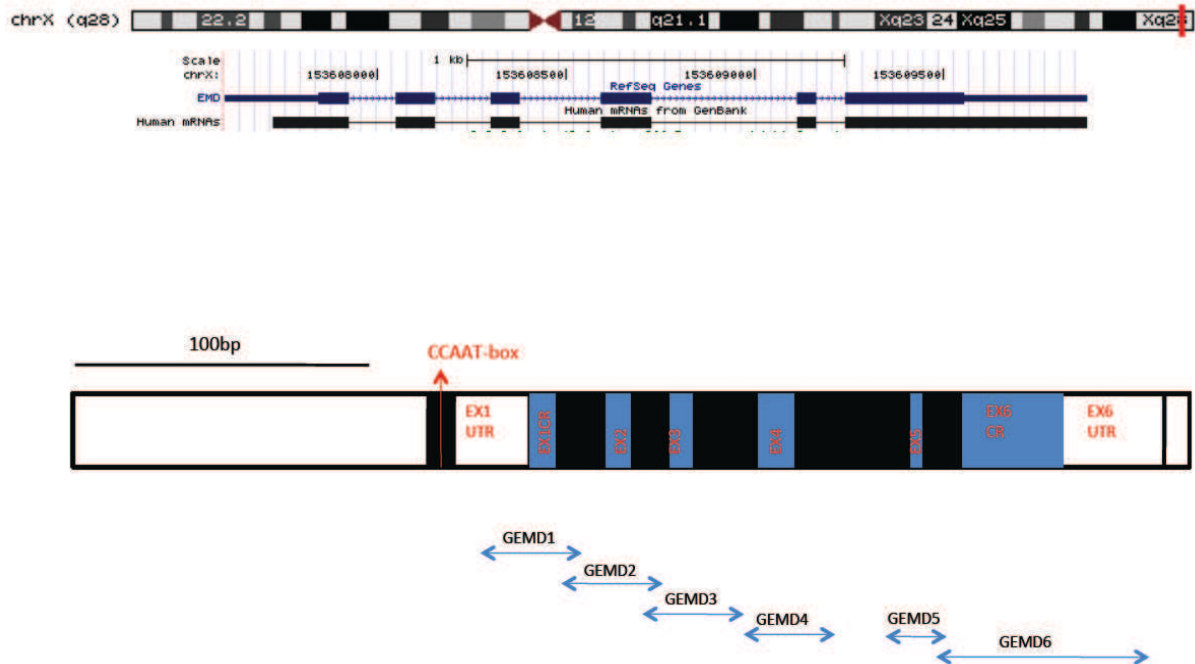


Table 1.1: Primers for emerin gene

Exon	Primer name	Sequence	Size (bp)
1	GEMD1 F	5'-AGCGGCCGTGACGCGACAACG-3'	320
	GEMD1 R	5'-GAGGTCGCGAGGCGGAGC-3'	
2	GEMD2 F	5'-CGCGACCTCCCCGCTGCC-3'	245
	GEMD2 R	5'-TGCCGCTCTCGACCGGCC-3'	
3	GEMD3 F	5'-GTCGAGAGCGGCACTGGA-3'	213
	GEMD3 R	5'-GTCTCAGGTCCTCCCTGTGAGC-3'	
4	GEMD4 F	5'-CCATCAGGCCAGGCGGGG-3'	216
	GEMD4 R	5'-TAGGTTGTACCCAAGTACAGG-3'	
5	GEMD5 F	5'-GTGGGTTCTGGCCTCTAACC-3'	200
	GEMD5 R	5'-GGAGCCTGGACCCAGAACC-3'	
6	GEMD6 F	5'-GCTCCTGGCCCACTTGCTCC-3'	396
	GEMD6 R	5'-CTAAGGCAGTCAGCCAGGAC-3'	
1 5'-UTR	EMD F	5'-CAGCCTCCAGCTCTCAG-3'	300
	EMD R	5'-TCGGTATCCGAAGATCTGC-3'	
	GAPDH F	5'-TGATGACATCAAGAAGGTGGTGAAG-	300
	GAPDH R	3' 5'-TCCTTGGAGGCCATGTGGGCCAT-3'	

F: forward; R: reverse

Cloning into chemically competent cells

The purified PCR products of exon 1 have been cloned into chemically competent cells using the TOPO TA pCR 2.1 Cloning kit (Invitrogen, UK).

Sanger sequencing

Fifty ng of the purified PCR product were sequenced by the DNA sequencing service in the MRC CSC Genomics Core Laboratory (Hammersmith Campus-Imperial College London).

Mutation interpretation analysis

I used the Alamut software (<http://www.interactive-biosoftware.com/alamut.html>) to interpret genomic mutations.

Pathway analysis to identify proteins interacting with emerin

I performed a pathway analysis to identify proteins interacting with emerin using String 9.0 database (<http://string-db.org/>) available online. String is a database of known and predicted protein interactions. The interactions include direct and indirect associations, derived from four different sources (genomic context, high-throughput experiments, co-expression and previous knowledge).

RESULTS

Chromowave

In silico analysis of expression microarray obtained from muscle of patients with PM, DM, sIBM and healthy controls, downloaded from GEO dataset (<http://www.ncbi.nlm.nih.gov/gds?term=myositis>) showed a statistically significant expression of several genes in sIBM compared to healthy controls (t-test 2.10596E-07) (Figure 1.1a, b), in PM versus healthy controls (t-test 1.26909E-06) (Figure 1.1c, d) and DM versus healthy controls (t-test 1.44259E-05) (Figure 1.1e, f).

Similarities between the three patterns of Chromowave analysis in sIBM, PM and DM microarray, involving mainly chromosome 6, showing predominantly up-regulations of genes involved in inflammation, were observed and confirmed previous reports of up regulation of cytokines, MHC class I and II molecules, granzymes, and immunoglobulins. Individual analysis of each gene expression on each chromosome compared for the three different conditions analysed allowed me to identify a down-regulation of the gene coding for emerin (EMD) in Xq28 in sIBM (expression level -0.74) compared to healthy controls and to DM (expression level -0.002) and PM (expression level 0.009) (Figure 1.2a, b).

A standard analysis confirmed low expression of EMD gene in sIBM compared to healthy controls, PM and DM.

No difference in expression was found for genes encoding for proteins interacting with emerin (Table 1.2).

Table 1.2: Expression of genes encoding for proteins interacting with emerin

Proteins interacting with emerin		Chromowave expression level sIBM vs control	Chromowave expression level DM vs control	Chromowave expression level PM vs control
EMD	Emerin	-0.742974	-0.002	0.009
LMNA	Lamin A/C	0.23814	-0.09	0.003
BANF1	Barrier-to-autointegration factor	0.20859	-0.05	0.009
LMNB1	Lamin B1	No difference	No difference	No difference
BCLAF1	Bcl-2-associated transcription factor 1	No difference	No difference	No difference
GUCA2A	Guanylin precursor	No difference	No difference	No difference
LEMD3	Inner nuclear membrane protein Man1	-0.082796	0.043	-0.08
SYNE2	Nesprin-2	-0.12016	0.019	0.002
LMNB2	Lamin B2	No difference	No difference	No difference
LMO7	LIM domain only protein 7	-0.275603	0.06	0.015
GUCY2C	Heat-stable enterotoxin receptor precursor	No difference	No difference	No difference

Figure 1.1: Cases obtained from GEO dataset. T-test between (a) sIBM cases and healthy control subjects; (c) Healthy controls and PM; (e) healthy controls and DM; (b, d, f) **chromosomal expression pattern extracted from Chromowave analysis** respectively for the three different conditions analysed. sIBM versus healthy control subjects (b); PM versus healthy control subjects (d); DM versus healthy control subjects (f). X-axis: gene distance on every chromosome; y-axis: gene differential expression (intensity and direction, log2 scale). In b and d positive values represent mRNA down-regulation in sIBM and PM patients respectively, while in f positive values represent mRNA up-regulation. Red arrow pointing to the emerlin gene. (next page)

Fig.1.1a T-test between sIBM cases and healthy control subjects

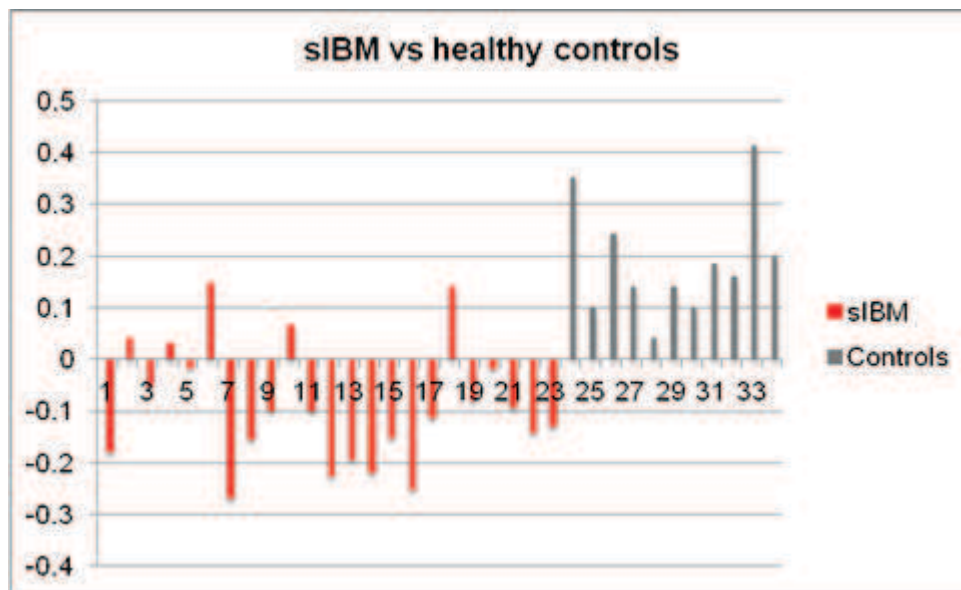


Fig.1.1b

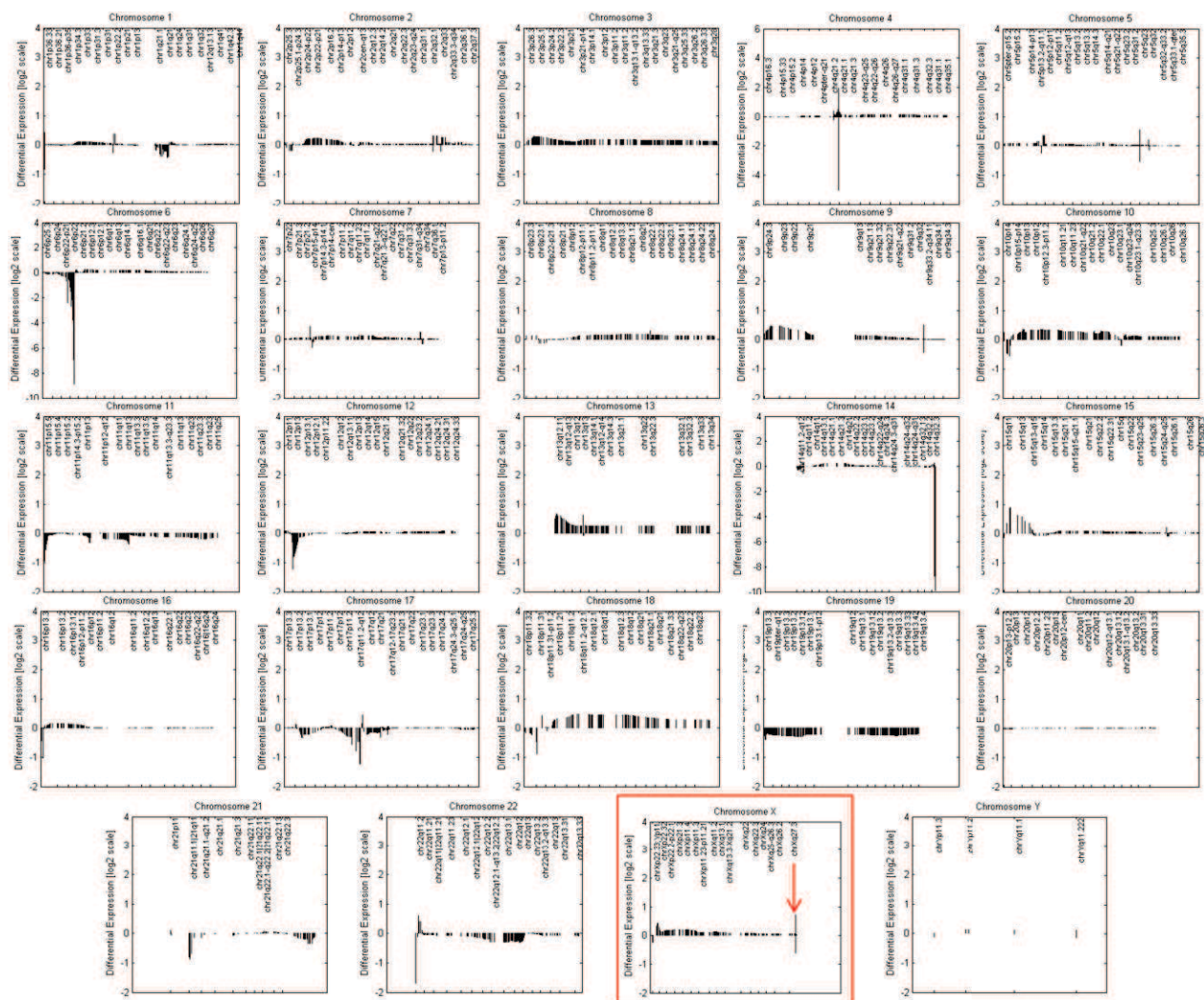


Fig.1.1c T-test between healthy controls and PM

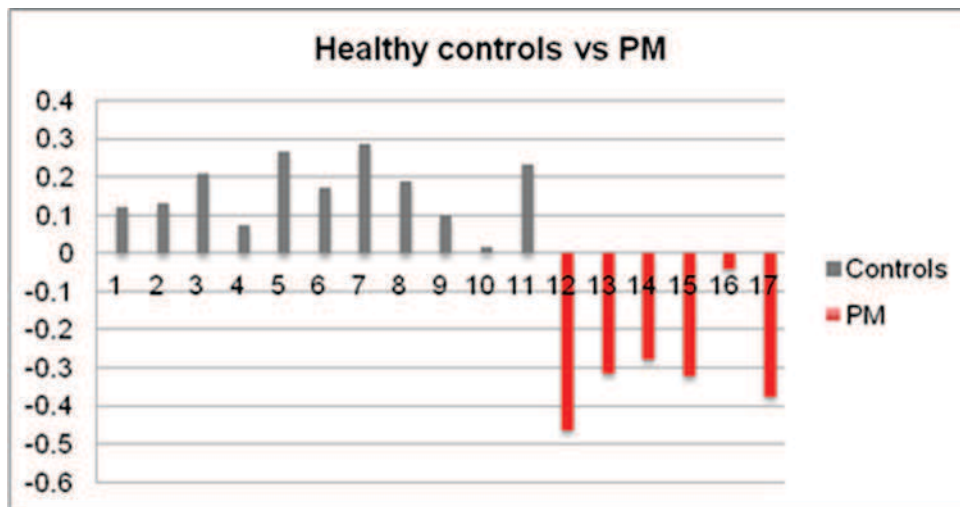


Fig.1.1d

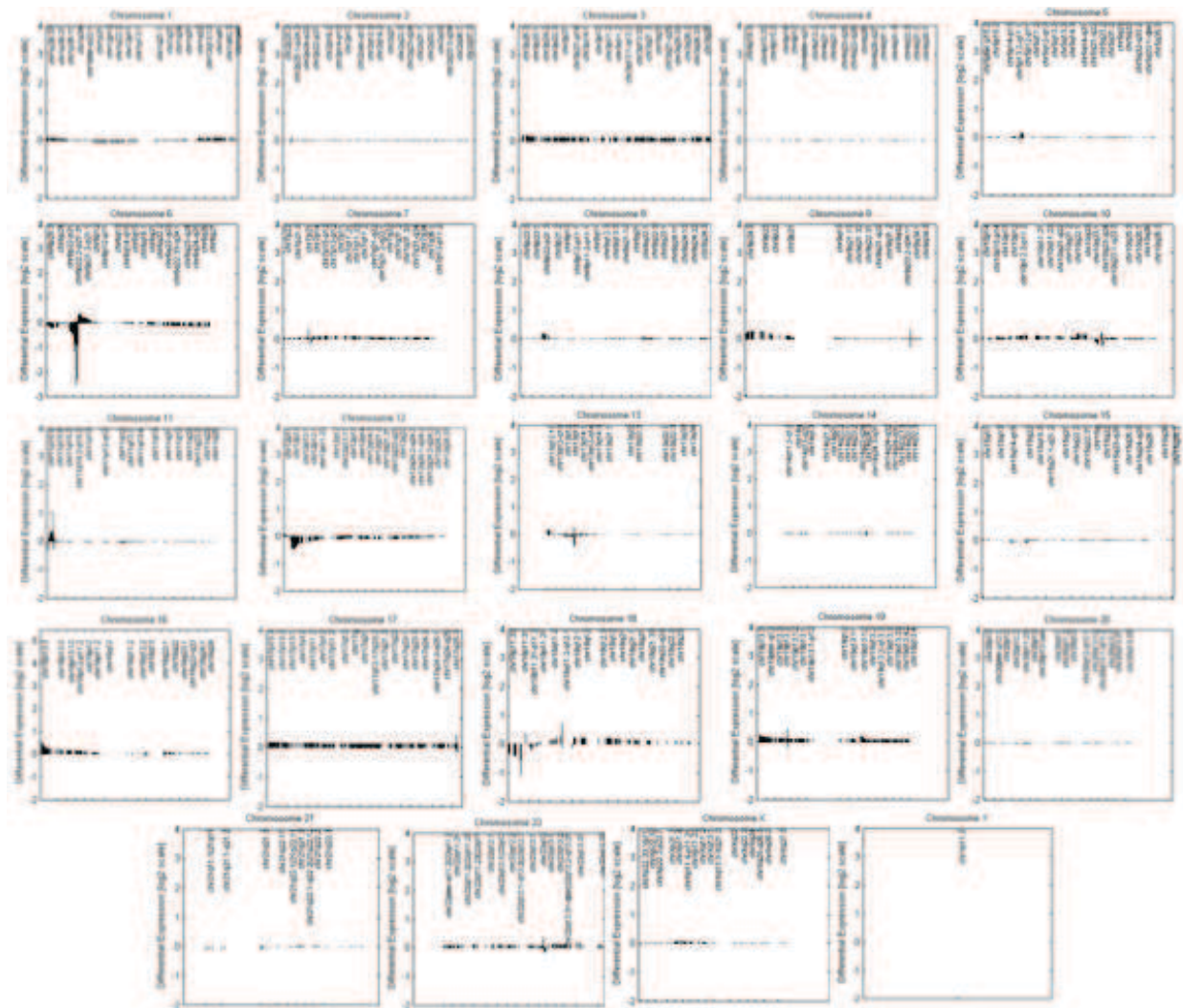


Fig.1.1e T-test between healthy controls and DM

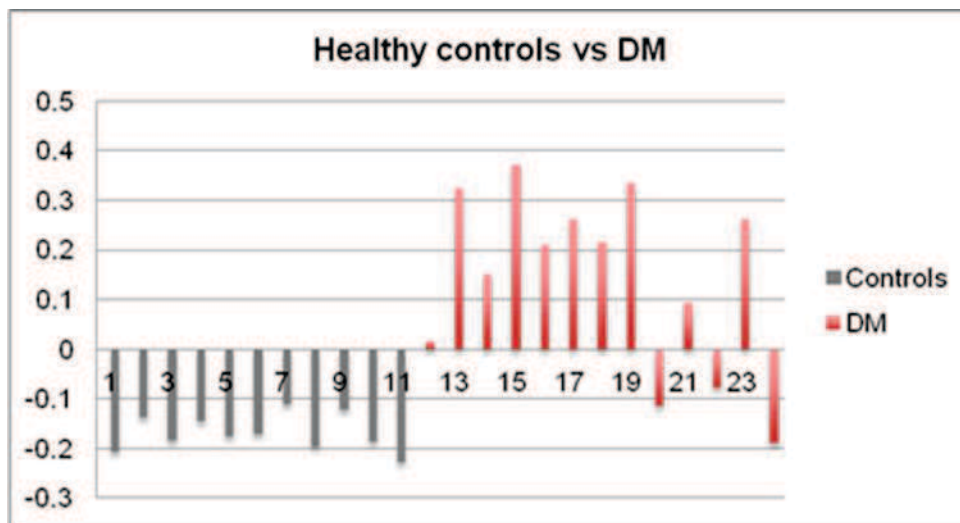


Fig.1.1f

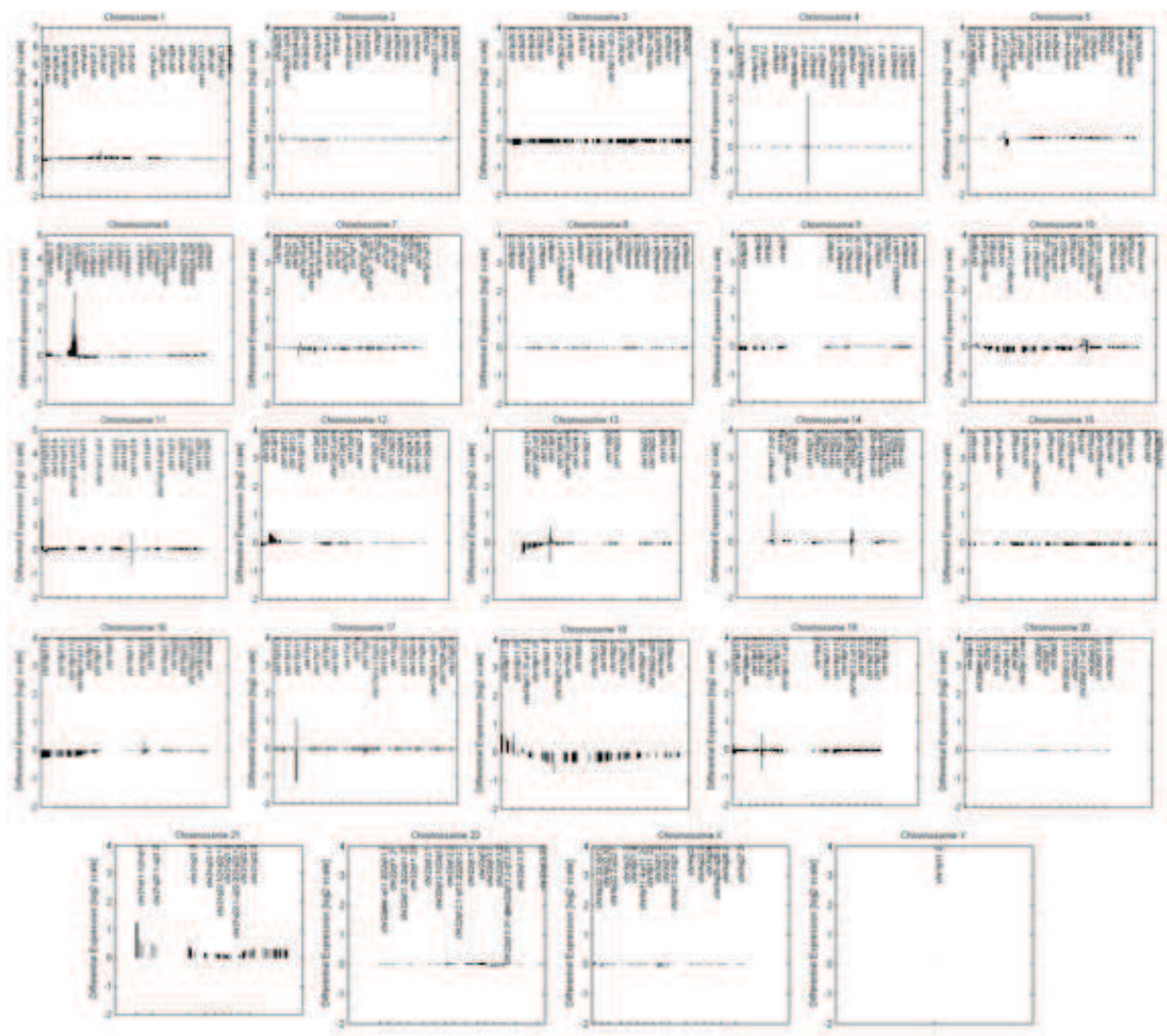


Figure 1.2a: Changes in expression of genes located on chromosome X in sIBM, DM and PM compared to healthy controls extracted from Chromowave analysis.

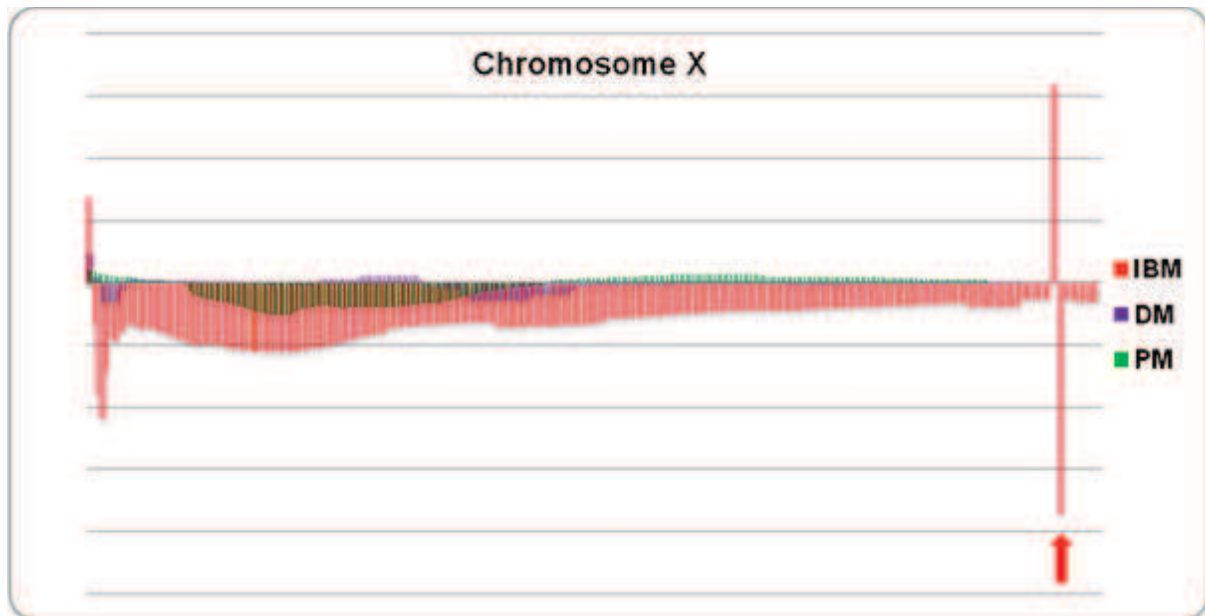
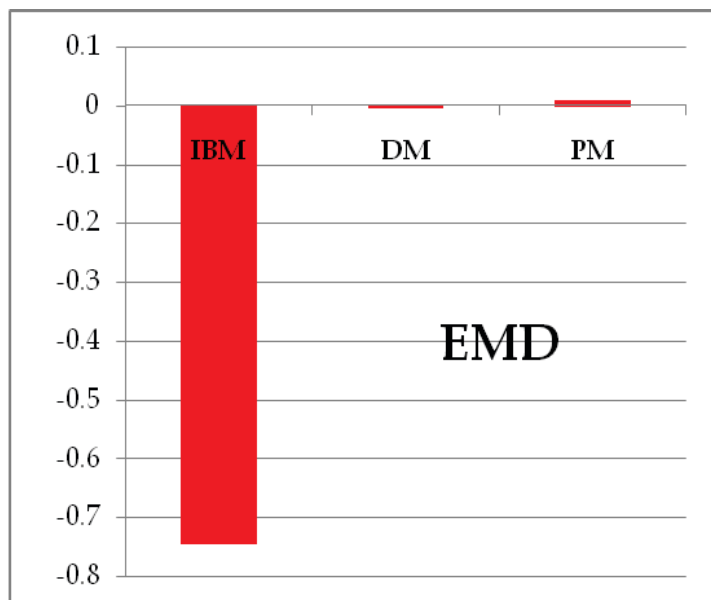


Figure 1.2b: Comparison of EMD gene expression in sIBM, DM and PM extracted from Chromowave analysis.

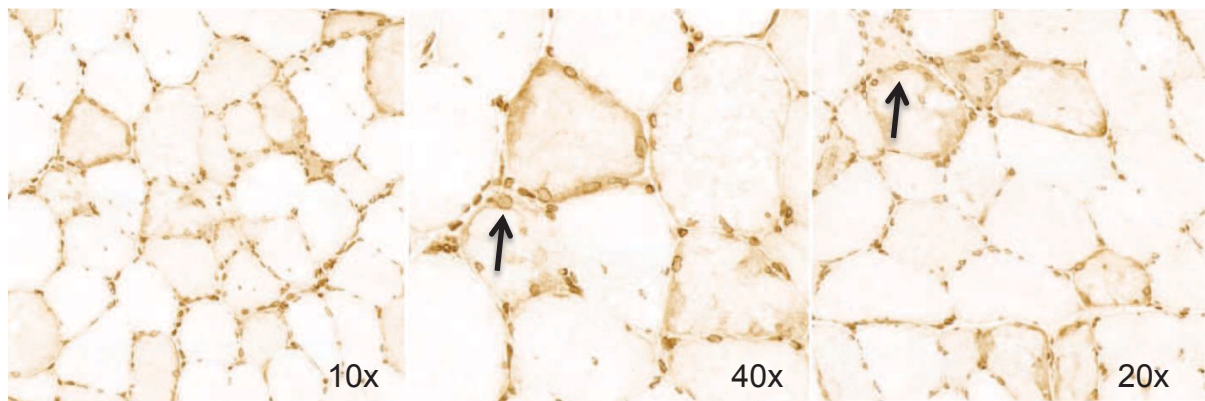
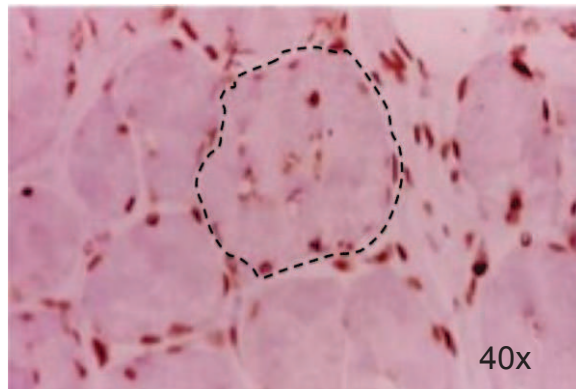


Immunohistochemistry and Immunofluorescence

Immunohistochemical and immunofluorescence stains with anti-emerin antibody revealed abnormal immunolabelling of emerin in myonuclei of sIBM patients compared to control muscle and positive immunolabelling in RVs (Figure 1.3a, b). Myonuclei stained with emerin appear like ground glass (Figure 1.3a) in sIBM patients. Further to this there is also positive sarcolemmal labelling of emerin in several fibres.

Figure 1.3a: Examples of immunoperoxidase staining using anti-emerin antibodies (Novocastra 1:100), weak immunolabelling in some nuclei, positive immunolabelling in RVs (dotted line highlighting one of the fibres with RVs) in sIBM patients as ground glass appearance of nuclei (arrow) (A) and normal immunolabelling in controls (B)

A



B

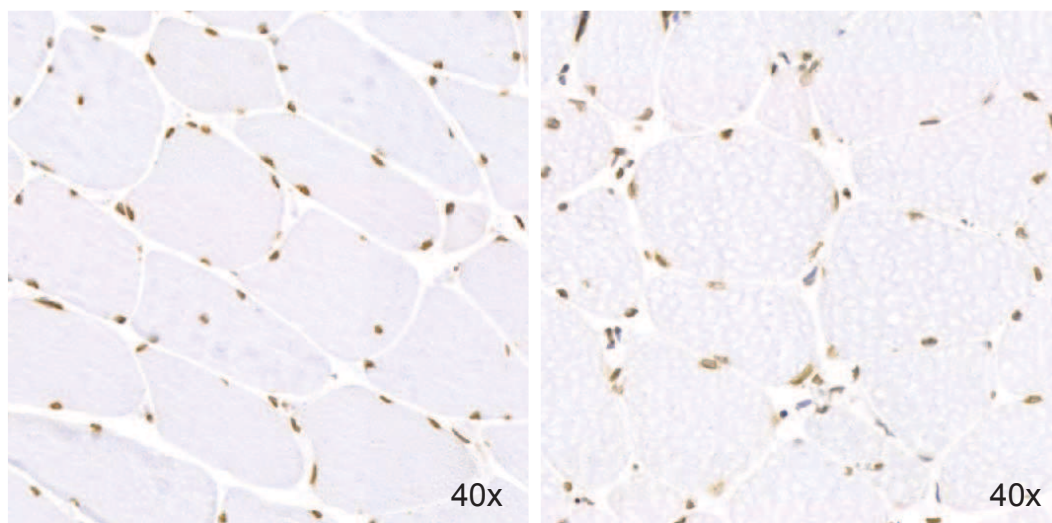
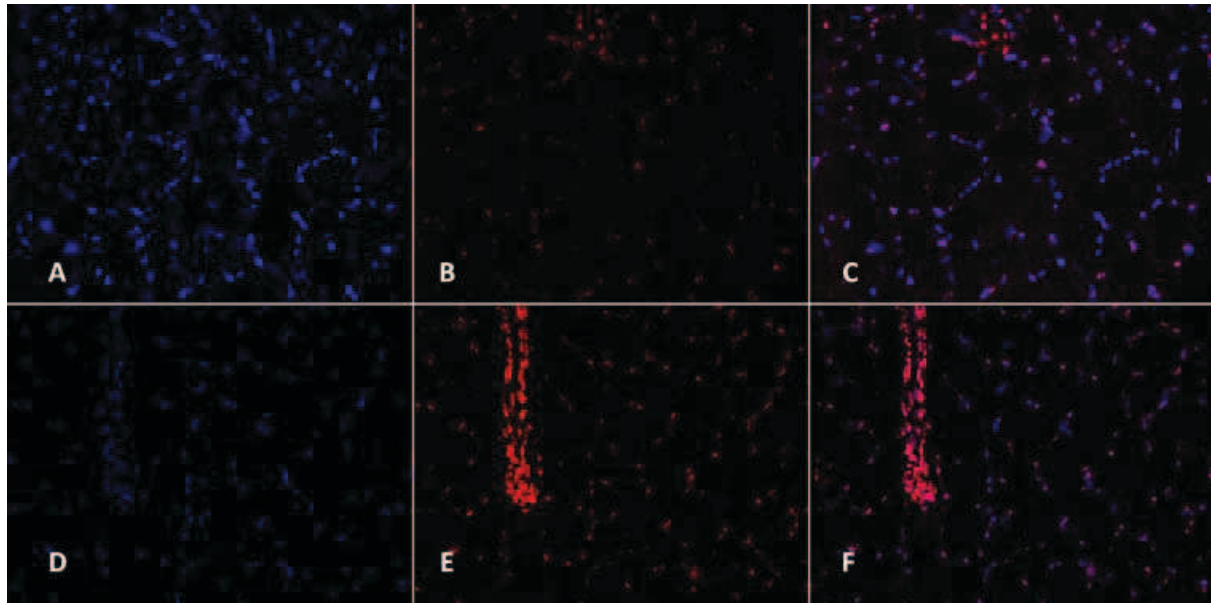


Figure 1.3b: Immunofluorescence study of emerin expression in sIBM (A, B, C) and DM (D, E, F), (20x). In **E** and **F** a blood vessel shows strong positive labelling of nuclei. **A** & **D**, Dapi; **B** & **E**, anti-emerin antibody (LabVision 1:100); **C** & **F**, merged.

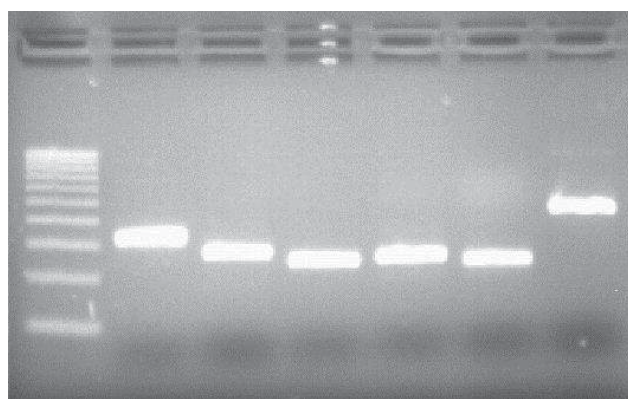


PCR

PCR for genomic DNA using GEMD1-6F/R primers has been performed on all patients and controls. For each exon the PCR product was of the expected molecular size in all patients (Fig.1.4).

Figure 1.4: Agarose gel showing PCR product for exon1-6 of the emerin gene.

Marker Exon1 Exon2 Exon3 Exon4 Exon5 Exon6



Sequencing and mutation interpretation analysis

Sanger sequencing was performed at the MRC CSC Genomics Core Laboratory (Hammersmith Campus-Imperial College London). Sequencing of emerin exon 1 revealed two point mutations (c.1-77C>T and c.1-5C>T) in the 5'-untranslated region (5'-UTR) in 2 different clones of patient 1. *In silico* analysis using Alamut software to predict mutation pathogenicity failed to show any predictable consequences.

These results prompted me to further investigate the significance of these mutations. I designed further primers to cover the entire emerin gene, including the 5'-UTR. The 5'-UTR region is a CG-rich region and hence can be particularly difficult to sequence. Sequencing of this region was performed initially with the same polymerase used for all other sequencing experiments in this section. This was then repeated a specific polymerase for GC-rich regions, AccuPrime GC-rich DNA polymerase (Thermo Scientific, USA). This is a robust enzyme designed for regions with a CG content

>65%.

Sequencing analysis was then performed on further 5 sIBM, 5 DM/PM and 5 normal control subjects. Sequencing analysis revealed single nucleotide substitutions in the 5'-UTR in all sIBM patients tested, in 3 DM/PM and in none of the healthy controls (Table 1.3). No mutations were identified in exon 2-6 of the emerin gene.

Table1.3 Sequencing results of the first exon of the emerin gene, including the 5'-UTR region showed that patients had various SNPs in the 5'-UTR region.

Patient	Identified variant in 5'UTR of EMD gene
Patient 1 (sIBM)	c.1-145A>G
Patient 2 (sIBM)	c.1-125T>C
Patient 3 (sIBM)	c.1-90C>T
Patient 4 (sIBM)	c.1-51T>C
Patient 5 (sIBM)	c.1-63T>C
Patient 6 (DM/PM)	c.1-69C>T
Patient 7 (DM/PM)	c.1-149T>C
Patient 8 (DM/PM)	c.1-198A>G

***In silico* analysis for mRNA secondary structure prediction using GeneBee**

In silico analysis using GeneBee database to predict mRNA secondary structure changes due to point mutations in -77C>T and -5C>T showed possible secondary structure changes (Figure 1.4a, b, c).

Figure 1.4a: mRNA emerlin predicted secondary structure

Free Energy of Structure = -354.9 kkal/mol

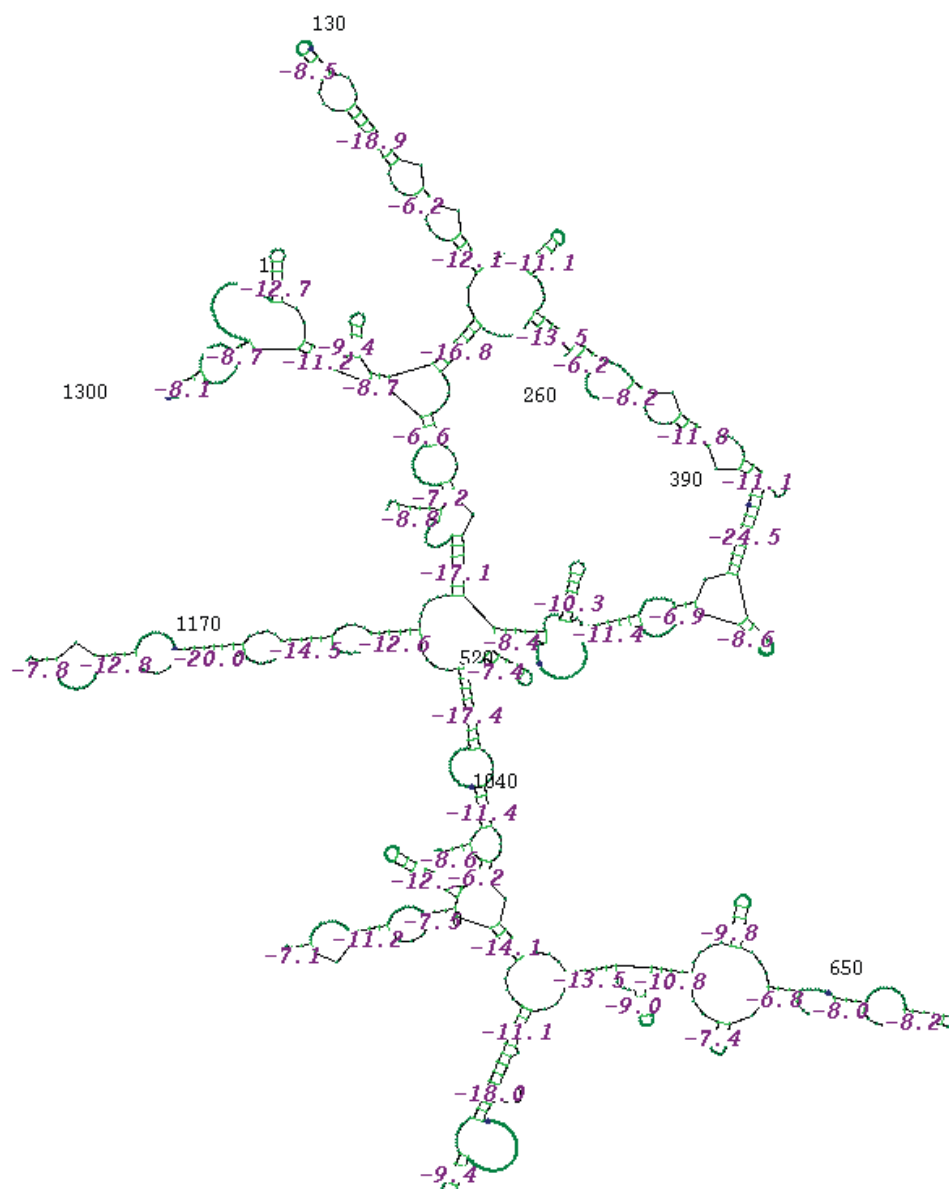


Figure 1.4b: mRNA emerlin predicted secondary structure with mutation in - 77C>T

Free Energy of Structure = -354.5 kkal/mol

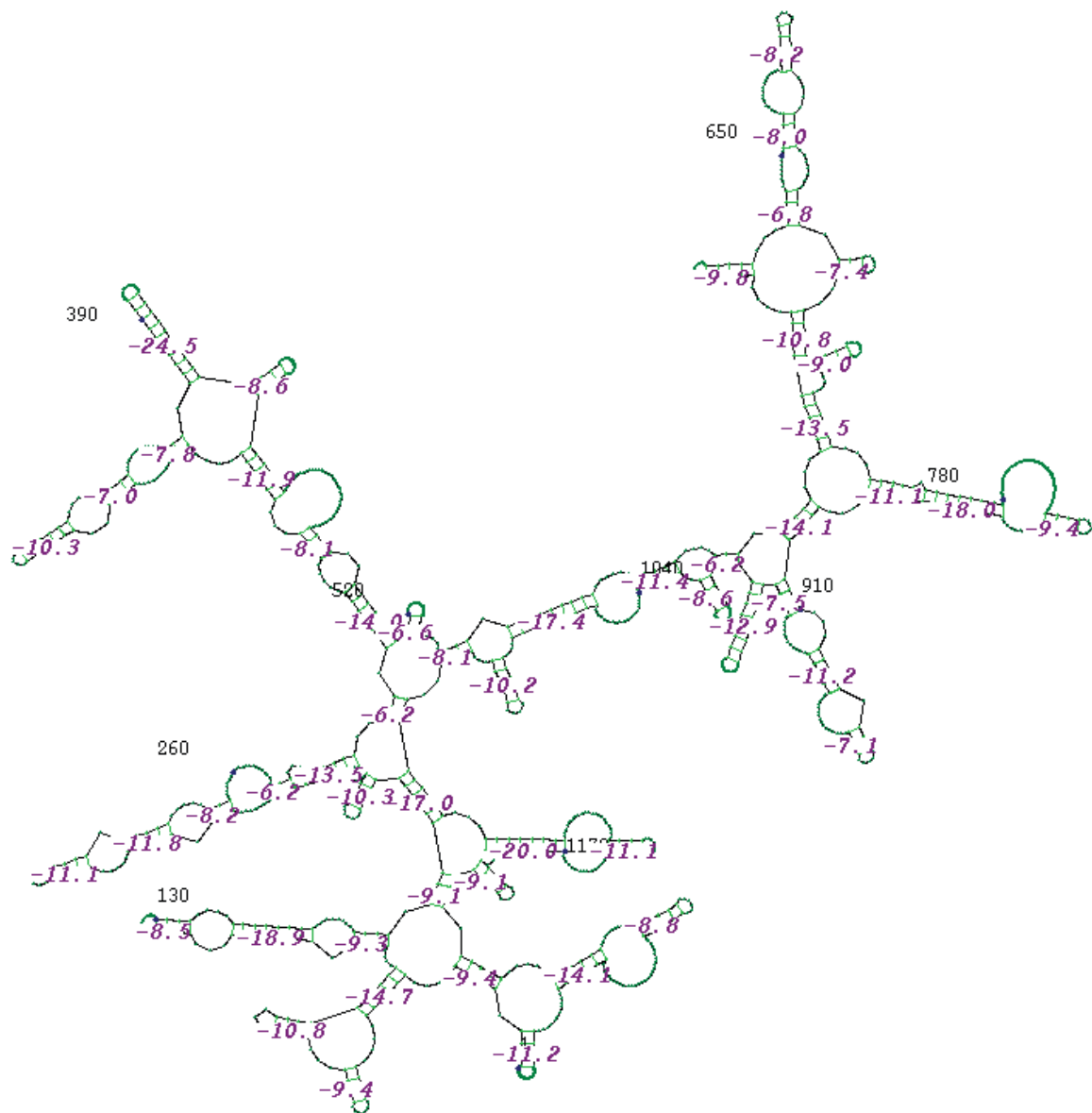
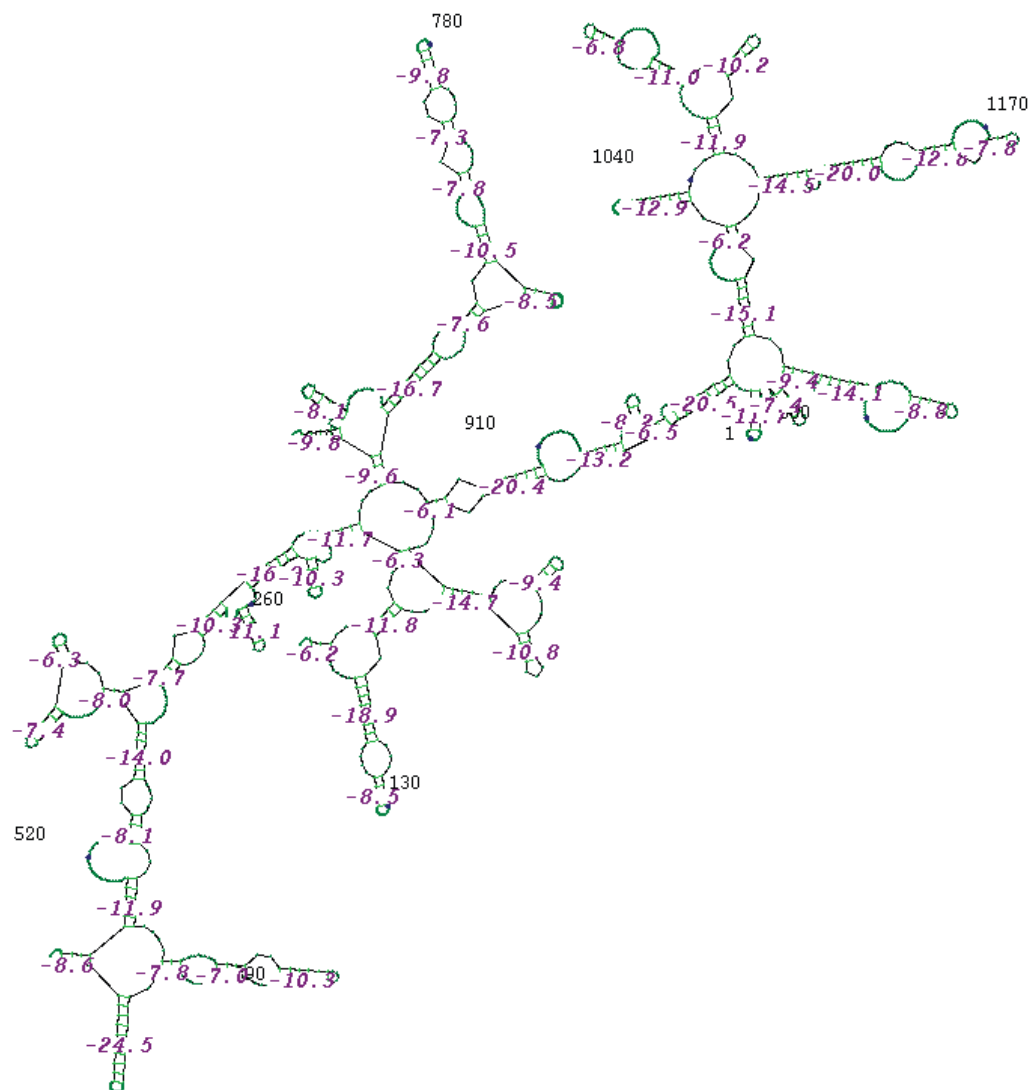


Figure1.4c: mRNA emerlin predicted secondary structure with mutation in - 5C>T

Free Energy of Structure = -365.3 kkal/mol



Pathway analysis to identify proteins interacting with emerin using String database

According to String database emerin interacts directly and indirectly with several proteins residing at the nuclear envelope, in the nucleus and cytoplasm (Figure 1.5). Predicted functional partners are specified in Table 1.4.

Figure 1.5: Emerin close interaction network extracted from String database.

The different line colours represent the types of evidence for the association. Colour legend is reported in the table below. Evidence is in most cases based on experiments and textmining.

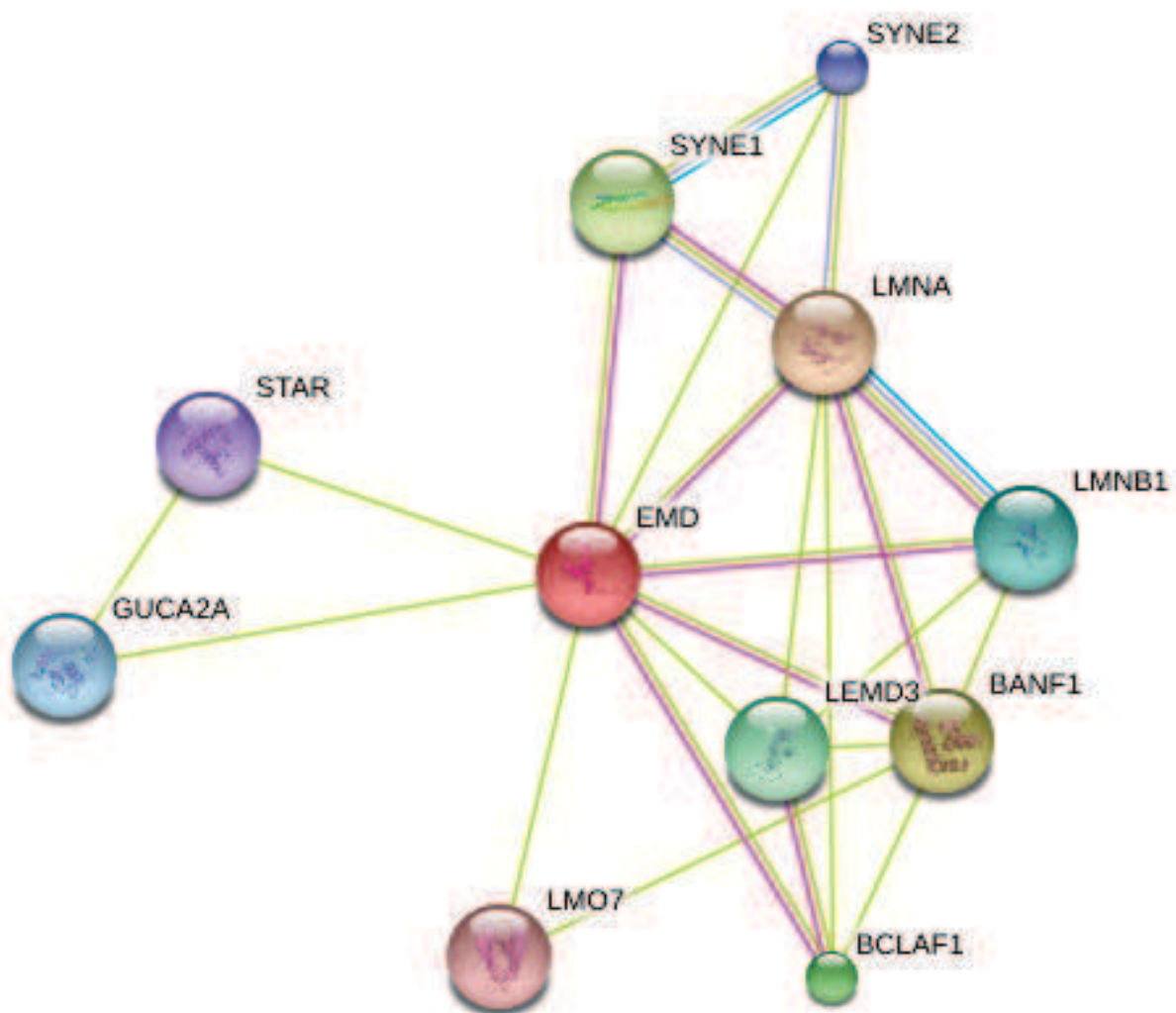


Table 1.4 Predicted Emerin Functional Partners extracted from String database

		Neighborhood	Gene Fusion	Cooccurrence	Coexpression	Experiments	Databases	Textmining	[Homology]	Score
	<u>LMNA</u>	lamin A/C; lamins are components of the nuclear lamina (664 aa)						•	•	0.999
	<u>BANF1</u>	barrier to autointegration factor 1; plays fundamental roles in nuclear assembly and chromatin organisation (89 aa)						•	•	0.999
	<u>SYNE1</u>	spectrin repeat containing, nuclear envelope 1; involved in the maintenance of nuclear organisation (8797 aa)						•	•	0.979
	<u>BCLAF1</u>	BCL2-associated transcription factor 1; death-promoting transcriptional repressor (920 aa)						•	•	0.966
	<u>LEMD3</u>	LEM domain containing 3; can function as a specific repressor of TGF-beta, activin, and BMP (911 aa)							•	0.965
	<u>LMNB1</u>	lamin B1 (586 aa)						•	•	0.964
	<u>GUCA2A</u>	guanylate cyclase activator 2A (guanylin); endogenous activator of intestinal guanylate cyclase (115 aa)							•	0.960
	<u>SYNE2</u>	spectrin repeat containing, nuclear envelope 2; involved in the maintenance of nuclear organisation (6907 aa)							•	0.952
	<u>STAR</u>	steroidogenic acute regulatory protein; plays a key role in steroid hormone synthesis (285 aa)							•	0.945
	<u>LMO7</u>	LIM domain 7 (1349 aa)							•	0.926

DISCUSSION TO CHAPTER 1

Changes in emerin gene expression seen in sIBM with Chromowave (Turkheimer et al., 2006) analysis and further supported by immunohistochemistry and immunofluorescence studies are in agreement with well-known sIBM features like morphologically abnormal myonuclei and with previous studies that identified nuclear proteins within RVs suggesting a nuclear damage. This finding highlights the complexity of sIBM and is consistent with the idea that sIBM is a degenerative myopathy rather than a primary inflammatory myopathy.

Chromowave analysis showed a statistically significant difference between the sIBM and control groups. In particular, expression of nuclear envelope protein emerin mapping on Xq28 was significantly lower compared to healthy controls and to DM and PM. No difference in expression was found for genes coding for the other proteins of the nuclear envelope including proteins interacting with emerin. In contrast, the expression of gene involved in the inflammatory response, including MHC on chromosome 6 was up regulated, as expected.

To look at the localization of emerin in sIBM patients, I then selected 10 biopsies with typical features of sIBM, 5 of DM, 5 PM and 75 control muscles from the Imperial College Healthcare Tissue Bank. As normal controls, we considered patients who underwent a muscle biopsy with a suspicion of vasculitis or with myalgia as only symptom but that appeared normal at pathological examination. Cases were stained for emerin using immunoperoxidase. Similar to previous studies (Karpati & Carpenter, 1993; Askanas & Engel, 2003; Raju & Dalakas, 2005; Greeberg et al, 2005, 2006; Greenberg 2007; Fidzianska et al, 2008; Nakano et al, 2008; Parker et al, 2009; Nishii et al, 2011) and in keeping with *in silico* analysis, we observed abnormal subsarcolemmal and intranuclear localization of the protein and accumulation in RVs.

Previous studies showed cases of X-linked Emery-Dreifuss Muscular Dystrophy, in which patients presented a typical pathological picture of sIBM (inflammatory infiltrate, Rvs, amyloid deposits, cytoplasmic and nuclear tubulo-filamentous inclusions), suggesting that sIBM pathological aspects are non-specific and probably present also in other neuromuscular disorders (Fidziańska et al., 2004, 2008).

The nuclear envelope (NE) is composed of inner and outer nuclear membranes (INM and ONM), nuclear pore complexes and the lamina, a thin fibrous support structure. The nuclear lamina and INM proteins are involved in fundamental nuclear activities like maintaining and integrating cell shape, anchoring chromatin, association with nuclear bodies, localization to and function in sites of DNA replication and mediating transcription. The nuclear lamina is composed mainly of nuclear lamins, lamin A type and B type (Stuurman et al. 1998). Several proteins have now been identified that specifically localize to the inner nuclear membrane. Almost all of the INM proteins have a large nucleoplasmic domain capable of binding lamins, chromatin or both. The NE has an important physiologic and pathologic role. Indeed, mutations in NE proteins are responsible for a group of diseases termed “nuclear envelopathies”. The clinical phenotype of these diseases is variable, ranging from cardiac and skeletal myopathies and partial lipodystrophy to peripheral neuropathy and premature aging. In this project *in silico* analysis showed low expression of emerin gene in sIBM, therefore I performed a pathway analysis using String database to highlight emerin’s direct and indirect interactions with other proteins and as expected the interaction network is very complex and vast (Figure 1.5).

Emerin is a 34 kDa, type II integral membrane protein, residing principally at the inner nuclear membrane (INM) (Manilal et al., 1998) and belongs to a family of nuclear proteins (LAP2, MAN1). Each member of this family has a 40 amino acids LEM (**L**AP2, **E**merin, **M**AN1) domain that binds barrier-to-autointegration factor (BAF) (Lee et al., 2001; Haraguchi et al., 2001), a conserved chromatin protein. Emerin interacts with proteins that are not only residing at the NE. Table 1.5 summarizes proteins interacting with emerin and their main function.

Table 1.5 Proteins interacting with emerin

Proteins interacting with emerin	Protein role in the interaction with emerin
Lamin A/C	Lamins form a stress-resistant structural support to the nuclear membranes. Emerin interacts in particular with lamin A.
Nesprins	Nesprin 1 and 2 bind both emerin and lamins A/C and form a network linking the nucleoskeleton to the INM, the ONM, membraneous organelles, the sarcomere and the actin cytoskeleton.
SUN1 and 2	SUN-emerin interaction occurs at the INM
LUMA	LUMA is an integral membrane protein that spatially and functionally organises protein complexes of the INM. LUMA interacts with emerin and participates in controlling its distribution along the nuclear membrane.
β -catenin	Down-stream target of the Wnt signalling pathway. Emerin is responsible for β -catenin export from the nucleus.
BAF	DNA binding protein. Binds emerin through LEM domain during telophase, important step for re-establishment of nuclear lamina.
β -tubulin	Cytoplasmic and nuclear protein. Interacts with emerin to anchor the centrosome at the ONM.

To date at least 70 different emerin mutations have been published and shown to be pathogenic, but mutations in the 5'-UTR has never been described (<http://www.hgmd.cf.ac.uk/ac/gene.php?gene=EMD>) perhaps because primers generally used for diagnostic do not cover the 5'-UTR.

Initial sequencing analysis of patient 1 showed two different point mutations in the 5'-UTR. *In silico* analysis using Alamut software to predict mutation pathogenicity did not show any predictable consequences. However, *in silico* analysis to predict secondary mRNA structure using GeneBee software showed that the mutations may possibly affect the secondary mRNA structure and hence translation. Sequencing analysis was then performed in 5 sIBM, 5 DM/PM and 5 normal control subjects. Sequencing analysis revealed single nucleotide substitutions in the 5'-UTR in 6/10 sIBM patients, 3 DM/PM and none of the healthy controls. No mutations in exon 2-6 were found.

The 5'-UTR region is the sequence that extends from transcription start site to just before the ATG translation initiation codon (Chatterjee & Pal, 2009). The UTRs are highly structured GC-rich sites composed of upstream open reading frame and internal ribosomes entry sites. The UTRs are site for protein expression regulation at the level of translation. Point mutations and truncations in UTRs may affect translation (Chatterjee & Pal, 2009).

Pathway analysis showed that emerin interacts with β -catenin, which is a downstream target of the Wnt-signalling pathway. Previous studies in sIBM suggested already a crucial role of the Wnt-signalling pathway in sIBM pathology (Morosetti et al., 2008). This pathway is involved in many diseases (i.e. colon cancer); the Wnt-signal promotes intranuclear translocation of β -catenin where it binds to transcription factor proteins and activates a transcription coactivation factor (Cadigan & Peifer, 2009). Emerin is responsible for β -catenin nuclear export and therefore influences β -catenin nuclear accumulation and reduced levels of emerin can lead to β -catenin nuclear accumulation suggesting a role of the Wnt-signalling pathway in sIBM (Markiewicz et al, 2006; Morosetti et al, 2008; Cadigan & Peifer, 2009).

Intranuclear β -catenin accumulation could be responsible for glycogen synthase kinase-3 β (GSK3 β) dysregulation. Indeed, GSK3 β is part of a cytoplasmic

destruction complex and is activated when bound to β -catenin. Dysregulation of GSK3 β could have a role in β -amyloid accumulation (Morosetti et al., 2008). In order to validate these studies and identify a diagnostic marker for sIBM, this pathway was explored as a separated project in Dr Roncaroli's laboratory by an MRes student, who I co-supervised, and we performed immunohistochemistry analysis for β -catenin on 50 consecutive cases of IIM including 10 sIBM. These immunostains did not prove any nuclear accumulation in sIBM patients (data not shown as per Imperial College London regulations). This might have been dependant on different technical approach since Morosetti and co-workers (2008) performed their studies on cell cultures from sIBM patients.

In conclusion, using an *in silico* approach I tried to identify target molecules that could shed light on the pathophysiology of sIBM. *In silico* analysis of already published expression microarray suggested that emerin could be a valid candidate, which led me to further explore its expression using immunochemistry and sequencing analysis.

Interestingly, immunochemistry studies revealed abnormal localization of emerin at subsarcolemmal level, intranuclear level and nuclei showed ground glass appearance without the typical rim observed normally with emerin staining. Emerin also stained positively in RVs as already reported in previous studies (Fidzianska et al, 2010). If further confirmed at larger scale, this finding suggests that emerin staining could be used as biomarker for sIBM diagnosis.

Sequencing analysis of the emerin gene revealed single nucleotide substitutions in the 5'-UTR of the emerin gene in all sIBM patients and three disease control subjects but none of the controls. As previously mentioned this specific region is usually not sequenced for diagnostic purpose. However, in recent years the UTR regions are gaining more interest as being responsible for protein expression regulation at translation level. Sequencing analysis of a larger sample is needed to further confirm this result.

CHAPTER 2

IDENTIFICATION OF A NOVEL AUTOANTIBODY AGAINST A SARCOLEMMAL ANTIGEN IN THREE PATIENTS WITH IIM

INTRODUCTION TO CHAPTER 2

IIM are heterogeneous, multi-factorial acquired conditions that can potentially cause irreversible muscle damage with resulting long-term and often severe disability. The activation of the immune system likely occurs in genetically predisposed individuals. More than 50% of individuals with IIM develop myositis specific MSA and MAA (Nagaraju, 2005). Although the pathogenic role of recognised MSAs and MAAs remains unclear, IIMs patients are increasingly classified by serological status, which closely associates with clinical phenotype (Finch, 2006). However, there is increasing evidence that IIMs may result from chronic immune activation in genetically susceptible subjects following specific but undefined environmental triggers (Rothwell et al, 2013). The majority of IIM genetic research is focused on the role of the MHC genes and their potential implications in autoantibody generation and disease expression. Further studies have also investigated the role of polymorphisms in the immunoglobulin genes, pro-inflammatory cytokines, and in genes that had been previously reported to have a role in other autoimmune disorders (Raju & Dalakas, 2005).

A revision of the Charing Cross Hospital database of patients with diagnosis of IIM between 2001 and 2011 resulted in the identification of three unrelated patients with remarkably similar phenotype characterised by symmetrical proximal weakness and early involvement of the forearm flexors but with relative quadriceps sparing, high CK levels (over 3,000 IU/L) and a muscle biopsy with features of sIBM but good response to steroid treatment and plasma exchange. Immunohistochemistry with anti-emerin antibodies showed normal localization. Analysis of their sera revealed novel autoantibodies to components of the sarcoglycan complex. Gene analysis of the sarcoglycans revealed that the three patients were heterozygous for a missense mutation in the β -sarcoglycan gene (c.799C>T) leading to the amino acid substitution p.Arg267Cys in the extracellular domain of the protein. Molecular modelling of the β -sarcoglycan protein suggests that this substitution causes a conformation change of the extracellular domain.

PATIENTS AND METHODS

Patients' phenotype and clinical investigations

All patients provided written informed consent for the genetic tests and functional and immunological analyses. The Ethics Committee of Imperial College London approved the study.

The three patients, two Caucasians and one Asian had a similar phenotype with high CK values and a muscle biopsy with features of sIBM. Sub-acute onset of generalised weakness and early and prominent symmetrical involvement of the forearm flexors and sparing of the quadriceps characterised the clinical phenotype of these patients. Other variable features included mild orbicularis oculi weakness, scapular winging, dysphagia, weakness of the neck muscles and anterior distal leg muscles. Muscle MRI of the thighs showed multifocal increased signal intensity, particularly in the forearms and proximal and distal leg muscles, on T2 and STIR sequences. EMG showed features consistent with an inflammatory response. CK levels were elevated at onset to over 2000 IU/L. Immunomodulation was beneficial with normalisation of CK and recovery from or stabilisation of weakness. In the differential diagnosis, a broad range of conditions from myositis and sIBM in particular, to adult-onset myopathies and dystrophies were considered. These conditions were however excluded following the results of immunohistochemistry, immunoblotting and genetic analysis. Tests for MAA and MSA, which included Jo-1, PL-7, PL-12, EJ, OJ, SRP, PM-Scl (75kD and 100kD), Ku, Mi-2 β , U1-RNP and HMGCR were negative. Anti-NT5C1A antibodies were positive only in patient 1.

Muscle biopsies showed remarkably similar features. The biopsies were characterised by atrophic and hypertrophic fibres, fibre necrosis, florid endomysial and perimysial, and perivascular lymphocytic infiltrates and aggregates of plasma cells. Some fibres contained RVs. A few COX-negative fibres were identified with the combined reaction COX/SDH. No sarcoplasmic inclusions were identified with congo-red and crystal violet. Lymphocytic infiltrates were composed of CD4 and CD8-positive T-cells in similar number, several CD20-positive B-cells and plasma cell infiltrate (CD138). MHC-I expression was ubiquitous and granular deposits of membrane attack complex (C5b-9) were present both on the sarcolemma and in endomysial capillaries and arterioles. Some fibres contained aggregates of p62. All

the other immunostains (dystrophins, caveolin 3, dysferlin, laminin- α 2) were within normal limits. Double immunofluorescence showed co-localisation of IgG and C5b-9 on several fibres suggesting an antibody-mediated pathogenesis of the condition (Figure 2.1B). In addition, staining with anti-emerin antibodies, performed on the basis of previous results in sIBM patients, showed normal nuclear localisation.

Patient 1. A 43-year-old Caucasian man presented with 18 months progressive generalized weakness and mild myalgia. Family and past medical history were unremarkable. On examination he had scapular winging, mild weakness of the orbicularis oculi, neck flexion and extension weakness (MRC 4/5), symmetrical limb-girdle weakness (4-/5) with clinically spared quadriceps, moderate finger flexor and mild foot dorsiflexion weakness. CK was 4000 IU/L (normal 40-320 IU/L). The patient responded well to prednisolone (60mg/day) and azathioprine (150mg/day). The steroid dose was tapered over 6 months and discontinued completely after 17 months.

At 6 months, CK was normal but reduction of the steroid dose resulted in CK increase and the re-emergence of the same pattern of weakness as previously, with particularly marked wasting of the forearm flexors (Fig. 2.1A), and distal leg weakness with bilateral foot drop.

Three years from presentation, CK was 1446 IU/L. He had generalised upper limb weakness and wasting and bilateral foot drop. Prednisolone and azathioprine were re-introduced. Six months later, CK was normal and proximal strength much improved. Review of the muscle pathology prompted a trial of plasma-exchange (PE), which produced further improvement in muscle strength.

He has since then remained on prednisolone (5mg/day) and azathioprine (100mg/day) and his strength and function has not deteriorated. His CK remains moderately elevated (520 IU/L).

Patient 2. A 76-year-old Caucasian woman presented with a 5-month history of progressive, symmetrical proximal limb and axial weakness. Family and past medical history were unremarkable. On examination, she had symmetrical generalised limb

and axial weakness (4/5). CK was 2200 IU/L. She responded well to prednisolone (0.8mg/kg/day) and methotrexate (20mg/week) but axial weakness persisted. She began to develop finger flexor weakness and wasting, and mild weakness of foot dorsiflexion. Methotrexate dose was further increased to 25 mg/week. On review after 8 months treatment, proximal limb strength remained normal but symmetrical weakness and wasting affecting wrist and finger flexors and extensors (4/5) was now more apparent. The patient has remained on weekly methotrexate and prednisolone (6mg/day) and her disease remains stable to date (6 years), with CK 300 IU/L.

Patient 3. A 44-year-old Asian woman presented with an 18-month history of dysphagia, progressive proximal weakness of the legs and generalised arm weakness. There was a family history of diabetes and Raynaud's phenomenon. On examination, she had mild weakness of the orbicularis oculi, neck flexion and extension weakness, symmetrical proximal arm weakness, weakness of the forearm flexors, and hip flexion weakness (4/5), but quadriceps strength was normal. CK was 3832 IU/L. She responded well to prednisolone (60mg/day) and azathioprine (150mg/day). On a review at one month, the dysphagia had resolved and limb strength had largely recovered. Following review of the muscle pathology, she underwent PE, with further improvement in muscle strength. One month later, muscle strength was normal except in triceps and finger flexors (4-/5). She was maintained on prednisolone (15mg/day) and azathioprine (150mg/day). Three months later, her strength and function had remained stable and CK was normal.

One year later, CK increased (574-900 IU/L) and she developed predominantly distal limb weakness. A course of IVIg (40g/day for 5 days) produced improvement. The patient is currently on azathioprine (100mg/day) and CK is within normal limits.

Myositis specific and associated antibodies line blot

To detect MSA and MAA, the sera were tested with a line-blot immunoassay system (Euroimmun, Luebeck, Germany). The system is able to detect IgG antibodies to the following 11 MSAs and MAAs: Jo-1, PL-7, PL-12, EJ, OJ, SRP, PM-Scl (75kD and 100kD), Ku, Mi-2 β , U1-RNP.

Sera were incubated with a strip coated with parallel lines of highly purified recombinant or native. If specific antibody is bound to an antigen, the bound conjugate is visualised by the presence of a coloured product at the location of the antigen.

Anti-HMGCR and anti-NT5C1A antibodies were tested in Dr A Pestronk' laboratory. Anti-HMGCR were negative in all three patients, while anti-NT5C1A were positive only in patient 1 and negative in patient 2 and 3.

Histochemistry and immunohistochemistry (IHC)

Quadriceps muscle biopsies were frozen in liquid nitrogen. Ten micron sections were cut and stained with haematoxylin and eosin, modified Gomori-trichrome, oxidative enzymes, Periodic Acid Schiff, oil red oil and NADH. IHC using a standard peroxidase technique was performed on frozen sections of the 3 initial biopsies and on the follow-up biopsies of patients 1 and 2. Normal control muscle was used as positive control. Immunostaining with omission of the primary antibody was performed as negative control. A standard peroxidase IHC technique was used to test the patients' sera (diluted 1:100) against five samples of frozen and formalin fixed and paraffin embedded normal control muscle made available by the Imperial College Healthcare Tissue Bank London, UK. In addition to the staining performed as part of the routine diagnostic service, additional staining with α - β -, γ - and δ -sarcoglycans (1:40, 1:50, 1:30, 1:40, mouse monoclonal, Novocastra, UK) and anti-filamin C (1:150, mouse monoclonal, Sigma-Aldrich, USA) was performed.

Immunofluorescence (IF)

IF using a standard technique was performed on frozen sections of the 3 initial biopsies and on the follow-up biopsies of patients 1 and 2. Immunostaining with omission of the primary antibody was also performed as negative control. Sections were incubated with anti-C5b-9 (1:30, mouse monoclonal, Novus Biologicals, USA) for 1 h. After washing with PBS, slides were incubated with anti-human IgG (Heavy and light chain) (1:200, Alexa Fluor 488, Invitrogen, USA) and anti-mouse polyclonal IgG (1:200, Alexa Fluor 555, Invitrogen, USA). Slides were mounted with mounting medium and 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Vector Labs, USA).

Western blot analysis

I performed a one-dimensional 12% SDS-polyacrylamide gel electrophoresis of muscle proteins extracts and recombinant α -, β -, γ - and δ -SG (full-length recombinant protein with glutathione S-transferase tag, (GST-tag) at N-terminal) (Abnova, Taiwan). The gel was electroblotted onto nitrocellulose (Invitrogen, USA). To prevent non-specific binding, blots were blocked for 3 hours at 4°C in blocking buffer (5% dry non-fat milk/0.1% Tween/PBS) (Tween20 for electrophoresis, Sigma, USA).

Western blot on muscle protein extracts

I tested the patients' sera (1:1000) on extracts of human normal control muscle, and human skeletal muscle membrane and cytoplasmic lysate (Abcam, UK). A healthy control serum and omission of patients' sera were used as negative controls. After washing with 0.1% Tween/PBS, membranes were incubated with biotinylated anti-human IgG and IgM (1:10000) (Vector Laboratories, USA) for 1 hour at room temperature. After washing, membranes were incubated with avidin-biotin peroxidase complex (Vectastain ABC complex, Vector Labs, USA) for 45 minutes, washed and developed with diaminobenzidine for 5 minutes.

Western blot on recombinant sarcoglycans

The three patients' sera, including pre- and post-PE sera, and additional 30 sera from a Swedish cohort (MyoNet) (diluted 1:1000), were tested against recombinant SGs. A healthy control serum and omission of patients' sera were used as negative controls. Anti- α -, β -, δ - and γ -SGs (1:100) (Novocastra, UK) were used as positive control. After washing, membranes were incubated with horseradish peroxidase conjugated anti-human IgG/M (1:10000) (Abcam, UK) for 40 minutes at room temperature. After washing, membranes were exposed to enhanced chemiluminescence reagent (ECL Prime Western Blotting Detection Reagent, GE Healthcare) and developed.

Western blot for anti- α -, β -, δ - and γ -SGs antibodies on patients' samples

Protein extraction from normal control and patients' muscle samples was performed using Radioimmunoprecipitation assay (RIPA) buffer and a protease inhibitor. Samples were squashed mechanically, heated for 3 minutes at 90°C and centrifuged at 14000 rpm for 10 minutes. The same procedure was repeated 3 times. Protein concentration was determined with the Bradford method using bovine serum albumin as a standard (BioRad Protein Assay Kit, Germany).

Nitrocellulose membranes were incubated with anti- α -, β -, δ - and γ -SGs antibodies, according to Anderson & Davison, 1999. After incubation, membranes were washed, incubated with anti-mouse polyclonal immunoglobulins (1:1000) (Dako, UK) for 40 minutes at room temperature, washed again, and developed using an ECL Western blotting detection system (GE Healthcare, UK).

Gene analysis for α -, β -, γ - and δ -SGs

Genomic DNA of the 3 patients was extracted from blood using the E.Z.N.A.TM Blood DNA kit (OmegaBiotek, USA) according to the manufacturer's instructions. The four genes were sequenced at Northern Genetics Molecular laboratory at The International Centre for Life (Newcastle upon Tyne, UK). Genomic DNA was also isolated from frozen muscle by phenol extraction and ethanol precipitation. The quality and quantity of DNA were assessed by spectrophotometer. Sanger sequencing analysis was then repeated in the laboratory.

Primers for SGCB and the biotinilated oligonucleotide probe recognising the mutation revealed by the initial gene analysis (forward primer 5'-GGTCAGCACCACTCGCCTA-3' and reverse primer 5'-CCACAGGGGTTGTCTGAGAT-3'; *wt*-probe 5'BIO-AGCACCAACCCGCCTACCCA-3'; *m*-probe 5'BIO-AGCACCACTGCCTACCCA-3') were designed using <http://frodo.wi.mit.edu/primer3/> and purchased from Invitrogen (USA). Genomic DNA amplification was performed in a 20µL reaction at 50°C and 30 cycles using 100ng of DNA template, 0.1 µL forward and reverse primers (100 µmole) respectively, and 18.8 µL of ReddyMix buffer (Thermo Scientific, USA).

The PCR product was visualised on an ethidium-bromide stained 0.8% agarose gel and purified using the Nucleo Spin Extract II (Macherey-Nagel, Germany) according to the manufacturer's instructions.

The gel was blotted on a positive charged nylon membrane (Ambion, USA) using a passive capillary transfer and alkaline buffer. The membrane was then incubated with the biotinilated probe (100 µM, 1:6000) overnight at 45°C on a shaking plate. The membrane was then developed with a chromogenic kit (ThermoScientific, USA) according to manufacturer instructions.

Modelling of β -sarcoglycan

To the best of my knowledge the three-dimensional structure of β -SG has not yet been resolved by X-ray crystallography or NMR spectroscopy. A PSI-BLAST analysis using the amino acid sequence of this protein did not reveal the presence of homologous proteins of known structure. Therefore, I used the fold recognition algorithm implemented in I-TASSER (Zhang, 2008; Roy et al, 2010; Yang & Zhang, 2015) (iterative threading assembly refinement algorithm) to predict a 3D model of both wild type (*wt*) and mutated (*m*) β -SG. To obtain a more reliable structure prediction of these membrane proteins, the amino acidic sequences were split into two domains: intracellular-transmembrane domains (residues 1-86) and extracellular domains (residues 87-318), which included the site of the mutation found in the initial sequencing. The domain boundaries of β -SG sequence were obtained from UniProt web site (UniProt Consortium, 2014).

The disulphide bonds between the cysteine residues of extracellular domain of the wild-type (*wt*) and *m* β -SG were predicted by the DiANNA (DiAminoacid Neural Network Application) algorithm. For the extracellular domain of the *wt* protein the disulfide bonds between Cys288 and Cys314 and between Cys290 and Cys307 were used as restraints in the I-TASSER structure assembly simulation. For the extracellular domain of *m* β -SG two models (*m1* and *m2*) were built; in *m1* the residue of Arg267 was substitute with the Cys267 and the same disulfide bonds of *wt* (Cys288-Cys314, Cys290-Cys307) were used as restraints whereas in the *m2* two different disulfide bonds were used as restraints, Cys267-Cys290 and Cys288-Cys307, as predicted by DiANNA algorithm relative to the mutant Arg267Cys of β -SG.

The best-scored model structure obtained by the I-TASSER prediction of each protein domain (intracellular-transmembrane, *wt* extracellular domain, *m1* extracellular domain and *m2* extracellular domain) was chosen for the molecular dynamics simulation of β -SG.

The N-glycosilation sites of β -SG were analysed using bioinformatic analysis that confirmed the presence of three glycosylation sites located in the extracellular domain at positions Asn158, Asn211 and Asn258.

Molecular Dynamics Simulation

We performed three atomistic 20ns MD simulations of *wt*, *m1* and *m2* β -SG starting from the structure of domains obtained from I-TASSER as described above. Initially, the models of intracellular-transmembrane and extracellular domains were equilibrated separately to relax the structure and subsequently the proteins were reconstituted by merging of the domains.

The model of intracellular-transmembrane domains of β -SG was embedded in a pre-equilibrated dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) bilayer, consisting of 275 molecules, using the INFLATEGRO program (Schmidt & Kandt, 2012). The transmembrane domain was oriented with the axis of α -helix parallel to the normal of lipid bilayer and translated along this axis to have the residue of Leu75, central residue of transmembrane domain, in the middle region of the bilayer.

The systems were solvated with water in a rectangular box of $9.5 \times 9.5 \times 11.0 \text{ nm}^3$, and the proper number of chloride ions was added to preserve electroneutrality. In addition, NaCl was added (150 mM) to simulate physiological conditions. The systems were energy minimized and equilibrated for 10 ns at 310 K.

The models of the extracellular domain of *wt*, *m1* and *m2* β -SG were solvated in three rectangular boxes with a minimum distance between the protein and the box wall of 1.0 nm and in the presence of chloride ions and NaCl 150 mM. After 8 ns of equilibration the residues of asparagine were glycosylated and the proteins were solvated in new rectangular boxes. Each system, consisting of glycosylated extracellular domain and water, chloride ions and NaCl 150 mM was energy minimized and then equilibrated for additional 6 ns.

The equilibrated structures of extracellular domains were merged to the intracellular-transmembrane domain and embedded in lipid bilayer to obtain the initial structure of *wt*, *m1* and *m2* β -SG for the molecular dynamics simulations. Each protein, embedded in the DMPC bilayer, was solvated in a rectangular box of $9.5 \times 9.5 \times 17.0 \text{ nm}^3$, neutralized by adding chloride ions in the presence of NaCl 150 mM.

All MD simulations were carried out with GROMACS v.4.0.5 package, using the GROMOS G53a6 force field for proteins and ions in conjunction with the Berger force field for the lipids.

Titrateable residues of proteins were modelled according to their protonation state in water at pH = 7.

The glycans $\text{GlcNAc}_2\text{Man}_8$, linked to residues of asparagine, were modelled with the G53a6 force field. Water was modelled with the SPC (Simple Point Charge) model.

Lennard-Jones and electrostatic interactions were calculated using a cut-off of 1.2 nm and the long-range electrostatic interactions were accounted by using the particle mesh Ewald method (PME).

All bonds were constrained using the LINCS algorithm whereas the geometry of water molecules was fixed with the SETTLE algorithm.

The simulations were carried out with a time step of 2 fs in the isothermal-isobaric (NPT) ensemble. The simulated systems were warmed up with five consecutive

unrestrained MD from 100 K to 310 K in 50 ps. The temperature of protein, lipids, water and ions was kept constant separately at 310 K using the velocity rescale method with a time constant of 0.1 ps. The pressure was controlled in the first 2 ns of equilibration using the Berendsen barostat ($P = 1$ bar, $\tau_p = 1.0$ ps) after which the Parrinello-Rahman barostat ($P = 1$ bar, $\tau_p = 4.0$ ps) was used to produce the correct ensemble. The pressure coupling was isotropic in the case of the simulations in water and semi-isotropic in the presence of the lipid membrane. Periodic boundary conditions were applied in all three dimensions.

The root-mean square fluctuation (RMSF) was calculated for the C_α atoms of the three different domains of the protein. The RMSF of the intracellular and extracellular domains increased rapidly in the first nanoseconds of simulation, and then reached a plateau within the first 10 ns. On this basis, the last 10 ns of the trajectories of *wt*, *m1* and *m2* β -SG were used for the analysis of the proteins structure.

The trajectory analysis was performed using the tools in the GROMACS package. Analysis of the secondary structure was performed with the program DSSP. VMD was used for trajectory visualization and graphical structure analysis.

Table 2 Summary of simulated systems

Protein	Net Charge of protein	Number of Water molecules	Number of Ions
β -SG <i>wt</i>	+5	35579	131 Na ⁺ , 136 Cl ⁻
β -SG <i>m1</i>	+4	35579	131 Na ⁺ , 135 Cl ⁻
β -SG <i>m2</i>	+4	35612	131 Na ⁺ , 135 Cl ⁻

Alignment of the extracellular amino acid sequence of β - with γ - and δ -SG

Alignment of the extracellular amino acid sequence of the 3 SGs using the Basic Local Alignment Search Tool (BLAST) showed homology and similarity of different regions (Fig. 2.5). Interestingly, the SNP c.799C>T in the β -SG induces a substitution p.Arg267Cys which increases the homology of β -SG with γ -SG in that

region. I have also aligned homologue and similar amino acid of the extracellular domains of *wt* and *m1* β -SG models in order to calculate most likely target sequence and displaced them from different angles (Fig. 2.6).

RESULTS

Analysis of patients' sera

To understand if the inflammatory response was driven by autoantibodies other than the known MSA and MAA, I then tested the serum of the three patients against sections of normal muscle and observed that positive immunolabelling of the sarcolemma suggesting an immune reaction against a sarcolemmal antigen. No immunoreactivity was conversely observed in the sarcoplasm and myonuclei.

I then investigated the patients' sera against lysates of whole muscle proteins extracted from a control sample and normal sarcolemma and sarcoplasm (Abcam, UK). The sera of ten healthy volunteers were also tested as controls. This experiment showed that patients' sera contained autoantibodies against a sarcolemmal antigen of about 35 kDa while none of the controls showed any immunoreactivity (Fig 2.3A,B).

From the molecular weight I hypothesized the β , γ - and δ -sarcoglycans (SGs) could be the most likely target proteins. I therefore tested the sera of patients (1 and 3) and healthy control against the four full-length recombinant SGs (Abnova, Taiwan) and confirmed autoantibodies against β -, γ - and δ -SG (Fig 2.3C). In contrast, no band was identified in the blot with recombinant α -SG. No auto-antibodies were detected with the sera collected after PE (Fig 2.3D) but notably, the serum of patient 3 tested 6 months after PE showed a weak positive reaction suggesting reactivation of the immune response against the β -, γ - and δ -SGs.

Frequency of anti- β -, γ -, δ -SG autoantibodies in other inflammatory myopathies

I tested 30 additional sera from a Swedish myositis patient cohort (MyoNet – Global Myositis Network) against recombinant SGs. Twenty were seronegative for the known MAAs and MSAs and 10 had anti-Jo1 antibodies. Two seronegative and one Jo-1 positive patient had antibodies against β -, γ -, δ -SGs (Fig.2.3E, F). ASO-PCR and sequencing analysis showed that these three patients were also heterozygous for the β -SG mutation (c.799C>T) (Fig. 2.3G).

Immunohistochemistry and immunoblotting for SG and filamin C

IHC and immunoblotting with anti- α -, β -, γ - and δ -SG antibodies (mouse monoclonal, Novocastra, UK) showed normal SG expression in all patients (Fig. 2.1B and 2.3).

Immunohistochemistry for filamin C

IHC for filamin C (rabbit anti-human FLNC; Sigma, USA) revealed abnormal subsarcolemmal distribution in several fibres in all 3 patients (Fig. 2.1B), possibly consequent to a conformation change in the SG complex.

Genetic analysis of the sarcoglycans

These results prompted me to sequence the genes of the four SGs using genomic DNA from blood and DNA from muscle biopsies. A heterozygous missense mutation c.799C>T (p.Arg267Cys) in the β -SG gene was found in the three patients by Sanger sequencing. *In silico* analysis (Alamut Mutation Analysis Software-Interactive Biosoftware and PolyPhen-2 Software-Polymorphism Phenotyping) predicted that the mutation might be pathogenic. This variant has been reported previously in the Leiden Muscular Dystrophy database in one patient but no clinical data are available. Further to this result, we performed an ASO-PCR using a *wt* and an *m* 5'-biotinilated oligonucleotide probe against genomic DNA extracted from the 3 patients and 16 healthy control subjects showed that all three patients but none of the 16 controls were also heterozygous for this mutation.

Protein modelling and p-BLAST

In order to understand the impact of p.Arg267Cys on the structure of β -SG I performed in collaboration with a colleague, Dr Stefano Borocci from Università della Tuscia, Viterbo (Italy), protein modelling of the β -SG. Using a bioinformatics approach we obtained the 3D model of *wt* β -SG and two models (*m1* and *m2*) for the mutation p.Arg267Cys. The residue 267 is close to the cysteine-rich motif and the substitution p.Arg267Cys introduces a potential site for the formation of disulfide bond. Actually, the model *m2* was built to evaluate the effect of the disulfide bond

between Cys267 and Cys290 located in the cysteine-rich motif and involved in the native disulfide bridge Cys290-Cys314. To evaluate the effect of this mutation on the structure and dynamics of β -SG we performed MD simulations (Figure 2.4). This showed that the mutation affects mainly the extracellular domain of the protein, causing a conformational change and may affect the motility of the intracellular domain (Fig.2.4). RMSF of the C α carbon atoms with respect to their average position over the MD trajectory was used as means to evaluate the structure flexibility of the proteins. Residues with large value of RMSF are more mobile with respect to residues with low value of RMSF (Fig. 2.4A and 2.5). A p-BLAST of the extracellular amino acid sequence of the $\beta/\gamma/\delta$ -SGs shows different regions of homology and similarity (Fig.2.6).

Figure 2.1 Representative patient and muscle biopsy

A: Patient 1, two years from presentation, showing scapular winging, forearm flexors wasting and relative quadriceps sparing.



B: Peroxidase IHC and IF on frozen and formalin fixed muscle samples.

(H&E) Variation in fibre size, atrophic and hypertrophic fibres, necrotic fibres and florid endo- and perimysial lymphocytes (CD4; CD8; CD20), and plasma cell infiltrate (CD138). Some fibres contained rimmed vacuoles (Gomori-Trichrome). Several COX-negative fibres were present (COX-SDH). There were p62 inclusions in some fibres. There was ubiquitous sarcolemmal expression of MHC-I antigen (MHC-I) and non-ubiquitous C5b-9 deposits on sarcolemma and endomysial arterioles and capillaries (C5b-9, Double IF anti-h-IgG -Alexa Fluor 488-and C5b-9- Alexa Fluor 555). γ -SG (Novocastra, UK) shows normal level of expression. FLNC (Sigma-Aldrich, USA) showed abnormal subsarcolemmal accumulation of FLNC in several fibres.

B

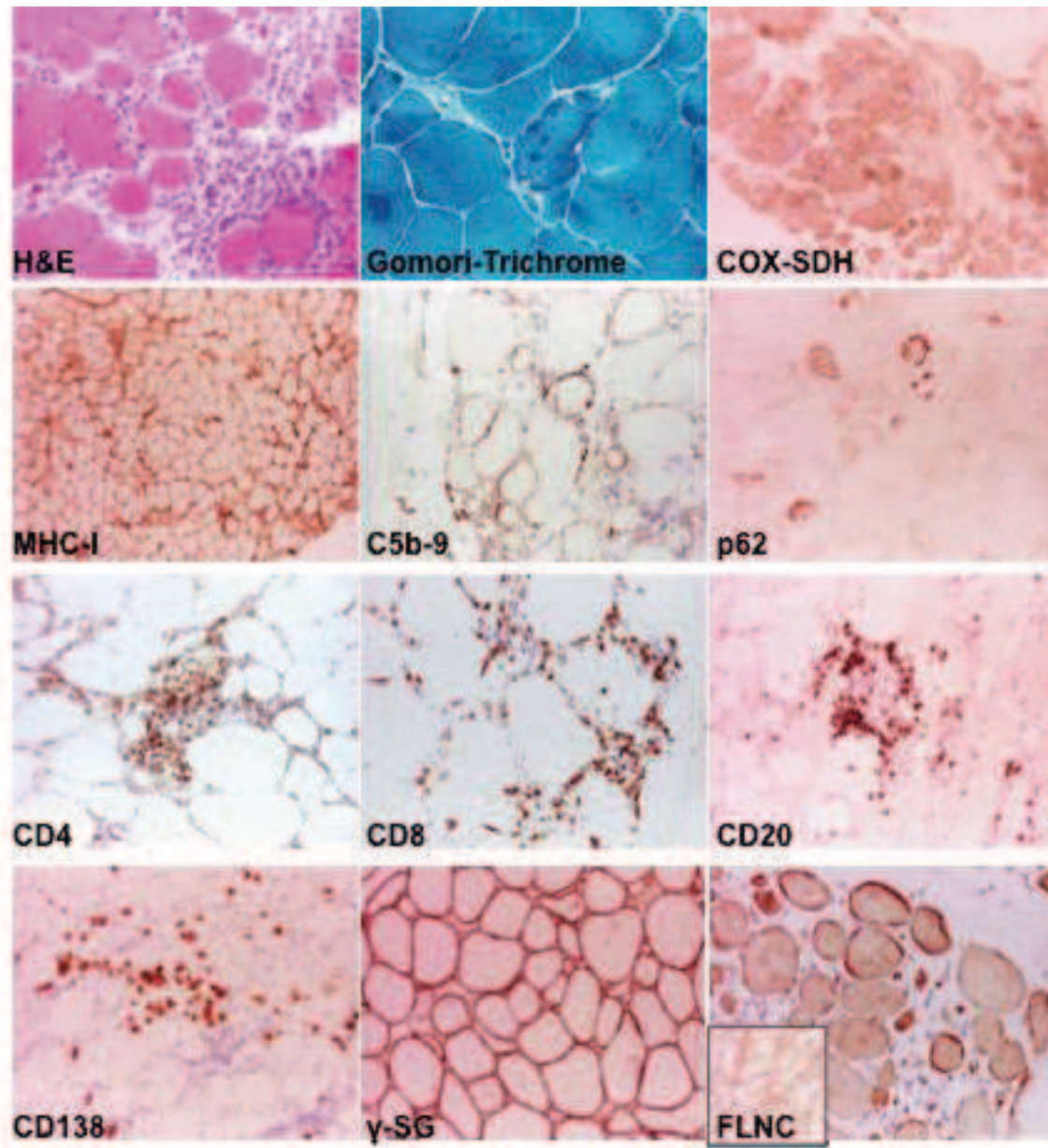


Figure 2.2 IHC and IF using patient's serum as primary antibody

A: The serum of patient 3 incubated on sections of control muscle decorates the sarcolemma of several fibres (immunoperoxidase – x20)

B: sera from normal controls do not show any immunoreactivity (immunoperoxidase – x20).

C – D: Immunofluorescence demonstrates IgG (C) but not IgM deposits (D) on muscle tissue from patient 1.

E: IgG co-localise with C5b-9 indicating the presence of immunocomplexes.

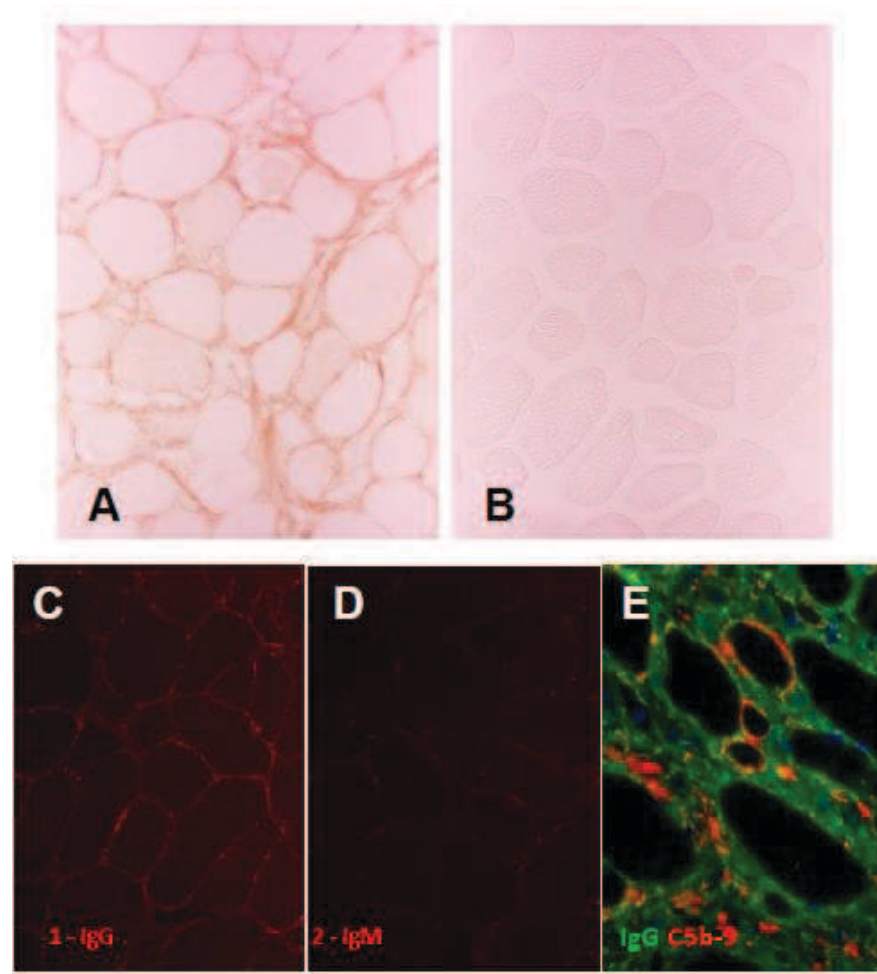
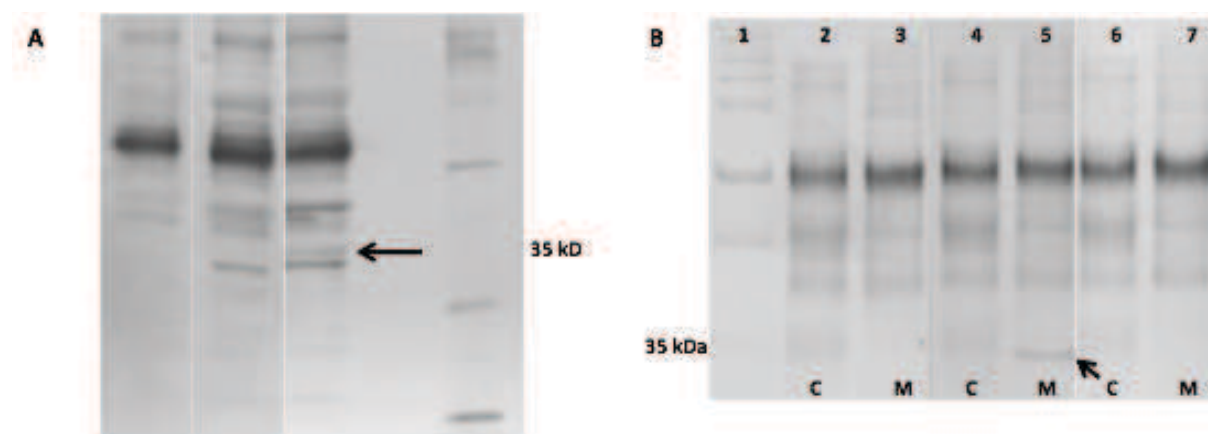


Figure 2.3 Western blots

Western blot (WB) study of sera. One-dimensional 12% SDS-PAGE of normal muscle protein extracts and recombinant SGs (Abnova, Taiwan) were electroblotted onto nitrocellulose (Invitrogen, USA) and the membranes were blotted with patients' and control sera as primary antibody, followed by anti-human IgG antibody.

A: WB of patients' sera and control serum (1:1000) tested against whole skeletal muscle proteins. Patients' sera showed a positive 35kDa band (arrows). Omission of serum and control serum were used as negative control.

B: WB on human skeletal muscle membrane (**M**) and cytoplasmic (**C**) lysate (Abcam, UK). Lane 1: Molecular weight markers. Lanes 2-3 were incubated only with secondary antibody and omission of serum; lanes 4-5 were incubated with serum of patient 3 (diluted 1:4000). Positive band around 35 kDa (arrow); lanes 6-7 were incubated with control serum (diluted 1:4000).



C: WB on recombinant α , β , γ , δ -SG (Abnova, Taiwan) patient 1 and 3's serum (1:1000) showed positive reaction for both β , γ , δ - recombinant SG

C (Patient 1)  **(Patient 3)** 

D: WB on recombinant γ - and δ -SG (Abnova, Taiwan). Lanes 1-2 were incubated with pre-PE serum; lanes 3-6 were incubated with serum obtained immediately after PE; lane 7 was incubated with 6 month post-PE serum. All sera were diluted 1:1000.

D 

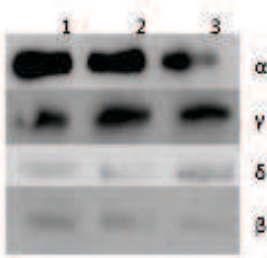
E: WB with one of the Jo-1 positive MyoNet sera tested on recombinant γ - and δ -SG showed positivity for recombinant γ - and δ -SG.

E 

F: WB with two of the MyoNet sera tested against recombinant α , β , γ , δ -SG, showed positivity against γ - and δ -SG. For positive control the recombinant δ -SG protein was tested with anti- δ -SG antibody (Novocastra, UK).

F 

G: WB with anti- α , β , γ , δ -SG antibodies (Novocastra, UK) on muscle extracts from the 3 patients showed normal α , β , γ , δ -SG levels.

G 

I: Genomic DNA was extracted from peripheral-blood leukocytes and frozen muscle. Sequencing analysis showed c.799C>T of the β -SG gene.

I

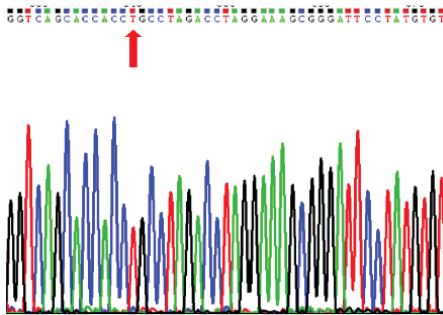


Figure 2.4 Protein modelling

A: Snapshots after 20ns of MD simulation of β -SG *wt* (I), *m1* (II) and *m2* (III). The proteins were embedded in a DMPC bilayer consisting of 275 molecules, in the presence of water, chloride ions to preserve the electroneutrality and NaCl (150 mM) to simulate physiological conditions.

The proteins are shown as ribbons coloured by secondary structure: *red*; α -helices, *yellow*; β -strands, *cyan*; loops. The residue 267 (Arg or Cys) is shown in space-filling model (spheres), the glycans and the cysteines of the two disulfide bonds are shown as sticks. The ammonium head groups of DMPC lipids are shown as sticks to indicate the position of the bilayer.

Structure of the extracellular domain of β -SG *wt* (IV), *m1* (V) and *m2* (VI).

Backbone representation of β -SG *wt* (VII), *m1* (VIII) and *m2* (IX). The line thickness and the colour scale (blue-green-red) represent the RMSF of the backbone atoms of protein.

The analysis of *wt* β -SG secondary structure showed β -strands organised in β -hairpin motifs in the extracellular domain and an α -helix in the transmembrane region (IV).

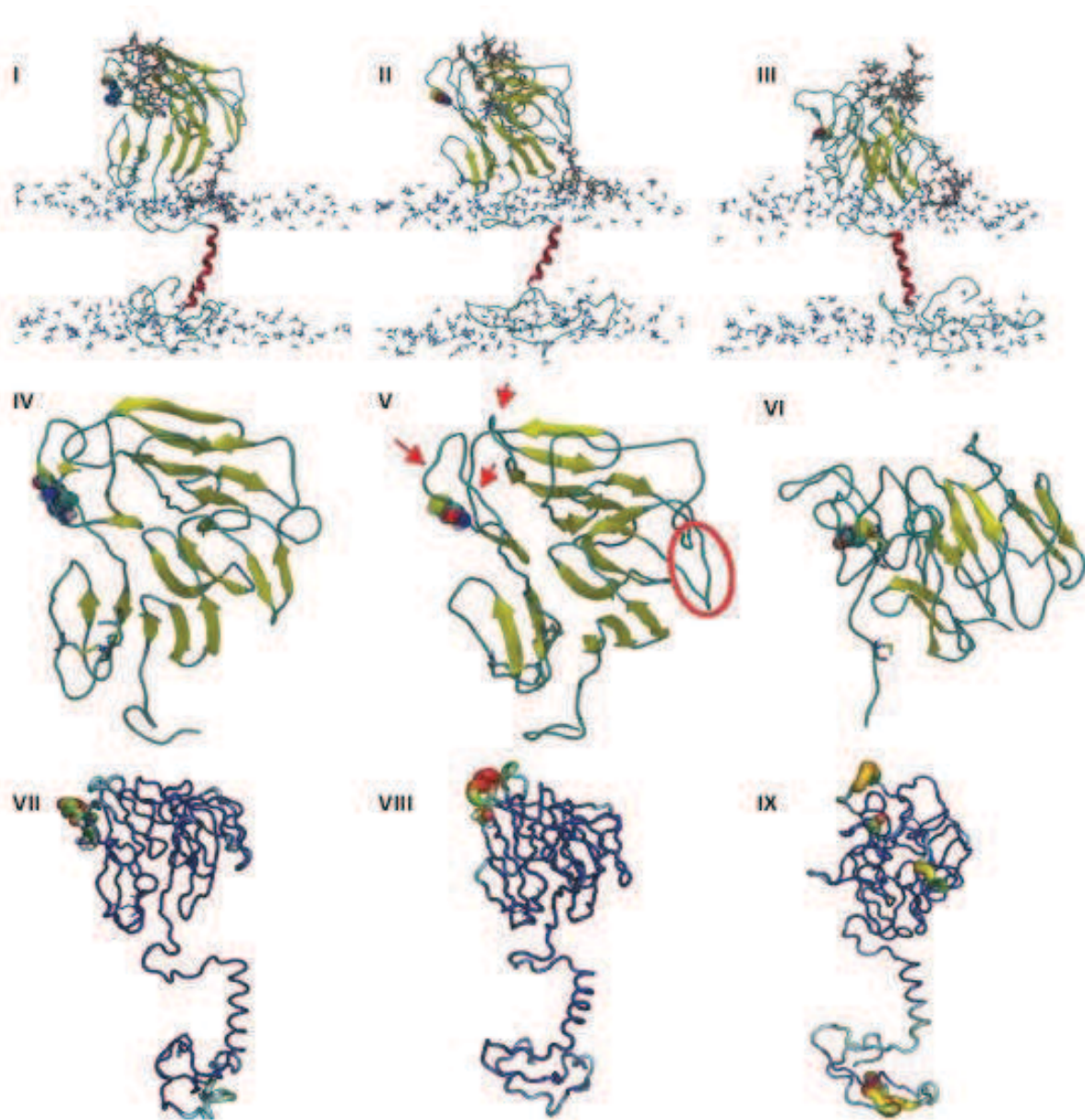
The model *m1* (V) maintains the main secondary structure of the *wt* β -SG, however, significant changes were observed (arrows). Interestingly, the structural changes induced by the mutation concern also regions far from the site of mutation as the region from the residue 153-160 (circle). The simple amino acid substitution Arg267Cys in *m1* affects the structure of extracellular domain (V) and the mobility of protein (VIII). It is worth to note that in *m1* the intracellular domain is less mobile compared to *wt* (VII, VIII).

The structure of *m2* (VI) is more compact with a reduced number of β -strands with respect to *wt* with a reduced mobility of the extracellular domain and an increased mobility of the intracellular domain with respect to *wt* protein (IX).

The analysis of RMSF of *wt* β -SG shows that the β -strands structures are stable with a low flexibility, whereas some loops connecting the β -strands are more flexible. In particular, the loop formed by the residues 266-276 has the highest mobility (VII). Residues with large value of RMSF are more mobile with respect to residues with low value of RMSF.

B: Alignment of homologue and similar amino acid of the $\beta/\gamma/\delta$ -SGs of the extracellular domains of *wt* and *m1* SG models. Ribbon and transparent surface representation for the extracellular domain of *wt* and *m1* β -SG after 20ns of MD simulation. Homology and similarity are represented in different colours of surface according to the degree of homology (see legend). *Wt* and *m1* models are displaced in different angles (top, front, reverse, lateral right and left view).

A



B

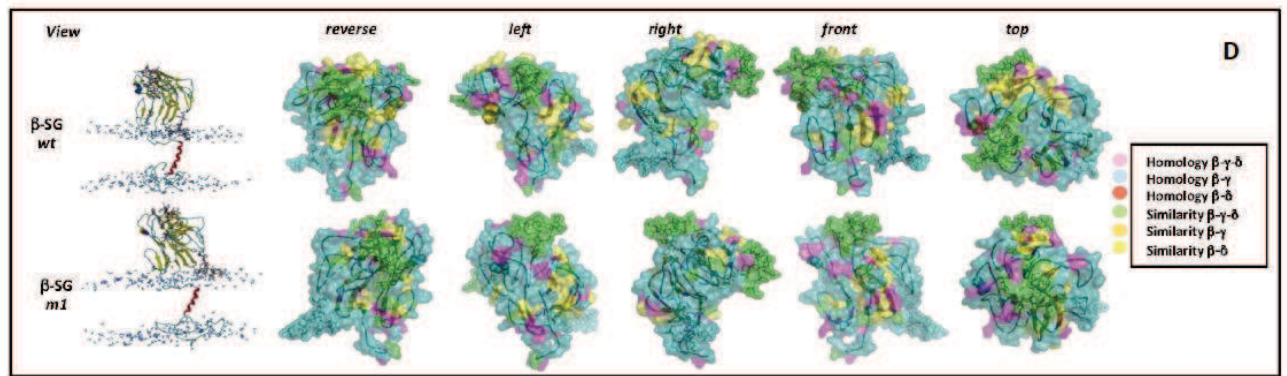
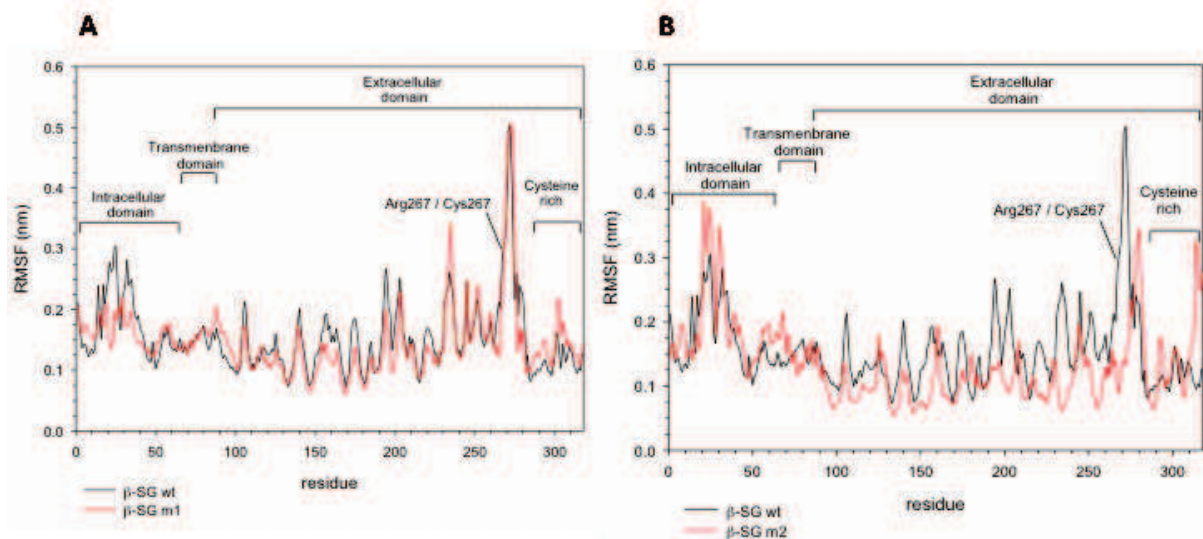


Figure 2.5 RMSF



A: The RMSF comparison between β -SG *wt* and β -SG *m1* models showed a reduced flexibility of the intracellular domain and increased mobility of the extracellular domain in *m1*. The residues 233-234 and the cysteine-rich motif (residues 285-318) are more mobile in *m1* in respect to *wt*. The loop 266-276 is the region with the highest RMSF in both *m1* and *wt*.

B: The RMSF comparison between β -SG *wt* and β -SG *m2* models showed an increased mobility of the intracellular domain and reduced mobility of the extracellular domain in *m2*.

Figure 2.6

P-BLAST of the extracellular amino acid sequence domain of the $\beta/\gamma/\delta$ -SGs.
Amino acids are highlighted in different colours according to different grades of homology/similarity (see legend).

				Extracellular domain			
β	Query	52	LHKTGLRGRGNLAICVILLFILAVINLIITLVINAVIRIGENGCDSELMHESGLLRFK	111			
			++K G+ G + ++LL I+ V+NL +T+ I				
γ	sbjct	23	VYKIGIYGWRKRCLYLFVILLIILVNNLALTINILKVMWFSBAGMGRICVTRDG-LRLR	81			
β	Query	51	RLHKTGLRGRGNLAICVILLFILAVINLIITLVINAVIRIGENGCDSELMHESGLLRF	110			
δ	sbjct	21	QVYKVGSIYGWRKRCLYFFVILLIILVNNLALTINILKVMWFTIDSMGNLAITEKGL---	77			
			++K G+ G + ++LL IL ++NL +T+ I V+				
	Query	112	QVSDMGVIMFLYKSTVGGRRNENLVITGNQPIV-----FQQGTTKLSVENNK	159			
	sbjct	82	GESEF--LFPLYAKEIHSRVDSLLLOSTQNVTVNARNSEGEVTGRLLKVGFPMVEVQNOQ	139			
	Query	111	KQVSIMGVIMFLYKSTVGGRRNENLVITG-----NNQPIVFQQ---STTKLSVENN	158			
	sbjct	78	KLEGDSFQFLYAKEIQSRPGNALYFKSARNVTVNILNDQTKVLTQITGPKAVEAYGK	137			
	Query	160	TSITSDIGMQFFDERTQNILFSTDYETHEFHLPSSGVKSLNVQKASTERITSNATSDLNK	219			
	sbjct	140	PQINSNDGKPLFTVDEKEVVVGTD----KLRVTGPEGALFEHSVETPLVRADPFQDLRL	195			
	Query	159	KISITSDIGMQFFDERTQNILFSTDYETHEFHLPSSGVKSLNVQKASTERITSNATSDLN	218			
	sbjct	136	KFEVKTVSGLLFSADNNEVVVGAE----RLRVLGAEGTVFPKSIETPNVRADPFKELRL	193			
	Query	220	VDGRAI-VRGNEGVFIMGKTIEFHMGGNMELKAENSIILNGS----VMYSTTRLPSSSS	273			
	sbjct	196	SPTRSLMDAPRGVHIQAH-----AGKIEALSQMDILFHSDDGMLVLDDETVCLEPLVQ	249			
	Query	219	KVDGRAIV-RGNEGVFIMGKTIEFHMGGNMELKAENSIIL---NFSVMV--STTRLPSSS	272			
	sbjct	194	ESPTRSLVMEAPRGVEINAE-----AGNMEATCRTELRLSEKDGRIKLDAAKIRLPLRP	247			
	Query	274	GDQLGSG-DWVRVYELCMCADGTLEKVVQVTSQNMGCQISDNFC	314			
	sbjct	250	GTWGPSSGSSQSLYEICVCPDGRKLY-LSVAGVSTTCQEMSHIC	290			
	Query	273	SGDQLGSGDWVR-YKLCMCADGTLEKVVQVTSQNMGCQISDNFC	314			
	sbjct	248	HGSYTPTGTROKVFEICVCANGRLFLSCAGAGST-CQINTSVC	289			

Homology β - γ - δ (Pink)

Homology β - γ (Light Blue)

Homology β - δ (Red)

Similarity β - γ - δ (Green)

Similarity β - γ (Yellow)

Similarity β - δ (Orange)

DISCUSSION TO CHAPTER 2

Reviewing the clinical database of patients with diagnosis of IIM between 2001 and 2011 at Charing Cross Hospital resulted in the identification of three unrelated patients with heterozygous missense mutation c.799C>T in the gene encoding β -SG, causing the amino acid substitution p.Arg267Cys. The patients developed an inflammatory myopathy associated with autoantibodies directed to $\beta/\gamma/\delta$ -SG. Protein modelling suggested that this substitution causes a conformational change of the extra-cellular domain of β -SG suggesting that the conformational change confers susceptibility to develop a pathogenic immune response against muscle fibres. For the first time, this study suggests that genes encoding structural proteins of skeletal muscle represent a novel class of susceptibility genes for IIMs.

The clinical phenotype of the three patients is characterised by sub-acute onset of generalised weakness and early and prominent involvement of the forearm flexors, suggestive of sIBM but with relative clinical sparing of the quadriceps. Variable features included mild orbicularis oculi weakness, dysphagia, and weakness of the neck and anterior compartment distal leg muscles. Furthermore, CK levels were raised at above 2000 IU/L at presentation, not typical of sIBM, and immunomodulation produced clear clinical benefit, with normalisation of CK levels.

Muscle biopsies showed remarkably similar features that were reminiscent of sIBM including rimmed vacuoles and p62 sarcoplasmic inclusions but also florid B-lymphocytes and plasma cells. Although there were sIBM-like pathological features, there was also clear evidence of a humoral component with florid endo- and perimysial T- and B-cell inflammatory infiltrate, plasma cells and deposits of C5b-9 on sarcolemma and endomysial capillaries (Fig. 2.1B) suggesting an innate and humorally mediated immune response. B-cell and plasma cell infiltrates have been demonstrated in sIBM and PM along with local production of immunoglobulins (Ig) suggesting a B-cell permissive microenvironment characterises IIMs. The evidence of IgG sarcolemmal deposits in our patients, their co-localization with C5b-9 is in keeping with an antibody-mediated process and strongly supports the pathogenic role of the autoantibodies identified in our patients. These observations are in keeping with recent studies that emphasized the role of B-lymphocytes and plasma cells in IIMs, and documented a B-cell permissive microenvironment in myositis. IF in

our cases showed co-localization of anti-human IgG with deposits of C5b-9 on the sarcolemma of many of the muscle fibres, strongly supporting the pathogenic role of the antibodies and consistent with the favourable response to PE.

This evidence along with the absence of known MSA and MAA led me to test further the serum of the three patients.

Analysis of patients' sera by immunohistochemistry and western blot allowed me to identify autoantibodies directed against the $\beta/\gamma/\delta$ -SGs. Sequencing analysis of the four SGs showed that the patients were heterozygous for the missense mutation (c.799C>T, p.Arg267Cys) of the β -SG. Interestingly, a subsequent survey of samples from patients in the MyoNet database revealed three further patients with anti-SG antibodies carrying the same mutation in the β -SG gene while no anti-SG were found in normal healthy controls. One of the patients of the MyoNet cohort with anti-SG antibodies tested also positive for anti-Jo1 suggesting that identification of anti-SG does not exclude the presence of other MSAs.

The present study is the first to identify a missense mutation in a structural sarcolemmal protein that appears to endow antigenicity and trigger an immune response. The mutation has been previously reported in the Leiden Muscular Dystrophy Database but the clinical phenotype is not reported. I performed an ASO-PCR on further 16 controls which were negative for the mutation c.799C>T. *In silico* analysis using PolyPhen and Alamut predicted the change to potentially affect the structure of β -SG but immunostains and WB showed normal expression and normal sarcoplasmic localisation of the four SG proteins. In addition, no changes were found in the expression of proteins related to SGs apart from focal subsarcolemmal accumulation of filamin C in some damaged fibres.

To explore further the impact of p.Arg267Cys on the conformation of β -SG, I performed, in collaboration with a colleague, Dr Stefano Borocci, from Università della Tuscia, Viterbo (Italy), a 3D molecular modelling of the protein using a bioinformatics approach.

SGs are transmembrane glycoproteins forming a complex with the dystrophin-associated glycoprotein complex. The assembly of the SG complex follows a specific pathway where the formation of β/δ -SG core structure is a critical step and its disruption affects the SG complex targeting to the sarcolemma. With the exception of

α -SG, the SGs consist of a short intra-cellular domain, a single transmembrane region and a large extracellular domain containing a carboxyl-terminal cluster of highly conserved cysteine residues forming intra-molecular disulphide bonds. The intracellular domain of γ/δ -SG can associate with FLNC while β -SG binds dystrophin. Structural characteristics suggest that the SG complex might be involved in signal transduction from the extracellular to the intracellular region (Barton, 2006; Constantin, 2013). The interaction of both γ - and δ -SG with FLNC provides a direct structural link from the SGs to the actin cytoskeleton and to the signal transduction pathway associated with FLNC.

According to the MD simulation, the substitution p.Arg267Cys induces a conformational change in the extracellular domain of β -SG. The conformational change of the *m1* extracellular domain also affects some regions of the amino acid sequence far from the site of mutation (residues 153-160) and produces a major exposure of the cysteine-rich loop, which is also more mobile than in the *wt*. Cysteine-rich motifs are found in different extracellular proteins and are highly conserved throughout different species. The cysteine-rich domain of the β -SG belongs to the epidermal growth factor-like family and appears to be important for membrane targeting but not for protein-protein interaction (Chen et al, 2006; Draviam et al, 2006). These conformational changes may expose an epitope that triggers an autoimmune reaction towards β -, γ -, δ -SG. Δ - and γ -SG share 70% homology and 50% homology to β -SG while α -SG differs considerably from these three proteins. Alignment of the extracellular amino acid sequence of β - with γ - and δ -SG showed homology of specific regions (Fig. 2.6).

The *m1* MD simulation showed that the substitution p.Arg267Cys also influences the mobility of the intracellular domain, which is less mobile than in the *wt*. Change in mobility may explain subsarcolemmal deposition of FLNC in some fibres. The *m2* MD simulation conversely showed major conformational changes in the extracellular domain of the protein with reduced extracellular mobility and increased mobility of the intracellular domain. Given the entity of change, I think that it is unlikely that a protein with such structure might reach the sarcolemma as the β -SG structure integrity is crucial for its' targeting to the membrane surface and for the β - δ -SG core formation (Draviam et al, 2006).

The identification of a novel autoantibody to structural sarcolemmal proteins in patients with IIM and a mutation in the β -SG, together with the observation of a B-cell permissive microenvironment, suggests and emphasises that the immunopathogenesis of some IIMs can involve both cytotoxic and humoral elements acting in concert. Although Stuhlmüller et al. described antibodies to a 50 kDa sarcolemmal protein in patients with polymyositis in 1996, using skeletal muscle extract as the antigen source (Stuhlmüller et al, 1996), this protein was not further characterised. Antibodies to sarcolemmal proteins have also been identified in other immune disorders, notably myasthenia gravis but also in myocarditis (Lindstrom et al, 1976; Pankuweit et al, 1995). In addition, there are very rare cases of immune-driven myopathies with antibodies to muscle extracellular matrix components (al-Lozi et al, 1995 and 1997; Yabe et al, 2002).

This study opens new frontiers to the understanding of the pathogenesis of IIM and their treatment, and emphasises the importance of careful analysis of muscle tissue to determine the elements involved in the immunopathogenesis in individual cases, since patients who exhibit a significant humoral element to the disease will likely respond to immunosuppressive therapies such as PE and IVIg as adjuncts to the standard interventions of steroids and immunosuppressant drugs. The use of B-cell blocking agents may be attractive alternative treatment.

CHAPTER 3

IDENTIFICATION OF NOVEL AUTOANTIBODIES TO SARCOLEMMAL PROTEINS IN IIM: A PILOT STUDY

INTRODUCTION TO CHAPTER 3

The previous identification of a novel autoantibody to sarcolemmal proteins, against three components of the sarcoglycan complex, in three patients led me to test sera of a further small number of consecutive patients with a query diagnosis or confirmed diagnosis of IIM and control subjects against recombinant sarcoglycans and other sarcolemmal proteins. The muscle biopsies of the selected patients were characterised by inflammatory infiltrate composed of T-cells and B-cells as well as deposits of MAC on sarcolemma and/or endomysial capillaries.

The recombinant sarcolemmal proteins were selected from those commercially available from Abnova (Taiwan). These were $\alpha/\beta/\gamma/\delta$ sarcoglycans (α -SG, β -SG, γ -SG, δ -SG), caveolin 3 (CAV3), sarcospan (SSPN), fukutin related protein (FKRP) and laminin- α 2 (LAMA2) (Table 3.2). A western blot with recombinant proteins was performed; proteins were blotted on a nitrocellulose membrane and then incubated with patients' sera used as primary antibody. Anti-human IgG was used as secondary antibody.

Aim of this pilot study was to identify novel autoantibodies to sarcolemmal proteins. Current methods to identify novel autoantibodies in IIM use mainly HeLa extracts as antigen source; however, HeLa cells do not express several proteins that are specific and selectively expressed in skeletal muscle. In this pilot study I have used sarcolemmal proteins in order to identify novel autoantibodies.

This pilot study led us to novel results that were filed as a patent.

MATERIALS AND METHODS

Patients and Samples

The sera of 10 patients were collected after signed informed consent. All patients were referred with either a query diagnosis of IIM or a confirmed diagnosis of IIM. Details of patients are reported in Table 3.1. I collected and tested the sera of 9 healthy individuals after informed consent and used these as control sera.

Table 3.1 Overview of patients' details.

Patients	Gender	Age (years)	Final diagnosis	CK (IU/L)	Treatment
1	F	31	DM	>600	azathioprine, methotrexate and prednisolone
2	F	63	IBM	751	steroids, methotrexate and IVIG
3	M	63	Rhabdomyolysis	8300	N/A
4	F	73	IBM and Sjogren's syndrome/SLE	194	methotrexate, prednisolone and hydroxychloroquine
5	F	76	IBM	150	mycophenolate mofetil, prednisolone, azathioprine and IVIG
6	F	75	IBM + Sjogren's syndrome	169	azathioprine
7	M	60	HIV related PM	664	azathioprine, prednisolone and IVIG
8	M	69	Myofibrillar myopathy	700	N/A
9	M	72	Possible statin induced necrotising myopathy	>1000	steroids
10	F	74	Necrotising myopathy	185	steroids

Western blot analysis

I performed a one-dimensional 12% SDS-polyacrylamide gel electrophoresis of recombinant α -, β -, γ - and δ -sarcoglycans, caveolin 3, fukutin related protein, laminin- α 2 and sarcospan (partial and full-length recombinant protein with glutathione S-transferase tag, (GST-tag) at N-terminal) (Abnova, Taiwan) (Table 3.2). One microgramme of protein per well was loaded in each well. The gel was electroblotted onto a 0.45 μ m pore nitrocellulose membrane (Invitrogen, USA). To prevent non-specific binding, blots were blocked for 3 hours at 4°C in blocking buffer (5% dry non-fat milk/0.1% Tween/5% Sheep serum/TBS) (Tween20 for electrophoresis, Sigma).

The patients' and controls' sera were diluted (1:1000) and tested against the recombinant proteins. Healthy control sera were used as negative controls. After washing, membranes were incubated with HRP-conjugated goat anti-human IgG Fc (goat) (Rockland, USA) (1:10000, 5% dry non-fat milk/0.1% Tween/1% Sheep serum/TBS) for 45 minutes at room temperature. After washing in TBS/T, membranes were exposed to enhanced chemiluminescence reagent (Li-Cor, UK) and acquired with the Li-Cor acquisition system.

Table 3.2 Recombinant sarcolemmal proteins from Abnova, UK.

Protein	Features	Proteins' concentration ($\mu\text{g}/\mu\text{l}$)	1 μg per well (Volume in μl)
α -SC: H00006442-P01	GST-Tag full-length	0.34 $\mu\text{g}/\mu\text{l}$	2.94 μl
β -SG: H00006443-P01	GST-Tag full-length	0.15 $\mu\text{g}/\mu\text{l}$	6.67 μl
γ -SG: H00006445-P01	GST-Tag full-length	0.3 $\mu\text{g}/\mu\text{l}$	3.33 μl
δ -SG: H00006444-P01	GST-Tag full-length	0.06 $\mu\text{g}/\mu\text{l}$	16.67 μl
CAV3: H00000859-P01	GST-Tag full-length	0.09 $\mu\text{g}/\mu\text{l}$	11.11 μl
FKRP: H00079147-Q01	GST-Tag full-length	0.09 $\mu\text{g}/\mu\text{l}$	11.11 μl
LAMA2: H00003908-Q01	GST-Tag partial-length	0.3 $\mu\text{g}/\mu\text{l}$	3.33 μl
SSPN: H00008082-P01	GST-Tag full-length	0.03 $\mu\text{g}/\mu\text{l}$	33.33 μl

Alignment of sarcolemmal proteins

Alignment of the amino acid sequence of sarcolemmal proteins was performed using online available software p-BLAST in order to establish level identity.

RESULTS

Patients' reactivity to recombinant proteins

This pilot study testing sera of patients with a diagnosis of IIM, including sIBM or query IIM against recombinant sarcolemmal proteins by WB showed that 7/10 patients that were studied had a positive reaction to one or more recombinant proteins. In particular, 3/10 patients failed to show any reactivity against the recombinant proteins; these patients had a diagnosis of DM, rhabdomyolysis and myofibrillar myopathy. Three out of 10 patients were positive for all the sarcoglycans and caveolin 3, fukutin related protein and sarcospan; these patients had respectively diagnoses of sIBM/systemic lupus erythematosus (SLE) and HIV related PM. One out of ten patients had positivity for β - and δ -SG and another patient was positive for γ -, δ -SG, caveolin 3, fukutin related protein and sarcospan; they both had a query diagnosis of sIBM. While one patient had a positive reaction to β - and δ -SG, caveolin 3 and another patient was positive for β -, γ - and δ -SG; both had a diagnosis of necrotising myopathy. Of the healthy controls only one patient out of nine showed positive reaction to all the sarcoglycans and caveolin 3; this control had been previously investigated for systemic lupus erythematosus because of a malar rash that is still present. However, none of the investigations confirmed the diagnosis of systemic lupus erythematosus. Another control reacted against laminin- α 2, however the band was not at the expected molecular weight and specificity of this result is unclear. This control had no history of autoimmune disorders. Results of patients' reactivity are summarized in Table 3.3. Immunoreaction is reported as positive (POS) or negative (NEG) defined by the presence or absence of a band on the blot; in cases where there was a positive band but not at the correct molecular weight this was defined as dubious positivity (POS?). Western blots of recombinant sarcolemmal proteins blotted with patients/healthy controls are shown in Figure 3.1.

To note that, two patients (n. 5 and n. 9) had strong reactive bands for some of the recombinant proteins, however, these were not at the expected molecular weight. It is unclear whether this is a non-specific binding or degradation bands.

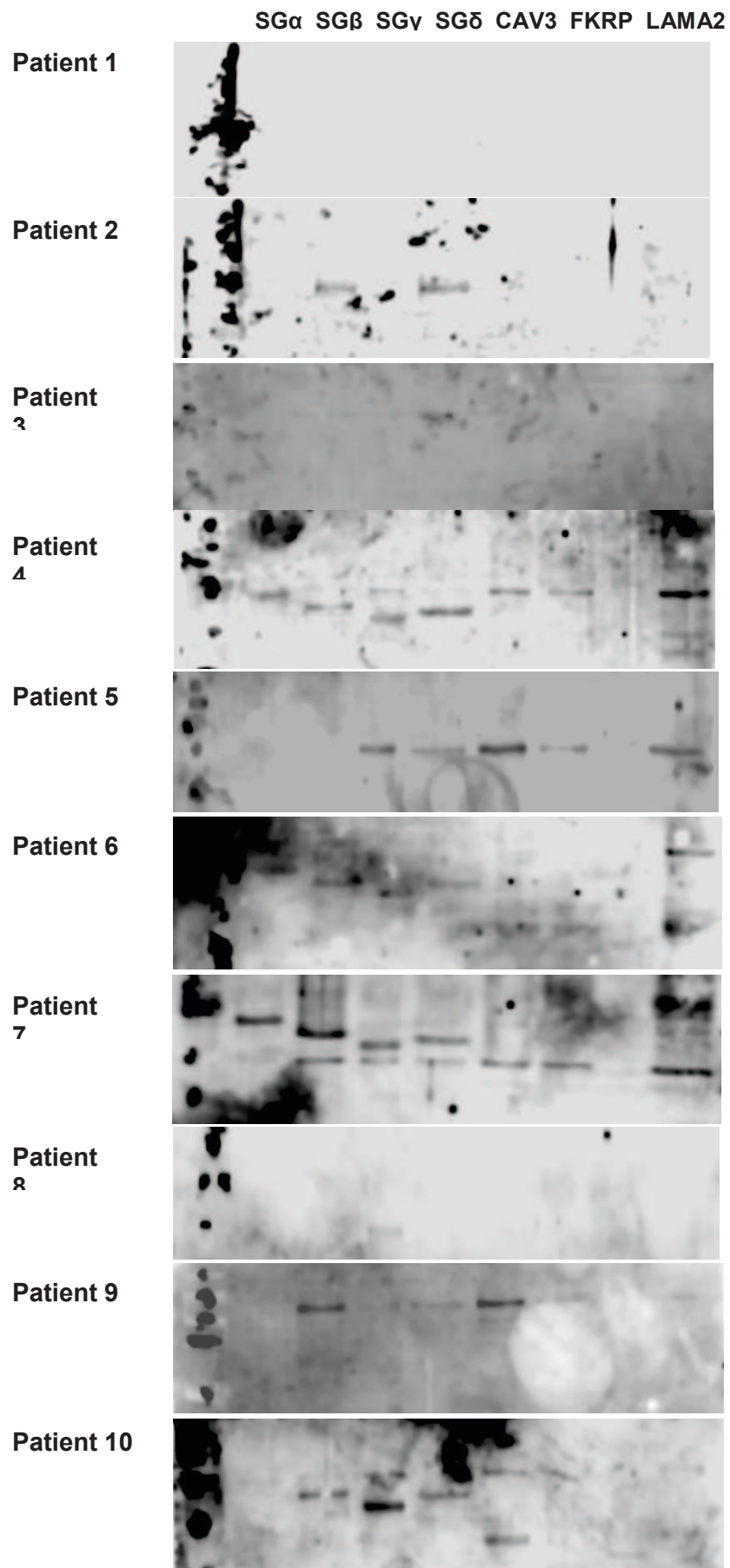
Table 3.3 Results of patients' and healthy controls reactivity to recombinant sarcolemmal proteins. Immunoreaction is reported as "positive" or "negative"

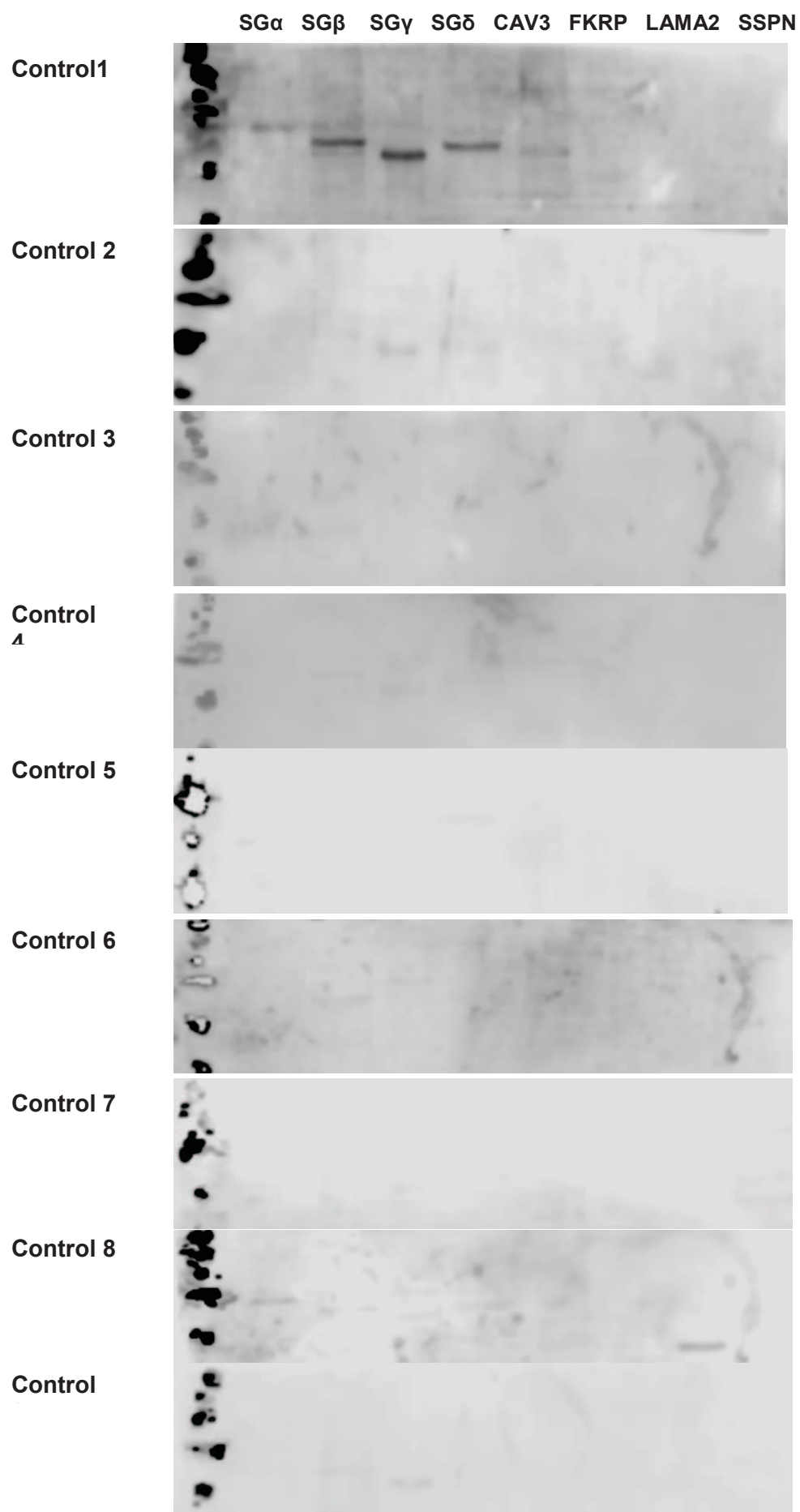
Patients	Recombinant Proteins							
	SGCA	SGCB	SGCG	SGCD	CAV3	FKRP	LAMA2	SSPN
1	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
2	NEG	POS	NEG	POS	NEG	NEG	NEG	NEG
3	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
4	POS	POS	POS	POS	POS	POS	NEG	POS
5	NEG	NEG	POS?	POS?	POS?	POS?	NEG	POS?
6	POS	POS	POS	POS	POS	POS	NEG	POS
7	POS	POS	POS	POS	POS?	POS?	NEG	POS?
8	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
9	NEG	POS?	NEG	POS?	POS?	NEG	NEG	NEG
10	NEG	POS	POS	POS	NEG	NEG	NEG	NEG
Controls								
1*	NEG	POS	POS	POS	NEG	NEG	NEG	NEG
2	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
3	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
4	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
5	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
6	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
7	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
8	NEG	NEG	NEG	NEG	NEG	NEG	POS?	NEG
9	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG

Positive (POS), negative (NEG), Dubious positivity (POS?)

*Healthy control with possible diagnosis of SLE

Figure 3.1 Western blots of recombinant sarcolemmal proteins blotted with patients and healthy control sera (next page).

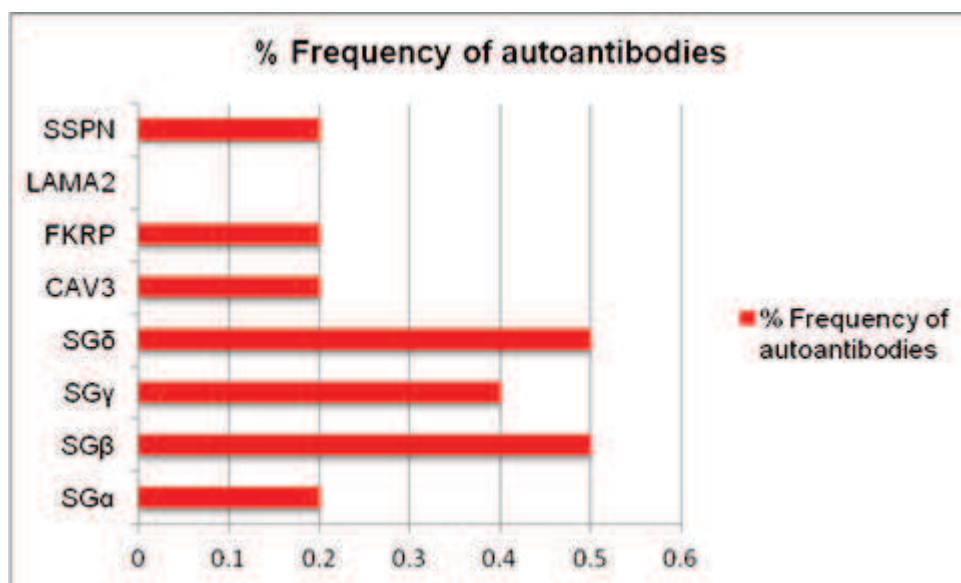




Frequency of the autoantibodies

In the studied patient cohort the most frequent autoantibodies were β -SG and δ -SG (50%), followed by γ -SG (40%), while all the others were found in 20% of the cases, apart from antibodies to laminin- α 2 that were not found in any of the patients. In some cases there was a dubious positivity and this was not considered. Although this is only a pilot study and the size of the studied cohort is small, these results show that the antibodies to $\beta/\gamma/\delta$ -SG are the most frequent autoantibodies in patients with query IIM or a definite diagnosis of IIM.

Figure 3.2 Frequency of autoantibodies in patients expressed as percentage



Alignments of sarcolemmal proteins

Using online available software (p-Blast) I have aligned the amino acid sequence of the proteins to establish identity of their sequences. The p-Blast analysis for alignment of two or more amino acid sequences is shown as a result of aligned sequences and hits with corresponding scoring. The smaller the E-value, which corresponds to the expected number of chance alignments, the better the match. This analysis showed that $\beta/\gamma/\delta$ -sarcoglycans have high homology and identity in

their amino acid sequence, while hits between the other proteins did not reveal significant levels of homology or identity.

DISCUSSION TO CHAPTER 3

The serological status of IIM patients together with clinic-pathological features is becoming more and more important for the classification of IIM. IIMs patients are increasingly classified by serological status, which closely associates with clinical phenotype. More than 50% of individuals with IIM develop MSA and MAA (Nagaraju, 2005). Although the pathogenic role of recognised MSAs and MAAs remains unclear, identification of autoantibodies can be useful in addressing patients' individual treatment (Finch, 2006). MSA and MAA are mainly being identified by testing patients' sera against proteins extracted from HeLa cell line and only recently other types of tissue, like skeletal muscle, is being used. HeLa cells in fact do not express several proteins that are tissue specific. As previously reported, recently we have identified novel autoantibodies targeting $\beta/\gamma/\delta$ -sarcoglycans in a small group of patients. Sarcoglycans may not be the only antigenic proteins to trigger an IIM. In fact, there are previous reports describing antibodies to the sarcolemma (Stuhlmüller et al, 1996) in patients with polymyositis; however this was not further characterised. Reports of antibodies to sarcolemmal proteins are also well known in other immune disorders including myasthenia gravis and myocarditis as well as very rare cases of immune-mediated myopathies with antibodies to extracellular matrix components (al-Lozi et al, 1995 and 1997; Yabe et al, 2002). MSA and MAA reported until now target intracellular antigens and interestingly some of these, i.e. Jo-1 and Mi-2, are expressed only in myotubes and in regenerating myofibres (Betteridge et al, 2009; Gunawardena et al, 2009), suggesting that they do not have a primary role in triggering the disease but are rather responsible for secondary events maintaining the inflammatory response.

Prompted by the previously identified antibodies against components of the sarcoglycan complex, I tested the serum of further 10 patients with a query diagnosis or diagnosis of IIM and 9 healthy controls against several recombinant commercially available sarcolemmal proteins.

Interestingly, the patients that were tested showed variable degree of positive reaction against the recombinant proteins, suggesting presence of autoantibodies. The only patient with a diagnosis of DM showed no reaction at all to the recombinant sarcolemmal proteins.

The most frequent autoantibodies were against the sarcoglycans complex with anti- δ -SG being the most frequent. Anti-sarcospan antibodies were also relatively frequent and always associated with anti-sarcoglycan antibodies. Antibodies to caveolin 3 and fukutin related protein were less frequent followed by antibodies to laminin- α 2. Unexpectedly, among the healthy controls one subject was found to have antibodies to the sarcoglycans. I discovered she had been investigated in the past for possible systemic lupus erythematosus for the presence of malar rash, which is still present but the diagnosis of SLE is not confirmed.

A few patients displayed positive bands at a different molecular weight to the expected one, whether this is true positivity or reaction or non-specific binding will need to be established in further studies with a larger cohort of patients and extended analysis. So far this is only a pilot study and false positive should also be considered and normal range of positivity will need to be established in further studies.

Correlation studies between autoantibody frequency in relation to their age or on gender predominance has not been performed, as it is difficult for the small sample size.

Alignment of the amino acid sequence of the different proteins using p-BLAST showed high degree of identity between SGCB, SGCG and SGCD and less with SGCA. SGCB, SGCG and SGCD are aligned for most of their lengths and have many identical and positive amino acids in common. For all other proteins no significant degree of homology or identity was identified. These data are in keeping with results obtained; in fact patients that showed positive reaction to SGCB were also positive for SGCG and SGCD.

In conclusion, this pilot study revealed novel autoantibodies, which may potentially be involved in the pathogenesis of IIM. Further studies on larger cohorts and functional studies are needed to better understand their role in the pathogenesis of IIM and the significance of these findings. If confirmed, these data could have also diagnostic and therapeutic implications, as antibodies could be used as biomarkers as well as give rise to novel therapeutic targets.

OVERALL DISCUSSION

Sporadic IBM is the most frequent inflammatory myopathy above the age of 50 years and despite enormous efforts from the scientific community, its pathogenesis still remains a dilemma and it is unclear whether it is a primarily inflammatory disorder with secondary degenerative pathological changes or vice versa.

Over the years the scientific community has invested efforts and resources in order to understand pathogenic mechanism of sIBM and in recent years identification of novel biomarkers has triggered initial excitement, followed by some disappointment since the newly identified autoantibodies, anti-NT5C1a were found not to be solely associated to sIBM (Larman et al, 2013; Pluk et al 2013; Lloyd et al, 2015).

The clinical and pathological heterogeneity of IIMs and in particular of sIBM represents a diagnostic and therapeutic challenge for the clinician and specific diagnostic markers are needed.

Main aim of this study was to identify molecules of interest that may shed light on the pathogenesis of sIBM by applying Chromowave, a recently developed mathematical model to process and analyse expression microarrays (Turkheimer et al., 2006), to analyse already published mRNA expression datasets of sIBM, DM and PM. When selecting patients from the Charing Cross Hospital IIM audit (2001-2011), I identified a smaller cohort of three patients that were initially diagnosed as sIBM based on the pathological features and some clinical features. Analysis of their sera allowed the identification of a novel autoantibody and target which prompted additional *in silico* and sequencing analysis, leading to the third part of this report. The third part of this report is a pilot study aiming to identify novel autoantibodies in patients with IIM using sarcolemmal proteins.

Patients reported here, identified from the cohort of IIM patients diagnosed and followed up at Charing Cross Hospital (Imperial College London), reflect the heterogeneity of IIM and the diagnostic challenges and highlight the need for validated diagnostic criteria.

In the first part of this study, I tried to identify molecules of interest that may shed light on the pathogenesis of sIBM and the re-analysis of already published mRNA datasets using a newly developed mathematical model allowed me to identify a

nuclear protein, emerin, as a possible key protein in the pathogenesis of sIBM. Chromowave analysis showed significant reduced expression of emerin in sIBM. Emerin has been previously reported together with other proteins in rimmed vacuoles. Immunohistochemistry studies confirmed previous reports, however I also observed misplacement of emerin at a sarcolemmal level and at intranuclear level as well, making the nuclei appear as ground glass, with less defined nuclear rim. If confirmed on a larger scale this observation could be used as a biomarker for the diagnosis of sIBM.

Sequencing analysis of the emerin gene of sIBM patients showed various SNPs in the 5'UTR of the gene. Although functional studies are needed to prove their pathogenicity, it is well known that 5'-UTR regions are crucial site for correct processing of the mRNA and point mutations and truncations in UTRs may affect translation. *In silico* analysis using GeneBee software, showed that these mutations may affect the secondary mRNA structure. This result could explain the mislocalisation of emerin at sarcolemmal level and intranuclear level. Additional studies to define the pathogenicity of variants identified in the 5'-UTR of the emerin gene are needed for example through a luciferase assay (Wang et al, 2007) since the 5'-UTR is not expressed.

Thanks to the revision of a previous audit of IIM patients of the Charing Cross Hospital cohort, three patients with sIBM-like phenotype have been identified and have been further investigated in view of their unusual phenotype. Further investigation of these patients led to the second chapter of this study. Serum analysis of these three patients tested against sarcolemmal proteins allowed me to identify a novel autoantibody and its antigen.

Skeletal muscle proteins have rarely been used to identify autoantibodies in IIM, and most of the antibodies so far identified are being detected using HeLa cell lysate, and notably HeLa cells may not express all muscle specific proteins. In addition, most of the known MSA target a cytoplasmic antigen and generally are not expressed in adult muscle, suggesting that they are unlikely to be the primary trigger. Recently proteins extracted from other types of tissue other than HeLa cell line, are being used and allowed the identification of a novel autoantibody anti-NT5C1a

(Larman et al, 2013; Pluk et al 2013). HeLa cells in fact do not express several proteins that are tissue specific.

The serum of these three patients with IBM-like phenotype was blotted against sarcolemmal proteins and resulted in the identification of a novel autoantibodies targeting $\beta/\gamma/\delta$ -sarcoglycans. This is the first time that autoantibodies to sarcolemmal proteins, the sarcoglycans, have been reported as in association to inflammatory myopathy. The three patients were eventually found to carry a missense mutation in the β -sarcoglycan gene which likely triggered an autoimmune reaction since the three patients were found to have antibodies targeting the sarcoglycan complex. Using an in silico approach we created a β -sarcoglycan protein model to explain potential changes to the 3D structure of the protein induced by this mutation.

Sarcoglycans may not be the only antigenic proteins to potentially trigger an IIM. Previous reports already described antibodies to the sarcolemma in patients with polymyositis, myasthenia gravis, myocarditis and very rare cases of immune-mediated myopathies with antibodies to extracellular matrix components (al-Lozi et al, 1995 and 1997; Stuhlmüller et al, 1996; Yabe et al, 2002). Autoantibodies reported until now target intracellular antigens and in some cases are expressed only in myotubes or in regenerating myofibres, suggesting that they do not have a primary role in triggering the disease but are rather responsible for secondary events maintaining the inflammatory response (Betteridge et al, 2009; Gunawardena et al, 2009).

This striking finding led me to design a pilot study (third part of this report) to study a further cohort of IIM patients and control subject and test their sera against recombinant sarcolemmal proteins.

Further analysis of IIM patients' sera against recombinant sarcolemmal protein allowed me to identify positive reaction in some patients suggesting presence of antibodies targeting sarcolemmal proteins. Further studies on a larger cohort of patients are needed to determine the specificity and prevalence of these antibodies are needed. Nonetheless this finding allowed us to file a patent for the development of a new diagnostic test able to identify novel antibodies in IIM patients.

GLOSSARY

Anti NXP2 anti-nuclear matrix protein 2

Anti-ARSs Anti-aminoacyl-tRNA synthetase

Anti-CADM-140/MDA5 antibodies anti melanoma differentiation associated gene 5

Anti-cN1A anti-cytosolic 5'-nucleotidase

Anti-EJ anti-glycyl synthetase

Anti-Ha anti-tyrosyl synthetase

Anti-HMGCR anti-3-hydroxyl-3-methylglutaryl-coenzyme A reductase

Anti-Jo1 anti-histidyl synthetase

Anti-KS anti-asparaginyl synthetase

Anti-OJ anti-isoleucyl synthetase

Anti-PL12 anti-alanyl synthetase

Anti-PL7 anti-threonyl synthetase

Anti-PM-Scl anti-polymyositis-scleroderma

Anti-SAE small ubiquitin-like modifier activating enzyme 1 & 2

Anti-SRP signal recognition particle

Anti-URNPs anti-small ribonucleoprotein

Anti-Zo anti-phenylalanyl synthetase

ASO allele specific oligonucleotide

BLAST Basic Local Alignment Search Tool

CAV3 caveolin 3

CK creatine kinase

DAPI 4',6-diamidino-2-phenylindole

DM dermatomyositis

DMPC 1,2-dimyristoyl-sn-glycero-3-phosphocholine

ECL enhanced chemiluminescence

EM electron microscopy

EMG elctromyography

FKRP fukutin related protein

FLNC filamin C

H&E Hematoxylin and eosin

HLA human leukocyte antigen

IF immunofluorescence

IFN interferon

IHC immunohistochemistry

IIM Idiopathic inflammatory myopathy

IL interleukin

ILD interstitial lung disease

IM-VAMP inflammatory myopathy – vacuoles, aggregates, mitochondrial pathology

INM inner nuclear membrane

IVIg intravenous immunoglobulins

jDM juvenile dermatomyositis

LAMA2 laminin- α 2

LDH lactate dehydrogenase

MAA myositis associated antibody

MAC membrane attack complex

MD molecular dynamics

MHC Major histocompatibility complex

MSA myositis specific antibody

NE nuclear envelope

PCR polymerase chain reaction

PE plamsa exchange

PM poymyositis

PME particle Ewald method

RIPA Radioimmunoprecipitation assay

RMSF Root mean square fluctuation

RVs rimmed vacuoles

SDS-PAGE Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis

SG sarcoglycans

sIBM sporadic inflammatory myopathy

SLE systemic lupus erythematosus

SPC simple point charge

SSPN sarcospan

Tasser Iterative Threading ASSEmbly Refinement

TNF tumour necrosis factor

UTR untranslated region

WB western blot

WT wavelet transform

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