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Investigating the molecular pathology of Dupuytren's disease

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Investigation of the Molecular Pathology of Dupuytren’s Disease

Robert Pearce FRACS

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ABSTRACT

The eponymous progressive palmar fibromatosis described by Guillaume Dupuytren in 1831 is a common and debilitating disease with limited treatment options and high rates of recurrence after intervention. Dupuytren’s disease has been generally accepted as predominantly affecting Northern European individuals; Dupuytren’s disease is sometimes referred to as “Vikings Disease” as an attribution to this ethnic origin. Its familial occurrence in all communities is suggestive of Mendelian inheritance as an autosomal dominant disease with incomplete penetrance. The genetic and molecular basis of Dupuytren’s disease (DD) has been the focus of many studies, with increasingly sophisticated approaches since the sequencing of the human genome in 2003. However, whilst the pathology of the progressive fibrosis is well understood, the etiology and molecular triggers of the disease remain unknown.

This study has investigated the genetic basis of Dupuytren’s disease in families and the population of Western Australia. The study involved a tripartite investigation in patients from three different cohorts. The DNA of four DD affected people within a four-generation family with autosomal dominant inheritance of DD was submitted for whole exome sequencing (WES) which initially identified 43 candidate genes with non-synonymous heterozygous rare variants. Two candidate genes, EXOG and GORASPI, were in regions of positive linkage whilst the remainder were not excluded by linkage analysis. Sanger sequencing was focused on three candidate genes, EXOG, GORASPI and COL6A5, but no variant segregated with Dupuytren’s disease in additional family members. No likely disease-causing variant was identified in all family members positive for the disease phenotype.

A second cohort consisted of the Busselton study population, a study initiated in the 1960s and following the health outcomes of nearly 5000 people in Busselton WA. Using a survey 48 unrelated individuals were identified as having had surgery for DD, whilst 409 people had evidence of early DD. DNA from the participants of the Busselton study was screened for SNPs using a GWAS chip approach and the data analysed to identify SNPs associated
with DD. This study lacked sufficient power to identify statistically significant SNPs associated with the disease. A shortlist of variants was identified using an arbitrary p-value cut-off. However, none of these SNPs were associated with genes in either the familial study or previous research. For the final genetic study DNA from another set of individuals with severe DD were subject to WES. The aim was to identify rare mutations in genes that may be associated with DD and that would promote the more aggressive phenotype in these individuals. Whilst no mutations in identical genes as previous studies were identified there was overlap in gene families and networks that suggests pathways that may be important in DD. Finally, fibroblasts isolated from some Dupuytren patients and control fibroblasts from the wrist were characterized using a modified ‘scar-in-a-jar’ protocol to investigate their collagen production. Interestingly, this model did not identify any differences between disease fibroblasts and control fibroblasts from the same patients. This may suggest that profibrotic signals in Dupuytren disease are external to the fibroblasts rather than autonomous. In addition it strongly suggests a need to develop more complex models to reproduce the disease state in vitro for further studies to understand the molecular basis of Dupuytren’s disease and

The complexity of the known genetic susceptibility together with environmental factors, as well as the likely importance of non-coding and epigenetic factors continues to make identification of the genetic changes underpinning Dupuytren’s disease difficult. In addition limited in vitro and animal models for Dupuytren’s disease will continue to hamper therapeutic development and understanding. However, the studies here suggest common pathways may be implicated from the familial and population study approaches. Further work to identify the functional changes that underpin DD will be critical to the development of more effective treatment and prevention of the disease.
This thesis is the work of Robert Pearce and has not been submitted or accepted for the award of any other degree or diploma at any other university or institution.

To the best of my knowledge this thesis contains no material previously published or written by another person except where due reference is made in the text of the thesis.

Signed

Robert Pearce FRACS
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CHAPTER 1

1.1 An overview of Dupuytren’s disease

Dupuytren’s disease is the most common heritable disorder of connective tissue (Hu et al., 2005). Since his lectures to the staff at Hotel Dieu in Paris in 1831 (Dupuytren, 1834) the name of Baron Dupuytren has received universal acceptance to describe this progressive palmar fibromatosis, with or without contractures. Although he was unable to identify the true pathophysiology or aetiology of this condition, attributing its occupational cause to prolonged flexion of the digits in a coach-driver, Guillaume Dupuytren (Fig.1.1) has been credited with the earliest description of his eponymous disease. Dupuytren’s disease is characteristically a late-onset fibromatosis affecting subcutaneous tissue of the palms and fingers (Fig. 1.2). It is relatively rare before the age of forty-five, with the mean age of onset in males of all races at fifty years (+/- five years) and fifty five years (+/- five years) in females (Pearce, 1991).

Figure 1.1 Guillaume Dupuytren

Progressive palmar fibromatosis was first described in 1831 by Baron Dupuytren In the pathogenesis of Dupuytren’s disease, subcutaneous fat is progressively replaced by relatively avascular fibrous tissue, which develops as nodules in the palm and extends distally into the digits as thickened cords. Tender palmar nodules and progressive shortening of cords across metacarpo-phalangeal and proximal interphalangeal joints, together with subsequent changes in other structures including collateral ligaments, cause functional disability (Fig. 1.2).
**Figure 1 2 Dupuytren's disease (DD (Palmar fibromatosis))**

DD is shown here affecting the palm in a 45 year old male with fibrous nodules in the palm extending as cords across the metacarpophalangeal and proximal interphalangeal joints causing flexion contractures.

Being a progressive condition that often presents in middle age with pain or digital contractures, Dupuytren’s disease is a common cause of disability interfering with manual dexterity in employment. In the majority of cases fibromatosis initially affects the palm and little finger, spreading in time across the palm radially, involving sequentially the ring, middle and index fingers. Less commonly there is a palpable band in the first web and isolated contracture across the metacarpo-phalangeal (MCP) joint of the thumb. A further unexplained occurrence is an isolated mid-palm cord extending into the middle finger with early contracture at the MCP joint, a finding statistically associated with diabetes mellitus (Ardic et al., 2003; Chammas et al., 1995; Eadington et al., 1989; Eadington et al., 1991; Early, 1962; Herzog, 1951). Both trigger finger and Dupuytren’s disease are common in the middle and ring fingers of the diabetic, sometimes referred to as the DDT syndrome (Burgess & Watson, 1987; McFarlane, 1991; Parker, 1979).

Untreated digital contractures, particularly affecting both hands, can be very disabling as they progress in association with other co-morbidities of ageing. While collagenase injection into fibrous bands of Dupuytren’s tissue has met with limited success when used
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in early stages of the disease, surgical intervention in established cases is currently the preferred treatment, with the best chance of functional recovery.

The clinical indications for medical or surgical intervention are related to disabling palmar nodules, which can be painful and often interfere with everyday activities of life, or fibrous cords extending across the small joints of the hand causing digital contractures with limited extension of the metacarpo-phalangeal or proximal interphalangeal joints.

In summary, Dupuytren’s disease is a late onset progressive fibromatosis, predominantly affecting males and with a disabling effect on the function of the hands. Whilst the clinical manifestations of disease progression are easily recognized and well understood, the molecular basis is not as clear.

1.2 Current clinical management of Dupuytren’s disease

1.2.1 Prevention
The aetiology of Dupuytren’s disease is not well understood, particularly in relation to the part played by lifestyle and environmental factors. It is therefore not possible at this stage to advise preventive measures beyond notifying those identified as being at risk (through familial background) of the potentially harmful effects of smoking and alcohol. The possible application of knowledge gained from an understanding of the genetic basis for this disease to its prevention should be worth consideration. While epidemiological studies provide often biased and conflicting evidence in relation to occupation as a causative factor in Dupuytren’s disease there is no sound basis for considering employment in terms of prevention (Mikkelsen, 1978).

1.3 Surgery to treat Dupuytren’s disease
Since the identification of Dupuytren’s disease as a progressive, debilitating condition, surgery has been the primary treatment option. The majority of Dupuytren's disciples used multiple open wounds, operating in an era of unconfined suppuration and the anticipated drainage of laudable pus. Without the benefits of asepsis or antibiotics, this procedure was
associated with significant mortality and morbidity. Another prominent French surgeon, Guillaume Goyrand, advocated the use of longitudinal incisions and open fasciotomies, while fifty years later William Adams appears to have pioneered the combination of post-operative splinting with a closed subcutaneous fasciotomy technique in an attempt to reduce post-operative infection (Adams, 1885; Goyrand, 1833).

Open operations did not become safe until the advent of antisepsis (following the work of Pasteur and Lister) and later developments of aseptic surgical technique. Fasciectomy, performed under general anaesthetic and tourniquet control, has subsequently supplanted fasciotomy as the operation of choice, although recent success without tourniquet and performed under local/regional anaesthetic will add a new dimension following the work of Lalonde of Toronto (Lalonde and Martin, 2014).

1.3.1 Limited or partial fasciectomy
Operating on only those areas of palpable fibrosis or contractures involving the small joints, dissection can be restricted to involved tissues that are safely excised through longitudinal incisions and under tourniquet. This approach has been widely favoured and recommended (Hamlin, 1952; Heuston, 1969; Skoog, 1963). Apart from some variations in operative and post-operative splinting, this operation is still the surgery of choice today (Hindocha, Stanley, Watson, & Bayat, 2006; Shih and Bayat, 2010). By avoiding or limiting dissection into normal tissue planes one avoids excessive scar tissue formation which inevitably transforms to Dupuytren’s tissue and results in recurrence (Bayat & McGrouther, 2006).

1.3.2 Dermofasciectomy
Sacrifice of skin is only contemplated when considered necessary to adequately remove underlying adherent Dupuytren’s tissue. It generally becomes an issue during the course of operation for recurrent disease. Extensive involvement of the palmar skin with adherence to underlying fibrosis sometimes necessitates excision of the skin and replacement with a full-thickness skin graft (dermofasciectomy). This technique was introduced by Maccallum in 1962 (Maccallum & Hueston, 1962) and has proved effective in reducing further recurrence (Hueston, 1984).
1.3.3 Z-Plasty repair

The use of Z-plasties in repair of the skin incision has three main objectives: to assist in closure with adequate skin cover by rearrangement of flaps and lengthening the wound; to avoid a longitudinal scar across skin creases which may lead to scar contracture; and thirdly to realign the subcutaneous tissue and thereby reduce the chance of recurrence of contracture (Baker & Watson, 1980).

Radical or total fasciectomy radical dissection of Dupuytren’s affected subcutaneous tissue across the palm is rarely performed. McFarlane has advocated an extensive removal of involved and potentially involved digital and palmar fascia (McFarlane, 1990), based on his description of three different types of cords found in relation to normal anatomical structures in the palm. Apart from being unnecessary in most cases, it implies removal of potentially normal tissue including the palmar aponeurosis. This surgery is also time-consuming with the other disadvantage of a high recurrence rate (Clarkson, 1963). Selective dissection along affected bands of Dupuytren’s while preserving normal anatomy is much preferred. Luck and other authors who have reported extensive dissections have removed only involved fascia rather than perform a prophylactic fasciectomy (Luck, 1959; McIndoe & Beare, 1958; Iselin, 1955; McGregor, 1985; Hueston, 1961; McCash, 1964).

1.3.4 Amputation

Considered as a last resort following failed attempts at release of debilitating digital contractures, amputation must be thought of as an acceptable means of restoring a functional grip. The difficulty in straightening the proximal interphalangeal joint, particularly in the little finger, was appreciated by Dupuytren in 1831. Since then there have been numerous operations proposed in an effort to avoid amputation, including proximal interphalangeal joint arthrodesis or arthroplasty, division of flexor tendons, and wedge osteotomy of the proximal phalanx (Moberg, 1973), generally associated with post-operative extension splinting (Ebskow et al., 2000; Larson and Jerosch-Herold, 2008). Amputation would be an appropriate option, however, if the complications of a useless
finger are to be avoided and vascularized dorsal skin flaps can be utilized to replace involved skin on the adjacent palm (Tonkin, 1984).

1.3.5 Percutaneous division of fibrous cords

This blind technique (to be distinguished from the open fasciotomy advocated by Astley Cooper in 1822) involves the use of a bevelled needle or fine scalpel to release a contracture, usually at metacarpo-phalangeal level, where a reasonably thin cord of Dupuytren’s can be palpated beneath the skin. Closed fasciotomy is almost confined to solitary contractures in the early stages of disease or in elderly patients who may not withstand more extensive surgery, as it can be performed under local anaesthetic. This minimally invasive procedure has good short-term results in mild or moderate contractures with a very low complication rate (Rowley et al., 1984; Foucher et al., 2003; Van Rijssen et al, 2006). While it may be considered a temporary measure (Van Rijssen & Werker, 2006), this approach is rarely considered appropriate in developing or progressive disease (Shaw et al., 1996). There are limited published data comparing medium-term outcomes of fasciectomy and closed percutaneous fasciotomy, but adherents to the latter procedure are aware of the complications involving skin tears and prolonged healing with frequent early recurrences.

1.4 Non-surgical treatment of Dupuytren’s disease

1.4.1 Physical manipulation of Dupuytren's contracture

Traumatic extension with rupture of fibrous cords responsible for digital contractures in Dupuytren’s disease was found to provide immediate though painful release of a flexion deformity (Hawkins, 1835). When performed as an elective procedure under anaesthetic the excessive force required by passive extension is quite traumatic. Used in conjunction with percutaneous section of the cords there is increased likelihood of skin dehiscence with the problem of prolonged healing and no lasting benefit (Colville, 1983).

A similar outcome results from sustained splinting with gradually increasing traction to release a flexion contracture. Where release of the contracture has apparently been successful there is almost universal evidence of recurrence within weeks of removal of
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the traction apparatus (Shaw et al., 2007). Although it becomes an essential stage in the management of contractures by injection, physical manipulation per se is therefore no longer recommended to treat contractures due to Dupuytren’s disease (vide infra).

1.4.2 Irradiation
As a non-surgical intervention radiotherapy has been trialled in early-stage Dupuytren’s disease to reduce fibroblast activity (Keilholz et al., 1996). An estimated 87% of patients reported a subjective relief of symptoms while symptomatic cords and nodules were reduced in 75% (Seegenschmiedt, Olschewski, & Guntrum, 2001). Long term results in one study showed no progression of disease after thirteen years (Betz et al., 2010). Apart from some minor late toxicity in the form of localized skin atrophy in 32% of cases, this would appear to be safe practice with no major complications. Significantly there has been no evidence of radiation-induced malignancy, contrary to early experience with exposure to X-rays. Whilst the results reported in the few studies on irradiation therapy have been positive, this treatment modality has not been widely adopted. The positive results are potentially due to the treatment of patients at a very early stage of localized disease development. Only a few patients present for medical advice at this early time, when surgery would not be considered as an option.

1.4.3 Extracorporeal shockwave therapy
Knobloch and others hypothesized in 2010 that focused extracorporeal shock wave therapy may be used as a non-invasive method of reducing palmar fibromatosis and progressive contractures in Dupuytren’s disease, on the basis of positive results obtained from a randomized-controlled trial in the management of Peyronie’s disease (Knobloch et al., 2011). The outcome of a similar trial with Dupuytren’s individuals suggests that this therapy may be useful in preventing or slowing the rate of contracture (without major complication), but long-term follow-up has not been reported.

1.5 Pharmacological treatments of Dupuytren’s disease
Other non-surgical or medical treatments have been attempted with variable degrees of success. Their rationale is based on the possible reversal or interruption of cellular activity
of abnormal myofibroblasts in Dupuytren’s tissue and the removal or prevention of the excessive collagen deposition characteristic of this disease. Such treatments might therefore be expected to have some beneficial effect through direct or indirect limitation of collagen deposition, thereby limiting disease progression. They include: injection of steroid, interferon or collagenase; ingestion of vitamin E, allopurinol or verapamil; Bassot (1965) and later Hueston advocated ‘enzymic fasciotomy’ as an alternative to surgery, but their results were not rewarding (Bassot, 1965; Hueston, 1974).

1.5.1 Intralesional steroid injection
The effect of steroids on Dupuytren’s disease has been investigated with reference to the role of programmed cell death; the progress of Dupuytren’s disease is seen as an imbalance between fibroblast activity and apoptosis. Both in vivo and with cultured Dupuytren’s cells, steroids were shown to increase the rate of apoptosis and to decrease rates of proliferation when compared with control fibroblasts (Meek et al., 2002).

1.5.2 Intralesional collagenase injection
Somewhat better results have followed various clinical trials using Clostridium histolyticum collagenase injections with release of limited contractures in the early stages of disease (Badalamente et al., 2002; Hurst et al., 2009). In America Clostridium histolyticum collagenase was approved by the FDA in February 2010 for use as an injectable treatment for Dupuytren’s disease (Shih & Bayat, 2010). Injection is followed by manipulation and attempted release of contracture after forty-eight hours. Results to date have been variable and outcomes depend heavily upon case selection, clinical expertise and patient compliance. Reliable longer-term recurrence rates have yet to be determined.

1.5.3 Intralesional gamma-interferon
Pittet et al. (1994) tested the effect of Gamma-Interferon injected into Dupuytren’s tissue, and also hypertrophic scars (Pittet et al., 1994). Gamma-interferon has been shown to decrease fibroblast replication, alpha smooth muscle actin expression and collagen
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production. Some decrease in lesion size has been observed in hypertrophic burn scars and Dupuytren’s nodules with no significant release of contractures.

Although there is some advocacy in recent years for pharmacological and other minimally invasive treatments as temporary or palliative control of limited fibrosis in selected cases of Dupuytren’s disease, none of these methods has been adopted universally as alternative treatment on a routine basis.

1.6 Summary

In summary, the primary treatment modality for Dupuytren’s disease is currently surgical intervention, with removal of the cord tissue, release of debilitating contractures and salvage of normal surrounding tissue. This has been effective also in slowing the progression of Dupuytren’s disease in many cases, but the progressive nature of the disease can lead to recurrence and continued disability. There are also potential risks inherent in the surgical procedures, most notably associated neurovascular trauma that can be avoided by careful dissection, and scar contractures that are often painful. Sympathetic dystrophy as a complication of hand surgery is disabling, inexplicable, resistant to treatment and fortunately rare.

Other conservative treatment options, predominantly pharmacological, have been developed and are subject to clinical controlled trials, long-term follow-up and assessment. These have generally shown limited efficacy, at least in part due to an incomplete understanding of the molecular pathology. In a recent review of surgical and medical advances in the management of Dupuytren’s disease (Mafi et al., 2012) the authors have emphasized the importance of genetic studies in elucidating the cause of the disease with the prospect of preventing its progression.

In line with current understanding of the clinical progression and treatment of Dupuytren’s disease therefore, there is reason to believe that further elucidation of its molecular pathology and aetiology might result in new treatments and potentially improved outcomes.
1.7 The Epidemiology of Dupuytren’s disease

Dupuytren’s disease is generally uncommon before the age of forty-five. Although the age at which palmar fibromatosis in adults first becomes apparent can be difficult to establish, the average age of onset is generally accepted as being 50+/- 5 years for males and 55+/- 5 years for females (Hindocha et al., 2006). Beyond the age of fifty years the prevalence of Dupuytren’s disease in any population study increases with age until about 80 years, when it tends to plateau (Early, 1962; Khan et al., 2004). There is a definite gender bias, generally accepted as approximately M:F = 6:1 in the 60-70 year age group, with variable figures beyond this age, but the observed convergence of male and female incidence after eighty years could be due to a combination of population changes, hormonal imbalance and the variable effects of age-related comorbidities and lifestyle factors.

Ageing itself could be instrumental in progressing the fibromatosis as with other neoplasms, possibly related to diminishing immune response and nuclear/chromosomal fragility. Specific neurovascular changes have not been identified in Dupuytren’s tissue, but could become secondary factors in the activity of fibroblasts as relatively avascular collagen deposits accumulate. With an ageing population raised triglyceride levels, hypercholesterolaemia and hypertension as well as Dupuytren’s disease become more prevalent - to date there is nothing to suggest that there is any common causal relationship.

The possible role of associated conditions in the aetiology of Dupuytren’s palmar fibromatosis has received much attention in world literature (P. Burge, Hoy, Regan, & Milne, 1997; P. D. Burge, 2004; Gordon, 1954; Hindocha, Stanley, et al., 2006; Mackenney, 1983; Carson and Clarke, 1993; Loos et al., 2007) Associated conditions referred to include: diabetes mellitus, osteoarthritis, hypercholesterolaemia, hypertension, and gout, all of which are closely related to ageing.

The relevance of some of these factors to the aetiology of Dupuytren’s, including alcohol abuse, epilepsy, hypercholesterolaemia, hepatic cirrhosis, smoking and diabetes mellitus,
Investigating the Molecular Pathology of Dupuytren’s Disease

has been questioned (Carson and Clarke, 1993; Schiavon et al., 2004; Loos et al., 2007) The association of Dupuytren’s with epilepsy is uncertain (and denied by some authors on a statistical basis; Mackenney, 1983) but the highest prevalence rate was reported in a study group of adult epileptic patients in the United Kingdom (56%, 1976). However there is a huge discrepancy with other similar studies (eg Germany, 20% and France, 9%; Hindocha, McGrowther and Bayat, 2009). Whilst there are a number of links associating the presence of Dupuytren’s disease with other conditions, to date no causative links between Dupuytren’s disease and other pathologies has been established, and in fact ageing may be the common link between the diseases rather than any specific molecular pathology.

1.7.1 Epidemiological evidence for a genetic component to Dupuytren’s disease.

Dupuytren’s disease is most common in Caucasian adults of Northern European ancestry (Bayat and McGrouther, 2006). Various surveys have been published to report the occurrence of Dupuytren’s disease among other ethnic communities; African Americans (Gonzalez et al., 1998; Mitra & Goldstein, 1994; Sladicka et al., 1996) Chinese-oriental (Egawa et al., 1990) Japanese (Abe et al., 2007); African (Makhlouf et al., 1987; Mennen, 1986; Muguti & Appelt, 1993; Richard-Kadio et al, 1997); Icelandic (Gudmundsson et al., 2000); Chinese (Liu & Chen, 1991); Thai (Vathana et al., 1990) and Indian (Srivastava et al., 1989) populations as well as Eastern European and Mediterranean cohorts (Zerajic & Finsen, 2004), with varying rates of incidence in these different populations. The ethnic influence becomes more apparent when migrant populations from Poland or Scandinavia examined in America are found to show similar rates of disease as found in their countries of origin (Soboeiro, 2000), suggesting the importance of genetics rather than environment in the incidence of Dupuytren’s disease. Nevertheless, environment is also likely to play a role, as evidenced by the study of Finsen and colleagues (2002) who studied two ethnic groups in northern Norway. Without identifying specific factors that might influence the occurrence of Dupuytren’s, the findings showed that family members were more likely to develop Dupuytren’s disease if they lived in the same geographic area as their affected relatives.
Despite the many studies linking Dupuytren’s disease to other environmental factors, family history is undoubtedly one of the strongest predictors of susceptibility. This clearly demonstrates that there is a substantial genetic influence on the development of Dupuytren’s disease. The inheritance pattern of Dupuytren’s disease was studied by Burge, who reported:

“Characterization of autosomal dominant inheritance in this condition is reinforced by the occurrence of affected individuals, both male and female, in consecutive generations, with evidence of transmission female to male, and variations in degrees of penetrance,” (Burge, 1999). The more recent ‘Rejkavik Study’ reported by Gudmundsson and colleagues that recorded a large Icelandic family pedigree with affecteds in three generations, reached similar conclusions (Gudmundsson et al., 2000). Therefore strong evidence exists for a significant genetic component to the occurrence of Dupuytren’s disease. Understanding the key genes that underpin this link will be important to understand the disease and ultimately to develop better treatments.

1.8 The Molecular and cellular pathology of Dupuytren’s disease

Dupuytren’s disease involves progressive, increasing deposition of stable collagen and extracellular matrix proteins, with subsequent contracture of the matrix through the activity of fibroblast subpopulations. Whether the increased density of the matrix is caused by excessive production or reduced degradation is as yet unclear, and the molecular signals that trigger this process unknown. There is some evidence that the immune system is important in Dupuytren’s disease, whilst other studies suggest that innervation and neuroinflammatory mediators may be involved. Nevertheless, whilst the molecular and cellular triggers remain difficult to elucidate, the progressive increase in extracellular matrix and subsequent contraction clearly involves myofibroblasts (Gabbiani & Majno, 1972). This section will review the evidence and possible roles of different cells and systems in the pathology of Dupuytren’s disease.

1.8.1 The role of myofibroblasts in Dupuytren’s disease

The cellular structure and biology of the myofibroblast were elucidated by Gabbiani, Majno and Ryan (Gabbiani & Majno, 1972; Gabbiani et al., 1973), and others (Tomasek and Haaksma, 1991; Tomasek et al., 1999; Skalli and colleagues, 1989). The contractile
mechanisms of the myofibroblasts in Dupuytren’s disease were first investigated by Badalamente and colleagues in relation to the pathogenesis of contractures in this disease (Badalamente et al., 1983). Factors involved in the differentiation of these cells at a molecular level and the activation of their innate contractility in the progression of Dupuytren’s disease have been further studied by Verjee et al. (2013).

Connective tissue contracture as occurs in Dupuytren’s pathology is “a sustained, low-energy shortening process which involves matrix-dispersed cells and is dominated by extracellular events such as matrix remodeling” (Tomasek, 2002; Glimcher & Peabody, 1990). Histological examination of Dupuytren’s tissue readily confirms the relative hypocellularity in a dense matrix of collagen in established disease. Multinucleated cells have been observed in all clinical stages of progressive fibromatosis. Whether due to fibroblast fusion or nuclear division without cytokinesis, the fact that multinucleated fibroblasts can originate via different mechanisms may influence their behaviour in various conditions including in vitro culture assays and in certain fibroblastic pathologies such as foreign body response, fibrosis, cancer and aged tissue (Holt and Grainger, 2011).

In Dupuytren’s disease, as in normal wound healing, collagen type I is largely replaced by type III collagen which is known to be present in remodeling tissues, or in normal tissues that are subject to mechanical stress (Gabbiani et al., 1976). The remodeling, which is an essential element in the progressive shortening process of contracture formation, involves removal of matrix molecules and is largely mediated by metalloproteinases which allow slippage and re-apposition of adjacent fibrils.

It is thought that the contractile force generated by myofibroblasts translates into collagen network shortening through fibronexus adhesion complexes that are linked to intracellular stress fibres. These factors (stress fibres and adhesion complexes with the extracellular matrix) are distinctive features for differentiated myofibroblasts. These cells generate sustained contractile force, which is generated by contractile stress fibres composed of bundles of actin microfilaments with associated non-muscle myosin and other actin-binding proteins. The production of matrix and subsequent response to the matrix to trigger contraction is critical to the pathology of Dupuytren’s disease.
Under mechanical stress, fibroblasts can undergo a two-stage differentiation process (Figure 1.3). The fibroblasts first differentiate into proto-myofibroblasts, which form cytoplasmic actin-containing stress fibres that terminate in fibronexus adhesion complexes (Tomasek et al., 1991; 2002). Proto-myofibroblasts also express and organize cellular fibronectin at the cell surface and are capable of generating contractile force. The combination of transforming growth factor β1 (TGF-β1) with cellular fibronectin and continued mechanical tension facilitates the final stage in formation of the differentiated myofibroblast (Fig. 1.3). Therefore it is likely that during the initiation and progression of Dupuytren’s disease the initial mechanical stress triggers fibroblast differentiation and production of TGF-β1, leading to increased mechanical stress and subsequently a negative cycle of increased matrix production, fibroblast differentiation to myofibroblasts and contraction. This cycle of degradation and production of the matrix leads to shortening and contracture (Fig. 1.4).

**Figure 1.3 Two stage model of myofibroblast differentiation** Mechanical tension and the expression of specific growth factors lead to fibroblast differentiation into myofibroblasts. Increasing myofibroblasts increases tension and propagates the transition (from Tomasek et al., 2002).
1.8.2 The role of the immune system in Dupuytren’s disease

The possibility of Dupuytren’s being a T-cell mediated autoimmune condition has been proposed and the finding of increased numbers of DR+ T-cells in the circulation may support an immunological mechanism in its pathogenesis (Gudmundsson et al. 1998). The role of the highly polymorphic human leucocyte antigen (HLA) has also been investigated, particularly in relation to genetic susceptibility to develop fibromatosis (Gudmundsson et al., 1998). A significant association between affected patients and HLA-DR3 and autoantibodies to proteins contained in the extra-cellular matrix (elastin and collagen types 1-4) suggests a correlation with the process of remodelling during the course of fibrosis (Neumuller et al., 1994).

McCarty and colleagues have explored the immunogenetic component of this condition and published a review of previous studies (McCarty et al., 2010). These authors concluded: “There is a clear association with specific HLA alleles and predilection or protection to Dupuytren’s disease. In particular, the class II HLA-DR locus is the most
promising region for a biomarker of Dupuytren’s disease. The ability to accurately perform an assessment of an individual’s potential immunogenetic susceptibility to Dupuytren’s disease may potentially lead to a more personalized approach to their diagnostic, therapeutic and prognostic management in the future”.

More recently, a characterization of the cells present in Dupuytren’s tissue using immunohistochemistry found significant numbers of immune cells, including classically activated macrophages. High levels of proinflammatory cytokines (eg tumour necrosis factor, TNF) were also detected in tissue from Dupuytren’s patients (Verjee et al., 2013). Verjee et al. also demonstrated a link between TNFa and the differentiation of fibroblasts into myofibroblasts, particularly in cells isolated from Dupuytren’s tissue. This suggests that the microenvironment may influence fibroblast phenotype, and that these changes could be mediated by the immune system. These studies indicate that the immune system may play an important role in the etiology and pathology of Dupuytren’s disease. However, whilst inflammation has a strong link to fibrosis during wound healing, the mechanistic link between immune activation and Dupuytren’s disease is not clear. Many of these observations may be indicative of immune responses rather than drivers of the disease, and the potential importance of other mechanisms cannot be overlooked.

1.8.3 A role for vascular and innervation changes in Dupuytren’s disease

The search for control factors that may influence the growth and activity of myofibroblasts and the production of collagen in the extracellular matrix has provided an important focus for extensive laboratory investigations over the closing decades of the 20th century. Morphological evidence consistent with localized microvascular ischaemia (Hussl, 1979; Maccallum & Hueston, 1962; Murrell & Hueston, 1990) in Dupuytren’s tissue has been observed.

Murrell and colleagues (Murrell et al., 1987; Murrell et al., 1989) also showed that superoxide free radicals and hydroxyl radicals, which are produced during the metabolism of adenosine triphosphate to xanthine and uric acid, are released in Dupuytren’s tissue. “A six-fold increase in the concentration of hypoxanthine in Dupuytren’s tissue and in the
activity of xanthine oxidase have been demonstrated and these have a stimulatory effect on the proliferation of fibroblasts” (Thurston, 2003). These findings are significant in light of the strong association between Dupuytren’s disease and gout, diabetes mellitus and smoking, with their inherent metabolic and peripheral vascular changes known to be associated with these diseases. In particular the link between Type II diabetes mellitus and the incidence of Dupuytren’s disease suggests the peripheral vascular changes may be important in this disease. Other studies have clearly shown that vascular changes can influence fibroblast phenotype (Misra et al., 2010; Watson et al., 2014), with hypoxia leading to a more myofibroblast phenotype and likely to be important in fibrosis in at least some tissue types. However, to date the evidence in Dupuyren’s disease is associative rather than causative, and no clear studies have demonstrated a link between changes in vascular function and Dupuytren’s pathology.

1.9 Evidence for candidate susceptibility genes in Dupuytren’s disease
Identification of proteins/genes important in Dupuytren’s disease has largely been focused on two approaches. One is the identification of genes associated with Dupuytren’s disease through standard genetic approaches. The other is the proteomic or transcriptomic analysis of disease tissue compared to control tissue (commonly in the same patients). Both these approaches provide an opportunity for insight into the molecular pathology of the disease, but to date no conclusive evidence for the mechanism of induction of Dupuytren’s disease or the specific mutations involved has been generated.

As early as 1984 Iwasaki and colleagues studied the histopathology of Dupuytren’s disease and concluded that growth factors induce proliferation of genetically abnormal myofibroblasts (Iwasaki et al.,1984). Subsequently, the studies of Baird (Baird et al., 1993) among others have implicated transforming growth factor β (TGFβ) cytokines and receptors in the regulation of cell proliferation, migration and differentiation, and therefore as a major factor in the pathogenesis of Dupuytren’s disease (Alioto et al., 1994; Badalamente et al., 1996; Berndt et al., 1995; Kloen et al., 1995; Tomasek et al., 1999).
These receptors were therefore seen to represent candidate susceptibility genes for Dupuytren’s disease and were submitted to further investigation (Bayat et al., 2003). A statistically significant difference in genotype frequency distributions between cases and controls for TGFβR1 polymorphisms in the recessive model was observed in these studies. The genotype or allele frequency distributions for other TGFβ receptors were not significantly different between controls and Dupuytren’s patients. Their conclusion therefore, was that Dupuytren’s appears to be a complex oligogenic condition in which the TGFβ signaling system plays an essential role. From a more recent study by Zhang et al. a gene expression analysis of Dupuytren’s cord tissue, over twenty unique genes (including several previously reported) were identified and found to be significantly upregulated, ie expressed uniformly at much higher levels than in the controls (Zhang et al., 2008). Shih et al. (2009) performed similar genetic studies for identification of biomarkers in Dupuytren’s tissue by comparative analysis with non-specific fibroblasts (Shih et al., 2009) while differentially expressed genes were identified in fibroblasts from Dupuytren’s tissue by Satish and colleagues (Satish et al., 2011). While increased expression of these genes suggests that they play a role in the disease process it is not clear if this is promoting the disease or a response to ameliorate the fibrosis.

Genetic approaches have led to the identification of several chromosomal regions thought to be associated with Dupuytren’s disease. One of the earlier genetic studies reported mapping of an autosomal dominant gene for Dupuytren’s to chromosome 16q in a Swedish family (Hu et al., 2005; Shih et al., 2011). However, this has not been supported by more recent investigations.

Shih et al. also noted the potential role for Matrix Metalloproteases (MMPs) and the Wnt signalling pathway in Dupuytren’s disease (Shih et al., 2012). This is supported by the findings of a large genome-wide association study (GWAS) into the genes associated with Dupuytren’s disease (Dolmans et al., 2011; Ojwang et al., 2010). This study, reported in the New England Journal of Medicine, involved a GWAS of 960 Dutch persons with Dupuytren’s phenotype and 3117 controls, to test for association between the disease and genetic markers. Nine different loci involved in genetic susceptibility to Dupuytren’s
disease were identified, six of them found to harbour genes encoding proteins involved in the Wnt-signalling pathway. This finding strongly suggests that aberrations in this pathway are key to the process of fibromatosis in this disease. However, whilst this data is convincing again the functional data linking the genes identified with Dupuytren’s disease remains lacking, and whilst Wnt signalling is known to be important in fibrosis (Piersma, 2015), again there is limited data directly linking specific genes within this pathway or mutations in specific genes to Dupuytren’s disease. Other signalling pathways such as the ‘sonic hedgehog pathway’ and the ‘TGFβ signalling pathway’ are also known to involve genes that are linked to Dupuytren’s development and progression of fibrosis (Fig.1.5), and are thought to converge and result in fibroblast to myofibroblast transition, thus promoting the progression of Dupuytren’s disease (Fig.1.5).

![Figure 1.5 Signaling pathways with evidence of involvement in Dupuytren’s disease](image)

**Figure 1.5 Signaling pathways with evidence of involvement in Dupuytren’s disease**

WNT, TGF and Mechontransduction pathways have all been implicated in the pathogenesis of Dupuytren’s disease through genetic, molecular and cellular studies (taken from Musumeci M et al, 2015)

Unravelling the signalling pathways promoting fibrosis in Dupuytren’s disease has revealed TNF (tumour necrosis factor) as the only pro-inflammatory protein mediator (cytokine) to selectively convert normal fibroblasts from the palm of patients with
Dupuytren’s disease into myofibroblasts via the Wnt signalling pathway (Verjee et al., 2013). The same authors reported reversal of the myofibroblast phenotype by blockade of TNF (by reducing expression of alpha smooth muscle actin and inhibiting contractile activity of myofibroblasts) and concluded that TNF inhibition may be considered a therapeutic target in the prevention of progression or recurrence of Dupuytren’s disease.

From analysis comparing fibroblasts with disease-specific phenotype from tissue biopsies to control cells Shih et al. found 25 candidate genes, further short-listed to six genes via functional annotation, and subsequently identified ADAM12, POSTN and TNC as potential biomarkers for Dupuytren’s disease. The review by same authors showed two genes highlighted by both gene-expression profiling and whole genome association studies – MAFB and PRKX (a sex-linked protein kinase), whilst MAFB has also been associated with Dupuytren’s disease in another study (Lee et al., 2006).

Tissue studies for gene expression in fibroblasts have also been used to demonstrate transcription profile differences between Dupuytren’s disease and normal control fibroblasts (Forrester, 2013). Results indicate that in Dupuytren’s disease there is an excess of collagen and other ECM that is not controlled due to a reduction in matrix metalloproteinases and other matrix remodelling proteins (Shih, 2009; Forrester, 2013). A reduction in the fibrotic control protein, follistatin, may also contribute to development of Dupuytren’s disease (Forrester, 2013). In summary, there have been a number of studies investigating the molecular pathology and genetics of Dupuytren’s disease. Whilst many of the studies have small sample numbers and limited power, the same consistent themes have emerged over time. A role for Wnt signaling has been supported in many genetic and expression-based studies, whilst the TGFβ pathway has also been heavily implicated, as in many other fibrotic diseases (Shih, 2012, Fig. 1.5)). Nevertheless, there remains a gap in the knowledge to demonstrate the molecular pathogenesis of Dupuytren’s disease. Genetic approaches remain one of the most likely options to progress our understanding of this disease.
Despite many years of investigation into the molecular and genetic factors that may be important in Dupuytren’s pathology, no clear mechanism has yet been described to explain the triggers that lead to Dupuytren’s fibrosis. The use of disease tissue is limited by the difficulty of identifying responsive and causative changes, whilst genetic studies to date remain inconclusive without the necessary additional functional validation. Therefore there is a need for further investigation into the possible causes of Dupuytren’s disease at the molecular and genetic level. Understanding the triggers and key pathways that are important in Dupuytren’s disease will be critical to change the treatment paradigm from one of symptom limitation to disease prevention. In this thesis, different genetic strategies have been used to identify genes that may be important in the pathogenesis of Dupuytren’s disease in the Western Australian population. The clear genetic basis for the disease in specific families provides an opportunity to identify causative rather than responsive genes involved in the pathology. Therefore the first study was to investigate potential causative mutations in an individual Western Australian family with inherited Dupuytren’s disease.
Hypothesis

That heritable Dupuytren’s disease is caused by a non-synonymous mutation leading to defective protein function and the onset of palmar fibromatosis.

Aims

1. To use whole exome sequencing (WES) of a Western Australian family with autosomal dominant inheritance of Dupuytren’s disease to identify putative causative mutations.

2. To investigate the presence of the putative mutations in a wider population-based cohort of people with Dupuytren’s disease.

3. To evaluate the putative mutations from Aims 1 and 2 in unrelated individuals with Dupuytren’s disease.

4. To investigate the functional consequences of the putative mutations identified in Aims 1-3 on fibroblast phenotype and pathobiology with relation to Dupuytren’s disease.
CHAPTER 2

Aim 1. To use whole exome sequencing (WES) of a Western Australian family with autosomal dominant inheritance of Dupuytren’s disease to identify putative causative mutations.

2.1 Introduction

The genetic defects underlying inherited traits have traditionally been investigated using linkage analysis. This technique involved the use of microsatellite markers located throughout the genome and found in linkage disequilibrium as the basis for identifying regions inherited in affected individuals within a family and not in those that were unaffected, ‘linking’ the causative defect to the regions identified (Chistiakov et al., 2006). In the case of Dupuytren’s disease, a previous linkage study has been performed in a Swedish population and suggested an important region was located on Chromosome 16. The single region identified was approximately 30cM in size, bound by markers D16S3131 and D16S514 with a LOD score of >1.5. The LOD score was improved to LOD 3.18 when two affected cousins were genotyped with additional microsatellite markers, supporting linkage to the region on chromosome 16 (Hu et al., 2005). However, traditional linkage studies such as this have been limited by low resolution and an inability to progress to identification of specific genes of interest.

More recent approaches using single nucleotide polymorphisms (SNPs) as markers throughout the genome (of which >2 million have been identified) to identify regions of interest have provided much higher resolution and greater ability to identify gene mutations involved in disease (Stranger et al., 2011). These studies, however, are still not optimal and significant challenges in identifying causative mutations and functional data nevertheless remain (Edwards et al., 2013).
One large population study reported in the New England Journal of Medicine identified nine possible Dupuytren’s disease-associated loci on three different chromosomes (7, 19 and 22), in a GWAS involving 960 Dupuytren’s patients and over 3000 controls (Dolmans et al., 2011). Six of the nine associated loci were found to harbour genes encoding proteins involved in the Wnt-signaling pathway. However no functional evidence supporting a role for the Wnt-signaling pathway or identified genes was provided, and the presence of multiple other genes residing in the identified regions leaves doubt as to the relative importance of Wnt-signaling to Dupuytren’s pathology.

An alternative approach that has recently been used in studies of inherited disease within families is the use of whole exome sequencing (WES) (Rabbani et al., 2014). This approach involves sequencing the part of the genome encoding for proteins to identify variants that are likely to affect protein function and therefore underlie disease. This approach is limited in that only functional variants (rather than those affecting expression levels, for example within promoter regions) can be identified. However, many disease-causing variants (mutations) have been identified using this approach, which has the significant advantage over GWAS methodology in actually identifying variants in specific genes that can be further characterized (Bao et al., 2014; Rabbani et al., 2014).

Here, we have used WES in four members of a Western Australian family with autosomal dominant Dupuytren’s disease. The four members of the family initially sequenced were all affected individuals with Dupuytren’s disease. Heterozygous, non-synonymous variants present in all 4 individuals were identified. These variants were subsequently investigated using PCR in other affected and unaffected members of the family to determine those most likely to underlie the Dupuytren’s pathology.
2.2 Methods

2.2.1 Ethics Approval
All human studies complied with the National Health and Medical Research Council statement on ethical conduct of human studies and were approved by the Notre Dame Human Research Ethics Committee. All participants received a patient information sheet and had any questions answered by Dr Pearce prior to providing informed consent (Appendix 1).

2.2.2 Participants
Members of a family related to a previous patient of Dr Pearce who had been identified as having Dupuytren’s disease were identified and examined for phenotypic evidence of Dupuytren’s disease by Dr Pearce. Related siblings, children of a second generation and spouses by marriage were included (Fig. 2.1). All members were interviewed and examined by Dr Pearce for the presence of Dupuytren’s disease. DNA was collected from all consenting participants.

2.2.3 Saliva collection for genomic DNA isolation
Sputum samples were collected from fifteen members who were clinically diagnosed with Dupuytren’s disease and from eight non-affected family members (Figure. 2.1) using Oragene saliva collection kits (DNAGenotek) and stored at 4 °C until DNA was isolated.

Figure 2.1: Deidentifed family pedigree of family with autosomal dominant inheritance of Dupuytren’s disease

Affected family members are highlighted with red, unaffected samples are completely white. Deceased family members have a strikethrough symbol as per standard representation.
DNA samples were collected from 23 members of the family including affected and non-affected individuals. Red indicates affected individual with clinical diagnosis of DD.

2.2.4 DNA isolation protocol

DNA was isolated using the manual purification protocol (as per manufacturer’s instructions). This procedure produced a median yield of 110µg of DNA from 2ml of saliva collected in a 4ml Oragene saliva collection tube.

2.2.5 DNA quantitation

DNA samples were quantified using a Nanodrop spectrophotometer (ND-1000, Thermoscientific Instruments). For each sample a 1µl aliquot was removed and DNA concentration calculated.

2.2.6 Whole exome sequencing (WES)

2.2.6.1 SOLiD Platform

WES was initially performed for 2 affected and one unaffected family member (D3, D12, D18, Fig. 2.1) at the Lotterywest State Biomedical Facility Genomics (LSBFG) node (Perth, Western Australia) in a previously described method (Ravenscroft et al., 2013). Sonication was used to fragment 3µg of DNA from each individual; DNA was fragmented by and ligated to sequencing adaptors (SOLiD). Exomic sequences were enriched from the resulting libraries (SeqCap EZ Human Exome Library v2.0 exome capture system; Nimblegen, Roche Diagnostics). Sequencing was performed using a 5500XL SOLiD™ system (Applied Biosystems). After alignment the exomes were covered to >20-fold for at least 80% of the targeted sequence.
Investigating the Molecular Pathology of Dupuytren’s Disease

LifeScope™ 2.5 (Life Technologies) was used to call variants against the GRCh37 human reference genome, which were subsequently filtered using ANNOVAR (Wang et al., 2010). Filtering included exclusion of variants in the 1000 Genomes database (Clarke et al., 2012) if variants had a minor allele frequency greater than 1%, and removal of SNPs in the dbSNP135 common database.

2.2.6.2 Ion Proton
A second whole exome sequencing approach was next undertaken using the Ion Proton™ platform (Ampliseq chemistry, Life Technologies) and was also performed at the LSBFG, as previously described (Todd et al., 2015). Four affected family members (D4, D6, D8, D31) were processed with this technique. Twelve PCR pools were used to create libraries from 100ng of DNA from each individual. Libraries were purified and amplified for two rounds using AMPure beads (Beckman Coulter) and Platinum® High-Fidelity Taq Polymerase respectively. An Ion Proton™ Template 200 v3 kit was used to attach Ion Sphere™ Particles to the libraries (diluted to 18-26pM). Sequencing was performed using a P1 sequencing chip with an Ion Proton sequencer™.

All exomes were covered to >20-fold for >90% of the targeted sequence. Sequence variants were analysed using in-house scripts, again based on ANNOVAR software. Variants were excluded if they appeared at a frequency of >0.5% in the Exome Sequencing Project, 1000 Genomes or ExAC Browser databases, and if they appeared in the dbSNP137 common database. Pathogenicity predictions were made using the online prediction software programs SIFT, PolyPhen (Flanagan et al., 2010) and MutationTaster (Schwarz et al., 2014). The GEMINI program was also utilized for variant analysis (Paila et al., 2013)

2.2.6.3 Illumina
Illumina whole exome sequencing (exome capture kit SureSelectXT V5+UTR; TheragenEtex, Korea) was performed on 2 affected family members (D4 and D8). All exomes were >90% covered to >20-fold. The file processing from FASTQ to VCF was performed as per the GATK best practices guidelines version 3.6 (https://software.broadinstitute.org/gatk). Variant annotation was performed with in-
house scripts using the ANNOVAR and GEMINI software programs, as per the Ion Proton section described above.

2.2.7 Linkage analysis

Variants were extracted from the Ion Proton whole exome sequencing data using the vcf2linkdatagen.pl script from LINKDATAGEN (Bahlo & Bromhead, 2009) to infer genotypes at the location of HapMap Phase II SNPs (https://www.ncbi.nlm.nih.gov /pmc/articles/ PMC2689609/). This allows linkage analysis to be performed without requiring genotyping arrays. Mendelian inheritance errors were identified and removed. MERLIN (Abecasis et al., 2002) was used to perform parametric multipoint linkage analysis, using a fully penetrant dominant disease model. Linkage calculations were performed using an assumed disease allele frequency of 0.0001.

2.2.8 Variant confirmation and prioritization

Variants identified were prioritized according to likely functional relevance to Dupuytren’s pathology and the predicted pathogenicity of the variants. Pathogenicity of single nucleotide variants was estimated by the use of the SIFT, PolyPhen2 and Mutation Taster tools (Adzhubei et al., 2010; Schwarz et al., 2010; Sherry et al., 2001). For selected variants PCR and Sanger sequencing confirmation of the presence/absence of the variant in affected/unaffected family members was used.

2.3 Results

2.3.1 Genes identified using the SOLiD platform

The initial experiment involved WES of 3 affected family members using the SOLiD platform. This process identified four hundred variants common to all three family members sequenced. This number was significantly reduced by selecting only those that were both non-synonymous (therefore more likely to be disease causing) and heterozygous (given the autosomal dominant nature of inheritance). Also by using two custom variant-filtering steps: (1) one against the 1000 Genomes database (February 2012
release) and eliminating any variants detected in >1% of the population, and (2) one against the dbSNP135 common database and again eliminating any common SNPs identified. This resulted in a final list of 43 identified genes containing non-synonymous heterozygous mutations (Table 2.1).

For further family member sequencing a subset of this total (9 candidates) was selected. The list was refined by using biological databases to identify those genes with the most likely relevance to what is known of Dupuytren’s pathology (for example search terms included collagen deposition/secretion; skin; male hormones; diabetes). Databases (including ENSEMBL and Genecards) were both used to investigate gene function. In addition each mutation was assessed for likely deleterious impact using in silico predictive tools. A list of nine candidate genes was ultimately selected using this approach (Table 2.2).

Table 2.1: list of candidate genes from WES analysis. Final candidate gene list with all genes containing a non-synonymous heterozygous variant as outlined in the methods section. These genes were identified by comparative analysis of only affected family member DNA samples.

All 43 genes contain a non-synonymous variant and are heterozygous in the family members analysed by WES
Table 2.2: Selected mutations for initial SNP analysis

<table>
<thead>
<tr>
<th>Known SNP?</th>
<th>3000 Genomes</th>
<th>EVS</th>
<th>PolyPhen2</th>
<th>Mutation Taster</th>
<th>sift</th>
<th>Polyphen2</th>
<th>N/A</th>
<th>0.01%</th>
<th>0.1%</th>
</tr>
</thead>
</table>
| NO         | N/A           | N/A | N/A       | N/A            | N/A  | N/A       | 0.66%| 0.99% | 0.9%
| NO         | N/A           | N/A | N/A       | N/A            | N/A  | N/A       | 0.97%| 0.98% | 0.9%
| NO         | N/A           | N/A | N/A       | N/A            | N/A  | N/A       | 0.1% | 0.99% | 0.9%
| NO         | N/A           | N/A | N/A       | N/A            | N/A  | N/A       | 0.1% | 0.99% | 0.9%
| NO         | N/A           | N/A | N/A       | N/A            | N/A  | N/A       | 0.1% | 0.99% | 0.9%
| NO         | N/A           | N/A | N/A       | N/A            | N/A  | N/A       | 0.1% | 0.99% | 0.9%

In-silico Prediction

- N/A
- 0.01%
- 0.1%
- 0.66%
- 0.97%
- 0.1%
- 0.1%
- 0.1%
- N/A

Population Genetic

- N/A
- 0.01%
- 0.1%
- 0.66%
- 0.97%
- 0.1%
- 0.1%
- 0.1%
- N/A

Function

- N/A
- 0.01%
- 0.1%
- 0.66%
- 0.97%
- 0.1%
- 0.1%
- 0.1%
- N/A

Variant
2.3.1.2 PCR fragment sequencing in additional family members

Sanger sequencing was then conducted on short fragments containing each gene mutation in additional family members. Heterozygous mutations were identified by the presence of two alternate reads at the single nucleotide position (Figure 2.2A). Non-affected and affected family members were sequenced.

None of the identified mutations segregated with the affected members of the family (Table 2.3). All mutations were not present in at least 2 affected family members, except for the ADAM15 gene mutation that was only not present in one affected family member (Table 2.3).

![Figure 2.2](image)

Figure 2.2: Sanger sequencing in family members for heterozygous non-synonymous variants in selected genes.

Heterozygous nucleotides are identified by the presence of two peaks at the individual sequencing site being investigated (A), compared to the single peak observed for homozygous nucleotides (B). Arrow indicates nucleotide position of interest.

No variants segregated in all affected family members (Y is a positive sample containing heterozygous variants, and N is a sample not containing the heterozygous variant).
Table 2.3: Sanger sequencing of putative causative variants in additional affected and non-affected family members

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>ADAM15</th>
<th>KLF11</th>
<th>CDCC80</th>
<th>ANKRD31</th>
<th>SERPINA1</th>
<th>VP541</th>
<th>VP513A</th>
<th>EXOC6B</th>
<th>JHDM1D</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>3</td>
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<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
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<tr>
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<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>6</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>8</td>
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<td>N</td>
<td>N</td>
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<td>Y</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>10</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>12</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>18</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>17</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

No identified genes contained the SNP of interest in all affected patients tested and mutations were identified in family members that continue to be unaffected at the time of the study making them unlikely candidates.

Prior to assessing other candidate variants in the family an additional clinical visit was made to confirm diagnosis of those family members that were affected. During this revisiting process a family member was identified that had been incorrectly classified as an affected family member when there was no evidence of the disease at the follow-up visit. Therefore additional WES was then conducted on 2 other family members that were identified as affected during the clinical visits.

2.3.2 Genes identified using the Ion Proton sequencing platform

The misdiagnosed exome sequence was removed from analysis and the identification process was repeated, this time using the Ion Proton sequencing platform. This resulted in a list of 28 variants being identified that were common to all four affected exomes sequenced (Table 2.4). All four affected family members sequenced for this analysis were positive for Dupuytren’s disease at the follow-up clinical visit.
**Investigating the Molecular Pathology of Dupuytren’s Disease**

Table 2.4: Variants identified in 4 family members by WES

List of 28 variants identified by WES of four affected family members with Dupuytren’s disease (all variants were heterozygous and present at <0.1% in the ExAC database). Table shows Chromosomal location, reference and actual base at this position, gene name, type of mutation and the % present in the ExAC database.

<table>
<thead>
<tr>
<th>Chr</th>
<th>Start</th>
<th>Ref</th>
<th>Alt</th>
<th>Gene</th>
<th>Mutation</th>
<th>ExAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>228596057</td>
<td>C</td>
<td>T</td>
<td>TRIM17</td>
<td>non-synonymous SNV</td>
<td>0.0002</td>
</tr>
<tr>
<td>10</td>
<td>135368906</td>
<td>G</td>
<td>C</td>
<td>SYCE1</td>
<td>synonymous SNV</td>
<td>0.0041</td>
</tr>
<tr>
<td>13</td>
<td>52693478</td>
<td>A</td>
<td>G</td>
<td>NEK5</td>
<td>nonsynonymous SNV</td>
<td>0.0001</td>
</tr>
<tr>
<td>13</td>
<td>46562926</td>
<td>C</td>
<td>T</td>
<td>ZC3H13</td>
<td>synonymous SNV</td>
<td>0.0089</td>
</tr>
<tr>
<td>15</td>
<td>63014680</td>
<td>G</td>
<td>A</td>
<td>TLN2</td>
<td>synonymous SNV</td>
<td>0.0032</td>
</tr>
<tr>
<td>17</td>
<td>8167600</td>
<td>TG</td>
<td>GC</td>
<td>PFAS</td>
<td>non-frameshift substitution</td>
<td>absent</td>
</tr>
<tr>
<td>17</td>
<td>4462264</td>
<td>G</td>
<td>A</td>
<td>GGT6</td>
<td>synonymous SNV</td>
<td>0.006</td>
</tr>
<tr>
<td>18</td>
<td>47778134</td>
<td>C</td>
<td>T</td>
<td>CFAP53</td>
<td>non-synonymous SNV</td>
<td>0.0024</td>
</tr>
<tr>
<td>22</td>
<td>19750797</td>
<td>C</td>
<td>T</td>
<td>TBX1</td>
<td>synonymous SNV</td>
<td>0.003</td>
</tr>
<tr>
<td>3</td>
<td>169485336</td>
<td>A</td>
<td>G</td>
<td>ACTRT3</td>
<td>non-synonymous SNV</td>
<td>0.0033</td>
</tr>
<tr>
<td>3</td>
<td>130107567</td>
<td>T</td>
<td>G</td>
<td>COL6A5</td>
<td>non-synonymous SNV</td>
<td>0.0034</td>
</tr>
<tr>
<td>3</td>
<td>52383001</td>
<td>G</td>
<td>A</td>
<td>DNAH1</td>
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</tr>
<tr>
<td>3</td>
<td>38565782</td>
<td>C</td>
<td>T</td>
<td>EXOG</td>
<td>non-synonymous SNV</td>
<td>0.0019</td>
</tr>
<tr>
<td>3</td>
<td>39145040</td>
<td>T</td>
<td>C</td>
<td>GORASP1</td>
<td>non-synonymous SNV</td>
<td>5.04E-05</td>
</tr>
<tr>
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<td>52836533</td>
<td>G</td>
<td>A</td>
<td>ITIH3</td>
<td>non-synonymous SNV</td>
<td>0.0087</td>
</tr>
<tr>
<td>3</td>
<td>129150385</td>
<td>C</td>
<td>G</td>
<td>MBD4</td>
<td>non-synonymous SNV</td>
<td>0.0054</td>
</tr>
<tr>
<td>3</td>
<td>140401440</td>
<td>C</td>
<td>T</td>
<td>TRIM42</td>
<td>non-synonymous SNV</td>
<td>0.0009</td>
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<tr>
<td>3</td>
<td>165547451</td>
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<td>T</td>
<td>BCHE</td>
<td>synonymous SNV</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>169555275</td>
<td>A</td>
<td>G</td>
<td>LRRIQ4</td>
<td>synonymous SNV</td>
<td>0.0045</td>
</tr>
<tr>
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<td>52507794</td>
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<td>A</td>
<td>NISCH</td>
<td>synonymous SNV</td>
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<tr>
<td>3</td>
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<td>G</td>
<td>COX18</td>
<td>non-synonymous SNV</td>
<td>0.0052</td>
</tr>
<tr>
<td>6</td>
<td>31238909</td>
<td>GT</td>
<td>AG</td>
<td>HLA-C</td>
<td>non-frameshift substitution</td>
<td>Absent</td>
</tr>
</tbody>
</table>
2.3.2.1 Linkage analysis

Of these 28 variants identified, 17 were non-synonymous (Table 2.4). In addition to the identification of variants of interest, linkage analysis was also used to screen for regions of positive linkage and also to screen those regions that were definitely not linked to the disease (Table 2.5. Fig. 2.3).

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position</th>
<th>SNP</th>
<th>Gene</th>
<th>Type</th>
<th>P-value</th>
</tr>
</thead>
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<td>C</td>
<td>OSBPL3</td>
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</tr>
<tr>
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<td>136419629</td>
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<td>A</td>
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</tr>
<tr>
<td>9</td>
<td>130472886</td>
<td>A</td>
<td>G</td>
<td>CFAP157</td>
<td>0.0002</td>
</tr>
<tr>
<td>9</td>
<td>130265116</td>
<td>C</td>
<td>T</td>
<td>LRSAM1</td>
<td>0.0001</td>
</tr>
<tr>
<td>9</td>
<td>126213026</td>
<td>T</td>
<td>C</td>
<td>DENND1A</td>
<td>0.0007</td>
</tr>
</tbody>
</table>
Table 2.5: Linkage analysis to eliminate candidate genes. Only 14 potential candidate genes remain after screening:

Regions could be eliminated using linkage scores across this chromosome, leading to the removal of VPRBP, DNAH1, NISCH and ITIH3 as potential candidate genes due to their location within negative regions (Table 2.5, Figure, 2.3). The remaining list contained 14 potential candidate genes. Of these, only EXOG and GORASP1 were within regions with positive linkage scores (Table 2.6).

The LOD score was calculated for individual SNPs across a region of chromosome 3. SNPs were assessed for positive (green), neutral (yellow) or negative (pink) LOD scores.
Figure 2.3: Linkage analysis of individual chromosomes.

Linkage analysis identifies positive and negative regions in WES of 4 affected family members. a/b show regions across Chromosome 3, which could then be used to eliminate candidate genes within the negative regions, including VBPRBP and DNAH1.
Investigating the Molecular Pathology of Dupuytren’s Disease

Table 2.6: Non-synonymous variants remaining after removal of those with a negative linkage score

Genes highlighted in bold are those with a positive linkage score, other genes not in bold were not excluded by linkage analysis.

<table>
<thead>
<tr>
<th>Chr</th>
<th>Start</th>
<th>Ref</th>
<th>Alt</th>
<th>Gene</th>
<th>Mutation</th>
<th>ExAC</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>228596057</td>
<td>C</td>
<td>T</td>
<td>TRIM17</td>
<td>non-synonymous SNV</td>
<td>0.0002</td>
</tr>
<tr>
<td>13</td>
<td>52693478</td>
<td>A</td>
<td>G</td>
<td>NEK5</td>
<td>non-synonymous SNV</td>
<td>0.0001</td>
</tr>
<tr>
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<td>C</td>
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<td>non-synonymous SNV</td>
<td>0.0033</td>
</tr>
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<tr>
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<td>A</td>
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<td>9</td>
<td>130265116</td>
<td>C</td>
<td>T</td>
<td>LRSAM1</td>
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<td>0.0001</td>
</tr>
</tbody>
</table>

12 genes were identified after refinement by linkage analysis, and only two were in regions of positive linkage (shown in bold).

2.3.2.2 In silico prediction

Using in silico prediction, the likelihood of each mutation being deleterious was assessed (Table 2.7). None of the 12 variants were predicted to be deleterious by all in silico analyses. The gene with the highest predicted value for being deleterious, and predicted to have a high impact on protein function, was COL6A5. Given the well-established importance of other collagen types in fibrosis, this gene was therefore investigated further. In addition, given that both GORASP1 and EXOG were also predicted to be potentially deleterious and were found in regions of positive linkage, these variants were also investigated further.
Use of in silico predictive tools to identify deleterious mutations and prioritise further analysis showed consensus across three different in silico platforms (SIFT, Polyphen and Mutationtaster) that the genes in a region of positive linkage contained mutations likely to be deleterious (D = deleterious, B or N benign or non-deleterious mutations; L and M suggest Low/Medium impact of the mutation)

<table>
<thead>
<tr>
<th>Gene name</th>
<th>SIFT_score</th>
<th>SIFT_p</th>
<th>Polyphen2_HDIV_score</th>
<th>Polyphen2_HVAR_score</th>
<th>Polyphen2_LRT_score</th>
<th>MutationTaster</th>
<th>MutationAssessor</th>
<th>MutationAssessor</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXOG</td>
<td>0.1 T</td>
<td>0.015 B</td>
<td>0.003 B</td>
<td>0.04 N</td>
<td>1 D</td>
<td>1.61 L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GORASP1</td>
<td>0 D</td>
<td>0.998 D</td>
<td>0.986 D</td>
<td>0 D</td>
<td>1 D</td>
<td>3.18 M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTRT3</td>
<td>0 D</td>
<td>0.996 D</td>
<td>0.958 D</td>
<td>0 D</td>
<td>0.999 D</td>
<td>3.31 M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFAP157</td>
<td>0.19 T</td>
<td>0.363 B</td>
<td>0.223 B</td>
<td>0 D</td>
<td>1 D</td>
<td>1.87 L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFAP53</td>
<td>0.03 D</td>
<td>1 D</td>
<td>0.999 D</td>
<td>0 D</td>
<td>1 D</td>
<td>2.045 M</td>
<td></td>
<td></td>
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<tr>
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<td>0 D</td>
<td>0.973 D</td>
<td>0.885 P</td>
<td>0.995 D</td>
<td>3.53 H</td>
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</tr>
<tr>
<td>COX18</td>
<td>0.12 T</td>
<td>0.61 P</td>
<td>0.3 B</td>
<td>0.43 N</td>
<td>1 N</td>
<td>1.355 L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LRSAM1</td>
<td>0.01 D</td>
<td>1 D</td>
<td>0.91 D</td>
<td>0 D</td>
<td>1 D</td>
<td>2.555 M</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0.998 D</td>
<td>0.983 D</td>
<td>0 D</td>
<td>1 D</td>
<td>1.445 L</td>
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<td></td>
</tr>
<tr>
<td>NEK5</td>
<td>0 D</td>
<td>1 D</td>
<td>0.998 D</td>
<td>0 D</td>
<td>1 D</td>
<td>2.965 M</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0.992 D</td>
<td>0 D</td>
<td>1 D</td>
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<td>0 D</td>
<td>1 D</td>
<td>0.981 D</td>
<td>0 D</td>
<td>0.967 D</td>
<td>1.34 L</td>
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<td></td>
</tr>
</tbody>
</table>

COL6A5 has the highest level of prediction to be deleterious across all predictive programs, and the variant was also predicted to have a high impact on protein function by Mutation Assessor scoring (Predicted types of mutation; T - tolerated, B - benign, D - deleterious; Impact of mutation predicted by Mutation Assessor as; L – low, M - medium, H – high).

2.3.2.3 Sequencing of COL6A5, GORASP1 and EXOG fragments in family members

To investigate whether these variants segregated within the family with Dupuytren’s disease, Sanger sequencing of PCR fragments was conducted using multiple family samples. Sequencing was focused on 3 candidate genes. GORASP1 and EXOG were both selected due to their identification in a region of positive linkage from initial analysis. In addition COL6A5 was selected for sequencing due to the fundamental link between collagen deposition and fibrotic disease. Initial sequencing showed that no variant segregated with the disease in additional family members (Fig. 2.4). Interestingly, no family member tested appeared to have a COL6A5 variant present (Fig. 2.4). Given the large number of siblings within the family being tested it was unusual to observe no heterozygotes in the PCR analysis. Investigation of the sequence underlying the original
primers used for PCR demonstrated the presence of additional SNPs that may have disrupted the amplification ability, leading to detection of only the wildtype allele. Therefore additional primers were designed and \textit{COL6A5} was again sequenced. However, \textit{COL6A5}, whilst positive for the variant in some family members, did not segregate with the disease in all family members tested, suggesting this variant is not disease-causating in the family being investigated (data not shown).

\textbf{Figure 2.4: Sanger sequencing of candidate gene fragments in additional family members.} Sequencing of \textit{COL6A5} (A), \textit{GORASP1} (B) and \textit{EXOG} (C) fragments in additional family members. No mutation appears positive in all disease positive family members tested. \textit{COL6A5} appears to have no heterozygotes present when tested with this primer set.
2.3.4 Genes identified using the Illumina platform

Illumina WES (exome capture kit SureSelectXT V5+UTR; TheragenEtex, Korea) was performed on DNA from two affected family members (D4 and D8). The variants identified using this platform included twelve new candidates (Table 2.8) in addition to those previously found by the use of SOLiD and Ion Proton sequencing platforms. Of the twelve novel variants identified using the new platform, none had been previously identified using other platforms, even if restricting the comparative sequencing to the two members sequenced using the Illumina platform (D4 and D8 only). Of the new genes five have previously been recorded in association with clinical conditions exhibiting inflammatory changes and immune reactions. No further investigation of these genes was undertaken at this point as a novel approach to triangulate alternative data sources was undertaken to further investigate the mutations underpinning Dupuytren’s disease.

Table 2.8: Additional genes identified that contained non-synonymous variants in family members sequenced using the Illumina WES platform.

<table>
<thead>
<tr>
<th>Gene Name</th>
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</thead>
<tbody>
<tr>
<td>ALPI</td>
<td>CYP2T3P</td>
<td>IRGC</td>
<td>PIK3R4</td>
<td>TCP11X3P</td>
<td>TRIM21</td>
</tr>
<tr>
<td>ANKRD36BP</td>
<td>HSPA12B</td>
<td>MEX3D</td>
<td>PSG8</td>
<td>TOLLIP</td>
<td>VNN3</td>
</tr>
</tbody>
</table>

The use of an alternative sequencing platform highlights new variants of interest, demonstrating the differences arising from the platform used. A number of these genes are related to inflammatory diseases, suggesting a possible role in Dupuytren’s disease.

2.3.5 Summary

WES of family members with diagnosed Dupuytren’s disease was used to investigate the presence of non-synonymous variants within the family that may be causative. Using three alternative sequencing platforms with subsequent PCR sequencing for mutations in the wider family, no individual variant was found to segregate with the disease in this family.
2.4 Discussion

2.4.1 Non-synonymous variants identified by WES in a family with Dupuytren’s disease

The data from the WES analysis of four related, affected individuals using the Ion Torrent platform identified non-synonymous heterozygous variants in 12 candidate genes that also conformed to the selection criteria. The three candidate genes that either showed positive linkage (GORASP, EXOG) or were known to have a strong likely functional link to fibrosis (COL6A5) were all subsequently tested using PCR for segregation within the family. None of the variants tested was shown to segregate with all affected members of the family, thereby ruling out these variants as being disease-causing. Therefore other variants identified should be followed up for this study.

COL6A5 was indicated as a strong possible candidate gene from the lists of genes containing non-synonymous variants due to the well-known important role of collagen in fibrosis (Wynn Ta, 2008). The COL6A1 subunit has previously been shown to be elevated in expression in Dupuytren’s tissue (Shih et al, 2009) and therefore other subunits of COL6 are potentially strong candidates. Collagen VI is a component of the extracellular matrix of almost all connective tissues, including cartilage, bone, tendon, muscles and cornea, where it forms abundant and structurally unique microfibrils organized into different suprastructural assemblies. The precise role of collagen VI is not clearly defined although it is most abundant in the interstitial matrix of tissues and often found in close association with basement membranes (Fitzgerald et al 2013).

A recent paper reviewing ‘Collagen Type VI-Related Disorders’ (Lampe, Flanigan, et al; 2004; 2012) discusses the clinical characteristics of several disorders that represent a continuum of overlapping phenotypes. These include Bethlem myopathy and Ullrich congenital muscular dystrophy (CMD) and two rare, less well-defined disorders, specifically autosomal dominant limb girdle muscular dystrophy and autosomal recessive myosclerosis myopathy. These phenotypes however share little overlap with
Dupuytren’s disease, and the subsequent findings that family members positive for the disease did not all share the mutation identified by WES suggest that this variant is unlikely to be causative for Dupuytren’s in this family.

However, it is important to consider that the lack of segregation does not necessarily preclude the variants from being important in the pathology of Dupuytren’s disease. It is possible, for example, that an individual member of the family could be positive for Dupuytren’s disease due to a somatic rather than an inherited mutation, particularly since Dupuytren’s disease is a relatively common disease. This would effectively mask the disease-causing mutation in most family members by providing a positive phenotype without the inherited mutation. It is also possible due to incomplete penetrance that there are members of the family who are ‘carriers’ of the causative mutation(s) but do not express the Dupuytren’s disease phenotype due to different environmental influences. Therefore, it is important to consider the genes identified as containing rare variants for potential functional relevance to the pathophysiology of Dupuytren’s disease.

The first list of potential candidates needed to be discarded due to errors in diagnosing family members, highlighting the importance of clinical diagnoses in genetic studies. Fortunately, the individual WES data for each person was still accurate and new comparative analyses could be performed based on the revised clinical diagnosis of the family member in question.

Subsequent to the Ion Proton sequencing used for 4 family members the additional Illumina sequencing of two affected individuals provided a list of a further 12 candidate genes with non-synonymous rare variants. These 24 potential candidate genes will need to be further investigated, with the focus initially on the 21 genes not yet investigated. Interestingly, the genes containing variants that may be associated with the disease appear to comprise subsets of genes with similar roles/functions. This may indicate the importance of certain processes in the pathogenesis of Dupuytren’s disease in this family.

Many of the genes containing non-synonymous variants have functions related to angiogenesis, immune responses and neural functions. Many of these processes have
important and well-established roles in wound healing and fibrosis, suggesting that the genes with non-synonymous variants may be good candidates for further investigation. Indeed, whilst it is clear that fibroblasts play an important role in fibrotic disease, it has also been suggested that rather than fibroblast dysfunction being the driver of fibrosis, it is external triggers of fibroblast collagen production that are altered (Fig. 2.5). As outlined in Figure 2.5, it has been shown that external signals impact significantly on fibroblast function and fibrosis. For example, keratinocytes have been shown to be important in keloid disease, whereby it is the epithelial cells that appear to drive the disease rather than changes to the fibroblasts. Similarly, immune drivers of fibrosis such as in sclerosis also occur external to the key collagen producing fibroblasts. Therefore, it is possible that the mutations that underpin Dupuytren’s disease exist in the current shortlist of candidate genes and influence the disease through an indirect influence on fibroblast activity. The possible role for these genes that have not been tested and the evidence for their likely involvement in Dupuytren’s pathogenesis are discussed in more detail below.

Figure 2.5: Factors influencing fibroblast differentiation and function.

Diagram illustrating the potential role for other physiological processes to influence fibroblast function and ultimately fibrosis in Dupuytren’s disease.
2.4.2 Genes involved in angiogenesis

The *HSPA12B* gene has an important role in endothelial cells and in angiogenesis (Steagall et al., 2006). Angiogenesis is an important process in tissue damage repair and has been implicated in fibrosis, although the role of angiogenesis remains controversial (Hanumegowda et al., 2012). It has been postulated that increased angiogenesis plays an important role in progressive fibrosis such as keloid scar formation (Zhang et al., 2015), but equally there is evidence that a lack of angiogenesis can induce hypoxia and a fibrotic response in damaged tissue (Lokmic et al., 2012). Despite the unclear role for angiogenesis in contributing to fibrotic disease, it is possible that this process is important in the progression or initiation of fibrosis. *HSPA12B* has also recently been shown to be protective against cardiac damage in a model of myocardial infarction (Li et al, 2013). In this study *HSPA12B* attenuated tissue damage through promotion of more rapid vascularization of damaged tissue and therefore faster repair. Therefore it could be hypothesized that a mutation in this gene could lead to increased or decreased vascularization, dependent on the mutation type, in response to the damage to tissue caused by extensive use of the hands over many years. A link between Dupuytren’s disease and vascular supply has previously been hypothesized due to the correlation between type II DM and the incidence of Dupuytren’s disease. Therefore *HSPA12B* remains a candidate gene for further investigation.

2.4.3 Genes with roles in inflammatory processes

Studies of genetic variants associated with susceptibility and mortality of idiopathic pulmonary fibrosis (IPF) have identified several rare mutations and one common SNP of *MUC5B* (Noth et al, 2013). Through a large international consortium and a genome-wide association study variants associated with the *TOLLIP* and *SPPL2C* genes were also found to be associated with IPF susceptibility. These associations and the reduced expression of *TOLLIP* in patients with IPF who carry *TOLLIP* SNPs emphasise the potential importance of this gene in IPF. The *TOLLIP* mutation identified by the Illumina platform in the affected Dupuytren disease family members has previously been reported in lymphoproliferative syndrome and inflammatory signaling (Cohen, 2014). Therefore
given the role of *TOLLIP* in IPF and its importance to immune function it is also a strong potential candidate gene for Dupuytren’s disease.

The genes *IRGC, TRIM21* and *VNN3* are all commonly highly expressed in a number of inflammatory and immune-related disorders. *VNN3* is one of two members of the vanin gene family found to be induced at the mRNA and protein level by psoriasis-associated pro-inflammatory cytokines (Jansen et al., 2009), whilst *TRIM21* has been investigated both for its role in physiological and pathological immunity (Yoshimi, 2012; McEwan, 2016). *TRIM21* has been shown to be protective against aberrant protein accumulation in a model of Alzheimer’s disease, whilst *TRIM21* is also known to have potent anti-viral effects. Given the diversity of roles for these genes and their importance to immune function it is reasonable to expect that significant mutations in these genes would lead to a more widespread phenotype than observed. Nevertheless there is evidence that the immune system may be an important contributor to the initiation of Dupuytren’s disease and these genes require further investigation.

**2.4.4 Candidate genes with neural functions**

The third ‘cluster’ identified appear to be genes associated with neural function. Whilst to date there is little evidence for a role of peripheral nerves in the development of Dupuytren’s disease, cutaneous innervation is well known to be important for wound healing and some recent evidence exists for a role in hypertrophic scar formation (Li, 2015; Lavardet, 2015; Roosterman, 2006; Cheret, 2013). Therefore it is important to assess mutations in genes with important roles in peripheral nerves as these may play an as yet unidentified role in Dupuytren’s pathology. The *LRSAM1* gene identified in this study has previously been associated with Charcot Marie Tooth (CMT) syndrome. This disorder begins with atrophy and weakness of distal muscles in the leg (e.g. peroneal), advancing insidiously to involve other muscles of the leg and arm. The hereditary polyneuropathy affects both sensory and motor pathways with disparate clinical presentations.
Peripheral neuropathy is a clinically variable and genetically heterogeneous disease. In a cohort of patients Gonzaga-Jauregui et al. identified causative variants in 45% of the families studied, proposed candidate disease genes for an additional three families, and recognized a significant variant burden in patients versus controls that likely contributes to phenotypic variability (Gonzaga-Jauregui et al., 2015). Whilst Dupuytren’s patients do not present with peripheral neuropathy, other peripheral nerve dysfunction may be important. For example the role that peripheral nerves can play in inflammation and the immune response may be important, similar to the studies to date suggesting immune dysfunction is central to the disease. However, at present with the limited evidence for an impact of innervation on Dupuytren’s disease candidate genes with functions relevant to peripheral nerves should be prioritized lower than those involved in other processes with stronger evidence such as the immune and vascular response.

2.4.5 The absence of genes identified in previous studies

The only known genome-wide analysis from members of one family of Dupuytren’s affecteds is the extensive investigation of a five generation Swedish family with autosomal dominant inheritance of the disease, reported by Hu et al. (2005). These investigators established linkage to a single 6cM region between markers D16S49 and D16S3032 on Chromosome 16q. There is no evidence from the current study to confirm this finding with no mutations observed in genes within this region.

Similarly the GWAS of 960 unrelated Dutch persons with the Dupuytren’s phenotype yielded an association with 11 SNPs from 9 different loci, six of which contain genes known to be involved in the Wnt-signalling pathway ie \( WNT4, SFRP4, WNT2, RSP02, SULF1 \) and \( WNT7B \). No mutations were found in any of these genes in the current investigation.

Meanwhile, several studies of gene expression analysis in Dupuytren’s disease, based on cord tissue samples from affected hands, have identified a larger number of genes with dysregulated expression levels in Dupuytren’s tissue (Zhang et al., 2008; Forrester et al., 2013). However no mutations in the genes with the most significant differences in
expression in these studies (for example in top 50 upregulated and down-regulated in Forrester et al) were found in this family.

As with many conditions where there is phenotypic variance, in this case related to age of onset, palmar involvement, degree of digital contractures, rate of progression of fibromatosis and racial differences, there is reason to consider multiple gene involvement and that it is changes to similar pathways that likely underlie the disease rather than common specific mutations in an individual gene or genes. It is also important to consider with the expression studies that the genes identified as being most dysregulated may not be related to the cause of the disease but rather their dysregulation may be a consequence of the disease, thus explaining the disparity between genetic studies and expression studies of this disease. Given the importance of environmental factors, the use of different populations in these studies compared to the West Australian family in this current study and the different approaches used it is not surprising that overlap has not been observed. However, WNT signaling has been extensively implicated in Dupuytren’s disease, and the absence of any genes with known critical functions in WNT signaling in this study is surprising. This may be due to the limitations of this study approach and the restrictions used in identifying mutations of interest (see section 2.4.7).

2.4.6 The advantages of using WES to identify causative mutations

One of the advantages of using a genetic approach is that it is potentially feasible to identify the trigger for Dupuytren’s disease. This is in contrast to a second common approach that uses diseased tissue as the source material for identifying transcriptome or proteomic changes related to Dupuytren’s disease. Because the use of diseased tissue invariably involves cells that have been cultured or isolated it is very difficult to identify causative changes as opposed to consequential changes. This is particularly true in diseases that are generally treated surgically at quite a late stage such as Dupuytren’s disease, where the time between disease initiation and obtaining tissue is substantial. During this period it is likely cells undergo many changes in phenotype and expression and therefore identifying the causative changes is a significant challenge.
The essential benefit of WES is its selective focus on the part of the genome encoding for proteins to identify non-synonymous mutations that are likely to affect protein function and therefore underlie disease. While only functional mutations (rather than those affecting expression levels, for example within promoter regions) can be identified, it reduces significantly the number of data points identified and therefore is more likely to be able to find disease-causing mutations. WES is restricted to mutations that change the coding sequence (although they can be synonymous) and therefore whilst this restricts the number of data points it also provides an opportunity to identify functional mutations in smaller populations such as the family investigated here.

Although Dupuytren’s disease can be sporadic, even within a pedigree exhibiting familial inheritance, use of the WES approach in a family cohort offers a strong likelihood of identifying the same disease-causing mutations in all affected samples – and similar phenotype of disease within the family would suggest a common cause. Compared with the GWAS type approach exome sequencing also provides a list of mutations with likely functional impact, rather than mapping results that link to likely regions of interest. This therefore provides a better opportunity for further functional investigation of the impact of the mutations and to demonstrate a functional link to the disease.

WES provides the possibility of locating rare functional changes within the protein-coding part of the genome (the exome) as using family members means the shared DNA is extensive and they will have the same causative mutation – whilst the exact mutation identified through WES in a family cohort may not apply to all affecteds the gene and/or pathway will be common to others and can therefore assist in better understanding the disease. The GWAS approach can only find common regions across large populations, and is not suitable to locate rare functional mutations.

2.4.7 Limitations of using the WES approach
The use of WES as a strategy to identify disease-causing mutations has been successful in many diseases and continues to be a mainstay of disease SNP identification strategies [Biesecker et al., 2011]. However, as with all strategies currently employed there are
limitations to this approach that can lead to misidentification or non-identification of mutations linked to disease (Sims et al., 2014). Less than 2% of the human genome contains protein-coding sequences and these regions must undergo exon-targeting capture process before being sequenced to identify clinically relevant alleles. It is important also to determine the coverage of these captured regions – i.e. depth of sequencing – as the higher the coverage the more likely the captured base will be accurate and not a false read due to technical error (Li et al., 2015). Inadequate coverage of certain exomes/genes could lead to false negatives and the omission of important variants. Equally, inadequate coverage can lead to false positive variant identification. As the goal of WES is to identify those mutations that may be potentially pathogenic it is important to have efficient strategies and appropriate variant analysis pipelines. Depending on filtering parameters applied it is possible for a pathogenic variant to be missed if it is not targeted for analysis. Targeted sequencing has the advantage of increased sequence coverage of regions of interest at lower cost and higher throughput compared with random sequencing methods.

Genome sequencing could avoid some of the technical challenges faced by WES but it remains expensive and requires more sophisticated analysis pipelines to filter and identify the increased number of potential variants. Indeed, it is unlikely that whole genome sequencing would provide suitable candidate lists for further investigation in a small family centric study such as that conducted here. However, filtering much larger datasets from small family studies such as that becomes increasingly difficult, and the relatively limited knowledge of the function of all non-coding bases in the DNA increases the complexity of identifying changes of interest when using whole genome sequencing.

Genetic variants identified using WES induce a phenotype that can vary between individuals in penetrance or physiological effect and may depend on (1) environmental factors; (2) modifier genes and/or the epigenome; and (3) the additive/synergistic effect from another genetic variant (digenic inheritance) (Majewski et al., 2011). High-penetrance variants induce a strong physiological effect and these alleles have usually been identified as causative for Mendelian monogenic disorders using linkage studies in families. Low penetrance variants have a weak phenotype and causative alleles are
typically identified in large case/control cohorts as part of the study of complex trait disorders. Common high-penetrance disease variants are rare as they would normally be eliminated from breeding populations, except in cases of balancing selection where heterozygotes have an advantage over homozygotes (e.g. sickle cell disease). The distinction between monogenic and complex diseases is therefore operational as all genetic variants are de-facto transmitted in a Mendelian fashion and thus amenable to discovery using WES, giving this technique far wider applicability than simply the study of rare monogenic disorders (Rabbani et al, 2014).

By limiting the further investigation to those mutations known to occur in <1% of the general population the majority of those considered unlikely to be involved with Dupuytren’s disease have been excluded. It remains possible, however, particularly in light of subsequent findings, that one or more relevant mutations may have been excluded by this means.

Since all of the first generation of affected family members had only one parent known to have Dupuytren’s disease, the mutation must be heterozygous in these patients and results in the familial pattern of autosomal dominance observed in the family pedigree. Significantly, only one previous study has proposed an autosomal recessive model for inheritance of Dupuytren’s disease (Bayat et al., 2003) based on a statistical analysis of transforming growth factor β receptor (TGFβR) gene polymorphisms obtained from DNA sequencing of peripheral blood samples. Their conclusion, however, based upon investigation of a small range of candidate susceptibility genes before it was possible to detect candidate gene loci, was that Dupuytren’s disease “appears to be a complex oligogenic rather than a monogenic condition that segregates into multiple modes of inheritance” (see also Burge, 1999). The pedigrees of other families with Dupuytren’s reported in the literature strongly support an autosomal dominant pattern of inheritance (Gudmundsson et al., 2000). Synonymous mutations, almost by definition, will not usually affect translation of otherwise normal amino acid sequences to abnormal protein, but the possibility of interaction between multi-purpose codons (DUONS) causing interference with normal processing during transcription and/or translation must be
considered (Stergachis, 2013). The study reporting the existence of codons with dual function may provide a mechanism by which synonymous variants may be more important in disease, and suggests that the strategy employed in this chapter of only investigating non-synonymous mutations is limited (Weatheritt, 2013).

There is indeed evidence to suggest that changes in a gene’s sequence that are silent with respect to protein sequence are not always silent with respect to function (Gingold & Pilpel, 2011). From their assessment of mutations obtained from large-scale sequencing of cancer genomes the authors of a recent review article have concluded that synonymous mutations within oncogenes represent 6-8 percent of all driver mutations due to single nucleotide changes, and that approximately half of synonymous drivers alter splicing, which in turn may influence cancer progression (Supek et al., 2014) Of further relevance is the fact that synonymous mutations can also alter protein folding, thereby contributing to tumour progression (Kimchi-Sarfaty et al., 2007; Zhang et al., 2009; Zhou et al., 2013).

Selection of priority genes of interest was based on either gene function being known to be relevant to Dupuytren’s pathology (for example ADAM15, KLF11); genes known to be involved or expressed in relevant cell types (for example CDCC80 expression in fibroblasts); genes that had been linked to conditions also linked by epidemiology to Dupuytren’s disease (for example JHDM1D) and genes that had previously been identified or were similar to those identified in other studies of Dupuytren’s disease (for example ADAM15). This selective process creates the potential for the elimination of other mutations that may be critical, but is necessary to restrict further investigation to a list that can be interrogated more fully. Nevertheless it is an important limitation that may lead to the omission of important mutations in this study.

2.5 Summary

Within the family members currently investigated a limited number of mutations potentially relevant to Dupuytren’s disease have been identified. However no overlap with the studies of others was identified and no mutations that were sequenced in other family members appeared to segregate with the disease phenotype. Therefore it has not been
possible at this stage to identify a causative mutation. The limitations of this approach include the use of WES that will exclude non-coding mutations as well as the assumptions made to restrict the number of mutations identified in the small number of exomes sequenced. Therefore additional approaches may facilitate a better understanding of the causes of Dupuytren’s disease in the Western Australian population. To follow-up this family based study the next chapter will focus on a population approach, using data derived from a community-based health survey in which people were identified as having Dupuytren’s disease. The aim of this study will be to then interrogate potential regions of the genome for overlap with the family study presented in this chapter to determine if a causative gene/mutation can be identified through the combination of these different approaches.
CHAPTER 3

Aim. 2 To investigate the presence of identified mutations in a wider population-based cohort of people with Dupuytren’s disease.

3.1 Introduction

Genome Wide Association Studies (GWAS) use a high-density map of SNPs across the entire genome to localize regions of interest with respect to disease. The relative occurrence of specific SNPs in affected and unaffected populations is identified and compared to locate regions of the genome that are in high linkage disequilibrium. That is the region strongly associated with the presence of the disease. These studies require large populations of both affected and unaffected individuals to be effective in isolation.

3.1.1 The Busselton Population Health Survey

Repeated cross-sectional health surveys of adults living within the south west coastal community of Busselton have been carried out from 1966 to 2009, although there have been only two major surveys since 1990, in 1994-95 and 2005-06. Busselton has a relatively stable population of predominantly European descent and participation rates ranged between 64 to 91 percent of eligible adults. Randomised participation was by invitation based on the electoral rolls with recruitment via letter of introduction and subsequent personal interview. The population recruited is approximately 5000 individuals, although not all individuals were assessed for all conditions in all surveys so not all subsequent studies have been able to use the entire population.

An essential element of the Busselton study has been the longitudinal follow-up of the same cohort at intervals of three to five years (Phase II)(eg Busselton Healthy Ageing Study, 2010; James et al, 2013). The elements of each periodic community survey have included a limited clinical examination, focusing on certain medical conditions which occur commonly and which are most likely related to lifestyle, and a comprehensive questionnaire in support of the long-term clinical progress through childhood and all stages of adulthood. In the 1994-95 survey, at the request of Dr Pearce, two additional
questions pertaining to the participant’s Dupuytren status (affected or unaffected) were included in the surveys. All adults included in the survey also submitted to examination of their hands for evidence of the clinical manifestations of Dupuytren’s disease. Those responsible for examining the hands were not trained or medically qualified and the results suggest a high degree of inaccuracy, thereby precluding further interpretation or analysis.

The questions specific to Dupuytren’s disease were:
1. Have you observed the appearance of palpable bands or cords in the palms or digits?
2. Have you had surgery for Dupuytren’s disease affecting your hands?

Whereas over four hundred individuals claimed to have palpable palmar bands or cords, suggesting a possible Dupuytren’s phenotype based on subjective self-examination, only forty-eight admitted to having surgery for a confirmed clinical diagnosis of Dupuytren’s disease. The veracity and therefore reliability of the former result must be questioned as it included all age groups, which suggests an incidence of Dupuytren’s disease that is not only highly improbable but virtually impossible. It would be difficult to relate this group to a cohort with a diagnosis of Dupuytren’s disease with any confidence. As their DNA has been made available, however, an analysis of association may be made with some comparisons with GWAS results from the surgically treated individuals.

Subsequently, many of these same individuals have had DNA collected and analysed using GWAS SNP profiling as part of the Busselton study. Here, we have identified the affected and unaffected individuals in the Busselton study from their responses in the 1994-95 survey. We have then used the collected GWAS data on these individuals to identify regions of interest that may be associated with the incidence of Dupuytren’s disease in this population. Whilst the population is somewhat small for a GWAS study, the information gained from this approach will be integrated with the findings from Chapter 2 to potentially identify genes of interest using two separate approaches.

3.2 Methods
Ethics approval for this study was obtained from the Busselton study committee and relevant institutional ethics committees (University of Western Australia Human Research Ethics Committee – RA/4/1/2203).

3.2.1 Survey Responses
In 1995 two questions were included in the Busselton community survey as outlined above. Out of a complete set of responses to the questionnaire, 409 individuals responded positively to question one, whilst only 48 positive responses to question 2 were received. Thus a cohort of 48 adults who had recorded a surgical history for Dupuytren’s disease (DD) was identified; 42 of them provided blood samples.

Of the 409 participants whose questionnaire provided suggestive evidence of palmar bands, 360 (ie 90 percent) gave blood for laboratory analysis. The analysis focused on those with a surgical history of DD as the positive affected cohort.

3.2.2 Statistical power for association analysis
Power analysis for the 513,727 Busselton GWAS SNPs available after quality control with suggestive genome-wide association after correction for multiple testing (i.e. requiring a p-value threshold of 1.0 x 10^{-4}), with a 10% prevalence rate, and a minor allele frequency of 0.19 for the risk variant demonstrates that we expect 80% power for a SNP that has an odds ratio $\geq 1.67$ for 409 developed thickened band cases and 4,262 controls and an odds ratio $\geq 4.0$ for 48 positive cases and 4623 controls. Although these odds ratios would be considered large for common variants from GWAS, these results were primarily applied to prioritize interrogation of WES data for Dupuytren’s disease. Gene- and pathway-centric burden tests were employed to compensate for low statistical power in variants that might have been missed due to low copy number.
3.3 Results

Both a dominant model (table 3.1) and additive model (table 3.2) were used to identify SNPs associated with DD in the Busselton cohort. The dominant model assumes that the risk of the disease phenotype is equivalent between heterozygotes and homozygotes with the relevant polymorphism. Inheritance of Dupuytren disease within families follows a dominant model with incomplete penetrance. The additive model, in contrast, assumes increased risk in homozygous compared to heterozygous SNP presence (linear increase therefore a homozygous affected would have 2x the effect of a heterozygous affected).

Using an arbitrary cut-off p value of $5 \times 10^{-5}$ 20 SNPs and 39 SNPs were identified for the dominant and additive models respectively (Tables 3.1, 3.2). Using UCSC Genome browser (https://genome.ucsc.edu/) a search for proximal genes was then conducted to identify potential genes of interest. A distance cut-off from the SNP of 50MB was used for identifying associated genes. Within 50MB of the SNPs identified there were 17 genes and 5 long non-coding RNAs (LINCs) for the dominant model (table 3.1). There were 18 genes and 2 LINC within this distance for SNPs identified using the additive model (table 3.2).

No genes identified using this methodology were identified that were also identified in the previous family study using whole exome sequencing (Chapter 2). However, genes with roles closely related to those identified in the previous family WES study were identified, including genes with known functions in immune responses, matrix remodeling and vascularization. This may implicate these processes in the molecular pathology of DD and are discussed in detail below.
Table 3.1: Single nucleotide polymorphisms (SNPs) identified as being associated with DD in the Busselton population using the dominant model. Table shows chromosomal location, SNP (A1) at this location, Odds ratio (effect size of the SNP identified), \( p \) value (not corrected) and genes identified at this location using UCSC genome browser together with other features of that region (within 50MB DNA of SNP).

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Table 3.2: Single nucleotide polymorphisms (SNPs) identified as being associated with DD in the Busselton population using the additive model. Table shows chromosomal location, SNP (A1) at this location, Odds ratio (effect size of the SNP identified), p value (not corrected) and genes identified at this location using UCSC genome browser together with other features of that region (within 50MB DNA of SNP).

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3.4 Discussion

3.4.1 Findings in other linkage and genome wide association studies for Dupuytren’s disease

Previous genetic studies in Dupuytren’s disease have targeted those genes involved in the regulation of fibroblast activity, including pathological collagen production and lattice contraction in the extracellular matrix, as well as apoptosis and proliferation and alpha smooth muscle actin expression (Vi et al., 2009; Shih et al., 2012). One previous GWAS study has been conducted using a large population including those affected (960) and not affected (3117 controls) with Dupuytren’s disease (Dolmans et al., 2011). This study combined with three additional independent case series comprising a total of 1365 individuals affected with Dupuytren’s disease and a further 8445 controls from Germany, the United Kingdom and the Netherlands to identify 35 SNPs most strongly associated with Dupuytren’s disease. From these large populations this study identified 11 SNPs from 9 regions of interest that were validated in two independent replication populations. Of particular interest, within 6 of these regions genes known to be involved in a single signaling pathway, the WNT signaling pathway, were located. This pathway has previously been implicated in other studies investigating the pathogenesis of fibrosis in Systemic Sclerosis, Idiopathic Pulmonary Fibrosis and Dupuytren’s disease, although to date no strong functional evidence exists to link dysfunctional Wnt signaling to progressive fibrosis (Moon et al., 2004; Vlad et al., 2008; Konigshoff et al., 2009; Bhattacharyya et al., 2012).

Despite the large population size and independent replication populations, the previous GWAS study of Dupuytren’s disease has provided support for a role of specific genes but no actual mutations involved in Dupuytren’s pathogenesis were identified and validated. This is a common limitation of the GWAS approach and supports the use of multiple research methods combined with the GWAS approach to identify disease-causing mutations.
3.4.2 Possible role for genes in regions of interest from the Busselton GWAS study

None of the same SNPs identified previously by Holman et al were identified in this study of Dupuytren’s disease although this may be solely due to limited statistical power of the current study. Of the genes located within regions showing positive association with SNPs using the p-value cut-off none have previous evidence directly linking the gene to DD. However, there were genes identified that have key biological functions related to fibrosis and may therefore play a role in DD.

SDE2 is a Telomere maintenance homologue (S Pombe) on chromosome 1. An important paralog of this gene is SF3A3 which may be responsible for development of intrahepatic cholangiocarcinoma (Wang et al., 2006). This gene has been identified among related genes by comparison with normal liver tissues, but no known disorders have been found for the SDE2 gene. Dupuytren’s disease has a reputation for an association with high alcohol intake, which seems to imply a metabolic basis that could potentially be related to alcoholic cirrhosis (Wolfe et al., 1956). Hnanicek et al (2008) found a high incidence of chronic liver disease in Dupuytren’s patients but demonstrated a lack of association with iron metabolism in Dupuytren’s disease. These findings, however, have not been confirmed consistently through large epidemiological studies, and there has been no positive relationship with other forms of hepatic fibrosis including haemachromatosis and post-hepatitis cirrhosis. Stremmel et al (1988) identified differing abnormalities in estrogen, androgen and insulin metabolism in idiopathic haemochromatosis compared with alcoholic liver disease, findings that could be relevant in Dupuytren’s pathology (Stremmel et al 1988).

LEFTY2 represents Left–Right determination Factor 2 and is a member of the TGF superfamily. The work of Bayat (2003) and many others has confirmed that TGFβ family members are multifunctional including a role in wound healing and fibrosis, although only TGFβRII polymorphisms were significantly different in genotype frequency distributions between Dupuytren’s patients and controls. There was no statistical difference in genotype or allele frequency distributions between cases and controls for TGFBRII and TGFβRIII SNPs (Bayat et al, 2003).
The *SSUH2* gene is a transcription regulator in Notch signaling and binds specifically to methylated DNA. It binds to the oxygen-responsive element of COX412 and activates its transcription under conditions of hypoxia (4% oxygen). (Aras et al 2012; Funayama et al, 2015). Since hypoxia is a known cause of tissue fibrosis and age-related peripheral vascular changes, diabetes and smoking are commonly associated with the development of progressive palmar fibromatosis there could well be a role for this gene in the pathogenesis of Dupuytren’s disease.

*NTRK3* (neurotropic tyrosine kinase receptor type 3) encodes a member of the neurotrophic tyrosine receptor kinase (NTRK) family. This kinase is a membrane-bound receptor that, upon neurotrophin binding, phosphorylates itself and members of the MAPK pathway. Signaling through this kinase leads to cell differentiation and may play a role in the development of proprioceptive neurons that sense body position.

In South America it has been investigated in association with Chaga’s Disease. Trypanosoma cruzi infestation promotes neuronal and glial cell survival (*in vitro*) by inhibiting TicC-induced apoptosis (Rodriguez and Mehlen, 2010).

*RBFOX1* has been associated with small joint osteoarthritis of the hand, familial neurodegenerative diseases, spino-cerebellar ataxia type 2, and in Japan it has been identified with primary biliary cirrhosis (Joshita et al., 2010) Its involvement with epilepsy could be significant in those cases that exhibit a Dupuytren’s phenotype, but the association of Dupuytren’s with epilepsy has not been consistently reported or confirmed.

### 3.4.3 Comparison of results from the Busselton and family studies

From the WES analysis of affected family members (Chapter 2) and the GWAS population study from Busselton there appears to be no overlap of genes identified from both cohorts. The two studies also do not provide overlap with other published studies looking at the genetic basis of Dupuytren’s disease. However in a population of unrelated individuals it would be expected that variable expression in terms of age of onset, degree of hand involvement (unilateral/bilateral), clinical evidence of digital contracture, and rate of progression of fibromatosis, gender differences, association with other disease profiles, would involve a variety of genetic pathways and genes. It is not therefore completely surprising that the genes obtained from such a diverse small population do not correspond with those identified from other studies.
Interestingly when comparing the family WES and Busselton study results there does appear to be overlap, whilst not in genes, but in the biological pathways that may be implicated. For example, in the WES family study (Chapter 2) a number of potential genes of interest that were identified had clear roles in vascularization/angiogenesis and there were also pro-inflammatory/immune modulating genes identified. In the investigation using the Busselton population data, CD226, PYHIN1 and MAVS genes which were proximal to identified SNPs all have roles in the immune system. In particular PYHIN1 and MAVS are both implicated in the interferon induced anti-viral response, which is of interest given that previous reports have associated viral infection to Dupuytren’s disease. There are other very strong candidate genes identified in the list of genes proximal to the SNPs identified. This includes ZMIZ1, which is important in androgen receptor signaling and also the regulation of SMAD3/4 expression. SMAD3/4 in particular are well-known to be important in fibrosis and their direct involvement in regulation of fibrosis suggests a good candidate for Dupuytren’s disease. Genes with important roles in matrix production and turnover were also identified including HPSE2 (a heparanase with no enzymatic activity but likely a regulator of other heparanase activity), TLN2 (Talin 2) and interestingly ADAMTS9-AS2. AS genes are anti-sense genes that are only recently described. The transcription of these genes appears to regulate the expression of the related gene, in this case ADAMTS9. The ADAM family of proteins is large and some have already been implicated in DD in previous studies.

Finally, tropomyosin I (TPM1) is also a potentially interesting candidate for further investigation. Tropomyosin is critical in regulating actin-myosin interactions and contractile activity. It is clear this activity could be important in the progression of DD. Despite this study being underpowered the candidate genes identified in proximity to SNPs associated with the DD phenotype do appear to have functional roles that are important in DD pathology. However, the fact the study is underpowered reduces the significance of any individual finding and further validation that these genes may be involved is critical. This also highlights the difficulty of GWAS type approaches, since these genes are only implicated by proximity to these SNPs in the same way as WNT pathway genes were implicated in the much larger GWAS conducted by Dolmans et al (2011). Therefore, whilst these genes are potentially of interest there is no clear functional
or expression difference to verify that the genes identified are altered in DD affected patients. The finding that many of these genes have roles in pathways also identified in the WES study (Chapter 2) and in other studies of DD, including angiogenesis and the likely role of hypoxia/circulation in DD pathology, support a non-fibroblast origin of DD pathology that has previously been hypothesized.

3.4.5 Significance of environmental factors and genetic susceptibility

Numerous environmental and genetic studies of the fibromatoses and related pathologies have produced evidence to support the familial nature of these conditions in the majority of cases. Inheritance of a susceptibility to develop a Dupuytren’s phenotype after middle age appears to follow an autosomal dominant pattern with variable expression and occasionally incomplete penetrance. The ethnic differences which demonstrate consistent but widely different incidence of Dupuytren’s pathology are most obvious between white Caucasians from Northern Europe and black Africans. The same difference in incidence exists between black and white communities living in the same country such as the United States. This would strongly suggest a predetermined genetic susceptibility that exists within the white population and to a far lesser degree among African Americans. A similar discrepancy exists between Europeans and most Asian populations.

With some allowance to be made for differences in lifestyle factors such as diet, climate, education, employment and living conditions, it is obvious that even the susceptibility to develop a particular disease must be genetically determined. From the many genetic studies that have attempted to identify genetic pathways and specific candidate genes responsible for the progression of fibrosis in a Dupuytren’s phenotype it has become obvious also that there must be several genes and pathways involved in this pathology, the spectrum of such combinations accounting to some extent for the variations observed in age of onset and degrees and speed of progress of the disease.

The experience of many epidemiologists tends to favour a range of environmental factors which might trigger the onset of Dupuytren’s, many of which can be related to the ethnic variations and differences in incidence observed (Geohegan et al., 2004; Gordon, 1954). Lifestyle factors such as smoking and alcohol abuse, both of which have been associated with significant levels of Dupuytren’s in certain populations, tend to be related to gender,
Investigating the Molecular Pathology of Dupuytren’s Disease

age, generation and employment, factors which affect the expression of this disease to a variable extent in different communities (Godtfredsen et al., 2004; Khan et al., 2004). Nevertheless, as with a number of co-morbidities that impose further metabolic and immunologic stresses on molecular function, the importance of environmental impact upon a susceptible organism must be significant.

“Transforming growth factor β is the pre-eminent signal for connective tissue synthesis, and is considered as a ‘core pathway’ in both normal wound healing and pathological fibrosis” (Bhattacharyya et al., 2012). However, the molecular pathways responsible for activation and progressive proliferation of fibroblasts and myofibroblast differentiation can be expected to involve a number of intrinsic and extrinsic cellular factors, including dysregulated beta-catenin and Wnt signaling glycoproteins (Vlad et al 2008), Akt and integrin pathways, and aberrant or dysregulated TGF-beta expression. Dysregulation may also be stimulated external to the fibroblasts which are clearly the drivers of the progressive disease.

The role played by TGFβ receptors in the genetic susceptibility to Dupuytren’s disease has previously been investigated with a statistically significant difference of TGFbR1 polymorphisms in DD in the recessive model. (Bayat 1;2003 These authors also conclude that “DD appears to be a complex oligogenic rather than a monogenic condition that segregates into multiple modes of inheritance”) (see also Burge P Genetics of DD Hand Clinics 1999;15(1):63-72) re proposed autosomal recessive mode of inheritance). While the recessive model is not in agreement with the majority of observed series reported with familial Dupuytren’s disease the suggestion of a complex polygenic inheritance involving several pathways is readily accepted in light of the variable expressivity and degree of phenotypic progression (Hu et al., 2005; Gudmundsson et al., 2001). The role of TGF beta2 in this pathology has been investigated also by Zhang et al (2008) with ‘gene expression analysis’; different isoforms of TGF-beta have been reported to have both profibrotic and antifibrotic effects.

Genetic linkage and whole genome association studies have also been reported, significantly associated loci being compared with the results of gene expression profiling studies (Shih et al., 2012). Two such genes, MAFB and PRKX (protein kinase X-linked),
were highlighted by both studies. *PRKX* contains a SNP that shows a positive association with Dupuytren’s disease (Li X. et al., 2011). The expression of *MAFB* in Dupuytren’s disease has been investigated by Lee et al (2006) who noted its complete absence in adjacent control fascia. MafB was found to be of particular interest because of its prominent role in tissue development and cellular (myofibroblast) differentiation, and a significant co-location in some cells with alpha-smooth muscle actin (Lee et al., 2006).

As with the puzzling array of genetic influence in the pathogenesis of Dupuytren’s disease, the combined effects of aging, associated changes in immune response, hormones and metabolism, together with the effects of co-morbidities such as diabetes mellitus, provides a wide range of environmental factors to influence the clinical outcome. Such a ‘pot pourri’ of active ingredients with possible influence on the development of a highly recognizable immuno-histo-chemical pathology would suggest, however, that the molecular pathway, once established, is little affected by environmental factors. Nevertheless, the environmental factors may play a very significant role in the establishment of the disease and therefore confound genetic studies aimed at identifying causative mutations or polymorphisms.

### 3.4.6 Limitations of this study

GWAS studies require large populations of both affected and non-affected individuals to be effective in isolation. The power of the Busselton study in relation to the current investigation is limited by the small number of identified affecteds – this does not represent a large enough population with Dupuytren’s disease for GWAS. It was, however, useful to provide a database for associations in the population study with possible areas of interest in common with mutations derived from the whole exome study. The DNA obtained from the Busselton 2006 population study encompassing a broad investigation of five thousand essentially unrelated adults became available for specific genetic analysis. Forty-eight adults were positively identified through the detailed questionnaire as having undergone surgery for Dupuytren’s disease, the clinical diagnosis thereby confirmed.

GWAS on this cohort were considered inadequate for statistical analysis or for comparison with results of previous studies, eg Dolmans et al, 2011. The Busselton results, however,
produced a list of mutations with an OR >1.0, and with locations identified to a number of chromosomes of interest. Our current study has found it useful to focus on these regions to determine associations with genes from the WES of family members as reported in Chapter Two. Other than small study size making the approach underpowered the other significant limitation of this approach is the inability to identify potential functional changes that can be further interrogated. Had there been direct overlap in genes identified in the WES study (Chapter 2) it may have been feasible to progress further investigation on a common candidate using functional and genetic approaches. However, in this study, whilst a number of genes with relevant functions were identified as being close to polymorphisms with a positive association with DD, there was no insight into whether there were expression of functional changes in the identified genes. This limits the ability to further interrogate these findings and is a significant limitation of all GWAS studies.

Taking into account the broad spectrum of clinical involvement as exhibited by the variable Dupuytren’s phenotype found among members of one family, and the unpredictable nature of disease progression, the variable severity of this disease may be due to different levels of interaction between genetic and environmental factors possibly triggering different levels of activated/suppressed signaling pathways.
CHAPTER 4

Aim 3

Investigation of the presence of rare mutations in unrelated individuals with the severe Dupuytren’s phenotype by whole exome sequencing, and comparison with findings from affected family members and the community-based study of unrelated affecteds.

4.1 Introduction

The previous work using Whole Exome Sequencing of family members and data from a population based study has identified a small number of candidate genes that may contribute to Dupuytren’s pathology. To further investigate the significance of specific genes to molecular changes underlying the development of this pathology and verify the range of genes involved it was considered advantageous to investigate the presence of rare mutations in unrelated individuals with severe Dupuytren’s disease using whole exome sequencing.

Whole exome sequencing is considered appropriate for the investigation of inherited pathology within a family cohort. The initial sequencing of DNA from family members with Dupuytren’s disease (Ch 2) has identified mutations common to all individuals; this number was reduced by a selection process which identified candidates which responded to a functional data base and excluded a large number of variants found in the general population and considered unlikely to be specific to this disease.

To further investigate the potential validity of these candidates the DNA from two unrelated individuals with a Dupuytren’s phenotype considered to be aggressive and severe has been included in this study for whole exome sequencing. Mutations identified from their DNA are then matched against those selected from the family affecteds to further reduce the possible mutations considered to have functional involvement in Dupuytren’s disease.

4.2 Methods

The protocols for collection of DNA and Whole Exome Sequencing were followed as described in Chapter 2.
Two unrelated individuals with aggressive disease were also included in this study. They were assessed as severe cases by the following criteria; early age of onset (40 or younger), bilateral hand involvement with Dupuytren’s disease, known positive family history of Dupuytren’s disease, and progressive active palmar fibromatosis with digital contractures. Analysis of data followed the protocols as described in Chapter 2.

4.2.1 Pathway analysis
For pathway analysis a combined gene list of all candidate genes of interest identified from all three studies (family WES, Busselton GWAS and Severe brother study) was generated. The gene list was uploaded to the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8 (1) to look for gene ontology groups and pathways enriched from the genes of interest. For the gene ontology analysis, the groups “GOTERM_BP_DIRECT”, “GOTERM_CC_DIRECT” and “GOTERM_MF_DIRECT” were selected and run with an EASE score of 0.2, a modification of the Fisher’s Exact test to measure whether independent groups can fall into one of two mutually exclusive categories. Resulting data was exported to excel. Pathway data was analysed in a similar way, using an EASE score of 0.2 and the “KEGG_PATHWAY” option. Resulting data was exported to excel, and images of pathways with genes involved also exported (Huang et al, 2008).

4.3 Results
The following table lists the genetic mutations identified as occurring in both the severe Dupuytren cases as heterozygous mutations.
**Table 4.1:** Non-synonymous polymorphisms identified in two related individuals with severe Dupuyren’s disease

List of mutations identified in both individuals and that had a predicted deleterious effect on the protein as medium or high for impact severity. These were identified and screened as described (methods section), with the focus on mutations likely to have a higher level of effect given that these patients were early onset and exhibited a severe progressive disease pattern.

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To assess this larger list of mutations for possible functional significance and correlation to the genes identified in previous chapters (2 and 3) gene set enrichment analysis (GSEA) was conducted using the online DAVID tool (Huang et al, 2008). GSEA provides a method to identify pathways and processes that are significantly over-represented in entity lists. This can help identify important themes in large datasets and overcomes some of the limitations of low powered studies that cannot identify significant changes at the individual gene/polymorphism level.

Pathways identified as being significantly over-represented in the combined studies in this thesis using gene ontology categorization include cell morphogenesis and protein turnover pathways (Table 4.2). Other pathways that were potentially enriched (but do not reach statistical significance) include cell proliferation, migration and apoptosis (Table 4.2).

Table 4.2: Gene ontology enrichment analysis. Processes including heart morphogenesis and cellular proliferation and apoptosis are enriched in the candidate gene lists identified in this study (table shows pathway identified by, % of pathway genes enriched in the target gene list, P value and genes from the target gene list identified in each pathway.)

<table>
<thead>
<tr>
<th>Term ID</th>
<th>Term Name</th>
<th>%</th>
<th>PValue</th>
<th>Genes</th>
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<tr>
<td>GO:0003007</td>
<td>heart morphogenesis</td>
<td>2.362205</td>
<td>0.016698</td>
<td>ZMIZ1, TBX1, LEFTY1</td>
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<tr>
<td>GO:0061157</td>
<td>mRNA destabilization</td>
<td>1.574803</td>
<td>0.036595</td>
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<td>GO:0051865</td>
<td>protein autoubiquitination</td>
<td>2.362205</td>
<td>0.038386</td>
<td>LRSAM1, TRIM17, TRIM21</td>
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<tr>
<td>GO:0001934</td>
<td>positive regulation of protein</td>
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<td>0.04433</td>
<td>MAVS, NTRK3, TBX1, STK4</td>
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<td>GO:0006623</td>
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<td>GO:0000902</td>
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<td>0.056509</td>
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<td>GO:0000902</td>
<td>sulfur amino acid metabolic process</td>
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<td>0.066076</td>
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<td>GO:0030855</td>
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<td>GO:0030212</td>
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<td>GO:0008285</td>
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<td>0.099541</td>
<td>NOX4, ENPP7, CYP27B1, BCHE, IGFBP7, STK4</td>
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Using KEGG pathway analysis significantly enriched pathways were for Huntington’s disease and Focal adhesions (Table 4.3).

Table 4.3: KEGG pathway analysis. Pathways enriched in the gene lists identified in this study include Focal adhesions and Huntington’s disease (table shows pathway identified by KEGG, % of pathway genes enriched in the target gene list, P value and genes from the target gene list identified in each pathway.

<table>
<thead>
<tr>
<th>Category</th>
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<td>KEGG_PATHWAY</td>
<td>Mucin type O-Glycan biosynthesis</td>
<td>1.575</td>
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</table>

The genes enriched in the focal adhesion pathway are highlighted in Figure 4.1 in the context of the whole pathway used for this annotation (Fig. 4.1). Genes identified in this pathway originate from the family WES study (COL6A5), the Busselton study (TLN2) and the WES of the severe affected individuals (ITGA6). This indicates that this pathway has a gene that may be disrupted in each of the studies and points to a potential common pathway that may in part underpin Dupuytren’s disease.
Investigating the Molecular Pathology of Dupuytren’s Disease

Figure 4.1: Focal Adhesion Pathway genes implicated in DD. The focal adhesion pathway is over-represented in the combined lists of target genes identified in the genetic studies in this thesis. Figure shows focal adhesion pathway with genes identified in the genetic studies highlighted with red star symbol.

4.4 Discussion

The reason for investigating the genetic profile of unrelated individuals with severe/aggressive Dupuytren’s phenotype was the potential to identify mutations (polymorphisms) in genes previously identified but that would be rare mutations (since severe Dupuytren’s disease is very uncommon in comparison to late onset disease) and that these mutations would have a more deleterious effect on protein function, thereby leading to the earlier onset and excessive nature of the disease in those with the severe phenotype. When restricting the list of non-synonymous mutations identified in both individuals with severe disease to those with a predicted moderate/severe effect on protein function, heterozygous and in less than 0.1% of the population, a total of 93 polymorphisms were identified. This is a large number of potential candidates and is an interesting observation given that these are rare non-synonymous mutations identified to be in common in two unrelated individuals. Despite this large number of potential candidate genes, as in the previous studies (Chapters 2 and 3), no overlap in candidate
genes was observed in the list of genes obtained from the unrelated individual analysis when compared to the previous studies.

4.4.1 Identification of potential target genes
Given the extensive list of potential candidate genes identified it is unsurprising that there are genes of interest with functions that can be hypothesized to be relevant to DD contained within. There are many different matrix associated proteins with functions that are likely or have been well described to be important in fibrosis, including Integrins (ITGA6 and ITGA7), members of the ADAMTS family (a disintegrin and metalloproteinase with thrombospondin motifs family), Collagen genes (including COL5A1) and cadherin related proteins (CDHR4). All of these genes have key roles in maintaining and regulating the ECM: cell interactions and are therefore plausible candidates for involvement in DD given the known pathology of the disease. Similar to the previous studies presented in Chapters 2 and 3, genes with known roles in immune responses (for example ITIH4 and LAIR1) and genes important in the regulation of vascularization (for example ANGPTL4) are also candidate genes that can be hypothesized to be involved in DD pathology. Interestingly, ANGPTL4 is associated with Type II Diabetes, which is itself associated with DD, and ANGPTL4 plays an important role in vascularization and a response to hypoxia, which has previously been hypothesized to be the underlying cause of DD. However, despite the presence of genes with relevant functions and polymorphisms predicted to be deleterious, these findings are weakened by the presence of many other rare polymorphisms in both individuals that were predicted to be moderately or severely deleterious but occur in genes with roles most likely unrelated to the disease. This makes interpretation of these findings alone difficult, as would be expected when using WES on only two unrelated individuals. However, in the context of the previous studies and combining the findings from these studies it becomes apparent that there are specific pathways that may be common across the individuals that have been investigated and this may provide insight into the pathogenesis of DD.

4.4.2 Pathway analysis of combined genes of interest
Given the lack of direct overlap between these studies a pathway analysis was used to identify any pathways that may be in common, albeit affected by polymorphisms in
different genes that may underpin DD. Using this pathway analysis focal adhesions appeared to be significantly enriched. Interestingly this pathway contained genes identified in each of the three independent studies, suggesting that in each study, family, Busselton and severe unrelated individuals, there was a gene identified of interest that is involved in focal adhesions and the signals generated by them. Focal adhesions are a type of adhesive contact between cells and the extracellular matrix. They are critical structures that facilitate communication between cells and the environment and can be involved in biomechanical sensing and signaling (for example changes in matrix physical properties such as stiffness) as well as biochemical signaling (mediating biological signals for cell:cell and cell:matrix communication). Focal adhesions are critical to many different processes and the intracellular signaling stimulated by focal adhesions is mediated by Focal adhesion kinase (FAK). FAK has been demonstrated to be critical to TGFβ signaling and in the induction of profibrotic genes in response to TGFβ signaling (Liu et al 2007). More recently targeting FAK has been demonstrated to ameliorate fibrosis in a number of animal models of fibrosis, suggesting this signaling pathway is critical (Lagares, 2013; Kinoshita K, 2013). Therefore strong evidence exists for the importance of focal adhesions in fibrosis and this suggests the identification of this pathway potentially being affected by polymorphisms identified in the studies presented here is important to follow-up with further work to characterize the impact of these polymorphisms and the role they may play in DD.

4.4.3 Summary
To identify gene mutations common to unrelated individuals exhibiting evidence of Dupuytren’s phenotype with a clinical picture suggestive of severe or aggressive disease, the DNA from two males with early-onset, progressive, bilateral palmar fibromatosis and a positive family history of Dupuytren’s disease was submitted to analysis by whole exome sequencing. No genes directly linked to the previous studies were identified. However, the importance of focal adhesions has been implicated by pathway analysis and this warrants further investigation. In addition, other polymorphisms with clear likely relevance to DD warrant further investigation from those identified in individuals with a severe disease phenotype. Further sequencing from other unrelated individuals with severe disease may facilitate identifying the likely causative mutations.
CHAPTER 5

*Aim 4 To investigate the functional consequences of the putative mutations identified in Aims 1-3 on fibroblast phenotype and pathobiology with relation to Dupuytren’s disease.*

**5.1 Introduction**

The aim of this work is to identify functional mutations in genes that contribute to the pathology of Dupuytren’s disease using genetic methods. However, whilst the genetic studies may facilitate identification of mutations that are important it is essential that the impact of these mutations is also tested in appropriate models. Currently there is no animal model for Dupuytren’s disease and therefore *in-vitro* models are required to investigate the impact of mutations on Dupuytren cells. In this study fibroblasts have been isolated from two patients with Dupuytren’s disease. The cells were isolated from the affected site (test sample) and an additional tissue sample was taken from the wrist (unaffected control fibroblasts). A scar-in-a-jar assay was used to investigate collagen I production by both disease (cord) fibroblasts and control fibroblasts to determine if there were significant differences in collagen production in vitro using this assay. If significant differences between disease and control cells can be detected this assay would potentially be suitable for investigating the effect of identified mutations on fibroblast activity.

**5.2 Methods**

5.2.2. Scar-in-a-jar model

The functional assays were carried out using a published protocol known as “Scar-in-a-Jar” with some modifications (Chen et al. 2009). This method involved culturing fibroblasts, collagen immunostaining, microscopic imaging and semi-automated analysis of collagen quantitation and orientation.

5.2.2.1. Cell culture and isolation

Tissue samples were collected from consenting participants in Falcon tubes containing Dulbecco’s Modified Eagle Media (DMEM) with 10% foetal bovine serum (FBS) and
500UI/ml penicillin and 500µg/ml streptomycin (5% pen/strep) (Life Technologies, USA) and processed within four hours. Fibroblasts were isolated from fresh tissue by a slightly modified explant method. (Keira et al. 2004, Tucci-Viegas et al. 2010). The samples were washed with PBS containing 100UI/ml penicillin and 100µg/ml streptomycin (1% pen/strep) (four times, 30 seconds each). Samples were then placed in sterile petri dishes containing DMEM and subcutaneous tissue trimmed using a scalpel and forceps. The tissue was cut into small fragments (5-10 mm²) and excess media on the fragments was removed by blotting on a petri dish lid. About 10-12 fragments were transferred to each petri dish which was marked with perpendicular lines made by a scalpel to facilitate fragment attachment to the dish with the dermis side placed down. The dish was left semi-open in the fume hood for 30 minutes at room temperature for the fragments to adhere to the surface. Six ml of DMEM with 10% FBS and 1% pen/strep was added gently to the dish and then incubated at 37°C in 5% CO₂. The media was changed every two days and any floating fragments were plated in a new dish. Outgrowths of fibroblasts were noticed after 7-14 days. When the cells reached 80% confluence the media and tissue fragments were discarded. The cells were washed with phosphate buffered saline (PBS) and trypsinized with 0.05% trypsin with EDTA (Life Technologies, USA), then seeded into a T75 flask.

KF and NF were seeded at a density of 5 x 10⁴ cells/well in 4-chamber slides (Lab-Tek, ThermoFisher Scientific, USA) in 1 ml of Dulbecco’s modified eagle medium nutrient mixture F-12 (DMEM/F12, Life Technologies, USA) with 10% fetal bovine serum (FBS, Invitro Technologies, Noble Park North, Australia) and 1% penicillin/streptomycin (P/S, GIBCO®, Life Technologies, USA). After 14 hours incubation at 37°C in 5% CO₂, KF and NF media was removed and replaced by 1 ml of Un-stim media (3 wells) or Stim media (3 wells). The Un-stim media contained DMEM with 1% P/S, 0.5% FBS and 1% of 100 mM L- ascorbic acid 2-phosphate (Sigma Aldrich, USA). The Stim media was Un-stim media containing 37.5 mg/ml Ficoll PM70 (Fc70, GE Healthcare, UK), 25 mg/ml Ficoll PM400 (Fc 400, GE Healthcare, UK) and 5 ng/ml-1 transforming growth factor beta 1 (TGFβ1, R & D Systems, USA). The cultured cells were maintained at 37°C in 5% CO₂ for 6 days before starting the staining.
5.2.2.2. Immunohistochemistry

The immunohistochemistry for COL1A was carried out using the “Scar-in-a-Jar” protocol (Chen et al. 2009) with some modifications. After 6 days of culturing with Un-stim or Stim media the media was removed and the cultured fibroblasts washed once with FluoroBrite DMEM (GIBCO®, Life Technologies, Grand Island, New York, USA). Samples were then blocked with 3% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, Missouri, USA) in FluoroBrite DMEM for 30 minutes at room temperature. The blocking solution was removed and primary antibody (10AB, monoclonal mouse anti-human collagen type I, Santa Cruz Biotechnology, Dallas, Texas, USA) diluted (1:1000) in 3% BSA was added to the cells and incubated at 37°C in 5% CO2 for 90 minutes followed by one wash with FluoroBrite DMEM. A solution of 4% paraformaldehyde (Sigma Aldrich, USA) in PBS was added to the cells and incubated for 10 minutes at room temperature followed by two washes with FluoroBrite DMEM. The secondary antibody (AlexaFluor 488 goat anti-mouse IgG, Life Technologies, Eugene, Oregon, USA) diluted (1:500) in FluoroBrite DMEM was added to the cells and then incubated for 30 minutes at 37°C in 5% CO2 followed by one wash with FluoroBrite DMEM. The nucleolus was stained for 10 minutes at room temperature with Hoechst (Life Technologies, USA) diluted (1:1000) in FluoroBrite DMEM. The Hoechst solution was then removed and followed by two washes with PBS. PBS was removed and coverslips were mounted on the slides using Prolong Gold antifade mounting solution (Life technologies, USA). Nail polish was added to fix the coverslips before imaging.

5.2.2.3 Assessment of collagen quantitation

The imaging of the experiments was undertaken using a Nikon inverted research microscope (TE 300) and the Nikon NIS-Elements software (Nikon, Japan). The entire well was tiled and scanned using a 4X objective, B2-A (488nm), DAPI (358nm) filter blocks; and using the same imaging alignments for both the collagen and nuclear staining (Figure 5.2). The exposure time was set at 300ms for the Hoechst stain and 1s for the collagen staining (488nm excitation).
For each well, several rectangular ROIs (3-6) were used to cover as much of the stained area of the well as possible. The same dimensions and number of rectangles were applied for both the collagen staining and its corresponding nuclear staining. Each rectangular area had a binary threshold applied to mark either the cell nuclei or the collagen fibers. Once the threshold was adjusted to minimize background and ensure an accurate read, the object count and binary area covered were measured (Figure 5.2). The obtained data was exported to Excel. For each well, the sum of binary areas obtained from measuring the collagen staining was divided by sum of its corresponding object count obtained from measuring the nuclear staining to give an estimation of the amount of collagen secreted per cell. The collagen per cell obtained from each ROI was used in the statistical analysis.

5.2.2.4 Fluorescence resonance energy transfer assay of procollagen

A Fluorescence Resonance Energy Transfer (FRET) assay was carried out to measure procollagen concentration in cell media using the human pro-collagen type 1, 10000 tests kit from Cisbio (Cat. No. 63ADK014PEH), according to the manufacturer’s instructions. 16μL of cell media was added to a single well of the 384 well plate, (Greiner 384 low volume white plates, high base 4-25ul working volume, Cat. No. 784075, Interpath), to which 2μL of the anti-human procollagen cryptate antibody and 2μL of the anti-human procollagen d2 antibody was also added. A standard curve was made up the same way, with the procollagen standards ranging from 0.78-100ng/ml, as well as a negative control and a cryptate control. Each sample was run in triplicate. The plate was incubated at room temperature overnight, then read on a BMG Clariostar microplate reader. Microplate reader settings were as follows: excitation filter – 330nm, emission filters – 620nm and 665nm, integration delay (lag time) 60μs, integration time 400μs, number of flashes – 200, gain – 2400. For each well, the ratio of the 665nm/620nm was calculated, then the mean ratio calculated using the triplicate wells for each sample and standard. The delta F% was then calculated by using the following equation – (ratio of the standard or sample – ratio negative control)/ratio negative control x 100. The delta F% values for the standard curve were then plotted, the equation of the line worked out and the procollagen concentration of each sample calculated.
5.2.2.5. Statistical analysis

For each experimental condition, the individual data from each ROI for both the quantitation and the coherency of collagen were used for statistical comparison. Statistical significance between all experimental conditions was calculated using the Kruskal-Wallis (non-parametric) test with corrections for multiple testing. Statistical significance between two selected groups was calculated using the Mann-Whitney U-test (nonparametric). \( P \)-value < 0.05 was considered significant. All statistical analysis was carried out using Prism software v.6.0 (GraphPad, La Jolla, CA).

5.3 Results

The scar-in-a-jar model was tested using normal skin fibroblasts from an unrelated individual and collagen production compared to fibroblasts isolated from a keloid scar. Keloid scar fibroblasts were used as an example of fibroblasts from a progressive fibrotic disease and were therefore expected to be similar in characteristics to Dupuytren disease fibroblasts. In unstimulated conditions normal skin fibroblasts produced minimal collagen I (Fig 5.3.1a, e), and this was significantly increased when stimulated with TGFβ (Fig 5.3.1b, e). In contrast keloid derived fibroblasts produced significantly higher levels of collagen I without TGFβ stimulation (Fig 5.3c, e, f) when compared to normal skin fibroblasts and also significantly increased collagen I expression in response to TGFβ stimulation (Fig 5.3d, e, f). This was true in two different keloid patient samples tested
and suggests collagen I production *in-vitro* is increased in keloid fibrosis derived fibroblasts when compared to normal skin derived fibroblasts.

The same experiment was then conducted using cells derived from two patients with Dupuytren’s disease and cells isolated from the cord and wrist (disease and control respectively). Similar to the observations with normal skin fibroblasts neither control nor disease fibroblasts produced significant levels of collagen I in normal culture conditions (Figure 5.3.2 a-d). However in response to TGFb stimulation all cells increased collagen I production significantly (Fig 5.3.2 e-h).

Figure 5.3.1: Collagen quantitation in scar-in-a-jar model with normal skin and keloid scar fibroblasts

Collagen I immnostaining and nuclei staining (green and blue respectively) for normal skin unstimulated (NF1-US) and stimulated (NF1-S) fibroblasts (a, b) and keloid unstimulated (KF-US) and stimulated (KF-S) fibroblasts (c, d). Keloid fibroblasts produce significantly more collagen in vitro without stimulation when compared to normal skin fibroblasts (e, f)
To further investigate collagen production in Dupuytren fibroblasts, cell supernatants were isolated and collagen I synthesis production analysed using a FRET assay (Fig. 5.3.3). Low levels of Collagen I were produced in unstimulated disease (a) and control (b) cells. Significantly increased collagen I production was observed after TGFβ stimulation (Fig. 5.3.3a, b). However, no difference was observed between control and disease fibroblasts in either unstimulated or stimulated conditions.

Figure 5.3.2: Collagen quantitation in scar-in-a-jar model with fibroblasts from the wrist (control) and dupuytren cord (test).

Collagen I immunostaining (green) and nuclei (blue) using unstimulated conditions and fibroblasts from patient 1 cord (a) and wrist (b) and from patient 2 cord (c) and wrist (d). No significant collagen I production was detected. After stimulation with TGFβ, cells from patient 1 cord (e) and wrist (f) and patient 2 cord (g) and wrist (h) all expressed significantly higher levels of collagen I. No differences between control and disease derived fibroblasts, or between these cells and those from unrelated normal skin samples, was observed.

To further investigate collagen production in Dupuytren fibroblasts, cell supernatants were isolated and collagen I synthesis production analysed using a FRET assay (Fig. 5.3.3). Low levels of Collagen I were produced in unstimulated disease (a) and control (b) cells. Significantly increased collagen I production was observed after TGFβ stimulation (Fig. 5.3.3a, b). However, no difference was observed between control and disease fibroblasts in either unstimulated or stimulated conditions.
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Collagen I production measured as concentration of propeptide in cell media. The production of collagen I is shown over 6 days in control (a) and disease (b) fibroblasts in unstimulated (red) and stimulated (+TGFb) media (blue). Increased collagen I synthesis is observed after stimulation with TGFb in both control and disease fibroblasts. No significant difference between control and disease fibroblasts was observed.

Discussion

This investigation was to determine whether the scar-in-a-jar assay and the measurement of collagen I production could be used as a tool to interrogate the effect of identified functional mutations on fibroblast function. Since DD, as with other fibrotic disease, is characterized by excess collagen production it was hypothesized that disease cells would produce higher levels of collagen than control normal skin fibroblasts. Interestingly, whilst keloid fibroblasts produced excess collagen when compared to normal skin cells, this was not the case with DD fibroblasts. In this assay DD fibroblasts appeared to produce very little Collagen I without TGFb stimulation, as seen with control and unrelated normal skin fibroblasts. The response to TGFb was also similar between disease and control cells.
with no significant difference in the elevated levels of collagen I produced. The levels of collagen production were confirmed using two different assays which correlated closely, suggesting the methodology of detection was valid and that there is no difference in the DD fibroblast collagen production in vitro when compared to normal skin controls. The use of alternative methodology or models may have shown a difference in Collagen production not observed in this study. However, the scar-in-a-jar protocol allows examination of fibrillar collagen deposition, rather than pro-collagen production (FRET or Western blot assays) or in the case of qPCR transcription of the gene of interest. Examining the end product of collagen synthesis (fibrillar collagen) is important since collagen deposition can be regulated both intracellularly as well as extracellularly. Therefore by examining the end product of synthesis this protocol allows measurement of the collagen production most similar to that observed in vivo in the case of fibrotic disease. This is potentially a better system than inferring increases in ECM collagen deposition by looking at earlier stages of synthesis that may or may not result in increased deposition. However, this method is limited, as are many others, by the investigation of de novo matrix production rather than mimicking the production of collagen by cells already within a matrix, as occurs in vivo.

The findings in this study are potentially interesting and important for a number of reasons. First, it is clear that other skin fibrotic disease such as keloid does lead to increased production of collagen I using this assay. This suggests that the fibroblast activity in keloid disease is at least in part autonomous, as without additional stimulation keloid fibroblasts produce elevated levels of collagen I in vitro. In contrast the DD cells do not produce elevated levels of collagen I in vitro, whilst it is clear that excess collagen deposition occurs in vivo in this disease. Therefore, it is likely that the excess collagen deposition observed in vivo may be caused by factors other than changes in the fibroblast. Factors affecting fibroblast collagen production are numerous and have been investigated in many fibrotic conditions. One key factor is the matrix interaction with the cell itself. It has been shown that fibroblasts will respond to changes in matrix stiffness, and part of this response includes changes in collagen production (Wells RG, 2013). Surprisingly, it appears that increased matrix stiffness stimulates increased collagen production, creating a positive feedback loop that may in part underpin progressive fibrosis (Duscher D et al,
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2015; Rittie L, 2015). The assays conducted in this chapter were done in-vitro with no control over the stiffness of the matrix the cells encountered. Therefore, it may be that in DD it is matrix changes that are important in fibroblast activity and this explains the absence of differences between control and DD cells observed here. However, tissue culture plastic, the surface on which cells are grown in this study and many others, has a stiffness much higher than any encountered in physiological matrices. Thus is may be that this very high level of stiffness has an impact on fibroblast phenotype. It would be interesting to pursue further studies of fibroblast activity using matrices designed to reflect physiological and increased stiffness conditions to determine if this impacts on DD fibroblasts.

Alternatively, it may be that other cell signals are important in driving fibroblast activity in DD. There are many inflammatory signals that are known to be profibrotic, but also recent evidence that epithelial cells, particularly in response to damage, can secrete profibrotic signals which in the context of normal wound repair promote restoration of the tissue barrier. However, these signals can be activated for prolonged periods or inappropriately and lead to excessive scarring or progressive fibrosis in this context. It is also possible that hypoxia may play a role, and this can also be replicated in vitro to determine whether this is an important factor in DD pathology and fibroblast activity. Evidence exists that hypoxia may be important in fibrosis and in DD, both from the epidemiological link to Diabetes and reduced peripheral circulation but also through more mechanistic studies.

These issues highlight the complexity of fibrosis and replicating a useful model of fibrotic disease in vitro. Clearly, in vivo, the Dupuytren disease fibroblasts produce excess collagen I and this is sustained. However, this could not be replicated in the in vitro model here using a culture of only fibroblasts and as outlined above this may be due to many different factors that could be important. The use of more complex co-culture models (to mimic the effects of inflammatory or epithelial cells), as well as physical constraints using gels to mimic stiffness or stretch effects on the cells are likely to be important to more closely mimic the in vivo situation and provide a significant difference in collagen production between disease and unaffected cells. The fact that this does not appear to be the case in keloid disease cells, which produce excess collagen I in vitro with no additional
stimulus, is a very interesting finding. It suggests that whilst closely related in the fibrotic pathology, keloid and Dupuytren’s disease are very distinct in their molecular pathology, and this is worthy of further investigation.

5.4.1 Summary
This study suggests that the scar-in-a-jar assay for collagen production is not suitable for assessing phenotypic variability between DD and normal skin fibroblasts. Therefore, other in-vitro assays are required for investigation of functional mutations that may contribute to DD. The findings also suggest that fibroblast activity in DD may be aberrant in-vivo due to other factors. These could be physical changes to the matrix or other signaling molecules produced from other cell types. Further investigation into the effects of matrix stiffness on DD cell activity may clarify whether DD cells are sensitive to matrix changes or whether other signaling is important. In addition, more complex in-vitro models involving the use of multiple cell types may be warranted to understand the potential role of cell signaling in promoting the progressive fibrosis observed in DD.
CHAPTER SIX

6.1 Summary

To place this study in context I will quote from an early investigation by Terek et al (1995) from Boston which sought to demonstrate that the gene for the B chain of platelet-derived growth factor is expressed in Dupuytren’s contracture. “The presence of platelet-derived growth factor B and its receptor in patients who have Dupuytren (sic) contracture suggests that platelet-derived growth factor B (PDGFB) acting through an autocrine and paracrine loop may account for the proliferation, contracture and abnormal biochemistry of fibroblasts in Dupuytren contracture. Future treatment strategies may be directed at reversing or counteracting this abnormality in gene expression”(Terek et al,1995). In over twenty years since that published article a large volume of research into Dupuytren’s disease has focused on trying to identify the molecular pathology underpinning Dupuytren’s disease. Whilst the fibromatous changes that occur during Dupuytren’s disease are well characterized in contrast, despite the many studies, a clear understanding of the molecular basis of the disease remains elusive. In 1994 Boerwinkle and Chan wrote “It is a developing paradigm in medical practice and research that both genetic and environmental factors are key contributors to disease aetiology and pathophysiology (Boerwinkle and Chan, 1994). Whilst it is undoubtedly the case that environmental factors play a role in the development of the disease, there is also strong evidence for a genetic basis for the disease. It is clear that a large proportion of individuals with the Dupuytren’s phenotype demonstrate a familial trait which is most commonly found to be inherited in an autosomal dominant fashion with variable expression and incomplete penetrance, based upon epidemiological studies. The difference in Dupuytren’s disease incidence observed between different ethnicities also highlights an important role for genetics.

In this thesis a combination of different genetic approaches has been used to investigate the molecular pathology of Dupuytren’s disease in Western Australia. The justification for further genetic investigation is the continued need to identify candidate genes underlying Dupuytren’s pathology. By using a previously untried combination of genetic
studies (Whole Exome Sequencing Analysis for family samples from those with mild and severe disease and a Genome Wide Association Study of a WA population) the aim was to use the combined datasets to identify genes that play functional roles in the development of Dupuytren’s disease.

The genetic defects underlying inherited traits have traditionally been investigated using linkage analysis which involved the use of microsatellite markers located throughout the genome as the basis for identifying regions inherited only in affected individuals within a family, ‘linking’ the causative defect to the regions identified. In the case of Dupuytren’s disease, a previous linkage study performed in a Swedish population was indicative of an important region being located on Chromosome 16. (Hu et al., 2005). However, traditional linkage studies such as this have been limited by low resolution and an inability to progress to identification of specific genes of interest.

More recent approaches using single nucleotide polymorphisms (SNPs) as markers throughout the genome to identify regions of interest have provided much higher resolution and greater ability to identify gene mutations involved in disease (Stranger, Stahl, & Raj, 2011). However, significant challenges in identifying causative mutations and functional data still remained (Edwards, Beesley, French, & Dunning, 2013).

Functional mutations can be identified in specific genes by using whole exome sequencing (WES) in studies of inherited diseases. This approach involves sequencing the part of the genome encoding for proteins to identify non-synonymous mutations that are likely to affect protein function and therefore underlie disease. It has the significant advantage over GWAS methodology in actually identifying functional mutations in specific genes that can be further characterized (Bao et al., 2014; Rabbani et al., 2014).

The DNA of four related individuals with a clinical diagnosis of Dupuytren’s disease was submitted for Whole Exome Sequencing (WES). Results obtained from sequencing these four family members with Dupuytren’s phenotype provided a set of potentially important mutations. However, no mutations of interest appeared to segregate with members of the
family that also had Dupuytren’s disease. No genes identified were similar to previous studies using a GWAS approach or linkage approach (Hu et al, 2005; Holman et al, 2011). Similarly, there was no overlap between the candidate genes identified and genes close to SNPs that were potentially associated with Dupuytren’s disease in the Busselton study (Chapter 3). Finally, related individuals with a severe disease phenotype were also investigated using WES. It was hypothesized that these individuals may have different mutations within the same genes as those with a mild phenotype that led to the more aggressive disease. However no overlap between the genes identified in this study and the previous studies was observed. Pathway analysis to identify potentially common pathways affected by genes across the studies did not clearly identify important pathways that were likely to be common across the individuals investigated. However, groups of genes with related functions within each study suggests the pathology may not be directly related to fibroblast activity but rather indirect influence on activity through other body systems.

### 6.2 Identification of causative mutations in Dupuytren’s disease

The current study has provided an analysis of DNA from three separate cohorts. The limitations of these methods have been discussed in individual chapters and are dominated by the inability to obtain sufficient statistical power for the GWAS study, limited numbers within each family study and the complexity of incomplete penetrance of the disease. Further whole exome sequencing of DNA obtained from two unrelated affecteds with a clinical history and phenotype suggesting severe or aggressive disease identified a set of different mutations, none of which demonstrated overlap with results of previous analyses. They did bring to light two groups of genes, however, that might suggest alternative pathways that could be significant in triggering this histology.

There are a number of key reasons that may contribute to the lack of commonality across the genetic studies and to the continued difficulty in identifying key mutations in this study. These include;

1. For the family and related individual WES studies the incomplete penetrance of Dupuytren’s disease may impact on the findings. Whilst only family members that were clinically assessed as positive cases of Dupuytren’s disease were initially sequenced to identify potential causative mutations, confirmation required sequencing of identified
mutations in both positive and negative DD family members to demonstrate segregation of the mutation with the disease. However, it is possible that either due to age of some individuals sequenced that they were yet to express the Dupuytren phenotype and were incorrectly assigned as negative for DD. In addition, there may be members of the family that were negative for DD but did inherit a contributing mutation and did not develop the disease due to other environmental/genetic factors. There is also the possibility, since Dupuytren’s disease is a common disease, that an individual family member with the disease may have a sporadic cause rather than the same genetic mutation prevalent in the family. There are also limitations of WES in the coverage and calling of each exon for every gene. This can result in specific genes with lower coverage for individual exons and lead to false negatives whereby mutations are not identified.

One key issue with using WES approach is that it will only identify mutations within the coding region of genes. This is a small fraction of the genome and recent studies of the human genome have clearly demonstrated the importance of entire regions of DNA that are non-coding and that influence expression of genes. Therefore, it is possible that the mutations do not lie within the exome at all and will be missed by this approach.

2. For the Busselton study which involved a GWAS type approach to a small population in Western Australia the key limitation is the lack of power of the approach given the small population and very small number of positive cases of Dupuytren’s disease. In addition, those identified as positive for Dupuytren’s disease were not assessed by plastic surgeons but were self-identified through questionnaires. Whilst this is unlikely to affect those that reported having an operation for DD, those that report having banding within the palm may be subject to misidentification. Another possible issue is the relatedness of the people within the study. This can lead to the identification of SNPS not associated with the disease but rather because a number of the affected individuals are more closely related than the control population. Finally, given the wide range in age, it is possible that many of the ‘unaffected’ in the Busselton study were not at the age where Dupuytren’s disease would be apparent. This would result in the control samples containing a number of those that are likely to develop DD in the future. However, this has a much smaller effect on the study than the likely relatedness of the positive individuals.
Two distinct approaches have largely been used to investigate DD. These are genetic approaches, as described here and including GWAS studies, or alternatively a transcriptome/proteomic approach interrogating disease-state cells for key changes when compared to control cells. These proteomic/transcriptomic studies have focused on isolating and investigating fibroblast activity as these are known to be the cells that drive collagen production in fibrosis, a hallmark of DD and other fibrotic diseases. However, the two key advantages of the genetic approach are that this does not assume a specific cell type is the driver of disease, and this may be important given our increasing understanding that fibrosis may be initiated and sustained by immune and other responses which drive fibroblast collagen production rather than by aberrant fibroblast activity itself. In addition, the advantage of the genetic approach is that the use of disease cells can inhibit the ability to find changes that drive disease when there are multiple other changes that are caused by the disease progression or are simply bystander changes associated with the pathology. Again the genetic approach avoids the difficulty of sorting disease-associated changes from disease-driving changes.

At this point in this study functional mutations have not been linked to the pathology of Dupuytren’s disease. This may be due to limitations in the study or alternatively indicate that rather than functional changes there are regulatory elements that drive DD pathology. These include specific elements controlling gene expression and epigenetic changes. The possible impact of these changes and methodology to investigate their involvement in DD is discussed below.

6.3 Identification of functional elements beyond the parameters of Whole Exome Sequencing – the ENCODE database.

There are many well defined elements within the human genome that influence the expression of genes within the exome, and these have been investigated over many years, with the discovery of Locus Control Regions (LCRs) and enhancer elements in DNA long before the human genome sequence was completed. These types of regulatory elements will not be identified using the WES approach described in this thesis. Since 2003 it has
been possible to identify and characterize these functional elements in much more detail in the human genome sequence and this work has been undertaken as part of a consortium pursuing the ENCODE project (ENCyclopedia Of Dna Elements), which reported preliminary results in 2007 and updated work in 2014 (Birney et al, 2007; Kellis et al, 2014). The findings of the ENCODE project demonstrate large numbers of conserved and non-conserved elements through evolution that have significant impact on gene expression. In addition, recent studies have increasingly focused on the roles of long non-coding RNA elements in regulating gene expression. This increasing awareness and knowledge of the diversity of regulation of gene expression adds significantly to the complexity of interrogating disease-causing mutations, particularly in diseases such as DD where there are likely to be many factors that contribute to the disease. As with our understanding of epigenetic regulation, this area is developing very rapidly and may lead to novel insights into DD and other diseases as the information becomes better understood and interpreted.

6.5 Epigenetic influences in control of gene expression.

In relation to fibroblast activity and collagen production there are many stages that contribute to the regulation of collagen levels in the extracellular matrix, from post-transcriptional processing through to secretion, cross-linking and stability as well as degradation. The control of gene expression is also a critical mechanism for normal cell function. Epigenetic changes, that is changes to the DNA that are not sequence changes but structural (commonly methylation status changes or histone changes in DNA packaging) have been shown to be involved in several complex diseases including cancer and in normal development and have been investigated in fibrotic and neural disorders. Epigenetic changes result in increased and decreased expression of genes and are now well described as being important in disease. From epigenetic studies several genes, including Rasal1 in renal fibrosis (Bechtel et al, 2010; RASAL1 encoding an inhibitor of the Ras oncoprotein), Fli1 in scleroderma (Wang et al, 2006) and Thy1 in pulmonary fibrosis (Saunders et al, 2008) have been demonstrated to be hypermethylated and to induce fibroblast proliferation and the production of collagen in fibrotic disease. Therefore, it is feasible that there are epigenetic changes in DD that are important in the
disease progression. However, to date no studies have clearly demonstrated a role for epigenetic changes in the molecular pathology of DD. It is also possible that the epigenetic changes described in fibrotic conditions arise as a result of cellular changes rather than drive cellular change. Therefore, it will be important to characterize and understand whether epigenetic changes and regulation play a role in DD.

6.6 Whole Genome Sequencing (WGS)

Kellis et al, (2014) have reviewed the strengths and limitations of biochemical, evolutionary and genetic approaches for defining functional DNA segments and have concluded that the elucidation of genome function will require a combination of all three approaches (Kellis et al, 2014). The extensive GWAS study by Dolmans et al (2011), published in the New England Journal of Medicine, implicated the Wnt signalling pathway in the process of fibromatosis (as did Ojwang et al, 2010, and Shih et al, 2012), but with the limitation of identifying a region of the genome but not the actual mutations involved or the molecular changes involved in the disease progression. WGS does provide an opportunity to interrogate the entire genome and any sequence changes that may contribute to disease. However, because of the increasingly large sets of data that require increasing numbers of patients to be interrogated, and often a lack of knowledge of the function of many non-coding elements in the DNA, it will not necessarily provide an opportunity to identify DD related mutations and their effects. This is in addition to the possibility that epigenetic factors play a role and the influence of environmental factors that may diminish the contribution of genetics to the disease phenotype. To date no WGS approach to DD has been attempted. This may change as decreasing costs and increasing speed of the approach, as well as better data analysis tools will make it more feasible for the investigation of the genetics of disease.

6.7 The identification of molecular changes in DD using disease derived cells.

As mentioned above, the alternative approach to genetics that has been used to try to identify important molecular changes in DD is cell isolation from diseased tissue and either transcriptomic or proteomic profiling of these cells. A comparison of gene expression in Dupuytren’s tissue against normal tissue has been reported by Forrester et
al (2014), also by Zhang et al (2008) and others (reviewed in Bayat, 2012). The latter demonstrated a dominant increase in TGFβ2 expression in Dupuytren’s cord tissue and suggested that this gene expression profile could prove useful in developing a specific gene therapy for this pathology. Shih et al (2009) also used a comparative analysis of Dupuytren’s fibroblasts with tissue biopsies in disease-specific phenotypes for the purpose of identifying biomarkers (Shih et al, 2009).

The study by Forrester et al (2014) analysed RNA isolated from five affected individuals, but identified over three hundred genes that were either up-regulated or down-regulated in the cultured Dupuytren’s fibroblasts. Their findings provide compelling evidence that the development and progression of Dupuytren’s disease is closely associated with significant up-regulation of a broad group of collagen genes and down-regulation of matrix metallo-proteinase and collagenase genes involved in extracellular matrix remodelling. These results are used to justify injection of Dupuytren’s with collagenase from Clostridium histolyticum, a non-surgical treatment that has resulted in short-term release of contractures in some cases while complicated by debilitating pain and significant deep tissue adhesions in the longer term (Rozen et al, 2012).

Possibly the most significant genes up-regulated in the Dupuytren’s cells were two Fibroblast Growth Factor genes KGF9 and FGF11, a suite of keratin genes (KRT34), and others which are also involved in inflammatory diseases (Tumour Necrosis Factor) and found in synovial fluid in osteoarthritis and rheumatoid arthritis (TNFAIP6 and VCAM1). A number of genes identified as up-regulated were expressed in Diabetes and other conditions sometimes associated with Dupuytren’s and are therefore not reliable biomarkers of this condition.

As with other studies of this type the major limitation in the use of diseased cells is the inability to separate driver and bystander changes in expression. In addition, these studies have focused largely on fibroblasts, which whilst they clearly are responsible for the increased collagen deposition observed, may not in themselves be pathogenic but rather responding to signals from other cell types that actually trigger and stimulate disease.
6.8 Possible non-fibroblast origin of Dupuytren’s disease
Several aspects of the familial inheritance and clinical and histological features of Dupuytren’s disease within a specific family strongly support an autosomal genetic component to the disease. Findings in other genetic based studies have often focused on genes involved in the regulation of fibroblast activity, including pathological collagen production and lattice contraction in the extra-cellular matrix, as well as apoptosis and proliferation and alpha-smooth muscle actin expression. Even with whole genome and linkage approaches the absence of definitive functional changes has led to the implicated genes often being highlighted as likely to have important functions in fibroblasts. In addition, those studies that have used Dupuytren tissue for transcriptome and proteomic approaches, in addition to many of the histology and immunochemical studies have focused on the fibroblast cells. This is understandable given the clear role of these cells in the pathology of the disease. However, whilst fibroblasts clearly play a role in the disease it is important to also focus on possible triggers and causes of the disease. This is feasible with genetic approaches that do not involve cell isolation or proteomic profiling and can lead to insight into processes that may underpin the changes that lead to Dupuytren fibrosis.

The suggestion of a complex polygenic inheritance involving several pathways is readily accepted in light of the variable expressivity and degree of phenotypic progression (Hu et al, 2005; Gudmundsson et al, 2001). Differences may well depend upon the nature and timing of trigger factors, some of which remain to be identified. The effect of direct trauma to the hands remains uncertain, while some series depict Dupuytren’s as an occupational hazard and others suggest that occupation involving repeated pressure on the palms may have a contributory role in its aetiology. The occurrence of Dupuytren’s disease in females and others with no history of manual labour would argue against the significance of occupational trauma, while the very unequal incidence of Dupuytren’s between males and females (6:1) invokes a possible hormonal influence and in particular fluctuations in relation to ageing and menopause.

A review of external factors and the possible significance of their role in this disease has reinforced the importance of certain ethnic, cultural and lifestyle factors, including northern European origins, on the incidence of Dupuytren’s. Certain habits and co-
morbidities such as smoking and diabetes, which have been associated with Dupuytren’s in many epidemiological surveys may well provide a trigger or facilitating role in genetically susceptible individuals. In this regard it would seem that a parallel may exist between Dupuytren’s fibromatosis and other diseases whose histochemical processes are initiated and controlled by neuronal activity.

6.9 Neuronal activity/disruption and Dupuytren’s disease

The possibility of neuronal involvement in Dupuytren’s disease may be implicated from the study findings. Rare mutations found in severe cases (Chapter 4) (e.g. BHMT, CACNA1A, PRX); and in the family study (LRSAM1) from Chapter 2 (Tandan et al, 1990) are known to play important role in peripheral innervation. In particular, LRSAM1 identified as a candidate for Dupuytren’s (Ch2) is involved in a peripheral neuropathy and this may be a significant factor if it has a role in DD.

Referring to the skin as a neuroimmunoendocrine organ Roosterman et al (2006) drew attention to the neuronal function of the skin in numerous clinical disorders including wound healing. The histochemical changes involving inflammation, cellular proliferation, fibroblast activity and remodelling, which are characteristic of normal wound healing, also occur in palmar fibromatosis. Neuropeptides released from the cutaneous innervation are essential to neuroendocrine and neurovascular activity responsible for the cell proliferation (abnormal myofibroblasts), cytokine and growth factor production, and neovascularisation observed in progressive Dupuytren’s disease.

The involvement of neuronal pathways in the triggering or progression of palmar fibromatosis is worth investigating further. Normal diagnostic histology has not identified abnormal nerve patterns in Dupuytren’s tissue but special stains and immunohistochemistry could be applied to fresh sections or to cultured fibroblasts to further investigate neuropeptides and growth factors produced by these cells. Specific cellular activity may be observed by electron microscopy and the functional response of myofibroblasts to a range of external stimuli may be tested in-vitro or in an animal model.

Further investigation of the role of sensory neurons and their secretomes in triggering fibrosis has been suggested because of the frequent expression of cells capable of producing neuropeptides commonly identified in all forms of fibrotic disease. There may
be a genetic susceptibility to all of these conditions through mutations caused by infective, immune, traumatic, neuroendocrine, metabolic or other physiological challenges, but the subsequent pathway to progressive fibromatosis could involve a limited number of stages which respond to variable functional mutations. Secretomes such as cytokines and growth factors provide a favourable environment for cell growth and proliferation, and much depends upon a balance between neurotrophins and apoptosis. Subsequent remodelling depends on the relative activity of cell changes and chemical structure of the extracellular matrix. The stages of proliferation and remodelling are impacted by both genetic and environmental factors which accounts for some of the variability in presenting phenotype.

A neuronal hypothesis could be tested by demonstration of in-vitro response of cultured fibroblasts to neuropeptides and other secretomes, and possibly reinforced by positive evidence of cell differentiation and proliferation. This work could be carried out using in vitro models that mimic stretch and/or matrix stiffness to more closely mimic the conditions of DD and provide insight into the interaction between innervation and fibrosis in DD.

There is increasing evidence that the peripheral nervous system and autonomic nerves may play an important role in mediating normal wound healing, a histochemical process involving inflammation, cellular proliferation, fibroblast activity and remodelling (Roosterman et al, 2006). These changes are also characteristic of the progressive fibromatosis of Dupuytren’s disease and their common molecular basis may be significant.

The processes involved in wound healing involve a combination of neuro-endocrine and neurovascular activity, vital changes which are dependent upon neuropeptides released from the cutaneous innervation. These changes are also characteristic of the progressive fibromatosis of Dupuytren’s disease. The fundamental processes of healing and scar formation include cell migration and proliferation, inflammation, synthesis and secretion of cytokines and extracellular matrix proteins and remodeling. In individuals with a familial predisposition, some of these changes can become excessive in formation of keloid and hypertrophic scars. In keloids keratinocytes induce fibroblasts to secrete Connective Tissue Growth Factor (CTGF), a downstream mediator of the role of
Transforming Growth Factor β (TGFβ) in enhancing fibrosis. Keratinocytes also directly increase the proliferation of underlying dermal fibroblasts while decreasing their production of collagen.

In summary, innervation plays an important role in wound repair and tissue maintenance and has been demonstrated to be critical to repair in the skin. It is possible that innervation also plays a role in the pathobiology of DD and this warrants further investigation.

6.10 Angiogenesis and a role for vascular changes in Dupuytren’s disease

The pathological remodelling of connective tissue characteristic of scleroderma is suggestive of fundamental interactions between microvascular damage and inflammation linked to tissue fibrosis (Abraham and Varga, 2005). This complex autoimmune disease results in abnormally fibrotic scars in areas of increased tension (cf hypertrophic scars) with induction of Connective Tissue Growth Factor (CTGF) by Transforming Growth Factor β (TGFβ), a process that may be suppressed by Tumour Necrosis Factor alpha. The anti-sclerotic effect of TGFβ antibody was demonstrated in a mouse model with bleomycin-induced scleroderma (Yamamoto et al, 2005). Angiotensin II (ANG II) appears to influence tissue repair via activation of angiotensin I receptor (AT1R) in fibroblasts, leading to collagen remodelling, collagen gel contraction, and upregulation of collagen-binding integrins in-vitro (Watson et al, 1998). Platelet-derived growth factor (PDGF) also plays an essential role in embryo development and is critical for tissue repair. Overactivity of PDGF has been linked to tumour progression of glioblastoma and sarcomas and has also been implicated in atherosclerosis and several fibrotic conditions, including pulmonary fibrosis, renal fibrosis, hepatic cirrhosis, and myelofibrosis (Heldin et al, 1999, 2013; Bonner, 2004). PDGFR alpha also facilitates infections by certain viruses (Soroceanu et al, 2008).

In Dupuytren’s contracture both PDGF A- and B-chain were expressed by primary cell cultures from palmar fibromatosis, and the expression increased when the cells were subjected to mechanical strain (Alman et al, 1995,1996). An immunohistochemical analysis revealed that PDGF A- and B-chains are present in infiltrating inflammatory cells. The synthesis of PDGF by inflammatory cells is often increased in response to
external stimuli such as exposure to low oxygen tension or stimulation by other growth factors and cytokines. PDGF receptors are upregulated, suggesting a role for PDGF in mesenchymal cell proliferation in some autoimmune diseases including rheumatoid arthritis and scleroderma, both conditions not normally associated with Dupuytren’s disease. The possibility that reduced innervation and hypoxia contribute to DD is also suggested by the association of Diabetes and concurrent reduction in peripheral circulation with the development of DD. In the studies reported here genes with roles in angiogenesis and vascularization were identified as being candidates of interest with functional mutations. These may be investigated further as this work is progressed to continue to try to identify functional changes involved in DD.

6.11 Fibroblasts, ECM changes and Dupuytren’s disease

Dermal fibroblasts (DF) represent the main cellular component of the dermis and are implicated in the homeostatic maintenance of skin extracellular matrix (ECM). They are metabolically active cells and their role is associated with the synthesis and secretion of collagens, proteoglycans, fibronectin, and metalloproteases. Dermal fibroblasts are involved in physio-pathological processes, including wound healing and fibrosis (Krieg et al., 2007; Werner et al., 2007) and have therefore been the dominant focus of research into fibrotic diseases, including DD. After injury, different factors, in particular TGFβ (Desmouliere et al., 2003), promote dermal fibroblast differentiation into myofibroblasts (DM), characterized by the expression of alpha Smooth Muscle Actin (α-SMA) (Hinz, 2007), which is therefore used as a myofibroblast differentiation marker. The dermal myofibroblasts are the key effector in injury/repair processes and fibrosis, as they control ECM component deposition, tissue contraction and wound resolution (Hinz, 2007), and their subsequent apoptosis is essential for tissue re-epithelization (Desmouliere et al., 2005). Whilst fibroblasts are clearly a key cell type involved in the pathology of DD, in this study candidate one key advantage of the genetic approaches used in this study (and others) is that unlike tissue based DD studies that have isolated fibroblasts from disease tissue to characterize the changes involved the genetic studies allow the identification of
genes that may be important but not related to fibroblast activity. This has clearly been seen in this study as not only matrix genes but other pathways have been highlighted. Interestingly one possible hypothesis that has not been extensively explored to explain DD pathology is the threshold effect that is seen in mitochondrial disorders (Bayat et al, 2004). Severe cases of fibromatosis affecting both hands and feet tend to be particularly common in individuals with a positive family history. Interestingly, a known familial condition affecting the palms and the soles (palmoplantar keratoderma) associated with sensorineural hearing loss is attributable to a specific mitochondrial mutation A7445G (Sevior et al, 1998). Mitochondrial DNAs with substantial mutations replicate more rapidly than normal mitochondrial DNAs, a disproportion more noticeable in some specific tissues (eg muscle) and considered to be related to increasing age (Weber et al, 1997). The variation in age of onset of Dupuytren’s fibromatosis may be related to the rate of accumulation and percentage of accumulated mutated mitochondrial DNA in affected tissues. This may be an alternative explanation for changes in fibroblast activity unrelated to changes in the cellular DNA or gene expression but linked instead to the mitochondria.

6.12 Infection and Immune system involvement in DD

A number of degenerative neurological disorders are also known to be caused by viruses, and it is this intimate association with gene mutations with functional diversity that provides a possible driver for the immunogenetic pathway of Dupuytren’s pathology (Li Zhou, 2013).

The presence of inflammatory cells, including multinucleated macrophages in healing wounds, scar tissue and the fibromatoses including Dupuytren’s disease, is considered an indication of immune response to either tissue antigens or foreign proteins including viruses. The significance of immunological pathways in any phase of disease where inflammation is apparent in response to viral infection or other stimulus is that they may represent a broader target in terms of prevention or treatment (Deleidi and Isacson, 2012).
Verjee et al (2013), in a study of freshly isolated tissue from Dupuytren’s patients, found that it contained significant numbers of immune cells including activated macrophages and released proinflammatory protein mediators (cytokines). Of the latter only TNF selectively converted normal fibroblasts from the palm of patients with Dupuytren’s disease into myofibroblasts via activation of the Wnt signaling pathway.

In 1990 Bower and co-workers published data on Dupuytren’s disease in a cohort of men with complications of severe HIV infection. Rather surprisingly this group of men with an age range of 19 to 54 years showed a prevalence of 36% with Dupuytren’s disease (compared with an expected 4-6% in the general population, even less in this younger age group). The purpose of their study was to investigate further the role of free radicals, as proposed by Murrell et al in 1987, in the pathogenesis of Dupuytren’s disease. Superoxide free radicals produced as a result of ischaemia-related conversion of xanthine dehydrogenase to xanthine oxidase are thought to induce proliferation of fibroblasts and the production of type III collagen (Murrell et al, 1987).

The rather high prevalence of Dupuytren’s among patients with HIV (Human Immunodeficiency Virus) infection was thus linked with disturbed metabolism of free radicals. Although HIV may have originated as early as 1920, the pivotal year appears to be 1981 when previously healthy young gay men in Los Angeles developed a rare lung infection called Pneumocystis carinii pneumonia (PCP), and by December of that year there were 270 reported cases of severe immune deficiency among gay men and injected drug users with 121 fatalities. There were also reports of men in New York and California with unusually aggressive Kaposi’s Sarcoma.

Although there are an estimated 35 million individuals worldwide now living with AIDS, the majority of them in sub-Saharan Africa, no further reference to an association with Dupuytren’s disease is available. But the potentially fatal Kaposi’s sarcoma, an aggressively malignant lymphovascular fibromatosis which is caused by Human Herpes Virus VIII, is most commonly found now in men with AIDS.
6.13 Future studies

The current study has included identification of thirty-one candidate genes from whole exome sequencing of DNA in four related family affecteds (Chapter 2; Table 2.4). These mutations were selectively screened against SNP databases and further restricted to twelve non-synonymous heterozygous mutations, of which two were in regions of positive linkage. Sanger sequencing of PCR fragments was focused on 3 candidate genes to determine whether these mutations segregated within the family in members with Dupuytren’s disease. GORASP1 and EXOG genes were both selected due to their identification in a region of positive linkage from initial analysis. In addition, COL6A5 was also selected for sequencing due to the fundamental link between collagen deposition and fibrotic disease. Initial sequencing showed that no mutation segregated with the disease in additional family members. Further investigation of the other non-synonymous mutations identified in four (affected) family members (Table 2.5) could possibly identify significant segregation with Dupuytren’s.

Whole exome sequencing from a limited number of family affecteds has possibly restricted the number of identifiable potential candidate genes for further investigation. Ideally the cohort could be expanded to include Dupuytren’s phenotype in other generations, as well as non-affecteds, which might provide evidence to reinforce the current findings.

The problem remains with the large number of possible candidates that have been excluded by the selection process, all of them identified in DNA from family members with Dupuytren’s and a number that were found to occur in all family members.

As expected, the WES has identified only those genes with a functional role, sequencing the part of the genome encoding for proteins to identify non-synonymous mutations that are likely to affect protein function rather than those affecting expression levels. Further sequencing of additional variants found in all family members to identify mutations that segregate with Dupuytren’s disease would be useful. Whole Genome sequencing has been mentioned previously as a possible approach while acknowledging the financial limitations and an anticipated burden of genetic information.
A further study of severe cases would be justified and more significant results could be obtained from an expanded cohort with aggressive Dupuytren’s disease, including related individuals with this condition. While different genes may be involved in severe cases their variance might be related to environmental or epigenetic factors and some gene overlap with those currently identified could still occur.

Comparison with other fibrotic diseases at a molecular level has already provided some understanding of the processes involved and it is reasonable to maintain a belief in the common pathways hypothesis while understanding that not only the characteristic tissue structures but also trigger mechanisms will account for phenotypic variations.

The eventual challenge of fibroblast activity and function in relation to chemical and neuronal responses will rest with laboratory tests in vitro, including epigenetic regulation of gene expression in relation to fibroblast activity.

Our ultimate goal must be to determine the molecular mechanisms that control the pathogenesis of Dupuytren’s disease with the aim of prevention and/or treatment by molecular control. For the functional annotation of genetic elements in a familial condition such as Dupuytren’s CRISPR would be ideal for identifying and targeting specific genetic mutations eg those candidate genes identified by exome sequencing (Chapters 2/4) and/or mutations identified in previous studies and presumed to be involved in the pathogenesis of Dupuytren’s disease. It would be useful to correct or modify these genes and observe changes in the cell phenotype and function in-vitro. This would provide strong evidence for a causative link between the mutations and the clinical disease state.

The application of CRISPR in genome-wide studies would also enable large-scale screening for therapeutic targets and other phenotypes and will facilitate the generation of engineered animal models that would benefit pharmacological studies and the understanding and treatment of the human condition.

**6.14 Summary**

Possibly for the first time an integrated approach has been taken to determine a genetic basis for Dupuytren’s disease. The combination of a familial cohort subjected to Whole
Exome Sequencing (WES), a larger cohort of affecteds from a community population survey whose DNA was sequenced by Genome Wide Association (GWAS), and a very limited number of unrelated individuals with evidence of a severe phenotype (WES), has not previously been reported. Although a number of candidate genes were identified as non-synonymous for Dupuytren’s in each cohort there was no overlap between these three groups. The hypothesis has not been proven. However, further studies of those mutations involving the application of specific biomarkers to Dupuytren’s fibroblasts in-vitro could determine specific cellular response and validate the ability of certain genes to regulate apoptosis, proliferation, myofibroblast differentiation, collagen and alpha smooth muscle actin expression and fibroblast-populated collagen lattice contraction. Comparisons with results from other similar studies including Dupuytren’s tissue DNA sequencing have also failed to identify positive overlap. The author agrees with previous researchers in concluding that the underlying cause of Dupuytren’s fibromatosis is a complex system of polygenic pathways facilitated or triggered by a variety of environmental factors. It remains possible, however, that further studies of neuronal pathways and their influence on aetiology and pathogenesis of fibromatosis could displace current theory regarding cell differentiation and proliferation in Dupuytren’s disease.
REFERENCES


Altun V. et al. (2001). Nerve outgrowth and neuropeptide expression during the remodeling of human burn wound scars: a seven month follow-up study of 22 patients. Burns


Investigating the Molecular Pathology of Dupuytren’s Disease


Bouzas-Rodriguez, J. and Mehlen, P. (2010). Neurotrophin 3 (NTF3); neurotrophic tyrosine kinase receptor 3 (NTRK3; TrkC) J Clin Invest 120:850-858


Investigating the Molecular Pathology of Dupuytren's Disease


Forrester, H., Temple-Smith, P., Ham, S. et al. (2013). Genome Wide analysis using exon arrays demonstrates an important role for expression of extracellular matrix, fibrotic control and tissue remodeling genes in Dupuytren’s disease. PLOS One, 8(3). e59056.


Gerdts, J, Brace, E.J. et al 2015. SARM1 activation triggers axon degeneration locally via NAD. Science 348(6233); 453-457.


Heilig R. and Weissenbach J (2003)., The DNA sequence and analysis of Human chromosome 14; Nature


Jonsson et al. (2013). Association of HLA DRB1 01 with Dupuytren’s disease. Scand J Rheumatology; 42: 45-47


Investigating the Molecular Pathology of Dupuytren’s Disease


operated hands from 1956 to 2006. BMC Musculoskeletal Disorders;8:60;1186:1471-1474.
Investigating the Molecular Pathology of Dupuytren’s Disease

Na Nui and Xuebin Qin (2013). New insights into IL-7 signaling pathways during early and late T cell development. Cellular & Molecular Immunology, 10, 187–189;


Investigating the Molecular Pathology of Dupuytren’s Disease


Investigating the Molecular Pathology of Dupuytren’s Disease


Tsai and Wu, 2010. A genome-wide association study identifies susceptibility variants for Type 2 Diabetes in Han Chinese, PLOS Genet


Wells RG. Tissue mechanics and fibrosis. Biochimica et Biophysica Acta (2013) 1832:884-890
Investigating the Molecular Pathology of Dupuytren’s Disease


