Clinical and Molecular Genetics

of the

Multiple Pterygium Syndromes

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Abstract

The multiple pterygium syndromes are a heterogeneous group of conditions in which arthrogryposis (joint contractures), pterygia (webbing) and a variety of other developmental anomalies are present. It is caused by lack of fetal movement in the womb. Mutations in *CHRNG*, the embryonic subunit of the acetylcholine receptor (AChR), cause some of the cases. *CHRNG* mutation analysis was undertaken in a large patient cohort of 100 families and the mutations identified were included in a new Locus Specific Database. Genotype phenotype analysis showed that pterygia were almost invariably present in the *CHRNG* mutation positive patients.

It was hypothesised that mutations in other genes necessary for fetal AChR function may cause fetal akinesia. Using a candidate gene approach a homozygous frameshift mutation in *RAPSN* was identified in one family and a homozygous splice site *DOK7* mutation in second family. Mild mutations in both *RAPSN* and *DOK7* have been previously identified in the congenital myasthenic syndromes (CMS). Thus, mild mutations in *RAPSN* and *DOK7* cause CMS whereas severe mutations cause fetal akinesia.

Finally, work was done to identify a novel cause of fetal akinesia in a consanguineous family using an autozygosity mapping approach. A region of homozygosity was located and candidate genes sequenced.

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List of Abbreviations

AChR	acetylcholine receptor
ACh	acetylcholine
ACTA-1	*ACTA-1* gene
AMC	arthrogryposis multiplex congenita
CHRNG	gamma subunit of the embryonic AChR gene
CHRNA1	alpha1 subunit of the embryonic AChR gene
CHRNB1	beta1 subunit of the embryonic AChR gene
CHRND	delta subunit of the embryonic AChR gene
cM	centimorgans
DOK7	downstream of kinase 7 gene
EVMPS	Escobar variant multiple pterygium syndrome
FADS	fetal akinesia deformation sequence
LMN	lower motor neurone
LMPS	lethal multiple pterygium syndrome
MPS	multiple pterygium syndrome
NEB	nebulin gene
NMJ	neuromuscular junction
RAPSN	gene encoding rapsyn
RYR1	ryanodine receptor gene
SEPN1	selenoprotein N gene
UMN	upper motor neurone

Chapter 1: General Introduction

1.1 Introduction

This project was designed to study the multiple pterygium spectrum disorders, a group of rare conditions characterised by joint contractures or arthrogryposis, pterygia or webbing across the joint and a variety of congenital anomalies. This spectrum of conditions has been considered in the context of congenital anomalies. Particular attention has been given to joint contractures, a consistent feature of this group of conditions, as the causes of arthrogryposis provide important insights into the underlying mechanisms for the clinical phenotype. This information can be also be utilized in selecting candidate genes for further study and in understanding the biological basis of disease.

This chapter reviews the literature up to 2010 when the clinical and laboratory work had been completed and the MD research thesis write-up commenced.

1.2 Congenital Anomalies

The word “congenital” is derived from the Latin “congenitus” first used in 1796, meaning present or existing at the time of birth. Babies may be born with a variety of structural developmental abnormalities known as congenital anomalies, congenital abnormalities, congenital malformations or birth defects. During the first 14 days after conception insults or injuries to the embryo are either repaired or result in an early fetal demise and miscarriage of the pregnancy. Embryopathies can arise up to around 8 weeks post conception when the rapidly developing embryo is especially vulnerable to damage because of the large numbers

of mitotic cell divisions. Fetopathies result from damage to the fetus after this time. However most organs, except for the brain, are formed by this time and are therefore less susceptible to injury. Defects in fetal development may be due to environmental, genetic, multifactorial or unknown factors. A recognisable pattern of developmental problems is referred to as a syndrome.

Congenital anomalies can arise through a variety of mechanisms. There are various classifications for congenital abnormalities. A primary abnormality is due to an inherent defect that affects organ development. Primary abnormalities can be divided into gene defects, which cause 7.5% of congenital anomalies, and chromosome aberrations, which account for approximately 0.5%. Secondary abnormalities are caused by factors that adversely affect development before birth. The development of a secondary congenital abnormality is influenced by the health of the mother, the nature of the toxin or insult, the timing of the damage and the genetic background of the fetus. Multifactorial anomalies are due to environmental factors acting on genetically susceptible individuals. Neural tube defects ranging from spina bifida through to anencephaly have been associated with genetic as well as environmental factors. The high recurrence risk for siblings of those with a neural tube defect supports a role for genetic susceptibility (Detrait et al., 2005, Harris and Juriloff, 2007) and the prevention and reduced recurrence of neural tube defects with preconception folic acid supplementation is evidence of the ameliorating effect of an environment factor (Blencowe et al., 2010).

Review of the likely mechanisms leading to a congenital anomaly can be helpful in elucidating the underlying cause of that developmental abnormality, providing information

about the aetiology and likelihood of recurrence for families as well as enabling classification of the data for the collection and public health monitoring purposes.

1.2.1 Mechanisms of congenital anomalies

Congenital anomalies can arise through four different mechanisms: malformation, disruption, deformation and dysplasia.

1.2.1.1 Malformations

Major malformations arise in the embryonic period of development, between two and eight weeks of gestation, and are due to inherent differences in the development of a tissue. These congenital anomalies can be due to altered processes in the development of the egg, sperm or the embryo which may occur before, during or after fertilisation. They can be due to single gene disorders as well as chromosome abnormalities.

A chromosome disorder may be suspected in an affected infant because of the presence of multiple congenital anomalies that may be accompanied by global developmental delay. A condition with recognisable pattern of developmental abnormalities is referred to as a syndrome. Examples include Down syndrome or trisomy 21, Edward syndrome or trisomy 18 (Edwards et al., 1960) and Patau syndrome or trisomy 13 (Patau et al., 1960), in each of which a whole extra chromosome is present. Smaller chromosome imbalances, microduplications or microdeletions, are increasingly detected due to the availability of higher resolution chromosome testing (microarrays) and new phenotypic associations are being described. An example is the 3q29 microdeletion syndrome (Willatt et al., 2005). Single gene disorders may affect the development of one or more organ. For example Holt-Oram

syndrome due to autosomal dominant mutations in TBX5 is characterised by congenital heart disease and radial ray abnormalities (Mori and Bruneau, 2004).

1.2.1.2 Disruption

Disruption is the adverse affect or interruption of the usual developmental processes by extrinsic factors and can arise at any time in the fetal period, commencing at eight weeks of gestation. An example is the loss of peripheral elements usually of the limbs due to amniotic bands. Approximately 10-15% of congenital anomalies are estimated to be the result of adverse environmental factors on prenatal development, the majority of which are due to maternal disease states and less than 1% to external agents such as drug or chemical exposure (Brent and Beckman, 1990). Other external factors which may disrupt the usual developmental processes include teratogens such as alcohol, drug treatments such as sodium valproate for epilepsy, diabetes and infections such as cytomegalovirus and rubella. Teratogens can have a wide range of affects depending on the timing and nature of the exposure. Teratogens can cause reduced fertility, intrauterine growth retardation, structural abnormalities, intellectual disability as well as pregnancy loss and fetal death (Gilbert-Barness, 2010).

1.2.1.3 Deformation sequence

This is the response of normal tissue to an unusual mechanical force that occurs in the fetal period of development and is more frequent in the third trimester due to greater fetal constraint. A sequence of fetal consequences can arise due to a single malformation or mechanical factor in the fetus or the mother. Potter’s sequence has an incidence of 1 in 4000

births**.** Oligohydramnios in utero due to fetal renal agenesis, polycystic kidneys or an obstructive uropathy results in the typical constellation of features which includes a recognisable facial appearance and severe usually fatal respiratory insufficiency due to pulmonary hypoplasia (Potter, 1946). Pierre-Robin sequence has an incidence of approximately 1 in 8500 live births (Bush and Williams, 1983) and is characterised by a triad of micrognathia, cleft palate and retroglossoptosis (Robin, 1994). Although the causes of the Pierre-Robin sequence are very heterogeneous, this triad of anomalies arises from a single malformation. The mechanical obstruction caused by mandibular hypoplasia results in the tongue being positioned high in the oral cavity preventing closure of the palatal shelves. Delay in the neurological maturation of the tongue musculature may contribute to the Pierre Robin sequence. This is supported by self-correction with time in the majority of cases. Fetal akinesia deformation sequence (FADS) is another example of a deformation sequence with an estimated incidence of 1 in 15,000 (Bayat et al., 2009). A recognisable pattern of developmental features attributable to fetal immobility includes joint contractures, pulmonary hypoplasia and a cleft palate. A lack of swallowing movements may also be associated with maldevelopment of the abdomen and gut in some patients and a dysmorphic appearance including hypertelorism.

1.2.1.4 Dysplasia

Dysplasia or congenital abnormalities of tissue development or differentiation may affect several organs at the same time. Examples are skeletal syndromes in which there may be a dysplasia of the developing bone or cartilage. However the cause may not be identified as it may be a non-specific effect.

1.2.2 Consequences of congenital abnormalities

Isolated congenital anomalies or multiple congenital anomalies affecting more than one system of the body may be present. There may be major or minor abnormalities. Minor congenital anomalies have no serious medical consequences whilst major anomalies may require medical or surgical treatments or result in early demise. Major congenital anomalies occur in 2-3% of fetuses and newborns. After immaturity they are the second most common cause of infant death accounting for 0.1% of deaths in liveborn children (Office for National Statistics, 2010). Following prenatal detection of fetal anomalies, a fifth of cases are lost through pregnancy termination, die after 20 weeks of gestation, are stillborn or die soon after birth. Approximately 80% are liveborn (Dolk et al., 2010). Chromosome abnormalities are identified in 0.36% of births, but account for almost a third of fetal deaths and a congenital anomaly diagnosed after 20 weeks is present in almost half of pregnancy terminations.

In the non chromosomal group, congenital heart defects occur most frequently in 0.63%. Cardiac disease is the commonest cause of death from a congenital anomaly. Limb anomalies are the next most frequent congenital anomaly occurring in about 0.36%, however unlike congenital heart disease they do not usually result in death. Urinary tract anomalies are identified in 0.28% and nervous system anomalies in 0.22%. Congenital anomalies in other systems occur less frequently (Office for National Statistics, 2010, (Dolk et al., 2010).

Particular attention has been given below to limb anomalies, as these are a major component of MPS / FADS, the subject of this research study.

1.2.3 Limb anomalies

Limb defects may be detected antenatally or immediately after birth. This group includes a wide variety of limb anomalies, such as upper and lower limb reduction defects, polydactyly and syndactyly, as well as arthrogryposis multiplex congenita and isolated joint contractures such as congenital clubfoot and congenital hip dysplasia and dislocation. These conditions occur with widely differing frequencies with complete absence of a limb occurring in 0.18 per 10,000 pregnancies whereas congenital club foot is recorded in 9.8 per 10,000 pregnancies (Dolk et al., 2010). Limb defects may be isolated or may occur with multisystem involvement as part of a syndrome. They may occur as a consequence of environmental or genetic conditions or as part of a deformation sequence. An example is the multiple joint contractures that occur as part of the Fetal Akinesia Deformation Sequence (FADS). Joint contractures / arthrogryposis are a major component of the Multiple Pterygium Syndromes (MPS) / FADS spectrum of conditions.

1.3 Limbs and joints

As development of the limbs and joints occurs early in gestation, genetic factors and very early environmental insults incur the maximal impact.

1.3.1 Limb development

The upper limb bud appears at 26 days of gestation and is followed on the 28th day by the lower limb bud. The limb buds are mainly comprised of mesenchymal cells which differentiate into the various limb tissues. Cells derived from somites form a distinct

population that become the myoblasts. Neural elements later invade the developing limb bud. The apical ectodermal ridge is a specialised layer of ectoderm which drives the differentiation of mesenchymal cells. Limb structures are formed in a proximal to distal direction, for example the humerus and femur before the digital structures. Development and maturation of the bones, muscles and nerves of the limbs occurs in parallel throughout gestation.

Early on in development the preaxial borders of the upper limbs face cranially but are medially positioned by the end of the embryonic period. In the lower limbs the soles of the feet are medially positioned in a "praying" stance however during the fetal and early postnatal period the foot position is modified so that the sole of the foot can rest on the ground. The future skeleton is laid down as a collagen template in the first month of fetal life onto which a mineralised bone is deposited from the second month. The process of chondrogenesis occurs in an oxygen poor environment whereas osteogenesis requires a good supply of oxygen. There are important differences between embryonic and postnatal bone. Embryonic bone contains irregularly arranged collagen fibres, is highly mineralised and contains a large number of osteocytes whereas lamellar bone is less mineralised, contains fewer osteocytes and gradually covers the connective tissue vascular spaces of the embryonic bone. All primary bone is remodelled by the age of 2 years. Ossification of the long bones begins in the perichondral region and proceeds distally. Ossification of the vertebrae occurs from three primary ossification centres and commences in the lower thoracic vertebrae from around the eighth week and proceeds cranially and caudally (Royer et al., 1981). Skull bones are the last to fuse, with some cranial sutures only fusing in adult life (Enlow et al., 1986).

Multinucleated muscle cells appear at about five weeks of gestation and early muscle fibres are present from around the eleventh week. By about 20 weeks most muscle fibres appear similar to adult muscle. Although initial muscle development occurs in the absence of nerve innervation, neurological stimulus is required from 20 to 24 weeks of gestation to enable further muscle development and differentiation. The two muscle fibre types, slow and fast twitch fibres, cannot be distinguished before 18 to 20 weeks of gestation. Slow twitch fibres are present in early development whilst fast twitch fibres increase after week 26 and approximately similar numbers of both fibre types are present at term. Muscle cells increase in number rather than size during the first 26 weeks. After this time the cell size increases and the cell number increases more slowly. Growth occurs by hypertrophy of existing fibres once muscle differentiation is complete.

From around week 10 primitive nerve fibres are found in the vicinity of the muscle fibres. Myoneural junctions are formed by week 11 and recognisable motor end plates can be identified. During development the several motor nerves innervating a single muscle fibre are replaced by a single axon. The precursor to the muscle spindle, the spindle nerve fibre complex can already be identified between 9 and 10 weeks of gestation in human embryos (Deriu et al., 1996).

1.3.2 Joint classification

Joints are derived from mesenchyme. There are three types of joint, fibrous joints, cartilaginous joints and synovial joints, each with different mechanical properties adapted to their function in the body. In the skull, the joints between the cranial sutures form as the mesenchyme is transformed into a dense fibrous connective tissue. The cranial sutures allow

for slight movement critical during childbirth and for brain growth in early childhood however these joints later become rigid to provide better protection for the brain. In cartilaginous joints the mesenchymal tissue between the bones becomes cartilage. Cartilaginous joints allow more movement between the joints than fibrous joints, but are less mobile than synovial joints. Examples are the manubrio-sternal joint which enables effective upward and outward chest movement that occurs with each breath, the intervertebral discs allowing spinal mobility and the pubic symphysis assisting delivery of the head during childbirth. The third type of joint is the synovial joint. These joints are the most mobile and allow the widest range of movement, but have the least joint stability. This is particularly well illustrated by the rotatory motion that can be achieved by the hip. Other synovial joints with a lesser range of movement include the elbow, the knee and the interphalangeal joints. Synovial joints are comprised of complementary shaped surfaces with an articular cartilage covering. The joint has a synovial lining and is encased in a synovial capsule. Stability and joint movement is provided by the ligaments in and around the joint. The various components of the joint are adapted to their particular roles. The articular cartilage is stratified into layers. The superficial zone produces lubricants to enable smooth joint movements. The long flat cells are embedded in a collagenous matrix parallel to the articular surface. The cells in the middle zone are round and ordered vertically. They give the material its toughness. The cells in the bottom zone interface between the articular cartilage and the underlying bone. The cells here are large and also involved in matrix production (Archer et al., 2003).

1.3.3 Joint development

The mammalian skeleton in embryos is formed from a continuous cartilaginous structure. Histologically the joint forms from a layer of avascular, compact mesenchymal tissue that

interrupts the adjacent cartilage. This interzone region expresses growth and differentiation factors essential for joint development, as removal of the interzone cells results in expansion and union of the cartilaginous segments. The cells in the interzone region are flat and tightly bound at right angles to the limb (Holder, 1977, Storm and Kingsley, 1996). These cells have been shown to be joint progenitor cells for a variety of tissues including articular cartilage, synovial lining and ligaments within the joint that remain functional in the adult. However other cells need to migrate from the surrounding tissues into the developing joint to enable joint development to proceed (Pacifici et al., 2006, Decker et al., 2014). A summary of the sequence of events in joint formation are outlined below in Fig 1.

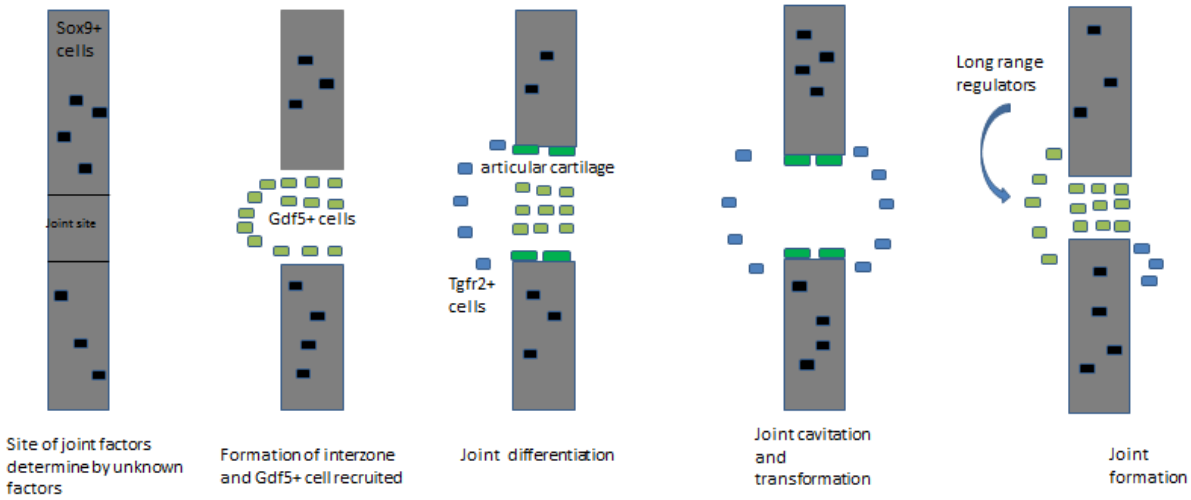


Figure 1: Generalised model of joint development

(Taken from Genesis and morphogenesis of limb synovial joints and articular cartilage, Decker et al., 2014).

The components of the bone mesenchyme are continuous throughout the structure. Unknown upstream mechanisms determine the site of future joint development. Initial formation of the

mesenchymal interzone cell population occurs through the expression and migration of *Gdf5* positive mesenchymal cells in the interzone to form both the cartilage and fibrocartilagenous elements of the joint. This process is dependent on the *Noggin, Wnt4/Wnt9a, Tgfbr2*, β-catenin, *TAK1* and *Ext2,* as defective joint formation and cartilage fusion due to early or excess chondrocyte differentiation have been observed in mice lacking these genes (Brunet et al., 1998, Spater et al., 2006, Spagnoli et al., 2007, Gunnell et al., 2010, Mundy et al., 2011). Differential expression of *Tgfbr2* and *matrillin-1* influences the development of the articular chondrocytes, ligaments and other joint structures as well as population of progenitor cells for repair and regeneration. Joint formation and development of the growth plate and bone shaft are closely coordinated. This is illustrated by the joint fusion seen in mice lacking *ihh* expression (St-Jacques et al., 1999) and the marked joint, meniscal and growth plate abnormalities in mice lacking the chondrogenic genes *Sox5* and *Sox6* (Dy et al., 2010). Other local regulators of joint formation include *Sox11, Osr1* and *Osr2* zinc transcription factors (Kan and Tabin, 2013, Gao et al., 2011). The fluid filled synovial joint space is formed by cavitation later in gestation and in early postnatal life. Immobilisation inhibits joint cavitation, whereas the earlier developmental processes in the joint such as specification of the cells, limb patterning and the growth of the joint occur even in the absence of muscular activity (Lamb et al., 2003), thus other genes are likely to drive joint cavitation. It has been suggested that the joint cavity appears by programmed cell death (Abu-Hijleh et al., 1997) however there is little evidence to substantiate this (Pacifici et al., 2005, Pitsillides and Ashhurst, 2008).

The development and growth of bones and joints is a dynamic process modified by movement. Load bearing movement significantly increased adult bone strength. This effect

has been shown to be more marked in slow growing animals. Bones with inherently slower rates of growth, which may be due to genetic factors, are more responsive to extrinsic stimuli (Patterson et al., 1986, Fleming et al., 1994). Experimental evidence has shown that movement has an important role in the formation and maintainance of the joint cavity. A lack of embryonic joint movement prevents formation of joint cavities and causes regression of the joint cavities that have been formed and fusion of opposing cartilaginous joint elements (Osborne et al., 2002). Nerve damage resulting in immobilization inhibits the formation of the joint cavity (Osborne et al., 2002) whereas movement stimulates the establishment of joint cells and joint lubricants (Dowthwaite et al., 2003). However experiments with agents causing irreversible neuromuscular blockade and muscle spasticity, and thus a continuous static load on the joints, have been shown to inhibit the formation of joint cavities and promote joint fusion (Kavanagh et al., 2006). In addition, mouse embryos without contracting muscles fail to activate Wnt/β-catenin signalling and develop normal joints. As certain joints appear to be more susceptible to this effect, there may be a differential joint response. This may due to a different mechanism and pattern of signalling molecules depending on the joint (Kahn et al., 2009). Factors such as the joint function and the timing of the immobility may be produce varying affects on the different joint types. In addition, earlier developmental processes such as limb outgrowth and limb patterning occur independently of limb movement and muscle activity and may be determined by underlying genetic programming (Lamb et al, 2003). Overall, although the formation of limb joints is a complex interplay between the cells in and adjacent to the interzone, the growth plate and many transcription and signalling factors at various stages of development, mechanical factors such as the action of skeletal muscles during movement are also essential for this process.

1.4 Joint contractures and arthrogryposis

Joint contractures can occur as an isolated finding or in association with a variety of congenital abnormalities as a component of a syndrome or in utero deformation sequence. Isolated joint contractures affecting a single joint type are common and may be present in healthy newborns. Conditions such as congenital talipes equinovarus or congenital dislocation of the hips are seen in more than 1% of newborns. There may be family history of similar joint contractures for example a positive family history of developmental hip dyplasia has been observed in over 20% of children requiring treatment for this condition (Marks et al., 1994). This inherited predisposition may be due to factors such as joint laxity or the skeletal development in the family. Environmental factors such as oligohydramnios and a breech presentation at birth are well recognised risk factors for congenital hip dislocation (Chan et al., 1997). Joint contractures may occur in association with other congenital abnormalities in a wide range of genetic syndromes notably Edward syndrome (Trisomy 18) and cerebro- oculo-cutaneous-skeletal syndrome (COFS), a neurodegenerative autosomal recessive disorder which also includes features of microcephaly, congenital cataracts, severe mental retardation and a dysmorphic facial appearance (Pena and Shokeir, 1974). They may occur as a result of central or peripheral nervous system insults, muscle disorders as well as environmental exposure to congenital infection. Progressive joint contractures may result from disease processes such as inflammatory myositis which affect the soft tissue components around the joint.

Arthrogryposis multiplex congenita or multiple joint contractures are less common. The incidence has been estimated to be 1 in 3000 live births (Fahy and Hall, 1990, Hall, 1985),

however a Swedish study suggested a birth prevalence of 1 in 5000 live births for multiple congenital contractures (Darin et al., 2002). AMC may be detected on ultrasound scan in utero, particularly if there is extensive joint involvement, congenital abnormalities and severe associated fetal akinesia. For the incidence of joint specific congenital contractures see Table 1.

All congenital contractures	**1/100 – 1/250**
Talipes equinovarus (Danielsson, 1992, Paton et al., 2010, Bridgens and Kiely, 2010)	1-2/1000
Congenital dislocated hips (Sewell et al., 2009)	1-3/100
AMC (Hall, 1985) (Darin et al., 2002)	1/3000

Table 1: Incidence of congenital contractures.

(Adapted from Arthrogryposis: A Text Atlas, edited by Staheli, Hall, Jaffe, Paholke, Cambridge University Press, 1998).

1.4.1 Aetiology of arthrogryposis

Arthrogryposis is a descriptive term derived from Greek in which ″Arthron″ means joint and ″Grypos ″ means curved. Thus arthrogrypotic joints are fixed in a curved position. Arthrogryposis multiplex congenita (AMC) is defined by the presence of multiple joint

contractures affecting more than one region of the body that are present from birth. Fetal movement is essential for normal joint formation and arthrogryposis occurs when there is decreased joint movement in the fetus. During development, whole body movements in the fetus commence from around 7-8 weeks of gestation (de Vries et al., 1982). Upper limb movements begin at about 9 weeks and lower limb movements start from around 10 weeks of gestation (Luchinger et al., 2008). Contractures result from factors that inhibit normal fetal movement and are associated with increased connective tissue around the joints. Immobilised joints surfaces become flattened, further limiting the range of joint movement. More severe contractures at birth are associated with an earlier onset and longer duration of fetal akinesia (Moessinger, 1983, Hall, 1986).

The aetiology of AMC is very heterogeneous and diverse (see figure 2), thus the character of the joint contractures, the associated features, prognosis, inheritance and subsequent recurrence risks vary widely. Fetal hypokinesia or akinesia can be due to factors in the mother or in the fetus. It can be due to extrinsic or environmental factors such severe fetal constraint due to lack of available space as well as or intrinsic or genetic factors, with arthrogryposis being a recognised component of more than three hundred conditions (Hall et al., 1982a). The affected limbs are typically cylindrical with thin overlying subcutaneous tissue, an absence of skin creases and the muscle may appear atrophic or absent (Wynne-Davies and Lloyd-Roberts, 1976). As well as rigidity of the affected joints there may be joint dislocations most frequently affecting the hips followed by the knees. Involvement of the temporomandibular joint frequently results in associated limited mouth opening or trismus. The contractures themselves may have different characteristics depending on whether they are due to intrinsic or external factors. In general, joint contractures caused by intrinsic factors are symmetrical

with tightness of the overlying skin and a lack of flexion creases over the joints. Additional accompanying features may include polyhydramnios and webbing across the joints. Contractures due to external forces may be associated with position limb anomalies, normal or prominent flexion creases lax or tight skin (Navarro et al., 2004). A sub-type of Ehlers-Danlos syndrome has been described in which individuals have recognisable craniofacial characteristics, multiple congenital contractures, progressive joint and skin laxity, and multisystem fragility-related manifestations (Kosho et al., 2010)**.** The most common cause of arthrogryposis is amyoplasia, accounting for a third of patients with AMC. A further third have an underlying central nervous system abnormality causing their arthrogryposis and the remaining third are due to a variety of disorders, see Fig 2. It has been estimated that a specific cause for the arthrogryposis can be identified in half of patients (Fahy and Hall, 1990).

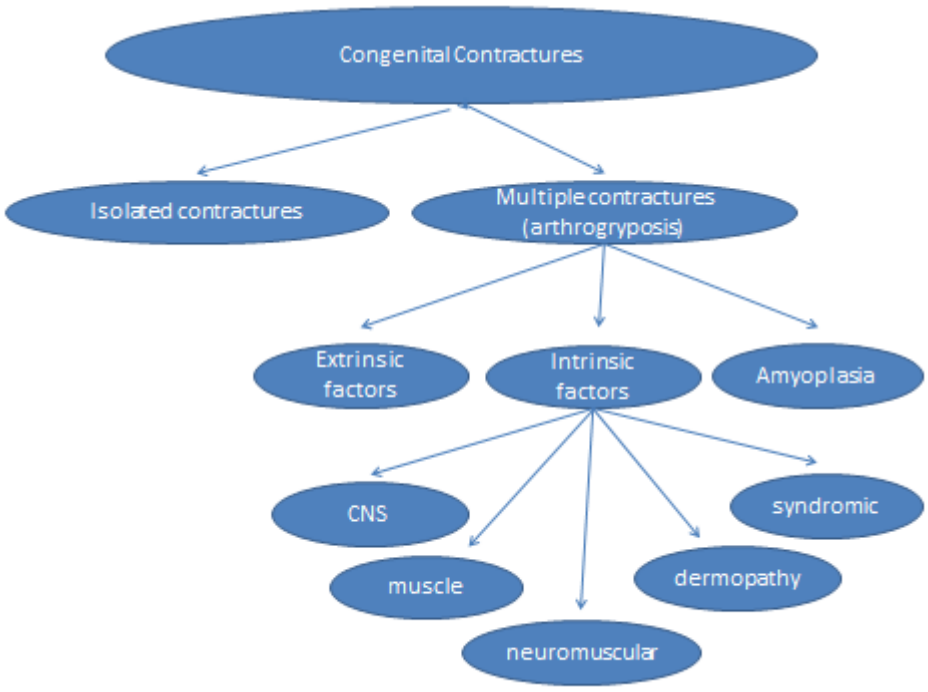


Figure 2: Aetiology of Congenital Contractures

1.4.1.1 Amyoplasia

Amyoplasia literally meaning a lack of muscular growth is the commonest cause of AMC, with an incidence of approximately 1 in 10,000 live births. In three quarters of patients all four limbs are involved and there is symmetric involvement of the upper and lower limbs in 60-80% of patients (Sells et al., 1996, Hall et al., 1983). However, less commonly there is involvement of the upper or lower limbs or an asymmetric pattern of limb involvement (Vanpaemel et al., 1997, Hall et al., 1983). Typically the shoulders slope downwards and are internally rotated and adducted. The elbows are usually extended and wrists are flexed and deviated to the ulnar side. Accompanying dimpling may be observed over the affected joints. There is fibrotic replacement of the muscle and a glabella haemangioma may be present. Intellectual development is normal. There is usually a good response to early physiotherapy. Amyoplasia is almost certainly not genetic and may be due to vascular compromise in early development. This is supported by the occurrence of amyoplasia in only one sibling of several monozygotic twin pairs, and the association of trunk wall defects and congenital bowel anomalies such as gastroschisis and bowel atresias in around 10%. Amyoplasia usually occurs sporadically and has a low recurrence risk (Hall et al., 1983).

1.4.1.2 Maternal Factors

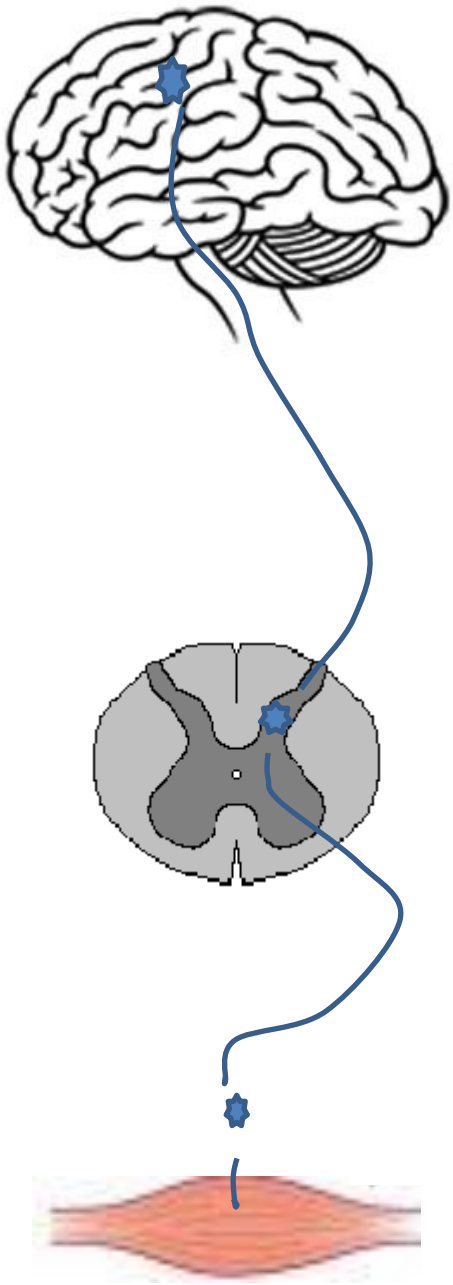
Arthrogryposis may be due to factors in the mother. Myasthenia gravis is an acquired autoimmune condition. It most commonly presents in females during adolescence or early adulthood and is characterised by progressive weakness and fatiguability of the voluntary muscles. It is caused by antibodies binding to nicotinic acetylcholine receptor (AChR) at the neuromuscular junction resulting in loss of these receptors (Drachman, 1994). Conditions such as maternal myasthenia gravis cause arthrogryposis by the transfer of maternal

acetylcholine receptor antibodies through the placenta to the fetus. IgG antibodies to the adult acetylcholine receptor subunit causes transient myasthenic syndrome symptoms in about 10% of newborns that subside after birth with the decrease in maternal IgG antibody levels (Namba et al., 1970). However the earlier passage of maternal antibodies to the fetal acetylcholine receptor or to the MuSK receptor, also involved in neurotransmission across the synapse, impairs early fetal movement and causes abnormal joint and muscle development (Vincent et al., 1995). Due to the presence of antibodies to the fetal neuromuscular junction components, asymptomatic women with myasthenia gravis can have children with arthrogryposis multiplex congenital and fetal akinesia sequence (Brueton et al., 2000). Decreased fetal movement and joint contractures can occur in association with maternal myotonic dystrophy (Stratton and Patterson, 1993, Robin et al., 1994). Other maternal causes of arthrogryposis include maternal hyperthermia in pregnancy caused by abnormal nerve growth or nerve migration (Reid et al., 1986, Edwards, 1986), limited space due to structural uterine abnormalities or multiple births and maternal drug ingestion or vascular compromise (Hall, 1996). Congenital fetal infection due to maternal cytomegalovirus infection may result in arthrogryposis with additional teratogenic features due to the viral infections. Other maternal infections in pregnancy, such as rubella, varicella and coxsackie virus infections can also cause multiple joint contractures through central or peripheral nerve destruction (Konstantinidou et al., 2007, Hall and Reed, 1982). Maternal diabetes and multiple sclerosis have been associated with the presence of joint contractures, although the mechanism is unknown (Livingstone and Sack, 1984). Severe bleeding in pregnancy or a failed pregnancy termination has been reported in association with multiple joint contractures, presumably due to an interruption to the blood supply to the developing joints (Hall, 1996, Wynne-Davies and Lloyd-Roberts, 1976). It is important to try to identify the underlying cause of the arthrogryposis, as there may be particular health

surveillance recommended for affected patients. This information is also important to inform families of the recurrence risk, which may vary considerably depending on the underlying cause.

1.4.1.3 Fetal Factors

As fetal movement is essential for normal joint development, conditions in the fetus that cause fetal hypo or akinesia can result in joint contractures and other features of the fetal akinesia deformation sequence (FADS). The intact motor pathway initiates and controls movement. Electrical nerve impulses originating from the primary motor cortex in the brain mainly pass along upper motor neurone to the contralateral side of the spinal cord where they synapse with the anterior horn cell. This electrical signal is transmitted down through the lower motor neurone and is chemically transmitted across the neuromuscular junction. On reaching the motor end plate, the nerve impulse causes the muscle to contract. Therefore, any factors that interrupt this pathway can potentially cause fetal akinesia and arthrogryposis (see Figure 2). AMC in the fetus is due to neurogenic factors in 80-90% (Banker, 1985, Hageman et al., 1987, Vuopala et al., 1994). This may include problems with the development of the brain, the spinal cord or the peripheral nerves. It has been estimated that the underlying cause of fetal akinesia deformation sequence can be determined with extensive neuropathological studies of the brain, spinal cord and muscles in about 50% of those affected (Witters et al., 2002).



Motor cortex of the brain

AMC due to developmental brain abnormalities

Upper motor neurone (UMN)

AMC due to UMN in LCCS1 (LMN and brainstem motor neurones also affected)

Spinal cord

Anterior horn cells affected in SMA

Lower motor neurone

Neuromuscular junction

Fetal akinesia due to AChR pathway conditions

Muscle

Primary myopathies associated with AMC

Figure 3: Motor nerve pathway from brain to muscle.

Defects in components along this pathway can result in fetal akinesia and arthrogryposis.

Developmental brain abnormalities

Brain abnormalities are the commonest cause of multiple joint contractures. A wide variety of brain pathologies can cause reduced or absent fetal movement. A developmental brain abnormality (Brodtkorb et al., 1994, Vuopala et al., 1994, Fedrizzi et al., 1993) may occur as an isolated problem or as part of a syndrome and is usually a sporadic occurrence. In general, the mortality, morbidity and developmental progress in this group is less favourable (Darin et al., 2002). Major structural brain abnormalities such as anencephaly and holoprosencephaly can be associated with arthrogryposis (Morse et al., 1987, Hockey et al., 1988). Arthrogryposis and seizures may be seen with neuronal migration disorders such bilateral perisylvian polymicrogyria (Kuzniecky et al., 1993, Gropman et al., 1997, Poduri et al., 2010). A lethal familial fetal akinesia syndrome has been identified in association with lissencephaly (Encha Razavi et al., 1996). Structural brain anomalies can be associated with joint contractures and there is evidence that CNS hypoxia or injury may be associated with secondary muscle changes and fetal akinesia (Rudzinski et al., 2010).

Spinal cord pathology

Anterior horn cell pathology occurs in Spinal Muscular Atrophy (Burglen et al., 1996). Homozygous *SMN1* deletions associated with a low *SMN2* copy number have a severe presentation. Patients may be born with generalised oedema, multiple joint contractures, micrognathia and a high arched palate, and die in the neonatal period or in early infancy (Devriendt et al., 1996, Garcia-Cabezas et al., 2004). Mutations in the Ubiquitin activating enzyme 1 (*UBE1*) have been identified with loss of anterior horn cells in severe lethal X-linked SMA. Patients with this condition have multiple joint contractures, hypotonia, respiratory insufficiency and die in infancy (Ramser et al., 2008).

The Finnish lethal congenital contractural syndrome 1 (LCCS1), the most severe human motor neurone disease, is characterised by marked loss of spinal motor neurones and fetal akinesia. Severe fetal immobility may be detected at about 13 weeks of gestation and is associated with fetal hydrops, multiple joint contractures, pterygia and lung hypoplasia. On neuropathological examination there is lack of anterior horn cells and severe atrophy of the ventral spinal cord. Skeletal muscle is severely hypoplastic or almost absent. It is due to autosomal recessive mutations in the mRNA export mediator *GLE1*, indicating the critical role of mRNA processing in the development and survival of anterior motor neurones (Nousiainen et al., 2008).

Lethal congenital contractural syndrome 2 (LCCS2) and LCCS3 have been described in Israeli-Bedouin families. Affected neonatal LCCS2 patients have decreased fetal movements, multiple joint contractures, severe muscle wasting and a neurogenic bladder due to atrophy of the anterior horn cells of the spinal cord. Homozygous mutations in Epidermal growth factor receptor 3 (*ERBB3*) have been identified (Narkis et al., 2007b). LCCS3 is similar to LCCS2, however the bladder abnormalities are not present. It is due to homozygous mutations in phosphatidylinositol-4-phosphate-5-kinase, type 1, gamma (*PIP5K1C*) (Narkis et al., 2007a).

Abnormalities of the peripheral nerves

Congenital hypomyelination neuropathy due to deficiency of myelin proteins has been observed in arthrogryposis multiplex congenita (Boylan et al., 1992). Congenital hypomyelination neuropathy or amyelination of the peripheral nerves is associated with multiple joint contractures, respiratory distress and infant demise (Gabreels-Festen, 2002).

The genetic basis of this condition is currently unknown, although genes known to cause progressive hypomyelination have been suggested as possible candidates.

Neuromuscular junction pathology

Neuromuscular junction disorders can be congenital or acquired. Examples of acquired disease include myasthenia gravis and Lambert-Eaton myathenic syndrome, both of which are due to autoimmune conditions causing antibodies to specific nerve components and muscle ion channels. Fetal akinesia and arthrogryposis secondary to maternal myasthenia gravis is discussed in section 1.4.1.2 maternal factors.

The congenital myasthenic syndromes (CMS) are a rarer group of inherited conditions with an estimated prevalence of 1 in 500,000 (Millichap and Dodge, 1960) that can also be associated with multiple joint contractures (Vajsar et al., 1995). Congenital myasthenic syndromes are characterised by muscle weakness and fatigability due to impaired transmission of nerve impulses across the neuromuscular synapse. Fluctuating extraocular, bulbar and respiratory symptoms are typically present. The phenotypic spectrum varies from a rare severe prenatal onset of arthrogryposis and neonatal respiratory compromise through to weakness present at birth or in early childhood, feeding difficulties, ptosis, abnormal eye movements and delayed motor milestones usually in the absence of joint contractures (Brownlow et al., 2001, Beeson et al., 2003). This group of conditions is genetically heterogeneous and most frequently due to autosomal recessive mutations in acetylcholine receptor pathway genes however autosomal dominant gene mutations may be implicated. CMS can be due to abnormalities of ACh release, AChR number or function as well as acetylcholinesterase activity. Mutations in the postsynaptic AChR genes may alter the kinetics of the response to ACh in the synapse and

can result in fast and slow channel phenotypes. In the fast channel syndromes there is brief infrequent ion channal activation resulting in reduced postsynaptic acetylcholine receptor opening times and thus a reduced response to acetylcholine. In slow channel syndrome there is prolonged opening of the acetylcholine receptors, resulting in a depolarising block of the muscle acion potential. Fast channel syndromes are due to autosomal recessive loss of function mutations whereas slow channel syndromes are caused by autosomal dominant gain of function mutations, (Ohno et al., 1997, Shen et al., 2005).

The genes most commonly implicated in CMS are *CHRNE, CHAT, COLQ, DOK7, GFPT* and *RAPSN*. Mutations in *CHRNE*, which encodes the epilson subunit of the adult acetylcholine receptor, are present in about half of those affected with CMS (Beeson et al., 2005). Mutations in *RAPSN* account for around 15% of cases (Gaudon et al., 2010). CMS is diagnosed from the clinical history, characteristic electrophysiology showing a decremental response of the compound muscle action potential to low-frequency stimulation, a positive response to anti-cholinesterase inhibitors and the absence of acetylcholine receptor antibodies. Although individuals affected with this condition have the hallmark features of CMS, particular distinguishing features in the clinical presentation may point to the underlying genetic aetiology. For example, autosomal recessive mutations in *DOK7* are associated with a predominantly limb girdle pattern of weakness, ptosis but no external ophthalplegia (Palace et al., 2007). Recessive mutations in the acetylcholine receptor subunit *CHRND* and *RAPSN* have been detected in children born with arthrogryposis (Brownlow et al., 2001, Burke et al., 2003, Banwell et al., 2004)**.**

The multiple pterygium syndromes can also result from neuromuscular dysfunction as discussed in Chapter 1.5.

Primary muscle conditions

Muscle conditions such as the congenital muscular dystrophies, congenital myopathies and myositis are estimated to account for 5-10% of the cases of fetal akinesia (Banker, 1985). Central core disease may be due to autosomal recessive and autosomal dominant *RYR1* mutations (Romero et al., 2003). Multi-minicore disease caused by *SEPN1* gene mutations can result in reduced prenatal fetal movements, polyhydramnios and arthrogryposis (Jungbluth et al., 2000, Ferreiro et al., 2000). A severe form of nemaline myopathy with absent spontaneous movement, arthrogryposis and death in early life has been described with autosomal recessive *NEB* mutations (Wallgren-Pettersson et al., 2002) and *ACTA1* mutations (Garcia-Angarita et al., 2009, Stenzel et al., 2010). (Stenzel et al., 2010)

X-linked myotubular myopathy caused by mutations in *MTM1* may present with reduced fetal movements and poyhydramnios. However, these patients usually have severe hypotonia and generalised muscle weakness (Laporte et al., 1996, Chang et al., 2008). Autosomal recessive mutations in Contactin-1 (*CNTN1*) have been identified in a consanguineous Egyptian family with fetal akinesia and lethal congenital myopathy. Contactin-1 is expressed in the central and peripheral nervous systems and the neuromuscular junction. However, abnormal localisation of contactin-1 at the skeletal muscle sarcolemma is associated with secondary dystroglycanopathy due to loss of beta2-syntrophin and alpha-dystrobrevin. Thus, this condition may be considered as a congenital myopathy or a neuromuscular junction disorder (Compton et al., 2008).

Connective tissue abnormalities

Connective tissue conditions such as restrictive dermopathies can lead to fetal akinesia, joint fixation and arthrogryposis through collagenous thickening of the joint capsule (Happle et al., 1992). Restrictive dermopathies may be due to autosomal dominant mutations in *LMNA* or more frequently to autosomal recessive mutations in *ZMPSTE24* (Navarro et al., 2004, Navarro et al., 2005, Smigiel et al., 2010). Autosomal dominant *FBN2* mutations can cause congenital contractural arachnodactyly or Beals syndrome, which can be associated with severe joint contractures, a characteristic crumpled appearance of the ears and a long, thin body habitus (Beals and Hecht, 1971, Ramos Arroyo et al., 1985, Putnam et al., 1995). Excessive joint laxity occurring in collagen disorders such as Larsen syndrome can be associated with congenital joint contractures, particularly talipes eqinovarus, and joint dislocations (Larsen et al., 1950, Krakow et al., 2004).Variants of Ehlers Danlos (EDS) syndrome such as CHST14-related EDS Kosho type can also present with a combination of joint flexibility and contractures (Miyake et al., 2010, Kosho et al., 2010).

Skeletal dysplasias

Skeletal dysplasias can also be associated with AMC (Hall et al., 1982a). Examples are the *TRPV4*-associated of disorders which are all characterized by brachydactyly, short stature with progressive spinal deformity and involvement of the long bones and pelvis and a recognisable radiological bone patten. Spondyloepiphyseal dysplasia is at the milder end of the spectrum with more severe phenotypic features seen in metatropic dysplasia. Arthrogryposis multiplex congenita-like contractures may be present in individuals with severe metatropic dysplasia secondary to the accompanying peripheral neuropathy. This

condition may be prenatally or perinatally lethal because of accommpanying extremely narrow chest and hypoplastic lungs (Unger et al., 2011)**.**

Metabolic Disorders

Glycogen storage disease type VII (phosphofructokinase deficiency) can occasionally be identified as the cause of fetal akinesia and multiple joint contractures (Moerman et al., 1995). Perinatally, glycogen storage disease type IV can present with a cervical cystic hygroma, fetal hydrops, fetal akinesia. The patient may have a cardiomyopathy at birth, phenotypic features of lethal multiple pterygium syndrome and die in the neonatal period (Nolte et al., 2008, Akman et al., 2007). It is due to autosomal recessive mutations in glycogen branching enzyme 1 (*GBE1*), (Ravenscroft et al., 2013).

In contrast the joint contractures seen in the mucopolysaccharidoses are not usually present at birth and are progressive. Stiffness and contractures preferentially affect the phalangeal joints of the hand resulting in a claw hand deformity however other joints can also be involved including the hips, knees ankles and spine (Aldenhoven et al., 2009).

Other fetal disorders

Factors in the fetus that modify the in utero environment can cause arthrogryposis. The presence of amniotic fluid protects the developing baby from compression and facilitates free fetal movement. Oligohydramnios due to fetal kidney disorders such as kidney agenesis result in reduced fetal movements, joint contractures, pulmonary hypolasia and other features of in utero compression such as micrognathia and intrauterine growth retardation (Potter's syndrome). Other causes of olighydramnios including leakage of amniotic fluid due cervical

insufficiency can result in similar features in the fetus (Vuopala et al., 1994, Wynne-Davies and Lloyd-Roberts, 1976).

Arthrogryposis can be due to constitutional factors or specific genetic syndromes in the fetus. Chromosomal syndromes such as trisomy 18 (Edward syndrome) can be associated with joint contractures in the context of other malformations (Reed et al., 1985). Partial duplications of chromosome 1q have been seen in babies with absent fetal movements, fetal hydrops, finger contractures and the Pierre-Robin sequence, suggesting there may be dosage sensitive genes in this region (Aboura et al., 2002). Germline chromosome imbalances may be detected on blood samples and somatic mosaicism is more usually detected on cultured fibroblasts.

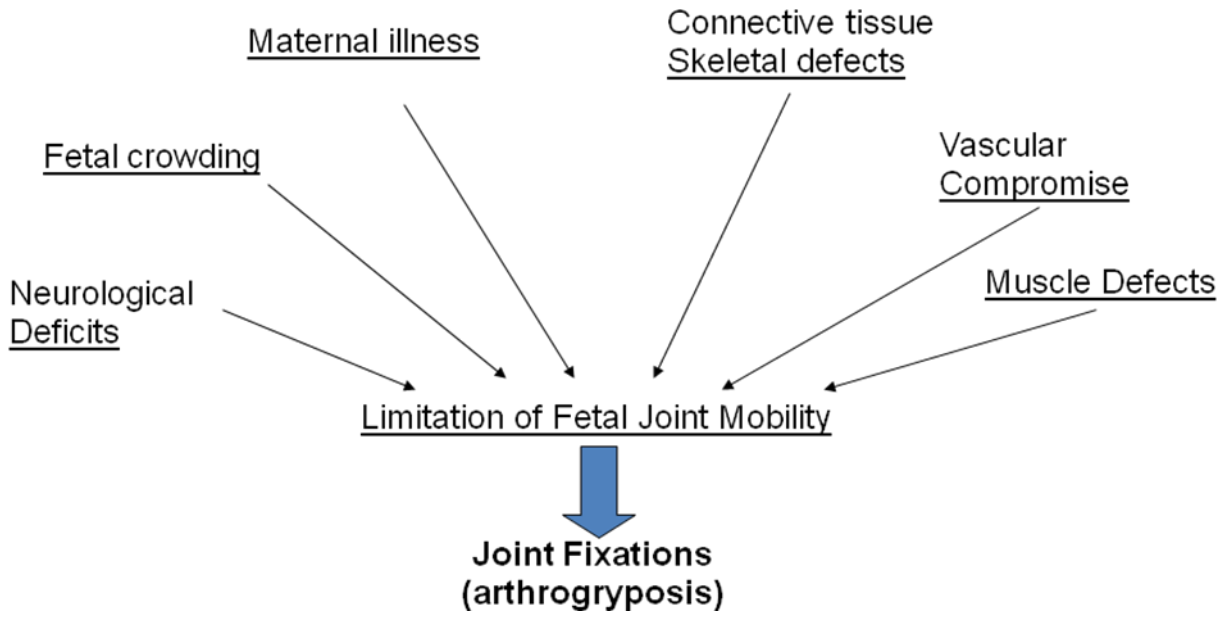


Figure 4: Causes of Arthrogryposis

(Adapted from Arthrogryposis: A Text Atlas, edited by Staheli, Hall, Jaffe, Paholke, Cambridge University Press, 1998).

1.4.2 Classification of arthrogryposis

Arthrogryposis can be classified according to the pattern of joint involvement. AMC usually has proximal as well as distal joint involvement whereas there are predominantly distal joint contractures in the distal arthrogryposis syndromes. Additional features such as ophthalmoplegia and cleft palate in the affected members of the family may help in determining the phenotypic subtype. Arthogryposis is a component of many genetic syndromes including the multiple pterygium syndomes.

1.4.2.1 Distal arthrogryposis

This group of conditions is characterised by distal joint involvement most frequently finger contractures and talipes. In the upper limb there may be ulna deviation of the wrist, overriding digits, camptodactyly or decreased ability to straighten the fingers with wrist extension (pseudocamptodactyly). The ventral surfaces of the palms and fingers may appear smooth with faint or absent flexion creases due to lack of movement of the underlying joints. Additional features such as a cleft palate, an ophthalmoplegia or the puckered mouth characteristic of Freeman Sheldon syndrome may be used to identify specific clinical phenotypes with a different molecular basis. The classification of distal athrogryposis has been reviewed in the paper by (Bamshad et al., 2009). The distal arthrogryposes are usually inherited in an autosomal dominant pattern of inheritance with inter as well as intrafamilial variability in the range and severity of the phenotypic features present. Although one or other parent and other relatives may be similarly affected, distal arthrogryposis may be due to an apparently de novo condition in the family.

The genetic basis of all the subtypes of distal arthrogryposis is not currently fully understood however mutations in the troponin-tropomyosin-myosin complex of the muscle sarcomere have been identified in this group of conditions (see Figure 5).

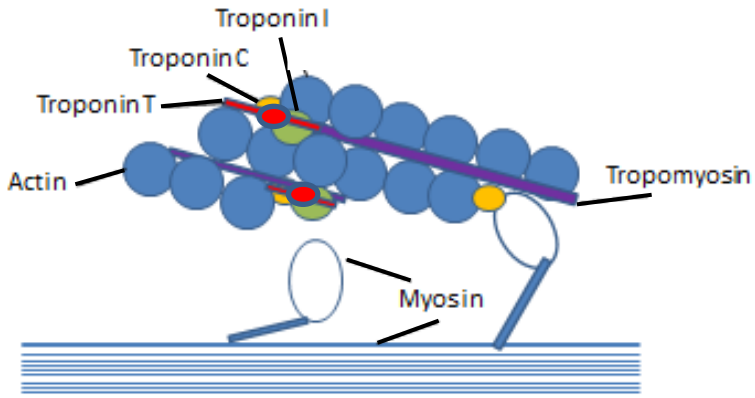


Figure 5: Diagram of the muscle sarcomere.

Distal arthogryposis is caused by mutations in genes that encode the sarcomeric proteins: *TNNI2* (Troponin I), *TNNT3* (Troponin T) and *MYH3* (embryonic myosin).

(Adapted from Toydemir et al., 2006).

DA1 is characterised by camptodactyly of the fingers and talipes. The severity of the joint involvement is very variable. Other proximal joints such as the shoulders and hips are less frequently affected. It is an autosomal dominant condition due to heterozygous mutations in TPM2 (encoding tropomyosin 2).

This pattern of joint contractures is also seen in Freeman Sheldon syndrome. This condition is associated with the characteristic “whistling face” because of the very small oral opening and puckered lips. Heterozygous mutations in *MYH3* are identified in 90% of affected patients

(Sung et al., 2003a). Sheldon Hall syndrome (SHS) is characterised by distal joint contractures, downslanting palpebral fissures, prominent nasolabial folds and a small mouth. Mutations in *MYH3* are also a frequent cause of SHS, however mutations in *TNN12* (encoding troponin I), *TNNT3* (encoding troponin T) and *TPM2* can also cause this condition (Sung et al., 2003b, Sung et al., 2003a).

DA5 is associated with distal arthrogryposis and ophthalmoplegia. This may be a genetically heterogeneous group of conditions as mutations in *MYH2* and *MYH13* have only reported in single families to date (Bamshad et al., 2009).

1.4.2.2 Syndromes associated with arthrogryposis

Arthrogryposis or multiple joint contractures can be associated with other features as part of a genetic syndrome. The following examples are single gene disorders associated with severe fetal akinesia and multisystem involvement.

Arthrogryposis-Renal dysfunction-Cholestasis (ARC) syndrome

ARC is an autosomal recessive condition due to mutations in the *VPS33B* and *VIPAR* genes (Gissen et al., 2004, Cullinane et al., 2010). It is multisystem disorder with features of neurogenic arthrogryposis multiplex congenita, renal tubular dysfunction and neonatal cholestasis. Currently there is no specific treatment for this condition and despite supportive care, most patients die in the first year of live. Liver cirrhosis and severe developmental delay are present in those with longer survival.

Fowler syndrome

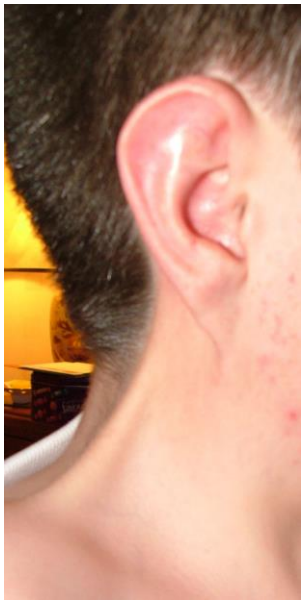
This is an autosomal recessive syndrome due to mutations in the *FLVCR2* gene (Meyer et al., 2010). The typical features of this condition are a proliferative vasculopathy and hydranencephaly–hydrocephaly. As well as joint contractures, webbing or pterygia across the joints indicating the presence of severe fetal akinesia has been noted in this condition. It has been recommended that this condition is considered in the differential diagnosis of the Lethal Multiple Pterygium syndromes (Williams et al., 2010).

Multiple Pterygium syndromes

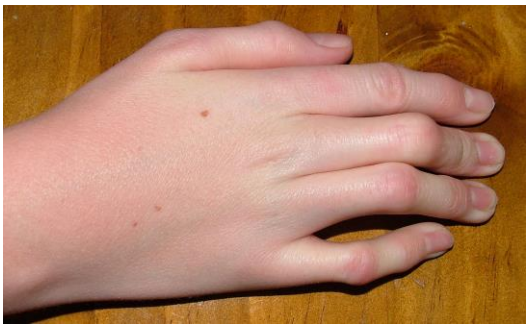
In addition to arthrogryposis and pterygia or webbing across the joints, individuals affected with the Multiple Pterygium syndromes may have a variety of congenital abnormalities and a characteristic facial appearance. The focus of this project was the non lethal Escobar variant Multiple Pterygium syndromes (EVMPS) and the lethal Multiple Pterygium syndromes (LMPS). This group of conditions is discussed in more detail in the next section.

1.5 Multiple pterygium syndromes (MPS)

The multiple pterygium syndromes (MPS) are a phenotypically and genetically heterogeneous group of disorders. They are characterised by arthrogryposis (joint contractures), pterygia (webbing across joints) and a variety of congenital developmental abnormalities, see figures 4 (i), (ii) and (iii).



(i)



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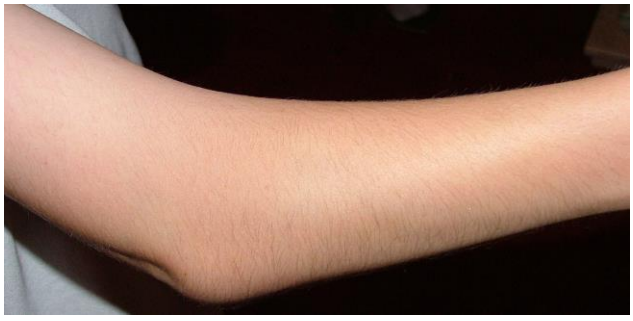


(iii)

Figure 6: (i), (ii) and (iii). Features in a patient with MPS-Escobar variant.

(Taken from Morgan et al., 2006).

(i) Pterygium in the neck

(ii) Arthrogryposis (joint contractures) of the fingers

(iii) Elbow contracture and associated pterygium

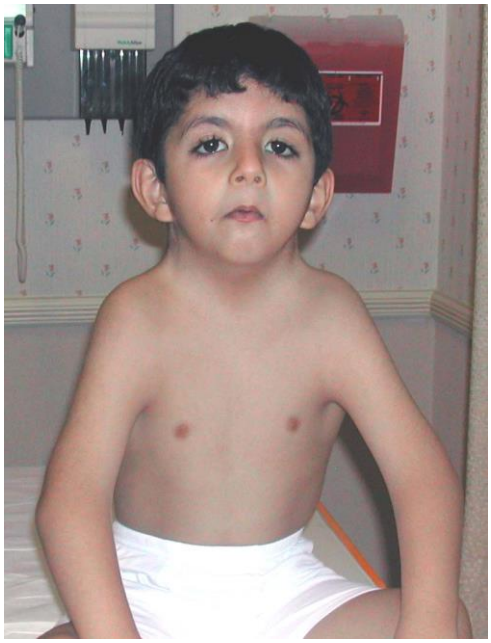
Pterygium is a Greek word meaning “wing”. The MPS are characterised by multiple pterygia or webs across joint. Pterygia may be present in the neck, the axillae, the antecubital and popliteal fossae and intercrural region (Escobar et al., 1978, Gillin and Pryse-Davis, 1976, Stoll et al., 1980). The number and type of pterygia identified may be very variable between patients with MPS and within families affected with this condition (Hall et al., 1982b). Pterygia are believed to result from immobility in early life and it has also been suggested that early fetal oedema causes cutaneous folds in the joint regions (Van Regemorter et al., 1984). An alternative explanation for the formation of pterygia is that a genetically determined insult results in delayed development in the connection between muscle and lymphatic vessels. Obstruction to the drainage of fluid from the internal jugular vein into the jugular lymphatic vessels results in oedema. Pterygia are left with the resolution of the excess subcutaneous fluid as the link between the venous an lymphatic systems matures (Isaacson et al., 1984). Although pterygia may be observed before or at the time of birth, they may only become apparent or more obvious with time, suggesting that progression in the formation of the pterygia has occurred (Thompson et al., 1987, Ramer, 1991).

Releasing the webbing across the affected joints does not usually increase the range of joint movement, as this is determined by the condition of the underlying joint. In addition, the muscle development around the affected joint is also abnormal. Older patients may be concerned about the cosmetic appearance of the ptergyia however care must be taken when considering surgical options as ptergia may contain the neurovascular bundle for that limb.

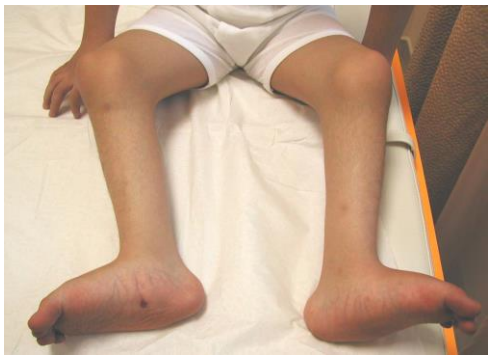
Multiple congenital anomalies such as cleft palate, cryptorchidism, intestinal malrotation, congenital heart anomalies and cardiac hypoplasia, diaphragmatic hernia, genito-urinary

abnormalities, obstructive uropathy, microcephaly and cerebellar and pontine hypoplasia have all been described in patients with this condition. Respiratory impairment may be caused by a small chest size and spinal deformity. Multiple joint contractures and rocker bottom feet with a vertical talus are frequent in this disorder. In addition, an expressionless face, with facial dysmorphism including ptosis of the eyelids, down slanting eyes, a small mouth, cleft palate and micrognathia may be present. However, patients with MPS usually have a normal intellect (Thompson et al., 1987, Fryns et al., 1988, Hall et al., 1982b).

The MPSs can be divided in to the milder Escobar variant (EVMPS, 265000), Figures 5 (i) and (ii), and the perinatally lethal MPS (LMPS, MIM 253290) (Hall et al., 1982b). Features of EVMPS include multiple pterygia, facial dysmorphism with downslanting palpebral fissures and bilateral ptosis, short stature and vertebra fusion, and other internal anomalies (Escobar et al., 1978, Rajab et al., 2005).



(i)



(ii)

Figure 7: (i) and (ii). Child with EVMPS variant MPS.

(Taken from Morgan et al., 2006).

(i) typical facies with downslanting palpebral fissures, bilateral ptosis and neck pterygia

(ii) arthrogryposis of knees and rocker bottom feet

LMPS is characterised by features of the fetal akinesia deformation sequence (FADS) including severe flexion contractures or arthrogryposis, multiple pterygia, micrognathia, cleft palate, lethal pulmonary hypoplasia and intrauterine growth retardation. Extensive subcutaneous oedema causing fetal hydrops with cystic hygroma and lung hypoplasia may be detected on ultrasonography in pregnancy, Figure 8.

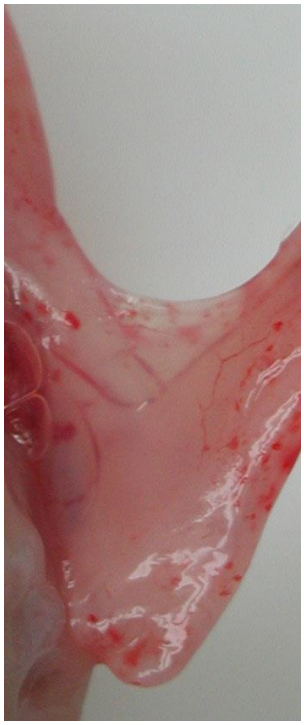


Figure 8: 13 week old LMPS fetus with a pterygium across an elbow contracture.

(Taken from Morgan et al., 2006).

The identification of mutations in the fetal acetylchloline receptor (AChR) subunit gene (*CHRNG*) in a proportion of patients indicated an underlying mechanism for the fetal akinesia at least in this subset of the patient group (Morgan et al., 2006), as AChRs are essential neurotransmitter receptors at the neuromuscular junction.

1.5.1 The neuromuscular junction (NMJ)

The NMJ is comprised of three highly specialised cells, the motor neurone, the muscle fibre and the Schwann cell. The motor nerve is adapted for neurotransmitter release by having a large number of ACh containing vesicles. The postsynaptic membrane has a high concentration of AChRs located on the crests and part-way down the sides of the post-synaptic membrane folds to enable a rapid reliable response to neurotransmitter release (Covault and Sanes, 1986, Flucher and Daniels, 1989). The processes of the Schwann cell provide an insulating cap and possibly trophic factors for the nerve terminal.

At the neuromuscular junction, motor neurone activity causes the release of ACh from vesicles in the presynaptic membrane (Figure 9). This results in end plate potentials which generate an action potential. Opening of voltage gated sodium channels causes a rapid sodium ion current inwards. Repolarisation of the membrane occurs by inactivation of the sodium ion and potassium ion channels. The signal is transmitted along the surface membrane to the deeper parts of the muscle fibre. The voltage gated pentameric calcium channel or dihydropyridine receptor senses membrane depolarisation, alters its conformation and activates the ryanodine receptor which releases calcium ions from the sarcoplasmic reticulum. Calcium ions bind to troponin and activate the contractile machinery resulting in muscle contraction. This mechanism is impaired by abnormalities in any of the critical components of this process.

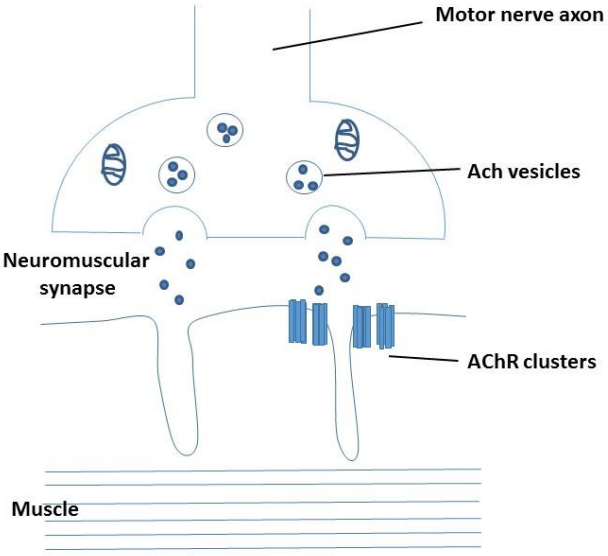


Figure 9: Diagram of the neuromuscular junction.

On activation of the motor nerve, vesicles containing the chemical acetylcholine (ACh) fuse with the presynaptic membrane. ACh is released from these vesicles into the synapse. The ACh binds with the AChRs on the postsynaptic membrane of the muscle fibre. This causes a cascade of reactions culminating in muscle contraction.

1.5.2 Acetylcholine receptors (AChR)

These neurotransmitter receptors are found in both neuronal and non neuronal tissues throughout the body and are activated by acetylcholine (ACh). The two types of AChR are subdivided into muscle (muscarinic) or neuronal (nicotinic) AChRs, depending on their site of expression and the local muscle innervation. Muscarinic receptors can be activated by Amanita mushroom muscarine toxin and are inhibited by the toxin atropine from the Atropa belladonna nightshade family. Structurally they have seven transmembrane domains and function as second messengers in G-protein coupled reactions. Activation of these receptors is

relatively slow and results in a direct effect on cellular homeostasis. They have a particular role in postganglionic parasympathetic nervous system reactions (Hulme et al., 1990).

Nicotinic AChR are activated by nicotine. The nicotinic AChR is a pentameric transmembrane protein comprised of four different subunits with a central pore (Noda et al., 1983, Shibahara et al., 1985). These receptors have ion channels which can be activated within microseconds and thus have the ability to provide a rapid reponse. Binding of an ACh agonist to these receptors causes a change in the flow of ions and thus a cellular response. Nicotinic AChRs are comprised of subunits with homologous structural units, a large terminal extracellular NH2 domain, three transmembrane domains, a cytoplasmic loop of a variable amino acid composition and size and a fourth transmembrane domain with a short variable and a terminal extracellular COOH sequence. In common with all ligand gated ionic channels, there are two cysteine residues separated by 13 intervening amino acids to enable an agonist to bind. The various domains are folded to optimise their specific cellular functional role. In particular, configuration of the subunits results in a wide channel at the mouth of the receptor constricted by the encircling extracellular domains to enable closure of the gated receptor.

AChRs subunits can be very diverse and the receptor pentamers can be composed of various subunits enabling highly specialised tissue specific signalling functions. Interestingly, the nicotinic AChRs are conserved throughout the various life forms because of their role in muscle contraction, and thus may be a target for attack. Hence the role of nicotine from the tobacco plant as a pesticide in farming and horticulture.

In skeletal muscle the nicotinic AChR mediates neurotransmission across the neuromuscular synapse. Release of the agonist ACh from vesicles on the presynaptic membrane and binding with AChRs on the postsynaptic membrane causes opening of a selective cation channel. This results in an ionic current that depolarises the membrane and leads to muscle contraction. Clustering of the AChRs on the postsynaptic membrane is critical to enable a significantly large synaptic potential to be achieved to initiate an action potential in a muscle fibre. Acetylcholinesterase inhibitors can be utilized to potentiate the effects of ACh in disease, by increasing the quantity and duration of the agonist in the synapse.

The nicotinic AChR exists in an adult forms and a fetal form. The embryonic AChR predominates in fetal and denervated muscle and consists of two alpha and one beta, one gamma and a delta subunit ($\alpha_2\beta\gamma\delta$), see Figure 10. The adult form is present in innervated adult muscle (Xu and Salpeter, 1997). In adult AChRs the gamma subunit is replaced by an epsilon subunit ($\alpha_2\beta\varepsilon\delta$). In humans the switch from γ to ε and the transition from a fetal to adult AChR occurs during fetal life and is complete by 31 weeks of gestation, whereas in rodents the switch occurs postnatally (Hesselmans et al., 1993, Bouzat et al., 1994, Kues et al., 1995, Yumoto et al., 2005). This switch is believed to be induced by the nerve derived chemical ARIA (acetylcholine receptor-inducing activity), thus there may be differential expression of ARIA when promoters of the γ and ϵ subunit genes are activated (Missias et al., 1996).

Embryonic and adult forms exist for many proteins in the body to support the different functional requirements in pre and postnatal life. The embryonal and adult AChR have differing conductance and gating suggesting the γ to ε subunit switch causes functional

alterations in the AChR during muscle maturation (Mishina et al., 1986). The embryonic AChR has long opening times allowing the release of acetylcholine to generate spontaneous muscle action potentials. This is in contrast to the adult receptor, which has shorter mean channel opening times and consequently short-lived mini end plate currents. Experimental work has shown that only embryonic miniature end plate currents last long enough to trigger contraction in fetal muscle. As fetal muscle must be active for postsynaptic membrane specialisation and muscle differentiation, it has been suggested that the characteristics of the embryonic AChR are essential for the spontaneous contractile activity necessary for normal development of the neuromuscular motor terminus in utero (Jaramillo et al., 1988, Bouzat et al., 1994). In γ-subunit deficient mice pre- and post synapses are abnormal and there is absence of spontaneous action potentials from the minature end plate potentials reinforcing the critical importance of γ containing AChRs in neuromuscular development (Takahashi and Alford, 2002). Further study has highlighted the role of the fetal AChR in ensuring an orderly innervation pattern in skeletal muscle. The functional end plate properties associated with adult AChRs consisted of shorter mean opening times, a larger conductance and increase in calcium ion flux, essential to preserve a functional neuromuscular synapse (Koenen et al., 2005). Mice deficient in ε-AChR subunits have persistence of the γ-AChRs at the end plates at reduced density with resulting impaired neuromuscular transmission, progressive muscle weakness and premature death illustrating the need for ε-AChR postnatally (Witzemann et al., 1996).

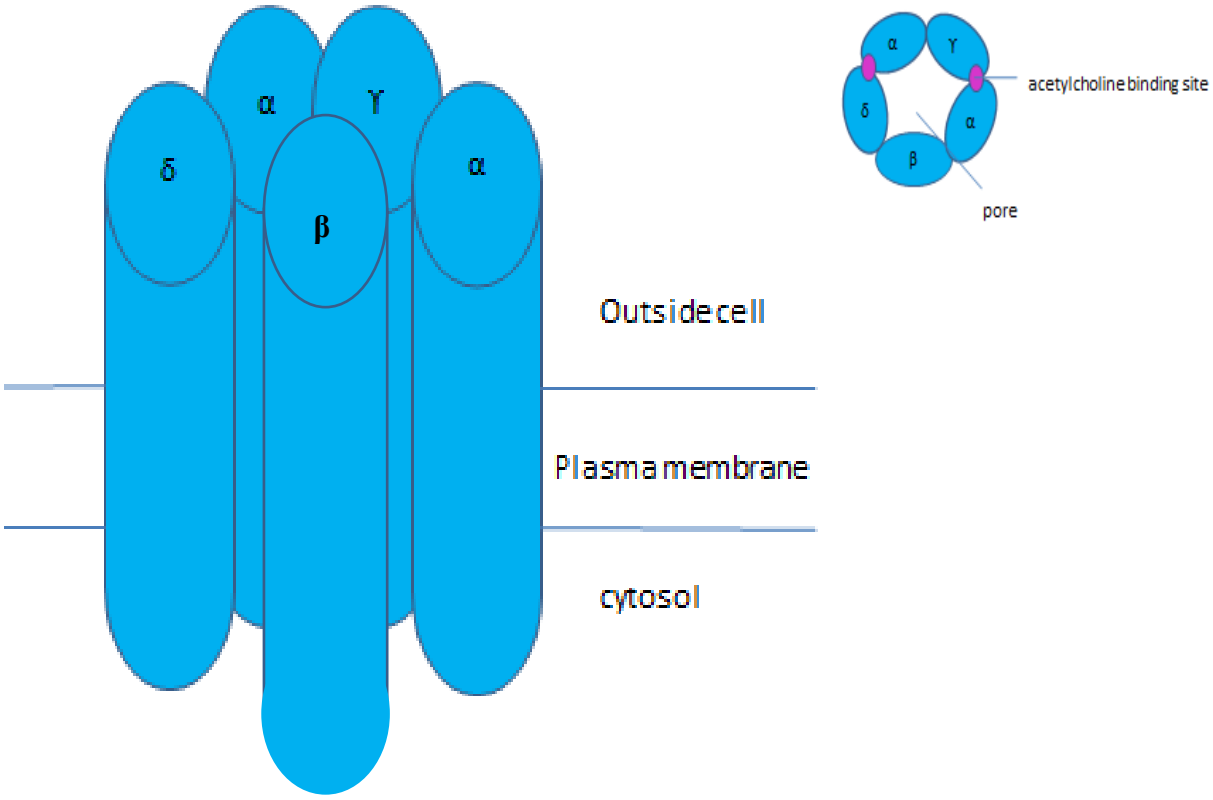


Figure 10: The subunits of the embryonic AChR.

(Adapted from Molecular cell biology, sixth edition, 2008. W.H. Freeman and company).

1.5.3 *CHRNG*

CHRNG encodes the gamma subunit of the embryonic AChR. The *CHRNG* gene is located at chromosome 2q33-q34. It is composed of 1545 base pairs and has 514 amino acid residues arranged in 12 coding exons. Structurally, the gamma subunit contains a large extracellular N terminus through which it binds acetylcholine, four transmembrane domains numbered M1 to M4, a large cytoplasmic domain between the M3 and M4 domains and a short extracellular C terminus (Shen et al., 2005), Figure 11. It is expressed in the post-

synaptic membrane of the face, limbs, paravertebral, intercostals, diaphragmatic and neck muscles in fetal life.

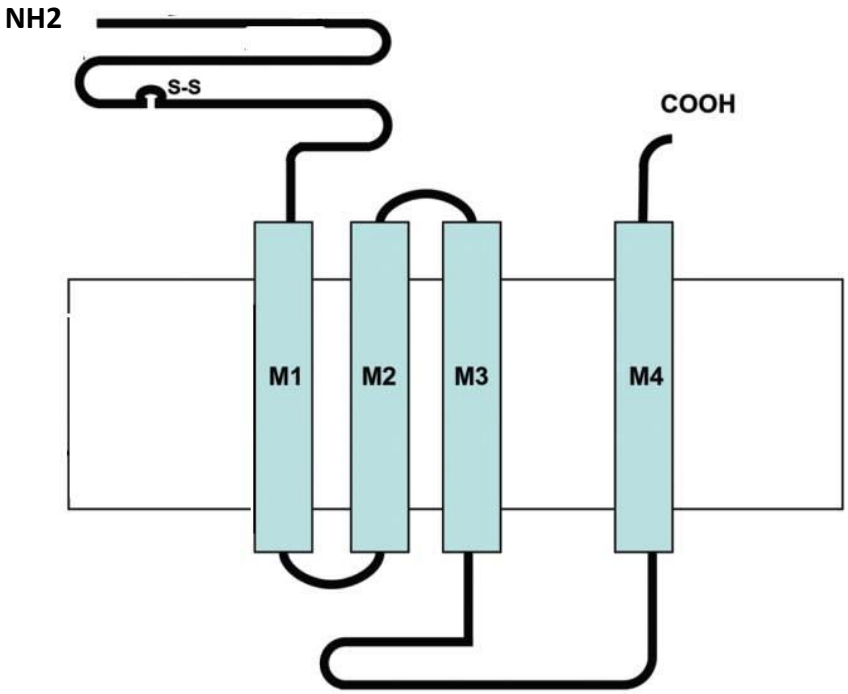


Figure 11: Diagram of *CHRNG.*

***(*Adapted from Morgan et al., 2006).**

The identification of *CHRNG* mutations in MPS patients suggested that pterygia resulted from early onset fetal akinesia. Although the embryonal AChR has a key role in normal prenatal muscle development, the γ subunit is not required postnatally thus Escobar variant MPS patients do not demonstrate marked progressive muscle weakness (Hoffmann et al., 2006). There remains however uncertainty about the mechanism and cause of the other variable associated developmental anomalies.

Following detection of *CHRNG* mutations in MPS I undertook a genotype-phenotype study to investigate the clinical features that may predict the presence of a *CHRNG* mutation and whether the *CHRNG* mutation spectrum differed between LMPS and EVMPS kindreds. *CHRNG* mutation analysis was performed in 100 families with a clinical diagnosis of MPS/FADS (see Chapter 3). Clinical data about the cohort was collected using a clinical features questionnaire. This work has also been included in the publication genotype-phenotype correlations in the multiple pterygium syndromes (Vogt et al., 2012).

1.5.4 Gene mapping in the Multiple Pterygium Syndromes

The multiple pterygium syndromes are most commonly, though not exclusively, autosomal recessively inherited. Gene identification in autosomal recessive disorders has been difficult, due to the rarity of individual conditions and family size, as families with more than two affected siblings within a sibship are infrequent. When considering gene discovery in MPS, techniques such as autozygosity mapping in autosomal recessive families, candidate gene analysis and second generation sequencing for novel genes have been utilized.

1.5.4.1 Autosomal Recessive Diseases

Autosomal recessively inherited diseases are responsible for a wide spectrum of developmental, metabolic and medical problems. Many of these conditions manifest in childhood and cause a significant excess morbidity and mortality in consanguineous families (Bundey and Alam, 1993). In autosomal recessive conditions the affected individual has mutations on both copies of a specific gene located on one of the autosomes. The parents of a person affected with an autosomal condition each have one copy of the mutated gene and are thus carriers of the condition. For a couple that are carriers of an autosomal recessive condition there is a 1 in 4 or 25% chance of having an affected child in each pregnancy. Males and females have an equal chance of being affected (Strachan and Read, 1999). Although autosomal recessive conditions are rare, the frequency of particular conditions may be increased in populations with a high carrier frequency or in communities that practice consanguineous marriages, with the greatest effect the more closely the parents are related (Smith, 1974). Identification of disease causing genes enables diagnostic, prenatal and carrier testing to be offered to affected families. In addition, the study of the disease causing genes

helps to improve the understanding of gene function, the pathogenesis of disease and the potential therapeutic targets for treatment.

1.5.4.2 Autozygosity Mapping

In 1987 a powerful approach to identifying disease genes from the linkage information yielded from the affected children born to consanguineous partnerships was described (Lander and Botstein, 1987). The technique of autozygosity mapping utilizes the fact that the consanguineous parents of a child affected with a rare autosomal recessive disease are likely to be autozygous for markers linked to the disease locus. The disease causing mutation and the adjacent chromosomal segments may be expected to be homozygous or identical by descent in the affected individuals (Lynex et al., 2004). First cousin parents would be expected to share 1/4 of their genes and their offspring autozygous at 1/16 of all loci because of their common ancestry. The offspring of second cousin parents would be estimated to be homozygous for 1/64 of their genome. The offspring of double-first cousins would be expected to be homozygous for 1/8 of their genome. However, greater homozygosity may be observed in populations with a long history of prolonged consanguinity and consequently a higher background level of homozygosity. In these circumstances it has been calculated that that for the offspring of first cousin parents on average 11% (range 5%–20%) of their genomes are homozygous (Woods et al., 2006).

These homozygous segments can be searched as a method for localising disease genes. It has previously been shown that most pathogenic mutations are identified in regions of autozygosity greater than 4 cM (Woods et al., 2006). Autozygosity mapping has been

used very effectively to map rare recessive genes, particularly in areas where consanguineous marriages are usual or families originate from isolated populations (Houwen et al., 1994). The power of this technique in comparison with the analysis of small nuclear families has been calculated mathematically. However, it is important to consider genetic heterogeneity when pooling the linkage information from different complex consanguineous families (Mueller and Bishop, 1993).

1.5.4.3 Candidate Gene Analysis

Evaluation of candidate genes is a method used to search for susceptibility genes in population based association studies. In this approach, markers such as microsatellites or SNPs (single nucleotide polymorphisms) are used to map Quantitative trait loci (QTLs), stretches of DNA containing or linked to the genes associated with that disease phenotype by comparing the patient population with healthy controls. This method may be successful if there is a disease causing genetic variant identified in the disease population that is not present in the control group. This technique may be more efficient when the pathogenesis of the condition is understood either in human or model organisms. Likely candidate genes can be selected and screened using Sanger sequencing for causative mutations. Although this strategy may be successful, it is relatively time consuming and expensive. In addition, new mechanisms of disease may not be identified with this method. The efficacy of candidate gene analysis depends on factors such as accurate clinical phenotyping, the size of the population to be studied and the number of available markers.

1.5.4.4 SNP arrays

SNP arrays utilize DNA sequence variation to study the variations throughout the whole genome. Humans carry the same base residue on both chromosome homologs at the majority of genomic sites. However in the remainder, single nucleotide polymorphisms (SNPs), two distinct alleles or nucleotides, are present in a proportion of the population. There are estimated to be approximately 10 million SNPs in the human genome (Kruglyak and Nickerson, 2001). Each SNP on the array is interrogated with different probes. These are labelled as SNP A and B. For an individual, their genotype at each SNP site is usually either AA, AB or BB. About one million SNPs are analysed in one assay. The genotyping accuracy is estimated to be greater than 99%.

The arrays contain hundreds of thousands of nucleotide probe sequences. Each probe is designed to bind a target DNA sequence, as nucleotide bases bind their complementary sequences. The signal intensity measured depends on the quantity of target DNA in the sample and the affinity between the target DNA and the probe. This method enables detection of stretches of homozygosity due to inheritance of haplotypes identical by descent.

1.5.4.5 Second Generation Sequencing

The development of high throughput sequencing technologies allows extensive mutation screening to be undertaken at increasingly lower costs. Massively parallel sequencing of large numbers of DNA amplicons has the advantage of accelerating detection of novel genes in the pathogenesis of disease, providing information about normal development and disease mechanisms and improving health care with the promise of personalised medicine. Whole

exome sequencing (WES) uses next generation sequencing technology to identify sequence variants in all the known coding exons. The human genome contains approximately $3x10^9$ coding and non-coding bases arranged in 180,000 exons, comprising about 1% or 30 Mb of the total human genome. WES covers over 95% of these exons, which has been estimated to contain about 85% of the mutations causing single gene disorders and single nucleotide polymorphisms (SNPs) predisposing to common diseases and cancer. Exome sequencing is therefore considered to be more efficient than genome sequencing as the majority of genetic variants in Mendelian disorders disrupt protein coding sequences. In addition, a large number of rare non synonymous substitutions are predicted to be deleterious and there is a high functional variation in splice site sequences. It has a higher coverage and a lower cost. These techniques can be used together to improve the detection rate of disease causing genes. For example homozygosity mapping using SNP arrays for genome wide linkage analysis in consanguineous families has successfully been used to target the exome sequencing region for interrogation and gene identification (Bolze et al., 2010). A limitation of this technique is the inability to detect variation in non-coding genomic DNA and epigenetic phenomenon which may cause or modify the clinical phenotype, and to reliably detect exon deletions and duplications. Due to the large number of human SNPs various strategies have been utilized to filter out variants unlikely to be disease causing using public databases such as dbSNP and HapMap. Inhouse and ethnicity-specific data collections can also be a useful adjunct to assist in identifying plausible variants. Categorisation to indicate the degree of pathogeneicity that may be attributed to each variant may help in current and future interpretation of genomic variation.

1.5.4.6 Identification of disease genes and bioinformatics

Following the identification of a suspected disease causing gene mutation, the variant is further evaluated for features consistent with pathogenicity. Supportive features include appropriate segregation within families and its absence (or very low frequency) in ethnically matched controls. The variant should be in a credible candidate gene. Properties of the variant that need to be assessed include the predicted effect on protein structure and function and the evolutionary conservation. Disease causing genes and the sequence variants are incorporated into several genetics databases. Examples include Ensembl, The Human Gene Mutation Database and OMIM (Online Mendelian Inheritance in Man). The information included on these gene resources enables further disease associated study. The Leiden Open Variation Database (LOVD) also includes patient specific clinical information with the mutation thus enabling genotype phenotype correlations to be made. With the advent of exome and genomic technologies, sophisticated sifting and analyzing of data will be essential for interpretation and clinical use.

1.5.4 Aims and objectives of the MPS/FADS study

The main aim of this project was to advance the identification and understanding of the molecular and biological basis and the phenotypic features of the MPS/FADS for families and their clinicians.

Information about the genetic basis of a condition is important for the management of families with autosomal recessive conditions. In addition to providing parents with information about the cause of the condition and the chance of recurrence, the possibility of early prenatal or

pre-implantation diagnosis may be made available for carrier couples at risk of having an affected child and carrier testing of relatives can be offered.

The objectives were thus to:

- Establish a large patient resource of MPS (Escobar-variant and perinatally lethal) families.

- *CHRNG* mutation analysis and a genotype phenotype correlation study

- Produce an online *CHRNG* mutations database resource detailing the type and position of the *CHRNG* sequence variants detected

- Identification of new MPS/FADS candidate genes

- Develop a practical molecular pathway to investigate MPS/FADS families

- Finally, the development of new technologies and how this can be incorporated into the search for and identification of further genes causing MPS will be considered.

Chapter 2: Materials and Methods

2.1 *CHRNG* Genotype Phenotype Correlations Study

2.1.1 Patients and Methods

100 families with at least one individual with clinical features of EVMPS, LMPS or FADS were recruited to this study. Entry criteria specified EVMPS/LMPS or FADS of unknown cause and so the cohort was representative of this group of families but not of all cases of these disorders. Ethical approval and valid consent was obtained. DNA samples from the probands and, where possible, the parents were collected. Mutation analysis of all 12 coding exons of *CHRNG* was performed by direct sequencing. Homozygous *CHRNG* mutations c.320T>G (p.Val107Gly), c.136C>T (p.Arg46X), c.401_402delCT (p.Pro134Argfsx43), c.753_754delCT (p.Val253AlafsX44), c.459dupA (p.Val154SerfsX24) and the possible mutation IVS4-9T>C (c.351-9T>C) in 6 families (15 affected individuals) have been described previously, (Morgan et al., 2006). A standardised questionnaire was used to gather phenotypic information including details of the family history, ethnicity, perinatal and postnatal history. The results of the relevant investigations, including brain scans and muscle biopsy results were recorded where available. Genotype-phenotype correlations were evaluated by subdividing the cohort into nonlethal and lethal patients and *CHRNG* mutation positive/negative groups. In families with multiple affected siblings, an analysis was undertaken to estimate the likelihood of a further affected pregnancy with a similar phenotype.

Statistics: Analysis was undertaken using Fisher's exact test. Statistical significance was taken at the 5% level.

2.1.1.1 Multiple Pterygium Syndrome Questionnaire

Name: ☐ Male ☐ Female
Date of Birth: Ethnic origin:
Hospital number:

Major features: fetal akinesia +/ multiple pterygia please delete as appropriate

Clinical History
Pregnancy:
Duration

*First detected in gestational week

	Yes		No	Unknown	Details (e.g. onset)
Intrauterine growth retardation	☐	__	☐	☐	
Polyhydramnios	☐	__	☐	☐	
Oligohydramnios	☐	__	☐	☐	
Cystic hygroma, oedema, hydrops fetalis	☐	__	☐	☐	
Reduced fetal movements	☐	__	☐	☐	
Joint contractures	☐	__	☐	☐	
Other abnormalities noted on USS	☐	__	☐	☐	
Other abnormalities	☐	__	☐	☐	

Neonatal period <1 month Bwt__ Length__ Head circumference__

	Yes	No	Unknown	Details
Respiratory problems	☐	☐	☐	
Cardiac problems	☐	☐	☐	
Other	☐	☐	☐	

Clinical Features
Growth Weight__ Height__ Head circumference__

		Yes	No	Unknown
Craniofacial anomalies		☐	☐	☐
Expressionless face		☐	☐	☐
Facial haemangioma		☐	☐	☐
Eyes:	downsloping palpebral fissures	☐	☐	☐
	ptosis	☐	☐	☐
	epicanthic folds	☐	☐	☐
	hypertelorism	☐	☐	☐
Nose:	depressed nasal bridge	☐	☐	☐
Oral:	cleft palate	☐	☐	☐
	high arched palate	☐	☐	☐
	small mouth	☐	☐	☐

	micrognathia		☐	☐	☐	
Ears:	low set		☐	☐	☐	
	malformed		☐	☐	☐	
		Yes	No	Mild	Moderate	Severe
Pterygia:	neck	☐	☐	☐	☐	☐
	axillae	☐	☐	☐	☐	☐
	elbows	☐	☐	☐	☐	☐
	groins	☐	☐	☐	☐	☐
	knees	☐	☐	☐	☐	☐
	other	☐	☐	Details:		

	Yes	No
Consent for photography	☐	☐

Flexion contractures: limitation of movement degrees of movement

	Yes	No	
Shoulders	☐	☐	_____
Elbows	☐	☐	_____
Wrists	☐	☐	_____
Fingers	☐	☐	_____
Hips	☐	☐	_____
Knees	☐	☐	_____
Ankles	☐	☐	_____
Other	☐	☐	Details:

Muscle weakness	☐	☐	
Muscle fatigability	☐	☐	
Hand abnormalities	☐	☐	Details:
Foot abnormalities:	☐	☐	Details:
Arachnodactyly	☐	☐	
Bony anomalies	☐	☐	
Vertebral fusion	☐	☐	
Scoliosis	☐	☐	
Ribs	☐	☐	
Other	☐	☐	
Cryptorchidism	☐	☐	
Other genital anomalies	☐	☐	

Hearing loss	☐	☐			
Soft vocalization /nasal speech	☐	☐			
Developmental delay	☐	☐			
Other features	☐	☐	Details:		

Family History (please enclose family tree)

	Yes	No	Male	Female	
Affected sibling	☐	☐	☐	☐	
Other affected relatives	☐	☐	☐	☐	Details:

relationship to proband

	Yes	No	Unknown	
Previous stillbirths / perinatal deaths	☐	☐	☐	Gestation: Details:

	Yes	No	Unknown
Consanguinity	☐	☐	☐

Laboratory results	Yes	No	N/A	Date	Details
Post mortem	☐	☐	☐		
Nerve studies	☐	☐	☐		
EMG	☐	☐	☐		
Biopsy	☐	☐	☐		
Maternal AChR Abs	☐	☐	☐		
Ratio anti-fetal /anti-adult AChR	☐	☐	☐		
Chromosomes	☐	☐	☐		
other	☐	☐	☐		

Thank you for your help.

Form completed by ……. Date ……

Contact details:

2.2 Candidate Gene Analysis of *CHRNA1, CHRNB1, CHRND, RAPSN* and *DOK7*

2.2.1 DNA samples

Patient DNA samples were obtained from a number of sources, including clinicians both within Europe and around the world. All families gave informed consent and the study was approved by the South Birmingham Research Ethics Committee. Where possible, completion of the clinical questionnaires was requested.

2.2.2 Primers

CHRNG	
Exon1-2F	CATCTCTCTCTGCCCCAGAC
Exon1-2R	ATGCTATCCCCAGCAGGTC
Exon3-4F	GTGGGGACTGGCACTGAAG
Exon3-4R	CAGAGTGCCTTGTTCTGCTG
Exon5F	CCTATCCACATGGGGCAC
Exon5R	CAGAGCCAAGGCCAGAAG
Exon6F	ATGCAGTCGTGGCAGGTC
Exon6R	TCCAGCCTTGACCACAGC
Exon7F	GGGATCTGCCTAGCTCACG
Exon7R	TCTGTTCCCCAGGCAGG
Exon8-9F	GCTACTTCTGGGGTAAGGGG
Exon8-9R	AGAGGGAGGCGTAGGGG
Exon10F	CAACCTCTGGCTTCTGCTC
Exon10R	ACTCATCTGCTGTGCCTGG
Exon11F	GAGAGGCCATCTTCTCTGCC
Exon11R	CTCTATCACGGTTCCAAGCC
Exon12F	CCCAAGGATGAGAACAGGAC
Exon12R	AAGACTCAGTGTGACCCACG

CHRNA1	
Exon1F	AGTGCCAGTGAGAAGCACAG
Exon1R	AGCAAGACTTTGATTTGGGG
Exon2-3F	TCATGTGTTTGGAGGGTGG
Exon2-3R	GGGCCCAGGTTTAATTTCAG
Exon4F	ATGCACATCAGGGCTCTTG

Exon4R	CTTTATTCCACCTGCCCC
Exon5F	AGCAGAATGGAAGGCTCATC
Exon5R	AAGGAAAAGTTGGAGACCCG
Exon6F	CTGATTACAAAACGCTGCCC
Exon6R	ATAGCAGCACGAGACCCATC
Exon7F	TATAAATGGTGGGGTGGGAG
Exon7R	GGGGAGGCCTGTAGATGTG
Exon8F	TGACTTACCCCAAATCACATTG
Exon8R	CAGACTCACCACCTGTGCTC
Exon9F	AGCACAAACGTTGTGAGTCC
Exon9R	ATCTTCCTCTCCTGCCCTTC

CHRND	
Exon1-2F	CACCCTCATTCCACAGCC
Exon1-2R	CCTGGTACTGGAAAGCCATC
Exon3F	GAGAGTGGGTGAATGTGTGC
Exon3R	CTTGATCTCCTGACCTCGTG
Exon4-5F	AGGAGCCTGGATGGCTG
Exon4-5R	AGGGTGCTTCAGTCACCTTC
Exon6F	CTTACCAGCCCTTCCCCAC
Exon6R	GACAGGGCCAGTGTGTCTG
Exon7F	AGCCCCTCATTCTGTCCC
Exon7R	GCTGGCTGTGCTCAGGTG
Exon8-9F	CAGCTGGACCCTCTAGGACC
Exon8-9R	GCCTCAGCCTCCTGAAATG
Exon10-11F	CAGCCTGGTGACAGAATGAG
Exon10-11R	TTGGTGTGTGCCTCTAAGGG
Exon12F	GGGCTTTGTGGCCTGAG
Exon12R	AGGAGTGGGTGTCATTAGGG

CHRND	
Exon1-2F	CACCCTCATTCCACAGCC
Exon1-2R	CCTGGTACTGGAAAGCCATC
Exon3F	GAGAGTGGGTGAATGTGTGC
Exon3R	CTTGATCTCCTGACCTCGTG
Exon4-5F	AGGAGCCTGGATGGCTG
Exon4-5R	AGGGTGCTTCAGTCACCTTC
Exon6F	CTTACCAGCCCTTCCCCAC
Exon6R	GACAGGGCCAGTGTGTCTG
Exon7F	AGCCCCTCATTCTGTCCC

Exon7R	GCTGGCTGTGCTCAGGTG
Exon8-9F	CAGCTGGACCCTCTAGGACC
Exon8-9R	GCCTCAGCCTCCTGAAATG
Exon10-11F	CAGCCTGGTGACAGAATGAG
Exon10-11R	TTGGTGTGTGCCTCTAAGGG
Exon12F	GGGCTTTGTGGCCTGAG
Exon12R	AGGAGTGGGTGTCATTAGGG

RAPSN	
Exon1F	ATTCCTGCCCCATGAGTG
Exon1R	GGAGCCTGGGAGACATCC
Exon2F	TGGGGTCCAAGGCTCAG
Exon2R	GTAGTGCCACAGGGTGTGTG
Exon3F	ACTTGGGGCAGCCTCTG
Exon3R	AAGAAGGAAGCCCTGCTGTC
Exon4-5F	GGTCTGTTGAGGGGAGGG
Exon4-5R	TAACCTACTGGCCCCAAGTG
Exon6F	GTGCTTCCCTGTGAGCAAG
Exon6R	CTTCCCCAGACCCAAGTTC
Exon7F	GACAGGGAAGTGGCTGAGAC
Exon7R	TCTCTGACTCCTCTTTGGGG
Exon8F	CAGGCAAGGAAGGAAGAGAG
Exon8R	TGCAGTGGAGAAAGAGCAGG

DOK7	
Exon1-2F	CTGCGAGCTATTTTGAAAGTG
Exon1-2R	CTCGGTAACCCGTGTCG
Exon3F	GGGTCTCTGCACTGTCACG
Exon3R	CTCCCACCTCTGCAGCC
Exon4F	TGATGTCCTCTCACCCTGC
Exon4R	CCGAAGCCAGCAGTTCTC
Exon5F	CTGTGTTCCTCCTCTTCAGG
Exon5R	CTAACTGGGGCTGACAAATC
Exon6F	ACTGAGTCAGGCTGGGC
Exon6R	ATGCCTCCTGAGCTCTCC
Exon7_1F	GAGACCAGAGAGTGCTGGC
Exon7_1R	CTGCGTGGTGTGTCATAGTG
Exon7_2F	CAGCCTCTCGTCCTACGC
Exon7_2R	CACAGAACACAGAGCACCAG

DAG1	
exon2F	GGTGTGCAGAGGGTGAGAAC
exon2R	CTTGAAAAGGAAAAGCCACC
exon3_1F	AAACCTGTCACACAATTCAGG
exon3_1R	GCACACTGTTCTGGTTCAGG
exon3_2F	CAGACTATTTGACATGTCGGC
exon3_2R	GGATACTCGTGGCTTCTTGG
exon3_3F	GATTCGCCCAACGATGAC
exon3_3R	GCCTGTGGACGTGGATCTC
exon3_4F	AGTTCAACAGCAACAGCCAG
exon3_4R	AGGTAGACATCATCCTCACTGC
exon3_5F	CGGCACCTACAGTTTATCCC
exon3_5R	CAACACCATGTCGTCTCCAG

MUSK	
exon1F	CTGTGGAGCCATTTTCCTTG
exon1R	AAAACCCAAGCCCACCATAC
exon2F	TTTGATTTCTCTTTCCTTTCA
exon2R	TGTATCAAAACATCTCATCTACTTCA
exon3F	TTCCTCATTAACAAGTCATCGG
exon3R	GTGAACCGAGATCATGCC
exon4F	TGCCTCAAATGATTCTCAAAAC
exon4R	CCAAATGAAATGCCATGCAG
exon5F	TTGGCAAATGTATCCTTGATAGAC
exon5R	TCACAGTAGACAGTGAGGCCC
exon6F	CACCCTAGCTTGACCATTTCC
exon6R	CCTGAAGTTCACCACCAATG
exon7F	TGCTTTTCCCTTCAAATCTACTG
exon7R	GGGAGATTTTCTCCAATGCTC
exon8_9F	TTTCTGAGACACAGATGTGAAAAC
exon8_9R	AAACTCCTTCTGTTGTGCAGATAG
exon10F	AACACAGAATTTAGGCTCTGCC
exon10R	GAAGCTTTAAGTTCACACCTTAGTTTC
exon11F	GCACTCACTCTCTCACAGAATATG
exon11R	ACACATGCATGAATCCAATTAC
exon12F	AATATATAACCAGGGAGAACCTGATAC
exon12R	TCCTCACGTCATACCAACTCC
exon13F	AAGTCTAAGTACAAAGATCTAACCTGC
exon13R	GCTAATAGTTTGGTAGCTTTTCAGAC
exon14F	AGCCTTTTACACAGGGGAGG
exon14R	AAAGCACGAACAAGGGGAG

exon15_1F	GCTTTTGGAGTGCTCTTTCC
exon15_1R	AAATGGACTCTGGTGGCATC
exon15_2F	GTGAAAATTGCCGACTTTGG
exon15_2R	CCTCTGGTGTAGGATTCCCC

2.2.3 Microsatellite markers

(Human Genome Assembly Build 36)

Marker	Start position (bp)	End position (bp)	Approximate size	Marshfield	deCODE (cM)
CHRNG					
D2S1363	226737850	226738038	164-204	227	229.1
D2S396	230391872	230392111	230-244	232.9	252.5
D2S427	231914508	231914752	243-263	236.7	238.2
D2S2193	231945632	231945898	239-271	236.7	238.2
D2S2344	233152938	233153148	193-221	238.3	239.3
D2S206	233416066	233416307	123-151	240.8	240
RAPSN					
D11S4109	47558064	47558236	155-185	58.4	63.8
D11S1344	46123506	46123786	273-293	58.4	63.5
DOK7					
D4S412	3417660		237-249	4.7	4.4
D4S2935	6678952		104-120	14	14
D4S2366	6602749		120-144	12.9	12.5

2.2.3 PCR Amplification for Sequencing

Standard conditions for PCR amplification were used. For each reaction 50-100 ng of genomic DNA, 1 x Taq polymerase buffer, 500 μM dNTP's, 5.0 pmol of forward and reverse primers and 1.5 U of ABgene Taq DNA polymerase were made up to 25 μl with dH_20. PCR conditions were: an initial denaturation of 95^oC for 5 minutes, followed by 34 cycles of 45 seconds denaturation at 95^oC, 45 seconds annealing at 50-65^oC (depending on the reaction)

and 45 seconds extension at 72^{o}C. This was followed by a final extension at 72^{o}C for 10 minutes. The PCR products were cleaned up using ExoSAP purification.

2.2.4 Sequencing of Candidate genes

The purified PCR product was sequenced in both forward and reverse directions using the relevant primers. The 10µl sequencing reaction was set up with 1 µl Big Dye Reaction Mix, 2 µl 5x Sequencing buffer, 3.5 µl Purified PCR product, 2 µl Forward or Reverse Primer (2 pmol/µl) and 1.5µl Water. The cycling conditions were 96^{o}C for 3 minutes followed by 30 cycles of 96^{o}C for 30 seconds, 50 ^{o}C for 15 seconds and 60^{o}C for 4 minutes.

Sequencing reactions were cleaned up to remove any unincorporated dye terminators prior to electrophoresis. 1 µl of EDTA (250 mM) was added and mixed to each 10 µl sequencing reaction before adding 30 µl of absolute ethanol. This was incubated for 15 minutes at room temperature and then centrifuged for 20 minutes at 2000 rpm at 20°C. The supernatant was removed by briefly spinning the plate upside down to 400rpm. 90 µl 70% ethanol was then added, mixed and the plate centrifuged at 2000 rpm for a further 10 minutes at room temperature. The supernatant was again discarded by inverting the plate and spinning to 400 rpm. The pellet was then left to air dry.

Sequencing reactions were resuspended in 10 µl Hi-Di Formamide and denatured for 2 minutes before snap chilling on ice.

2.2.5 Microsatellite Marker studies

PCR amplification was performed in 10 µl reactions containing 20 ng of genomic DNA, 1 x *Taq* DNA polymerase buffer, 1.5 mM $MgCl_2$, 0.5 U *Taq* Polymerase, 2 mM dNTP and 2.0

pmol each primer (forward fluorescently labelled and reverse). After an initial denaturation at 95^{o}C for 5 minutes, a standard PCR protocol was followed consisting of 28 cycles of 95^{o}C for 30s, annealing at 55^{o}C for 30s and extension at 72^{o}C for 30s, followed by a final extension step at 72^{o}C for 5 minutes.

2.3 Mapping New Genes

2.3.1 SNP arrays

In consanguineous families with autosomal recessive conditions, the assumption is that the causative gene will be located in a region of homozygosity shared between the affected individuals in the family because of homozygosity by descent for the disease gene. A 10cM genome-wide linkage scan was carried out by Louise Tee using the Affymetrix 250K SNP chip in two affected siblings from the branch of the a family (II.3 and II.4) and a similarly affected cousin (II.5) from the b branch of the family, in order to identify common regions of homozygosity. In particular regions larger than four megabases were sought, as these are most likely to contain the pathogenic gene (Woods et al., 2006). Further analysis and fine mapping of the homozygous region was undertaken using microsatellite markers in the available parents and offspring for whom DNA was available.

2.3.2 Microsatellite markers

Marker	Start position (bp)	End position (bp)	Approximate size	Marshfield	deCODE (cM)
D3S1300	60484947	60485189	217-241	80.3	82.2
D3S1481	60633630	60633711	104		82.58
D3S1239	62034672	62035006	189-193	82.2	84.7
D3S1312	62381446	62381761	215-225	82.2	85.1
D3S3566	62517629	62517997	218-234	81.4	85.1
D3S3698	63094791	63095178	270-278	84.9	86.4
D3S1600	63294878	63295304	182-198	86	86
D3S3644	64772879	64773050	164-176	91.2	89.3
D3S1285	64914199	64914434	232-242	91.2	89.3

2.3.3 Primers

Synaptoporin	
1A_F	AAAGAGGGACAAGTGGCTG
1A_R	CCAAATGTGCCTATGGAGAC
2A_F	CCCTCAATCCAGACATTAAGC
2A_R	TGTTTACCCAGAAGTCCCTG
3A_F	CCAGATCACATCCCATTTTG
3A_R	GGGCTCAGGTAACTCCAAAG
4A_F	CTTGTTTCCAGTGCTATGGG
4A_R	GGCCTAGTTTTGTAGGCAAC
5A_F	AAAGTGAAAAGTGATCTTTTGTATTG
5A_R	TGCCACTGAAATGGAAGATG
1-2B_F	AGGACGGTGGTGCCAAG
1-2B_R	GACCTCTCCTCACCTGATAGC
1C_F	TTTGAATGAAGCATTTTGAAAC
1C_R	TGTGCACATAAAAGTAAGAAGGG

FEZF2	
2_1_F	CTTTTCCGGAATGCAGAC
2_1_R	AGCCTGGTTGATGACCTG
2_2_F	AAAACCAACTGTGGCGTG
2_2_R	AAAAGGGGCATTCCAGG
3-4_F	GATTGTAGGCCCTCGGAC
3-4_R	AGATCCCTCTTTGCCTTCC
5_F	CTTCCTCCCCACCATTCTAC
5_R	TGTGTGATCTGTTTTCAGGTG

ATXN7	
3_F	GAAAGCGAAAGCTAGCCC
3_R	CAAACGATAGCATCTCTGCC
4_F	GTGTGCGTCACACTCCTG
4_R	CGGTCCCAGTGAACTGTC
5_F	AATGGTTTTATATCAGTCAGAACC
5_R	CATGCCATCACTGTTCCTTAG
6_F	AGAATTACATTTGAGATTCGCC
6_R	TCTCTTTTATATTACTCACAGGCAC
7_F	TCTGGCTCACCATTCACC
7_R	ATTTGCTCCCCACGAATG
8_F	AAAATACATGGAGGAGAGTTTG

8_R	CCCAGTCACCCATGTTTATC
9_F	TTTTGGGATATAAGGCAGACC
9_R	ACAGTATCTGCAAGTCTCATCTC
10_F	AGTTAAGGTGGCTGATTCCC
10_R	CCCTGTCCATGCTGTGTG
11_F	CTGGGCATGCCAGTGTG
11_R	CATCAGTCTCACAGGAAATAAAAG
12-1_F	AAGTCTCTGTGAATGGCTGTG
12-1_R	TGTTACCGTTGAGGGGTAGG
12-2_F	CCAGTAGCAGTACCAGTGGC
12-2_R	TACAAACCAACCAACCAACC
13_F	CCAGCTTCCTTTGCTCTAAC
13_R	GCTGGCATTTGAGTTAAGTTC
14_F	AGTGCAGTGTACAAACGCTTAC
14_R	TCCTCGTTAAAACAACAGCC

PRICKLE	
2_F	GCCAAGGAATGTGTTTATGG
2_R	CCTCATTTTCCACTGGGG
3_F	CCCAGTAAGTGGGAATAGGC
3_R	AAGGTAACAATTCATAGAAGGCAC
4_F	CTCAGAGTCATGGGCTTCAG
4_R	ATGGGTGAAAAGATGCCAG
5_F	TCATTTATGCACAGCACTGG
5_R	AGACCATGTGTTGGCTATGG
6_F	AGGACCCCATAGCTTTCTTC
6_R	AAACAAAGGTGACCGCATC
7-1_F	CTATGTGTTTCACGTCCCAG
7-1_R	AGTTCTGATGTTGCACTGGC
7-2_F	CAGACACCCAGCCTCAAC
7-2_R	CAGTCACCCAGAGACTACCC
8-1_F	ACAGTGATACCCATTGCTGC
8-1_R	GCCTATCTTTTAACCGGGAG
8-2_F	CACAGGTCCAGGCGTTC
8-2_R	GCGCTTTAACATTTAAAACAGTG

2.3.2 Exome sequencing

1-3μg DNA from an affected proband from family branch 1a (II:4) was used for further testing. Exome sequencing was undertaken by our collaborative group at the Beijing Genomics Institute (BGI) (Beijing, China) with the Agilent Technologies SureSelect Human All Exon Kit. The sequences obtained were aligned to the hg18 reference genome build. The sequence variants were compared with the various sources including NCBI dbSNP build 129 and our in-house database. Variants were categorised and tabulated according to whether they were exonic, splicesite or intronic, whether they were heterozygous or homozygous and the sequence variant was classified as frameshift, nonsense, missense non synonymous or synonymous. Particular scutiny for potentially pathogenic sequence variants was focussed on regions of homozygosity previously identified on the SNP arrays.

Chapter 3: *CHRNG* Genotype Phenotype Correlations in the Multiple Pterygium Syndromes

3 *CHRNG* Genotype Phenotype Correlations in the Multiple Pterygium Syndromes

A substantial part of this chapter has been published in Vogt et al., 2012.

3.1 Introduction

The MPS are a spectrum of phenotypically heterogeneous disorders ranging from the non lethal Escobar variant through to lethally affected fetuses with fetal akinesia and pterygia at the most severe end of the spectrum. The aetiology of MPS is also heterogeneous. A diagnosis of a specific primary myopathy, metabolic or neurodevelopmental disorder is made in a few cases, but until recently, in the underlying aetiology remained unknown in many cases (Cox et al., 2003).

MPS is most commonly inherited in an autosomal recessive fashion, though autosomal dominant, (McKeown and Harris, 1988, Prontera et al., 2006) and X-linked recessive pedigrees have been described, (Tolmie et al., 1987). Recently, genetic studies in autosomal recessive families have identified mutations in *CHRNG*, which encodes the gamma subunit of the embryonic nicotinic acetylcholine receptor (AChR) in families with LMPS and/or EVMPS, (Morgan et al., 2006, Hoffmann et al., 2006).

3.2 Results

3.2.1 Clinical cohort and *CHRNG* mutation detection

A cohort of 100 multiple pterygium syndrome (MPS) families of unknown cause were tested for the presence of a *CHRNG* mutation. There were 41 families in which the proband

presented with Escobar variant multiple pterygium syndrome (EVMPS). These MPS families contained 54 affected individuals. The affected proband presented with lethal multiple pterygium syndrome (LMPS) / fetal akinesia deformation sequence (FADS) in 59 families. These families comprised 84 affected individuals. A *CHRNG* mutation was identified in eleven (27%) of the EVMPS families and five (8%) of the LMPS/FADS families. Parental consanguinity was noted in 31% of families with *CHRNG* mutations (3 out of 11 with EVMPS and 2 out of 5 with LMPS/FADS). Parents were unrelated in 69% of these families (8 out of 11 with EVMPS and 3 out of 5 with LMPS/FADS). The clinical features present were collected using a questionnaire tool. Tick boxes were utilised to enable easy completion of the questionnaire and to aid analysis of the data.

3.2.2 Clinical features present in the cohort

The clinical features in the 41 Escobar variant MPS families and the 59 LMPS/FADS families were compared. Joint contractures were universally present in both groups. Pterygia were noted in 50/54 (93%) of the Escobar patients and 58/84 (69%) of those with LMPS/FADS. The prenatal features were compared between the two groups. In the LMPS/FADS cases cystic hygroma was present in 42/84 (50%), fetal hydrops in 39/84 (46%) and pulmonary hypoplasia in 30/84 (36%). However, in contrast, cystic hygroma and fetal hydrops were rarely reported 1/54 (2%) in the nonlethal patients ($P< 0.0001$ and $P<0.0001$) and pulmonary hypoplasia 2/54 (4%) was only occasionally recognised ($P< 0.0001$). One of these severely affected infants had diaphragmatic eventration and respiratory distress from birth and died at 4 months of age. The other was a 3 day old neonatal death. Reduced fetal movements were more frequently reported in the LMPS/FADS patients 49/84 (58%) compared with the 14/54

(26%) EVMPS patients (P=0.0002). Intrauterine growth retardation was present in 35/84 (42%) of the LMPS/FADS cases and 8/54 (15%) of the EVMPS cases (P=0.0012). Abnormalities were detected on ultrasound examination in 56/84 (67%) of the lethal cases compared with 3/54 (6%) of the non lethal families (P<0.0001), Figure 12.

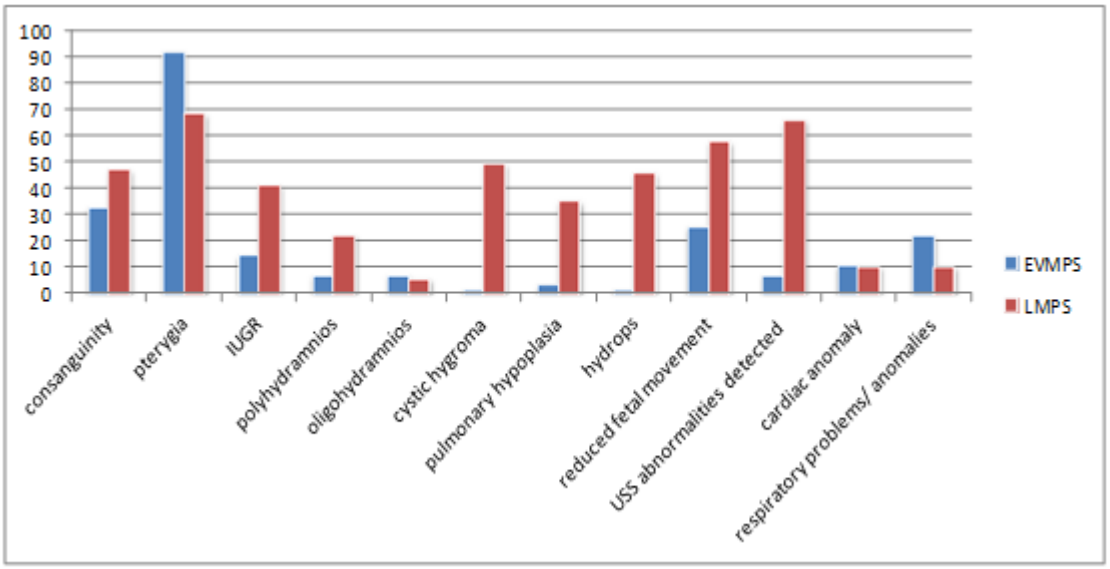


Figure 12: The prenatal features in the lethal and nonlethal MPS families.

(Taken from *CHRNG* Genotype Phenotype Correlations in the Multiple Pterygium Syndromes, Vogt et al., 2012).

Cleft palate was identified in over a third 32/84 (38%) of the lethal patients compared with 7/54 (13%) of the nonlethal group (P=0.0017). Central nervous system manifestations were twice as common in the lethal patients (14/84 (17%) and 4/54 (7%) in the nonlethal group (P>0.1)), but the difference was not statistically significant. Reduced muscle bulk was observed in 24/84 (29%) of the lethal families, compared with 4/54 (7%) of the Escobar cases (P=0.0023). Poor postnatal growth was reported in 20/54 (37%) of the non lethal MPS families.

3.2.3 Clinical features in EVMPS patients with and without a detectable *CHRNG* mutation

CHRNG mutation positive and negative EVMPS cases generally had a similar pattern of clinical features (see Table 2).

Feature	EVMPS (N=17) *CHRNG* mutation positive	(%)	EVMPS (N=37) *CHRNG* mutation negative	(%)
pterygia	16	94	34	92
IUGR	0	0	8	22
polyhydramnios	2	12	2	5
oligohydramnios	0	0	4	11
cystic hygroma	1	6	0	0
pulmonary hypoplasia	2	12	0	0
hydrops	1	6	0	0
reduced fetal movement	4	24	10	27
USS abnormalities detected	1	6	3	8
cardiac anomaly	2	12	4	11
respiratory problems/ anomalies	6	35	6	16
growth	7	41	13	35
craniofacial dysmorphisms	12	71	27	73
expressionless face	6	35	16	43
cleft palate	1	6	6	16
low set ears	6	35	8	22
malformed ears	2	12	4	11
reduced muscle bulk	1	6	3	8
proximal joint contractures	13	76	28	76
distal joint contractures	13	76	30	81
vertebral anomalies	9	53	21	57
thoracic anomalies	2	12	10	27
genital anomalies	3	18	17	46
CNS anomaly	0	0	3	8

Table 2: Features in *CHRNG* mutation positive and negative EVMPS patients.

(Taken from *CHRNG* Genotype Phenotype Correlations in the Multiple Pterygium Syndromes, Vogt et al., 2012).

3.2.4 Clinical features in LMPS/FADS patients with and without a detectable *CHRNG* mutation

Developmental central nervous system anomalies were not present in the patients with detectable *CHRNG* mutations (0/7). However, a variety of CNS malformations were identified and more than one structural abnormality was described in several patients in the *CHRNG* mutation negative group 14/77 (18%) (P> 0.1). Cerebellar involvement was present in approximately 30% of those with CNS anomalies. Cerebellar hypoplasia was reported in 5/77 (6%) of cases. Polymicrogyria was identified in 4/77 (5%) and ventricular dilatation in 3/77 (4%). Although cleft palate was not observed in our *CHRNG* positive cohort, it was frequently noted in those patients without a *CHRNG* mutation, occurring in 32/77 (42%) (P=0.041), see Table 3.

Feature	LMPS/FADS (N=7) *CHRNG* mutation positive	(%)	LMPS/FADS (N=77) *CHRNG* mutation negative	(%)
pterygia	6	86	52	68
IUGR	3	43	32	42
polyhydramnios	3	43	16	21
oligohydramnios	0	0	5	6
cystic hygroma	4	57	38	49
pulmonary hypoplasia	3	43	27	35
hydrops	5	71	34	44
reduced fetal movement	4	57	45	58
USS abnormalities detected	6	86	50	65
cardiac anomaly	1	14	8	10
respiratory problems/ anomalies	0	0	9	12
growth	0	0	0	0
craniofacial dysmorphisms	6	86	54	70
cleft palate	0	0	32	42
low set ears	3	43	41	53

malformed ears	1	14	8	10
reduced muscle bulk	1	14	23	30
proximal joint contractures	5	71	68	88
distal joint contractures	6	86	55	71
vertebral anomalies	3	43	15	19
thoracic anomalies	3	43	21	27
genital anomalies	0	0	16	21
CNS anomaly	0	0	14	18

Table 3: Features in CHRNG mutation positive and negative LMPS/FADS patients.

(Taken from *CHRNG* Genotype Phenotype Correlations in the Multiple Pterygium Syndromes, Vogt et al., 2012).

3.2.5 Correlation of the clinical features present in the *CHRNG* mutation positive and *CHRNG* mutation negative MPS patients.

Pterygia were present in 21/24 (86%) of the *CHRNG* mutation positive EVMPS and LMPS patients compared with the mutation negative group where pterygia were observed in 86/114 (75%) (P=0.28). Cleft palate was only seen in 1/24 (4%) of the *CHRNG* mutation positive patients but was noted in 38/114 (33%) of the *CHRNG* negative MPS patients (P=0.0025). CNS malformations were not associated with the presence of a *CHRNG* mutation (0/24) and were more frequent in the *CHRNG* mutation negative group 20/114 (P=0.024).

3.2.6 Spectrum of *CHRNG* mutations identified in the MPS cohort

Table 4 shows the 20 *CHRNG* mutations detected in the cohort. Homozygous mutations were identified in 12 patients whilst compound heterozygous *CHRNG* mutations were detected in 4 patients. 7 of the 20 *CHRNG* mutations identified were novel. Although *CHRNG* mutations

were found throughout the gene, there was a hotspot identified in exon 5 with a recurrent *CHRNG* mutation, and a further recurrent mutation in our cohort detected in exon 7 (see Figure 13).

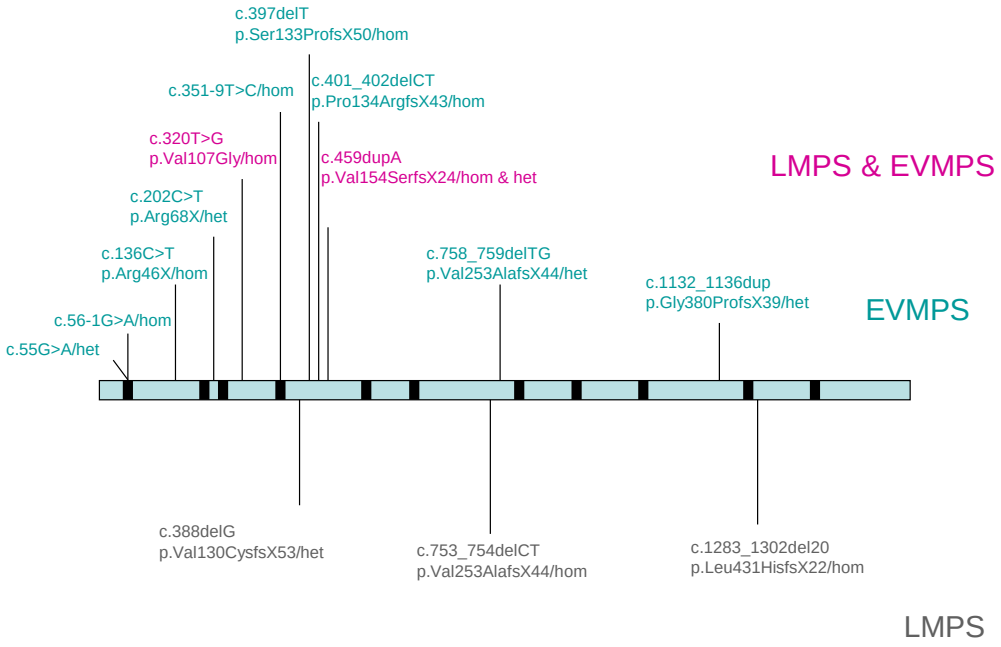


Figure 13: Distribution and characteristics of *CHRNG* mutations in MPS patients.

(Adapted from *CHRNG* Genotype Phenotype Correlations in the Multiple Pterygium Syndromes, Vogt et al., 2012).

The recurrent mutation c.459dupA (p.Val154SerfsX24) was identified in 6 families from different ethnic backgrounds. Affected patients had EVMPS as well as LMPS phenotypes. This *CHRNG* mutation occurred in the homozygous state and in association with a second *CHRNG* mutation in two compound heterozygous individuals. Another recurrent *CHRNG* mutation c.753_754delCT (p.Val253AlafsX44) in exon 7 was less frequently observed. It was

present in 3 families with both the non lethal and lethal phenotypes. It occurred as a homozygous mutation and in compound heterozygous individuals. Data from the public database, Exome Aggregation Consortium (ExAC), which summarises the exome data from over 60,000 unrelated individuals, was used to acertain the allele frequencies of the *CHRNG* mutations where available. The recurrent *CHRNG* mutations identified in my study c.459dupA (p.Val154SerfsX24), c.753_754delCT (p.Val253AlafsX44) had a high allele frequency of less than 1 in 10, 000 compared to an allele frequency of less than 1 in 100,000 or no listing for the other mutations detected (Exome Aggregation Consortium (ExAC), Cambridge, MA (URL:http://exac.broadinstitute.org) [March, 2016].

Pedigree	Phenotype	Ethnic Origin	Parental Consanguinity	Nucleotide Alterations	Alterations in Coding Sequence	Homozygous or Heterozygous	Exon/ Intron	Exac database allele frequency
MPS001	Nonlethal and lethal	Arab	Yes	c.320T>G	p.Val107Gly	homozygous	4	not listed
MPS002	Nonlethal	Pakistani	Yes	c.136C>T	p.Arg46X	homozygous	2	$8.2x10^{-5}$
MPS006	Nonlethal	Pakistani	Yes	IVS4-9T>C (c.351-9T>C)	Unknown	homozygous	IVS4	not listed
MPS015	Nonlethal	White	No	c.401_402delCT	p.Pro134Argfsx43	homozygous	5	$9.8x10^{-5}$
MPS031	Nonlethal	S. American/White	No	c.397delT	p.Ser133ProfsX50	homozygous	5	$8.2x10^{-6}$
MPS033	Nonlethal		No	c.459dupA	p.Val154SerfsX24	homozygous	5	$2.6x10^{-4}$
MPS034	Nonlethal	White[a]	No	c.459dupA	p.Val154SerfsX24	homozygous	5	$2.6x10^{-4}$
MPS037	Nonlethal[b]	S. American/White	No	c.202C>T	p.Arg68X	heterozygous	3	$2.5x10^{-5}$
MPS037	Nonlethal[b]	S. American/White	No	c.753_754delCT	p.Val253AlafsX44	heterozygous	7	$1.8x10^{-4}$
MPS038	Nonlethal	White (Irish)	No	c.56-1G>A	splicesite	homozygous	2	not listed
MPS042	Nonlethal[b]	White	Unknown	c.459dupA	p.Val154SerfsX24	heterozygous	5	$2.6x10^{-4}$
MPS042	Nonlethal[b]	White	Unknown	c.753_754delCT	p.Val253AlafsX44	heterozygous	7	$1.8x10^{-4}$
MPS044	Nonlethal[b]	White[a]	No	c.55G>A	p.Gly19Arg	heterozygous	1	not listed
MPS044	Nonlethal[b]	White[a]	No	c.1132_1136dup	p.Gly380ProfsX39	heterozygous	10	not listed
MPS008	Lethal	Turkish	Yes	c.753_754delCT	p.Val253AlafsX44	homozygous	7	$1.8x10^{-4}$
MPS011	Lethal	White[a]	No	c.459dupA	p.Val154SerfsX24	homozygous	5	$2.6x10^{-4}$
MPS035	Lethal[b]	White	No	c.388delG	p.Val130CysfsX53	heterozygous	5	not listed
MPS035	Lethal[b]	White	No	c.459dupA	p.Val154SerfsX24	heterozygous	5	$2.6x10^{-4}$
MPS036	Lethal	Algerian	Yes	c.1292_1311del20	p.Leu431HisfsX22	homozygous	11	not listed
MPS039	Lethal	White[a]	No	c.459dupA	p.Val154SerfsX24	homozygous	5	$2.6x10^{-4}$

Table 4: Details of MPS-affected families with *CHRNG* mutations and sequence variants. (Adapted from *CHRNG* Genotype Phenotype Correlations in the Multiple Pterygium Syndromes Vogt et al., 2012). a patients from the United Kingdom; b compound heterozygotes patients. Note 1 MPS001, MPS002, MPS006, MPS008, MPS011, MPS015, (Morgan et al., 2006).

Haplotype analysis with microsatellite markers (D2S1363, D2S2193 and D2S2344) suggested that the three families with the *CHRNG* c.459dupA mutation did not share a common founder mutation (see Table 5).

Pedigree	MPS033	MPS034	MPS011		MPS015	MPS031	
CHRNG mutation	c.459dupA	c.459dupA	c.459dupA		c.401_402delCT	c.397delT	
Marker	(affected)	(affected)	(affected)	(mother)	(affected)	(affected 1)	(affected 2)
D2S1363	182	186	178 182	178 182	178	186	186
D2S2193	261	251 263	253 241	253 239	255 267	253 255	253 257
D2S2344	196	196	196	196	196	190	190

Table 5: Haplotype analysis of *CHRNG* c.459dupA mutation positive families.

(Taken from Vogt et al., 2012).

In addition sequence variants of uncertain pathogenicity were detected during the analysis. These are shown below in Table 6.

Pedigree	Phenotype	Ethnic Origin	Nucleotide Alterations	Alterations in Coding Sequence	Homozygous or Heterozygous	Exon/ Intron	Parental Consanguinity
MPS028	Lethal	? African	IVS7-13 T>C	unknown	heterozygous	7	Unknown
MPS028	Lethal	? African	IVS7-23insCCTGCCTG	unknown	heterozygous	7	Unknown
MPS029	Lethal	Turkish	c.367G>A	p.Glu123Lys	heterozygous	5	Yes
MPS30	Lethal	White	c.55+27G>A	unknown	heterozygous	1	Yes
MPS032	Nonlethal	S. American	c.299T>G	p.Leu100Arg	homozygous	3	Unknown
MPS043	Nonlethal	African	c.1259C>T	p.Pro420leu	heterozygous	10	Unknown
MPS046		Brazil	c.82C>T	p.Arg28Cys	heterozygous	1	Unknown

Table 6: Details of MPS-affected families with *CHRNG* sequence variants.

3.2.7 Intrafamilial Variation in *CHRNG* mutation positive families

The MPS phenotype was assessed in the affected patients with *CHRNG* mutations incorporated into our study. Within the EVMPS cohort, all 10 affected siblings subsequently born into these 41 families were affected with Escobar variant MPS. Although the 1st and 2nd siblings affected with MPS in one *CHRNG* positive family died in the neonatal period and early infancy, the 3rd affected sibling was not affected with lethal MPS, (Morgan et al., 2006). However the significance of the family history in this single family is unclear. Factors such as the severity of the congenital malformations and the availability of medical care may have influenced the outcome in these individuals. In this family there were 4 other cousins with Escobar variant MPS and one deceased cousin. In another family, two siblings with nonlethal MPS had a cousin who was severely affected and died in infancy. In the *CHRNG* mutation negative group there was one family with an affected child who died at 3 years old (about which little detail is available) who had an affected sibling who survived. Three siblings affected with Escobar variant MPS in one family had 2 cousins that died in early childhood, about which there are no further details.

In our cohort the proband presented with LMPS/FADS in 59 of the families. All of the 25 affected siblings had an LMPS/FADS phenotype (25/25). This included 6 families with 3 lethally affected siblings. In one *CHRNG* mutation positive kindred a sibling died in the neonatal period while the other was diagnosed with fetal hydrops in early pregnancy. Although the presentations were variable, they were both in the lethal spectrum. Overall, the chance of a similar MPS phenotype arising in further affected siblings is estimated to be 10/12+25/25=35/37 = 95% (95% CI 81-99 %).

3.3 Discussion

The discovery of gamma subunit (*CHRNG*) acetylcholine receptor mutations in MPS have provided valuable insights into the pathogenic mechanisms underlying this condition. Mutations in the embryonal AChR, *CHRNG*, result in severe fetal akinesia in early to mid-pregnancy, causing pterygia and other developmental anomalies in MPS. As the switch to the adult AChR occurs in the third trimester of pregnancy, postnatal muscle weakness and fatigability is not a feature of C*HRNG* positive MPS. This contrasts with the congenital myasthenic syndromes, another group of AChR deficiency conditions.

CHRNG mutations were found in 27% of EVMPS and 8% of LMPS/FADS kindreds. The actual detection rate may be lower because patients with alternative diagnoses, estimated to be present in about half of cases (Witters et al., 2002), were not included in our cohort. However, *CHRNG* heterozygous exonic deletions and mutations outside the coding exons and flanking sequences would not have been detected by our mutation analysis.

The EVMPS and LMPS/FADS cases had a similar *CHRNG* mutation spectrum, thus the severity of the phenotype did not appear to correlate with the mutation type or its position in the gene. The same *CHRNG* mutation (for example, the c.459dupA mutation) was identified in both the lethal and Escobar variant families. As interfamilial phenotypic variability was more marked than intrafamilial variability, this may have been due to the effect of genetic and environmental modifiers. The *CHRNG* mutations c.459dupA and c.753_754delCT were recurrent in our cohort. However, there was no evidence to support a common haplotype and a founder mutation affect.

Cystic hygroma and fetal oedema are frequent in LMPS/FADS, (de Die-Smulders et al., 1990, Hall, 1984), and the absence of these features in the EVMPS cohort suggests that they are markers for more severe fetal akinesia. Pulmonary hypoplasia was not observed in the EVMPS probands. It was detected in 2 of the 12 siblings however this may have been due to a more detailed assessment following the birth of an affected child. Cleft palate was identified in over a third of the LMPS/FADS patients but was less common in the EVMPS cases, thus it may represent more severe akinesia in that group. Cleft palate was more often observed in the *CHRNG* negative EVMPS patients occurring in 6/37 (16%) and 1/17 (6%) of the *CHRNG* positive patients. Pterygia arise as a consequence of hypokinesia and have been observed before the development of fetal oedema (Machin, 1989). However, fetal oedema may inhibit movement further and exacerbate muscle hypoplasia resulting in the associated congenital anomalies and characteristic craniofacial features, (Hall, 1984).

Central nervous system (CNS) anomalies in LMPS/FAD suggest that a *CHRNG* mutation is unlikely. Although CNS malformations were not reported in *CHRNG* positive patients, a fifth of the *CHRNG* negative group had one or more CNS anomalies. Cerebellar hypoplasia, polymicrogyria or ventricular dilatation were most frequently noted. Thus CNS abnormalities may indicate a different underlying pathogenesis for the MPS phenotype in these patients.

Growth retardation is frequent in LMPS/FADS, as previously reported (Gillin and Pryse-Davis, 1976, Chen et al., 1984, Chen et al., 1980). However in the EVMPS families poor postnatal growth was observed in over a third. Pterygia were almost universal in MPS patients with a *CHRNG* mutation (21/24), thus pterygia predicted the presence of a *CHRNG* mutation.

Phenotypic variation of affected children within families as well as between families is well documented in MPS, (Ramer, 1991). Clinically it is important where possible for parents to have information about the chance of a lethally affected sibling being born to a family with a child with EVMPS, or the chance of a nonlethally affected baby arising in a family who have previously had a lethally affected baby. From the analysis there is a 95% chance that a subsequent sibling will have the same MPS phenotype (EVMPS or LMPS) as the proband, although there is less concordance for more distant relatives.

The *CHRNG* mutations were included in the new Locus Specific Database as outlined in the following chapter. As a significant proportion, approximately two thirds of our cohort remained without a molecular diagnosis, further candidate gene studies were undertaken on the *CHRNG* negative families as described in the following chapters.

Chapter 4: Mutations in *CHRNG* – A New Locus-Specific database (LSDB)

4.1 Introduction

4.1.1 *CHRNG* Database

A Locus-Specific sequence variation Database (LSDB) has been established for *CHRNG* mutations using the Leiden open (Source) Variation Database (LOVD) software (Fokkema et al., 2005). The aim of the database was to provide clinicians, laboratory scientists and researchers with the currently available *CHRNG* mutation information.

The embryonic acetylcholine receptor subunit gene *CHRNG* is located on chromosome 2 and comprises 12 exons. Mutations in *CHRNG* have been detected in a significant proportion of those affected with lethal and Escobar variant multiple pterygium syndrome (MPS) (Morgan et al., 2006, Hoffmann et al., 2006, Vogt et al., 2012). A variety of homozygous and compound heterozygous *CHRNG* mutations have been detected throughout the gene. Follow-up investigations of the *CHRNG* sequence variants detected may confirm segregation in the family and the absence of the *CHRNG* variant in ethnically matched control samples. However, as this gene is only expressed in fetal life, the lack of functional studies can make it difficult to determine pathogenicity of a sequence variant. This has implications for genetic counselling, the availability of prenatal diagnosis and carrier testing for affected families. The establishment of a locus specific database (LSDB) was considered to record the *CHRNG* mutations detected and to to try and improve our understanding of *CHRNG* sequence variants and aid in interpretation of the results for families.

The LSDB database enables easy submission, curation and maintenance of the *CHRNG* variants online, to ensure that the nomenclature complies with standardised Human Genome Variation Society (HGVS) guidelines. Numbering of the cDNA sequence was undertaken using the National Centre for Biotechnology Information (NCBI) accession number NM_005199.4, where *CHRNG* cDNA sequence +1 equalled A of ATG in accordance with the Human Genome Variation Society (HGVS) guidelines. The database included details about the DNA sequence variant, the exon, the subsequent predicted effect on the protein sequence, the type of mutation and its pathogenicity. Mutations were graded according to the type of mutation. Pathogenicity was assigned based on the classification of the mutation and its association with disease. All the sequence variants were detected in affected patients, segregated with the disease and were not present in controls. As *CHRNG* is only expressed in fetal life, RNA studies were not undertaken in any of the cases, due to the lack of availability of appropriate samples. The source of the material, the technique for mutation detection as well as clinical phenotype was linked to a unique patient identifier.

The database included published and unpublished novel as well as recurrent *CHRNG* sequence variants. Previously reported *CHRNG* mutations were renamed to concur with the HGVS nomenclature, however published mutation annotation is included for cross-referencing purposes. A PubMed hyperlink to the reference was added for published sequence variants where possible. Each *CHRNG* entry was allocated a unique identifier and the likely pathogenicity and clinical features information was added. Unpublished mutations and variants can be submitted by other contributors following registration for a login and password. Before this information is shared, it is curated as previously described (Fokkema et al., 2005, Bayley et al., 2005). The *CHRNG* LSDB of published and unpublished sequence

variants together with information about the likely pathogenicity and clinical phenotype thus provides an invaluable resource for clinician and scientists working with families with this group of conditions. This database is registered on the Human Genome Variation Society (HGVS) locus specific database list and can be accessed online at http://www.lovd.nl/CHRNG (Figure 14).

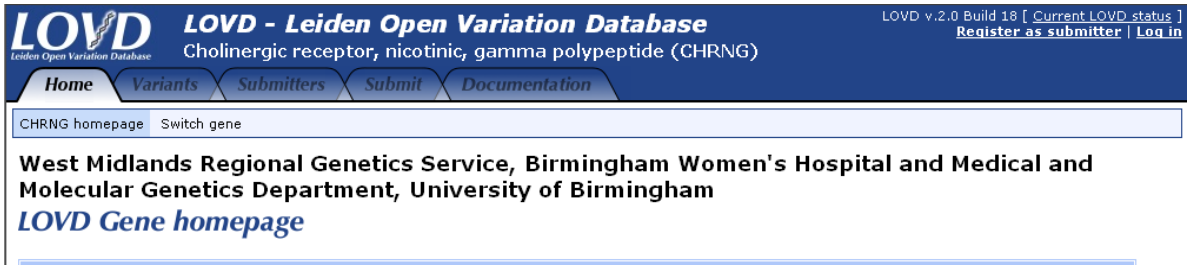


Figure 14: *CHRNG* mutation public access database

4.1.2 Contents of *CHRNG* Database

The database currently contains 34 entries from 29 families: 32 pathogenic mutations and 7 variants of unknown clinical significance in the *CHRNG* gene (Table 7). The DNA sequence variants were classified in 24% as deletions, in 26% as duplications and in 50% as missense substitutions. The protein variants are classified as nonsense in 12%, frameshift in 47%, duplications in 3%, substitutions in 32% and unknown in 6%.

Homozygous pathogenic *CHRNG* mutations from 19 families and pathogenic compound heterozygous mutations from 11 families are included on the database. However the second variant c.481G>A; W139X from EG-3 (Hoffmann et al., 2006) was omitted due to difficulties in annotating the variant. 13 previously published *CHRNG* mutations were included: 4

frameshift, 3 nonsense, 1 duplication, 2 splicesite and 3 missense mutations (Morgan et al., 2006, Hoffmann et al., 2006).

4.1.3 Novel *CHRNG* mutations

12 novel *CHRNG* mutations were added to the database: 1 nonsense, 4 frameshift and 2 splicesite mutations (included in our recent publication (Vogt et al., 2012) and 5 missense sequence variants of unknown pathogenicity.

4.1.3.1 Frameshift mutations

Four frameshift mutations were identified.c.388delG;p.Val130CysfsX53 in exon 5 was detected with the c.459dupA;p.Val154SerfsX24 mutation in a patient with lethal MPS. A homozygous c.1292_1311del20;p.Leu431HisfsX22 mutation in exon 11 was also identified in a lethally affected individual. The remaining three mutations were detected in Escobar variant families. The c.397delT;p.Ser133ProfsX50 mutation in exon 5 was identified in the homozygous state. The mutation c.1132_1136dup;p.Gly380ProfsX39 in exon 10 was identified in a compound heterozygote together with a novel splicesite mutation c.55G>A;pGly19Arg.

4.1. 3.2 Nonsense mutations

One Escobar variant patient identified was a compound heterozygote with a novel nonsense mutation, c.202C>T;p.Arg68X) in exon 3 and a frameshift *CHRNG* mutation c.753_754CT;p.Val253AlafsX44 mutation in exon 7.

4.1.3.3 Splicesite mutations

Two splicesite mutations were identified in *CHRNG*. A homozygous splicesite mutation was located at the 5'end of the gene at the exon 2 intron/exon boundary (c.56-1G>A) in a patient with Escobar variant MPS. The second splicesite variant c.55G>A;pGly19Arg was located at the most 3' nucleotide of exon 1. This sequence variant was occurred with an exon 10 frameshift mutation c.1132_1136dup;p.Gly380ProfsX39 in an Escobar variant MPS patient.

4.1.3.4 Missense Mutations

The homozygous missense mutation c.299T>G;p.Leu200Arg in exon 4 was detected in a patient with Escobar variant MPS. Four other heterozygous missense variants of unknown pathogenicity were identified. c.82C>T;p.Arg28Cys in exon 2 was noted in a patient with Escobar variant MPS and c.1259C>T;p.Pro420Leu in exon 10 was detected in another affected individual. c.367G>A;p.Glu123Lys in exon 5 and c.1516C>T;p.Pro506Ser in exon 12 were each present in a patients with lethal variant MPS.

In total 25 unique *CHRNG* sequence variants are included in the database (Table 7). Of these 18 are reported as pathogenic and there are 7 variants of unknown significance.

100 entries per page

Exon	DNA change	Protein change	Type	Variant remarks	DB-ID
01	c.13C>T	p.Gln4X	Nonsense	Homozygote Previously reported:c.DNA 13C>T, Mature protein after signal peptide cleavage Q-18X.	CHRNG_00042
01	c.55G>A	p.Gly19Arg	Splicesite	compound heterozygote	CHRNG_00053
02	c.56-1G>A	-	Splicesite	homozygote	CHRNG_00056
02	c.82C>T	p.Arg28Cys	Missense	Heterozygote	CHRNG_00052
02	c.136C>T	p.Arg46X	Nonsense	Homozygote	CHRNG_00002
03	c.202C>T	p.Arg68X	Nonsense	Compound Heterozygote	CHRNG_00035
04	c.256C>T	p.Arg86Cys	Missense	Compound heterozygote Previously reported:cDNA 256C->T, Mature protein after signal peptide cleavage R64C.	CHRNG_00047
04	c.299T>G	p.Leu100Arg	Missense	Homozygote	CHRNG_00032
04	c.300_308dup	p.Arg101_Pro103dup	Other	Duplication. Compound heterozygote. Previously reported:CDNA 300dup(9), mature protein after signal peptide cleavage 79dup(3).	CHRNG_00037
04	c.320T>G	p.Val107Gly	Missense	Homozygote	CHRNG_00001
IVS 4	c.351-9T>C	-	Splicesite	Homozygote Previously reported as IVS4-9T->C, possible splicesite mutation.	CHRNG_00044
05	c.367G>A	p.Glu123Lys	Missense	Heterozygote	CHRNG_00051
05	c.388delG	p.Val130CysfsX53	Frameshift	Compoound	CHRNG_00049

				heterozygote	
05	c.397delT	p.Ser133ProfsX50	Frameshift	Homozygote	CHRNG_00031
05	c.401_402delCT	p.Pro134ArgfsX43	Frameshift	Homozygote Previously reported: c.401_402delCT, p.Pro134ArgfsX 34.	CHRNG_00048
05	c.459dupA (Reported 6 times)	p.Val154SerfsX24	Frameshift	Homozygote	CHRNG_00033
07	c.715C>T (Reported 2 times)	p.Arg239Cys	Missense	Homozygote Previously reported:cDNA 715C->T, Mature protein after signal peptide cleavage R217C.	CHRNG_00043
07	c.753_754delCT (Reported 4 times)	p.Val253AlafsX44	Frameshift	Homozygote. Previously reported p.Pro251ProfsX4 6	CHRNG_00008
08	c.807dupT	p.Gly270TrpfsX28	Frameshift	Homozygote Previously reported:cDNA 807insT, Mature protein after signal peptide cleavage âˆ†248-274, 275X.	CHRNG_00045
10	c.1132_1136dup	p.Gly380ProfsX39	Frameshift	compound heterozygote	CHRNG_00054
10	c.1249G>C	p.Glu417Gln	Splicesite	Homozygote Previously reported:cDNA 1249G->C, Mature protein after signal peptide cleavage âˆ†395-418, 419X.	CHRNG_00046
10	c.1259C>T	p.Pro420Leu	Missense	Heterozygote	CHRNG_00055
11	c.1292_1311del20	p.Leu431HisfsX22	Frameshift	Homozygote	CHRNG_00041
12	c.1408C>T	p.Arg470X	Nonsense	Compound heterozygote. Previously reported:CDNA	CHRNG_00038

				1408C->T, mature protein after signal peptide cleavage R448X.	
12	c.1516C>T	p.Pro506Ser	Missense	Heterozygote	CHRNG_00050

Table 7: LOVD *CHRNG* database unique sequence variants.

4.2 Database Analysis

Although most *CHRNG* positive MPS patients studied to date have homozygous *CHRNG* mutations, six affected patients were compound heterozygotes. This may reflect the original recruitment methods favouring consanguineous families for autozygosity mapping studies. However, this trend can be reassessed in the future with the collection of further patient data.

Mutations have previously been reported throughout *CHRNG* the gene, with no observed mutational hot spot (Hoffmann et al., 2006, Morgan et al., 2006). However, recurrent mutations have more recently been identified suggesting previously unrecognised mutational hotspots (Vogt et al., 2012). The exon 5 frameshift mutation c.459dupA;p.Val154SerfsX24 mutation has been reported in the published literature (Morgan et al., 2006) and was identified in six patients from unrelated white families. Escobar-variant and lethal phenotypes were associated with both 459dupA;p.Val154SerfsX24 homozygotes and compound heterozygotes. The c.753_754CT;p.Val253AlafsX44 mutation in exon 7 was detected in four unrelated patients. It occurred in a homozygous state as well as in compound heterozygotes. Homozygous c.753_754CT;p.Val253AlafsX44 mutations were associated with an Escobar variant phenotype in one family and lethal MPS in another. Compound heterozygous c.753_754CT;p.Val253AlafsX44 mutations were identified in two other Escobar families. The homozygous mutation c.715C>T;p.Arg239Cys in exon 7 was reported in two families with lethal and Escobar-variant MPS.

Lethal and non lethal MPS phenotypes occurred in association with four *CHRNG* mutations (c.320T>G;p.Val07Gly, c.459dupA;p.Val154SerfsX24 and c.753_754CT;p.Val253AlafsX44,

c.715C>T;p.Arg239Cys). Although the lethal phenotype was not recorded in associated with mutations 5' to exon 4, this is more likely to be due to the small sample size rather than a positional effect of the mutation.

Five missense variants of unknown pathogenic significance were detected. A homozygous missense variant c.299T>G;p.Leu100Arg was observed in an Escobar patient. However, this family was not available for further study to clarify the significance of this *CHRNG* variant. Four missense variants were determined in the heterozygous state. This may be due either to failure to detect a second *CHRNG* mutation (e.g. an exon deletion), the presence of a mutation in a second as yet unidentified gene, or because the variant is not implicated in MPS.

CHRNG sequence variants have been included in the Locus-Specific database. This may be a useful tool for clinicians and laboratory scientists analysing *CHRNG* sequence information. Information obtained from the pooling of genetic variants for rare conditions can aid with interpretation of patient results. Confirmatory diagnostic genetic testing and *CHRNG* prenatal diagnosis is available for a proportion of families. However, the inter- and intrafamilial phenotypic variability and lack of clear genotype phenotype correlation continues to cause difficulties in genetic counselling. The shared database of *CHRNG* mutations may assist this process for families affected with this group of conditions. It is envisaged that this will be a useful tool for laboratory staff, clinicians and researchers working with patients with this group of conditions. With the transfer of the *CHRNG* testing to the diagnostic genetic testing laboratory service, curating and maintenance of database may be more efficiently managed by the diagnostic service laboratory team in the future.

Chapter 5: Candidate Gene Analysis of *CHRNA1*, *CHRNB1*, *CHRND* and *RAPSN*

Chapter 5: Candidate Gene Analysis of *CHRNA1*, *CHRNB1*, *CHRND* and *RAPSN*

A substantial part of this chapter has been published in Vogt et al., 2008.

5.1 Introduction

Multiple pterygia are occasionally present in children with arthrogryposis and in fetuses with fetal akinesia syndrome (Hall et al., 1982b). The embryonal gamma acetylcholine receptor subunit gene (*CHRNG*) has an important role in prenatal muscle development, thus the detection of *CHRNG* mutations in a significant proportion of patients with lethal MPS patients suggested that pterygia resulted from early onset fetal akinesia. Fetal akinesia deformation sequence syndrome (FADS) is characterised by a variable combination of fetal akinesia, intrauterine growth retardation, developmental defects (e.g. cystic hygroma, lung hypoplasia, cleft palate, cryptorchidism, intestinal malrotation, cardiac defects), arthrogryposis and, in some cases, limb pterygia (Hall et al., 1982b, Hall, 1984). FADS is clinically and genetically heterogeneous. Due to the phenotypic variability observed in the *CHRNG* positive cohort it was hypothesised that mutations in other AChR-related genes predicted to cause prenatal as well as postnatal neuromuscular transmission deficits might also cause fetal akinesia and pterygia. Patients referred for *CHRNG* mutation analysis without pterygia were also included in the analysis.

5.1.1 Selection of candidate genes

A candidate gene approach was used to select genes, mutations in which were likely to result in severe fetal akinesia. From the knowledge of the critical importance of the AChR, other

genes involved in neuromuscular junction transmission were considered. The skeletal muscle nicotinic AChR is a pentameric transmembrane protein that exists in two forms. The adult AChR consists of two alpha and one beta, a delta subunit and an epsilon subunit ($\alpha_2\beta\varepsilon\delta$) whereas the embryonic AChR has two alpha and one beta, one delta and a gamma subunit, ($\alpha_2\beta\gamma\delta$) (Mishina et al., 1986, van der Slot et al., 2003, Noda et al., 1983, Shibahara et al., 1985). As our patients had features of fetal akinesia, genes encoding *CHRNA1, CHRNB1, CHRND* prenatally expressed components of the embryonic AChR were selected for investigation. *RAPSN* was also included because it encodes a post synaptic protein involved in the development of specialised post-synaptic neuromuscular junction structures and has an essential role in AChR clustering (Sanes and Lichtman, 2001).

5.2 Results

Fifteen probands with no evidence of a germline *CHRNG* mutation were selected for mutation analysis of the coding sequence and flanking intronic sequence of *CHRNA1, CHRNB1, CHRND* and *RAPSN*. The clinical features of the 15 families are summarised in Table 8.

Family ID	Ethnic Origin	Parental Consanguinity	Phenotype	*RAPSN* Sequence Variant	*CHRND* Sequence Variant
MPS013	Pakistani	Yes	LMPS		
MPS012	Bengali	Yes	AMC, no pterygia		
MPS014	White	No	LMPS		
MPS016	White	No	LMPS		
MPS017	White	No	LMPS		IVS8-37G>A het
MPS018	White	No	LMPS		IVS8-37G>A het
MPS019	Indian	Yes	LMPS		
MPS020	White	No	LMPS		
MPS021	White	No	LMPS		
MPS022	Afrikaner	No	LMPS	IVS2-15C>T het	
MPS023	White	No	LMPS		
MPS010	Pakistani	Yes	AMC, no pterygia		
MPS009	Pakistani	Yes	LMPS		
MPS024	Palestinian	Yes	LMPS		
MPS025	Ethiopian	No	LMPS		

Table 8: Clinical features and sequence variants detected in *RAPSN* and *CHRND*.

Sequence variants were not detected in the acetylcholine receptor subunit genes *CHRNA1* or *CHRNB1*.

No pathogenic mutations were detected in *CHRNA1*, *CHRNB1* or *CHRND*. However, a *RAPSN* frameshift mutation was detected in a consanguineous family with three affected children (Figure 15). Both parents were heterozygous for a C.1177_1178delAA mutation and all three affected children were homozygous for the mutation.

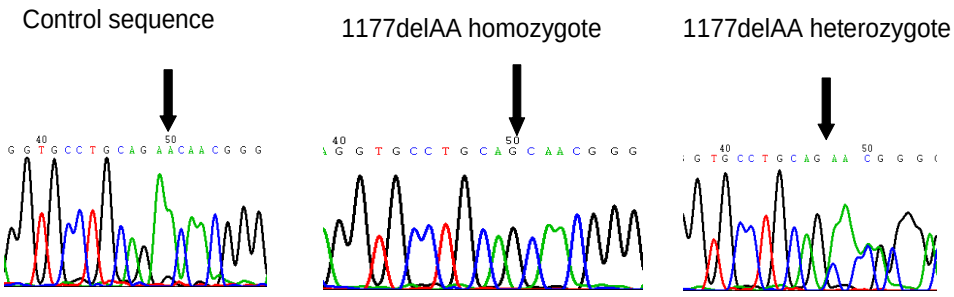
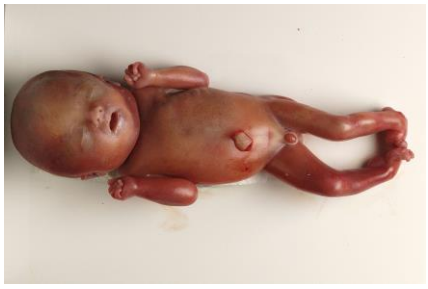


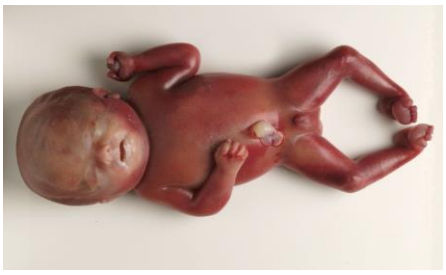
Figure 15: Sequence traces for normal control, homozygote and heterozygote carrier of *RAPSN* frameshift mutation.

Review of the clinical records revealed that the consanguineous Dutch couple presented when evidence of the fetal akinesia sequence was detected at 19 weeks of gestation in twin male fetuses. On ultrasound examination both fetuses had features of the fetal akinesia deformation sequence. They had joint contractures affecting the hands, elbows and feet and micrognathia. There were no respiratory movements and the thorax appeared small. There was mild hydrops. The pregnancy was terminated at 23 weeks of gestation. Post mortem examination revealed monochorionic, monoamniotic twins with no evidence of growth retardation [Twin 1 0.587 kg (~50 centile) and Twin 2: 0.665 kg (>50 percentile)]. They had mild hydrops with hydrothoraces and subcutaneous oedema of the head, neck, back of the shoulders and proximal extremities. Both twins appeared dysmorphic with a flat facial profile, hypertelorism, moderate micrognathia and a short broad neck. The lungs were hypoplastic

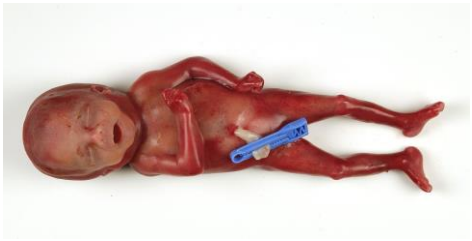
and the diaphragm was thin and membranous. Both twins had hyperextended wrists and flexed fingers. The ankles were fixed in a varus position and the toes hyperextended. No pterygia were detected. The couple had a further similarly affected female pregnancy. Fetal akinesia sequence was detected on ultrasound at around 19 weeks of gestation and the pregnancy was terminated at 23 weeks of gestation. On post mortem examination the fetus had subcutaneous oedema of the head, neck and shoulders. Facially there was micrognathia, low set ears, a short nose, a short philtrum, thin lips and a short broad neck. The elbows were flexed and the wrists hyperextended. There was overlapping of the second and third fingers on both sides. No pterygia were identified. The lungs were hypoplastic (Figure 16).



(i)



(ii)



(iii)

Figure 16: Clinical presentation of fetal akinesia defomation sequence in siblings

Homozygous *RAPSN* mutations (i) and (ii) affected male twins and (iii) affected singleton fetus.

Neuromuscular histopathological examination failed to identify a specific underlying cause for the fetal akinesia. The brain and spinal cord were of normal morphology on macroscopic and microscopic examination. The muscle histopathology revealed muscle fibres of varying

diameters (considered to be normal for the gestational age) with a normal striated pattern and no fibrosis in the male twins. The muscle fibres were of normal diameter for gestational age in the female singleton. No abnormalities were detected on enzyme analysis. On electron microscopy the striated muscle appeared well developed. All three cases showed occasional muscle fibres with centrally located nuclei, but this appearance was observed in <5% of the muscle fibres examined and was not diagnostic of a myotubular myopathy. Chromosome analysis was normal and molecular testing for congenital myotonic dystrophy was negative. The couple also have two healthy children.

The c.1177-1178delAA mutation was predicted to cause a frameshift after residue 392 of rapsyn resulting in a mutant rapsyn that is 62 amino acids longer than the wild type (Figure 17 and 18). Functional analysis of the *RAPSN* mutation associated with fetal akinesia sequence below was undertaken by Professor Beeson's team (Hayley Spearman and Judy Cossins), as published in the clinical report by (Vogt et al., 2008).

Figure 17: DNA sequences and translations of exon 8 for wild-type and mutant human rapsyn.

In the wild-type sequence, the two nucleotides that are deleted in the mutant are shown in red. As a consequence of the frameshift, mutant rapsyn is 62 amino acid residues longer than wild-type (asterisk indicates termination codon).

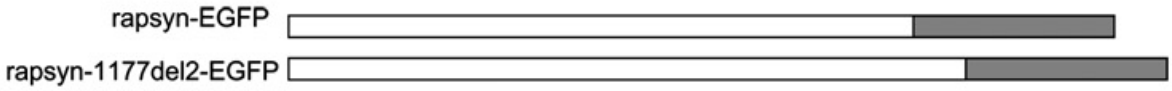


Figure 18: Diagram of wild-type and mutant rapsyn tagged with EGFP at the carboxyl terminus. Wild-type or mutant rapsyn cDNA without a stop codon were cloned into pEGFP-N1 (Clontech) so that EGFP was in-frame with rapsyn. Mutagenesis to delete the two adenine residues was carried out with the QuikChange mutagenesis kit purchased from Stratagene and was confirmed by DNA sequencing. The frameshift in the c.1177-1178delAA mutant extends the length of the protein by 62 amino acid residues, and so the corresponding 30UTR was included in this construct.

Professor Beeson's group tagged wild-type and mutant rapsyn with EGFP in-frame at their C termini and cotransfected with cDNAs encoding the human AChR into the muscle cell line TE671. TE671 cells were maintained at 37_C in DMEM (Sigma-Aldrich) supplemented with 10% FCS (TCS Cellworks) and 100 units/ml each of penicillin G and streptomycin (PS) purchased from Invitrogen. TE671 cells do not express detectable levels of endogenous rapsyn polypeptide. After transfection, effects on AChR clustering were studied. Thus, TE671 cells were seeded at 2.3 x 10^5 cells per well on coverslips in 6-well plates and the following day were transfected with a total of 3 mg DNA per well via calcium phosphate precipitation. Amounts of DNA used per well was 1 mg AChR α-subunit DNA, 0.5 mg each of AChR β-, δ- and ε-subunit cDNA, and 0.5 mg of rapsyn cDNA or pcDNA3.1-hygro for ''no rapsyn'' control transfections. Transfections with only rapsyn and no AChR were carried out with 3 mg/well of rapsyn expression plasmids. The next day, the media was replaced with fresh medium. Whereas transfection with wild-type rapsyn resulted in the colocalization of rapsyn

and AChR on the cell surface in small dense clusters, transfection with rapsyn-392+82-EGFP resulted in low levels of EGFP fluorescence within the cells and unclustered AChR expressed uniformly along the cell surface (Figure 19).

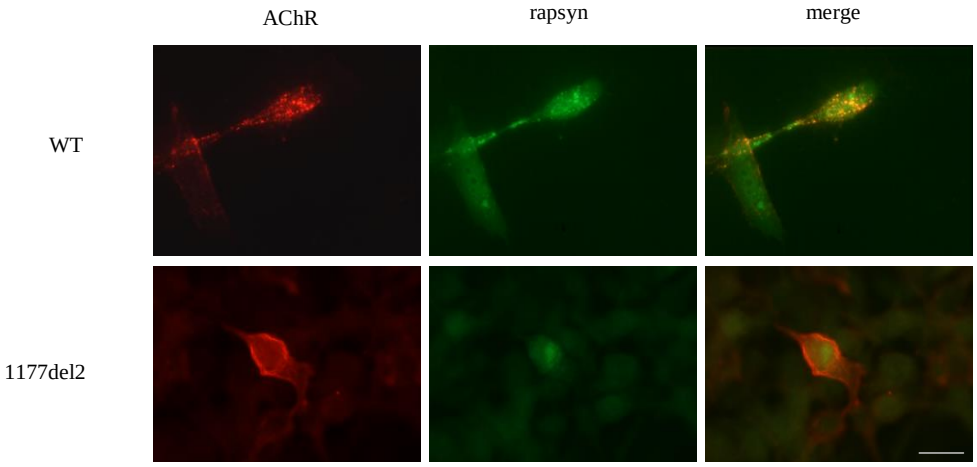
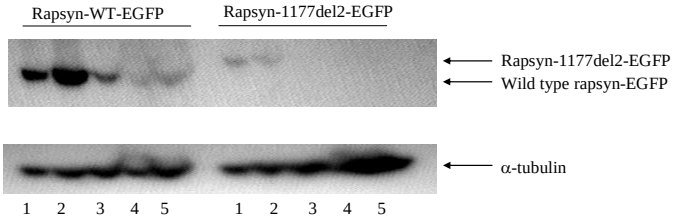


Figure 19: Rapsyn mutation c.1177-1178delAA does not cluster the AChR.
TE671 cells were cotransfected with cDNAs encoding rapsyn-EGFP or rapsyn-1177delAA-EGFP and the human AChR subunits. AChR was detected with mAb B3 directed against the AChR β subunit. 28 Cells were fixed with 3% paraformaldeyde at room temperature for 20 min, washed three times with PBS, and incubated with secondary antibody Alexa Fluor 594 goat anti-mouse IgG (HþL) diluted 1:1000 in PBS containing 1% BSA (Molecular Probes). Cells were washed 33 in PBS and mounted in fluorescent mounting media (Dako Cytomation). Microscopy was performed on an Olympus BX60 wide-field fluorescence microscope, and images were captured with Openlab software (Improvision).

To examine this observation further, a time course of rapsyn expression was performed following transfection by western blots on cell extracts. Confirming the observation from

fluorescence microscopy the mutant rapsyn was present at much lower levels and by three days post-transfection could not be detected (Figure 20 and 21). The results suggest that the mutant rapsyn is rapidly degraded. It is likely that in the patient fetal AChR are not clustered due to the loss of rapsyn, which results in the disruption of the neuromuscular synapse formation and neuromuscular transmission, which in turn results in severe fetal akinesia.

(Figure 20)



(Figure 21)

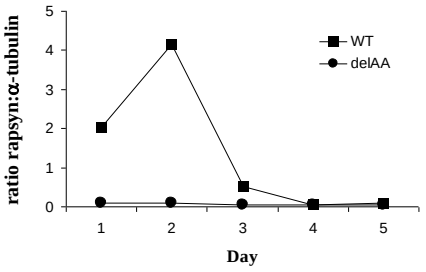


Figure 20: Western blot of rapsyn-EGFP and rapsyn-1177delAA-EGFP expressed in TE671 muscle cells. TE671 cells were transfected with wild-type or mutant rapsyn-EGFP, and 48 hr later, total cell lysate was analysed by Western blotting. Rapsyn was detected by mAb clone 1234 (Abcam) followed by anti-mouse-HRP and ECL (Amersham). As a control, a-tubulin was detected on the same western blots with a mAb (Sigma-Aldrich) followed by anti-mouse-HRP and ECL. The experiment was performed twice; one example is shown.

Figure 21:The ratio of rapsyn:a-tubulin was obtained by densitometric scanning of western blots. Whereas rapsyn-EGFP gave robust expression on days 1 and 2, rapsyn-1177delAA-EGFP was barely detectable throughout the time course.

5.3 Discussion

After identifying that mutations in the *CHRNG* gene that encodes the gamma subunit of the embryonal acetylcholine receptor may cause the non-lethal Escobar variant (EVMPS) or the lethal form (LMPS) of multiple pterygium syndrome (MPS), it was also considered that *CHRNG* mutations and mutations in other components of the embryonal acetylcholine receptor may present with fetal akinesia deformation sequence (FADS) without pterygia.

Mutations in AChR subunits have previously been associated with the congenital myasthenic syndromes (CMS). These are a heterogeneous group of inherited neuromuscular transmission disorders characterised by fatiguable muscle weakness. CMS can be caused by mutations in *CHRNA1, CHRNB1, CHRND* and *CHRNE,* as altered AChR channel kinetics or severely reduced AChR in the postsynaptic membrane results in AChR deficiency (Engel et al., 2003, Jurkat-Rott and Lehmann-Horn, 2005). Mutations in *CHRNE,* the postnatally expressed subunit of the AChR, most commonly result in a mild phenotype, whereas AChR deficiency mutations in the prenatally expressed subunits *CHRNA1, CHRNB1* and *CHRND* are rare and cause a severe phenotype. Although the clinical presentation of CMS and MPS are distinct, two siblings with a fast channel CMS due to heteroallelic mutations in *CHRND* had arthrogryposis (Brownlow et al., 2001). One sibling had reduced fetal movements in utero. At birth he was noted to have joint contractures in his hands and bilateral ptosis. He died at the age of 5 months from bronchopneumonia. His sister had also reduced in fetal movements and was noted to have flexion contractures of the interphalangeal joints at birth. She was floppy with a weak cry and feeding difficulties. She had recurrent severe respiratory compromise. Neurophysiology was consistent with CMS and there was improvement in strength with

pyridostigmine treatment. Genetic analysis identified the presence of two pathogeneic compound heterozygous *CHRND* mutations (Brownlow et al., 2001).

CMS can also result from mutations in other postsynaptic proteins involved in neuromuscular transmission or in the formation and maintenance of the neuromuscular junction. Recessive germline mutations in *RAPSN* have been reported in patients with CMS and AChR deficiency, (Ohno et al., 2002, Burke et al., 2003, Maselli et al., 2003, Muller et al., 2003). *RAPSN* has been mapped to chromosome 11p11.2p11.2 and contains 8 exons (Buckel et al., 1996). The *RAPSN* gene product (Rapsyn; receptor-associated protein of the synapse) has a key role in the clustering of AChRs at the neuromuscular synapse (Apel et al., 1995).

Rapsyn is a 43kDa complex protein. It is found at the postsynaptic membrane of the neuromuscular junction of skeletal muscle in association with cytoplasmic, transmembrane anchoring proteins and signalling molecules. A major feature of the neuromuscular junction is the presence of a high density of neurotransmitter receptors due in part to myonuclei at the synapse selectively transcribing AChR genes, and partly because of the clustering of the AChRs (Burden, 1998, Sanes and Lichtman, 1999). A high density of AChRs is critically important for transmission of a nerve impulse across the neuromuscular junction. Rapsyn and another protein, agrin, activate a muscle-specific kinase (MuSK) and have been shown to be essential for AChR clustering at the neuromuscular junction.

Although the tertiary protein structure of rapsyn is unknown, the structural domains of rapsyn have been shown to have distinct functions. The primary structure of rapsyn contains a consensus sequence for N-myristoylation. Fatty acid modification of the N-terminus has been

shown to enable rapsyn to be localised to the plasma membrane. In addition the 10 amino acids at the N-terminus are conserved across the species, suggesting this region may be important in protein interactions. Rapsyn contains 7 tetratricopeptide repeats (TPRs) (Figure 22). These are 34-amino acid alpha helical repeats believed to be involved in protein-protein interactions. A minimum of two TPRs are required for self-association to take place. Self-association of rapsyn enables the AChR to be linked to the cytoskeleton (Ramarao and Cohen, 1998).

The coiled-coil rapsyn domain is required for AChR clustering and rapsyn binds the long cytoplasmic loop of the AChR through the coiled-coil domain (Huebsch and Maimone, 2003). Cytoplasmic AChR domains are positioned close to rapsyn (Burden, 1998). Clustering of the AChRs appears to occur through the interaction of the coiled-coil domain with the cytoplasmic loop of either the beta subunit of the AChR (Bartoli et al., 2001), the alpha subunit of the AChR (Maimone and Enigk, 1999) or the delta subunit of the AChR (Muller et al., 2006). The Ring-H2 domain at the cysteine-rich COOH-terminus of rapsyn has not been shown to be necessary for rapsyn self-association or AChR clustering. However, the Ring-H2 domain interacts with the cytoplasmic tail of beta-dystroglycan resulting in dystroglycan clustering, providing a link between the extracellular matrix and the cytoskeleton. This is believed to be important in the organisation of the post synaptic membrane (Bartoli et al., 2001, Cartaud et al., 1998). The ability of rapsyn to cluster dystrophin in the absence of AChRs suggests it may stabilize AChR clustering by connecting the receptors to the cytoskeleton anchored dystrophin-glycoprotein complex at the neuromuscular junction (Lin et al., 2001). Agrin also plays an important role in maintaining the postsynaptic architecture (Apel et al., 1995). The amino acid 361-412 motif is found in other transcription factors

localised to the nucleus. It has been suggested that in the absence of a membrane-targeting signal, interaction with other nuclear associated proteins may localise it to the nucleus. It has also been suggested that this domain may have a role in determining the size of the AChR-rapsyn clusters (Ramarao et al., 2001).

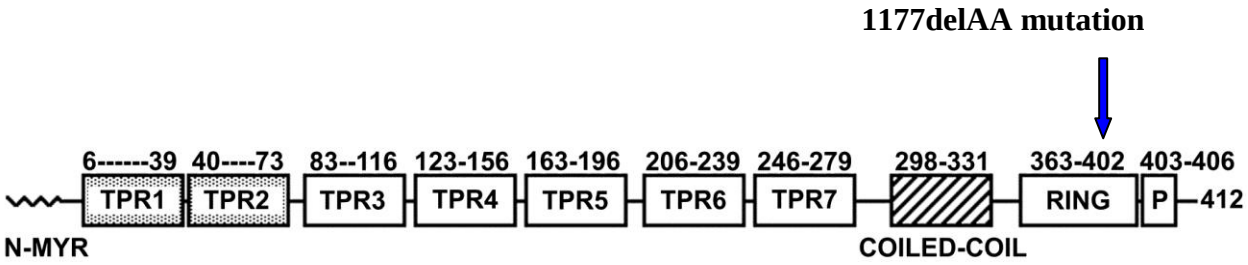


Figure 22: The structure of rapsyn illustrating the position of the c.1177delAA mutation.

Schematic diagram adapted from Ramarao et al., 2001.

The importance of rapsyn and a muscle-specific kinase (MuSK) for the initiation of postsynaptic differentiation has been demonstrated in transgenic mice. In mouse models, transgenic mice with targeted disruption of the rapsyn gene maintained synapse specific transcription of AChR genes. However, no detectable AChR clusters were seen, indicating rapsyn was essential for AChR aggregation at the neuromuscular junction. These mice had muscle weakness, severe respiratory distress, and died within hours after birth (Gautam et al., 1995).

Genotype-phenotype correlations have been described for CMS-associated *RAPSN* mutations, such that individuals who are homozygous for the most frequent *RAPSN* mutation (p.N88K missense mutation) associated with CMS (Muller et al., 2003) have less severe disease than compound heterozygotes with a single p.N88K allele (Dunne and Maselli, 2003). Previously

it was reported that the rapsyn missense mutation p.N88K showed a partial loss of function with reduced, less stable agrin-induced AChR clusters than wild type rapsyn (Cossins et al., 2006). Thus p.N88K may cause a less severe defect than mutations that produce a truncated protein or altered membrane attachment. Homozygosity (or compound heterozygosity) for *RAPSN* mutations predicted to cause a truncated protein have not been described in CMS to date. The c.1177_1178delAA mutation identified in our family has not been observed in samples from 340 control chromosomes (140 in the current study and 200 reported previously). It has previously been reported (in combination with the p.N88K allele) in patients with CMS (Burke et al., 2003). Affected patients had decreased fetal movements, flexion contractures, craniofacial anomalies, hypotonia and intermittent respiratory failure (Burke et al., 2003, Banwell et al., 2004). The c.1177_1178delAA frameshift mutation was predicted to abort the stop codon at residue 392 in the Ring-H2 domain extending the protein by 62 C-terminal missense amino acids. It was anticipated that the larger rapsyn molecule may be sequestered in the endoplasmic reticulum during protein trafficking or that the altered protein structure may impair interaction with other proteins resulting in reduced AChRs at the neuromuscular junction. Thus it was suspected that the c.1177_1178delAA mutation caused a more severe deficit in rapsyn function than p.N88K and so homozygosity for c.1177_1178delAA caused a fetal akinesia rather than a CMS phenotype. This was supported by the studies of the c.1177_1178delAA mutation on rapsyn function undertaken by Professor Beeson's team, which showed that homozygosity for this frameshift *RAPSN* mutation severely impaired protein stability (Vogt et al., 2008).

Thus *RAPSN* mutations may cause a spectrum of phenotypes ranging from later onset (third decade) CMS with "mild mutations" through neonatal CMS with arthrogryposis, to fetal

akinesia with lethality in the presence of homozygous severe truncating mutations (Burke et al., 2004) . Although the development of neuromuscular junction differs in mice and humans (in mice the switch from fetal to adult acetylcholine receptors takes place in the first two weeks of life, but in humans the switch occurs earlier and is apparently complete by 31 weeks gestation), (Kues et al., 1995, Hesselmans et al., 1993), the phenotype of Chrng and Rapsn gene inactivation in mice are similar. Homozygous mutant transgenic mice with targeted disruption of the rapsyn gene died within hours of birth with respiratory insufficiency and profound muscle weakness (Gautam et al., 1995). These mice showed no detectable AChR clusters along the length of muscle fibres. When these findings are considered together with *CHRNG* mutations, functional AChR deficiency will be a significant cause of MPS/fetal akinesia (Morgan et al., 2006, Hoffmann et al., 2006).

Although germline mutations in *CHRNA1*, *CHRNB1* and *CHRND* were not identified in our cohort of patients, mutations in *CHRNA1* and *CHRND* have been identified in MPS patients (Michalk et al., 2008), supporting the role of the various AChR genes in disorders of the neuromuscular junction (NMJ).

Chapter 6: Candidate Gene Analysis *DOK7*

6 Candidate Gene Analysis *DOK7*

A substantial part of this chapter has been published in Vogt et al., 2009.

6.1 Introduction

Germline mutations in *CHRNG*, encoding the fetal gamma subunit of the acetylcholine receptor (AChR) have been reported to account for ~30% of lethal and non-lethal (Escobar variant) multiple pterygium syndrome (LMPS and EVMPS) (Morgan et al., 2006, Michalk et al., 2008, Hoffmann et al., 2006). Subsequently mutations in genes that encode other fetal AChR subunits (*CHRNA1*, *CHRND*) or the crucial AChR clustering protein rapsyn (*RAPSN*) have been detected in FADS/LMPS (Vogt et al., 2008, Michalk et al., 2008). Mutations in *CHRNA1*, *CHRND*, and *RAPSN* have also been described in congenital myasthenia syndrome (CMS), a disorder that is characterised by muscle fatigability and, rarely, arthrogryposis (Brownlow et al., 2001, Burke et al., 2003, Beeson et al., 2005). Thus it was hypothesised that mutations in *DOK7*, a gene that has been implicated in CMS and fetal AChR function (Selcen et al., 2008), might also cause a FADS/LMPS phenotype.

6.2 Results

Fourteen families with features of FADS/LMPS (see Table 9) and no evidence of *CHRNG*, *CHRNA1*, *CHRNB1*, *CHRND* and *RAPSN* mutations were examined for germline mutations in the exons and flanking intronic sequences of *DOK7* by direct sequencing on an ABI 3730 DNA Analyser.

Family ID	Ethnic Origin	Parental Consanguinity	Phenotype
MPS012	Bengali	Yes	No pterygia
MPS013	Pakistani	Yes	LMPS
MPS016	White	No	LMPS
MPS017	White	No	LMPS
MPS019	Indian	No	LMPS
MPS020	White	No	LMPS
MPS021	White	Yes	LMPS
MPS022	Afrikaner	No	LMPS
MPS023	White	No	LMPS
MPS027	Afrikaner	No	LMPS
MPS028	Afro Caribbean	No	LMPS
MPS010	Pakistani	Yes	No pterygia
MPS009	Pakistani	Yes	LMPS
MPS029	Turkish	Yes	LMPS

Table 9: 14 families with FADS/LMPS.

These patients had no evidence of *CHRNG, CHRNA1, CHRNB1, CHRND* and *RAPSN* mutations and were examined for germline mutations in the exons and flanking intronic sequences of *DOK.*

A homozygous *DOK7* splice site mutation (c.331+1G>T) was identified in a consanguineous Bengali family with three children affected with the fetal akinesia deformation sequence (Figure 23). Both parents and the two unaffected siblings were heterozygous for the mutation. The mutation was not detected in 378 ethnically matched control chromosomes. Because this nucleotide substitution affects the consensus donor splice site motif, it is likely to abolish correct RNA splicing and an *in silico* splice site prediction programme (http://www.fruitfly.org/seq_tools/splice.html) suggested that the consensus donor splice site was lost in the mutant sequence. It was not possible to directly examine the effect of the

mutation on RNA splicing. This is because there was no fetal tissue available from which RNA could be extracted and I was unable to detect *DOK7* expression on the cDNA PCR studies undertaken on blood. However, this mutation was predicted to result in exon 3 skipping of an in frame deletion of 77 amino acids from the PH domain of *DOK7*. This in frame skipping of exon 3 has previously been confirmed and it had been shown that Dok-7 harbouring this mutation impairs MuSK activation in cultured cells (Selcen et al., 2008).

We postulated that the c.331+1G>T mutation caused a severe deficit in Dok-7 function resulting in fetal akinesia, rather than a CMS.

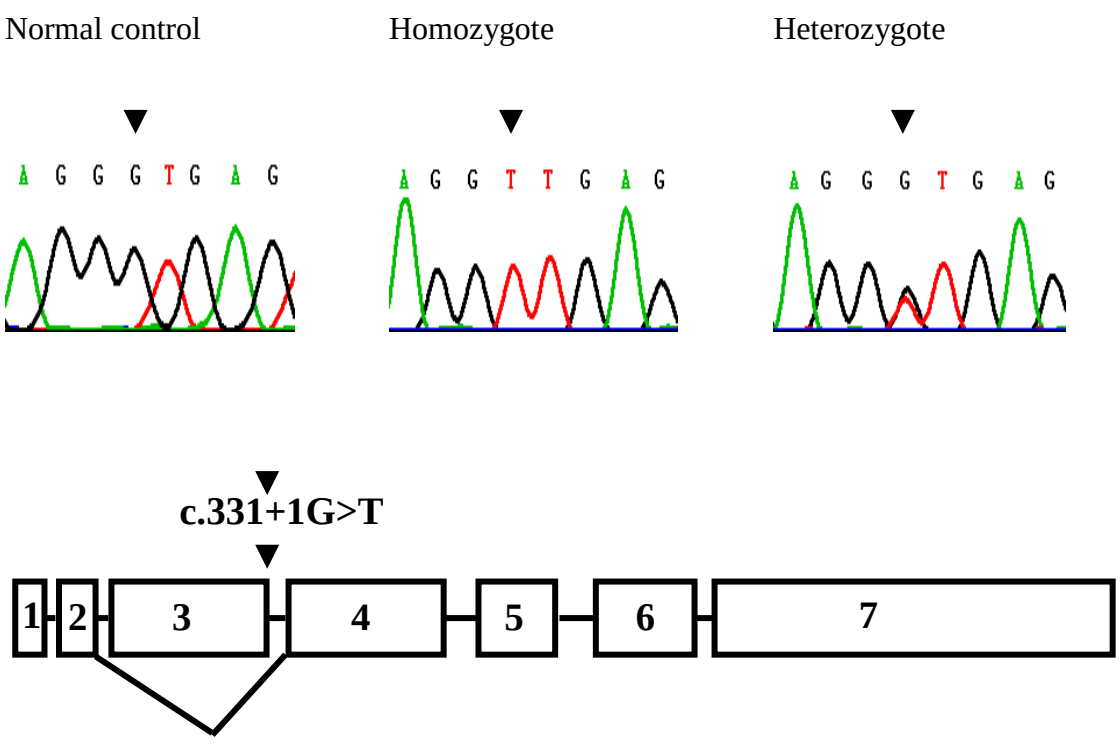


Figure 23: Sequence traces for normal control, homozygote and heterozygote carrier of *DOK7* splicesite mutation (c.331+1G>T).

Review of the clinical records revealed that the first fetus was stillborn at 32 weeks gestation and had signs of a neuromuscular developmental abnormality. The second affected fetus miscarried spontaneously at 22 weeks of gestation. Post mortem examination revealed that the fetus had downslanting palpebral fissures, a small jaw and a short neck. The limbs had extended extremities, overlapping fingers, normal palmar creases, bilateral talipes and rockerbottom feet with reduced muscle bulk. Bilateral hydrothoraces and severe generalised oedema was present. Histopathological examination detected features of a muscle denervation type lesion. Thus the muscle appeared immature, with irregularly shaped muscle cells, and there was a disparity in the slow to fast muscle density with patchy foci of slow fibres and atrophy of the fast fibres, Figure 24 (i) and (ii). The third affected fetus was detected by ultrasonography at 24 weeks of gestation when no fetal movements were observed. None of the affected fetuses had evidence of pterygia.

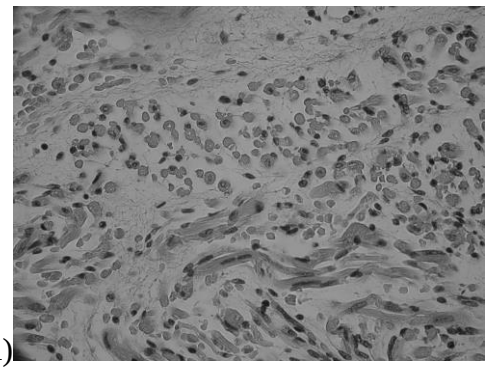
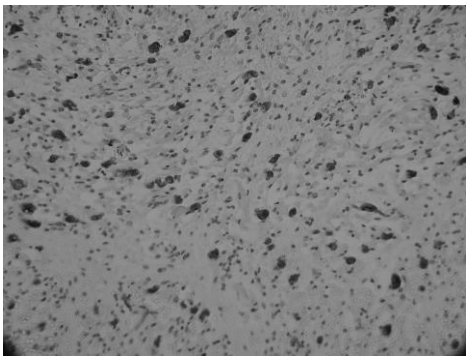
(i)  (ii) 

Figure 24: (i) and (ii). Immature muscle with irregularly shaped muscle cells.
There was a disparity in slow to fast muscle fibre density with patchy foci of slow fibres and atrophy of fast fibres, considered to be compatible with a neuromuscular disease.

6.3 Discussion

DOK7 (Downstream of kinase) is a seven exon gene located on chromosome 4. It encodes a 55-kD AChR clustering protein comprised of 504 amino acids (Okada et al., 2006). Like the other Dok family proteins Dok-7 contains a pleckstrin homology (PH) and phosphotyrosine binding (PTB) domain in the N-terminal region and Src homology 2 (SH2) domain target motifs in the C-terminal portion (Figure 25).

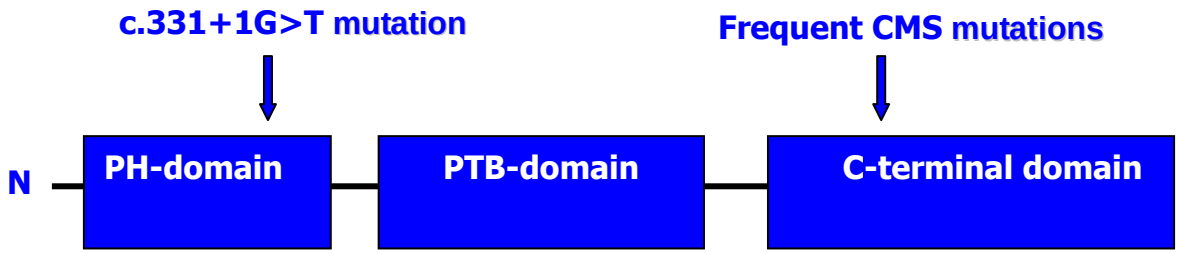


Figure 25: Structure of Dok-7 protein.

In C2C12 mouse myotubes Dok-7 binds to the MuSK phosphotyrosine binding domain target motif through its PTB domain and induces the tyrosine phosphorylation of MuSK, This results in numerous differentiated AChR clusters and co-clustering of Dok-7 with the AChR (Okada et al. 2006), Figure 26 below.

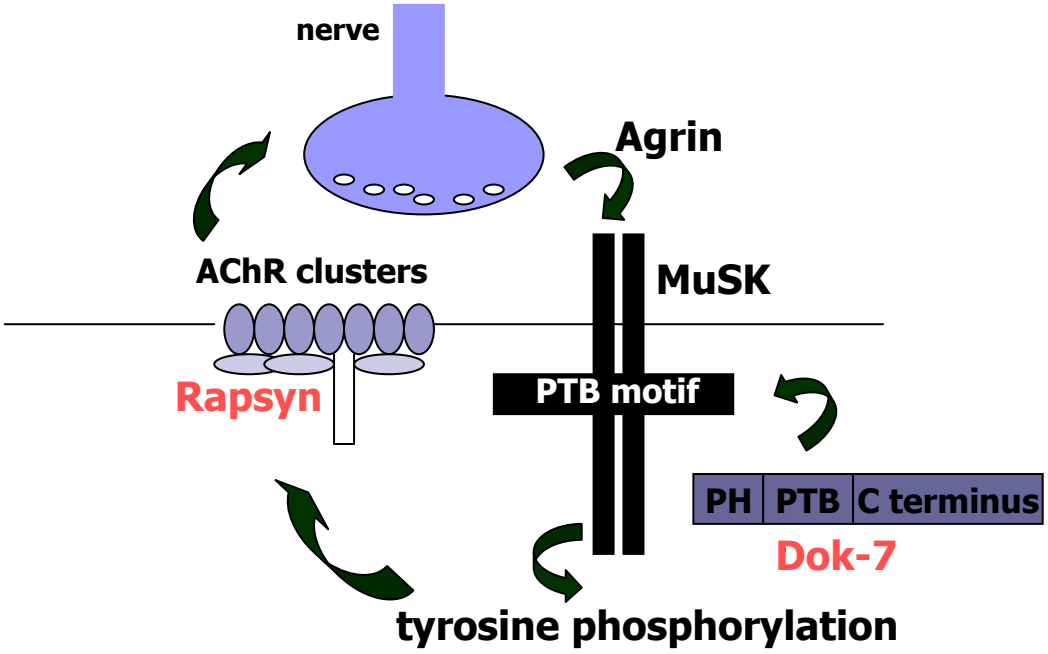


Figure 26: Role of Dok-7 in AChR clustering.

(Adapted from Palace et al., 2007).

Homozygous *DOK7* mutations have previously been detected in autosomal recessive congenital myasthenic syndrome (CMS) (Beeson et al., 2006, Muller et al., 2007, Palace et al., 2007), where they have been associated with small neuromuscular junctions, reduced postsynaptic folding but normal AChR density and function (Slater et al., 2006). Patients with *DOK7* mutations, almost always have at least one allele with a mutation, commonly the frameshift mutation 1124_1127dupTGCC, in exon 7 of the C-terminal region. CMS compound heterozygotes with the recurrent exon 7 mutation and a N-terminal mutation either in the PH or PTB domain have infrequently reported with early childhood onset limb girdle CMS (Palace et al., 2007). However severe homozygous or compound heterozygous mutations in the PH region of *DOK7* have not been described in CMS. Truncating mutations in the C terminal domain of Dok-7 result in an impaired ability to induce the phosphorylation of MuSK and to form the specialization of the postsynaptic structures (Beeson et al., 2006).

CMS patients homozygous for these mutations do not usually present at birth but in early childhood. This may suggest that there is little effect on initial synapse formation but impaired maturation and maintenance of synaptic structures (Beeson et al., 2006).

Although variable in severity, CMS patients with *DOK7* mutations typically have a limb girdle pattern of muscle weakness with the muscles of the limbs and trunk mainly affected. Facial and bulbar weakness is frequent, ptosis is common and eye movements are usually unaffected. Although respiratory impairment may be present from early life, *DOK7* mutations are more frequently associated with progressive respiratory deterioration over time necessitating nocturnal ventilation. Congenital joint contractures seen in CMS patients with *RAPSN* mutations are not a feature of *DOK7*-associated CMS (Palace et al, 2008).
The severe homozygous intron 3 splice site mutation in the PH domain was lethal in uterine life in the affected fetuses. This may suggest an effect on early synaptic development and severe disruption of Dok -7 function. It is possible that the mutation in the PH region may interfere with the docking of other signal transducing molecules or other neuromuscular junction proteins in the neuromuscular junction. Although the development of neuromuscular junction differs in mice and humans, the phenotype of *Chrng*, *Rapsn* and *Dok7* knockout mice are similar. Thus *Dok7* deficient mice were unable to breathe or move and died shortly after birth, suggesting a severe neuromuscular transmission defect, whereas their heterozygous littermates appeared normal. The *Dok7* deficient mice had no detectable AChR clusters in the endplate region of the diaphragmatic muscle, consistent with Dok-7 having a critical role in neuromuscular synaptogenesis (Ohno et al., 2002). This is consistent with the hypothesis that incomplete loss of Dok-7 function causes a CMS phenotype whereas complete loss of function is lethal.

The identification of germline mutations in various AChR neuromuscular junction (NMJ) genes supports their role in the aetiology in MPS / FADS. Mutations in genes only expressed in fetal life result in a severe in utero phenotype of LMPS or FADS whereas mutations occurring in genes expressed late in gestation or postnatally cause a postnatal onset CMS. Mutations in genes expressed throughout pre and postnatal life can cause both MPS / FADS and CMS as summarised in Table 10 below.

Gene	MPS / FADS	Congenital Myasthenic Syndrome
CHRNG	✓	×
CHRNE	×	✓
CHRNA1	✓	✓
CHRND	✓	✓
RAPSN	✓	✓
DOK7	✓	✓

Table 10: Phenotypic variability and allelic heterogeneity in inherited NMJ disease.

Based on the current understanding of the underlying molecular pathology, a molecular genetic diagnostic pathway was designed for the investigation of MPS/FADS. It was proposed that *CHRNG* mutation analysis should be offered to families with likely autosomal recessive EVMPS, as the detection rate was estimated to be in the order of one quarter. In the

LMPS/FADS families where central nervous system malformations were present, the detection rate for the acetylcholine receptor pathway genes was considered likely to be low, and therefore an alternative pathology should be considered. For LMPS/FADS patients with pterygia, *CHRNG* mutation analysis would be recommended followed by *CHRNA1* and *CHRND* if negative. In the absence of pterygia, *RAPSN* and *DOK7* mutation analysis may be pursued before *CHRNG, CHRNA1* and *CHRND* (Figure 27).

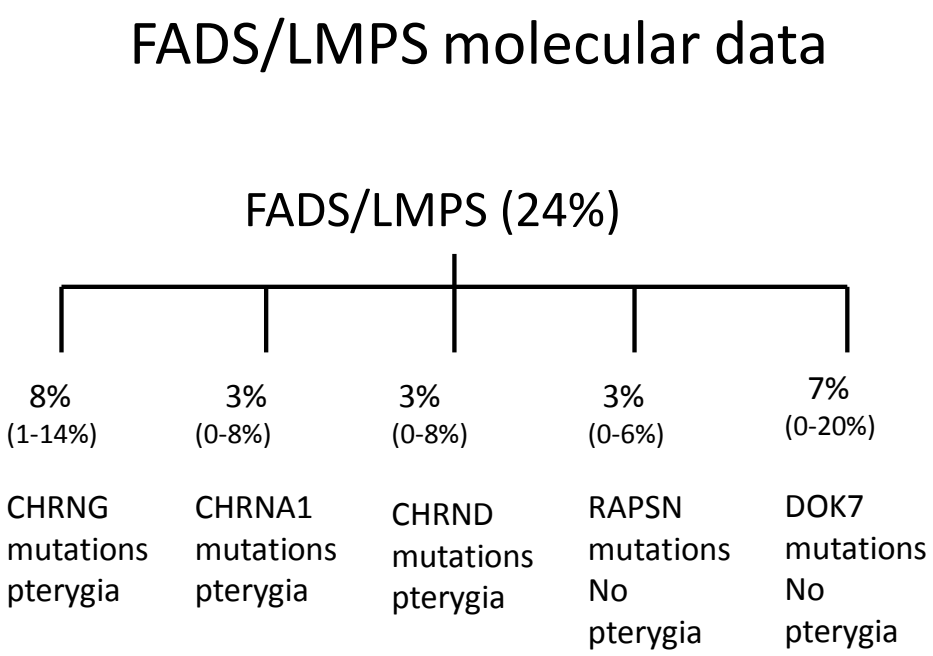


Figure 27: Molecular Genetic Data for LMPS / FADS.

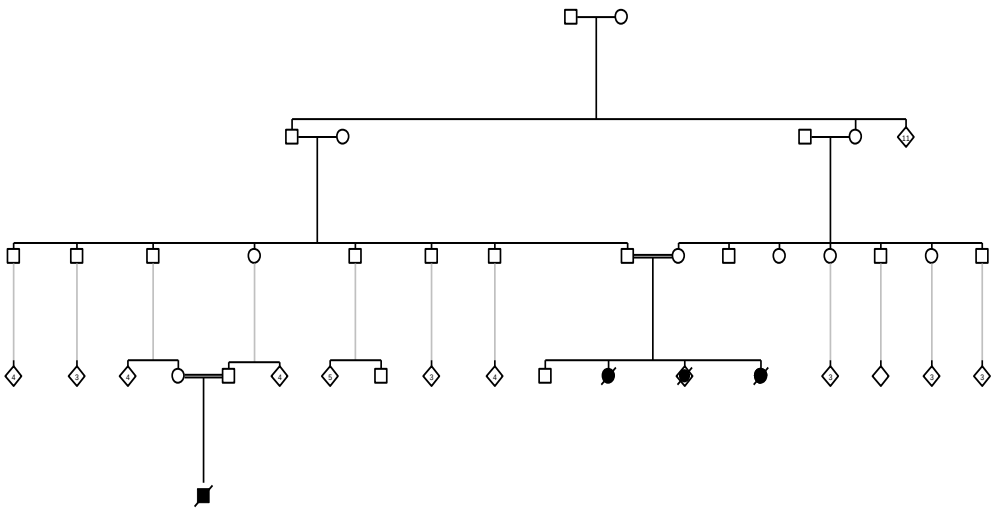
This figure shows the relative contribution of mutations in the AChR genes and denotes the presence or absence of pterygia. Note the wide confidence intervals due to small number of data points in some cases, (Vogt et al., 2012).

The FADS/LMPS mutation detection rate for each individual gene is not high however this is likely to improve with the identification of other molecular causes of these conditions in the future.

Chapter 7: Mapping New Genes

7.1 Introduction

In the multiple pterygium syndrome (MPS) / fetal akinesia deformation sequence (FADS) cohort there were many families for which the underlying molecular cause had not been elucidated despite screening for mutations in the known candidate genes. Families were assessed for their suitability for other gene mapping techniques. Families selected for possible further study included those with a well-defined phenotype, a history of consanguinity, multiple affected relatives including cousins rather than solely within sibships and availability of DNA samples from the affected individuals as well as other family members. The family below was selected for further study:



II.5 II.1 II.2 II.3 II.4

Family branch b Family branch a

Figure 28: Affected children in two branches of a consanguineous family.

7.1.1 Clinical Details

The consanguineous Pakistani family above (Figure 28) was referred by Dr Geoff Woods, for autozygosity mapping work. There were three affected pregnancies in two branches of the family. All the affected children had a similar phenotype with reduced fetal movements evident by 20 weeks of pregnancy, bilateral fixed talipes and fixed flexion of the arms across the chest. At birth they had severe multiple contractures with pterygia of the knees and elbows. All the affected children that had been examined had a small u-shaped midline cleft palate and renal pelviceal dilatation. One of the children that survived for several months had urinary reflux and recurrent urinary infections. This child was physically delayed and had severe intellectual disability. Microcephaly was a feature however intracranial imaging was normal.

Family branch a

First pregnancy was a healthy son. The second pregnancy was an affected female with congenital arthrogryposis, pterygia of the elbows and knees, a cleft palate and renal pelvic dilatation. The third pregnancy was interrupted because it was affected. The fourth pregnancy was an affected female born with severe congenital arthrogryposis, multiple pterygia, a cleft palate and renal pelvic dilatation. She had severe developmental delay and was not very responsive.

The family later went on to have a fifth pregnancy in which there was polyhydramnios and a reduced βHCG level. There was increased nuchal translucency, bilateral flexion contractures at the elbows but more arm movements than in previous pregnancies. The affected female was born post term with a birth weight of 7lb. She was stable in the neonatal period and could

bottle feed in the first year. However, she developed recurrent chest infections and was diagnosed with gastroesophageal reflux and aspiration pneumonia. She underwent a Nissen fundoplication and an insertion of a gastrostomy feeding tube. She has been gaining weight. She has had no seizures but frequent jittery episodes. Developmentally she smiles occasionally. She cannot focus but is reactive to bright light and there is some response to loud sounds. Her muscle strength appeared to be improving and she can move all four limbs spontaneously.

At the age of 2½ years her weight was above the 75 percentile and she had severe microcephaly. Her tone was reduced and she had generalised oedema, particularly affecting the feet. She had mild hypotelorism, large low set posteriorly rotated ears, micrognathia and a cleft palate. She had a short neck with a pterygium between her mandible and chest, elbow pterygia and a mild popliteal pterygium on the left. She had widely spaced nipples and a normal cardiac examination. She had clenched hands with short tapering fingers and bilateral single palmar creases. She had short toes with mild syndactyly between toes 2 and 3 on the left.

A postnatal echo was normal. A micturating cystogram showed a markedly distended neuropathic bladder that with a significant second bladder wall, trabeculations and multiple diverticulae. The patient had suspected cortical visual impairment with a normal ophthalmology examination. A hearing test was normal. A skeletal survey showed 11 pairs of ribs. Acetylcholine receptor antibodies were negative and her karyotype was 46,XX.

A mutation was not identified in *CHRNG* however the presence of a heterozygous single nucleotide polymorphism (SNP) in this gene was noted. *RAPSN* was also screened and no mutation was identified. Heterozygous SNPs were observed in *CHRNA1* and *CHRNB1*. There are no further clinical details currently available on the family.

Family branch b

This branch of the family had a single similarly affected deceased male child. There was little clinical information about this pregnancy.

7.2 Results

Autozygosity mapping techniques were utilized to investigate novel candidate genes for MPS/fetal akinesia in this consanguineous family. A 10cM genome wide linkage scan was undertaken using the Affymetrix 500K SNP Array to genotype three affected children (2 siblings and 1 cousin), II.3, II.4 and II.5 from the two branches of the family with similarly affected children. Four regions of homozygosity were identified in the three affected patients. However, only one of these regions extended to four megabases (Figure 29). This was a region on chromosome 3 between rs161661 and rs9814327; bp position g.60,958,320-g.64,091,416; hg18, NCBI 36.3. As it has been shown previously that regions of this magnitude have a one in six chance of containing the causative candidate gene, this region was targeted for fine mapping (Woods et al., 2006).

II.5 II.4 II.3

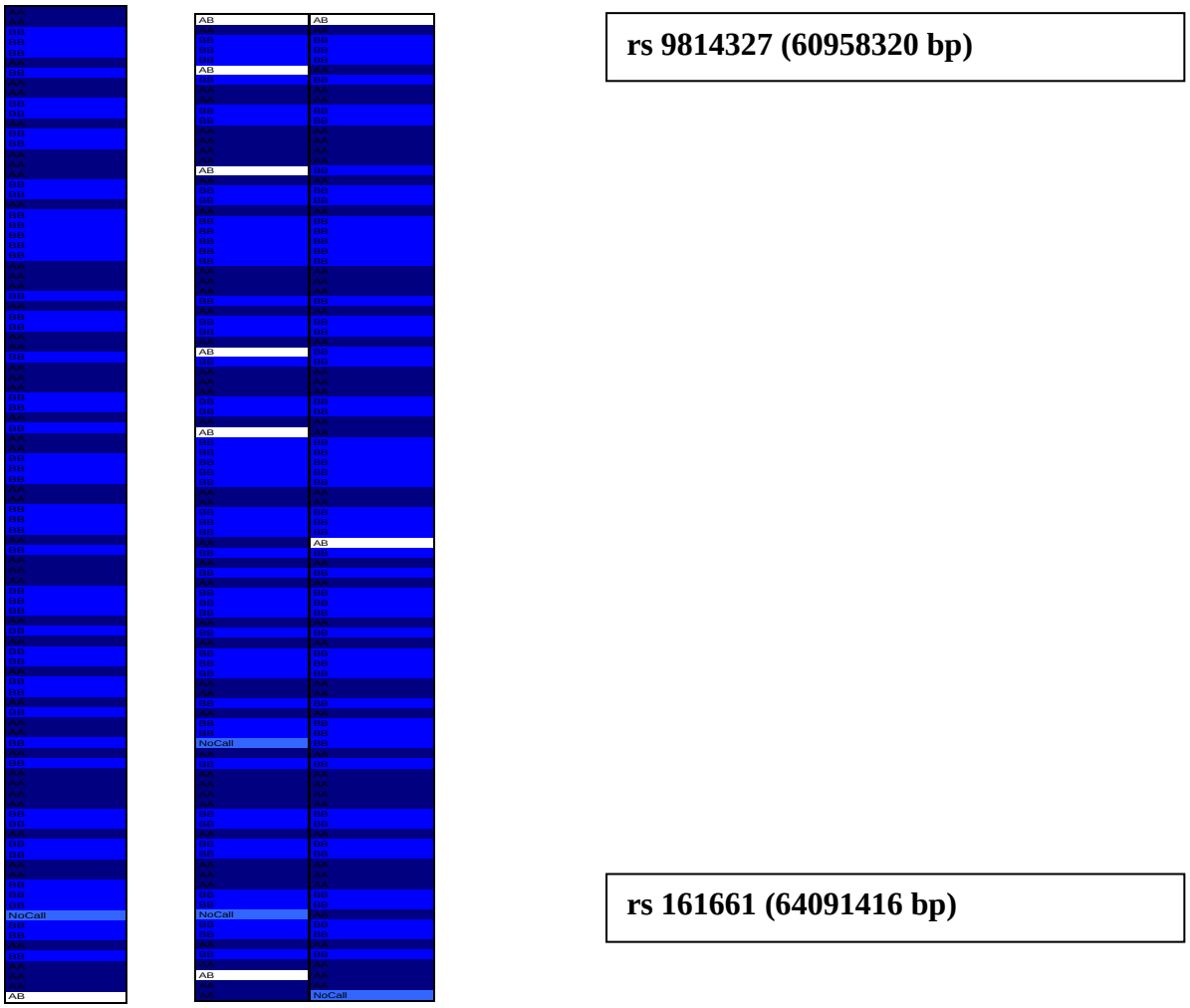


Figure 29: Affymetrix 500K SNP Array: 4Mb region of homozygosity extending from 60958320 bp to 64091416 bp, seen on analysis of the DNA from the 3 affected children II.3, II.4 and II.5.

Fluorescently labelled microsatellite markers were chosen at 0.5-1Mb intervals along the length of this region. From the results of the analysis, linkage to this region could not be excluded (Figure 30 below).

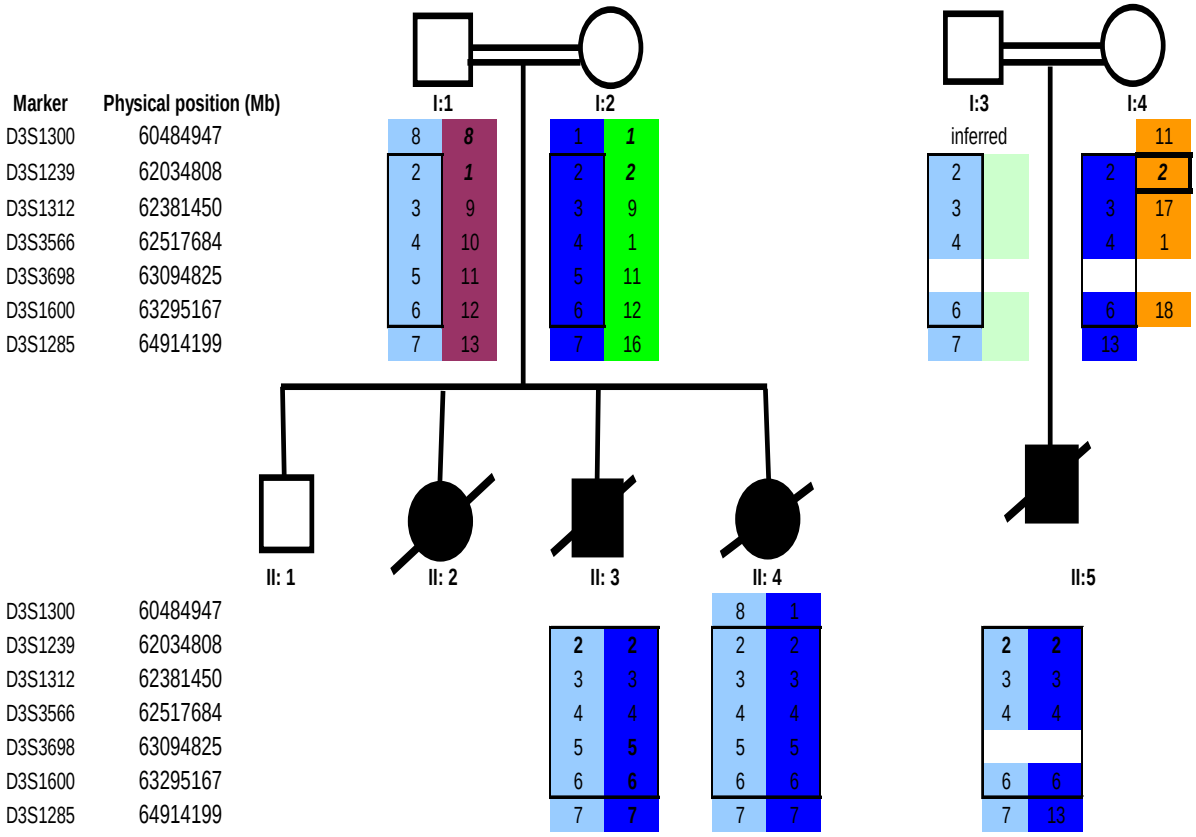


Figure 30: Fine mapping of region of homozygosity in family branches a and b.

7.2.1 Candidate Genes

The known genes located in the region were assessed (see table 11 below). The following four genes were considered to be promising candidate genes and were therefore prioritised for sequencing in the first instance in this family:

SYNPR

Synaptoporin is a five exon gene with two splice variants. It belongs to the synaptophysin family and is a membrane protein integral to synaptic vesicles. It has 97% homology with rat

synaptoporin and 81% homology with that of the chicken (Dai et al., 2003). It has been shown to be differentially expressed in the dorsal root ganglia of the rat. It was expressed in just over half of a subset of small neurones. In the spinal cord, rat synaptoporin is strongly expressed in the afferent fibres in laminae III and IV and weakly present in the other layers. Upregulation of synaptoporin occurs following peripheral nerve injury. It was deduced to be a major synaptic vesicle protein in A and C fibres in physiological and neuropathic pain (Sun et al., 2006). Human synaptoporin is specifically expressed in brain tissue (Dai et al., 2003).

FEZF2

FEZ family zinc finger 2 is a five exon gene. The 503 amino acid protein product contains six highly conserved zinc fingers at the C terminus (Matsuo-Takasaki et al., 2000). In mice it is not expressed in muscle but is selectively expressed by the pyramidal neurones in layers V and VI of the brain. Decreased FEZ2 expression reduced and altered the morphology of the subcortical axonal projections of the pyramidal neurones whereas misexpression resulted in ectopic subcortical axonal projections. In null mice the corticospinal tracts and Ctip2 the transcription factor for formation of these tracts was not expressed. Thus FEZ2 was shown to regulate differentiation on the fifth layer subcortical projection neurones and therefore to have an important role in the differentiation of subcortical projection neurones (Chen et al., 2005, Chen et al., 2008). There were no subcerebral projections and no cortical projections into the brain stem and spinal cord in null mice and no other neuronal cells were affected (Molyneaux et al., 2005).

PRICKLE2

This gene is located on chromosome 3p14. It is an eight exon gene comprised of 844 amino acids. It contains an N-terminal PET domain, three LIM domains and a prickle homology domain at the C-terminus. *PRICKLE2* is 50% homologous with *PRICKLE1* with 80% homology in the N-terminal PET and LIM domains**.** *PRICKLE2* and *PRICKLE1* are coexpressed in the brain, the eye and the testis. *PRICKLE2* is also expressed in the fetal brain, adult cartilage, pancreatic islet cells, gastric cancer and uterine tumour cells (Katoh, 2003). Heterozygous missense mutations in *PRICKLE2* have been identified siblings with progressive myoclonic epilepsy and unrelated patients with myoclonic seizures. It has been suggested that *PRICKLE2* has a role in the development of a neural network through its effect on cell polarity or affects calcium signalling providing an alternative mechanism for the seizure condition. In the animal model, heterozygous *prickle1* and *prickle2* null mice had a susceptibility to seizures.

ATXN7

The ATXN7 gene maps to chromosome 3p14.1. It has thirteen exons ranging from 69 to 979 bp in size with the CAG repeat located in exon 3. The introns vary in size from 233 bp to about 40 kb, so that the overall size of *ATXN7* is about 140 kb (Michalik et al., 1999). ATXN7 is a transcription factor that appears to have an important role in histone acetylation for chromatin remodeling and deubiquitination (Sopher et al., 2011). Heterozygous CAG triplet repeat expansions of this gene are associated with spinocerebellar ataxia type 7 (SCA7). Patients with SCA7 have highly unstable and variable CAG repeats ranging from 38 to 130, compared with 7 to 17 repeats on control alleles (David et al., 1997). However,

diseases states have not been reported in association with homozygous frameshift or nonsense mutations in this gene.

BLAST The Human Genome

Genes On Sequence All Sequence Maps next

Total Genes On Chromosome: 1638 [8 not localized] Region Displayed: 60,960K-64,090K bp Genes in Region: 18

start	stop	Symbol	Description
59710076	61212164	FHIT	fragile histidine triad gene
61522285	62254738	PTPRG	protein tyrosine phosphatase, receptor type, G
61703110	61718860	LOC100128936	similar to ribosomal protein L10a
62084054	62085426	ID2B	inhibitor of DNA binding 2B, dominant negative helix-loop-helix protein
62280436	62294361	C3orf14	chromosome 3 open reading frame 14
62330387	62334230	FEZF2	FEZ family zinc finger 2
62359061	62836094	CADPS	Ca2+-dependent secretion activator
62886941	62887626	LOC389127	similar to 40S ribosomal protein S10
63057090	63162129	LOC132205	similar to tau tubulin kinase 2
63163934	63164164	LOC100129031	hypothetical LOC100129031
63404064	63577637	SYNPR	synaptoporin
63613384	63625933	FLJ44379	similar to S-100 protein, alpha chain
63713311	63773851	LOC100130345	hypothetical protein LOC100130345
63775826	63809352	C3orf49	chromosome 3 open reading frame 49
63794586	63824637	THOC7	THO complex 7 homolog (Drosophila)
63825273	63961367	ATXN7	ataxin 7
63971271	63984160	PSMD6	proteasome (prosome, macropain) 26S subunit, non-ATPase, 6
64054587	64186171	PRICKLE2	prickle homolog 2 (Drosophila)

Table 1: NCBI Map ViewHomo sapiens (human)Build 36.3 (Current).
Candidate genes *PRICKLE2, ATXN7, SYNPR, CADPS* and *FEZF2* were selected for from the region of interest. Sequencing of these genes was undertaken on affected child and parent pairs. No pathological mutations were identified in these genes.

Sanger sequencing was undertaken on these genes however a pathogenic mutation was not identified in this family.

Exome sequencing was undertaken in this family on DNA from a single affected female proband II.4. It was predicted that homozygous mutations would be detected in the affected patients. Data from the genome wide linkage was used to focus the analysis to the largest regions of homozygosity already identified. A total of 28,384 sequence variants were identified. 204 were classified as homozygous and novel. Of these 5 were nonsense variants, 185 were missense and 14 were splicesite. 95 were located on chromosome 3 however only none of these variants were located in the candidate region. Within this region there was only a single homozygous intronic variant in *PRICKLE2*.

Work done by HP Vogt, 2010 using autozygosity mapper established two further regions of homozygosity: a region on chromosome 7 of approximately 3 megabases extending from 2,999,096 base pairs to 5,853,557; and a 2 megabase region extending from 122,803,491 to 124,735,879 on chromosome 12. On further interrogation of the WES data a single homozygous novel exon 5 A>G splicesite variant was detected in position 4181929 within the region of interest on chromosome 7.

The variant identified was in a gene called *SDK1*, sidekick of drososphila, homolog of, 1 (OMIM number 607216). SDK1 and SDK2 are homologous transmembrane molecules belonging to the immunolglobulin superfamily. The signal sequence is comprised of 6 immunoglobulin C2 motifs, 13 fibronectin type III motifs, a single transmembrane domain

and a 200 amino acid cytoplasmic domain. There is a conserved C-terminal hexapeptide sequence GFSSFV in all SDKs. The SDK proteins are believed to be evolutionary conserved in size and structure in species ranging from C.elegans through to Drosophila and vertebrates. SDKs are concentrated in synaptic regions where pre and post synaptic SDK expressing cells connect. In vitro studies in retinal cells have shown that ectoptic production of SDK redirects cell processes. It is therefore plausible that SDKs are important target recognition molecules in synaptogenesis that can influence cell connectivity (Yamagata et al., 2002). SDKs 1 and 2 been shown to have a role in cell adhesion between neurones and are believed influence specific connectivity in the eye and also other parts of the central nervous system (Yamagata and Sanes, 2008). It is thus conceivable that biallelic mutations in SDK1 may be detrimental to development of the central nervous system and could result in a fetal akinesia phenotype, due to impaired brain development, as noted in our family.

Unfortunately I was unable to undertake further laboratory work on *SDK1* as my research attachment was completed. However the variant detected in *SDK1* appears to be a good candidate gene that may warrant further investigation including confirmation with conventional Sanger sequencing, segregation studies, analysis of ethnically matched controlled chromosomes, and *SDK1* sequencing of the fetal akinesia cohort for other affecteds with homozygous or compound heterozygous *SDK1* sequencing variants. Future functional studies may be helpful for validation. Splice sites are at the junction of intron and exons. The 5' (splice donor site) and 3' (splice acceptor site) ends have recognisable consensus sequence for mRNA. The splice donor site is AG and at the splice acceptor site is GT in 98.71% of mammalian genes (Burset et al., 2000). The homozygous splice variant A>G detected may therefore be significant. Overall, no definite causative pathogenic mutations were detected in

this family. In the future further analysis in planned. In addition, regions not well covered by the exome sequencing can be studied in more detail and other regions of homozygosity explored.

7.3 Discussion

Due to the phenotypic and molecular genetic heterogeneity and the diverse mechanisms of disease that are evident in MPS/FADS, various genetic strategies have been employed for gene identification. In addition to screening a large cohort of patients for mutations in *CHRNG*, which was previously identified as a significant cause of lethal and nonlethal MPS, a candidate gene approach which included testing of other neuromuscular junction pathway genes was also done. Consanguineous families without an identifiable mutation in a known gene were scrutinised to see if they would be suitable for an autozygosity mapping approach. This could potentially result in identification of a novel cause of FADS / MPS and provide further insights into other mechanisms of disease. The family above was selected for further study.

Despite employing a combined approach of SNP arrays to localise large regions of homozygosity likely to harbour the disease gene, analysis of suitable candidate genes and next generation technology using exome sequencing, the causative pathogenic gene was not identified in this family. There are a variety of possible reasons why this may have been the case. These can be divided into technical and human factors. The technical reasons may include poor coverage of the regions of interest and an inability to examine non coding sequences. The gene of interest may also in a minority of cases

lie within small regions of homozygosity (<4Mb). Human error in the clinical interpretation of phenotypic features and failure to recognise significant data are other possibilities. Certainly exome sequencing of more than one affected individual in the family may have increased the power of this technique and reduced errors or oversight due to both technical and human factors. With the advancement in next generation sequencing technology and bioinformatics support many of these difficulties may be minimised and more successful gene discovery expected.

Chapter 8: Discussion

8: Discussion

This project was designed to investigate the clinical and molecular genetics of the multiple pterygium syndromes (MPS), as up until the time of this research study our understanding of the underlying genetic causes of this group of conditions and the phenotypic correlations has been limited.

From the previous studies of families with clinical features of MPS it is recognised that the non-lethal Escobar variant of the condition as well as lethal MPS is usually inherited in an autosomal recessive fashion. Mutations in the fetally expressed gamma subunit of the acetylcholine receptor encoded by *CHRNG* have been identified as a significant cause of Escobar and lethal MPS, accounting for approximately a third of a small group of families tested. In the initial phase of the study my patient cohort comprising 100 families was sequenced in order to determine the frequency of *CHRNG* mutations in the largest group of MPS/FADS patients to date. This analysis confirmed that *CHRNG* mutations were the significant cause of MPS / FADS. In this large cohort study *CHRNG* mutations were detected in 27% of the Escobar variant patients and in 8% of lethal LMPS / FADS families. This was a slightly lower *CHRNG* mutation detection frequency than that in the original gene identification reports. This may have been because the previous studies were undertaken on a small sample size of highly selected predominantly consanguineous patients with very similar phenotypic features. Thus the detection rate in our large patient cohort is more likely to reflect the overall detection rate of *CHRNG* mutations in a wider spectrum of patients.

A genotype phenotype study was undertaken to investigate the correlation between the clinical features present in the affected patients and the molecular findings in this cohort. When the clinical features were analysed, the presence of pterygia was associated with detection of a *CHRNG* mutation. *CHRNG* encodes the fetally expressed gamma subunit of the acetylcholine receptor, critical for in utero transmission of nerve impulses across the neuromuscular junction and consequently for fetal movement. Thus the severe, early onset fetal akinesia caused by mutations in the embryonal acetylcholine receptor may provide a mechanism for development of pterygia. The development of joint contractures without webbing may instead be due to partial or later onset fetal immobility, hypokinesia rather than akinesia or inhibition of fetal movement later in gestation possibly due to different underlying molecular mechanisms. Fetal hydrops was also a poor prognostic sign in keeping with severe immobility early in gestation and indicating a severe manifestation of the condition. It is also possible that included within this cohort were fetuses with unrecognised congenital cardiac malformations either as a component of the LMPS or as part of another unrecognised syndromic condition which may have have contributed to the poor prognosis in the hydropic group.

Central nervous system malformations can cause reduced or abnormal fetal movements however central nervous system malformations were not associated with the presence of a *CHRNG* mutation. The MPS / FADS are phenotypically and genetically heterogeneous and thus abnormal intracranial development is likely to indicate a different pathology and an alternative molecular or other mechanism may need to be sought. The presence of a cleft palate was not highly associated with the detection of a *CHRNG* mutation. This may be because although a high or cleft palate

may occur as a result of the fetal akinesia sequence, an alternative mechanism or different molecular pathology may underlie the clefting process in some patients. Identification of in-utero features of fetal akinesia correlated with lethality, particularly in the absence of a family history of this condition. This is likely to be a reflection of the severity of the phenotype and thus its detection.

This study also examined the distribution of *CHRNG* mutations across the gene and the associated clinical phenotype to see if there was a correlation between particular developmental features and specific mutations. It was anticipated that this may enable the phenotype to be predicted from the *CHRNG* mutation and this in turn would provide information about the biological role of various *CHRNG* domains. However *CHRNG* mutations were detected throughout the gene and neither the position nor the character of the mutation appeared to correlate with the severity of the phenotype. Interfamilial variation and intrafamilial phenotypic variation was noted in the cohort with the same *CHRNG* mutation for example the recurrent c.459 dupA mutation was identified in patients affected with a non lethal Escobar phenotype as well as in lethally affected fetuses. This indicates that genetic modifiers or compensatory mechanisms as well as environmental factors may be important in determining the degree of in utero akinesia, the phenotypic consequences of these mutations and the prognosis for the patient. This opens up the possibility of future developments that could convert a severe phenotype into a milder condition through modulation of other genes in the fetus or the local environment.

Although local uterine environmental factors such as physical constraints resulting in the fetal akinesia sequence are well recognised, other physiological or therapeutic

factors in pregnancy may enhance fetal movement. For example there is a suggestion that caffeine based products increase fetal activity (Devoe et al., 1993). There is currently no evidence to suggest that fetal movement in early pregnancy is increased or this has an effect on ameliorating the phenotype. The use of such products is not currently recommended and will need to be evidence based, as the potential benefits have to be balanced against the adverse effects, for example fetal growth restriction with the consumption of caffeine in pregnancy (CARE Study Group, (2008). Importantly the observation that there is apparently less phenotypic variability between affected siblings than between affected relatives in the wider family may be of practical help in the genetic counselling of MPS / FADS families.

The genotype phenotype study utilized a clinical features questionnaire comprising prenatal, antenatal and postnatal questions based on a review of the current literature with the input of others working in the field. This therefore had the potential disadvantage of focussing the questions and information collected around already recognised associated clinical features. In addition, although the largely "yes" "no" format was designed for ease of completion to ensure that as many responses as possible were obtained, this may have affected the quality of the information supplied. In order to encourage the reporting of other possibly interesting or unusual findings, opportunities for "other" comments was incorporated into the structure however the information provided may have been limited. In addition, due to the sensitivity factors related to the nature of the conditions studied, recruitment of affected patients was not systematic or complete, but was dependent on referral from the local clinicians if appropriate. Nevetheless, despite these limitations, this study enabled important clinical correlations to be established on a large MPS / FADS patient cohort.

An initiative from this study has been the development of shared Locus-Specific databases (LSDB). The *CHRNG* sequence variant data has been incorporated onto this open access database along with other published data. This will enable the cumulative *CHRNG* sequence variant information and the associated clinical features to be shared with others. This database will continue to be populated with information from our laboratory and others undertaking *CHRNG* analysis. It is anticipated that this web-based resource will provide a more complete picture of the spectrum of *CHRNG* variants detected and the associated phenotypes, which may in turn enhance our understanding of the biological properties of the protein encoded genetic regions. This is particularly helpful for the *CHRNG* gene which is fetally expressed making study of the sequence variants to establish pathogenicity impractical in most situations. Thus the LSDB will provide an invaluable resource that can be utilised by clinicians and researchers in this field.

As MPS / FADs patients are recognised as being phenotypically and genetically heterogeneous, further research work was undertaken to try and identify other molecular causes of MPS / FADS. A candidate gene approach was adopted and AChR pathway genes were selected for sequencing in this condition. In particular genes encoding neuromuscular junction components expressed in fetal life were considered as plausible candidates, as they would be predicted to cause early onset, severe fetal akinesia. The genes encoding the AChR subunits expressed in fetal and postnatal life, *CHRNA1*, *CHRNB1* and *CHRND* were selected. In addition, *RAPSN*, which encodes rapsyn known to be critical for AChR clustering, and *DOK7*, which has a role in the formation of the neuromuscular junction, were also chosen. Homozygous pathogenic mutations in *RAPSN* and *DOK7* genes were detected in families with severe lethal

fetal akinesia using this strategy. Although germline mutations in *CHRNA1, CHRNB1* and *CHRND* were not identified in our cohort of patients, mutations in *CHRNA1* and *CHRND* have been identified in other MPS patients (Michalk et al., 2008), supporting the role of the various AChR genes in disorders of the neuromuscular junction. It is envisaged that mutations in *CHRNB1* as well as other AChR-related genes will also be detected in this patient group in the future.

Thus mutations in several AChR genes and AChR-related genes have been identified in MPS/FADS. Patients affected with another group of conditions the congenital myasthenic syndromes (CMS) may also have autosomal recessive mutations in AChR pathway genes. The CMS are disorders of neuromuscular transmission. They are due to defects in presynaptic, synaptic and post synaptic neuromuscular junction proteins (Engel et al., 2002). The majority of post synaptic CMS syndromes are due to a deficiency of AChRs at the muscle endplate due to mutations in *CHRNE* (Engel et al., 1996). This gene encodes the epsilon subunit of the AChR. As *CHRNE* is only expressed towards the end of gestation and in postnatal life, mutations in this gene do not cause a fetal phenotype. Conversely, the gamma receptor subunit of the AChR is not expressed postnatally and therefore *CHRNG* mutations are not associated with postnatal muscle weakness and have not been identified in CMS.

Mutations in other AChR genes can also cause CMS. Interestingly, mutations in *RAPSN* have been associated with a late onset phenotype as well as an early onset presentation evident at birth with arthrogryposis, bulbar dysfunction and respiratory difficulties. Patients harbouring one common *RAPSN* mutation N88K and a second severe mutation including 1177del2 may represent this intermediate presentation

(Burke et al., 2003). Thus there may be a spectrum of acetylcholine receptor mutations with phenotypes ranging from CMS, CMS with arthrogryposis and breathing difficulties to severe fetal akinesia.

The acetylcholine receptor pathway genes therefore play a crucial role in the development of the neuromuscular junction and in muscle contraction in fetal life. Factors interfering with normal NMJ development and maturation have a detrimental effect on the execution of movement. The phenotypic consequences of autosomal recessive mutations in the AChR-related genes depend on the timing and the severity of the neuromuscular junction disturbance. Thus AChR genes may cause a spectrum of conditions from lethal MPS/FADS, (Vogt et al., 2008) to postnatal CMS (Beeson et al., 2003).

As well as providing insights about the mechanisms of disease, genotype phenotype information can be utilized to guide the rationale for genetic testing in families. *CHRNG*, *RAPSN* and *DOK7* mutations have been identified in LMPS/FADS (Morgan et al., 2006, Vogt et al., 2008, Vogt et al., 2009). However, unlike *CHRNG* mutation positive patients, in whom pterygia were almost always present, the small number of cases identified with *RAPSN* and *DOK7* mutation did not have pterygia. In addition, as growth retardation was not observed in our *RAPSN* patients the mutations identified may have had a less severe effect on early fetal development and fetal growth. Siblings reported in a further family were not lethally affected, although they had severe respiratory distress. *CHRNA1* and *CHRND* mutations have been found in cases of LMPS/FADS associated with pterygia and congenital anomalies such as cleft

palate and cardiac defect. However, central nervous system anomalies were not present (Michalk et al., 2008).

Based on the current understanding of the underlying molecular pathology, a molecular genetic diagnostic pathway was designed for the investigation of EVMPS/LMPS/FADS. *CHRNG* analysis was recommended for all families with likely autosomal recessive EVMPS, as the detection rate was estimated to be in the order of one quarter. For the LMPS/FADS families the presence of central nervous system malformations may suggest an alternative pathology and therefore the identification of an acetylcholine receptor pathway gene was considered to be low. *CHRNG* mutation analysis was also recommended for LMPS/FADS patients with pterygia, followed by *CHRNA1* and *CHRND* if this was negative. In the absence of pterygia, it was suggested that *RAPSN* and *DOK7* mutation analysis should be pursued before *CHRNG, CHRNA1* and *CHRND* although the mutation detection rate for each individual gene is not expected to be high.

In summary, mutations in AChR pathway component genes are relevant for a wide range of FADS spectrum families. The identification of the molecular basis of these disorders may be of therapeutic relevance for survivors, for example the attenuation of the respiratory symptoms due to AChR pathway gene mutations by the development of specific targeted treatments. It is known that *RAPSN*-associated CMS may respond to acetylcholine esterase inhibitors which increase the quantity and duration of acetylcholine in the synapse and 3,4-diaminopyridine which the enhances the presynaptic release of acetylcholine. Although administration of 3,4-diaminopyridine may be limited because of central as well as its peripheral nervous system effects,

treatment may be lifesaving for patients presenting with severe disease such as respiratory failure at birth and during periods of intercurrent illnesses when clinical deterioration may occur, (Schara and Lochmuller, 2008). Empiric treatment of *DOK7* myasthenia with β-adrenergic agonists such as salbutamol is effective and may ameliorate the symtoms for survivors identified with severe disease (Engel et al., 2007). Ephedrine has also been reported to be effective in enhancing neuromuscular junction transmission in this patient group however the mechanism of action is unclear (Lashley et al., 2010). In addition, persistence of fetal acetylcholine receptor subunits in the postnatal neuromuscular junction in CMS may indicate that up-regulation of other molecules may lead to a partial therapeutic rescue strategy (Ohno et al., 1997). However these strategies are unlikely to improve the prognosis for severe in utero disease because of the adverse effects on early fetal development of very impaired neuromuscular junction transmission, and the presence of bilallelic null mutations which may limit the therapeutic response even if an adequate in utero dosage can be achieved with minimal maternal side effects.

There is likely to be extensive locus heterogeneity in LMPS/FADS, and as further EVMPS/LMPS/FADS genes are identified, utilisation of second generation sequencing techniques for diagnostic testing is likely to be a more efficient and cost effective genetic testing strategy. Discovery of novel genes causing this phenotype was commenced using next generation sequencing technologies. Although not successful in identifying a definite pathogenic cause in the family selected for study, this provides important insights into some of the challenges that have to be overcome in using this technology. It is clear that there are likely to be other AChR genes implicated in this group of patients as well as severe mutations in other genes, for

example the congenital myopathy genes, critical for development of the fetal motor pathway.

Future Work

Using our MPS / FADS cohort, further study of the underlying molecular mechanisms of this group of conditions is underway. Further analysis of other genes implicated in CMS, such as *MuSK* (Chevessier et al., 2004), may further expand the role of defective signal transmission at the neuromuscular junction in the MPS/fetal akinesia sequence. A recent single case of Escobar syndrome with nemaline myopathy due to a homozygous *TPM2* mutation (Monnier et al., 2009) suggests muscle sarcomere genes may be implicated in the development of the MPS/FADS phenotype.

The application of next generation sequencing technologies is likely to expedite the identification of novel candidate genes. However it will be important to continue to collect clinical phenotypic information, to help in clarifying the significance of any sequence variants detected, enable the study of genotype phenotype correlations and to inform the interpretation of the molecular results. Strategies such autozygosity mapping in consanguineous families may continue to provide valuable information about the genetic regions to be targeted for efficient mutation detection.

There is considerable overlap in the genetic causes of FADS and arthrogryposis. In the West Midlands region we have developed an expertise in the diagnosis and management of arthrogryposis-related conditions. We have a regional multi-disciplinary arthrogryposis service for adult and paediatric patients and access to established fetal medicine departments and fetal pathology expertise. In order to provide the optimum service for patients and their families it will be important to develop effective collaboration between the clinicians, the diagnostic genetics service

laboratory and the research scientists. This will facilitate collection of phenotyping information, efficient diagnostic investigation pathways and appropriate genetic counselling advice, as well as the development of more effective diagnostic testing strategies using multi-gene panels, the identification of novel disease genes, genetic mechansims and genetic modifiers of disease, and therapeutic targets for future study.

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