Identification of biomarkers and whole genome scanning in Dupuytren’s Disease

A thesis submitted to the University of Manchester for the degree of Doctor of Medicine in the Faculty of Medical & Human Sciences

2013

Sandip Hindocha

Faculty of Medical and Human Sciences

School of Medicine

Plastic and Reconstructive Surgery Research
List of Contents

List of Abbreviations .................................................. 6
List of Figures ............................................................. 7
List of Tables ............................................................... 8
List of peer reviewed publications, presentations and prizes related to this thesis ........................................ 10-12
Declaration ...................................................................... 13
Copyright Statement ...................................................... 14
Acknowledgements and dedications .................................. 15
Abstract ........................................................................... 16
Chapter 1: Introduction & Literature review. On the history, anatomy, epidemiology, genetics and pathology of DD .................................................. 17-84
  1.1 Introduction .................................................................. 18-19
  1.2 The history of Dupuytren’s Disease ................................. 20-21
  1.3 The anatomy, histology and clinical presentation of DD ....... 22-24
  1.4 Clinical severity and Dupuytren’s Diathesis ....................... 25-28
  1.5 Epidemiological evaluation of Dupuytren’s disease incidence and prevalence rates in relation to aetiology .......... 29
    1.5.1 Identification of epidemiological studies ....................... 30
    1.5.2 Review of significant studies ..................................... 31
    1.5.3 Misinterpretation of incidence and prevalence in DD .... 31
    1.5.4 Analysis of 62 epidemiological studies reveals the most reliable data sets and the difficulty of study comparison .......... 33
    1.5.5 Prevalence of associated aetiological factors in DD ...... 48-56
    1.5.6 Prevalence rates calculated in a clinical and community setting .................................................. 56-57
    1.5.7 Prevalence rates and its relation to assessor of disease ........ 59
    1.5.8 Cases of DD in various atypical geographical locations .... 59
    1.5.9 Age and gender specific epidemiology of DD .............. 60
    1.5.10 Comparing epidemiological data for DD from different sources .................................................. 61-62
  1.6 Genetics of Dupuytren’s Disease .................................... 63
    1.6.1 Identification of manuscripts discussing the genetic nature of DD .................................................. 63
    1.6.2 Familial aggregation studies & segregation analysis ......... 64-65
    1.6.4 Molecular genetic studies ......................................... 65-70
    1.6.5 Genetics and pathology concepts ............................ 70-72
  1.7 Identifying the source of cellular DD: Investigating stem cell markers .................................................. 81-82
  1.8 Summary ...................................................................... 83
  1.9 Hypotheses ................................................................... 84
  1.10 Aims .......................................................................... 84
Chapter 2. Study 1. Estimating penetrance and revising the sibling recurrence risk ratio in DD .................................................. 85-105
  2.1 Introduction ................................................................... 86-87
  2.2 Materials and methods .................................................. 88
    2.2.1 UK patient database and DD pedigree ascertainment .... 88
    2.2.2 Observing a pattern of inheritance in DD and evaluating environmental factors in relation to positive family history ........ 89
2.2.3 Pedigree analysis: Calculating the level of penetrance

2.2.4 Pedigree analysis: Revising the sibling recurrence risk ratio (λs)

2.2.5 Statistical analysis

2.3 Results

2.3.1 Family description

2.3.2 Pattern of inheritance of DD

2.3.3 Assessment of environmental risk factors on family history of DD

2.3.4 Quantification of penetrance in DD

2.3.5 Revised λs in DD

2.4 Discussion on the genetic epidemiology of DD and its application in clinical practice

Chapter 3. Study 2a. Whole genome scanning and linkage analysis in DD. Study 2b. Long term follow up of Icelandic pedigree, further linkage analysis and sequencing in DD

3.1 Introduction

3.2 Materials and methods

3.2.1 The road to Iceland: The search for a large multi-case DD pedigree

3.2.2 Identifying a collaborative research group in Iceland

3.2.3 Identifying a study cohort. Identification of the largest DD family to date

3.2.4 Whole genome scan – platform options

3.2.5 DNA collection and deliverance

3.2.6 DNA extraction

3.2.7 Genotyping and Quality Control (QC)

3.2.8 Statistical analysis

3.3 Results

3.3.1 Clinical data on the large Icelandic Dupuytren’s pedigree

3.3.1.1 Comment on the Icelandic pedigree

3.3.2 DNA Quality Control Analysis

3.3.3 Linkage analysis results

3.3.3.1 Linkage analysis results for chromosome 1

3.3.3.2 Linkage analysis results for chromosome 2

3.3.3.3 Linkage analysis results for chromosome 3

3.3.3.4 Linkage analysis results for chromosome 4

3.3.3.5 Linkage analysis results for chromosome 5

3.3.3.6 Linkage analysis results for chromosome 6

3.3.3.7 Linkage analysis results for chromosome 7

3.3.3.8 Linkage analysis results for chromosome 8

3.3.3.9 Linkage analysis results for chromosome 9

3.3.3.10 Linkage analysis results for chromosome 10

3.3.3.11 Linkage analysis results for chromosome 11

3.3.3.12 Linkage analysis results for chromosome 12

3.3.3.13 Linkage analysis results for chromosome 13

3.3.3.14 Linkage analysis results for chromosome 14

3.3.3.15 Linkage analysis results for chromosome 15
3.3.3.16 Linkage analysis results for chromosome 16

3.3.3.17 Linkage analysis results for chromosome 17

3.3.3.18 Linkage analysis results for chromosome 18

3.3.3.19 Linkage analysis results for chromosome 19

3.3.3.20 Linkage analysis results for chromosome 20

3.3.3.21 Linkage analysis results for chromosome 21

3.3.3.22 Linkage analysis results for chromosome 22

3.3.4 Summary of important SNPs identified

3.4 Discussion

3.5 Study 2b. Long term follow up of Icelandic pedigree, further linkage analysis and sequencing in Dupuytren’s Disease

Chapter 4. Study 3. Abnormal stem cell markers found in DD.

4.1 Introduction

4.2 Materials and Methods

4.2.1 Patient selection

4.2.2 Tissue extraction

4.2.3 Selection of stem cell markers to be investigated

4.2.4 Immunohistochemistry (IHC)

4.2.5 Qualitative Real-Time Polymerase Chain Reaction (QRT-PCR)

4.2.6 Fluorescence Activated Cell Sorting (FACS)

4.2.7 Statistical Analysis

4.3 Results

4.3.1 DD tissue is observed to have markers consistent with mesenchymal and haematopoietic stem cell populations

4.3.2 Elevated expression of CD13+ and CD29+ link adipocytes to DD pathogenesis

4.3.3 CD29 is observed to be a more predominant marker in DD in comparison to carpal tunnel control

4.3.4 CD34 shows high expression in DD skin

4.3.5 Frequency of CD44+ and CD90+ cells were observed to be consistent in the nodule and cord

4.3.6 FACS analysis suggested the possibility of population of MSC’s and HSC’s in DD

4.4 Discussion

Chapter 5. Discussion and suggestions for future study

5.1 Overview

5.2 Familial studies in DD (chapter 2) – discussion and future work

5.3 Whole genome scanning in DD (chapter 3) – discussion and future work

5.4 Characterisation of stem cell markers in DD (chapter 4) – discussion and future work

5.5 Conclusions

References

First page of peer reviewed publications

Appendix 2.1 Dupuytren’s Disease study: Patient consent form

Appendix 2.2 Genetics of Dupuytren’s Disease: Data collection proforma
Appendix 2.3  Genetics of Dupuytren’s Disease: Family Questionnaire  230-231
Appendix 2.4  Family pedigrees from the UK DD cohort  232-300
Appendix 3.1  Letter of intent. Icelandic Collaborative Agreement.  301-302
Appendix 3.2  DNA extraction protocol  303-304
Appendix 3.3  Whole genome scan protocol  305-312
Appendix 3.4  Clinical photographs of Icelandic pedigree  313-342
Final Word Count  50463
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>4',6-diamidino-2-phenylindole</td>
<td>DAPI</td>
</tr>
<tr>
<td>Bone Morphogenic Protein</td>
<td>BMP</td>
</tr>
<tr>
<td>Complimentary Deoxyribonucleic Acid</td>
<td>cDNA</td>
</tr>
<tr>
<td>Deoxyribonucleic Acid</td>
<td>DNA</td>
</tr>
<tr>
<td>Deoxyribonucleotide Triphosphate</td>
<td>dNTP</td>
</tr>
<tr>
<td>Dupuytren’s Disease</td>
<td>DD</td>
</tr>
<tr>
<td>Genotyping Console</td>
<td>GTC</td>
</tr>
<tr>
<td>Haematopoietic Stem Cell</td>
<td>HSC</td>
</tr>
<tr>
<td>Human Leucocyte Antigen</td>
<td>HLA</td>
</tr>
<tr>
<td>Logarithm (base 10) of odds</td>
<td>LOD</td>
</tr>
<tr>
<td>Matrix Metalloproteinase</td>
<td>MMP</td>
</tr>
<tr>
<td>Mesenchymal Stem Cell</td>
<td>MSC</td>
</tr>
<tr>
<td>Metacarpo-Phalangeal</td>
<td>MP</td>
</tr>
<tr>
<td>Proximal Inter-Phalangeal</td>
<td>PIP</td>
</tr>
<tr>
<td>Quality Control</td>
<td>QC</td>
</tr>
<tr>
<td>R Spondin 2</td>
<td>RSPO2</td>
</tr>
<tr>
<td>Real Time Polymerase Chain Reaction</td>
<td>RT PCR</td>
</tr>
<tr>
<td>Reference Single-Nucleotide Polymorphism Identification</td>
<td>rs ID</td>
</tr>
<tr>
<td>Ribonucleic Acid</td>
<td>RNA</td>
</tr>
<tr>
<td>Sibling recurrence risk ratio</td>
<td>λs</td>
</tr>
<tr>
<td>Single-Nucleotide Polymorphism</td>
<td>SNP</td>
</tr>
<tr>
<td>Tissue Inhibitor of Metalloproteinase</td>
<td>TIMP</td>
</tr>
<tr>
<td>Transforming Growth Factor</td>
<td>TGF</td>
</tr>
<tr>
<td>Transmission Disequilibrium Test</td>
<td>TDT</td>
</tr>
<tr>
<td>Tyrosine-protein kinase transmembrane receptor</td>
<td>ROR2</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1.3.1</td>
<td>Anatomy of the palmar fascia</td>
</tr>
<tr>
<td>1.3.2</td>
<td>Extensive anatomy of normal fascia and DD</td>
</tr>
<tr>
<td>1.3.3</td>
<td>Histology of DD</td>
</tr>
<tr>
<td>1.3.4</td>
<td>Clinical presentation of DD</td>
</tr>
<tr>
<td>1.5.1</td>
<td>Global distribution of DD</td>
</tr>
<tr>
<td>1.5.2</td>
<td>The worldwide prevalence of DD</td>
</tr>
<tr>
<td>1.5.3</td>
<td>Mean global DD prevalence</td>
</tr>
<tr>
<td>1.5.4</td>
<td>Prevalence of DD in epileptics</td>
</tr>
<tr>
<td>1.5.5</td>
<td>Prevalence of DD in diabetics</td>
</tr>
<tr>
<td>1.5.6</td>
<td>Varying prevalence rates among different communities in the same country</td>
</tr>
<tr>
<td>1.6.1</td>
<td>Signalling pathways of WNT and B catenin</td>
</tr>
<tr>
<td>2.3.1</td>
<td>Age at onset of DD</td>
</tr>
<tr>
<td>2.3.2</td>
<td>Alcohol consumption in DD</td>
</tr>
<tr>
<td>2.3.3</td>
<td>Distribution of digits affected with DD</td>
</tr>
<tr>
<td>3.3.1.1</td>
<td>Pedigree of the Icelandic family</td>
</tr>
<tr>
<td>3.3.2</td>
<td>Linkage analysis data output example</td>
</tr>
<tr>
<td>3.3.3</td>
<td>Representative graph for chromosome 1</td>
</tr>
<tr>
<td>3.3.4</td>
<td>Representative graph for chromosome 2</td>
</tr>
<tr>
<td>3.3.5</td>
<td>Representative graph for chromosome 3</td>
</tr>
<tr>
<td>3.3.6</td>
<td>Representative graph for chromosome 4</td>
</tr>
<tr>
<td>3.3.7</td>
<td>Representative graph for chromosome 5</td>
</tr>
<tr>
<td>3.3.8</td>
<td>Representative graph for chromosome 6</td>
</tr>
<tr>
<td>3.3.9</td>
<td>Representative graph for chromosome 7</td>
</tr>
<tr>
<td>3.3.10</td>
<td>Representative graph for chromosome 8</td>
</tr>
<tr>
<td>3.3.11</td>
<td>Representative graph for chromosome 9</td>
</tr>
<tr>
<td>3.3.12</td>
<td>Representative graph for chromosome 10</td>
</tr>
<tr>
<td>3.3.13</td>
<td>Representative graph for chromosome 11</td>
</tr>
<tr>
<td>3.3.14</td>
<td>Representative graph for chromosome 12</td>
</tr>
<tr>
<td>3.3.15</td>
<td>Representative graph for chromosome 13</td>
</tr>
<tr>
<td>3.3.16</td>
<td>Representative graph for chromosome 14</td>
</tr>
<tr>
<td>3.3.17</td>
<td>Representative graph for chromosome 15</td>
</tr>
<tr>
<td>3.3.18</td>
<td>Representative graph for chromosome 16</td>
</tr>
<tr>
<td>3.3.19</td>
<td>Representative graph for chromosome 17</td>
</tr>
<tr>
<td>3.3.20</td>
<td>Representative graph for chromosome 18</td>
</tr>
<tr>
<td>3.3.21</td>
<td>Representative graph for chromosome 19</td>
</tr>
<tr>
<td>3.3.22</td>
<td>Representative graph for chromosome 20</td>
</tr>
<tr>
<td>3.3.23</td>
<td>Representative graph for chromosome 21</td>
</tr>
<tr>
<td>3.3.24</td>
<td>Representative graph for chromosome 22</td>
</tr>
<tr>
<td>4.2.1</td>
<td>Method of DD tissue collection</td>
</tr>
<tr>
<td>4.3.1</td>
<td>FACS and IHC data for DD Cord</td>
</tr>
<tr>
<td>4.3.2</td>
<td>FACS and IHC data for DD Nodule</td>
</tr>
<tr>
<td>4.3.3</td>
<td>FACS and IHC data for DD peri-nodular fat</td>
</tr>
<tr>
<td>4.3.4</td>
<td>FACS and IHC data for DD distant palmar fat</td>
</tr>
<tr>
<td>4.3.5</td>
<td>FACS and IHC data for DD skin</td>
</tr>
<tr>
<td>4.3.6</td>
<td>Relative gene expression of each of the 6 markers investigated in the diseased and control tissue</td>
</tr>
</tbody>
</table>
List of Tables

Table 1.4.1 The severity of Dupuytren’s Disease (DD) ................................................. 26
Table 1.5.1 Quality assessment list used ................................................................. 35
Table 1.5.1 Epidemiological studies conducted in Dupuytren’s disease .............. 36-47
Table 1.6.1 Genetic Studies ....................................................................................... 73-80
Table 2.3.1 Frequency of associated risk factors among probands ...................... 97
Table 2.3.2 The overall frequency of the number of affected siblings among male probands (n=77) .............................................................................. 103
Table 2.3.3 The overall frequency of the number of affected siblings among female probands (n=12) .............................................................. 103
Table 2.3.4 Sibling recurrence risk ratio using population prevalence of DD previously calculated in UK (Early 1962) .......................................................... 103
Table 3.3.1 Icelandic pedigree clinical demographics ............................................. 116
Table 3.3.2 DNA QC Results .................................................................................... 121
Table 3.3.3 Areas on chromosome 1 to exclude and consider ............................... 123
Table 3.3.4 Areas on chromosome 2 to exclude and consider ............................... 124
Table 3.3.5 Areas on chromosome 3 to exclude and consider ............................... 125
Table 3.3.6 Areas on chromosome 4 to exclude and consider ............................... 126
Table 3.3.7 Areas on chromosome 5 to exclude and consider ............................... 128
Table 3.3.8 Areas on chromosome 7 to exclude and consider ............................... 130
Table 3.3.9 Areas on chromosome 8 to exclude and consider ............................... 131
Table 3.3.10 Areas on chromosome 9 to exclude and consider ............................. 133
Table 3.3.11 Areas on chromosome 10 to exclude and consider ........................... 134
Table 3.3.12 Areas on chromosome 11 to exclude and consider ......................... 135
Table 3.3.13 Identified genes with LOD score 1.8 on chromosome 11 ................... 136
Table 3.3.14 Areas on chromosome 12 to exclude and consider ........................... 137
Table 3.3.15 Areas on chromosome 13 to exclude and consider ........................... 138
Table 3.3.16 Areas on chromosome 14 to exclude and consider ........................... 140
Table 3.3.17 Areas on chromosome 15 to exclude and consider ........................... 141
Table 3.3.18 Areas on chromosome 16 to exclude and consider ........................... 142
Table 3.3.19 Areas on chromosome 17 to exclude and consider ........................... 143
Table 3.3.20 Areas on chromosome 18 to exclude and consider ........................... 144
Table 3.3.21 Areas on chromosome 19 to exclude and consider ........................... 145
Table 3.3.22 Areas on chromosome 20 to exclude and consider ........................... 146
Table 3.3.23 Areas on chromosome 21 to exclude and consider ........................... 147
Table 3.3.24 Areas on chromosome 22 to exclude and consider ........................... 148
Table 3.3.25 Important SNPs identified and related chromosome ....................... 149
Table 4.2.1 Description of markers used to investigate stem cells in DD ......... 159
Table 4.2.2 Genes used for QRT-PCR and respective forward and reverse primer sequences ............................................................................................... 162
Table 4.2.3 Primary antibodies used for FACS analysis ....................................... 163
Table 4.2.4 Isotype control antibodies used for FACS analysis ........................... 163
Table 4.3.1 Illustration of immunohistochemical data identifying the mean percentage (and standard deviations) of positive cells in each tissue type for each individual marker ................................................................. 171
Table 4.3.2 Probabilities of differences of mean relative gene expression for each marker investigated in comparison to control carpal tunnel tissue. 174
List of peer reviewed publications, presentations and prizes related to this thesis

Royal College of Surgeons of England Research Fellowship; awarded 2008-2009

Peer reviewed publications


Peer reviewed presentations and associated prizes


Frequency of primary and incidence of recurrent Dupuytren’s disease significantly impacts on the cost to the health service. International Symposium on Dupuytren’s Disease, Miami, USA, May 2010 (podium).


Declaration
No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.
Copyright statement

i. The author of this thesis (including any appendices and/or schedules to this thesis) owns any copyright in it (the “Copyright” and he has given The University of Manchester the right to use such Copyright for any administrative, promotional, educational and/or teaching purposes.

ii. Copies of this thesis, either in full or in extracts, may be made only in accordance with the regulation of the John Rylands University Library Manchester. Details of these regulations may be obtained from the Librarian. This page must form part of any such copies made.

iii. The ownership of any patents, designs, trade marks and any and all other intellectual property rights except for the Copyright (the “Intellectual Property Rights”) and any reproductions of copyright works, for example graphs and tables (“Reproductions”), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property Rights and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property Rights and/or Reproductions.

iv. Further information on the conditions under which disclosure, publication and exploitation of this thesis, the Copyright and any Intellectual Property Rights and/or Reproductions described in it may take place is available in the University IP Policy (see http://www.campus.manchester.ac.uk/medialibrary/policies/intellectual-property.pdf), in any relevant Thesis restriction declarations deposited in the University Library, The University Library’s regulations (see http://www.manchester.ac.uk/library/aboutus/regulations) and in The University’s policy on presentation of Theses.
Acknowledgements & dedications

I would like to dedicate this work to my wife Haneesha and my baby daughter Hema. Haneesha has provided continual, unconditional support and encouragement throughout this research.

I would like to thank my current supervisors Professor’s Gus McGrouther and Paul McArthur for their continual support, guidance and encouragement, especially during times of difficulty.

I would like to thank the head of school of translational medicine Professor Ashley Woodcock for his guidance, direction and support especially during the latter stages of this work.

I would like to thank the international collaborators; Professor Reynir Argrimsson; Dr Kristian Gudmundsson and Professor Thorbjorn Jonsson for their support, guidance, kindness and hospitality during my visits to Iceland.

I would like to thank all the members of staff at the University of Manchester and collaborators from Manchester, Derby and New York in this work, namely; Dr Ardeshir Bayat; Professor Ralf Paus; Professor Gillian Wallis; Professor Graeme Black; Dr Jill Urquart; Professor Joe Lee; Dr Rachel Watson; Professor JK Stanley; Mr Tommy Lindau; Mrs Melanie Arundell; Dr Sameer Farhatullah; Dr Amir Iqbal.
Abstract
Identification of biomarkers and whole genome scanning in Dupuytren’s Disease
Sandip Hindocha. The University of Manchester. Degree: MD. September 2013.

Background: Dupuytren’s Disease (DD) is a common fibroproliferative disease of unknown origin affecting the hand. The hypotheses of this thesis are; i) DD is a complex polygenic disease and ii) the cells that comprise DD tissue may be derived not only from fascia but also from fat and dermis. On this basis, the overall aims of this thesis were to: i) estimate the level of penetrance in a UK DD population; ib) present the largest DD family available to date (from Iceland) and attempt linkage analysis following whole genomic scanning; ii) observe stem cell markers as potential biomarkers in DD fascia, fat and skin compared to appropriate controls.

Methods: Patients diagnosed with DD (n = 135) were randomly selected from hospitals in the UK. One family was identified in Reykjavik, Iceland. Family pedigrees were compiled for each patient and subsequently analysed to calculate a revised sibling recurrence risk ratio (λs) and estimate the level of penetrance.

Members of the Icelandic family from whom DNA was available were genotyped using the Affymetrix 6.0 SNP chip and linkage analysis performed to identify susceptible genetic regions for DD. Biopsies of DD patients (n=9) with digital fixed flexion deformity were taken at operation from the diseased cord, nodule, peri-nodular fat, distant palmar fat and skin. Fluorescence Activated Cell Sorting (FACS), immunohistochemistry and Quantitative Real Time Polymerase Chain reaction (QRT-PCR) were used to identify expression of five mesenchymal (CD’s 13, 29, 44, 90, 166) and two haematopoietic (CD’s 34,117) stem cell markers.

Results: i) The DD status of 1156 relatives of the 135 probands was established. Patients with a family history had a greater severity of disease than those who did not (p<0.05). Despite published studies pointing towards DD being a polygenic disease, DD appears to show a near autosomal pattern of inheritance with variable penetrance (estimated penetrance level: 18%). The revised sibling recurrence risk ratio for the UK, λs=6.2.

ib) The Icelandic family had 25 affected members. The pedigree approximated autosomal dominance. Results identified 8 candidate genes with susceptibility loci over 3 chromosomes (GRK4, ADD1, SH3BP2 on chromosome 4; SGCZ on chromosome 8; NAV2, MRGPRX1, SAAL1, MYOD1 on chromosome 11).

ii) There was a significantly higher expression of selected stem cell markers in DD tissue over control: CD13 protein expression was increased in all DD tissue compared to controls (p=0.02), while CD44 was significantly over expressed in the cord and nodule (p=0.02). CD34 in the skin was also significantly enhanced (p=0.008). The mean number of positive cells expressing the stem cell markers was significantly greater in DD cord tissue compared to healthy carpal tunnel fascia (p= 0.003).

Conclusions: i) DD appears to show a near autosomal pattern of inheritance with variable penetrance (estimated at 18%), and the sibling recurrence risk ratio was revised. These calculations provide a more up to date evaluation of familial aggregation and more accurate estimates for complex bio-informatic DD models.

ib) A large DD pedigree has been identified and a whole genome scan has found 8 potential candidate genes within susceptibility loci on chromosomes 4, 8 and 11.

ii) The analysis of stem cell markers in various DD tissue components questions the previously proposed origin of abnormal DD fibroblasts from the fascia alone and suggests that the surrounding palmar fat and skin are also involved in DD pathobiology. Future work may involve functional analysis of these stem cell markers and potential use as biomarkers.
Chapter 1

Introduction & Literature Review:

On the history, anatomy, epidemiology, genetics and pathology of DD
1.1 Introduction

Dupuytren’s Disease (DD) is a benign, fibro-proliferative disease of unknown aetiology. The disease predominantly affects the palm of the hands causing permanent digital contracture of affected digits (Bayat, Cunliffe et al. 2007; van Dijk, Finigan et al. 2013). DD is a late onset disease and often progressive, irreversible and commonly bilateral. The disease, described as psychologically and physically disabling has a significant impact on healthcare economy, which not only affects the individual but society as a whole (Hindocha, Stanley et al. 2008; Werker, Pess et al. 2012; Baltzer and Binhammer 2013; De Salas-Cansado, Cuadros et al. 2013).

DD-like fibrotic tissue can occur on the dorsum of the hand over the proximal interphalangeal joints (Garrod’s pads), sole of the feet (Ledderhose’s disease) and penis (Peyronie’s disease) (Thurston 2003). DD is reported to be a common connective tissue disorder in Northern Europe with a frequent family history (Early 1962; Mikkelsen 1972; Bayat and McGrouther 2006; Shih, Watson et al. 2012).

The mainstay of treatment for DD is surgical, excising the diseased palmar fascia. The condition does recur and can do so with greater severity (Hindocha, Stanley et al. 2008; Werker, Pess et al. 2012). The aetiology is unknown, however one prominent associated factor is the frequent but not invariable familial nature of the disease with increasing evidence of genetic susceptibility (Picardo and Khan 2012).

The results of familial aggregation studies in DD have provided evidence of genetic susceptibility (Hindocha, John et al. 2006; Capstick, Bragg et al. 2012). The possibility of interaction between genetic and environmental factors has been
extensively discussed (Hindocha, John et al. 2006; Shih, Watson et al. 2012). Studies have examined potential genetic and cellular markers in DD in an attempt to provide a foundation upon which novel treatments can evolve (Hentz, Watt et al. 2012; Sedic, Pavelic et al. 2012; Worrell 2012). Epidemiological and genetic studies will help in identifying those patients who are at high risk of developing DD, and predict prognosis and recurrence after surgery.

This literature review will provide an introduction to this thesis; a brief history of DD; DD anatomy; clinical diagnosis, severity and the DD diathesis. A review of the epidemiology and genetics will describe the extent to which DD has been investigated. A brief account of the reasons to investigate stem cells in DD is provided. The points of focus in this thesis are; i) searching DD susceptibility loci and candidate genes and, ii) identifying biomarkers which may aid in sourcing the cellular nature of DD.
1.2 The history of Dupuytren’s Disease

The first possible description of DD in the medical literature was in 1614 when Felix Plater described contractures of the fingers in a patient with history of hand trauma (Plater 1614). Dupuytren’s main contribution to the understanding of the disease was to give a detailed anatomical description of the disease, including its specific features. Dupuytren is cited to have performed the first surgery for DD by palmar fasciectomy (Devitt, Yeo et al. 2012). Dupuytren’s lecture on DD recognised that contraction of the fingers, predominantly the ring finger, had been identified for many years before his own description (Dupuytren 1834; Dupuytren and Clark 1847; Glicenstein 2012; Ellis 2013).

Dupuytren suggested that people who used their hands for manual labour (coach men or masons) were more likely to be affected with DD, but the familial nature of the disease was not discussed (Gudmundsson, Jónsson et al. 2003). Since the first description of DD, many studies have been conducted to identify the disease pathogenesis. To date it is still a disease of unknown aetiology. However one factor that seems to stand out is the familial nature of the disease (Burge 1999; Michou, Lermusiaux et al. 2012). This has led to a number of complex genetic studies in recent years (see below). The studies described in this thesis aim to advance our knowledge and specifically to contribute to the cellular origin and genetics of DD.
1.3.1 The anatomy of the palmar fascia and formation of DD

As the histopathology of DD involves various tissue components (fascia, fat and skin), it is important to review key features of the normal anatomy of the relevant structures. The palmar fascia (PF), synonymous with palmar aponeurosis, is an extension of the Palmaris longus (PL) tendon, when present. If this tendon is absent, the palmar fascia interweaves with fascial structures overlying the carpal canal. The central fascia fans distally towards the digits (figure 1.3.1).

**Figure 1.3.1 Anatomy of the palmar fascia**
The figure illustrates normal palmar fascia anatomy with clear labels for key structures; 1=insertion of adductor pollicis brevis on tendon of Palmaris longus; 2=Palmar cutaneous branch of the median nerve; 3=proximal commissural ligament of the first web space; 4=proximal transverse ligament; 5=distal commissural ligament of the first web space; 6=natatory ligament; 7=adipose filled triangular space; 8=pre-tendinous bands; 9=Palmaris longus tendon Figure reproduced from the textbook Dupuytren’s Disease (Tubiana, Leclercq et al. 2000).
As DD tissue extends to the digits, the diseased cords that develop from the PF have specific names (figure 1.3.2). The cords around the joints of the digits create digital contracture. This diseased tissue is excised during surgery and as highlighted in figure 1.3.2, great care is taken so as not to damage underlying neurovascular structures.

**Figure 1.3.2: Extensive anatomy of normal fascia and DD**

This figure (Green’s Operative Hand Surgery 6th Edition) shows the normal anatomy and diseased anatomy (Hurst 2011).
1.3.2 Histopathology of DD

The cell responsible for digital contracture in DD is the myofibroblast. The myofibroblast has a contractile protein, α smooth muscle actin. It is this cell which typically causes contraction (Tomasek, Gabbiani et al. 2002). Histologically, DD fascia is more cellular with an increased amount of extracellular matrix in comparison to non diseased fascia (figure 1.3.3) (Shih and Bayat 2010). In the DD nodule, fibroblasts are not aligned. In DD cord cellular mass decreases and fibroblasts are more aligned (figure 1.3.3) (Shih and Bayat 2010).

**Figure 1.3.3: Histology of DD**

(Shih and Bayat 2010).

- **Normal fascia before development of DD**
- **Abnormal fascia after development of DD**
- **Histological features (nodule)**
  - Rich in fibroblasts
  - Fibroblasts are not aligned along lines of stress
- **Histological features (cord)**
  - Alignment of fibroblasts along stress fibers
  - Reduced cellularity
1.3.3 Clinical presentation of DD

The development of DD within fascial aponeurotic fibers causes the formation of the well known cords, nodules and palmar pits (Luck 1959; McFarlane 1974) (figure 1.3.3). Further disease causes contracture at MP and PIP joints of the hand.

**Figure 1.3.4: Clinical presentation of DD**
The clinical presentation of a palpable cord and nodule (left). As the disease progresses permanent digital contracture at the joints ensues (right) (Rehman, Goodacre et al. 2011).
1.4 Clinical diagnosis, severity and Dupuytren’s diathesis

DD diagnosis and severity

DD is rarely painful (von Campe, Mende et al. 2012). A patient presenting with severe DD (clear fixed flexion deformity) is fairly straightforward to diagnose for the majority of clinicians (Knobloch 2012). Mild DD, which presents with no digital contracture and only palmar signs, can be missed. This is an important consideration when studies assessing the epidemiology of the condition. When epidemiological studies rely on self-diagnosis, mild DD may be missed, under-reported and therefore prevalence or incidence rates underestimated. In addition, patients’ estimation of the age of onset of disease is frequently imprecise. This creates issues around ascertainment bias for data collection as age at onset (which is patient reported), is likely to be at a younger age (van Dijk, Finigan et al. 2013).

Severity is often based upon measurement of flexion deformity using a goniometer (Wagner, Roman et al. 2012). Another common assessment of severity of disease is the table top test; unfortunately these methods of assessment are not sufficient in planning surgical management as factors which may affect recurrence of disease may not have been considered (Werker, Pess et al. 2012). Severity of DD based upon the measurement of contracture of an affected digit was a system introduced by Tubiana and has been used by clinicians (Tubiana 1999). This method does not objectively associate other relevant risk factors with disease severity, which may alter treatment plans (Hindocha, Stanley et al. 2008). The Tubiana staging system was revised and clinical assessment criteria were added (table 1.4.1). These included frequency of surgery, recurrence rate per digital ray, the number of palmar pits and nodules and the presence of ectopic DD lesions. A severity score based upon the presence or absence
of these factors was calculated. This scoring system was designed to aid the clinician treating DD on the timing of surgery (Hindocha, Stanley et al. 2008). This scoring system has not yet been validated and can only be used as an observational tool.

**Table 1.4.1: The severity of Dupuytren’s Disease (DD)**
The clinical factors which can be considered in addition to Tubiana’s staging system (degree of total flexion deformity) when predicting disease severity (Hindocha, Stanley et al. 2008).

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total surgical procedures for DD.</td>
<td>Total for left and right hand</td>
</tr>
<tr>
<td>Recurrence of DD in affected digit.</td>
<td>Total for each digit</td>
</tr>
<tr>
<td>Number of digits affected</td>
<td>Total for left and right hand</td>
</tr>
<tr>
<td>Number of nodules</td>
<td>Total for left and right hand</td>
</tr>
<tr>
<td>Number of pits</td>
<td>Total for left and right hand</td>
</tr>
<tr>
<td>Garrod’s pads</td>
<td>Presence scores 1</td>
</tr>
<tr>
<td>DD on feet</td>
<td>Presence scores 1</td>
</tr>
<tr>
<td><em>For each digit stage DD.</em></td>
<td><em>Score according to stage (1-4). Total score for all digits. Flexion deformities to be measured with goniometer.</em></td>
</tr>
<tr>
<td>Stage 1= Total Flexion Deformity</td>
<td>1</td>
</tr>
<tr>
<td>Stage 2=TFD</td>
<td>2</td>
</tr>
<tr>
<td>Stage 3=TFD</td>
<td>3</td>
</tr>
<tr>
<td>Stage 4=TFD</td>
<td>4</td>
</tr>
<tr>
<td>Bilateral/unilateral DD</td>
<td>1 for unilateral; 2 for bilateral</td>
</tr>
<tr>
<td>TOTAL SEVERITY SCORE</td>
<td></td>
</tr>
</tbody>
</table>

*DD diathesis*

The term DD diathesis was coined to identify factors which would predispose an individual to DD (Hueston 1963). The link between the DD diathesis and genetic risk has been calculated (Dolmans, de Bock et al. 2012). Diathesis indicated degree of disease severity and is a potential predictor of post-operative outcome (Hueston 1963; Kan and Hovius 2012). DD diathesis originally described four characteristics as a
marker of disease severity and prognosis following surgery; bilateral disease (described as bilateral palmar lesions), family history of DD, ectopic lesions (DD found outside the palmar surface) and ethnicity (Hueston 1963). These were updated to include young age at onset (age at onset less than 50 years of age). Those patients with more diathesis factors were at a higher risk of disease recurrence (Hindocha, Stanley et al. 2006; Degreef and De Smet 2011).

Hueston’s study on the DD diathesis divided patients into those who developed recurrence and extension, with recurrence occurring more frequently (Hueston 1963). Recurrence can be divided into true and false recurrence. False recurrence can include scar and joint contracture whereas true recurrence is the development of new DD tissue within the area of previous surgery. Extension describes the development of disease away from the area of surgery (Iselin 1974). Recurrence at five and a half years follow up has been stated at 41% with extension occurring in 39% giving total disease activity in 55% (Foucher, Medina et al. 2003). Recurrence rates reported following fasciectomy range between 12% and 73% (Werker, Pess et al. 2012).

A recent study by Dolmans et al investigated the presence of DD diathesis factors and the predictability of genetic risk in 933 patients across 5 hospitals in the Netherlands (Dolmans, de Bock et al. 2012). The study found that 3 of the revised diathesis factors (age at onset less than 50 years, positive family history and Garrod’s pads) were associated with a high genetic risk (Dolmans, de Bock et al. 2012). Dolmans et al added the presence of Ledderhose’s disease as a factor associated with a high genetic risk (Dolmans, de Bock et al. 2012). The genetic risk was not increased if Garrod’s pads were present in the same patient. The authors therefore concluded that the
The highest risk ectopic lesion was the Garrod’s pad and should remain as part of the revised DD diathesis (Dolmans, de Bock et al. 2012). The paper concluded that the presence of diathesis factors is more likely to lead to the presence of genetic SNP’s associated with DD, which could then be linked to the clinical presentation of DD. This conclusion is based on the fact that the higher the number of diathesis factors, the higher the disease severity (Hindocha, Stanley et al. 2006). If a patient carries more risk alleles it would be expected that disease would increase in severity (Dolmans, de Bock et al. 2012).
1.5 Epidemiological evaluation of Dupuytren’s disease incidence and prevalence rates in relation to aetiology

The epidemiology of DD has been previously studied extensively (Ross 1999; Gudmundsson, Arngr msson et al. 2000; Thurston 2003; Lanting, van den Heuvel et al. 2013). DD is frequently labeled as the “Vikings” or “Nordic” disease. However, other than the common prevalence of DD in Scandinavia, no objective scientific evidence has been found to support and substantiate the “Nordic” origin of the disease (Burge 1999; Thurston 2003). The global presence of DD and patterns of migration are consistent with the hypothesis that DD is a genetic disease (McFarlane 2002).

DD is found to be most prevalent in Northern European Caucasians (Bayat and McGruther 2006) where it is one of the most common inherited connective tissue disorders with a prevalence that has been reported to reach up to 30% in the Norwegian population aged over 60 years (Early 1962; Mikkelsen 1972). By contrast, the prevalence rate of DD is reported to be just over 4% in the male population in England (Early 1962). These particular prevalence rates have been quoted in many epidemiological studies. With evolving population dynamics these prevalence values may or may not be accurate in relation to the present day. Increasing changes in the environment, working patterns and social structure may also impact on the observed changes in the epidemiology of DD (Descatha, Bodin et al. 2012).

In the subsequent section, therefore, the published evidence on the epidemiology of DD is critically reviewed.
1.5.1 Identification of epidemiological studies

Studies evaluating the epidemiology of DD were identified using the search engines Medline, Embase, PubMed, Web of Science and subsequent cross referencing to earlier articles. The following keywords were used to search for relevant articles: Dupuytren’s, disease, contracture, history, population, prevalence, incidence, and epidemiology.

Inclusion criteria:
The search included all case reports, letters, communications, prospective and retrospective studies. Inclusion criteria were based on incorporating any study investigating the prevalence or incidence of DD in any population group.

Exclusion criteria:
Studies were excluded if they investigated the prevalence or incidence of a risk factor within a group of DD patients.

A total of 62 studies, dating from 1951 to 2013, were identified as relevant to the epidemiology of DD. A relevant study was one that calculated a prevalence or incidence rate of DD. These studies were categorised as cross-sectional, longitudinal, observational, review or cohort, and the results tabulated (table 1.5.2). The study setting was noted as to whether, for example, patients were examined as inpatients or outpatients. In addition to this the data collector and the person who examined the DD patients were tabulated to estimate the likely accuracy of disease diagnosis.

The prevalence or incidence rates of DD for each study were tabulated and where possible compared between males and females with reference to age groups within
each population. Familial aggregation which has previously been confirmed in DD is considered to be important in relation to the epidemiology of DD (Hindocha, John et al. 2006). Therefore family history was explored in each study. Risk factors (e.g. diabetes and smoking) for DD investigated in each study were also examined and tabulated.

1.5.2 Review of significant studies

The 62 studies identified and tabulated (table 1.5.2) date from 1951 to 2013 covering a range of countries from North America to East Asia and Australasia (figure 1.5.2). Forty-nine studies were cross-sectional studies that included 3 cohort, 2 longitudinal and, 5 observational studies, 2 questionnaire surveys and 1 review. Assessment of the methods used in each study was assessed using a validated scoring system (table 1.5.1). The prevalence rate of DD was calculated in 61 studies with only one study presenting an incidence rate. The incidence rate of DD for the British population in 2004 was calculated as 34.4 per 100,000 men between the ages of 40 and 84 years (Khan, Rider et al. 2004).

1.5.3 Misinterpretation of incidence and prevalence in DD

Review of studies in table 1.5.2 has shown that most quoted “incidence” rates were in fact referring to “prevalence” rates. Incidence rate is defined as the number of new cases of a disease over a specified time period as a percentage of the population as opposed to the prevalence rate which is the current number of cases of disease at a single point time.
Figure 1.5.1: Global distribution of DD
Distribution of prevalence rates globally, figures extracted from table 1.5.2.
1.5.4 Analysis of 62 epidemiological studies reveals the most reliable data sets & the difficulty of study comparison

By providing each of the 62 studies with a score based on a known scoring system it has been possible to identify the best data sets from the studies evaluated. The scoring system used a high quality assessment list to carry out a meta-analysis on the effect of work on DD, this assessment tool was adapted in this literature review (Descatha, Jauffret et al. 2011). Scores ranged from 6 to 16 and were based on criteria in table 1.5.1. Three studies appear to give the most accurate prevalence rates for DD; the most recent Dutch study (Lanting, van den Heuvel et al. 2013), the Icelandic study (Gudmundsson, Arngrimsson et al. 2000) and Norwegian study (Finsen, Dalen et al. 2002). These studies give the most comprehensive epidemiological data including age, gender prevalence rates, links to family history and associated risk factors.

As shown in table 1.5.2 there is a huge variation in the methodologies used to calculate epidemiological data for DD. This is discussed further in sections 1.5.7 and 1.5.10. In addition methods of obtaining the data prior to analysis also differ. This makes comparisons of studies extremely difficult due to the varying risk factors that were investigated or the location of study (e.g. nursing home versus general community). As a result prevalence rates are skewed in particular studies due to the specific population group(s) studied.

The studies with the higher scores that have taken into account aetiological factors, were deemed to have a good study design and analysed accurately (tables 1.5.1 and 1.5.2).
From this analysis we can see that the prevalence of DD in men is between 5.6% and 26.4% and in women between 1.5% and 18.6% (Gudmundsson, Arngr msson et al. 2000; Finsen, Dalen et al. 2002; Lanting, van den Heuvel et al. 2013).
Table 1.5.1: Quality assessment list used.
Assessment of methodological quality used in epidemiological studies for DD (adapted from Descatha A et al (Descatha, Jauffret et al. 2011). Constructed using criteria from the Cochrane Centre, and recent reviews on musculoskeletal disorders at work adapted to Dupuytren’s contracture (Descatha, Jauffret et al. 2011). Criteria: study population, assessment of exposure, outcome, study design and analysis were adapted for DD. E.g. accounting for reporting age & gender.

<table>
<thead>
<tr>
<th>Criteria and attribution of points</th>
<th>STUDY POPULATION</th>
<th>ASSESSMENT OF EXPOSURE</th>
<th>ASSESSMENT OF OUTCOME</th>
<th>STUDY DESIGN</th>
<th>ANALYSIS AND DATA PRESENTATION</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 = yes if both age/gender are reported 0 = else</td>
<td>Positive if items in both groups were reported at baseline: a. Age (mean (SD or CI), or dichotomised groups) and gender b. Alcohol and/or smoking c. Heredity and/or diabetes and/or epilepsy</td>
<td>Positive if the participation of the exposed group and unexposed group was ≥ 80% Positive if the total number of cases was ≥ 50</td>
<td>1 = yes 0 = no</td>
<td>Adequate description of exposure a. Positive if the exposure was clearly defined b. Positive if the assessment of exposure was described c. Positive if the exposure was assessed by an independent person and was not based on self-reported exposure</td>
<td>Positive if the study design was prospective or a retrospective cohort/case control Positive if inclusion and exclusion criteria were described Positive if the follow-up period was ≥ 1 year Positive if personal information was given for completers and withdrawals</td>
<td>0 to 20</td>
</tr>
<tr>
<td>1 = yes alcohol OR tobacco 0 = else</td>
<td></td>
<td></td>
<td>2 = all three 0 = none 1= else</td>
<td>Positive if risk estimates were presented or when raw data were given which allow the calculation of risk estimates, such as: Odds ratios, prevalence ratios or relative risks Identifying confounders (at least 2, in addition to age/gender) a. Positive if the confounders considered were described b. Positive if the method used to control for confounding was described</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Author</td>
<td>Date</td>
<td>Location</td>
<td>Study Design &amp; Analysis score</td>
<td>Geographical Location</td>
<td>Data Collector</td>
<td>Total Prevalence</td>
</tr>
<tr>
<td>-------------------------</td>
<td>--------</td>
<td>-------------------</td>
<td>-------------------------------</td>
<td>-----------------------</td>
<td>-----------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Herzog EG</td>
<td>1951</td>
<td>England</td>
<td>Cross-sectional study of 1134</td>
<td>New York, USA</td>
<td>Manual laborers and clerks</td>
<td>Score 6</td>
</tr>
<tr>
<td>Gordon S</td>
<td>1954</td>
<td>Canada</td>
<td>Cross-sectional study of 2715</td>
<td>Cross-sectional study of 5162</td>
<td>Hospital inpatients</td>
<td>Score 8</td>
</tr>
<tr>
<td>Yost JR, Winters et al.</td>
<td>1955</td>
<td>New York, USA</td>
<td>Cross-sectional study of 3345</td>
<td>Cross-sectional study of 5162</td>
<td>Hospital inpatients</td>
<td>Score 11</td>
</tr>
<tr>
<td>Hueston JT</td>
<td>1960</td>
<td>Australia</td>
<td>Cross-sectional study of 3700</td>
<td>Cross-sectional study of 3700</td>
<td>Hospital inpatients</td>
<td>Score 11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Author</td>
<td>Year</td>
<td>Country</td>
<td>Study Type</td>
<td>Sample Size</td>
<td>Score</td>
<td>People in Community</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td>---------</td>
<td>------------</td>
<td>-------------</td>
<td>-------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Chaunt JC</td>
<td>1963</td>
<td>UK</td>
<td>Cross-sectional study of 180 stone masons</td>
<td>No mention</td>
<td>10</td>
<td>No mention</td>
</tr>
<tr>
<td>Mikkelsen OA</td>
<td>1972</td>
<td>Norway</td>
<td>Cross-sectional study of 16,000 inpatients</td>
<td>No mention</td>
<td>11</td>
<td>No mention</td>
</tr>
<tr>
<td>Critchley EM</td>
<td>1971</td>
<td>UK</td>
<td>Cross-sectional study of chronic epileptics</td>
<td>No mention</td>
<td>9</td>
<td>No mention</td>
</tr>
<tr>
<td>Stuhler T</td>
<td>1977</td>
<td>Germany</td>
<td>Cross-sectional study of 524 epileptics</td>
<td>No mention</td>
<td>8</td>
<td>No mention</td>
</tr>
<tr>
<td>Author</td>
<td>Cross-sectional study of 959 diabetes: Score: 7</td>
<td>Author</td>
<td>Cross-sectional study of 6888 men and 4120 women. Study of 1972</td>
<td>Author</td>
<td>Cross-sectional study of 919 patients attending the orthopaedic clinic: Score: 6</td>
<td>Author</td>
</tr>
<tr>
<td>------------------------</td>
<td>-----------------------------------------------</td>
<td>------------------------</td>
<td>----------------------------------------------------------------</td>
<td>------------------------</td>
<td>-----------------------------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>Mikkelsen O</td>
<td>Increased risk of DD in older diabetic patients in manual workers.</td>
<td>Mackenney RP</td>
<td>Positive correlation between stretching of palm and DD</td>
<td>Bennet et al</td>
<td>Positive correlation with manual work.</td>
<td>Arafa M</td>
</tr>
<tr>
<td>Mnickelsen O</td>
<td>Increased risk of DD in older diabetic patients in manual workers.</td>
<td>Mackenney RP</td>
<td>Positive correlation between stretching of palm and DD</td>
<td>Bennet et al</td>
<td>Positive correlation with manual work.</td>
<td>Arafa M</td>
</tr>
</tbody>
</table>

No mention

No mention

No mention

No mention

No mention

No mention

No mention

No mention

No mention
<table>
<thead>
<tr>
<th>Author</th>
<th>Score</th>
<th>Cross-sectional study of</th>
<th>Author</th>
<th>Score</th>
<th>Cross-sectional study of</th>
<th>Author</th>
<th>Score</th>
<th>Cross-sectional study of</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egawa T 1985</td>
<td>6</td>
<td>Cross sectional study of 3383</td>
<td>Egawa T 1985</td>
<td>6</td>
<td>Score: 6 Clinic orthopaedic patients attending study of 197</td>
<td>Egawa T 1985</td>
<td>6</td>
<td>Score: 6 Clinic orthopaedic patients attending study of 197</td>
</tr>
<tr>
<td>Stewart HD (Stewart, Innes et al. 1985)</td>
<td>7</td>
<td>Cross sectional study of 235</td>
<td>Stewart HD (Stewart, Innes et al. 1985)</td>
<td>7</td>
<td>Score: 7 Cross sectional study of 235</td>
<td>Stewart HD (Stewart, Innes et al. 1985)</td>
<td>7</td>
<td>Score: 7 Cross sectional study of 235</td>
</tr>
<tr>
<td>Study</td>
<td>Region</td>
<td>Design</td>
<td>Sample Size</td>
<td>Follow-Up</td>
<td>Age, Sex, &amp; Risk Factors</td>
<td>Author</td>
<td>Score</td>
<td>Notes</td>
</tr>
<tr>
<td>-------</td>
<td>--------</td>
<td>--------</td>
<td>-------------</td>
<td>-----------</td>
<td>-------------------------</td>
<td>--------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>Disease</td>
<td>Increased risk of DD in patients with liver</td>
<td>No</td>
<td>&lt;30 to 70 years</td>
<td>&lt;30 to 70 years</td>
<td>-</td>
<td>Author</td>
<td>28%</td>
<td>UK</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------------------------------</td>
<td>----</td>
<td>----------------</td>
<td>----------------</td>
<td>---</td>
<td>--------</td>
<td>------</td>
<td>----</td>
</tr>
<tr>
<td></td>
<td>Link DD and epilepsy</td>
<td>No</td>
<td>mention</td>
<td>mention</td>
<td></td>
<td>Author</td>
<td>38%</td>
<td>44%</td>
</tr>
<tr>
<td></td>
<td>Increased prevalence of DD</td>
<td>No</td>
<td>mention</td>
<td>mention</td>
<td></td>
<td>Author</td>
<td>11%</td>
<td>13%</td>
</tr>
<tr>
<td></td>
<td>Vibration white finger</td>
<td>No</td>
<td>mention</td>
<td>&lt;45 years</td>
<td>&gt;45 years</td>
<td></td>
<td>Author</td>
<td>13.6%</td>
</tr>
<tr>
<td></td>
<td>Increased risk of DD in diabetics and smokers</td>
<td>No</td>
<td>years</td>
<td>36-84 years</td>
<td>-</td>
<td>Author</td>
<td>24%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HIV on DD, Significant impact of 19-54 years</td>
<td>No</td>
<td>mention</td>
<td>years</td>
<td></td>
<td>Author</td>
<td>36%</td>
<td>-</td>
</tr>
<tr>
<td>Study</td>
<td>Year</td>
<td>City</td>
<td>Design</td>
<td>N</td>
<td>Age</td>
<td>Exposures</td>
<td>Risk Factors</td>
<td>Score</td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
<td>------</td>
<td>--------</td>
<td>---</td>
<td>-----</td>
<td>-----------</td>
<td>--------------</td>
<td>-------</td>
</tr>
<tr>
<td>Kelly SA et al.</td>
<td>1992</td>
<td>Derby, UK</td>
<td>Case-control</td>
<td>235</td>
<td>47-79 yrs</td>
<td>25% consumed large amount alcohol</td>
<td>8.5%</td>
<td>France</td>
</tr>
<tr>
<td>Cross-sectional study of 235 patients with Colles’ fracture.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Author: Kelly, Burke et al.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lennox IAC</td>
<td>1993</td>
<td>Aberdeen, Scotland</td>
<td>Cross-sectional</td>
<td>70</td>
<td>55 yrs</td>
<td>Mean age 55 yrs</td>
<td>No mention</td>
<td>10%</td>
</tr>
<tr>
<td>Score: 14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Author: Lennox, et al.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bergenudd H et al.</td>
<td>1993</td>
<td>Malmo, Sweden</td>
<td>Longitudinal</td>
<td>574</td>
<td>55 yrs</td>
<td>No mention</td>
<td>No mention</td>
<td>6%</td>
</tr>
<tr>
<td>Score: 6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Author: Bergenudd, H et al.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovenzi M</td>
<td>1994</td>
<td>Italy</td>
<td>Cross-sectional</td>
<td>570</td>
<td>&lt;60 yrs</td>
<td>Mean age 39 yrs</td>
<td>No mention</td>
<td>10%</td>
</tr>
<tr>
<td>Score: 14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Author: Bovenzi, M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Renard E et al.</td>
<td>1994</td>
<td>France</td>
<td>Cross-sectional</td>
<td>120</td>
<td>&gt;79 yrs</td>
<td>Type 1, 30% Type 2, 35%</td>
<td>No mention</td>
<td>35%</td>
</tr>
<tr>
<td>Score: 14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Author: Renard, Jacques et al.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Niezborala M et al.</td>
<td>1995</td>
<td>Poland</td>
<td>Case-control</td>
<td>324</td>
<td>&lt;60 yrs</td>
<td>No mention</td>
<td>No mention</td>
<td>5.3%</td>
</tr>
<tr>
<td>Score: 12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Author: Niezborala, M et al.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Author</td>
<td>Year</td>
<td>Country</td>
<td>Study Design</td>
<td>Subjects</td>
<td>Score</td>
<td>Score 2</td>
<td>Score 3</td>
<td>Score 4</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------</td>
<td>---------</td>
<td>----------------</td>
<td>--------------------------------------------------------------------------</td>
<td>--------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>Chammas M.</td>
<td>1995</td>
<td>France</td>
<td>Cross-sectional study of 120 diabetic patients.</td>
<td>Increase of DD risk in diabetic patients.</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arkilla PE.</td>
<td>1996</td>
<td>Finland</td>
<td>Longitudinal study of 207 type 1 diabetic patients.</td>
<td>Vibration exposure of DD risk increases with duration of DM.</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gudmundsson KG.</td>
<td>1999</td>
<td>Iceland</td>
<td>Cohort study of 2165 inpatients.</td>
<td>No mention</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gudmundsson KG.</td>
<td>2000</td>
<td>Iceland</td>
<td>Cohort study of 1297 males in the community.</td>
<td>No mention</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Author</td>
<td>Year</td>
<td>Country</td>
<td>Study Type</td>
<td>Sample Size</td>
<td>Diagnosis</td>
<td>Prevalence</td>
<td>Gender</td>
<td>Age</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td>---------</td>
<td>------------</td>
<td>-------------</td>
<td>-----------</td>
<td>------------</td>
<td>--------</td>
<td>-----</td>
</tr>
<tr>
<td>Smith SP</td>
<td>2001</td>
<td>UK</td>
<td>Cross-sectional</td>
<td>58 patients</td>
<td>Frozen shoulder</td>
<td>1.3%</td>
<td>Surgeon</td>
<td>Mean 54.9 years</td>
</tr>
<tr>
<td>Omari A</td>
<td>2001</td>
<td>UK</td>
<td>Cross-sectional</td>
<td>75 patients</td>
<td>Frozen shoulder</td>
<td>7%</td>
<td>Surgeon</td>
<td>Mean 54.9 years</td>
</tr>
<tr>
<td>Finsen V</td>
<td>2002</td>
<td>Norway</td>
<td>Cross-sectional</td>
<td>456 people</td>
<td>Frozen shoulder</td>
<td>9.5%</td>
<td>Surgeon</td>
<td>&gt; 50 years</td>
</tr>
<tr>
<td>White HA</td>
<td>2003</td>
<td>Wales</td>
<td>Observational</td>
<td>197 inpatients</td>
<td>Frozen shoulder</td>
<td>6.6%</td>
<td>Physician</td>
<td>-</td>
</tr>
</tbody>
</table>

**Notes:**
- DD: Degenerative Disease
- Cross-sectional study of 172 patients with shoulder pathology.
- Score: 16 for Kilian O (49) study of 172 patients with shoulder pathology.
<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Location</th>
<th>Type of Study</th>
<th>Score</th>
<th>Incidence Rate Per 100,000 Men</th>
<th>Age Range</th>
<th>Mention of Diabetes as Risk Factor</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Khan AA</td>
<td>2004</td>
<td>Oxford, UK</td>
<td>Review of National Mortality Survey</td>
<td>14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No mention</td>
</tr>
<tr>
<td>Geoghegan JM</td>
<td>2004</td>
<td>West Midlands, UK</td>
<td>Cross-sectional study of 383,000 GP patients</td>
<td>14</td>
<td>0.2%</td>
<td>24-97</td>
<td>-</td>
<td>Diabetes is a strong risk factor for DD</td>
</tr>
<tr>
<td>Zerajic D</td>
<td>2004</td>
<td>Bosnia</td>
<td>Cross-sectional study of 1207 people in community appearing to look over 50 years</td>
<td>16</td>
<td>0.15%</td>
<td>&gt;50</td>
<td>-</td>
<td>Significant higher prevalence in diabetics.</td>
</tr>
<tr>
<td>Ardic F</td>
<td>2004</td>
<td>Turkey</td>
<td>Cross-sectional study of 78 type II diabetics</td>
<td>14</td>
<td>0.05%</td>
<td>46-70</td>
<td>-</td>
<td>Diabetes is risk factor for DD</td>
</tr>
<tr>
<td>Khan AA</td>
<td>2004</td>
<td>Oxford, UK</td>
<td>Review of National Mortality Survey</td>
<td>14</td>
<td>0.1%</td>
<td>24-97</td>
<td>No mention</td>
<td>No mention</td>
</tr>
<tr>
<td>Geoghegan JM</td>
<td>2004</td>
<td>West Midlands, UK</td>
<td>Cross-sectional study of 383,000 GP patients</td>
<td>14</td>
<td>0.05%</td>
<td>24-97</td>
<td>No mention</td>
<td>No mention</td>
</tr>
<tr>
<td>Zerajic D</td>
<td>2004</td>
<td>Bosnia</td>
<td>Cross-sectional study of 1207 people in community appearing to look over 50 years</td>
<td>16</td>
<td>0.05%</td>
<td>&gt;50</td>
<td>No mention</td>
<td>No mention</td>
</tr>
<tr>
<td>Ardic F</td>
<td>2004</td>
<td>Turkey</td>
<td>Cross-sectional study of 78 type II diabetics</td>
<td>14</td>
<td>0.05%</td>
<td>46-70</td>
<td>No mention</td>
<td>No mention</td>
</tr>
<tr>
<td>Khan AA</td>
<td>2004</td>
<td>Oxford, UK</td>
<td>Review of National Mortality Survey</td>
<td>14</td>
<td>0.1%</td>
<td>24-97</td>
<td>No mention</td>
<td>No mention</td>
</tr>
<tr>
<td>Geoghegan JM</td>
<td>2004</td>
<td>West Midlands, UK</td>
<td>Cross-sectional study of 383,000 GP patients</td>
<td>14</td>
<td>0.05%</td>
<td>24-97</td>
<td>No mention</td>
<td>No mention</td>
</tr>
<tr>
<td>Zerajic D</td>
<td>2004</td>
<td>Bosnia</td>
<td>Cross-sectional study of 1207 people in community appearing to look over 50 years</td>
<td>16</td>
<td>0.05%</td>
<td>&gt;50</td>
<td>No mention</td>
<td>No mention</td>
</tr>
<tr>
<td>Ardic F</td>
<td>2004</td>
<td>Turkey</td>
<td>Cross-sectional study of 78 type II diabetics</td>
<td>14</td>
<td>0.05%</td>
<td>46-70</td>
<td>No mention</td>
<td>No mention</td>
</tr>
<tr>
<td>Study</td>
<td>Year</td>
<td>Country</td>
<td>Design</td>
<td>Score</td>
<td>No. of Participants</td>
<td>Data Collection Method</td>
<td>Age</td>
<td>Sex</td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
<td>---------</td>
<td>--------</td>
<td>-------</td>
<td>---------------------</td>
<td>-----------------------</td>
<td>------</td>
<td>-----</td>
</tr>
<tr>
<td>Godtfredsen, Lucht et al. (2004)</td>
<td>2004</td>
<td>Denmark</td>
<td>Cohort study of 7254 subjects.</td>
<td>14</td>
<td>Trained nurse/med student 11%</td>
<td></td>
<td>20-93 years</td>
<td></td>
</tr>
<tr>
<td>Logan AJ, Mason et al. (2005)</td>
<td>2005</td>
<td>UK</td>
<td>Questionnaire survey of 1100 climbers.</td>
<td>12</td>
<td>Patient 19.5%</td>
<td></td>
<td>20-93 years</td>
<td></td>
</tr>
<tr>
<td>Burke, Proud et al. (2007)</td>
<td>2007</td>
<td>UK</td>
<td>Cross-sectional study of 97537 miners.</td>
<td>13</td>
<td>Trained doctors 8.1%</td>
<td></td>
<td>25-99 years</td>
<td></td>
</tr>
<tr>
<td>Lucas G, Shroyer et al. (1997)</td>
<td>2008</td>
<td>France</td>
<td>Cross-sectional study of 2406 male civil servants.</td>
<td>14</td>
<td>Physician 8.8%</td>
<td></td>
<td>Mean age 50.7 years</td>
<td>20% had family history of DD</td>
</tr>
<tr>
<td>Degreef I et al. (2010)</td>
<td>2010</td>
<td>Belgium</td>
<td>Observational study of 500 people in the market place over the age of 50 years.</td>
<td>14</td>
<td>Hand specialist 32%</td>
<td></td>
<td>Mean age 65 years</td>
<td></td>
</tr>
<tr>
<td>Dibenedetti DB et al. (2011)</td>
<td>2011</td>
<td>USA</td>
<td>Population based study of 23,103 people on internet online survey.</td>
<td>14</td>
<td>Self-reported 0.5%</td>
<td></td>
<td>Mean age 50 years</td>
<td>18% positive family history</td>
</tr>
<tr>
<td>Nguyen et al. (2010)</td>
<td>2010</td>
<td>USA</td>
<td>Population based study of 103 people on internet online survey.</td>
<td>13</td>
<td>Self-reported 0%</td>
<td></td>
<td>Mean age 50 years</td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Year</td>
<td>Country</td>
<td>Study Type</td>
<td>Score</td>
<td>Diagnosis</td>
<td>Population</td>
<td>Patients</td>
<td>Hand Injury</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------</td>
<td>-----------------</td>
<td>---------------------</td>
<td>-------</td>
<td>-----------</td>
<td>------------</td>
<td>----------</td>
<td>-------------</td>
</tr>
<tr>
<td>Plastic Surgeons</td>
<td>2011</td>
<td>USA</td>
<td>Retrospective study</td>
<td>14</td>
<td>Hispanic</td>
<td>New York</td>
<td>2011</td>
<td>Yes</td>
</tr>
<tr>
<td>Ferry N et al</td>
<td>2012</td>
<td>France</td>
<td>Comparative study</td>
<td>13</td>
<td>Hispanic</td>
<td>Women</td>
<td>2012</td>
<td>No</td>
</tr>
<tr>
<td>Descatha A et al</td>
<td>2012</td>
<td>France</td>
<td>Cross sectional study</td>
<td>15</td>
<td>Workers</td>
<td>Manual workers</td>
<td>2012</td>
<td>No</td>
</tr>
<tr>
<td>Lanting R et al</td>
<td>2013</td>
<td>Netherlands</td>
<td>Cross sectional study</td>
<td>15</td>
<td>Upper limb surgeons</td>
<td>Netherlands</td>
<td>2013</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Findings from the literature review (table 1.5.2) were further sub-classified into age and gender. Prevalence rates of DD ranged from 0.2% to 56% in varying age groups and depended on methods of data collection. The prevalence of DD increased with age (table 1.5.2, figures 1.5.2-1.5.3) The highest prevalence rate (56%) was seen in a study group of epileptic (Critchley, Vakil et al. 1976) patients.

**Figure 1.5.2 The worldwide prevalence of DD**
Comparing worldwide prevalence rates in males and females. The diagnosis of DD in each study was standard. Prevalence rates in this figure have been taken from table 1.5.2.
1.5.5 Prevalence of associated aetiological factors in DD

Two elements in the aetiology of DD clearly continue to stand out. One is the familial nature of the disease (Lanting, van den Heuvel et al. 2013) and the other is that DD appears to be an extremely common disorder affecting Caucasians of Northern European ancestry (Bayat, Ferguson et al. 2002), although this may reflect the geography of the actual studies. The heritable nature of DD has been of great interest, with reports of the disease present in as many as 3 generations (Hu, Nystrom et al. 2005) and studies suggesting a possible autosomal dominant inheritance pattern (Ling 1963). However, it is possible that the multi-factorial aetiology of DD also includes a strong environmental factor based on the results of Finsen et al., who found that family members were more likely to develop DD if they were residing in the same geographical area as their diseased relative (Finsen, Dalen et al. 2002).

The wide variation in prevalence of DD in HIV infected patients (6% - 36%) may not be secondary to HIV infection but secondary to other aetiological factors. Neither study of the association between HIV and DD mentioned familial cases (Bower, Nelson et al. 1990; French, Kitchen et al. 1990). The association of HIV and DD requires further independent validation.

The origin and spread of DD is presumed to be from Northern Europe (McFarlane 2002). If this is the case we can see that the DD gene(s) may have spread with its migrating population (figure 1.5.1, 1.5.2). It is surprising that the genetic nature of DD has been discussed for over 50 years, yet epidemiological studies conducted have not stringently analysed the genetic aspect of the disease (table 1.5.2). The presence of sporadic cases of DD around the globe suggests there may be spontaneous genetic
mutations. The report of 10 cases of DD in patients from the Indian subcontinent living in the UK for several years suggests that environmental factors may also play a role (Srivastava, Nancarrow et al. 1989). DD may not only be a disease of Northern European Caucasians. The highest prevalence (across all age groups) in males was seen in Bosnia and in females, Canada (figure 1.5.2).

The presence of DD in children may be secondary to a genetic or environmental influence (Kraus, Alzen et al. 2012; Zheng and Liu 2013). The report of DD present in only one identical twin (Mandalia and Lowdon 2003; Beleta and Fores 2012) may be the result of sole environmental factors such as rock climbing (Beleta and Fores 2012) or that the disease may have developed at a later date in the other sibling. Cases of DD in the younger population are likely to have a genetic predisposition and this should be sought in these patients (Paller and Hebert 1986; Rao and Luthra 1988; Rhomberg, Rainer et al. 2002).

Early literature had stated that DD did not occur in women (Matthews 1979), however, the male to female ratio of DD varies between 7:1 and 15:1 (Gudmundsson, Arngrimsson et al. 2002). Females are older at the time of their first operation, and have a higher recurrence rate (Hindocha, John et al. 2006) compared to males (Wilbrand, Ekbom et al. 1999).

DD is a condition which has been linked to many risk factors including a history of smoking (Burge 2004), alcohol consumption (Noble, Arafa et al. 1992), frozen shoulder (Smith, Devaraj et al. 2001), epilepsy (Skoog 1948), diabetes mellitus (Arkkila, Kantola et al. 1997), carpal tunnel syndrome (Bonnici, Birjandi et al. 1992),
a history of manual labour (de la Caffiniere, Wagner et al. 1983) and hand injury (Kelly, Burke et al.; McFarlane 1991). All of these reports are controversial and frequently based on selective data. Factors associated with increased severity include male gender and a young age at onset, which are often reported although the evidence appears to be weak (Khan, Rider et al. 2004). A summary of the evaluation of the significance of presumed aetiological risk factors is listed in table 1.5.2 (see also figures 1.5.4 and 1.5.5).

Alcohol consumption and its association with DD is controversial. It is thought that alcohol consumption has been increasing over time and this has been linked to rising prevalence of DD (Noble, Arafa et al. 1992; Lanting, van den Heuvel et al. 2013). The rising reported prevalence of DD might be due to increased recognition by health professionals and patients (Geoghegan, Forbes et al. 2004). This raises doubts on the direct impact that alcohol may have on DD.

Since the prevalence of smoking is decreasing (Burge, Hoy et al. 1997), it is unlikely that increased risk is associated with smoking habits. In spite of this, cigarette smoking and increased alcohol consumption are more likely to result in surgical, rather than conservative management of DD (Burge, Hoy et al. 1997). Epidemiological studies have attempted to identify the risk of mortality, in particular cancer mortality in patients with DD. Gudmundsson concluded that mortality in DD patients is higher (Gudmundsson, Arngrimsson et al. 2002). This may not have any actual significance as DD severity increases with advancing age, as does the incidence of cancer.
Hypercholesterolemia is known to increase with age as does the prevalence of DD, it is also known that patients with DD are more likely to have raised serum triglyceride concentrations (Sanderson, Morris et al. 1992). With an aging population both hypercholesterolemia and DD are likely to become more prevalent and hence an increased prevalence of hypercholesterolemia may not be directly related to an increased prevalence of DD.

The prevalence of diabetes mellitus is increasing (Arkila, Kantola et al. 1997) and there is a positive association between diabetes mellitus and DD (table 1.5.2, figure 1.5.5). A large retrospective study evaluating the experience of treating DD in one centre concluded that there was no significant correlation between DD and diabetes mellitus, alcohol consumption, heavy smoking or epilepsy. However the prevalence of these risk factors is greater in DD patients than in the general population (Burge, Hoy et al. 1997). All these factors associated with DD can be debated as to their role in the pathogenesis of the disease and therefore the change in prevalence rate of DD cannot be confidently attributed to the change in prevalence of these associated factors.

Hand trauma, a history of manual labour hand injury, hand infection, elective hand surgery or vibration exposure have been suggested as precipitating DD (Luck 1959). Other musculoskeletal conditions have an increased prevalence in manual workers; a recent study has concluded that patients with a history of frozen shoulder are 8 times more likely to develop DD (Smith, Devaraj et al. 2001). Since this finding, a further study has concluded that occupational history and social class has no bearing on DD development or progression.
A history of rheumatoid arthritis has a negative correlation with DD (Alara, Steingold et al. 1984). There may be a common genetic pathway in the development of carpal tunnel syndrome and DD with chromosomal instability present in the palmar fascia in both of these conditions (Bonnici, Birjadi et al. 1992).

The changes in estimated prevalence of DD may be attributed to an increase in awareness of disease. Finsen in 2002 noted that there was a higher prevalence of DD in family members living within the same geographical area as their affected relative. A further genetic component of relevance to the epidemiology of DD was noted in the cross-sectional study from Manchester, UK carried out in 1984 which examined the hands of 392 patients with rheumatoid arthritis (Alara, Steingold et al. 1984). This study noted that there was a significantly reduced prevalence rate of DD in those with a diagnosis of rheumatoid arthritis, suggesting a genetic protective factor against the disease (Alara, Steingold et al. 1984). Yost in 1955 identified one family of DD with a comment on the development of early age at onset of DD (Yost, Winters et al. 1955). More recently, the Dutch study carried out in 2013 identified a positive family history as a significant prevalent factor in DD (Lanting, van den Heuvel et al. 2013).

Herzog (Herzog 1951) in 1951 and Early (Early 1962) in 1962 concluded that there was no significant difference in the prevalence of DD in manual and non-manual workers. A similar result was concluded by Yost (Yost, Winters et al. 1955) in 1955 with a negative correlation between DD and hand trauma. Contrary to these findings, Gudmundsson et al (Gudmundsson, Arngrimsson et al. 2000) concluded a significant association between manual work and DD. Thomas (Thomas and Clarke 1992) found a positive correlation between vibration white finger and DD. A more recent study to
determine whether DD is more prevalent following repetitive trauma found that rock climbing increases the risk of disease development (Logan, Mason et al. 2005). Lanting et al identified hand injury as a significant risk factor for DD, but not manual labour (Lanting, van den Heuvel et al. 2013)

Prevalence rates of DD were compared globally as illustrated in figures 1.5.1-1.5.2. Of the seven studies compared, the highest prevalence in males was seen in Bosnia and in Canada for females. The high numbers of affected individuals in both of these studies suggest the diagnostic criteria may be different. Generally the prevalence of DD increases with age (figure 1.5.2 and 1.5.3). From all the reported studies the prevalence in males and females is similar up to 45 years of age after which the rate is significantly greater in males (figure 1.5.3). This is an interesting finding as the mean age at onset of DD is in the fifth decade of life (Hindocha, John et al. 2006)
There is conflicting data on the significance of HIV and DD (Bower, Nelson et al. 1990). There is more consistent data in the findings for a link between DD, diabetes mellitus and epilepsy, with many studies finding a higher prevalence of DD in the diabetic population (Arkkila, Kantola et al.; Arafa, Noble et al. 1992; Arkkila, Kantola et al. 1997). The prevalence of DD in global epileptic (figure 1.5.4) and diabetic cohorts (figure 1.5.5) was compared. All studies showed a positive association between DD, epilepsy and diabetes.
Figure 1.5.4 Prevalence of DD in epileptics
Comparison of prevalence rates in epileptics. Data extrapolated from table 1.5.2.

Figure 1.5.5 Prevalence of DD in diabetics
Prevalence of DD within diabetic communities. Data extrapolated from table 1.5.2.

1.5.6 Prevalence rates calculated in a clinical and community setting

Of the 62 studies evaluated 24 were conducted in a hospital setting and 38 studies carried out in the community of which 2 were performed in a nursing home. Studies carried out in the nursing home identified a higher prevalence rate of DD compared to
those in a hospital or other community setting. Prevalence data from the community studies on the whole identified a higher prevalence rate than those conducted in a hospital setting (table 1.5.2), with the exception of one study that calculated a very low prevalence rate in 2004 (Geoghegan, Forbes et al. 2004).

The differences in the findings can be potentially based upon the diagnostic criteria used by the various groups as discussed in 1.5.7 and 1.5.10. The prevalence of DD in one country among different communities or regions may differ considerably (figure 1.5.6). Although a high prevalence of DD in Bosnia has been documented, there is a lower prevalence in the Bosnian Muslim community compared to the Serbian and Croat cohort (Zerajic and Finsen 2004). This may be due to migration patterns, with the possibility of the Serbian Croat population migrating from Northern European ancestry which is thought to be the origin of DD (McFarlane 2002). Similarly in Norway, after 60 years of age the prevalence of the disease in the Norwegian community is significantly greater when compared to that of the Sami population. In the UK it appears that prevalence is much higher in Scotland when compared to England. The migratory pattern of DD from Northern Europe to the rest of the world is reflected in the varying prevalence rates (figure 1.5.6).
Figure 1.5.6 Varying prevalence rates among different communities in the same country
Comparison of prevalence rates of DD in different communities/regions in Bosnia, Norway and the UK. Data extrapolated from table 1.5.2.
1.5.7 Prevalence rates and study methodology

Of the 62 studies represented in table 1.5.2, DD was diagnosed by a variety of clinicians. The most accurate method was probably in the most recent study by Lanting et al where diagnosis was made by plastic surgeons with a special interest in hand surgery and in particular DD (Lanting, van den Heuvel et al. 2013). The study by Logan et al (Logan, Mason et al. 2005) in 2005 identified a high rate of DD in rock climbers however diagnosis of DD was made by patients via a questionnaire survey. A high rate of DD identified in the Bosnian community by Zerajic (Zerajic and Finsen 2004) in 2004 may have resulted from the diagnosis of DD being made by a junior clinician or non hand surgery specialist. On the contrary, diagnosis made by a medical student or a nurse in a Danish study (Godtfredsen, Lucht et al. 2004) identified a prevalence rate of 11% a possible underestimate. Diagnosis is likely to be underestimated when not made by a specialist hand surgeon. This is highlighted in a study where the prevalence rate of DD was 18% when patients were examined by a physician and 42% when examined by a hand surgeon (Peto, Darby et al. 2000).

1.5.8 Cases of DD in various geographical locations

There are several reports of cases of DD around the world where one would not expect to find the disease, such as the African or Asian continents. Sladicka, Plasse and Zaworski each identified DD in an African American patient (Plasse 1979; Zaworski and Mann 1979; Sladicka, Benfanti et al. 1996; Swartz and Lalonde 2008). These cases were sporadic with no evidence of familial clustering or interracial marital relationships. The patient reported by Zaworski in 1979 did however have epilepsy and worked as a manual labourer (Zaworski and Mann 1979).
Similar sporadic cases were identified by Furnas in East Africa in 1979 (Furnas 1979), by Mennen, Richard-Kadio and Aladin in 1990 and 2001 respectively in an African patient (Richard-Kadio, Guedegbe et al.; Mennen 1986; Aladin and Oni 2001) and by Maes and Pai in 1979 and 1994 in Taiwan (Maes 1979; Pai and Tseng 1994). Muguti in 1993 identified DD in 4 indigenous Zimbabwean patients (Muguti and Appelt 1993). Three cases were male and one female. There was a history of manual trauma in the male patients and a history of epilepsy in the female patient. None of the cases had a family history of DD (Mitra and Goldstein 1994).

Liu in 1991 identified 41 cases of DD in the Oriental patient between 1970 and 1988 with 35 cases undergoing fasciectomy. There was no mention of family history in this case series (Liu and Chen 1991). A similar case series has been reported by Vathana (Vathana, Setpakdi et al. 1990). Srivastava in 1989 reported a series of 10 cases of DD operated on in a UK hospital, with all 10 cases being male (age range 45-68 years) originating from the Indian sub-continent (Srivastava, Nancarrow et al. 1989). None of the cases had a positive family history, 3 had recurrent disease, and 8 out of 10 had a history of repetitive hand trauma. This raises questions of a possible long-term environmental factor. Mitra reviewed 23 patients with DD of non-Caucasian origin and commented on these patients having a less extensive degree of DD diathesis (Mitra and Goldstein 1994). Again in this series of patients there was no mention of family history.

The review confirms the high prevalence of DD in patients from Northern European extraction and although this review provides a geographical representation of the epidemiology of DD, it may not be complete.
1.5.9 Age and gender specific epidemiology of DD

Population studies conducted over the last half century show that DD increases with age (table 1.5.2, figure 1.5.2 & 1.5.3). Interestingly, DD has also been recognised in the younger population (Hindocha, Stanley et al. 2006). The differential diagnosis of DD made in a child who presents with a nodule in the hand, contracture of a digit or a joint knuckle pad on the dorsum of the hand would include sarcoma of the upper limb (Lane and Hankin 1988). A non familial, sporadic case of DD has been identified in a child as young as 6 months old (Bebbington and Savage 2005) which may suggest a new genetic mutation(s) responsible for disease development.

The majority of studies have found that DD is more prevalent in the male population (table 1.5.1, figure 1.5.2), with a male to female ratio of approximately 5.9:1 (Hindocha, John et al. 2006). There appears to be a significantly stronger genetic element of DD in women with familial cases of the disease predominantly in women (Matthews 1979). This is further confirmed with the higher familial aggregation (sibling recurrence risk) of DD in females compared to males (Hindocha, John et al. 2006; Capstick, Bragg et al. 2012).

1.5.10 Comparing epidemiological data for DD from different sources

Each study has provided evidence for contributing aetiological risk factors. However, studies are not standardised making epidemiological comparisons rather difficult. Each study assessed population groups of different age ranges. A problem with the calculation of prevalence rates for DD is the inconsistency with which these studies have been carried out. For example Geohagan et al in 2004 calculated the prevalence of DD for patients between the ages of 24 and 97 years of age in the West Midlands,
UK (Geoghegan, Forbes et al. 2004). The prevalence rate for a population of 383,000 was 0.2%. Data collection was not based on clinical examination of every subject but was dependent on the coding for DD within general practice databases. We know that in the UK, there are a number of different generic codes for DD including the umbrella term musculoskeletal disorders. This may lead to inaccurate data collection.

One complication of several studies evaluating the epidemiology of DD was the experience of the investigator/s involved in diagnosing the disease in patients as highlighted in section 1.5.7. The diagnosis of advanced disease is relatively straightforward in subjects with extensive signs of digit contracture and prominent nodules, cords and palmar pits. However, in those with minor disease diagnostic criteria can be difficult to interpret. The highest rate of misdiagnosis of DD is most likely when asking a patient to self-diagnose. Therefore studies attempting to find the prevalence of DD by questionnaires have to be interpreted with great caution; e.g. a study in rock climbers via a patient questionnaire survey may have missed mild DD (presence of palmar nodules only), leading to a potentially inaccurate prevalence rate. (Logan, Mason et al. 2005)
1.6 Genetics of DD

The genetics of DD has been investigated by several approaches: observing that the condition runs in families and has familial aggregation, identifying a pattern of inheritance, calculating modes of heritability and complex genetic analysis. This section will review these methods, which have contributed to the understanding of the genetic nature of DD.

1.6.1 Identification of manuscripts discussing the genetic nature of DD

Studies evaluating the genetic and heritable nature of DD were searched using Medline, Embase, PubMed, and Web of Science and subsequent cross-referencing to earlier articles. Keywords were used to identify relevant articles and included Dupuytren’s, disease, genetics, gene, heritability, and inheritance.

*Inclusion criteria*

All prospective and retrospective studies, case reports, letters, communications and reviews. A study was seen as relevant when evaluating the genetic aspect of DD whether it be; i) identification of an inheritance pattern, ii) genomic or karyotype analysis or iii) identification of specific gene(s) as biomarkers.

*Exclusion criteria*

i) Studies evaluating the epidemiology of DD. This has already been extensively reviewed in section 1.5. ii) Studies identifying biomarkers to understand the pathophysiology of DD. iii) Clinical studies investigating the role of external associated factors in DD development.
1.6.2 Familial aggregation studies & segregation analysis

The heritable nature of DD has been recognised for many years (Goyrand 1833; Lyall 1993). The first familial study carried out by Skoog in 1948 showed that DD appeared to run in families in 44% of cases (Skoog 1948), a finding which was further confirmed in 1963 (Ling 1963). At around this time large families were also being identified with affected members spanning several generations (Demers and Blais 1960).

The earlier studies, describe DD as having a Mendelian autosomal dominant pattern of inheritance (Manson 1931; Stackebrandt 1932; Schroder 1934; Skoog 1948; Ling 1963; Kozlowski, Kafar et al. 1980; Nyberg, Bias et al. 1982). Following on from these observations a case report of a large family with DD was published but many of the affected members were female and it was suggested that DD may be sex-linked in certain cases (Matthews 1979). A reported x-linked syndrome comprises DD joint contractures, keloids, large optic cup-to-disc ratio and renal stones (Heyen, Delk et al. 2008).

Twin studies have also confirmed the genetic basis of DD with initial reports suggesting that DD was a purely genetic condition based solely on clinical examination (Bergh; Jentsch 1937; Couch 1939). However, a case series has shown that environmental factors are involved in disease pathogenesis as only one twin was affected with DD (Lyall 1993; Beleta and Fores 2012).

To estimate the genetic component of DD, familial clustering of DD has been calculated (sibling recurrence risk ratio). This was initially calculated in 2006
(Hindocha, John et al. 2006) and then revised in 2012 (Capstick, Bragg et al. 2012). The most recent sibling recurrence risk ratio calculated is equal to 4.5 (Capstick, Bragg et al. 2012).

1.6.3 Molecular genetic studies (Table 1.6.1)

HLA typing in several DD cohorts suggested DD may have an autoimmune basis because of the significance of HLA typing studies in other musculoskeletal conditions such as rheumatoid arthritis (Tait and Mackay 1982; Williams, Dann et al. 1983; Spencer and Walsh 1984; Pereira, Black et al. 1986; Neumuller, Menzel et al. 1994). However, there was no concrete evidence to support the HLA hypothesis. More recently, HLA-DRB1*01 allele has been observed more frequently in DD patients (frequency 11%) than controls (frequency 4%) (Jonsson, Gudmundsson et al. 2012). This recent study was an observation, and the allele HLA-DRB1*01 cannot be used as a biomarker until further functional studies have been carried out.

Karyotype analyses from cultured cells of DD tissue were thought to be an ideal way to identify macro structural abnormalities as initial thoughts were that DD was monogenic (Bonnici, Birjandi et al. 1992; Ling 1963). Karyotype analyses were carried out with conflicting data (Sergovich, Botz et al. 1983; Bonnici, Birjandi et al. 1992; Casalone, Mazzola et al. 1997; Pilato, Viotto et al. 1997; Dal Cin, De Smet et al. 1999). The most recent study investigating trisomy 8 has suggested that those present with the karyotype may be at a higher risk of DD recurrence following surgery (Dal Cin, De Smet et al. 1999).

The first study to identify candidate genes in DD using microarray technology was carried out in 2003. This study used the Atlas microarray system (Palo Alto, CA) with
an array of 1176 genes (Human 1.2k array II membrane contained 1176 genes). The results identified 23 genes as biomarkers and suggested possible links with causative environmental and demographic factors such as alcohol use and gender (Pan, Watson et al. 2003). This microarray study of 6 DD patients has highlighted two genes that may be linked to the associated risk factor of alcohol.

Since this initial study, several chromosomal regions, through linkage and case control association studies have been associated with DD pathogenesis. A Swedish family with many affected members underwent a genome scan and linkage analysis. It was subsequently found that a susceptibility locus was found on chromosome 16. However, further functional analysis was required to confirm this (Hu, Nystrom et al. 2005).

A microarray study was conducted by Lee et al. (2006) to identify up-regulated genes in DD from samples of DD tissue (Lee, Zhang et al. 2006). They identified several novel genes associated with the disease including MafB, collagen type V and type VIII. The MafB gene was focused upon in this study due to its role in tissue development. MafB was seen in 50% of DD tissue samples but not at all in control tissue by immunohistochemistry. However, the lack of functional studies meant MafB could not be exclusively associated with disease pathogenesis nor be used as a potential biomarker (Lee, Zhang et al. 2006).

Further genetic analysis to identify susceptible genes is comparative genomic hybridization to identify up and down regulated genes in the development of proliferating fibroblasts. Kaur et al conducted this on 19 samples and found that there
were no significant deletions or amplifications in the progression of the abnormal fibroblast. They did however identify 24 over expressed genes and 22 under expressed genetic targets which may be used for future reference when identifying susceptible genes in a whole genomic scan (Kaur, Forsman et al. 2008).

On a similar note gene expression profile analysis has been a further method to identify susceptible genes, but, yet again although identification of over expressed genes has been identified these are not correlated with disease severity (Rehman, Salway et al. 2008). Another study using microarray analysis identified 18 up regulated and 51 down regulated genes. Three genes coding proteoglycan, fibulin and type XV collagen were considered of high significance with RT-PCR, (Satish, Laframboise et al. 2008). As will be seen below, TGF β has been a biomarker of interest in many studies. Zhang et al conducted cDNA microarray analysis to evaluate the role of TGF β and found that TGF β2 was significant in the aetiopathogenesis of DD compared to TGF β1 and TGF β3 (Zhang, Fong et al. 2008).

A further recent microarray study using the Affymetrix platform has found that up to 127 genes may play a significant role in the pathogenesis of DD including a role of down regulation of myoglobin and up regulation of the tyrosine kinase-like orphan receptor 2 (ROR2). Myoglobin transports oxygen to tissue in times of hypoxia, ROR2 is under expressed in late DD and maybe associated with severe disease (Forsman, Paakkonen et al. 2008). ROR2 is involved in chondrocyte and cartilage development and an increase in extracellular matrix may increase the size of diseased cords or nodules causing more severe digital contracture (Forsman, Paakkonen et al. 2008).
More recently genome wide array studies have been carried out. Unfortunately most of these studies have small sample sizes as shown in table 1.6.1. One study does have a large sample size and conveys an important pathway in the potential development of DD (Dolmans, Werker et al. 2011). The large sample size from different European geographical locations makes this study the most accurate to date. Eleven SNPs in 9 DD risk loci were identified of which 4 candidate genes implicated the WNT pathway. The WNT proteins that bind to specific receptors result in the reduction of β catenin degradation (figure 1.6.1) and have been shown to have a genetic influence on what may be an abnormal pathological pathway in DD.

**Figure 1.6.1 Signaling pathways of WNT and β catenin** WNT signaling and DD study (Dolmans, Werker et al. 2011). 9 loci were identified including one containing genes coding for the frizzled receptor and RSPO2. All loci contained candidate genes coding β catenin were over expressed increasing risk of fibromatosis.
Other recent micro-array studies include one assessing the microRNA profile of DD fibroblasts in 29 patients to assess regulation of genes in the beta catenin, WNT5a and TGF pathways. This study did find that the WNT5 gene was regulated in the beta catenin pathway, which is involved in DD pathogenesis. (Mosakhani, Guled et al. 2010).

A genome wide association study of 40 unrelated DD patients identified 9 significant regions on 9 chromosomes and susceptible SNPs on chromosomes 6, 11 and 16 (Ojwang, Adrianto et al. 2010). This finding of involvement of chromosome 16 in the pathology of DD supports the work of Hu et al (Hu, Nystrom et al. 2005) but neither implicated the beta catenin or WNT pathways and no functional analysis was performed. A micro-array study in 25 affected DD patients identified 5 DNA copy variations on chromosomes 1,7,14,17 and 20 (Shih, Tassabehji et al. 2012). The study size is not sufficient to draw conclusions.

A micro-array study of cultured cells from 6 DD patients found different transcriptomic profiles of DD fascia compared to controls (Satish, LaFramboise et al. 2012). The results of this study do not provide new information on the differences between DD fascia and carpal tunnel fascia.

A study by Ratkaj et al has combined the micro-array and stem cell approach in investigating DD (Ratkaj, Bujak et al. 2012). This study analysed gene expression of TGF-β1 and expression of stem cell markers. The P38MAPK signaling pathway involved in fibroblast proliferation is suggested to be involved in DD (Ratkaj, Bujak et al. 2012). The identification of 3 up regulated genes (THBS1, GADD45B,
NUAK1) in this signaling pathway is relevant to DD pathology as they are markers of cellular proliferation but the study size of 45 is unlikely to be enough to provide consistent evidence of DD pathogenesis unless repeated in a larger cohort.

Many candidate genes have therefore been reported in DD but further work is required to study the role of these genes in the formation of DD.

### 1.6.4 Genetic and pathology concepts

Abnormal regulation of apoptosis has been suggested in the development of DD (Jemec, Grobbelaar et al. 1999). Mitochondria have a possible role in apoptosis (Raha and Robinson 2000; Bayat, Walter et al. 2005). A high number of mitochondria have been identified in diseased tissue and mitochondrial genes are involved in the regulation of apoptosis (Bayat, Walter et al. 2005).

Transforming Growth Factor (TGF) β1 is over expressed in cells which undergo mitochondrial stress (Amuthan, Biswas et al. 2001) and it has been shown that TGF β1 is over expressed in DD tissue (Kloen 1999). TGF β1 and β2 have also been identified in the pathogenesis of DD (Badalamente, Sampson et al. 1996). In the active and dormant phases of the disease TGF β2 has a profound effect on DD fibroblast proliferation (Badalamente, Sampson et al. 1996). Treatment with a known stimulator of cAMP (Forskolin) reduces the accumulation of TGF β in DD cells and is suggested as a form of therapy (Satish, Gallo et al. 2011). A micro-array analysis, identified miRNA’s in the β-catenin pathway including TGF β in DD patients (Mosakhani, Guled et al. 2010). The β-catenin signaling pathway increases cellular density (Bowley, O'Gorman et al. 2007). TGF β is known to be a regulator of the β-catenin pathway (Cheon, Nadesan et al. 2004). High levels of TGF β in DD therefore
have a direct link with fibroblastic accumulation via the β-catenin pathway (Varallo VM 2003, Moon RT 2004, Degreef 2009).

The matrix metalloproteinase (MMP) family has been investigated for their role in DD pathogenesis and as potential biomarkers. The Tissue Inhibitor of Metalloproteinase (TIMPs) have been studied in DD and it has been shown that an imbalance between MMPs and TIMPs causes increased collagen deposition and development of fibrotic tissue resulting in a greater degree of contracture in the digits (Ulrich, Ulrich et al. 2008). An MMP inhibitor, ilomastat was found to reduce contraction of fibroblasts (Townley, Cambrey et al. 2008).

It has been postulated that MMP genes are correlated with outcome following surgical treatment (Johnston, Larson et al. 2008). Recent gene expression studies have identified specific MMPs and suggestions of potential biomarkers. Knockdown of MMP1, 2, 3, 13 and 14 appear to reduce fibroblast contraction (Wilkinson, Davidson et al. 2012). Similarly the elevated expression of MMP2 in DD tissue is a predictive marker of fibroblast contracture and a target for collagenase therapy in vitro (Ratajczak-Wielgomas, Gok et al. 2012; Verhoekx, Beckett et al. 2013). It is evident from these MMP studies that these proteins may have a role in the development of DD.

It is known that α smooth muscle actin expressed by myofibroblasts is a protein involved in cellular contraction in DD. The WNT pathway and β catenin might have a role in this pathway, but further functional evidence is required (Bowley, O’Gorman et al. 2007). A study of α smooth muscle actin and DD in 6 patients confirmed the
over expression of a smooth muscle actin in DD cells (Satish, O'Gorman et al. 2013) and suggested this as a biomarker.

This section has reviewed the current whole genome and gene expression studies published. There have been many studies conducted in the last 5 years that have identified candidate genes in DD via various methods including gene expression and genotype associations. Many of the earlier studies were observational, however recent studies are assessing the function of pathways in abnormal gene expression to understand the role of specific proteins in the development of fibroblast proliferation or contraction. This work is now leading to hypotheses surrounding potential drug targets and non-surgical therapy. However, more work is required to clarify the functional roles of genes and their pathways in relation to DD pathology. In addition, their interaction with environmental factors recognised to impact on DD will be necessary to fully understand DD pathology.
Table 1.6.1: Genetic Studies

<table>
<thead>
<tr>
<th>Author</th>
<th>Date</th>
<th>Study Design</th>
<th>Type of genetic identification</th>
<th>Scientific Relevance</th>
<th>Clinical Relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tait BD et al (Tait and Mackay 1982)</td>
<td>1982</td>
<td>HLA typing methods - Experimental study of DD tissue biopsies.</td>
<td>HLA-DR4 presence in DD tissue.</td>
<td>Further study required to identify definite HLA-DR4 antigen.</td>
<td>Possibility that HLA-DR3 may be used as prognostic biomarker.</td>
</tr>
<tr>
<td>Williams PL et al (Williams, Dann et al. 1983)</td>
<td>1983</td>
<td>HLA typing in 40 DD patients undergoing DD release.</td>
<td>No MHC antigens found.</td>
<td>No association between MHC and DD tissue.</td>
<td>MHC not associated with DD.</td>
</tr>
<tr>
<td>Spencer JD (Spencer and Walsh 1984)</td>
<td>1984</td>
<td>HLA typing in 37 DD patients.</td>
<td>HLA-DR associated with DD development.</td>
<td>Further study required to identify definite HLA-DR antigen.</td>
<td>Possible role of DD as an autoimmune condition.</td>
</tr>
<tr>
<td>Pereira RS et al (Periera, Black et al. 1986)</td>
<td>1986</td>
<td>HLA typing methods from sera of 16 patients with DD.</td>
<td>Increase in IgG antibody formation to type II collagen but no increase in HLA-DR4.</td>
<td>No increase in HLA-DR4 in DD tissue compared to control.</td>
<td>HLA-DR4 not specific as biomarker for DD.</td>
</tr>
<tr>
<td>Bonnici AV et al (Bonnici, Birjandi et al. 1992)</td>
<td>1992</td>
<td>Chromosomal analysis of cell cultures from DD tissue.</td>
<td>Trisomy 8 in DD and control.</td>
<td>Chromosomal instability in pathway of fibrosis.</td>
<td>Suggestion that cancer biomarkers (c-myc) may have a role in the pathogenesis of DD.</td>
</tr>
<tr>
<td>Neumuller J et al (Neumuller, Menzel et al. 1994)</td>
<td>1994</td>
<td>HLA typing in 46 patients with DD.</td>
<td>HLA-DR3 associated with increased risk of developing DD.</td>
<td>Further study required to identify definite HLA-DR3 antigen.</td>
<td>Possibility that HLA-DR3 may be used as prognostic biomarker.</td>
</tr>
<tr>
<td>Badalamente MA et al (Badalamente, Sampson et al. 1996)</td>
<td>1996</td>
<td>Role of TGF-β1 and TGF-β2 in DD via immunologically important roles in pathogenesis.</td>
<td>Both TGF-β1 and TGF-β2 play a role in DD pathogenesis.</td>
<td>HLA-DR1 and HLA-DR2 play a major control role in DD pathogenesis.</td>
<td>TGF-β2 is a potent biological mediator in DD.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Author</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tait BD et al (Tait and Mackay 1982)</td>
<td>1982</td>
</tr>
<tr>
<td>Williams PL et al (Williams, Dann et al. 1983)</td>
<td>1983</td>
</tr>
<tr>
<td>Spencer JD (Spencer and Walsh 1984)</td>
<td>1984</td>
</tr>
<tr>
<td>Pereira RS et al (Periera, Black et al. 1986)</td>
<td>1986</td>
</tr>
<tr>
<td>Bonnici AV et al (Bonnici, Birjandi et al. 1992)</td>
<td>1992</td>
</tr>
<tr>
<td>Badalamente MA et al (Badalamente, Sampson et al. 1996)</td>
<td>1996</td>
</tr>
<tr>
<td>Author(s) and Year</td>
<td>Study Details</td>
</tr>
<tr>
<td>--------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Pilato G et al. (1997)</td>
<td>No evidence of high frequency of trisomy 8. Chromosomal abnormalities exist in DD but exact malfunction is uncertain. Clinical staging and prognostic indication is still best predictor of outcome.</td>
</tr>
<tr>
<td>Casalone R et al. (1997)</td>
<td>Chromosomal instability in DD tissue but no trisomy 8. Chromosomal changes seen in 18 cases. No significant difference in over half samples including non-dividing cells. Common TGFβ1 polymorphisms are not associated with DD. Results only look at a specific polymorphism of TGFβ2 and thus TGFβ1 should not be ruled out as a biomarker for DD.</td>
</tr>
<tr>
<td>Dal Cin P et al. (1999)</td>
<td>Chromosomal abnormalities exist in DD but exact malfunction is uncertain. Clinical staging and prognostic indication is still best predictor of outcome.</td>
</tr>
<tr>
<td>Bayat A et al. (2002)</td>
<td>Chromosomal abnormalities exist in DD but exact malfunction is uncertain. Clinical staging and prognostic indication is still best predictor of outcome.</td>
</tr>
<tr>
<td>Pan D et al. (2003)</td>
<td>Microarray analysis of DD tissue in 6 DD patients. 22 susceptible genes, 5 over expressed and 18 under expressed. 9 genes were further expressed and verified. Links to these genes and associated risks of DD.</td>
</tr>
<tr>
<td>Bayat A et al. (2002)</td>
<td>Chromosomal abnormalities exist in DD but exact malfunction is uncertain. Clinical staging and prognostic indication is still best predictor of outcome.</td>
</tr>
<tr>
<td>Dal Cin P et al. (1999)</td>
<td>Chromosomal abnormalities exist in DD but exact malfunction is uncertain. Clinical staging and prognostic indication is still best predictor of outcome.</td>
</tr>
<tr>
<td>Pilato G et al. (1997)</td>
<td>No evidence of high frequency of trisomy 8. Chromosomal abnormalities exist in DD but exact malfunction is uncertain. Clinical staging and prognostic indication is still best predictor of outcome.</td>
</tr>
<tr>
<td>Study</td>
<td>Methods</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>Shin SS et al. (2004)</td>
<td>Role of Bone Morphogenetic proteins in DD via qPCR and Western blotting</td>
</tr>
<tr>
<td>Kaur S et al. (2008)</td>
<td>Array Comparative Genomic Hybridization (aCGH) to identify significant genes</td>
</tr>
</tbody>
</table>

**Table Notes:**
- DD: Diabetic Dermopathy
- QRT-PCR: Quantitative Reverse Transcriptase PCR
- aCGH: Array Comparative Genomic Hybridization
- BMPs: Bone Morphogenetic Proteins
<table>
<thead>
<tr>
<th>Study Authors</th>
<th>Year</th>
<th>Summary</th>
<th>Outcome</th>
<th>Gene Expression Role</th>
<th>MMP Expression Role</th>
<th>Other Specified Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rehman S et al. (Rehman, Salway et al. 2008)</td>
<td>2008</td>
<td>Chromosomal imbalances.</td>
<td></td>
<td></td>
<td></td>
<td>2008</td>
</tr>
<tr>
<td>Satish L et al. (Satish, LaFramboise et al. 2008)</td>
<td>2008</td>
<td>Transcriptome profiling of DD tissue with validation using RT-PCR in 6 DD patients.</td>
<td></td>
<td></td>
<td></td>
<td>2008</td>
</tr>
<tr>
<td>Zhang AY et al. (Zhang, Fong et al. 2008)</td>
<td>2008</td>
<td>Gene expression analysis of TGF-β from DD tissue biopsies.</td>
<td></td>
<td></td>
<td></td>
<td>2008</td>
</tr>
<tr>
<td>Forsman M et al. (Forsman, Paakkonen et al. 2008)</td>
<td>2008</td>
<td>Microarray analysis and identification of myoglobin and ROR2 in DD.</td>
<td></td>
<td></td>
<td></td>
<td>2008</td>
</tr>
<tr>
<td>Brown JJ et al. (Brown, Ollier et al. 2008)</td>
<td>2008</td>
<td>Role of MHC genes in DD development.</td>
<td></td>
<td></td>
<td></td>
<td>2008</td>
</tr>
<tr>
<td>Ulrich D et al. (Brown, Ollier et al. 2008)</td>
<td>2009</td>
<td>Role of MMPs and TIMPs imbalance in DD development.</td>
<td></td>
<td></td>
<td></td>
<td>2008</td>
</tr>
</tbody>
</table>
| Therapy                                                                 | Prognostic factor | Genes involved in development of DD and non-familial involvement of WNT and TGF-β. No difference of expression in development of DD. | Genes known susceptible DD and their regulation of expression. Genes in DD patients. | MicroRNA profile of DD
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ulrich, Ulrich et al. 2009</td>
<td></td>
<td>B-catenin pathway again identified in DD.</td>
<td>ZIC1 and TGF-β. No correlation pathway: WNT5A.</td>
<td>MicroRNA profile of DD</td>
</tr>
<tr>
<td>Townley WA et al. (Townley, Cambrey et al. 2009)</td>
<td></td>
<td>3 different microRNAs were found to be different in familial and non-familial cases of DD.</td>
<td>3 different microRNAs were found to be different in familial and non-familial cases of DD.</td>
<td></td>
</tr>
<tr>
<td>Degreef I et al. (Degreef and De Smet 2009)</td>
<td></td>
<td>Overexpression of β-catenin as a biomarker for disease recurrence using PCR.</td>
<td>Differential gene expression of DD tissues in 5 DD patients.</td>
<td></td>
</tr>
<tr>
<td>Mosakhani N et al. (Mosakhani, Guled et al. 2010)</td>
<td></td>
<td>Microarray analysis of DD fibroblasts in 29 patients and regulation of known susceptible DD genes.</td>
<td>No difference of expression in familial and non-familial cases.</td>
<td></td>
</tr>
<tr>
<td>Fidilainen et al. (Fidilainen et al. 2010)</td>
<td></td>
<td>Microarray analysis of DD fibroblasts in 29 patients and regulation of known susceptible DD genes.</td>
<td>No difference of expression in familial and non-familial cases.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Tassabehji et al. 2010)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
<tr>
<td>Shih BB et al. (Shih, Brown et al. 2009)</td>
<td></td>
<td>High-resolution genome-wide screening for whole-genome DNA variation in 4 DD patients.</td>
<td>3 copy number variations on 10q22, 16p12, and 17p12.</td>
<td></td>
</tr>
</tbody>
</table>

**Notes:**
- **DD:** Dislocated Hip
- **MMP:** Matrix Metalloproteinase
- **miRNA:** microRNA
<table>
<thead>
<tr>
<th>Study Authors and Year</th>
<th>Study Details</th>
<th>Results and Discussion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ojwang JO et al. 2010</td>
<td>Genome-wide association scan of DD in 40 unrelated patients. Significant regions identified on chromosomes 1, 3-6, 11, 16, and 23. Linkage disequilibrium analysis identified ancestry regions in chromosomes 2, 6, 8, 11, 16, and 20. Bi-allelic analyses revealed significant regions in chromosomes 1, 11, and 13.</td>
<td>Bi-allelic analyses revealed significant regions in chromosomes 1, 11, and 13. In comparison to previous familial studies, chromosome 16 may harbor significant SNPs relating to the pathogenesis of DD.</td>
</tr>
<tr>
<td>Satish L et al. 2011</td>
<td>Expression of TGFβ and interaction with cAMP signaling in DD. Increased cAMP levels inhibit the formation of myofibroblasts and accumulation of ECM proteins. cAMP signaling is a protein which may be aberrant in the development of DD tissue.</td>
<td>Drug therapy that can increase the levels of cAMP in DD may slow progression or recurrence of DD.</td>
</tr>
<tr>
<td>Krause C et al. 2011</td>
<td>Expression of TGFβ in DD treated fibroblasts with BMP6 and SB-431532 in 3 DD patients. Increased expression of TGFβ in DD fibroblasts treated with BMP6 and SB-431532. TGFβ pathway involved in DD. This study confirms the findings of previous work.</td>
<td>TGFβ plays a role in the pathogenesis of DD. This study confirms the findings of previous work.</td>
</tr>
<tr>
<td>Dolmans GH et al. 2011</td>
<td>Genome-wide association study in 2325 DD patients. Loci identified associated with WNT signaling pathway. 6 SNPs at 9 loci. 6 involved in WNT pathway. 11 SNPs at 9 loci. Pathway identified in DD. First study to identify significant genetic SNPs in the pathogenesis of DD.</td>
<td>First study to identify significant genetic SNPs in the pathogenesis of DD. First study to identify significant genetic SNPs in the pathogenesis of DD.</td>
</tr>
<tr>
<td>Shih et al. 2012</td>
<td>Genomic array study to identify DNA copy number variations in DD, in 25 DD patients. 5 common copy number variations identified on chromosomes 1, 7, 14, 17, and 20.</td>
<td>Potential genetic targets which require further investigation. Results of this study cannot be related to clinical practice.</td>
</tr>
<tr>
<td>2012</td>
<td>(Satish, LaFramboise et al.)</td>
<td>Cell cultured microarray analysis from tissue biopsies of 6 DD patients.</td>
</tr>
<tr>
<td>2012</td>
<td>(Ratkaj, Bujak et al.)</td>
<td>Microarray analysis of DD cells and expression of stem cell markers in 45 DD patients.</td>
</tr>
<tr>
<td>2012</td>
<td>(Jonsson, Gudmundsson et al.)</td>
<td>Association of HLA-DRB1*01 of 121 males with DD and 51 controls.</td>
</tr>
</tbody>
</table>

| 2012 | (Ratajczak-Wielgomas, Gosk et al.) | Gene expression study of MMP-2, TIMP-1, TGF-β, DCN and periostin in 50 DD patients. | MMP-2 elevated in DD tissue. MMP-2, TIMP-1, TGF-β, DCN and periostin highly expressed in DD tissue. MMP-2, TIMP-1, TGF-β, DCN and DCN and expression in DD. | Balance of MMP-2, TIMP-1, TGF-β, DCN and periostin disrupted in DD tissue. Expression of DCN and TIMP-1 elevated in DD. MMP-2, TIMP-1 inhibitor, ADAMTS1, 14 inhibitor, TIMP-1, 2, 7, MMP-2, 2, TGF-β, 1, 3-13, Knockdown of |

<p>| 2012 | (Jonsson et al.) | Gene expression study of MMP-2, TIMP-1, TGF-β, DCN and periostin in 50 DD patients. | MMP-2 elevated in DD tissue. MMP-2, TIMP-1, TGF-β, DCN and periostin highly expressed in DD tissue. MMP-2, TIMP-1, TGF-β, DCN and DCN and expression in DD. | Balance of MMP-2, TIMP-1, TGF-β, DCN and periostin disrupted in DD tissue. Expression of DCN and TIMP-1 elevated in DD. MMP-2, TIMP-1 inhibitor, ADAMTS1, 14 inhibitor, TIMP-1, 2, 7, MMP-2, 2, TGF-β, 1, 3-13, Knockdown of |</p>
<table>
<thead>
<tr>
<th>Study Authors</th>
<th>Year</th>
<th>Methodology</th>
<th>Findings</th>
<th>Implications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Satish L et al.</td>
<td>2013</td>
<td>Gene expression study of CCT-eta and SMA in 6 DD patients and 6 CT patients as control using siRNA transfection</td>
<td>CCT-eta expression increased in active DD and normal palmar fascia of same patient compared to CT patients.</td>
<td>CCT-eta marker for latent and active disease. Maybe used as a biomarker to predict future prognosis in other digital rays of the same patient.</td>
</tr>
<tr>
<td>Zyluk A et al.</td>
<td>2013</td>
<td>SNP frequencies of the ALDH2 and DHDH genes in 300 DD patients and 300 matched controls</td>
<td>No difference of SNP frequencies between DD and controls for ALDH2. Higher frequency of DHDH gene in DD patients with a positive family history.</td>
<td>ALDH2 not associated with increased susceptibility for DD. ALDH2 and DHDH linked with malignancies. Unable to use these genes as biomarkers for DD.</td>
</tr>
<tr>
<td>Verhoekx JS et al.</td>
<td>2013</td>
<td>5 DD patients and 5 CT controls. Tissue biopsies following force tension</td>
<td>Increase of all markers in the DD nodule. Nodule cells more contractile than cord cells.</td>
<td>Treatment should be targeted at nodule to reduce degree of contracture.</td>
</tr>
</tbody>
</table>
1.7 The role of stem cells in DD

Besides genetic and environmental factors, the cellular origin of the abnormal tissue in DD has become a recent focus of research interest in the field (Hindocha, Iqbal et al. 2011; Iqbal, Manning et al. 2012; Ratkaj, Bujak et al. 2012). These studies have attempted to locate and characterize populations of potential stem cells in DD fascia, fat and skin overlying disease. The aims of these studies is to further understand aetiology, recurrence, and guide future therapy.

What is a stem cell?

Stem cells are defined as having capacity to self-renew and generate differentiated cells (Weissman, Anderson et al. 2001). This definition can be expanded in to further categories by defining whether a stem cell is pluripotent (differentiating into any cell line i.e. blood, neural, bone), multipotent (differentiating into different cells of the same cell line (eg haematopoietic cells) or unipotent (making only one cell type) (Kolios and Moodley 2013; Reddien 2013). In answering this question the definition of a stem cell therefore has the properties of replication, clonality and potency (Kolios and Moodley 2013).

Stem cells, fibrotic disease and relevance to DD

New masses of cellular tissue appear in DD but their cell of origin is not known. The renal, pulmonary and hepatic architecture have all been shown to have cells that differentiate into myofibroblasts (Zeisberg and Kalluri 2004; Bataller and Brenner 2005; Hinz, Phan et al. 2007). Carcinomas have mesenchymal stem cell populations in their stroma that differentiate in to fibroblasts (Quante, Tu et al. 2011).
Myofibroblasts which have contractile properties are involved in the development of DD (Eyden 2001). The cellular mechanics and biochemical properties of the DD myofibroblast have been extensively studied (Badalamente and Hurst 1999; Tomasek, Vaughan et al. 1999; Rayan 2007). The accumulation of DD myofibroblasts contributes to the nodules in the palm and puckering of the skin resulting in palmar pits and eventual contracture of the affected digit. However, the exact cell of origin of DD tissue remains inconclusive despite biochemical (Badalamente and Hurst 1999), cellular (Tomasek, Vaughan et al. 1999) and genetic studies (Dolmans, Werker et al. 2011). The differentiation of fibroblasts to myofibroblasts as a step in tissue repair is stimulated by TGFβ1 in cells expressing CD90 (Brenmoehl, Miller et al. 2009).

Fibroproliferative disorders such as lung fibrosis have been attributed to cells named as fibrocytes identified by specific expression of stem cell markers (Bucala, Spiegel et al. 1994). Identification of stem cell populations in colon cancer has then developed further to seek potential drug targets (Potten, Booth et al. 2003).

DD tissue shares cellular and biochemical properties with granulation tissue (Howard, Varallo et al. 2004). Some cell populations in wound healing are derived from bone marrow or from immune cells (Mori, Bellini et al. 2005; Barisic-Dujmovic, Boban et al. 2010).

The cell of origin of DD remains unknown but it seems reasonable to explore the possibility that it arises from a stem cell population in one or other of the various tissues of the hand – fascia, palmar fat and skin (Iqbal, Manning et al. 2012).

The palmar fat has been suggested as having a role in the pathogenesis of DD (Rabinowitz, Ostermann et al. 1983).
1.8 Summary

This literature review has described studies on the epidemiology and genetics of DD. Prevalence and incidence studies have been limited and selective such that it is not really known how common the disease may be. Studies have been conducted in different age groups, different population cohorts (i.e. HIV patients, nursing home residents, rock climbers, with different methods of data collection (self diagnosis of DD, diagnosis by a non-hand surgeon, diagnosis by a specialist). The vast changes in population structure, changes in prevalence of associated diseases and the change in diagnostic criteria of DD makes the epidemiology of limited value.

Genetic analytical techniques such as micro-array studies and gene association studies have increased in the last decade. Further studies are required to ascertain whether there are numerous genetic defects that result in the same clinical picture, and to identify the cell of origin and the molecular pathways that determine the disease.
1.9 Hypotheses

1. DD is a complex polygenic condition with many candidate genes.

2. The investigation of stem cell markers in DD will aid in determining the cellular source of the condition.

1.10 Aims

ia) Study 1 (chapter 2) aims to estimate penetrance and revise the sibling recurrence ratio in DD.

ib) Study 2 (chapter 3) aims to identify DD susceptibility genes in a large family with a high incidence of DD by linkage analysis

ii) Study 3 (chapter 4) aims to identify possible stem cell populations in different DD tissues by assessing selected mesenchymal and/or haematopoietic stem cell markers in palmar fascia, fat and skin.
Chapter 2

Study 1

Estimating penetrance and revising the sibling recurrence risk ratio in DD
2.1 Introduction

A genetic contribution to the aetiology of DD has been suggested in the literature over the last 60 years (Michou, Lermusiaux et al. 2012). The DD phenotype is variable and heterogeneous with respect to age at onset and distribution of affected digits (Lanting, van den Heuvel et al. 2013). The phenotypic heterogeneity suggests an aetiologic heterogeneity as has been confirmed in many studies (chapter 1). Historically studies of family pedigrees with a near Mendelian pedigree have tended to favour an autosomal dominant pattern of inheritance (Stackebrandt 1932; Schroder 1934; Skoog 1948; Ling 1963; Kozlowski, Kafar et al. 1980). The most significant of these carried out by Ling in 1963 (Ling 1963) is one of the few studies where probands and family members in each pedigree have been examined for evidence of DD. The study suggested an autosomal dominant pattern of inheritance with variable penetrance. However, the quantification of the variable penetrance was not calculated which left a void for the clinician attempting to estimate the risk of DD in family members of an affected individual. Later studies have suggested that DD is a complex polygenic disease and not one following a Mendelian pattern. With a near Mendelian inheritance pattern it is valuable to genotype these pedigrees to identify the candidate genes.

As a result of studies reporting varying modes of inheritance the surgeon is really unable to counsel the patient and their relatives on the inherent risk of DD. There are many current studies as identified in chapter 1 that are aiming to identify contributory molecular genetic loci which may be involved in disease aetiology. Genetic epidemiology does provide insight into molecular genetic studies (Sedic, Pavelic et al. 2012). In particular accurately defining the penetrance level to identify the genetic or
environmental quantity in a multifactorial disease such as DD can confirm familial aggregation (Capstick, Bragg et al. 2012). This can be used to determine the power to detect linkage by calculating the sibling recurrence risk ratio ($\lambda_s$) (Risch 1990). This study will report on a current population cohort used to calculate $\lambda_s$ as a revision.

With the number of associated factors related to DD it is likely that this disease is not a simple disease with a monogenic cause but one that is polygenic, multifactorial and complex (Dolmans, de Bock et al. 2012). The purpose of this chapter is to address this issue and provide more clarity as to why the disease is not Mendelian in nature and provide a basis for chapter 3 on genotyping a family with DD. Environmental risk factors will be taken into account to assess the potential contribution of environment in DD aetiology.

The objectives of this chapter are to; i) observe the pattern of inheritance of DD in the UK population; ii) evaluate the environmental factors in relation to family history in DD; iii) quantify the level of penetrance, if DD was presumed to have a near autosomal dominant pattern of inheritance and iv) revise the previously calculated sibling recurrence risk ratio ($\lambda_s$) in order to provide power for the subsequent linkage study (chapter 3). The revision of $\lambda_s$ will be based on an extension of the previous study (Hindocha, John et al. 2006). The cohort of probands will be increased in order to improve the accuracy of the previous calculation.
2.2 Materials and methods

2.2.1 UK patient database and DD pedigree ascertainment

Family histories were recorded from probands who were patients being treated for DD in 3 specialised hand surgery units at; South Manchester Teaching Hospital, Wrightington Hospital and the Pulvertaft Hand Centre, Derby. Patients from Derby were added to the cohort as an extension of the previous study conducted in 2006 (Hindocha, John et al. 2006). Ethical approval for the study was granted and included collection of DNA for genetic study. Consent for enrollment to the study was sought from each proband (appendix 2.1).

450 DD patients across all three sites were approached preoperatively to participate in the study. 135 (30%) agreed and consented. Demographic and clinical details were collected (appendix 2.2). A consultant hand surgeon confirmed the clinical diagnosis of DD. In addition all probands had adequate severity of disease to be enrolled as all were to undergo or had undergone surgical treatment of DD for digital contracture.

A family history questionnaire was completed by probands and this questionnaire was validated in order to minimize ascertainment bias. The family history questionnaire (appendix 2.3) elicited affection data. Families were classified as multiplex (at least one affected relative) or simplex (no reported affected family members).

DD status in family members was confirmed in 46 pedigrees and therefore allowed validation of the family questionnaire.
2.2.2 Inheritance in DD and aetiological factors

A family pedigree was constructed for each pro-band (appendix 2.4) using the PED 6.0.2 Pedigree software package (Plendl 2009). Each pedigree was reviewed and the pattern of inheritance identified. Phenotypes for each pro-band were confirmed. Phenotypes for family members were confirmed for those who were examined. The phenotype of the other family members was established using the questionnaire. Pro-bands were educated on the signs of DD prior to completing the questionnaire. The presence of associated DD factors was documented in addition to age at onset of DD and number of digits affected.
2.2.3 Pedigree analysis: Calculating the level of penetrance

The penetrance level cannot be calculated directly, only estimated (Bodmer and Bonilla 2008). Before variant penetrance is calculated, population attributable risk (PAR) is determined as a measure of the multi-factorial inherited component of a disease which is considered to have a multi-factorial aetiology (Bodmer and Bonilla 2008).

The PAR is calculated by relating the disease incidence in the cohort and the disease incidence in the absence of the genetic variant. In this situation the incidence of DD in this cohort in those with and without a positive family history:

\[ R = \frac{(K-y)}{K} \]

Where \( R \) is the PAR, \( K \) is the total number of probands and \( y \) is the total number of probands who do not have a positive family history of DD. \( R \) is seen as an approximate measure of the disease incidence which is attributed to genetic factors (Bodmer and Bonilla 2008).

If we were to assume that DD is a monogenic disease and were to calculate the level of penetrance, this would be carried out using the following formula: (Bodmer and Bonilla 2008)

\[ f = \frac{y(\alpha-1)}{1+y(\alpha-1)} \]

Where \( y \) is the probability of developing the disease in the absence of the genetic variant and \( f \) is the independent gene frequency of that variant (Bodmer and Bonilla 2008). Alpha is the odds ratio, previously calculated for disease recurrence following surgery in those with a positive family history and is equal to 1.35 (Hindocha, Stanley et al. 2006).
The above two formulae have been used to estimate the proportion of disease contributed by genetic factors and to quantify the level of penetrance in a near dominant model used for familial DD.
2.2.4 Pedigree analysis: Revising the sibling recurrence risk ratio ($\lambda_s$)

The sibling recurrence risk ($K_s$) is defined as the probability that a sibling of an affected individual is also affected (Olson and Cordell 2000). The ratio of $K_s$ to the disease prevalence in a population is termed the sibling recurrence risk ratio ($\lambda_s$) (Olson and Cordell 2000). This value is a pre-requisite for linkage studies. The $\lambda_s$ for DD has been previously calculated for DD in a cohort of unrelated individuals and their family members (Hindocha, John et al. 2006).

In the current study the cohort included those in the above study plus further unrelated individuals and their family members. The $\lambda_s$ was recalculated in this larger sample to provide a more accurate measure of the genetic contribution to DD. Other affected-relative risk ratios such as parent-offspring or grandparent-grandchild were not calculated. The reason for this is that DD is a later onset disease and it is more likely that DD would be present among siblings as they are more likely to be of a similar age group than other relative pairing.

The $\lambda_s$ was calculated using a population approach (Olson and Cordell 2000) and the standard formula:

$$\lambda_s = \frac{K_s}{K}$$

Where $K_s$ is the sibling recurrence risk (the proportion of affected siblings among all siblings of affected persons in a population) and $K$ is the normal population prevalence of the condition. $K$ was estimated for the current time from what was felt to be the most accurate prevalence rate of DD in the UK. The rate used was calculated in 1962 and was calculated by assessing DD in 6979 individuals in the community (Early 1962). The prevalence was found to be 3.5% (3.8% in males and 2.3% in females) among a population between the ages of 17 and 75 years. These figures may
be outdated and more recent values for the male population (8.1%) between the age of 25 and 99 years have also been included (Burke, Proud et al. 2007).
2.2.5 Statistical analysis

Data was entered and analysed using the SPSS 13.0 (Chicago) software package. The above formulae have been used to calculate estimated variant penetrance levels (Bodmer and Bonilla 2008) and the sibling recurrence risk ratio (James, Flack et al. 1971; Rybicki and Elston 2000). The significance of associated risk factors in relation to positive family history was calculated using the Chi squared and Student’s t-test.
2.3 Results

2.3.1 Family description

The cohort consisted of 118 (87%) males and 17 (13%) females. For the 135 probands a total of 1156 relatives were assessed of which there were 302 siblings. The mean age of the probands was 66 ± 10 years (range 37-88 years) with a mean age at onset of DD of 51 ± 11 years (range 16-77 years) (figure 2.3.1).

**Figure 2.3.1 Age at onset of DD**
Histogram showing the age at onset of DD among probands examined. The x-axis gives the age at onset of disease in years and the observed frequency on the y-axis.

![Age at onset of DD](image)

The mean age at onset of 51.7 years has an element of recall bias. DD is a gradual and progressive disease. The disease is usually painless and develops with a thickened cord or palmar nodule. Given that a patient may not accurately remember this early stage of disease and recollect age at onset at the time of digital contracture, it is likely that the actual age at onset of DD is lower than described here.
The family questionnaire distributed to probands to determine the DD status of relatives. 46 patients, who identified 430 relatives, completed this questionnaire. Probands reported 122 (28%) of relatives had positive DD status. Following examination of 108 relatives it was found that 41 (38%) of relatives had positive DD status. Proband-diagnosis was correct in 77 of 108 (71%) of cases. Mild disease with minimal or no contracture appeared to have been missed after examination of the 108 relatives, giving the impression that DD prevalence is likely to be underestimated.

2.3.2 Pattern of inheritance of DD

Following analysis of all ascertained pedigrees (appendix 2.4) the inheritance was compatible with a dominant pattern in 59/63 (94%) of cases. The remaining families showed a dominant pattern with variable penetrance. These pedigrees may harbor many candidate genes on varying susceptible loci causing DD and therefore, although showing near Mendelian inheritance, they are likely to be polygenic.

Of 1156 relatives identified by questionnaire 310 (27%) had positive DD status. Of those examined there was an underestimate of DD status by 10% (2.3.1). This shows ascertainment bias in that DD is underreported. As a result, the total number of reported cases has been increased from 27% to 37% (428 affected family members) to allow for this ascertainment bias. The number of affected family members has been increased and also reflected in the quantification of penetrance calculations in section 2.3.4.
2.3.3 Assessment of environmental risk factors on family history of DD

Associated risk factors of DD were ascertained by enquiry and tabulated.

Table 2.3.1: Frequency of associated risk factors among probands.

<table>
<thead>
<tr>
<th>Associated risk</th>
<th>Frequency</th>
<th>Percentage</th>
<th>Significance in relation to positive family history</th>
<th>Significance in relation to age onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manual job</td>
<td>97</td>
<td>71.9</td>
<td>( p=0.51 )</td>
<td>( p=0.11 )</td>
</tr>
<tr>
<td>Insulin dependent diabetic</td>
<td>6</td>
<td>4.4</td>
<td>( p=0.52 )</td>
<td>( p=0.60 )</td>
</tr>
<tr>
<td>Non-Insulin dependent diabetic</td>
<td>15</td>
<td>11.1</td>
<td>( p=0.77 )</td>
<td>( p=0.81 )</td>
</tr>
<tr>
<td>Epilepsy</td>
<td>1</td>
<td>0.7</td>
<td>( p=0.25 )</td>
<td>( p=0.55 )</td>
</tr>
<tr>
<td>Carpal Tunnel Syndrome</td>
<td>4</td>
<td>3.0</td>
<td>( p=0.81 )</td>
<td>( p=0.90 )</td>
</tr>
<tr>
<td>Frozen Shoulder</td>
<td>23</td>
<td>17.0</td>
<td>( p=0.37 )</td>
<td>( p=0.16 )</td>
</tr>
<tr>
<td>Hypertension</td>
<td>47</td>
<td>34.8</td>
<td>( p=0.25 )</td>
<td>( p=0.86 )</td>
</tr>
<tr>
<td>Rheumatoid Arthritis</td>
<td>3</td>
<td>2.2</td>
<td>( p=0.62 )</td>
<td>( p=0.67 )</td>
</tr>
<tr>
<td>Ledderhose’s disease</td>
<td>12</td>
<td>8.9</td>
<td>( p=0.97 )</td>
<td>( p=0.88 )</td>
</tr>
<tr>
<td>Garrod’s pads</td>
<td>20</td>
<td>14.8</td>
<td>( p=0.05 )</td>
<td>( p=0.35 )</td>
</tr>
<tr>
<td>Peyronies Disease</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Smoker or ex smoker</td>
<td>79</td>
<td>58.5</td>
<td>( p=0.42 )</td>
<td>( p=0.61 )</td>
</tr>
<tr>
<td>Hand Injury</td>
<td>10</td>
<td>7.4</td>
<td>( p=0.81 )</td>
<td>( p=0.05 )</td>
</tr>
</tbody>
</table>

Alcohol consumption among probands was documented as a continuous variable (figure 2.3.2). With a mean alcohol consumption of 14.3 units per week there was no significant relation to age at onset of disease \( (p=0.07) \) and positive family history \( (p=0.63) \). As can be seen from the above table, interestingly, the only factors significant in association with a positive family history of DD are the presence of Garrod’s pads and the fact that DD can be seen at a young age at onset in those who have sustained a hand injury.
Figure 2.3.2 Alcohol consumption in DD
Histogram showing the distribution of alcohol consumption among probands. The x axis shows the total number of alcohol units consumed in one week and the y axis the observed frequency.

The mean number of digits (out of 10) affected among the probands was 3.17 ± 2.2 SD (figure 2.3.3). There was no correlation between the number of digits affected and alcohol consumed (p=0.07). However there was a significantly greater chance of a proband having more digits affected in the presence of a positive family history (p=0.04).
Figure 2.3.3 Distribution of digits affected with DD
Histogram showing the distribution of digits affected with DD among probands. The x axis shows the total number of digits affected and the y axis the observed frequency.

![Histogram showing the distribution of digits affected with DD among probands. The x axis shows the total number of digits affected and the y axis the observed frequency.](image)

Mean = 3.17
Std. Dev. = 2.174
N = 135
2.3.4 Quantification of penetrance in DD

The total number of individuals assessed was 1156 of which there were 428 affected.

The population attributable risk (R) is calculated (Bodmer and Bonilla 2008):

\[ R = \frac{(K-y)}{K} \]
\[ R = \frac{135-72}{135} \]
\[ = 0.47 \text{ (47\%)} \]

The variant penetrance \((f)\) is calculated (Bodmer and Bonilla 2008):

\[ f = \frac{y(\alpha-1)}{1+y(\alpha-1)} \]
\[ y = \frac{728}{1156} \]
\[ = 0.63 \]

(Estimate of chance of developing disease in the absence of a positive family history)

\[ f = \frac{0.63(1.35-1)}{1+0.63(1.35-1)} \]
\[ = 0.18 \text{ (18\%)} \]

The population attributable risk is 47%, which is the percentage of cases with familial penetrance. From this value a further calculation is done to estimate the risk of a family member developing the condition in a previously negative family history (Bodmer and Bonilla 2008). This figure was calculated as 18%. This means that the risk of offspring developing DD in the absence of a previous family history is 18%.
2.3.5 Revised λs in DD

The analysis included all 135 probands. The pedigree size ranged from 4 to 21 individuals (appendix 2.4). Data for 1156 relatives were obtained as “DD affected” or “non-DD affected”. A total of 428 (37%) relatives were affected with DD. Forty-one male (35%) and 5 female (29%) probands had no siblings. A total of 249 siblings were assessed of which 238 (96%) were siblings of a male proband and 11 (4%) of a female proband. Using a population model where the sibling recurrence risk (Ks) is the proportion of affected siblings among total number of siblings in the cohort (Olson and Cordell 2000), the distribution of affected siblings between male and female probands was tabulated to derive the sibling recurrence risk (tables 2.3.2 and 2.3.3).

Ks among the male probands is equal to 20.5% and among female probands is equal to 45.5%. The sibling recurrence risk ratio is calculated as λs by dividing Ks by the population prevalence (table 2.3.4). For males the prevalence from Early’s study in 1962 is used as it represents a similar population in terms of geography to the one collected in this study. However, given that this prevalence may be outdated due to an aging population, an additional prevalence from 2007 (Burke, Proud et al. 2007) is also calculated. The revised λs for the UK population is now given as 6.2; for a male proband as 5.4 and for a female proband as 19.8.

The sibling recurrence risk ratio calculated in this study has been questioned in a similar study (Capstick, Bragg et al. 2012). It has been suggested that the method of measuring λs was incorrect. There are two methods to calculate λs. One is based upon single sib-ships (i.e. based on the recurrence risk of one sibling developing the
disease) (Capstick, Bragg et al. 2012). This method is useful in that it limits the degree of ascertainment bias as only one sibling is examined. The method used in the study of (Rybicki and Elston 2000) calculates the $\lambda$s based upon the presence of disease of all siblings. The sibling recurrence risk $K_s$ is defined as the proportion of affected siblings among all siblings of affected persons in a population (Olson and Cordell 2000). This method has been used in another unrelated study assessing retinal detachment (Mitry, Williams et al. 2011). This study investigating $\lambda$s in retinal detachment in a Scottish population used the methods as were used in this study, confirming an alternative but correct statistical analysis.
Table 2.3.2. The overall frequency of the number of affected siblings among male probands (n=85)

<table>
<thead>
<tr>
<th>Number sibling male proband</th>
<th>Number of affected siblings</th>
<th>Observed frequency / 85</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.3.3. The overall frequency of the number of affected siblings among female probands (n=4)

<table>
<thead>
<tr>
<th>Number sibling female proband</th>
<th>Number of affected siblings</th>
<th>Observed frequency /4</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.3.4. Sibling recurrence risk ratio using population prevalence of DD previously calculated in UK in 1962 (Early 1962) and 2007 (Burke 2007)

<table>
<thead>
<tr>
<th>Proband gender</th>
<th>Ks (%)</th>
<th>Prevalence rate (%)</th>
<th>λs</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>21.7</td>
<td>3.5</td>
<td>6.2</td>
</tr>
<tr>
<td>Male (1962)</td>
<td>20.5</td>
<td>3.8</td>
<td>5.4</td>
</tr>
<tr>
<td>Male (2007)</td>
<td>20.5</td>
<td>8.1</td>
<td>2.5</td>
</tr>
<tr>
<td>Female (1962)</td>
<td>45.5</td>
<td>2.3</td>
<td>19.8</td>
</tr>
</tbody>
</table>
2.4 Discussion on the genetic epidemiology of DD and its application in clinical practice

This chapter quantifies the level of penetrance and suggests that certain familial DD cases, although most likely polygenic, show a near autosomal dominant pattern of inheritance. In addition a more updated measure of familial aggregation is provided which can provide further power to proceed to linkage analysis in DD.

Although DD may appear to be autosomal dominant with variable penetrance, it has been reported that DD is a complex polygenic disease (Dolmans, de Bock et al. 2012). Therefore results calculating the estimated penetrance can be used academically, but not in clinical practice as penetrance levels assume a monogenic condition. The estimated level of penetrance in DD is 18% and the approximate number of familial DD cases in any one cohort is 47%. Again, this figure cannot be used in clinical practice to counsel patients and their relatives as to the likelihood of other family members being involved.

Like many chronic cutaneous or musculoskeletal conditions, DD represents a heterogeneous spectrum of disease phenotypes (Bayat and McGrouther 2006). This chapter has identified variables associated with a risk of DD in relation to positive genetic exposure. The epidemiologic analyses of the risk of DD in relation to a positive family history identified the presence of Garrod’s pads and the number of digits affected as associated factors.

Ascertainment bias is a complication when investigating a late onset disease such as DD. The disease can be difficult to identify by the layman, especially in those with mild palmar disease and many familial studies rely on family members identifying the
presence of DD in elderly relatives (van Dijk, Finigan et al. 2013). The aim was to reduce this bias by educating all individuals enrolled in the study prior to data collection. Ascertainment of families was accurate in that a validated family questionnaire was used. The genetic measure identified from this study is likely to be an underestimate as it has been shown that DD is likely to be under reported by family members (Hindocha, John et al. 2006). Analysis of this cohort confirms the findings of other studies that less than half of all cases of DD show a definite familial history (Ling 1963).

DD is multi-factorial and several risk factors have been associated with the disease including a history of epilepsy (Skoog 1948), diabetes mellitus (Ardic, Soyupek et al. 2003), alcohol (Burge, Hoy et al. 1997), smoking (Burge, Hoy et al. 1997), manual labour (de la Caffiniere, Wagner et al. 1983), frozen shoulder (Smith, Devaraj et al. 2001), carpal tunnel syndrome (Bonnici, Birjandi et al. 1992), hand injury (McFarlane 1991) and rheumatoid arthritis (Guðmundsson, Arngrimsson et al. 1999). Not all cases of DD show a familial pattern and penetrance in familial cases is not complete. Some risk factors appear to be more significant in DD aetiology than its familial nature, and factors such as the presence of a hand injury increase the risk of early onset DD (Lanting, van den Heuvel et al. 2013).

All of this analysis suggests that DD is not a monogenic disease. Therefore unraveling the genetic basis of DD will be a complex but necessary process to aid understanding of disease aetiology and pathogenesis and thereby to identify novel targets for DD treatment. The results from this analysis will provide a foundation for the next chapter, whole genomic scanning and linkage analysis on a large DD pedigree.
Chapter 3

Study 2a

Whole genome scanning and linkage analysis in Dupuytren’s Disease

Study 2b

Long term follow up of Icelandic pedigree, further linkage analysis and sequencing in Dupuytren’s Disease
3.1 Introduction

Once a condition is seen to have familial aggregation and has a form of heritability, the next logical genetic step would be to carry out linkage analysis. In chapter 2 we have seen that DD has familial aggregation and has heritability. Parametric linkage analysis is also based upon familial studies with affected and non-affected individuals. The aim of linkage studies is to identify a genetic locus that may reveal a candidate gene(s), which contributes to genetic susceptibility.

Linkage analysis is performed to investigate loci linked to a disease of interest (Hu, Nystrom et al. 2005). Linkage analysis examines the coinheritance of a trait and an allelic variant at a specific locus. Genes which are situated on the same chromosome, can show linkage. During meiosis a degree of cross over or recombination takes place and the level of linkage is dependent upon this (Hu, Nystrom et al. 2005). Linkage analysis tends to be conducted in large multi-affected families (Hu, Nystrom et al. 2005). Such families can be difficult to identify in late onset conditions such as DD. Further to linkage association, studies can be carried out which show the differences in allele and genotype frequencies between affected individuals that are unrelated and unaffected controls (Michou, Lermusiaux et al. 2012). This kind of study tends to be logistically less demanding as large pedigrees do not need to be sourced.

During linkage analysis, the recombination frequency, i.e. the frequency with which a single chromosomal cross over will take place between 2 genes during meiosis, will give a measure of the distance between markers on a chromosome. The percentage of recombination is measured in the unit centimorgan (cM). One cM is the equivalent of
1% recombination. Meaning, 1 cM is equal to a 1% possibility that one genetic locus will be separated by one genetic marker at a second locus due to crossing over in a single generation. 1 cM is equal to 1 million base pairs. Linkage analysis is a complex process and requires specialist statistical analysis. Further to this the prospect of finding large enough families with a high enough number of affected individuals is difficult. In addition the recombination and gene penetrance need to be known (Ollier and Worthington 1997; Pelttari, Kiiski et al. 2012).

Prior to linkage analysis being carried out data acquisition by genotyping needs to be carried out, i.e. genotypes need to be generated within the family from across the genome. The human genome containing the best part of 3 million base pairs with approximately 30,000 genes (Kidd, Cooper et al. 2008) represents a huge mass array of data to scan. However, once a suitable platform is found to scan the genome, areas can be examined for potential linkage. This method can detect loci linked with the disease candidate gene(s); however, a large enough family needs to be identified to achieve statistical significance as explained in section 3.2.1.

This chapter will describe the process that was undertaken to identify a suitable family to conduct a whole genome scan and linkage analysis.
3.2 Materials and methods

3.2.1 The road to Iceland: The search for a large multi-case DD pedigree

Clinics were contacted in the UK at 10 hospitals in the Northwest of England, the Pulvertaft centre in Derby, and a local GP on the Isle of Skye. Over a two-year period between 2006 and 2008 personal visits were made to these clinics with the aim of finding a suitable DD family to carry out whole genomic scanning. This was defined as a family with at least 15 affected members. In linkage analysis, the aim is to achieve a LOD score >3, therefore >15 informative meioses are required with no evidence of recombination (Knight, Abo et al. 2012).

In DD, the penetrance is low (see chapter 2). Therefore, when choosing a pedigree for linkage analysis it is impossible to ascertain this cut off point of > 15 informative meioses as the non-affected individuals in the pedigree maybe non penetrant. To carry out linkage analysis in DD a pedigree with at least 15 affected members is inevitable.

A sufficiently large multi-case DD family was not found in the UK. Following review of several epidemiological studies carried out by a group in Reykjavik, Iceland, identifying large DD pedigrees (Gudmundsson, Arngrimsson et al. 2000), a personal visit was arranged with the aim of setting up an international collaboration for this MD thesis project.
3.2.2 Identifying a collaborative research group in Iceland

The personal visit to Iceland identified a potential collaborative group consisting of a GP, a geneticist and immunologist. This visit resulted in an initial collaboration agreement. A family would be found with at least 20 affected members. The terms of the collaboration are outlined in appendix 3.1.

3.2.3 Identifying a study cohort. Identification of the largest DD family to date.

Genetic linkage studies require appropriate family pedigrees which will provide enough statistical power to identify areas of genetic interest, harboring potential predisposing genes (Knight, Abo et al. 2012). A DD pedigree that is expanding and the largest reported to date and ideal for further genetic analysis is presented here.

A patient with a strong family history of DD was identified in Iceland. Consent and ethical approval was sought and granted to examine this pedigree.

Each family member underwent a medical assessment; the family pedigree was constructed and clinical photographs taken. Those with DD were examined for DD diathesis and severity, following a rigorously defined diagnostic and severity classification (table 1.4.1) (Hindocha, Stanley et al. 2008).

3.2.4 Whole genome scanning – platform options

Two platforms were available for use (one from Affymetrix, the other from Illumina) and follow-on studies after linkage analysis had to be planned. The sample size for the WGS consisted of 17 affected and 8 unaffected individuals from the 48-member pedigree. All had varying phenotypes of DD as outlined in the results.
The cost of using the Affymetrix was less and thus this option was chosen. The Affymetrix chip was the Affy 1 million SNP chip.

### 3.2.5 DNA collection and deliverance

Venous blood samples were collected from family members in Reykjavik. Two 10ml EDTA samples were collected. These were then transported to the University of Manchester. Samples were couriered on dry ice and delivered within 24 hours.

### 3.2.6 DNA extraction

DNA extraction was carried at the University of Manchester’s clinical genetics laboratory (appendix 3.2)

### 3.2.7 Genotyping and Quality Control (QC)

This step was followed using the Affymetrix (Affymetrix, Santa Clara, CA, USA) protocol supplied (appendix 3.3) at the genomic facility at The University of Manchester and St Mary’s University Hospital. Data obtained from the Affymetrix whole genome scan was computed using Merlin software (Abecasis, Cherny et al. 2002). Further assistance was made available via the Affymetrix technical support line and www.affymetrix.com. Expertise from Affymetrix was used to carry out:

- Quality checks on the whole genomic scan
- Identify rs# in relation to the Affy SNP ID
- Understand the result output from Merlin
Quality Control analysis will inform how well the experiments resolve SNP signals into 3 genotype clusters. In a high quality data set the homozygote distributions will be well resolved from the heterozygote clusters.

The Genotyping Console (GTC) version 4.3.3.1 was downloaded from the Affymetrix website (www.affymetrix.com). Intensity Quality Control (QC) was carried out to determine basic data quality. The QC analysis provides an estimate of the quality based on an approved QC algorithm. For the Genome-Wide Human SNP Array 6.0 platform a total of 3022 SNPs are used for QC. In addition to the QC analysis a gender analysis is also performed. The QC call rate needs to be 86% or greater. The contrast QC threshold is 0.4.

In the event of negative results, i.e. no areas of linkage with a LOD score of >3, the highest LOD score regions will be considered (Knight, Abo et al. 2012). Susceptible genetic loci marker positions and relevant genes were retrieved from Affymetrix using NetAffx™ (Liu, Loraine et al. 2003). The cut off to exclude linkage is a LOD score of -2. In this analysis of 1 pedigree the highest and lowest LOD scores will be interrogated using the Affymetrix NetAffix query tool available on the GTC that lists candidate genes in that region.
3.2.8 Statistical analysis

Initial analysis was carried out with the guidance from the department of clinical genetics at The University of Manchester and St Mary’s University Hospital, Manchester. MERLIN (Abecasis, Cherny et al. 2002) was used to carry out linkage analysis to try and identify candidate genes on susceptibility loci. This would then provide areas of genetic susceptibility.

Parametric linkage analysis using an autosomal dominant model, initially with a penetrance level of 30% was carried out. As the pedigree appeared to follow an autosomal dominant pattern, parametric linkage analysis was carried out first. This did not reveal any meaningful results and thus the penetrance level was reduced to 10%. This analysis took place prior to the calculation of the penetrance level in Chapter 2 (study 1). Repetition of the analysis with an 18% penetrance level (calculated in chapter 2) was not possible due to time and funding constraints.
3.3 Results

3.3.1 Clinical data on the large Icelandic Dupuytren’s pedigree

The family consisted of 48 individuals (figure 3.3.1), spanning five generations. The DD status of the founder members of the family was unknown. Throughout the remaining four generations 25 members suffered with DD of which 21 were male and 4 female. The pedigree appears to show an autosomal dominant pattern of inheritance with complete penetrance. Of the 48 individuals in the family 25 were able to attend for clinical assessment during the time of the visit to Iceland. Of these 25, 17 had developed DD (3 females and 14 males). The clinical assessment of each family member of the pedigree is presented below. Family member 17 did not attend for assessment, giving a total of 25 assessed members.

Four members had Ledderhose’s disease and one presented with Garrod’s pads (table 3.3.1). The severity of DD ranged from palmar disease with no contractures to severe digital contracture causing reduced hand function (appendix 3.4). Five of the 17 who had evidence of DD underwent surgical treatment. DNA was collected from each family member examined, following ethical approval, to allow genetic analysis.
Figure 3.3.1. Pedigree of the Icelandic family

The individual ID is at the top left, the age (years) of the family member at the bottom left. Squares represent males and circles females. Shaded shapes represent affected individuals and a diagonal line means the individual is deceased.
<table>
<thead>
<tr>
<th>Pedigree ID</th>
<th>Age</th>
<th>Gender</th>
<th>DD present</th>
<th>Palmar disease only/contracture</th>
<th>Ledderhose's disease</th>
<th>Photograph (Appendix 3.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dup_20</td>
<td>72</td>
<td>Male</td>
<td>Yes</td>
<td>Palmar</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Dup_8</td>
<td>66</td>
<td>Male</td>
<td>Yes</td>
<td>Palmar</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Dup_4</td>
<td>88</td>
<td>Male</td>
<td>Yes</td>
<td>Contracture</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Dup_14</td>
<td>87</td>
<td>Male</td>
<td>Yes</td>
<td>Contracture</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Dup_18</td>
<td>86</td>
<td>Male</td>
<td>Yes</td>
<td>Contracture</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Dup_9</td>
<td>83</td>
<td>Female</td>
<td>Yes</td>
<td>Palmar</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Dup_25</td>
<td>81</td>
<td>Male</td>
<td>Yes</td>
<td>Contracture</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Dup_19</td>
<td>70</td>
<td>Male</td>
<td>Yes</td>
<td>Palmar</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Dup_6</td>
<td>66</td>
<td>Male</td>
<td>Yes</td>
<td>Contracture</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Dup_1</td>
<td>60</td>
<td>Male</td>
<td>No</td>
<td>-</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>Dup_7</td>
<td>50</td>
<td>Female</td>
<td>No</td>
<td>-</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>Dup_11</td>
<td>39</td>
<td>Female</td>
<td>No</td>
<td>-</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>Dup_24</td>
<td>43</td>
<td>Female</td>
<td>No</td>
<td>-</td>
<td>-</td>
<td>No</td>
</tr>
<tr>
<td>Dup_22</td>
<td>55</td>
<td>Female</td>
<td>Yes</td>
<td>Contracture</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Dup_15</td>
<td>62</td>
<td>Male</td>
<td>Yes</td>
<td>Palmar</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Dup_10</td>
<td>64</td>
<td>Male</td>
<td>No</td>
<td>-</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>Dup_5</td>
<td>59</td>
<td>Female</td>
<td>Yes</td>
<td>Palmar</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Dup_16</td>
<td>51</td>
<td>Female</td>
<td>No</td>
<td>-</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>Dup_3</td>
<td>54</td>
<td>Male</td>
<td>Yes</td>
<td>Contracture</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Dup_2</td>
<td>53</td>
<td>Male</td>
<td>Yes</td>
<td>Contracture</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Dup_26</td>
<td>51</td>
<td>Male</td>
<td>Yes</td>
<td>Contracture</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Dup_23</td>
<td>49</td>
<td>Male</td>
<td>Yes</td>
<td>Contracture</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Dup_13</td>
<td>44</td>
<td>Male</td>
<td>Yes</td>
<td>Palmar</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Dup_12</td>
<td>46</td>
<td>Male</td>
<td>Yes</td>
<td>Palmar</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Dup_21</td>
<td>47</td>
<td>Female</td>
<td>No</td>
<td>-</td>
<td>-</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 3.3.1 Icelandic pedigree clinical demographics
Photographs of each family member were taken with consent (appendix 3.4). Three individuals of the pedigree did not consent to clinical photographs being taken. The age, presence of disease and ectopic disease is summarised in table 4.3.1. Below is a more descriptive account of each family member based upon the clinical photographs taken.

*Dup_1*

This 60-year old gentleman showed no signs of DD. As seen in the pedigree his mother had DD and he has 2 brothers with DD (Dup_6 and Dup_19). Both siblings were older than this individual. DD may yet develop in Dup_1.

*Dup_2*

As shown in the clinical photograph this man had severe bilateral DD with an element of ulnar deviation to the right little finger. Both ring fingers were mainly affected with a contracture at the metacarpal phalangeal joint (MCPJ) of the right ring finger.

*Dup_3*

Garrod’s pads are shown well in this 54-year old male. They can be clearly seen over the proximal interphalangeal joints (PIPJ) of both index fingers. It would be expected that with ectopic lesions such as Garrod’s pads this individual would have severe DD but only mild palmar disease was apparent.

*Dup_4*

Severe DD of the left hand affected all fingers, the severest on the ulnar side of the hand. This person also had palpable disease on the right hand but contractures only affected the MCPJs. It can be seen that the disease was recurrent with evidence of a scar to the left hand.
Dup_5
This lady had very mild, palpable palmar disease with no contracture formation. Although knuckle pads were documented on the pedigree (figure 3.3.1) on further examination it was confirmed that she did not have evidence of Garrod’s pads.

Dup_6
Bilateral palmar disease was noted with contracture at the MCPJ and PIPJ of the left ring finger.

Dup_7
There was no evidence of DD in this individual. However, given that she is one of the younger members of this family she may yet develop the disease.

Dup_8
Palmar pits are shown well in the fourth ray of this man’s left hand. He had mild disease with minimal contracture at the MCPJ.

Dup_9
This lady showed evidence of mild disease to both hands. As seen in the pedigree she had no offspring.

Dup_10
This man showed no evidence of DD.

Dup_11
This lady showed evidence of mild disease to both hands. As shown in the pedigree she was the youngest member of the family to be assessed. At 39 years old she may still yet develop the disease.
**Dup_12**

This 46-year old man also showed no signs of DD. However given his youth and with the mean age at onset in the fifth decade, a follow up assessment may reveal an alternative clinical picture.

**Dup_13**

This 44-year old man, a sibling of Dup_12 and Dup_21 who are unaffected, had mild palmar disease and no evidence of a contracture.

**Dup_14**

This 87-year old man who had an affected daughter (Dup_22) had severe bilateral recurrent disease. Evidence of previous surgery was seen on the right and left hands. Dup_14 did not wish to pursue further surgery and thus contractures had progressed especially on the right little finger and the 3 left ulnar digits.

**Dup_15**

Mild palmar disease was seen in this 62-year old man. As seen in the pedigree he had no offspring.

**Dup_16**

This lady showed no evidence of DD. As seen in the pedigree she had no offspring.

**Dup_18**

This 86-year old man, father of Dup_15,10,5 and 16 had evidence of mild recurrent disease. This individual showed evidence that operative treatment can be a success.

**Dup_19**

This 70-year old man showed evidence of mild palmar disease. Although not apparent on the clinical photograph he had palpable nodules.
Dup_20

This 72-year old man showed evidence of mild palmar disease. He had no offspring but had a sibling (Dup_8) who also had mild disease.

Dup_21

This man showed no evidence of DD. As seen in the pedigree she had no offspring.

Dup_22

This 55-year old lady whose father (Dup_14) showed severe DD had evidence of mild DD with contractures of the PIPJs of both little fingers. One might have expected her to have more severe disease.

Dup_25

This 81-year old man who had had 4 sons, all of whom have developed DD, showed evidence of recurrent disease with a scar visible where previous disease had been excised.

3.3.1.1 Comment on the Icelandic pedigree

On examination of the pedigree it can be seen that Dup_15 and his 4 offspring (Dup_3,2,26 and 23) are all affected by DD. The mother who could not be examined was presumed to be unaffected. This would suggest that, given that all the offspring were affected, she might carry the genotype and not express disease phenotype. However, if examined she may have shown signs of mild, undiagnosed disease which is difficult to ascertain.

The sub-family of Dup_18 who had 4 children (Dup_19, 6, 1 and 7) showed a typical autosomal dominant-like pattern of inheritance.
3.3.2 DNA Quality Control Results

Table 4.3.2 DNA QC Results

<table>
<thead>
<tr>
<th>Family member</th>
<th>Genotyped Gender</th>
<th>Phenotype gender</th>
<th>Contrast QC</th>
<th>QC Call rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dup_2</td>
<td>Male</td>
<td>Male</td>
<td>2.63</td>
<td>97.55</td>
</tr>
<tr>
<td>Dup_4</td>
<td>Male</td>
<td>Male</td>
<td>2.77</td>
<td>98.38</td>
</tr>
<tr>
<td>Dup_5</td>
<td>Female</td>
<td>Female</td>
<td>2.85</td>
<td>97.78</td>
</tr>
<tr>
<td>Dup_6</td>
<td>Male</td>
<td>Male</td>
<td>2.63</td>
<td>98.84</td>
</tr>
<tr>
<td>Dup_7</td>
<td>Female</td>
<td>Female</td>
<td>2.87</td>
<td>98.91</td>
</tr>
<tr>
<td>Dup_8</td>
<td>Male</td>
<td>Male</td>
<td>1.98</td>
<td>98.01</td>
</tr>
<tr>
<td>Dup_9</td>
<td>Female</td>
<td>Female</td>
<td>3.54</td>
<td>99.01</td>
</tr>
<tr>
<td>Dup_10</td>
<td>Male</td>
<td>Male</td>
<td>2.77</td>
<td>98.64</td>
</tr>
<tr>
<td>Dup_11</td>
<td>Female</td>
<td>Female</td>
<td>3.55</td>
<td>99.54</td>
</tr>
<tr>
<td>Dup_12</td>
<td>Male</td>
<td>Male</td>
<td>2.29</td>
<td>98.71</td>
</tr>
<tr>
<td>Dup_13</td>
<td>Male</td>
<td>Male</td>
<td>3.31</td>
<td>98.31</td>
</tr>
<tr>
<td>Dup_14</td>
<td>Male</td>
<td>Male</td>
<td>2.47</td>
<td>97.58</td>
</tr>
<tr>
<td>Dup_15</td>
<td>Male</td>
<td>Male</td>
<td>2.84</td>
<td>96.82</td>
</tr>
<tr>
<td>Dup_16</td>
<td>Female</td>
<td>Female</td>
<td>2.55</td>
<td>97.02</td>
</tr>
<tr>
<td>Dup_18</td>
<td>Male</td>
<td>Male</td>
<td>2.77</td>
<td>97.62</td>
</tr>
<tr>
<td>Dup_19</td>
<td>Male</td>
<td>Male</td>
<td>3.09</td>
<td>98.74</td>
</tr>
<tr>
<td>Dup_20</td>
<td>Male</td>
<td>Male</td>
<td>2.75</td>
<td>98.94</td>
</tr>
<tr>
<td>Dup_21</td>
<td>Female</td>
<td>Female</td>
<td>3.54</td>
<td>98.81</td>
</tr>
<tr>
<td>Dup_22</td>
<td>Female</td>
<td>Female</td>
<td>2.83</td>
<td>95.17</td>
</tr>
<tr>
<td>Dup_24</td>
<td>Female</td>
<td>Female</td>
<td>2.81</td>
<td>98.21</td>
</tr>
<tr>
<td>Dup_25</td>
<td>Male</td>
<td>Male</td>
<td>3.10</td>
<td>98.44</td>
</tr>
<tr>
<td>Dup_26</td>
<td>Male</td>
<td>Male</td>
<td>2.44</td>
<td>98.38</td>
</tr>
</tbody>
</table>
3.3.3 Linkage analysis results

Linkage analysis was carried out using Merlin (Multipoint Engine for Rapid Likelihood Inference) (Abecasis 2002, Michigan). LOD scores were computed for each position on each autosome. Text files were generated with chromosome number, position SNP identification, the pedigree model used to carry out the analysis and the LOD score (figure 4.3.2). Parametric linkage analysis was carried out using a dominant model.

Figure 3.3.2 Linkage analysis data output example
This figure represents an example of the linkage analysis data output. For each autosome a line graph was created to the distribution of significant areas along each chromosome.

<table>
<thead>
<tr>
<th>CHR</th>
<th>POS</th>
<th>LABEL</th>
<th>MODEL</th>
<th>LOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.0008</td>
<td>SNP A-2217754</td>
<td>Dominant_Model</td>
<td>-1.0186</td>
</tr>
<tr>
<td>4</td>
<td>0.0008</td>
<td>SNP A-2155703</td>
<td>Dominant_Model</td>
<td>-1.0214</td>
</tr>
<tr>
<td>4</td>
<td>0.0009</td>
<td>SNP A-8706012</td>
<td>Dominant_Model</td>
<td>-1.0215</td>
</tr>
<tr>
<td>4</td>
<td>0.0009</td>
<td>SNP A-2159136</td>
<td>Dominant_Model</td>
<td>-1.0254</td>
</tr>
<tr>
<td>4</td>
<td>0.0013</td>
<td>SNP A-2182481</td>
<td>Dominant_Model</td>
<td>-1.0529</td>
</tr>
<tr>
<td>4</td>
<td>0.0017</td>
<td>SNP A-8336754</td>
<td>Dominant_Model</td>
<td>-1.0816</td>
</tr>
<tr>
<td>4</td>
<td>0.0017</td>
<td>SNP A-1822303</td>
<td>Dominant_Model</td>
<td>-1.0882</td>
</tr>
<tr>
<td>4</td>
<td>0.0017</td>
<td>SNP A-1940593</td>
<td>Dominant_Model</td>
<td>-1.0893</td>
</tr>
<tr>
<td>4</td>
<td>0.0018</td>
<td>SNP A-8454556</td>
<td>Dominant_Model</td>
<td>-1.0904</td>
</tr>
</tbody>
</table>
3.3.3.1 Linkage analysis results for chromosome 1

The highest LOD score was 1.8 and the lowest -2.4. As seen in figure 4.3.3 the majority of this chromosome represents a negative LOD score.

**Figure 3.3.3 Representative graph for chromosome 1**
The y-axis represents the LOD score and the x-axis the chromosome position in cM.

Table 3.3.3 highlights those areas which are less than -2 and those areas which are the highest for this chromosome.

**Table 3.3.3 Areas on chromosome 1 to exclude and consider**

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position / position range</th>
<th>LOD range</th>
<th>Affy SNP ID or number of SNPs</th>
<th>Area to exclude / consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>67.0-77.0</td>
<td>-2.4 – -2.0</td>
<td>1069 SNPs</td>
<td>Exclude</td>
</tr>
<tr>
<td>1</td>
<td>270.0</td>
<td>1.8</td>
<td>29 SNPs</td>
<td>Consider</td>
</tr>
</tbody>
</table>

Although the LOD score of 1.8 may not be significant, this narrow area of 29 SNPs is considered. NetAffx batch query of the 29 SNPs revealed that all these SNPs coded for the kinesin family member 26B. Kinesins are microtubule-dependent molecular motors which play a part in intracellular transport and cell division (Bandyopadhyay, Chiang et al. 2010).
### 3.3.3.2 Linkage analysis results for chromosome 2

The highest LOD score was 0.8 and the lowest -2.4. It is highly unlikely that there are any areas of linkage on this chromosome. The highest LOD scores on this chromosome will not be considered.

**Figure 3.3.4 Representative graph for chromosome 2**  
The y-axis represents the LOD score and the x-axis the chromosome position in cM.

![Graph showing LOD scores](image)

Table 3.3.4 highlights those areas which are less than -2.

**Table 3.3.4 Areas on chromosome 2 to exclude and consider**

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position / position range</th>
<th>LOD range</th>
<th>Affy SNP ID or number of SNPs</th>
<th>Area to exclude / consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>161.6-255.6</td>
<td>-2.4 - -2.0</td>
<td>3705 SNPs</td>
<td>Exclude</td>
</tr>
</tbody>
</table>
3.3.3.3 Linkage analysis results for chromosome 3

The highest LOD score was 0.8 and the lowest -2.4. It is highly unlikely that there are any areas of linkage on this chromosome. The highest LOD scores on this chromosome will not be considered.

**Figure 3.3.5 Representative graph for chromosome 3**
The y-axis represents the LOD score and the x-axis the chromosome position in cM.

![Graph](image)

Table 3.3.5 highlights those areas which are less than -2.

**Table 3.3.5 Areas on chromosome 3 to exclude and consider**

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position / position range</th>
<th>LOD range</th>
<th>Affy SNP ID or number of SNPs</th>
<th>Area to exclude / consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>96.2-155.7</td>
<td>-2.4 - -2.0</td>
<td>1962 SNPs</td>
<td>Exclude</td>
</tr>
</tbody>
</table>
3.3.3.4 Linkage analysis results for chromosome 4

The highest LOD score was 1.8 and the lowest -2.4. The highest LOD scores on this chromosome will be considered.

**Figure 3.3.6 Representative graph for chromosome 4**
The y-axis represents the LOD score and the x-axis the chromosome position in cM.

Table 3.3.6 highlights those areas, which are less than -2, and those areas that are the greatest for this chromosome.

**Table 3.3.6 Areas on chromosome 4 to exclude and consider**

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position / position range</th>
<th>LOD range</th>
<th>Affy SNP ID or number of SNPs</th>
<th>Area to exclude / consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>97.8-175.2</td>
<td>-2.4 - -2.0</td>
<td>3123 SNPs</td>
<td>Exclude</td>
</tr>
<tr>
<td>4</td>
<td>3.7-4.1</td>
<td>1.8</td>
<td>51 SNPs</td>
<td>Consider</td>
</tr>
</tbody>
</table>

Although the LOD score of 1.8 may not be significant, this area of 51 SNPs is considered. NetAffx batch query of the 47 SNPs were found to be codes for the genes;
Huntington, G protein-coupled receptor kinase 4, Adducin 1(alpha) and SH3-domain binding protein 2.

The Huntington gene is related to Huntington’s disease (Muhlau, Winkelmann et al. 2012) which was not present in this pedigree. The G protein-coupled receptor kinase 4 (GRK4) SNP was identified as SNP_A-8320450 or dbsSNP rs ID rs1801058. This gene has been linked to hypertension (Irvin, Lynch et al. 2010).

The Adducin 1 (alpha) (ADD1) SNP was identified as SNP_A-4237149 or dbsSNP rs ID rs878931. The adducins are a family of proteins which are found in brain and haematopoietic tissue. The ADD1 gene has been linked to memory loss and hypertension (Polonikov, Ushachev et al. 2011).

The SH3-domain binding protein (SH3BP2) was identified as SNP_A-8316733 or dbsSNP rs ID rs1264316. This gene regulates transcriptional activity in natural killer and basophilic cells. A mutation in this gene can cause a rare genetic disorder, cherubism where the lower portion of the face becomes prominent (Teixeira, Horz et al. 2011). This was not identified in this pedigree.
3.3.3.5 Linkage analysis results for chromosome 5

The highest LOD score was 0.8 and the lowest -2.4. It is highly unlikely that there are any areas of linkage on this chromosome.

Figure 3.3.7 Representative graph for chromosome 5
The y-axis represents the LOD score and the x-axis the chromosome position in cM.

Table 3.3.7 highlights those areas which are less than -2.

Table 3.3.7 Areas on chromosome 5 to exclude and consider

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position / position range</th>
<th>LOD range</th>
<th>Affy SNP ID or number of SNPs</th>
<th>Area to exclude / consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>25.7-206.5</td>
<td>-2.4 - -2.0</td>
<td>7788 SNPs</td>
<td>Exclude</td>
</tr>
</tbody>
</table>
3.3.3.6 Linkage analysis results for chromosome 6

The highest LOD score was 0.7 and the lowest -1.9. It is highly unlikely that there are any areas of linkage on this chromosome. The highest LOD scores on this chromosome will not be considered. The lowest LOD score cannot be excluded as it does not reach a significance level of -2.

**Figure 3.3.8 Representative graph for chromosome 6**
The y-axis represents the LOD score and the x-axis the chromosome position in cM.
3.3.3.7 Linkage analysis results for chromosome 7

The highest LOD score was 0.8 and the lowest -2.4. It is highly unlikely that there are any areas of linkage on this chromosome. The highest LOD scores on this chromosome will not be considered.

Figure 3.3.9 Representative graph for chromosome 7
The y-axis represents the LOD score and the x-axis the chromosome position in cM.

Table 3.3.8 highlights those areas which are less than -2.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position / position range</th>
<th>LOD range</th>
<th>Affy SNP ID or number of SNPs</th>
<th>Area to exclude / consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>15.5-173.3</td>
<td>-2.4 - -2.0</td>
<td>10826 SNPs</td>
<td>Exclude</td>
</tr>
</tbody>
</table>
3.3.3.8 Linkage analysis results for chromosome 8

The highest LOD score was 1.8 and the lowest -2.1. It is unlikely that there are any areas of linkage on this chromosome. The highest LOD scores on this chromosome will be considered.

**Figure 3.3.10 Representative graph for chromosome 8**
The y-axis represents the LOD score and the x-axis the chromosome position in cM.

![Graph showing LOD scores and chromosome positions.]

Table 3.3.9 highlights those areas which are less than -2.

**Table 3.3.9 Areas on chromosome 8 to exclude and consider**

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position / position range</th>
<th>LOD range</th>
<th>Affy SNP ID or number of SNPs</th>
<th>Area to exclude / consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>59.1-165.2</td>
<td>-2.1 - -2.0</td>
<td>2180 SNPs</td>
<td>Exclude</td>
</tr>
<tr>
<td>8</td>
<td>7.1-7.4</td>
<td>1.8</td>
<td>72 SNPs</td>
<td>Consider</td>
</tr>
<tr>
<td>8</td>
<td>14.1-14.5</td>
<td>1.8</td>
<td>58 SNPs</td>
<td>Consider</td>
</tr>
<tr>
<td>8</td>
<td>17.8-18.6</td>
<td>1.8</td>
<td>159 SNPs</td>
<td>Consider</td>
</tr>
<tr>
<td>8</td>
<td>23.1-26.2</td>
<td>1.8</td>
<td>664 SNPs</td>
<td>Consider</td>
</tr>
</tbody>
</table>
Between position 7.1 and 7.4 the SNPs all code for the CUB and Sushi multiple domains (CSMD1) and have been isolated to have a link with the pathogenesis of decline of lung function (Imboden, Bouzigon et al. 2012).

The following 58 SNPs between position 14.1 and 14.5 also code for the CSMD1 gene and also the microcephalin 1 gene (MCPH1). The MCPH1 gene encodes a DNA damage response protein. It is suggested the protein plays a role in the inhibitory phosphorylation of cyclin dependent kinase 1. Mutations of this gene have said to be pathognomonic of the autosomal recessive disease microcephaly 1 (Singh, Wiltshire et al. 2012).

The 159 SNPs that follow this region with a LOD score of 1.8 between positions 17.8 and 18.6 coded for the SGK223 or Pragmin gene and the claudin 23 (CLDN23) gene. The pragmin gene is involved in intracellular signal transduction (Vinayagam, Stelzl et al. 2011). CLDN23 codes for an integral membrane protein and components of tight junction strands. The gene is found to be expressed in colon cancer and B cells (Lal-Nag and Morin 2009).

The last group of SNPs investigated between positions 23.1 and 26.2 consisting of 664 SNPs coded highly for the sacroglycan-zeta gene (SNP_A-2248293 dbSNP rs ID rs17117333). The sacroglycan-zeta protein is one of 6 proteins which is involved in bridging between the cytoskeleton and the extra-cellular matrix (Aurino, Piluso et al. 2008). It has been advocated in platelet aggregation, muscular dystrophy and febrile seizures (Baulac, Gourfinkel-An et al. 2008); all perhaps relevant to DD.
3.3.3.9 Linkage analysis results for chromosome 9

The highest LOD score was 0.8 and the lowest -2.4. It is highly unlikely that there are any areas of linkage on this chromosome. The highest LOD scores on this chromosome will not be considered.

Figure 3.3.11 Representative graph for chromosome 9
The y-axis represents the LOD score and the x-axis the chromosome position in cM.

Table 3.3.10 highlights those areas which are less than -2.

Table 3.3.10 Areas on chromosome 9 to exclude and consider

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position / position range</th>
<th>LOD range</th>
<th>Affy SNP ID or number of SNPs</th>
<th>Area to exclude / consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>19.4-126.1</td>
<td>-2.4 - -2.0</td>
<td>4322 SNPs</td>
<td>Exclude</td>
</tr>
</tbody>
</table>
3.3.3.10 Linkage analysis results for chromosome 10

The highest LOD score was 1.8 and the lowest -2.4. It is highly unlikely that there are any areas of linkage on this chromosome. The highest LOD scores on this chromosome will be considered.

**Figure 3.3.12 Representative graph for chromosome**
The y-axis represents the LOD score and the x-axis the chromosome position in cM.

![Graph of LOD scores and chromosome positions](image)

10

Table 3.3.11 highlights those areas which are less than -2 and those areas which are the greatest for this chromosome.

**Table 3.3.11 Areas on chromosome 10 to exclude and consider**

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position / position range</th>
<th>LOD range</th>
<th>Affy SNP ID or number of SNPs</th>
<th>Area to exclude / consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>119.4-138.8</td>
<td>-2.4 - -2.0</td>
<td>298 SNPs</td>
<td>Exclude</td>
</tr>
<tr>
<td>10</td>
<td>136.4-136.5</td>
<td>1.8</td>
<td>29 SNPs</td>
<td>Consider</td>
</tr>
</tbody>
</table>

The 29 SNPs considered on chromosome 10, code for pancreatic lipase-related protein 3 (PNLIPRP3) which is responsible for the manufacture of pancreatic lipase (Davis, Diep et al. 1991). No family member in this pedigree was known to have lipase deficiency.
3.3.3.11 Linkage analysis results for chromosome 11

The highest LOD score was 1.8 and the lowest -2.0. It is unlikely that there are any areas of linkage on this chromosome. The highest LOD scores on this chromosome will be considered.

Figure 3.3.13 Representative graph for chromosome 11
The y-axis represents the LOD score and the x-axis the chromosome position in cM.

Table 3.3.12 highlights those areas which are less than -2.

Table 3.3.12 Areas on chromosome 11 to exclude and consider

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position / position range</th>
<th>LOD range</th>
<th>Affy SNP ID or number of SNPs</th>
<th>Area to exclude / consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>115.4-115.8</td>
<td>-2.0</td>
<td>73 SNPs</td>
<td>Exclude</td>
</tr>
<tr>
<td>11</td>
<td>25.9-43.6</td>
<td>1.8</td>
<td>1700 SNPs</td>
<td>Consider</td>
</tr>
<tr>
<td>11</td>
<td>57.5-58.2</td>
<td>1.8</td>
<td>180 SNPs</td>
<td>Consider</td>
</tr>
<tr>
<td>11</td>
<td>89.2-89.3</td>
<td>1.8</td>
<td>78 SNPs</td>
<td>Consider</td>
</tr>
</tbody>
</table>

Between position 25.9 and 43.6, 1700 SNPs were represented along varying positions of this section of the chromosome. A number of genes were identified and these are summarised in table 3.3.13.
Table 3.3.13 Identified candidate genes with LOD score 1.8 on chromosome 11 between positions 25.9 and 43.6

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Gene</th>
<th>SNP ID &amp; dbSNP rs ID</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>Neuron Navigator 2 (NAV2)</td>
<td>SNP_A-4249891 rs16937251</td>
<td>May play a role in cellular growth and migration (Bandyopadhyay, Chiang et al. 2010).</td>
</tr>
<tr>
<td>11</td>
<td>MAS related GPR X1 (MRGPRX1)</td>
<td>SNP_A-8518230 rs3100768</td>
<td>Unknown</td>
</tr>
<tr>
<td>11</td>
<td>Serum Amyloid A-like 1 (SAAL1)</td>
<td>SNP_A-2008173 rs951624</td>
<td>Over expression linked to synovitis (Sato, Fujii et al. 2011).</td>
</tr>
<tr>
<td>11</td>
<td>Myogenic differentiation 1 (MYOD1)</td>
<td>SNP_A-8335058 rs3901226</td>
<td>Regulates muscle cell differentiation by arresting the cell cycle (Noy, Suad et al. 2012).</td>
</tr>
</tbody>
</table>

As highlighted in table 3.3.13, the genes which have been identified play a role in the apoptotic process which is a key aspect of DD.

The next group of 180 SNPs between positions 57.5 and 58.2 were identified as for the heterogeneous nuclear ribonucleoprotein K pseudogene. This gene has been related to axonal guidance but has no confirmed function (Sokolowski, Wasserman et al. 2010).

The last group of SNPs searched between positions 89.2 and 89.3 was coding for the gene Ankyrin repeat domain 42 (ANKRD42) and does not have a specified function.
3.3.3.12 Linkage analysis results for chromosome 12

The highest LOD score was 0.8 and the lowest -2.4. It is highly unlikely that there are any areas of linkage on this chromosome. The highest LOD scores on this chromosome will not be considered.

**Figure 3.3.14 Representative graph for chromosome 12**

The y-axis represents the LOD score and the x-axis the chromosome position in cM.

Table 3.3.14 highlights those areas which are less than -2.

**Table 3.3.14 Areas on chromosome 12 to exclude and consider**

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position / position range</th>
<th>LOD range</th>
<th>Affy SNP ID or number of SNPs</th>
<th>Area to exclude / consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>39.1-172.1</td>
<td>-2.4 - -2.0</td>
<td>985 SNPs</td>
<td>Exclude</td>
</tr>
</tbody>
</table>
3.3.3.13 Linkage analysis results for chromosome 13

The highest LOD score was 1.8 and the lowest -2.4. It is unlikely that there are any areas of linkage on this chromosome. The highest LOD scores on this chromosome will be considered.

**Figure 3.3.15 Representative graph for chromosome 13**
The y-axis represents the LOD score and the x-axis the chromosome position in cM.

![Graph showing LOD scores for chromosome 13](image)

Table 3.3.15 highlights those areas which are less than -2.

**Table 3.3.15 Areas on chromosome 13 to exclude and consider**

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position / position range</th>
<th>LOD range</th>
<th>Affy SNP ID or number of SNPs</th>
<th>Area to exclude / consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>2.9-12.2 &amp; 120.0-130.0</td>
<td>-2.0 - -2.4</td>
<td>614 SNPs</td>
<td>Exclude</td>
</tr>
<tr>
<td>13</td>
<td>71.9-104.9</td>
<td>1.8</td>
<td>645 SNPs</td>
<td>Consider</td>
</tr>
</tbody>
</table>

Although there are a number of spikes on figure 3.3.15 identifying several areas which may be considered with a LOD score of 1.8, as they are in close proximity to one another they will be considered together. The SNPs coded for glypican 5 (GPC5)
and SLIT and NTRK like (SLITRK) family of genes. The SLITRK genes have been investigated in the pathogenesis of Tourette’s syndrome (Abelson, Kwan et al. 2005) and the glypican gene is hypothesised to play a role in cellular proliferation (Li, Shi et al. 2011).
3.3.3.14 Linkage analysis results for chromosome 14

The highest LOD score was 0.8 and the lowest -2.0. It is highly unlikely that there are any areas of linkage on this chromosome. The highest LOD scores on this chromosome will not be considered.

Figure 3.3.16 Representative graph for chromosome 14
The y-axis represents the LOD score and the x-axis the chromosome position in cM.

Table 3.3.16 highlights those areas which are less than -2.

Table 3.3.16 Areas on chromosome 14 to exclude and consider

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position / position range</th>
<th>LOD range</th>
<th>Affy SNP ID or number of SNPs</th>
<th>Area to exclude / consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>113.5-117.0</td>
<td>-2.0</td>
<td>200 SNPs</td>
<td>Exclude</td>
</tr>
</tbody>
</table>
3.3.3.15 Linkage analysis results for chromosome 15

The highest LOD score was 0.8 and the lowest -2.0. It is highly unlikely that there are any areas of linkage on this chromosome. The highest LOD scores on this chromosome will not be considered.

Figure 3.3.17 Representative graph for chromosome 15
The y-axis represents the LOD score and the x-axis the chromosome position in cM.

Table 3.3.17 Areas on chromosome 15 to exclude and consider

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position / position range</th>
<th>LOD range</th>
<th>Affy SNP ID or number of SNPs</th>
<th>Area to exclude / consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>133.1-133.7</td>
<td>-2.0</td>
<td>45 SNPs</td>
<td>Exclude</td>
</tr>
</tbody>
</table>
3.3.3.16 Linkage analysis results for chromosome 16

The highest LOD score was 1.8 and the lowest -2.0. The highest LOD scores on this chromosome will be considered.

Figure 3.3.18 Representative graph for chromosome 16
The y-axis represents the LOD score and the x-axis the chromosome position in cM.

Table 3.3.18 highlights those areas which are less than -2 and those areas which are the greatest for this chromosome.

Table 3.3.18 Areas on chromosome 16 to exclude and consider

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position / position range</th>
<th>LOD range</th>
<th>Affy SNP ID or number of SNPs</th>
<th>Area to exclude / consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>86.7</td>
<td>-2.0</td>
<td>1 SNPs</td>
<td>Exclude</td>
</tr>
<tr>
<td>16</td>
<td>68.5</td>
<td>1.8</td>
<td>5 SNPs</td>
<td>Consider</td>
</tr>
</tbody>
</table>

The 5 SNPs with the highest LOD score were coding for the colorectal neoplasia gene (CRNDE)(Graham, Pedersen et al. 2011).
3.3.3.17 Linkage analysis results for chromosome 17

The highest LOD score was 1.8 and the lowest -1.8. The highest LOD scores on this chromosome will be considered.

Figure 3.3.19 Representative graph for chromosome 17
The y-axis represents the LOD score and the x-axis the chromosome position in cM.

Table 3.3.19 highlights those areas which are 1.8.

Table 4.3.19 Areas on chromosome 17 to exclude and consider

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position / position range</th>
<th>LOD range</th>
<th>Affy SNP ID or number of SNPs</th>
<th>Area to exclude / consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>7.3</td>
<td>1.8</td>
<td>22 SNPs</td>
<td>Consider</td>
</tr>
</tbody>
</table>

The 22 SNPs code for the Smg-6-homolog (SMG6) gene which is involved in the telomerase ribonucleoprotein complex and thought to be involved in mRNA decay (Kashima, Jonas et al. 2010).
3.3.3.18 Linkage analysis results for chromosome 18

The highest LOD score was 1.8 and the lowest -1.9. The highest LOD scores on this chromosome will be considered. None of the SNPs on this chromosome can be excluded.

Figure 3.3.20 Representative graph for chromosome 18
The y-axis represents the LOD score and the x-axis the chromosome position in cM.

Table 3.3.20 highlights those which are the greatest for this chromosome.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position / position range</th>
<th>LOD range</th>
<th>Affy SNP ID or number of SNPs</th>
<th>Area to exclude / consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>61.7-62.2</td>
<td>1.8</td>
<td>104 SNPs</td>
<td>Consider</td>
</tr>
</tbody>
</table>

The 104 SNPs with the highest LOD scores coded for the RAS-like without CAAX (RIT2) gene which has been shown to have a response to disease modifying drugs for the treatment of rheumatoid arthritis (Wang, A et al. 2012).
3.3.3.19 Linkage analysis results for chromosome 19

The highest LOD score was 0.8 and the lowest -2.4. It is highly unlikely that there are any areas of linkage on this chromosome. The highest LOD scores on this chromosome will not be considered.

Figure 3.3.21 Representative graph for chromosome 19
The y-axis represents the LOD score and the x-axis the chromosome position in cM.

Table 3.3.21 highlights those areas which are less than -2.

Table 3.3.21 Areas on chromosome 19 to exclude and consider

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position / position range</th>
<th>LOD range</th>
<th>Affy SNP ID or number of SNPs</th>
<th>Area to exclude / consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>70.8-101.5</td>
<td>-2.4 - -2.0</td>
<td>1723 SNPs</td>
<td>Exclude</td>
</tr>
</tbody>
</table>
### 3.3.3.20 Linkage analysis results for chromosome 20

The highest LOD score was -0.1 and the lowest -2.4. It is highly unlikely that there are any areas of linkage on this chromosome. The highest LOD scores on this chromosome will not be considered.

**Figure 3.3.22 Representative graph for chromosome 20**

The y-axis represents the LOD score and the x-axis the chromosome position in cM.

![Graph for chromosome 20](image)

**Table 3.3.22 Areas on chromosome 20 to exclude and consider**

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position / position range</th>
<th>LOD range</th>
<th>Affy SNP ID or number of SNPs</th>
<th>Area to exclude / consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>8.2-113.5</td>
<td>-2.4 - -2.0</td>
<td>1002 SNPs</td>
<td>Exclude</td>
</tr>
</tbody>
</table>

As can be seen from the range of a LOD score of at least -2.0 the majority of this chromosome can be excluded.
3.3.21 Linkage analysis results for chromosome 21

The highest LOD score was 0.8 and the lowest -2.0. It is highly unlikely that there are any areas of linkage on this chromosome. The highest LOD scores on this chromosome will not be considered.

Figure 3.3.23 Representative graph for chromosome 21

The y-axis represents the LOD score and the x-axis the chromosome position in cM.

Table 3.3.23 Areas on chromosome 20 to exclude and consider

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position / position range</th>
<th>LOD range</th>
<th>Affy SNP ID or number of SNPs</th>
<th>Area to exclude / consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>3.6-12.8</td>
<td>-2.0</td>
<td>531 SNPs</td>
<td>Exclude</td>
</tr>
</tbody>
</table>
3.3.3.22 Linkage analysis results for chromosome 22

The highest LOD score was 0.6 and the lowest -2.4. It is highly unlikely that there are any areas of linkage on this chromosome. The highest LOD scores on this chromosome will not be considered.

**Figure 3.3.24 Representative graph for chromosome 22**

The y-axis represents the LOD score and the x-axis the chromosome position in cM.

Table 3.3.24 highlights those areas which are less than -2.

**Table 3.3.24 Areas on chromosome 22 to exclude and consider**

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position / position range</th>
<th>LOD range</th>
<th>Affy SNP ID or number of SNPs</th>
<th>Area to exclude / consider</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>2.8-74.6</td>
<td>-2.4 - -2.0</td>
<td>2427 SNPs</td>
<td>Exclude</td>
</tr>
</tbody>
</table>
3.3.4 Summary of important SNPs identified

The results of this genome scan have not revealed any significant areas of linkage with the highest LOD score of 1.8. Areas with LOD scores of 1.8 which were thought to be important for the pathogenesis of DD have been considered as important SNPs (table 3.3.25). If these areas were to be analysed further, they are likely to confirm that DD is a polygenic disease as a LOD score of >3 has not been achieved to support DD as a Mendelian disease.

Table 3.3.25 Important SNPs identified and related chromosome

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Gene</th>
<th>rs ID</th>
<th>Potential function in DD</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>GRK4</td>
<td>1801058</td>
<td>Candidate gene related to increased blood flow and increase in free radicals. Potentially linked to DD pathogenesis as free radicals associated with DD pathology (Rehman, Goodacre et al. 2011).</td>
</tr>
<tr>
<td>4</td>
<td>ADD1</td>
<td>878931</td>
<td>Candidate gene related to increased blood flow and increase in free radicals. Potentially linked to DD pathogenesis as free radicals associated with DD pathology (Rehman, Goodacre et al. 2011).</td>
</tr>
<tr>
<td>4</td>
<td>SH3BP2</td>
<td>8316733</td>
<td>Known gene in musculo-skeletal disease (Tuna, Shimizu et al. 2012).</td>
</tr>
<tr>
<td>8</td>
<td>SGCZ</td>
<td>1711733</td>
<td>Known to interact with the extracellular matrix and cytoskeleton (Mendell, Rodino-Klapac et al. 2012).</td>
</tr>
<tr>
<td>11</td>
<td>NAV2</td>
<td>16937251</td>
<td>Involved in cellular growth, mutation may create aberrant growth pattern (Bandyopadhyay, Chiang et al. 2010).</td>
</tr>
<tr>
<td>11</td>
<td>MRGPRX1</td>
<td>3100768</td>
<td>Unknown function.</td>
</tr>
<tr>
<td>11</td>
<td>SAAL1</td>
<td>951624</td>
<td>Involved in synovitis; if involved in DD may link pathology to an inflammatory nature (Sato, Fujii et al. 2011).</td>
</tr>
<tr>
<td>11</td>
<td>MYOD1</td>
<td>3901226</td>
<td>Known to arrest cell cycle, mutation in DD may stop this function leading to DD (Noy, Suad et al. 2012).</td>
</tr>
</tbody>
</table>
3.4 Discussion

A large DD pedigree from Iceland, in which DD was present in each generation, has been presented, but showed negative linkage analysis results. Further members will be added and current members in the pedigree may still develop DD so that linkage may still become apparent over time. Although the pedigree approximates to an autosomal dominant pattern, it may do so because of the high prevalence of DD in Iceland (Gudmundsson, Arngrimsson et al. 2000). The pedigree is unlikely to be Mendelian (as there were no LOD scores >3), and the cases within the pedigree may be sporadic, but possess several of the candidate genes that may predispose these individuals to DD. This may explain the phenotype variability in this Icelandic family.

The gender index is skewed in this pedigree, as is the general rule for DD at the population level (Lanting, van den Heuvel et al. 2013), the family having male preponderance. As seen from the linkage analysis results 8 susceptible genes (those with the highest LOD scores) have been highlighted over 3 chromosomes, although no significant LOD scores have been identified. Previous studies have suggested DD is not a monogenic, but more likely to be a polygenic disease with the influence of environmental factors (Hindocha, John et al. 2006). Others studies have suggested that the dominant model is less likely and that the “Dupuytren’s gene” is actually autosomal recessive (Burch 1966).

Dated studies i.e. pre 1990 reported that the pattern of DD inheritance was autosomal dominant with variable penetrance (Demers and Blais 1960; James 1985). There has been a long standing interest in the role of genetic influence in DD, including studies identifying DD in identical twins (Couch 1939; Lyall 1993; Beleta and Fores 2012),
collections of multiple multi-generation families presenting with DD (Skoog 1948; Ling 1963) and families identified for genetic mapping using modern molecular technology (Dolmans, Werker et al. 2011).

This current pedigree has also shown that DD severity can vary widely within one family. A strong genetic link may increase the susceptibility to develop DD but not necessarily predict the severity (Dolmans, de Bock et al. 2012). In this family half of the affected individuals had bilateral disease, few had Ledderhose’s disease and one presented with Garrod’s pads reflecting the variability in the expression of the underlying molecular pathology.

The penetrance is influenced by age with DD being a late onset condition and full penetrance may be observed if every family member is followed up over a lifetime. Further studies are required to evaluate the relation between family history of DD and severity of the disease, age-of-onset and influence on expression.

The linkage analysis carried out on this pedigree has shown a few regions that may contain candidate DD genes. It is more likely however, that these susceptible loci are present in this pedigree by chance. A previous study on a large Swedish pedigree identified a susceptible region on chromosome 16q at position 67.2cM (Hu, Nystrom et al. 2005). The HLA system has been suggested as being involved in the pathology of DD (Brown, Ollier et al. 2008; Jonsson, Gudmundsson et al. 2012; Jonsson, Gudmundsson et al. 2013); linkage on chromosome 6 in this pedigree did not reveal any susceptible areas.
It is clear from this initial linkage study in DD that further linkage or genome wide association studies would be required in the Icelandic patients to clarify the genetic component of disease. A large genome wide association study on Dutch DD patients revealed 11SNPs of potential importance linking the WNT gene-signaling pathway to DD (Dolmans, Werker et al. 2011). The present study did reveal an association of one SNP identified in the WNT study and will be discussed in chapter 5. The WNT gene family is associated with extracellular signaling molecules and is associated with many cancer related diseases (Dolmans, Werker et al. 2011). It is possible that the involvement of WNT is associated with an imbalance of apoptosis and cellular proliferation. It is possible that DD has a different genetic basis in different geographical areas and communities and therefore a wider association across several gene pools is necessary.

This study is a preliminary one and warrants repetition in additional, large Icelandic DD families. This can then confirm or refute candidate susceptibility genes in the current and in other DD studies.
Study 2b. Long term follow up of Icelandic pedigree, further linkage analysis and sequencing in Dupuytren’s Disease

The Icelandic pedigree identified is currently under long-term follow up. Over a 10-year period more family members will be added. DD may develop in individuals who currently do not show signs of DD. The study has commenced in collaboration with Professor Lee, Associate Professor of Clinical Epidemiology, Sergeivsky Centre, New York. Professor Lee is currently validating the Affymetrix chip results and conducting further more detailed bioinformatics to clarify the linkage analysis results.

Once this is complete a manuscript will be submitted for peer review followed by a strategy to either; i) conduct a case control association study or ii) whole exome sequencing in certain members of this pedigree.

A personal active role will be taken in this analysis and publication (study 2b). The work is ongoing and it is envisaged that it will continue for a number of years.
Chapter 4

Study 3

Abnormal stem cell markers found in Dupuytren’s disease.
4.1 Introduction

The cellular origin of DD is unknown. Identification of relevant biomarkers can aid in understanding the pathology and potentially link with genetic studies. Identification of stem cell markers and their use as biomarkers in DD has not been widely discussed in the literature.

The investigation of potential stem cells in DD may provide clues to cellular origin. In chapter 1, the definition of a stem cell was given. In relation to DD, any cell type around the diseased fascia could potentially be a cell contributing to the DD process. I.e. stem cells in DD could arise from a number of different tissue types within the palm; the palmar fascia, the fat surrounding this or the skin overlying the disease.

The palmar fat has been implicated in the pathogenesis of DD (Rabinowitz, Ostermann et al. 1983). Previous studies have investigated palmar fat cells (Shih, Wijeratne et al. 2008; Shih, Wijeratne et al. 2009) although the exact role of adipocytes and fat stromal cells in relation to DD pathology remains unclear.

Myofibroblasts which have combined properties of smooth muscle cells and fibroblasts are involved in DD development (Eyden 2001). The cellular mechanics and biochemical properties of the DD myofibroblast have been extensively studied previously (Badalamente and Hurst 1999; Tomasek, Vaughan et al. 1999; Rayan 2007). Importantly, the proliferation of DD myofibroblasts is believed to cause the nodular like lesions in the palm along with puckering of the skin resulting in palmar pits and eventual contracture of the affected digit. The precise cell of origin of the
myofibroblasts that contribute to DD pathology is not known despite extensive studies (Badalamente and Hurst 1999) (Ling and Edinburgh 1963; Burge 1999).

Mesenchymal stem cell’s (MSC’s) and haematopoietic stem cells (HSC) are potential sources of DD myofibroblasts. MSC’s are multipotent cells found in the bone marrow, skin and adipose tissue (Delorme B 2007). Therefore, the identification and profiling of stem cells in DD tissue may help unravel disease origin and provide further clues as to the disease pathology. In this chapter it is hypothesised that DD is not only a disease of the palmar fascia but that the palmar skin and fat may have a role in the disease pathology. Therefore this study aims to identify and profile stem cells in DD nodule, cord, peri-nodular fat, distant palmar fat and skin and to compare it to normal control tissue.
4.2 Methods

4.2.1 Patient selection

Patients diagnosed with DD (n=9, 5 male, 4 female) and those diagnosed with carpal tunnel syndrome (CTS) (n=4, all female, age range = 45-52 years) were enrolled in the study. Individuals with CTS were screened for signs, a family history of DD, and associated risk factors. If the screen was negative they were enrolled into the study as control subjects. All DD patients were Caucasians of Northern European extraction (age range = 42-68 years), with digital contracture. Full ethical approval and consent were obtained to conduct the study.

4.2.2 Tissue extraction

All patients with DD underwent a standard fasciectomy procedure with no adverse peri-operative complications. Skin overlying the diseased region was excised but limited to extent to allow direct closure of the operative wound. DD tissue was dissected en masse, followed by careful macroscopic dissection of the cord, nodule, skin, and perinodular fat. A biopsy of the distant palmar fat was also taken at least one digital ray away from the diseased cord (figure 4.2.1).

Control subjects enrolled in the study undergoing surgery for CTS had biopsies of palmar fat, palmar fascia and skin. The tissue was divided and immersed into 3 media types; i) Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen, UK) for fluorescent activated cell sorting (FACS), ii) formalin (Sigma, UK) for immunohistochemistry or iii) RNA later (Ambion, UK) for QRT-PCR (figure 4.2.1).
Figure 4.2.1 Method of DD tissue collection

Tissue was dissected at the time of operation and the nodule and cord were separated. The peri-nodular fat was identified and a further biopsy of fat from the palm away from the disease process was taken. Carpal tunnel patients underwent biopsies of the palmar fascia, palmar fat and skin. All tissue was placed into 3 different types of media; formalin for immunohistochemical analysis, RNA later for QRT-PCR and media for FACS analysis.

4.2.3 Selection of stem cell markers to be investigated

In the absence of published studies investigating the role of stem cells in DD, we selected specific stem cell markers based upon previous studies that had investigated the role of stem cells in similar fibrotic disorders such as keloid disease (Akino, Akita et al. 2008; Moon, Kwak et al. 2008). Five MSC and 2 HSC markers were selected for the purpose of this study (table 4.2.1).
Table 4.2.1. Description of markers used to investigate stem cells in DD

<table>
<thead>
<tr>
<th>Marker</th>
<th>Description</th>
<th>Method of detection</th>
<th>Relevance to DD</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD13</td>
<td>MSC marker. Adipocyte marker. Also described as a myeloid antigen commonly found to be expressed in fibroblasts and endothelial cells.</td>
<td>FACS, qPCR, immunostaining</td>
<td>Known to be expressed in skin derived stem cells.</td>
<td>(Hunyadi J 1993; Delorme B 2007) (Hunyadi J 1993)</td>
</tr>
<tr>
<td>CD29</td>
<td>MSC marker. Adipocyte marker. Also known as integrin beta-1. Known to be involved in cell adhesion and recognition.</td>
<td>FACS, qPCR, immunostaining</td>
<td>Involved in the adhesion of extra-cellular matrix.</td>
<td>(Weitzman JB 1995; Delorme B 2007)</td>
</tr>
<tr>
<td>CD34</td>
<td>HSC and vascular endothelial tissue marker. Important adhesion molecule.</td>
<td>FACS, qPCR, immunostaining</td>
<td>Clinical use as a vascular and cutaneous neoplasm marker.</td>
<td>(Fina L 1990; Traweek, Kandalaft et al. 1991; Aiba, Tabata et al. 1992; Abenoza P 1993; Drew E 2005; Delorme B 2007)</td>
</tr>
<tr>
<td>CD44</td>
<td>MSC marker. Important for cell adhesion and cell-cell interaction. Certain isoforms associated with malignancy.</td>
<td>FACS, qPCR, immunostaining</td>
<td>Important marker for cellular matrix adhesion which encourages cell migration in the extra cellular matrix.</td>
<td>(Lesley, Hyman et al. 1993; Naor, Sionov et al. 1997; Zhu H 2006)</td>
</tr>
<tr>
<td>CD90</td>
<td>MSC marker. Also known as Thy-1. Shown lack of expression in vascular malignancies and soft tissue fibroses, marker for necrosis.</td>
<td>FACS, qPCR, immunostaining</td>
<td>Regulator of cell-cell interactions in apoptosis.</td>
<td>(Rege and Hagoood 2006; Majeti R 2007)</td>
</tr>
<tr>
<td>CD117</td>
<td>HSC marker. Also known as c-kit. Known oncogene for fibrous tumours.</td>
<td>FACS, qPCR, immunostaining</td>
<td>Used as a tumour marker in breast cancer.</td>
<td>(Sarlomo-Rikala, Kovatich et al. 1998; Delorme B 2007; Lorenz K 2008)</td>
</tr>
<tr>
<td>CD166</td>
<td>MSC marker. Involved in angiogenesis and tumour metastasis.</td>
<td>FACS, qPCR, immunostaining</td>
<td>Has been found on adipose derived fibroblasts.</td>
<td>(Delorme B 2007; Lorenz K 2008; Joyce and Pollard 2009)</td>
</tr>
</tbody>
</table>
4.2.4 Immunohistochemistry (IHC)

DD tissue biopsies were immersed in 10% buffered formalin and fixed for 24 hours. Samples in formalin were paraffin wax embedded into blocks which were sectioned to provide 5µm and placed on Superfrost plus slides (Thermo Scientific, UK). Slides were first de-waxed in xylene (Fisher Scientific, UK) for thirty minutes and then gradually re-hydrated in 50%, 70%, 85%, 95% and 100% ethanol. Slides were then washed in de-ionised water, tissue was identified on each slide and encircled with a Dako pen.

Tissue sections were then incubated in a pepsin based solution made up of; 20mg pepsin, 5ml de-ionised water and 100µl hydrochloric acid. Tissue incubation time was 45 minutes at 37°C. Sections were washed in Dulbeco’s phosphate buffered saline (PBS) (Sigma Aldrich, UK), incubated in 1% blocking serum antigen for 20 minutes and washed again with PBS. Slides were then incubated in primary antibody; either CD13, CD29, CD34, CD44, CD90, CD117 or CD166 (BD bioscience, UK). These sections were left overnight at 4°C for adequate antibody exposure. Slides were washed again with PBS and secondary fluorescent antibody anti-mouse FITC or anti-rabbit (Jackson immunodiagnostics, USA) applied to each slide for a total of 45 minutes. Following a further wash with PBS, tissue sections were soaked in 4’6-diamidino-2-phenylindole (DAPI) (Invitrogen, UK) to accurately stain DNA in each cell.

Slides were examined by fluorescent microscopy at a magnification of 40x. Phase contrast images to identify the structure of the tissue were taken in addition to images of antibody and DAPI staining.
4.2.5 Qualitative real-time polymerase chain reaction (QRT-PCR)

Each biopsy sample placed in RNAlater (Ambion, UK) was stored over night at 4°C. All RNA experimental work was carried out in a laboratory used exclusively for RNA work and within a sterile tissue cabinet. Tissue biopsy samples were minced and approximately 10mm³ of tissue was immersed in 1mL of Trizol (Invitrogen, UK) in a 2mL Eppendorf tube containing an alcohol flamed sterilized steel ball bearing. The tissue was homogenised at 35 oscillations per second for a total of 15 minutes. The resulting suspension was transferred to a second Eppendorf tube and centrifuged at 13,604g for 10 minutes. The supernatant was carefully transferred to a further Eppendorf tube and mixed with 0.2mL of chlorophorm and left to stand for 2 minutes at room temperature. This mixture was centrifuged at 13,604g for 15 minutes. The upper most part of the supernatant (RNA) was carefully transferred to a third Eppendorf tube containing an approximate equal volume of 70% ethanol. This was processed using the RNEasy mini kit (Qiagen, UK) according to the manufacturer’s instructions followed by DNA treatment with DNAsse using the DNA free kit (Ambion, UK) as per the manufacturer’s protocol. RNA was estimated using the Nano drop 1000 UV spectrophotometer (Labtech International, UK). RNA was stored at -80°C until used for cDNA synthesis.

Complementary DNA (cDNA) was synthesised using SuperScript II Reverse Transcriptase kit (Invitrogen, UK). A total reaction volume of 20µl was made and included cDNA supermix (containing primer, deoxynucleotide triphostates (dNTPs), magnesium chloride and buffer), RNA and nuclease-free water (Ambion, UK). Total RNA was normalised to 1 mg in each reaction. Finally Reverse Transcriptase was
added and incubated for 5 minutes at 25°C, then at 42°C for 60 minutes and inactivated at 70°C for 5 minutes.

Real time quantitative polymerase chain reaction (RT-qPCR) was carried out using the StepOne platform (Applied Biosystems, USA). Each RT-qPCR reaction was done with a total volume of 10µl (4µl cDNA, 0.2µl forward primer, 0.2µl reverse primer, 0.1µl probe obtained from the Universal Probe Library (Roche Diagnostics, Germany), 5µl of probe Mastermix and 0.5µl of nuclease free water. Forward and reverse primers for each probe were tabulated (table 5.2.2). Each reaction was done in triplicate and duplicated in 96 well plates. Human Rpl2 was used as a housekeeping gene to demonstrate equal loading of RNA and to normalise gene expression.

Table 4.2.2: Genes used for QRT-PCR and respective forward and reverse primer sequences. At the time of study, suitable primer sequences were not available for CD117

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD13</td>
<td>5’ CATCCATCAGAGATGGCAGAC 3’</td>
<td>3’ TGCTGAAGAGATCGTTCTGG 5’</td>
</tr>
<tr>
<td>CD29</td>
<td>5’ CAGTTACTGAAGAATTTTCAGCCTGT 3’</td>
<td>3’ GCAGATAATGCCCTACTGCTGAC 5’</td>
</tr>
<tr>
<td>CD34</td>
<td>5’ ACTCGGTGCTCCTCTCTAGG 3’</td>
<td>3’ GTCAAGCTCATGAACCAGAA 5’</td>
</tr>
<tr>
<td>CD44</td>
<td>5’ TCAACAGACCCCCTCTAGAAAT 3’</td>
<td>3’ CAGATAAATAGCTACCCCTTGT 5’</td>
</tr>
<tr>
<td>CD90</td>
<td>5’ AGGACGAGGGCACCTACAC 3’</td>
<td>3’ GCCCTCATACCTGACCAGTT 5’</td>
</tr>
<tr>
<td>CD166</td>
<td>5’ AATCGTTAGGGAATGGCAAC 3’</td>
<td>3’ TTATTCCTCAGGGCTGCCT 5’</td>
</tr>
</tbody>
</table>

4.2.6 Fluorescence activated cell sorting (FACS)

Cells were extracted from tissue after Dispase II (Roche Diagnostics, UK) and Collagenase A treatment (Roche Diagnostics, UK). 2x10⁵ cells were placed in FACS tubes after being rinsed twice with 5%FBS/HBSS, and were incubated with primary antibody (1:50 dilution) conjugated to fluorophores for 30min on ice. Cells were washed once with 5%FBS/HBSS and resuspended in the same buffer. Propidium iodide (PI) was added to the determine cell viability. To assess non-specific binding,
isotype-matched primary antibodies conjugated to respective fluorophores were used as controls, as summarised in tables’ 4.2.3 and 4.2.4. Cell viability in all multiple labelled experiments was determined through Sytox blue (Invitrogen, UK) exclusion. Analysis was done on BD Bioscience FACS Aria equipped with 3 lasers. The data was analyzed using FACSDiva 5.1 software (BD Bioscience, UK).

**Table 4.2.3: Primary antibodies used for FACS analysis**

<table>
<thead>
<tr>
<th>Primary Abs</th>
<th>Fluorophore</th>
<th>Clone</th>
<th>Dilution</th>
<th>Source</th>
<th>Cat no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ms anti Hu CD13</td>
<td>FITC</td>
<td>WM-15</td>
<td>1:50</td>
<td>eBiosciences</td>
<td>11-0138-73</td>
</tr>
<tr>
<td>Rat anti Hu CD29</td>
<td>PE</td>
<td>TS2/16</td>
<td>1:50</td>
<td>eBiosciences</td>
<td>12-0299-73</td>
</tr>
<tr>
<td>Ms anti Hu CD34</td>
<td>APC</td>
<td>-</td>
<td>1:50</td>
<td>BD Pharmingen</td>
<td>555824</td>
</tr>
<tr>
<td>Rat anti Hu CD44</td>
<td>APC Alexa Fluor 750</td>
<td>IM7</td>
<td>1:50</td>
<td>eBiosciences</td>
<td>27-0441-82</td>
</tr>
<tr>
<td>Ms anti Hu CD90</td>
<td>PerCP-Cy5.5</td>
<td>eBio5E10</td>
<td>1:50</td>
<td>eBiosciences</td>
<td>45-0909-73</td>
</tr>
<tr>
<td>Ms anti Hu CD117</td>
<td>PE-Cy5.5</td>
<td>-</td>
<td>1:50</td>
<td>Invitrogen</td>
<td>CD11718</td>
</tr>
<tr>
<td>Ms anti Hu Cd166</td>
<td>PE</td>
<td>-</td>
<td>1:50</td>
<td>BD Pharmingen</td>
<td>559263</td>
</tr>
</tbody>
</table>

**Table 4.2.4: Isotype control antibodies used for FACS analysis**

<table>
<thead>
<tr>
<th>Isotype Abs</th>
<th>Fluorophore</th>
<th>Clone</th>
<th>Dilution</th>
<th>Source</th>
<th>Cat no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ms IgG1</td>
<td>FITC</td>
<td>-</td>
<td>1:50</td>
<td>Immunostep</td>
<td>ICIGIF-100T</td>
</tr>
<tr>
<td>Ms IgG1</td>
<td>PE</td>
<td>-</td>
<td>1:50</td>
<td>BD Pharmingen</td>
<td>555787</td>
</tr>
<tr>
<td>Ms IgG2a</td>
<td>APC</td>
<td>-</td>
<td>1:50</td>
<td>Immunostep</td>
<td>ICIGG2AA-100T</td>
</tr>
<tr>
<td>Ms IgG1</td>
<td>PE-Cy5.5</td>
<td>P3</td>
<td>1:50</td>
<td>eBiosciences</td>
<td>35-4714-73</td>
</tr>
<tr>
<td>Ms IgG1</td>
<td>PerCP-Cy5.5</td>
<td>P3</td>
<td>1:50</td>
<td>eBiosciences</td>
<td>45-4714-82</td>
</tr>
<tr>
<td>Rat IgG2</td>
<td>APC Alexa Fluor 750</td>
<td>eB149/10H5</td>
<td>1:50</td>
<td>eBiosciences</td>
<td>27-4031-81</td>
</tr>
</tbody>
</table>

**4.2.7 Statistical analysis**

Data obtained from the three laboratory techniques; immunohistochemistry, FACS and QRT-PCR were transcribed onto a data sheet. Student’s t-test was used to assess the significance of the data obtained. Statistical analyses were calculated using the SPSS software package (SPSS Inc., Chicago, IL).
4.3 Results

4.3.1 DD tissue is observed to have markers consistent with mesenchymal and haematopoietic stem cell populations

Results from FACS analysis

The findings obtained from the FACS analysis are presented in figures 4.3.1 to 4.3.5. The two markers to be least prevalent across all anatomical sites were CD117 and CD166. As seen in figures 4.3.1-4.3.5 the frequency of observed markers in the perinodular and distant palmar fat appeared to be similar for CD13, 117 and 166. There appeared to be a higher prevalence of CD29, 34, 44, and 90 positive cells in the peri-nodular fat compared to distant palmar fat.

The most prevalent markers in DD skin were CD29 and CD34. The high number of CD34 positive cells is consistent with the QRT-PCR findings (see below). Levels of markers in the cord and nodule appeared similar with the most prevalent markers being CD29 and CD44. Results from this observation are for single markers and not a combination of markers and thus conclusions from this set of data have to be viewed with caution. A further reason for drawing conclusions with caution from this data set is the absence of control measures from carpal tunnel biopsies as there were not enough live cells to analyse via FACS.

Results from immuno-histochemistry

The high expression of CD29 in the perinodular fat in comparison to carpal tunnel control was observed with immuno-staining. There were fewer positive cells for CD117 and 166 in DD tissue across all anatomical sites. CD90 was observed to show
a lower number of positive cells in comparison to CD13, 29, 34 and 44 positive cells across all anatomical sites (tables 4.3.1, figures 4.3.1-4.3.5). It must be noted that results from immuno-staining are qualitative rather than quantitative.
Figure 4.3.1 FACS and IHC data for DD Cord

Expression of all seven stem cell markers investigated using FACS and IHC in DD cord. Each graph shows the number of cells counted and those that were found to be positive for each marker. The y-axis demonstrates the cell count and the x-axis the fluorophore marker. The top left graph shows the percentage of live cells selected as sytox blue; in this case 55%. The subsequent graphs give the percentage of live cells positive for that particular marker (e.g. 8% live DD cord cells positive for CD13). The immunohistochemistry pictures identify individual cells (DNA marked with DAPI coloured blue) and those cells positive highlighted green or red (for CD117) fluorescence. Arrows indicate cells that were deemed positive for the selected marker.
Figure 4.3.2 FACS and IHC data for DD Nodule

Expression of all seven stem cell markers investigated using FACS and IHC in DD nodule. Each graph shows number of cells counted and those that were found to be positive for each marker. The y-axis demonstrates the cell count and the x-axis the fluorophore marker. The top left graph shows the percentage of live cells selected as sytox blue; in this case 55%. The subsequent graphs give the percentage of live cells positive for that particular marker (e.g., 13% live DD nodule cells positive for CD13). The immunohistochemistry pictures identify individual cells (DNA marked with DAPI coloured blue) and those cells positive with highlighted green or red (for CD117) fluorescence. Arrows indicate cells that were deemed positive for the selected marker.
Figure 4.3.3 FACS and IHC data for DD peri-nodular fat

Expression of all seven stem cell markers investigated using FACS and IHC in DD peri-nodular fat. Each graph shows number of cells counted and those that were found to be positive for each marker. The y-axis demonstrates the cell count and the x-axis the fluorophore marker. The top left graph shows the percentage of live cells selected as sytox blue; in this case 64%. The subsequent graphs give the percentage of live cells positive for that particular marker (e.g. 33% live peri-nodular fat cells positive for CD13). The immunohistochemistry pictures identify individual cells (DNA marked with DAPI coloured blue) and those cells positive with highlighted green or red (for CD117) fluorescence. Arrows indicate cells that were deemed positive for the selected marker.
Figure 4.3.4 FACS and IHC data for DD distant palmar fat

Expression of all seven stem cell markers investigated using FACS and IHC in DD distant palmar fat. Each graph shows number of cells counted and those that were found to be positive for each marker. The y-axis demonstrates the cell count and the x-axis the fluorophore marker. The top left graph shows the percentage of live cells selected as sytox blue; in this case 75%. The subsequent graphs give the percentage of live cells positive for that particular marker (e.g. 28% live DD distant palmar fat cells positive for CD13). The immunohistochemistry pictures identify individual cells (DNA marked with DAPI coloured blue) and those cells positive with highlighted green or red (for CD117) fluorescence. Arrows indicate cells that were deemed positive for the selected marker.
Figure 4.3.5 FACS and IHC data for DD skin
Expression of all seven stem cell markers investigated using FACS and IHC in DD skin. Each graph shows number of cells counted and those that were found to be positive for each marker. The y-axis demonstrates the cell count and the x-axis the fluorophore marker. The top left graph shows the percentage of live cells selected as sytox blue; in this case 50%. The subsequent graphs give the percentage of live cells positive for that particular marker (e.g., 82% live DD skin cells positive for CD13). There were not enough live cells to carry out FACS analysis for CD117 and CD166. The immunohistochemistry pictures identify individual cells (DNA marked with DAPI coloured blue) and those cells positive with highlighted green or red (for CD117) fluorescence. Arrows indicate cells that were deemed positive for the selected marker.
Table 4.3.1: Illustration of immunohistochemical data identifying the mean percentage (and standard deviations) of positive cells in each tissue type for each individual marker. Percentages are means of 3 field microscopic sections taken at 40x magnification

<table>
<thead>
<tr>
<th>Marker</th>
<th>CD13</th>
<th>CD29</th>
<th>CD34</th>
<th>CD44</th>
<th>CD90</th>
<th>CD117</th>
<th>CD166</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodule</td>
<td>13.6±2.3</td>
<td>9.4±2</td>
<td>18.3±2</td>
<td>50.1±6</td>
<td>3.1±0.5</td>
<td>3.3±0.5</td>
<td>17.8±2</td>
</tr>
<tr>
<td>Cord</td>
<td>31.2±3.5</td>
<td>1.6±0.3</td>
<td>21.9±2.3</td>
<td>26.7±2.1</td>
<td>11.8±1</td>
<td>18.5±3</td>
<td>6.3±2.5</td>
</tr>
<tr>
<td>PN Fat</td>
<td>42.0±1.8</td>
<td>47.2±5</td>
<td>27.5±3.1</td>
<td>34.8±3.2</td>
<td>42.9±5</td>
<td>6.7±0.5</td>
<td>36.7±4.5</td>
</tr>
<tr>
<td>DP Fat</td>
<td>33.6±2.6</td>
<td>27.1±3.5</td>
<td>15.8±2</td>
<td>39.5±3</td>
<td>28.6±2.3</td>
<td>12.0±2</td>
<td>20.0±1</td>
</tr>
<tr>
<td>DD Dermis</td>
<td>11.2±3.6</td>
<td>37.8±3.5</td>
<td>30.0±3</td>
<td>20.2±4</td>
<td>0.0±0</td>
<td>12.0±2</td>
<td>0.0±0</td>
</tr>
<tr>
<td>CT Fascia</td>
<td>0.0±0</td>
<td>3.9±0.5</td>
<td>3.4±0.4</td>
<td>5.8±1.5</td>
<td>7.7±1.5</td>
<td>6.7±1.2</td>
<td>0.0±0</td>
</tr>
<tr>
<td>CT Fat</td>
<td>12.5±1.1</td>
<td>24.6±1.2</td>
<td>31.5±2.3</td>
<td>13.3±2</td>
<td>39.5±2.5</td>
<td>10.3±0.7</td>
<td>8.3±0.5</td>
</tr>
<tr>
<td>CT Dermis</td>
<td>8.3±2.3</td>
<td>8.3±0.7</td>
<td>21.5±4</td>
<td>15.0±2</td>
<td>28.6±3.5</td>
<td>30.0±2</td>
<td>4.2±1</td>
</tr>
</tbody>
</table>
Results from QRT-PCR

Gene expressions of the stem cell markers investigated via FACS were measured except for CD117 (figure 4.3.6). The results from gene expression do not match those from frequency of positive cells seen in FACS analysis. The highest gene expression of CD13 was seen in carpal tunnel fascia. Similarly there is a high expression of CD34 in carpal tunnel fat. The expression of CD44 was greater in the cord and nodule in comparison to control tissue ($p<0.05$). The expression of CD90 was significantly less in the cord and nodule in comparison to control tissue ($p<0.05$). Expression of CD34 was significantly greater in DD compared to control tissue ($p=0.0008$) (table 4.3.2).
Figure 4.3.6 Relative gene expression of each of the 6 markers investigated in the diseased and control tissue

Bar graph showing relative expression (RQ; y-axis) for each tissue modality (x-axis) for 6 of the stem cell markers. There were no primers available for CD117. DD nodule and cord was compared with CT (carpal tunnel) fascia. DD distant palmar fat (DPF) and peri-nodular fat (PNF) was compared with CT fat and DD skin with CT skin.
Table 4.3.2: Probabilities of differences of mean relative gene expression for each marker investigated in comparison to control carpal tunnel tissue. CD44 was significantly over-expressed in the cord and nodule (↑), while CD90 was significantly under-expressed (↓). CD34 was significantly over expressed in DD skin (↑↑).

<table>
<thead>
<tr>
<th></th>
<th>CD13 p value</th>
<th>CD29 p value</th>
<th>CD34 p value</th>
<th>CD44 p value</th>
<th>CD90 p value</th>
<th>CD166 p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodule v CT fascia</td>
<td>0.25</td>
<td>0.4</td>
<td>0.1</td>
<td><strong>0.03 ↑</strong></td>
<td><strong>0.02 ↓</strong></td>
<td>0.24</td>
</tr>
<tr>
<td>Cord v CT fascia</td>
<td>0.38</td>
<td>0.83</td>
<td>0.12</td>
<td><strong>0.02 ↑</strong></td>
<td><strong>0.02 ↓</strong></td>
<td>0.7</td>
</tr>
<tr>
<td>PNF v CT fat</td>
<td>0.34</td>
<td>0.77</td>
<td>0.1</td>
<td>0.29</td>
<td>0.43</td>
<td>0.74</td>
</tr>
<tr>
<td>DPF v CT fat</td>
<td>0.95</td>
<td>0.52</td>
<td>0.07</td>
<td>0.43</td>
<td>0.06</td>
<td>0.4</td>
</tr>
<tr>
<td>Skin v CT skin</td>
<td>0.07</td>
<td>0.89</td>
<td><strong>0.0008 ↑</strong></td>
<td>0.42</td>
<td>0.72</td>
<td>0.4</td>
</tr>
</tbody>
</table>
Particular observations from the 3 laboratory techniques used are highlighted below:

4.3.2 Elevated expression of CD13+ and CD29+ link adipocytes to DD pathogenesis

The elevated expression of CD13+ and CD29+ in the peri-nodular fat in addition to the diseased palmar fascia suggest that the surrounding palmar fat may be involved in DD pathogenesis (tables 4.3.1-4.3.2, figures 4.3.1-4.3.6). In addition to this, the elevated expression seen in the so called “normal” distant palmar fat may suggest that palmar fat in Dupuytren’s patients is abnormal and provides an added reason for disease recurrence. This hypothesis requires further study, as gene expression levels were not significant in comparison to carpal tunnel controls.

4.3.3 CD29 is observed to be a more predominant marker in DD in comparison to carpal tunnel control

There was an observed elevated number of CD29 positive cells in all DD tissues. The cord, nodule, peri-nodular fat, distant palmar fat and skin all have elevated numbers of CD29 positive cells in comparison to carpal tunnel control from non DD subjects. Although expression of CD29 was greater with FACS and immuno-histochemistry data, this expression was not significant when analysed with QRT-PCR (table 4.3.2, figures 4.3.1-4.3.6)

4.3.4 CD34 shows high expression in DD skin

The expression of CD34+ in the DD skin was significantly greater in comparison to control carpal tunnel (table 4.3.2) skin ($p=0.0008$). This was noted with FACS, immuno-histochemistry and QRT-PCR data (figures 4.3.1-4.3.6). These CD34+ cells appear to be either HSC or fibrocytes that were found to be positive for CD13 and CD29.
4.3.5 Frequency of CD44+ and CD90+ cells were observed to be consistent in the nodule and cord

There were a higher number of CD44 positive cells in nodule, cord, peri-nodular and distant palmar fat in comparison to carpal tunnel control tissue. The elevated number of CD44 positive cells in the nodule and cord was significantly higher compared to carpal tunnel controls ($p=0.03$ and 0.02 respectively). On the contrary, the nodule and cord had a significantly lower expression of CD90 ($p=0.02$). The number of CD90 positive cells appeared lower in the DD fat, but this was not significant (table 4.3.1-4.3.2, figures 4.3.1-4.3.6).

4.3.6 FACS analysis suggested the possibility of a population of MSC’s and HSC’s in DD

In the nodule CD29 (60%) was the highest frequency marker followed by CD44 (52%). In the peri-nodular fat CD29 (50%) and CD44 (44%) again were frequent markers followed by CD34 (36%), CD13 (33%), CD90 (32%), CD166 15%) and CD117 (12%). The distant palmar fat had a similar pattern with lesser frequencies (figures 4.3.1-4.3.6). Tissue sample sizes to carry out all FACS analyses on all seven markers was not adequate and therefore CD117 and CD166 were not analysed in DD skin and none were analysed in carpal tunnel tissue (figures 4.3.1-4.3.6).


4.4 Discussion

This study provides observational data that raises the possibility of involvement of MSC and HSC in the various anatomical sites thought to be involved in DD, namely; cord, nodule, peri-nodular fat, palmar fat and the skin. The possibility that mesenchymal progenitor cells in DD may be predominantly located in the cord questions the widely presumed fascial origin of abnormal DD fibroblasts (McFarlane 1984). DD may rather represent a disease resulting from an abnormal expansion of mesenchymal progenitor cells rather than a primary inflammatory disorder. If confirmed, adipodermofasciectomy would be a treatment added to the debate on how best to manage primary DD.

This study shows the presence of MSC and HSC markers in different DD sites suggesting that the disease may house or create a niche for the homing and growth of abnormal mesenchymal cells contributing to DD development and or recurrence. This further supports the notion that DD exhibits important similarities with a (benign) quasi-neoplastic disorder.

The two HSC markers investigated, CD 34 and CD 117 are reported to be mediators of stem cell attachment (Delorme B 2007). Although CD34 is known to be expressed by early normal haematopoietic progenitor cells and clinically identified as a marker in vascular neoplasm’s, (Traweek, Kandalaft et al. 1991) it has been positively recognised in solitary fibrous neoplasms in the respiratory tissues (van de Rijn, Lombard et al. 1994). CD34 expression in skin is also associated with fibrocytes that are considered to be a variety of MSC (Barth and Westhoff 2007).
CD34 is currently used as a biomarker in other fibrotic skin diseases (dermofibroma sarcoma protrubans, DFSP) (Aiba, Tabata et al. 1992) and has been shown to be useful in identifying those patients at higher risk of recurrence and increased severity of DFSP (Abenoza P 1993). The over expression of CD34 in DD skin may suggest its use as a marker to predict those patients who may develop recurrent disease. Further evidence to this effect is provided by the fact that recurrence rates post surgery are reduced following dermofasciectomy a procedure that removes the skin overlying the disease and a layer of the sub-dermal fat. This area of full thickness skin defect, is then usually replaced by a skin graft harvested from the patient’s arm (Bayat and McGrouther 2006).

The identification of CD117 and its use as a specific tumour marker (Sarlomo-Rikala, Kovatch et al. 1998) may have potential in patient testing to recognise patients at risk of fibroproliferative diseases such as DD. CD117 is a tumour marker and predictor of prognosis in breast cancer (Charpin C and Allasia C 2009). DD is a recurrent disease and if, CD117 could be used as a predictor of outcome, it could have potential as a prognostic biomarker for DD.

The investigation of HSC markers in DD may clarify the process of recurrence or extension of the disease. This has been investigated in other recurrent dermatofibrotic disorders such as keloids (Akino, Akita et al. 2008; Lau, Paus et al. 2009).

The presence of CD13 in DD cells supports the hypothesis that DD has a possible neoplastic nature, as CD13 has been found to be a staging marker in non-small cell lung cancer (Ju S and DE 2009). In addition, the presence of adipocytes and poorer
prognosis in dermatofibrous tumours, squamous cell carcinoma and fibrosarcoma has been described (Pérez I and Irazusta J 2009; Wang X 2009). CD29 has been found to have an association with adipocytes in malignant (pancreatic cancer) and benign (hemangioma) neoplastic conditions (Seeberger KL 2006).

The high expression of CD13 in the carpal tunnel fat (control tissue) raises questions as to whether CD13 positive cells are significant in DD. CD13+CD29+ cells have been observed in the palm of DD tissue and in view of previous suggestions regarding the role of palmar fat in DD pathogenesis, our recent findings will need further validation (Rabinowitz, Ostermann et al. 1983).

The expression of CD44 has been previously associated with tumour invasiveness and increased cell adhesion (Wielenga, Heider et al. 1993). The high expression of this marker in DD cord, nodule and peri-nodular fat in comparison to carpal tunnel controls may suggest that DD has locally infiltrating properties whereby the aberrant myofibroblasts in the cord and nodule are also causing abnormal differentiation in the surrounding fat cells.

Under-expression of CD90 which is associated with cell necrosis and apoptosis (Rege and Hagood 2006) may signify the resilience of the DD fibroblasts to apoptosis. Significant under-expression of CD90 in the DD cord and nodule in comparison to the carpal tunnel control fascia has been identified. There is a general consensus that there appears to dysregulation in apoptosis in the development of DD (Jemec, Grobbelaar et al. 1999; Vi, Feng et al. 2009).
Angiogenesis is an important aspect of neoplasia. Research into other neoplastic conditions has identified CD166 as a key marker (Delorme B 2007), in addition to its role as a marker for bone marrow derived MSC’s (Shih, Lee et al. 2005). The expression of CD166 in DD tissue may indicate local invasive properties of the disease.

All the markers were assessed using various tissue modalities in the diseased tissue and compared to an appropriate control, except in FACS analysis. An attempt has been made to compare different tissue modalities using different laboratory techniques. Due to factors out of personal control a thorough comparative analysis was not possible nor was repetition of techniques followed by functional analysis. Had this been possible more concrete conclusions could probably have been drawn. The results of this work have, however, provided a useful foundation that subsequent studies can build upon.

**Limitations of the present study**

Although this study identified a number of individual markers in Dupuytren’s tissues, an analysis of multiple markers on individual cells is required to provide evidence of stemness. In addition in vitro differentiation of Dupuytren’s cells in to different cell lines would support the concept of stem cell aetiology. This study could have used more MSC markers to identify potential stem cells, based upon a consensus among MSC researchers over which marker combination to use, given the circumstances it was deemed appropriate to focus on CD13, CD29, CD44, CD90 and CD166. An increase in the number of markers for a particular tissue modality may provide a more accurate quantification of profiled cells. Internal controls from diseased hands were
taken. Unfortunately there was inadequate tissue volume to conduct all experiments and rather than attempt to analyse the few cells that were extracted and risk perplexity, the tissue was excluded. An alternative for control tissue for future work may be tissue from hand trauma cases rather than patients elected for carpal tunnel release. For FACS analysis there were no adequate numbers of cells from carpal tunnel controls.

All control tissue was taken from female patients undergoing release of the carpal tunnel. DD is a disease more common in males (Hinodcha, John et al. 2006), disease free males may be a better control group.

In summary this study has observed the presence of HSC and MSC markers in DD. The findings question the source of abnormal fibroblasts implicated in DD pathology. The possible clinical implication of this study may advocate the use of adipodermofasciectomy as a potential primary therapy. With certain markers suggesting a proliferative nature and possibly an abnormal apoptotic process it would be rational to identify certain apoptotic markers as biomarkers for DD.
Chapter 5

Discussion & Suggestions for future study
5.1 Overview

This thesis has explored 2 areas of uncertainty in the aetiology of Dupuytren’s Disease. The familial incidence of the disease has been undefined in previous literature and an attempt was made to clarify this by review of the prevalence and subsequently to identify and study the genetics of a family pedigree in Iceland.

Secondly the cellular of origin of the abnormal tissues is unknown and a search for possible stem cells in Dupuytren’s tissues was embarked upon.
5.2 Familial studies in DD (chapter 2) – discussion and future work

The family history of DD has been investigated to clarify the pattern of inheritance (Ling 1963; Capstick, Bragg et al. 2012) At the time of embarking upon this thesis the current popular view was of a Mendelian pattern of inheritance (Ling 1963).

Familial aggregation in DD has been previously calculated (Hindocha, John et al. 2006). There are, however some ascertainment issues in the investigation of the familial nature of DD, given the variation in diagnostic criteria. As the disease can be mild and of late onset (van Dijk, Finigan et al. 2013), it can easily be missed, therefore the population prevalence can be underestimated as the disease may not be clinically apparent for many years.

A more recent study has suggested an alternative method of calculating the sibling recurrence risk ratio (Capstick, Bragg et al. 2012). Rather than using all siblings within a family, this study investigated single sibling pairs. It has been suggested that this methodology reduces ascertainment bias (Olson and Cordell 2000). This is offset by an underestimate of the sibling recurrence risk as not all sibling relationships within a family are considered. The authors of this study did attempt to further reduce ascertainment bias by clinical examination of all siblings included in the calculation. A further improvement in this more recent study in comparison to the methodology described in chapter 2 was the calculation of the sibling recurrence risk ratio in different age groups. Whereas the methodology in this thesis examined the difference between different genders, Capstick et al calculated recurrence risk in different age groups. This is relevant as it is known that prevalence of DD increases with age (Hindocha, McGrouther et al. 2009).
To improve on this data repeated studies of this nature would need to be carried out at expanded (every 5-10 years) time intervals as the study population ages. A repetition of such a study would also benefit from the inclusion of a larger cohort. A comparison of recurrence risk between different genders and age groups could be calculated by combining the methods from the two previous studies (Hindocha, John et al. 2006; Capstick, Bragg et al. 2012). Given the heterogeneity of modern populations any further epidemiological study would need to take account of ethnicity, and it is unlikely that resources will be applied in future to the epidemiology of non-fatal disease. Future studies are likely to be based on genetic methodology Perhaps through such study the hypothesis of a Viking gene may be clarified (McFarlane 2002).

Epidemiological studies to date have been blighted by imprecise diagnostic criteria, a lack of an accepted classification of disease severity and disagreement on what constitutes recurrence Research in DD has now progressed away from epidemiological fieldwork to advanced genomic research to identify susceptible genetic loci and form the foundations of future novel prognostic markers and possible therapy.
5.3 Whole genome scanning in DD (chapter 3) – discussion and future work

It was decided to try to identify the genetic nature of the disease by employing current methodology, against a background of logistic and financial constraints. The methodology chosen was to identify a family with a high incidence of DD for whole genome scanning and linkage analysis.

One of the reasons for choosing a large multi case family is that the members would have a higher probability of developing the disease at an early stage as they have a strong diathesis (Hindocha, Stanley et al. 2006; Dolmans, de Bock et al. 2012). This predictive risk recently calculated by Dolmans et al also highlights the fact that those patients with a strong DD diathesis are more likely to carry more risk alleles for the reported DD SNPs (Dolmans, de Bock et al. 2012). It was for this reason that the largest DD family to date was used to carry out whole genomic scanning.

The results of the whole genome scan carried out in this thesis did not reveal any significant LOD scores i.e. a LOD score > 3 for any genetic region. The highest LOD score from this study was 1.8 and identified 8 genes over 3 chromosomes (GRK4, ADD1, SH3BP2 on chromosome 4; SGCZ on chromosome 8; NAV2, MRGPRX1, SAAL1, MYOD1 on chromosome 11).

A genome-wide association study (GWAS) has already been reported as a landmark finding in the pathology of DD (Dolmans, Werker et al. 2011). This study was a case controlled association study examining the genetic material of many multi-case
families. This genome wide association study in 960 DD patients identified 8 susceptible genes linked to the WNT pathway.

It is appropriate to discuss the reasons for the difference in genetic findings between the subject of this thesis and the report of Dolmans et al (2011).

Putting aside the possibility of experimental error, which can never be discounted, the gene pool in this study is different from that studied by the Dolmans group making this a possible reason why genes in the WNT pathway were not highlighted in the linkage analysis. The Swedish whole genome scan published in 2005 revealed different genes from those reported by Dolmans (Hu, Nystrom et al. 2005).

A possible reason could be that different gene pools have different underlying metabolic disturbances resulting in a similar end stage phenotype i.e. DD. Alternatively, DD may have many metabolic pathways with interlinking genes ultimately leading to a similar clinical presentation. If this were to be the case, the genes identified with the highest LOD scores in this study could be related to those in the WNT pathway.

On exploring this concept it was found that the NAV2 gene identified in this study with a LOD score of 1.8 is related to the WNT pathway by a member protein TCF7C1 which has an abnormal binding domain associated with the development of colon cancer (Muzny, Bainbridge et al. 2012).

The second gene identified in this study with a link to the WNT pathway is MyoD1. Again MyoD1 revealed a LOD score of 1.8 in this study. An in-vitro study of cultured
chick cells has revealed that cholesterol depletion by methyl-B-cycLODextrin (MCD) enhances differentiation of myogenic cells involving the WNT pathway. It is possible that MCD increases p53 and MyoD1 promoting cellular proliferation by the WNT pathway (Portilho, Soares et al. 2012). Further functional studies of the genetic switch MyoD1 and its involvement with the WNT pathway in DD may provide a link.

None of the other 6 genes with LOD scores of 1.8 (GRK4, ADD1, SH3BP2, SGCZ, MRGPRX1, SAAL1) has clear links with the WNT pathway. It has to be highlighted that although the 2 genes identified as having links with the WNT pathway had LOD scores of 1.8, which is considered insignificant, further repetition of this linkage analysis in more multi-case families followed by functional genetic analysis would need to be carried out to demonstrate whether or not there is an association. The single analysis reported here in a single DD pedigree may be the reason for low LOD scores and the addition of more family members from Iceland may have revealed significant results.

Suggestions for future work would include a case control association study with more larger families or fine mapping of these genetic regions that may reveal new genetic markers for DD.

Clustering of DD in families confirms a genetic nature. Studies such as that described by Dolmans et al (Dolmans, de Bock et al. 2012) highlight that the genetic basis for a condition such as DD is complex and influenced by the environment by correlating the DD diathesis with genetic risk.
Further funding would have allowed further analysis of the data and whole genome scanning in a further 2 families in Iceland. Analysis may include Transmission Disequilibrium Testing (TDT) moving from parametric to non-parametric linkage analysis. TDT will examine the transmission of an allele across a pedigree and can confirm association. TDT can follow up areas of linkage with susceptible loci.

Two further large multi case families had already been identified in Iceland. Over 200 cases in Iceland and in the UK were identified which would have allowed a case-control association study to take place.

GWAS studies tend to take part in conditions where there is a complex array of environmental factors and an unclear familial trait. Conditions such as schizophrenia have seen a huge increased interest in the genetics of the condition (Lee, Kim et al. 2013). In this study 1351 cases were used and potential pathological pathways were identified. This was supported by a further study suggesting that GWAS studies have the ability to explain the missing heritability link in complex multi-factorial conditions (Andreassen, Djurovic et al. 2013; Andreassen, Thompson et al. 2013).

Once susceptible genes are identified, sequencing of these genes can be carried out to assess function and associated disease pathology. Previous studies have identified the genes for matrix metalloproteinases (MMPs) and fibroblast growth factor as potential genes in association with DD (Satish, Gallo et al. 2011; Satish, O'Gorman et al. 2013). Transcription of genes and thus the functional outcome of these susceptible genes has now been investigated (Forrester, Temple-Smith et al. 2013). The genes identified from this exome sequencing study have suggested potential candidates in the
aetiology of DD and other fibrotic diseases. These include MMP1, 3, 16, STAT1 and fibroblast growth factor (FGF9, 11).

Future work and collaborations are in place to further analyse the data from chapter 3, as described. This will be followed by a case control association study or whole exome sequencing if any candidate genes with a significant LOD score are identified.
5.4 Characterisation of stem cell markers in DD (chapter 4) –
discussion and future work

This study addressed the possibility that there may be stem cell populations in DD
tissues. The hypothesis was that stem cell markers may be identified by FACS or
histology. Two markers consistent with haematopoietic stem cells (HSC) (CD34, 117)
and five associated with mesenchymal stem cells (MSC) (CD13, 29, 44, 90, 166)
were investigated.

The main weakness of this study was the inability to identify the presence of multiple
stem cell markers in any one cell type. Cells from the nodule, cord, fat and skin were
only observed for one stem cell marker at a time. Multiple markers in individual cells
were not examined thus preventing the positive identification of any cell as a stem
cell. In addition cells were not cultured and studied for differentiation in to different
lineages. The lack of resource in undertaking this work did not enable this follow up
work to take place making this chapter a simple observational study of single markers
in different DD tissues which might be indicative of stem cells. The availability of
multi-colour FACS analysis and culturing of cells to examine for differentiation
would have provided better evidence.

Further limitations of this study were the size of population cohort investigated and a
comparison of tissue samples from UK and Icelandic populations. A particular issue
with tissue collection was the small sample sizes from carpal tunnel control.
Following tissue processing, especially for FACS analysis there were not enough live
cells to carry out analysis leaving no control to compare to diseased tissue, especially
in the case of carpal tunnel skin. Carpal tunnel decompression is an elective procedure
and surgical exposure of the carpal tunnel limits the tissue sample size. An alternative for future work would be to use hand surgical procedures where tissue is excised as part of therapy to provide a supply of normal control tissue.

A technical improvement in this study would be to carry out multi-colour FACS analysis to show multiple cell markers on potential stem cells. Observation of differentiation in culture along different lineage pathways is required to conclude that the cells observed are stem cells.

Manual counting of positively stained cells was the method used to analyse immunostaining fields. This would be more accurate if done by computational quantitative analysis. Formal quantitative immunohistochemical analysis using commercially available software improves accuracy (Matkowskyj, Schonfeld et al. 2000; Matkowskyj, Cox et al. 2003).

It is not possible on the basis of this data to shed further light on the cellular origin of the disease, whether starting in the palmar fascia and disseminating to the fat, skin or vice versa. Additionally the definitive source of the myofibroblast is still unknown. Further characterisation of cells with stem cell markers would enhance knowledge of DD cells which may be targeted for future clinical therapy (Verhoekx, Mudera et al. 2013).
5.5 Conclusions

1. The near autosomal dominant pattern of inheritance in familial DD has been reviewed. A penetrance level of 18% has been estimated which can be used in genetic studies.

2. A revised sibling recurrence ratio has been calculated, $\lambda_s = 6.2$.

3. In the study of probands within a single pedigree, three susceptible loci with a LOD score $>1.8$ have been identified. Within these, 8 candidate genes (GRK4, ADD1, SH3BP2 on chromosome 4; SGCZ on chromosome 8; NAV2, MRGPRX1, SAAL1, MYOD1 on chromosome 11) have been selected. These genes require further validation as to their role in DD.

4. Using the results from three laboratory techniques; immunohistochemistry, QRT-PCR and FACS analysis, cell surface markers consistent with MSC or HSC have been observed. Further study is merited to ascertain whether or not stem cells contribute to the abnormal tissue in Dupuytren’s Disease.
References
Akino, K., S. Akita, et al. (2008). Human mesenchymal stem cells may be involved in keloid pathogenesis. 47: 1112.


Loos, B., V. Puschkin, et al. (2007). 50 years experience with Dupuytren's contracture in the Erlangen University Hospital – A retrospective analysis of 2919 operated hands from 1956 to 2006, BioMed Central Ltd. 8: 60.


Satish, L., W. A. LaFramboise, et al. (2012). Fibroblasts from phenotypically normal palmar fascia exhibit molecular profiles highly similar to fibroblasts from active disease in Dupuytren's Contracture. BMC Med Genomics 5: 15.


Skoog, T. (1948). Dupuytren’s contracture with special reference to aetiology and improved surgical treatment, its occurrence in epileptics, note on


Chondrogenic Differentiation of Adult MSCs

M. Griffin1, S. Hindocha2,3 and W. Khan4

1Academic Foundation Trainee, Rheumatology, London, UK; 2Department of Plastic Surgery, Whiston Hospital, Warrington, UK; 3Department of Orthopaedics and Rheumatology, University College London Institute of Orthopaedics and Musculoskeletal Sciences, Royal National Orthopaedic Hospital, Stanmore, Middlesex, HA7 4LP, UK

Abstract: Chondrogenesis is a vital part of adult life, as cartilage is important not only for articulation of joints but also maintenance functions of the body. Chondrogenesis is a five-stage process initiated by specific genes and cell-cell interactions which has been documented over recent years. This review highlights the current literature regarding the process of endochondral ossification and covers the different levels of control at genetic, transcriptional and post-transcriptional levels. Due to the proliferative nature of chondrogenesis and using chondrocytes for self-renewal and repair, current research involves finding ways in which to improve and replicate the expansion of chondrocytes. The review summarises ways in which ex vivo expansion can be manipulated using growth factors, external sources and scaffolds.

Keywords: Cartilage, chondrocytes, chondrogenesis, endochondral ossification, mesenchymal stem cell

INTRODUCTION

Cartilage is a type of connective tissue that is composed of a collagen and proteoglycan matrix with a sparse population of chondrocytes [1, 2]. Chondrogenesis, the formation of cartilage is a vital part of embryogenesis as well as in adult life. Chondrogenesis leads to the establishment of different types of cartilage including hyaline, fibrous and elastic cartilage [2].

Cartilage is a connective tissue that is composed primarily of matrix (usually collagen and proteoglycan) containing relatively sparse populations of chondrocytes, which perform matrix-generation and maintenance functions [2]. Cartilage is also useful for the articulation of joints and the maintenance of the loading capacity of the intervertebral discs [2]. Chondrogenic precursors are involved in the formation of mature connective tissue cartilage, which is eventually replaced by bone, which is achieved through a sequence of events called endochondral ossification. This process is closely regulated by specific genes and cell-to-cell and cell-matrix interactions [1, 2].

The endochondral ossification process can be divided into five main stages. Firstly the Mesenchymal Stem Cells (MSCs) are committed to become cartilage by certain paracrine factors becoming chondroblasts in the process called condensation [3]. Secondly, the MSCs then develop into adult chondrocytes under the influence of transcription factors specifically Sox9 during the process of differentiation [3]. Then the rapid division of chondrocytes causes the production of the Extracellular Matrix (ECM) [3]. The proliferation stage of the chondrocytes is then overtaken by hypertrophy, in which stage the ECM is mineralised with calcium salts [3]. Then the invasion of blood vessels takes place as the hypertrophic chondrocytes die by apoptosis [3]. The ossification process then mimics the tissue and starts forming bone by replacing the cartilaginous matrix with a mineralized matrix [3].

This review aims to explain the regulatory mechanisms that occur during developmental chondrogenesis including the control at both extrinsic and intrinsic levels. Furthermore, the review will briefly explain ex vivo expansion of chondrocytes and the ways in which it can be manipulated.

Mesenchymal Stem Cell Condensation and Determination of Chondrogenic Precursors

Chondrogenesis can only begin when MSCs have been recruited effectively. Mesenchymal cells for chondrogenesis are recruited from three main sites:

1. The neural crest cells that form the craniofacial bones [4].
2. The sclerotome of the paraxial mesoderm forming the axial skeleton [4].
3. The somatopleur of the lateral plate mesoderm forming the skeleton of the long bones [4].

After recruitment the MSCs aggregate into chondrogenic mesenchymal cells to form pericellular condensations [3]. To form mesenchymal condensations the MSCs increase their cellular interaction with the ECM and the surrounding cells as well as increasing their cell adhesion and formation of gap junctions and changes in the cytoskeletal architecture [5].

Condensation is initiated due to cell-to-cell matrix interactions [6]. Before condensation, MSCs produce extracellular matrix which contains hyaluron and collagen type I as well
Recent Surgical and Medical Advances in the Treatment of Dupuytren’s Disease - A Systematic Review of the Literature

R. Mafi¹, S. Hindocha² and W. Khan³

¹The Hull York Medical School, Hartford Building, Hull, HU6 7RX, UK
²Department of Plastic Surgery, Whiston General Hospital, Liverpool, L35 5DR, UK
³University College London Institute of Orthopaedics and Musculoskeletal Sciences, Royal National Orthopaedic Hospital, Stanmore, Hillingdon, HA7 4LP, UK

Abstract: Dupuytren’s disease (DD) is a type of fibromatosis which progressively results in the shortening and thickening of the fibrous tissue of the palmar fascia. This condition which predominantly affects white-northern Europeans has been identified since 1614. DD can affect certain activities of daily living such as face washing, combing hair and putting hand in a glove. The origin of Dupuytren’s contracture is still unknown, but there are a number of treatments that doctors have come across throughout the years. Historically, surgery has been the mainstay treatment for DD, but not the only one. The objective is to make a structured review of the most recent advances in treatment of DD including the surgical and medical interventions. We have looked at the most relevant published articles regarding the various treatment options for DD. This review has taken the previous data into consideration which have met the inclusion criteria. The most recent treatments used are multi-needle aponeurotomy, extensive percutaneous aponeurotomy and lipografting, inserting collagenase Clostridium histolyticum, INF-gamma and shockwave therapy as well as radiotherapy. Each of these treatments have certain advantages and drawbacks and cannot be used for every patient. In order to prevent this condition, spending more time and money in the topic is required to reach better and more consistent treatments and ultimately to eradicate this disease.

Keywords: Dupuytren contracture, dupuytren disease, medical, surgical, treatment, advances.

BACKGROUND INFORMATION

The first doctor who came across this condition was Pister in 1614 [1]. In 1831 a French military surgeon called Guillaume Dupuytren became famous for describing and operating on palmar fibromatosus, which is now commonly known as Dupuytren’s disease. The disease is described as a type of fibromatosis characterized by nodular and/or distributed aggregates of immature fibroblasts dispersed in a dense collagen [2]. The progressive and irreversible flexion contractures of the palmar digital joints of the hand are the nature of this disease. These flesions which predominantly affect the small and ring finger are due to the proliferation of myofibroblasts in the fascia of the hand. Myofibroblasts were the first responsible cause for contracture in this disease. It was first due to their ultrastructural identification in transmission electron-microscopic studies but later on scientists showed that the contracture was mainly due to the expression of Alpha-smooth muscle actin (SMA) in cells from tissue explants. Furthermore, it was shown that myofibroblasts can generate contractile force [3, 4].

EPIDEMIOLOGY

Looking at the incidence of Dupuytren’s disease (DD), white-northern Europeans have the highest rate whereas dark-skinned individuals have the lowest number of occurrences [5, 6]. Twin studies have shown that there is some evidence supporting the theory that this disease could be a familial disorder [7]. It is still uncertain whether Dupuytren’s disease is a monogenic or a polygenic condition as this condition has variable inheritance patterns and different levels of gene expression.

Scandinavians and people with Northern European ancestry were mainly responsible for the spread of this disease hence it is being called the “Viking disease” [8]. The theory of Nordic origin of the disease can be supported by the high incidence rate among the people in Denmark as well as in the northern part of the UK [9].

Both age and sex have an effect on the occurrence of Dupuytren’s disease. The incidence is very low among women and people in their twenties but the risk of having this condition increases each decade. According to Mikkelsen et al. at the onset of Dupuytren’s contracture (DC) is indirectly proportional to the recurrence and progression of this disease. In other words the earlier the onset of the disease the more likely the recurrence and progression of Dupuytren’s contracture in the future [10, 11]. Men are up to 15 times more likely to suffer from this disease. DC however, is less severe in women and may even remain unnoticed. During the 6th and 9th decade of life the ratio between affected men and women is equal [11].

There is much evidence as to what the causes and consequences of Dupuytren’s contracture may be. Scientists
Characterization of stem cells in Dupuytren’s disease

S. Hindocha1, S. A. Iqbal1, S. Farhatullah1, R. Paus2,4 and A. Bayat1,2,3

1Plastic and Reconstructive Surgery Research, Manchester Interdisciplinary Biocentre, 1School of Translational Medicine, University of Manchester, and 2Department of Plastic and Reconstructive Surgery, St George’s Hospital Medical School, Manchester, UK. and 3Department of Dermatology, University of Luebeck, Luebeck, Germany

Correspondence to: Dr A Bayat, Plastic and Reconstructive Surgery Research, Manchester Interdisciplinary Biocentre, University of Manchester, 131 Princess Street, Manchester M1 7DN, UK (e-mail: andular.bayat@manchester.ac.uk)

Background: Dupuytren’s disease (DD) is a common fibroproliferative disease of unknown origin. The source of abnormal cells leading to DD formation remains undetermined. In addition to fascia, palmar skin and fat-derived cells may be a potential source of cells causing DD. This study aimed to profile haemopoietic and mesenchymal stem cells in different DD tissue components compared with tissue removed at carpal tunnel surgery (control).

Methods: Biopsies were taken from the diseased cord, nodule, perinodular fat and skin overlying the nodule of ten patients with DD and compared with control tissue from seven patients having surgery for carpal tunnel syndrome. Fluorescence-activated cell sorting (FACS), immunohistochemistry and quantitative real-time polymerase chain reaction (QRT-PCR) were used to identify expression of selected stem cell markers.

Results: FACS and QRT-PCR analysis identified the highest RNA expression and number of cells positive for adipocyte stem cell markers (CD13 and CD29) in the DD nodule in comparison with carpal tunnel control tissue (P = 0.003). CD34 RNA was overexpressed, and a higher percentage of these cells was present in DD skin compared with carpal tunnel skin (P = 0.001).

Conclusion: Each structural component of DD (cord, nodule, perinodular fat and skin) had distinct stem cell populations. These findings support the hypothesis that DD may result from mesenchymal progenitor cell expansion.

Paper accepted 2 September 2010
Published online 22 November 2010 in Wiley Online Library (www.bjs.co.uk). DOI: 10.1002/bjs.7307

Introduction

Dupuytren’s disease (DD) is a benign fibroproliferative disease of unknown aetiology affecting the palmar fascia. It is one of the most common connective tissue disorders, affecting over 4% of the UK population, and over 25% of the Celtic population over the age of 60 years. Several environmental factors are associated with DD, including alcohol5,6, smoking6, diabetes6,7, epilepsy, hypercholesterolaemia and injury or microtrauma. Although these factors appear to have a significant association with the aetiology of DD, none has convincingly been shown to be causative.

The typical clinical progression of DD is the development of palmar nodules and pits leading to a gradual contraction of the affected digit. The treatment is surgical, where the digital contracture is released by fasciectomy, yet this common operation is associated with a high rate of recurrence11, which may reach 75 per cent in individuals with a positive family history, ectopic DD (Dupuytren-like tissue found in other anatomical sites), young age at onset and bilateral disease11. Dermofasciectomy is an alternative treatment that involves excision of the skin overlying the diseased fascia along with the subdermal fat, which is then replaced with a full-thickness skin graft. This method has reduced recurrence rate, below 12% per cent in the most severe cases12. The palmar fat has been implicated in the pathogenesis of DD13-15, although the exact roles of adipocytes and fat stromal cells remain unclear. Therefore, the skin overlying the diseased palmar fascia, as well as the surrounding palmar fat, is of scientific and clinical relevance.

Myofibroblasts, which have combined properties of smooth muscle cells and fibroblasts, are thought to be involved in the pathogenesis of DD14-17. The proliferation of myofibroblasts is believed to cause the nodular lesions

© 2010 British Journal of Surgery Society Ltd
Published by John Wiley & Sons Ltd
British Journal of Surgery 2011; 98: 308–315
212
Dupuytren’s Disease Shows Populations of Hematopoietic and Mesenchymal Stem-Like Cells Involving Perinodular Fat and Skin in Addition to Diseased Fascia: Implications for Pathogenesis and Therapy

Syed Amir Iqbal, Sandip Hindocha, Syed Farhatullah, Ralf Paus, and Ardeshr Bayat

Contents
21.1 Introduction .................................................. 167
21.2 Materials and Methods ...................................... 168
21.3 Results .......................................................... 169
21.4 Discussion ...................................................... 171
21.5 Conclusion ....................................................... 172
References ............................................................ 173

21.1 Introduction

Dupuytren’s Disease (DD) is a common, benign fibroproliferative disease of unknown origin that primarily affects the palmar fascia (Brickley-Parsons et al. 1981). DD is a progressive, irreversible, and highly recurrent condition which is often treated surgically (Bayat 2010). Myofibroblasts are considered to be the causative cellular elements involved in the formation of DD; however, their origin remains unknown to date (McCann et al. 1993; Bernderet et al. 1994).

DD typically involves the palmar fascia, but in progressive cases can extend into the digital fascia and subcutaneous tissues. The two principal tissue components commonly described in the fascia to be pathognomonic of the disease are the nodule and the cord (Shih et al. 2009b; Verjee et al. 2010). Myofibroblasts are found in both the nodule and the cord (Dave et al. 2001; Bisson et al. 2003). Other tissue components adjacent to the diseased fascia including the perinodular fat and the skin overlying the nodule have also been implicated in the pathogenesis of DD (Rubinowitz et al. 1983; Shih et al. 2009a). Previous studies have investigated palmar fat cells (Shih et al. 2009a, b), although the exact role of adipocytes and fat stromal cells in relation to DD pathology remains unclear. Therefore, the exact nature of the skin overlying the diseased palmar fascia plus the surrounding fat is of scientific and clinical relevance.

Mesenchymal Stem Cells (MSCs) are obvious candidates as a potential source for DD myofibroblasts but

S. A. Iqbal • S. Hindocha • S. Farhatullah • A. Bayat
Plastic & Reconstructive Surgery Research, Manchester Interdisciplinary Biocentre, Manchester, UK
E-mail: ardeshr.bayat@manchester.ac.uk

R. Paus
Epithelial Sciences Research Group, School of Translational Medicine, University of Manchester, Manchester, UK

C. Evan et al. (eds.), Dupuytren’s Disease and Related Hypoproliferative Disorders, DOI 10.1007/978-3-642-27678-0_21, © Springer-Verlag Berlin Heidelberg 2013
High Prevalence of Dupuytren’s Disease and Its Treatment in the British National Health Service: An Ongoing Demand

Karen Zaman, Sandip Hindocha, and Ardeshir Bayat

Contents

4.1 Introduction .................................................................................. 27
4.2 Material and Methods .................................................................. 28
4.2.1 Spotlighting the High Prevalence of DD .................................. 28
4.2.2 Estimation of the Types of Surgical Procedures and Cost of DD to the Health Care System ......................... 28
4.3 Results ....................................................................................... 28
4.3.1 Global Incidence of Epidemiology of DD ............................... 28
4.3.2 The Types of Surgical Procedures and Cost of DD to the Health Care System ................................................. 29
4.4 Discussion .................................................................................. 32
4.5 Conclusion ................................................................................ 34
References ...................................................................................... 34

4.1 Introduction

Dupuytren’s Disease (DD) is a common fibroproliferative disorder of unknown etiology affecting 3–5% of the UK population (Gerber et al. 2011). The disease is often progressive, irreversible, and commonly bilateral. DD is a late-onset disease with a mean age at onset of 55 years but can occur in younger age groups in particular in those with a strong family history of DD (Hindocha et al. 2006a). The rate of progression from palmar nodules to extreme digital contracture can vary between several months to many years. There is a general consensus that the greater the DD diathesis, the more severe the disease presentation (Hindocha et al. 2006b). DD diathesis refers to the presence of a number of factors including bilateral disease, oesophageal disease presentation, early age of onset and family history which may lead to a worse prognosis in disease outcome.

There are several therapeutic modalities available for treatment of DD. The standard treatment for DD is surgery. There are a variety of surgical approaches, which include fasciectomy (needle or open aponeurotomy) also referred to as release fasciectomy, fascioectomy (segmental, limited, radical) which can be either digital (surgery confined to the digits) or palmar (surgery confined to the palm of the hand), or dermofasciectomy. Although previous clinical studies have quoted a range of recurrence rates for these surgical interventions (Armstrong et al. 2000; Foucher and Navarro 2003), it is anticipated that a patient presenting with a strong DD diathesis has a higher recurrence rate (Hindocha et al. 2006b). As the disease is progressing, an observational approach can be taken, resulting in the disease being monitored in the outpatient department until the correct time for non-surgical (such as enzymatic injection) or surgical intervention is deemed appropriate. However, timing for surgery can be controversial, often the degree of disability and rate of progression of the disease are considered to be the determining factors (Bayat and McGrouther 2006). With no cure for the condition and high rates of disease recurrence, the cost of treating DD can be exponential. In addition, an ageing as well as a healthier, more informed population demanding functional surgical outcomes is likely to increase demand for early surgical...
Sources of Adult Mesenchymal Stem Cells Applicable for Musculoskeletal Applications – A Systematic Review of the Literature

R. Mafi¹, S. Hindocha¹,²,³, P. Mafi¹, M. Griffin¹ and W.S. Khan⁴

¹The Hull York Medical School, Hertford Building, Hull, HU6 7RX, UK
²Manchester Interdisciplinary Biocentre, University of Manchester, 131 Princess Street, M17DN, UK
³Department of Plastic Surgery, Whiston General Hospital, Liverpool, L35 5DR, UK
⁴University College London Institute of Orthopaedics and Musculoskeletal Sciences, Royal National Orthopaedic Hospital, Stanmore, Middlesex, HA7 4LP, UK

Abstract: Mesenchymal stem cells (MSCs) were first discovered by Friedenstein and his colleagues in 1976 from bone marrow. The unique property of these cells was their potential to develop into fibroblastic colony forming cells. Since Friedenstein’s discovery of these cells the interest of adult MSCs has been progressively growing. Nowadays MSCs are defined as undifferentiated biological cells capable of proliferation, self renewal and regenerating tissues. All these properties of MSCs have been discovered in the past 35 years. MSCs can play a crucial role in tissue engineering, organogenesis, gene therapy, transplants as well as tissue injuries. These cells were mainly extracted from bone marrow but there have been additional sources for MSCs discovered in the laboratories including: muscle, dermis, trabecular bone, adipose tissue, periosteum, pericyte, blood, synovial membrane and so forth. The discovery of the alternative sources of MSCs helps widen the application of those cells in different areas of medicine. By way of illustration, they can be used in various therapeutic purposes such as tissue regeneration and repair in musculoskeletal diseases including osteonecrosis of femoral head, stimulating growth in children with osteogenesis imperfecta, disc regeneration, osteoarthrosis and Duchenne muscular dystrophy. In order to fully comprehend the characteristics and potential of MSCs future studies in this field are essential.

Keywords: Mesenchymal stem cells, muscular dystrophy, musculoskeletal applications, tissue engineering.

Thirty five years ago Friedenstein et al. discovered mesenchymal stem cells (MSCs) in the bone marrow where he observed a certain population of cells that developed into fibroblastic colony forming cells (CFU-F) [1]. MSCs account for a very small ratio of the bone marrow and it has been estimated that 1/10000 to 1/100000 of the bone marrow nuclear cells are MSCs [2, 3]. Since the ground breaking discovery of these cells, the interest in adult MSCs has been growing and other sources of the adult MSCs have been identified by scientists. The new sources of adult MSC can be used for various therapeutic purposes such as tissue regeneration and repair in musculoskeletal diseases [4]. Despite their therapeutic and clinical significance, there are no articles bringing together and comparing the sources of MSCs and their various applications in musculoskeletal conditions. In this systematic review, studies have been searched for different sources of adult MSCs as well as the recent musculoskeletal applications using adult MSCs. These studies were predominantly searched using AMED, CINAHL, EMBASE, Medline, PubMed and ZETOC. The aim of this study is to summarize all the available literature relating to sources of adult MSCs and their application in musculoskeletal diseases.

Stem cells are defined as undifferentiated biological cells capable of proliferation, self renewal, conversion to differentiated cells, and regenerating tissues. There are two main types of stem cells in mammals [5]; embryonic stem cells (ESC) are pluripotent cells derived from the inner cell mass of blastocyst which are formed several days after an egg is fertilised, and non-embryonic stem cells (non-ESC) also known as adult stem cells as they are usually obtained from the bone marrow of adults. There are two types of stem cells from these sources: Haematopoietic, that differentiate into blood cells, and MSCs. Less mature tissue sources including umbilical cord blood, placenta and fetal somatic tissues are ranked among the non-ESC. Furthermore, the fetal stem cells (FSC) are regarded as an intermediate cell type between ESCs and adult stem cells. FSCs are obtained from the gonads in the first trimester of development [5, 6].

It has been more than 130 years that the existence of non-hematopoietic stem cells was proposed by the German pathologist Cohnheim. He suggested that bone marrow could aid the wound healing process in various peripheral tissues [7, 8]. This theory was confirmed by Friedenstein in the 1970’s. He discovered that the bone marrow consisted of fibroblastoid cells with clonogenic potential in vitro which are capable of forming colonies (CFU-F). Furthermore, Friedenstein proved that it was achievable to regenerate heterotopic bone tissues in different transplants. This way he provided evidence in support of the self renew potential of these cells [1]. Ever since, numerous scientists have
Tissue Engineering for Bone Production - Stem Cells, Gene Therapy and Scaffolds

E.G. Khaled1, M. Saleh1, S. Hindocha1,2,3, M. Griffin3 and Waseem S. Khan1

1Department of Plastic Surgery, Ain Shams University, Plastic Surgery Department, Ramsis Street, Abbassia Square, Cairo, Postal Code 11566, Egypt
2Department of Plastic Surgery, Whiston Hospital, Whiston Road, Liverpool, L35 5DR, UK
3University of Manchester, Manchester Interdisciplinary Biocentre, 151 Princess Street, Manchester, M1 7DN, UK
4University College London Institute of Orthopaedics and Musculoskeletal Sciences, Royal National Orthopaedic Hospital, Simmone, Midherset, HA7 4LP, UK

Abstract: A bone graft has been the gold standard treatment for repairing bone defects. However, due to bone grafts associated donor site morbidity several alternative bone substitutes options have been made available but with their added expense and limited osteoinductive properties they are not ideal. Therefore, research has begun in tissue engineering to investigate stem cells, which are one of the body's own mechanisms used to repair bone. Stem cells are clonogenic undifferentiated cells capable of self-renewal. Readily available from numerous bone marrow stem cells have the potential to differentiate into osteoblasts and chondrocytes showing capability to repair both bone and cartilage. The known immunologic properties of stem cells further enhance their therapeutic appeal. Stem cells have shown to be excellent carriers for gene transfer having the capability to be transduced. Gene transfer could enable growth factors and bone morphogenetic proteins to enhance bone repair. Stem cells are implanted onto scaffolds, which are structures capable of supporting tissue formation by allowing cell migration, proliferation and differentiation. Research aims to produce scaffolds that deliver and retain cells, allow for cell attachment has adequate biodegradability, biocompatibility and non-immunogenicity. However, having played a role in testing numerous materials including synthetic and natural products research into the perfect scaffold product continues. This review aims to explain how stem cells were discovered, the techniques used to isolate stem cells, identify and manipulate them down different cell lineages and discuss the research into using stem cells to reconstruct bone using genetic modification and scaffolds.

Keywords: Adult stem cells, bone marrow, bone reconstruction, gene therapy, scaffolds, tissue engineering.

Bone defects exceeding critical size usually heal with fibrous tissue (callus), missing complete bone re-union. The critical size is defined as atraumatic defect that will not heal with more than 10% new bone formation within life expectancy of the patient (human or mouse) [1].

Reconstruction of bone defects is dependent on certain mechanisms, which can be summarized into osteoconduction, osteoinduction and osteogenesis mechanisms. Osteogenesis is the formation of new bone from osteoblastic cells. Osteoconduction is the formation of bone along the scaffold of a biologic or alloplastic substance where the bone forming cells originate from pre-existing host osteoblastic cells. Osteoinduction is the formation of new bone by differentiation and stimulation of mesenchymal cells by bone inductive proteins [2].

In principle, bone grafts contain all the key elements required for bone repair; as they provide osteoconductive scaffold, growth factors for osteoinduction, and cells with osteogenic potential [3]. The use of bone grafts in the clinical practice presents several major inconveniences [4]. Although the percentage of success is high, complications as resorption, fracture, infection and non-union are still present [5]. Additionally harvesting of autologous bone often results in donor site morbidity, the extent of which may vary according to the location and the intervention technique including hemorrhage, nerve damage, cosmetic disability, pain, infection, and loss of function [6].

The use of human cadaver or animal bone grafts from bone banks prevents the problem of the donor site morbidity, but presents the potential risk of viral or bacterial infections and an immune response of the host tissue towards the implant. However, the continuous advancement of the processing techniques for grafts is significantly reducing the risk of infection [6].

Alternatively, guided bone regeneration and several biomaterials have been considered and used as bone substitutes, including calcium phosphate ceramics, polymers and bioglasses. All have the advantage of unlimited availability and good osteoconductivity properties. On the other hand, they are not osteoinductive, thus limiting their application to repair large bone defects [8]. On the contrary, bone distraction takes the advantage of bone regeneration potential, avoiding the troubles associated with the graft
**Adult Mesenchymal Stem Cells and Cell Surface Characterization - A Systematic Review of the Literature**

P. Mathi1, S. Hindocha2,3, R. Mathi1, M. Griffin1 and W.S. Khan4

1The Hull York Medical School, Huddersfield, York YO10 SDD, UK
2Manchester Interdisciplinary Biocentre, University of Manchester, 131 Princess Street, M1 7DN, UK
3Department of Plastic Surgery, Whiston General Hospital, Liverpool, L553DR, UK
4University College London Institute of Orthopaedics and Musculoskeletal Sciences, Royal National Orthopaedic Hospital, Stanmore, Middlesex, HA7 4LP, UK

Abstract: Human adult mesenchymal stem cells (MSCs) were first identified by Friedenstein et al. when observing a group of cells that developed into fibroblastic colony forming cells (CFU-F). Ever since, the therapeutic use and clinical applications of these cells have increased research and interest in this field. MSCs have the potential to be used in tissue engineering, gene therapy, transplants and tissue injuries. However, identifying these cells can be a challenge. Moreover, there are no articles bringing together and summarizing the cell surface markers of MSCs in adults. The purpose of this study is to summarize all the available information about the cell surface characterization of adult human MSCs by identifying and evaluating all the published literature in this field. We have found that the most commonly reported positive markers are CD105, CD90, CD44, CD73, CD29, CD13, CD34, CD166, CD106, CD54 and CD68. The most frequently reported negative markers are CD34, CD14, CD45, CD11a, CD40, CD16, CD106, CD10 and CD51. A number of other cell surface markers including STRO-1, S100, SH2, SH3, HLA-A, HLA-B, HLA-C, HLA-DR, HLA-E, DP, EMA, DQ (MC7 Class II), CD95, Oct-4, Oct-4A, Nanog, Sox-2, TERT, Stats, fibroblast surface antigen, smooth muscle alpha-actin, vinculin, integrin subunits alpha4, beta1, integrins alpha, beta1 and alpha, beta5 and ICAM-1 have also been reported. Nevertheless, there is a great discrepancy and inconsistency concerning the information available on the cell surface profile of adult MSCs and we suggest that further research is needed in this field to overcome the problem.

Keywords: Bone marrow derived multipotent progenitor cells; cell surface profile; mesenchymal stem cells; surface markers.

**INTRODUCTION**

About 130 years ago, the German pathologist Cohnheim proposed the existence of non-hematopoietic stem cells in the bone marrow. He suggested that these cells could contribute to wound healing as they can be a source of fibroblasts [1]. Later, Friedenstein et al. identified human adult mesenchymal stem cells when observing a group of cells that developed into fibroblastic colony forming cells (CFU-F) [2]. Friedenstein provided strong evidence for the self-renewal potential of stem cells by demonstrating their ability to regenerate heterotopic bone tissue. These findings have been confirmed and expanded by many further laboratory studies which have shown that the cells isolated by Friedenstein can also be found in human bone marrow and could differentiate into a range of different mesenchymal lineage cells including chondrocytes, adipocytes, myoblasts and osteoblasts [1-6].

Simmons et al. found that stromal cells supporting hematopoiesis were different to hematopoietic cells by showing that sex-mismatched HLA-identical cells from patients solely expressed the host genotype [7]. This again supports Friedenstein's findings showing hematopoietic stem cells were physically different from transplanted sex-mismatched cells capable of hematopoietic stem cell formation [8].

Now that these cells are found to be different, research is being done to identify whether there exist specific cell surface antigens to identify phenotypic differences between mesenchymal and hematopoietic stem cells. Even though many mesenchymal stem cell surface antigens have been cultured, there have been very few in vivo phenotypic characterization of MSCs [9]. Bianco *et al.* conducted one of the first studies with the intention of characterizing MSC-like cells both histologically and phenotypically. Interestingly, it has been shown that a wide range of anti-hematopoietic stem cells exist in the bone marrow and that MSC are merely a subset of this population. These include "multipotent adult progenitor cells" (MAPCs), "endothelial progenitor cells" (EPCs), "mature isolated adult multineurone inducible cells" (MINIs), "very small embryonic-like stem cells" (VESCs) [9-13].

Circu *et al.* has found that multipotent mesenchymal stem cells exist in many different human organs [14]. To support this fact, it has been shown that MSCs are not confined to bone marrow and can also be found in placenta, dental pulp, tonsils, skeletal muscle, fat, umbilical cord blood and amniotic fluid [14-22]. Despite the common belief that mesenchymal stem cells are derived from the embryonic mesoderm, a recent study by Takashima and colleagues showed that the earliest lineage of MSC-like cells are
Epidemiological Evaluation of Dupuytren’s Disease Incidence and Prevalence Rates in Relation to Etiology

Sandip Hindocha • Duncan Angus McGregor • Ardishar Bayat

Received: 19 November 2008 / Accepted: 15 December 2008 / Published online: 15 January 2009
© American Association for Hand Surgery 2009

Abstract Dupuytren’s Disease (DD) is a common, fibroproliferative disorder affecting the palmar surface of the hands which is often irreversible and progressive. Understanding the epidemiology of DD is important in order to provide clues to its etiopathogenesis. This review aims to evaluate the epidemiological studies carried out in DD since 1951. Studies evaluating the epidemiology of DD were searched using Medline, Pubmed, and Scopus which dated back from 1951 to current date. Inclusion criteria were any studies investigating the prevalence or incidence of DD in any population group. A total of 620 articles were cited. Forty-nine studies were subsequently identified as relevant to evaluating the epidemiology of DD. The prevalence of DD in all studies increased with age with a male to female ratio of approximately 5:1. Prevalence rates ranged from 0.2% to 50% in varying age, population groups, and methods of data collection. The highest prevalence rate was reported in a study group of epileptic patients. Although, only one study calculated the incidence (as opposed to prevalence) of DD to be equal to 34.3 per 100,000 mm (0.003%). In conclusion, the prevalence of DD in different geographical locations is extremely variable, and it is not clear whether this is genetic, environmental, or a combination of both. The majority of the prevalence studies have been conducted in Scandinavia or the UK, and the vast changes in population structure, the changes in prevalence of associated diseases, and the change in diagnostic criteria of DD makes understanding the epidemiology of this condition difficult.

Keywords Dupuytren’s disease • Epidemiology • Etiology • Prevalence

Introduction

Dupuytren’s Disease (DD) is a common, fibroproliferative disorder affecting the palmar surface of the hands which can present itself as a clinically challenging disorder for the patient and the surgeon alike. The disease is often progressive, irreversible, and commonly bilateral. DD can be a psychologically and physically disabling condition which can also have a significant impact on healthcare economy [65]. It is therefore, considered important to time surgical operative intervention appropriately [45, 59]. The disease is thought to involve abnormal tissue contraction, shown to be mediated by the myofibroblast in the palmar fascia causing a digital flexion deformity [101]. Therefore, knowledge of the exact causation of DD may provide clues to its etiology.

By looking at the medical literature, it soon becomes apparent that the epidemiology of DD has been previously studied extensively albeit only in limited geographical areas [23]. There has been a much quoted concept of DD being labeled as the “Vikings” or “Nordic” disease. Other than the common prevalence of DD in Scandinavia, no objective scientific evidence has been found to date to support and substantiate the “Nordic” origin of the disease.
Revised Tubiana’s Staging System for Assessment of Disease Severity in Dupuytren’s Disease—Preliminary Clinical Findings

Sandip Hindocha · John K. Stanley · James Stewart Watson · Ardenh Bayat

Received: 13 May 2007 / Accepted: 8 August 2007 / Published online: 11 September 2007
© American Association for Hand Surgery 2007

Abstract There are few objective staging systems to assess severity of Dupuytren’s disease (DD). Previous methods to assess severity of DD were based primarily on the degree of contracture of an affected digit measured using a goniometer. Nonetheless, this method of assessment alone may be incomplete, and other factors should be considered. White (n=92) patients diagnosed with DD from northwest of England were assessed for DD. Objective criteria for evaluating severity incorporated quantified variables. The revised severity stage was correlated to a known staging system of DD (Tubiana’s staging system) which measures total flexion deformity for a single affected digit. Total revised severity staging scores ranged between 4 and 53 (mean 18.7) and revealed significant positive correlation to Tubiana’s original staging system (r=0.8, p<0.001). There was significant difference between severity staging scores in those with a positive family history compared to those without (p<0.01). In current practice, often, the degree of contracture in an affected digit is used solely as a measure of disease severity. Additional objective clinical information may provide useful prognostic indices for disease progression as well as postoperative outcome.

Keywords Dupuytren’s disease · Contracture · Dupuytren diathesis · Ectopic dupuytren disease · Carpal’s pad · Disease prognosis · Risk factor · Family history · Severity

Introduction

Dupuytren’s disease (DD) is a benign condition that can present with varying severity. DD is a progressive fibro-proliferative disorder resulting in abnormal “scar-like” tissue in the palmar fascia [6] leading to irreparable, permanent, and progressive contracture of the involved digits. DD is commonly bilateral, and “Dupuytren-like” fibrotic tissue can occur on the dorsum of the hand over the knuckles (Garrod’s pads), feet (Lederman’s disease), and penis (Peyronie’s disease) [28]. DD is not only physically and psychologically disabling [12], but can also be aesthetically displeasing.

The decision to carry out surgical correction is often dependent upon the surgeon’s evaluation of clinical severity of the disease. Severity is often based upon measurement of flexion deformity using a goniometer. Another common assessment of severity of disease is the table top test; unfortunately, these methods of assessment may not be sufficient in planning surgical management, as factors which may affect recurrence of disease may not have been considered. Severity of DD and outcome after surgery based upon the measurement of contracture of an affected digit [10] (Table 3) [30] was a system introduced by Tubiana and has been used by clinicians in aiding surgical
radius removed to simulate an unstable extrarticular fracture. Each construct, with six plates from each system, was tested to failure in axial compression. Six separate constructs with each plate type then underwent 10,000 cycles applying 100% of compression to simulate physiology wrist motion and were then tested to failure in axial compression. Two plates from each system were also implanted in fresh frozen cadaver wrists with a section of distal radius removed in an identical fashion for testing. The cadaver wrists were tested in axial load to failure for comparison to the sawbones. The four plates used were the Hand Innovations DVR-A, Avanta SCSV, Wright Medical Lo-Con VLS, and Synthes volar plate.

Results All groups were loaded to failure. All failed with an apex volar angulation. The Hand Innovations DVR-A plate demonstrated significantly more strength in load to failure and failure after fatigue cycling (p = 0.00007 for single load and p = 0.0019 for fatigue failure) in a sawbones model. In cadavers there was no significant difference among the groups (p = 0.85) in axial compression failure. The cadaveric model demonstrated an identical failure mode to sawbones, but forces were approximately a magnitude higher to cause failure of the bone. The Avanta SCSV plates were the only noted to have any amount of pullout of the distal fixation, occurring in two of twelve plates.

Conclusions Volar fixation of unstable distal radius fractures with a fixed angle device is a reliable means of stabilization. The Hand Innovations DVR-A plate fixation system was the most rigid of the systems tested.

Why Plate? Fractures of the Distal Radius: A Unique Approach

Institution where the work was prepared: M. Ather Mirza MD PC, Smithtown, NY, USA

M. Ather Mirza, MD; Mary Kate Reinhardt, CNP; M. Ather Mirza, MD, PC

Purpose To assess the radiographic, clinical and functional outcome of patients with distal radius fractures treated with a minimally invasive, non-bridging external fixator.

Methods Over an 18 month period, patients with distal radius fractures (DRF's), extra-articular; displaced, non-displaced and intra-articular; non-displaced, reducible displaced fractures were treated with a minimally invasive cross pin fixation (CPX) system with an unstable lightweight non-bridging external fixator/strut. A removable, custom splint applied 5--7 days post-operatively allowed early mobilization of the wrist. Radiographic measurements radial height, radial inclination, and palmar tilt were recorded post reduction, post removal of fixation and at 6 months post-op. Outcome instrument scores were obtained 5--10 days, 3, 6 and 12 months post-op using the DASH and Patient Rated Wrist Hand Evaluation (PRWH).

Wrist range of motion (ROM), grip and pinch strengths were measured at specific intervals by an Occupational/Certified Hand Therapist.

Results 26 consecutive patients were treated with the CPX external non-bridging system. 1 patient was excluded in the early postoperative period due to non-compliance. The remaining 25 patients, 19 females and 6 males, mean age 60 (range 28--87) presented as 11 right, 15 left dominant, 10 non-dominant, 1 mixed dominance DRF's. Anatomic reduction was maintained. Wrist ROM was compared to the contralateral side, at 12 weeks dorsiflexion 77%, volarflexion 66%, pronation 94%, supination 83%, and at 1 year DF 92.5%, VP 87%, pronation 96%, supination 102%, was achieved. At 6 months, mean grip strength was 84% of the contralateral side and 98% at 1 year. Instrument outcomes at 6 months and 1 year revealed mean scores respectively, DASH 17.5 and 9.43, PRWHE 22 and 15.

Conclusion This study demonstrates that the CPX external non-bridging system is an effective minimally invasive surgical procedure for stabilization of DRF's. Radial height, palmar tilt and radial inclination are maintained. Good clinical and functional results were obtained as well as comparable DASH and PRWHE outcome scores.

Dupuytren's Diathesis Revisited: Modification of an Important Prognostic Indicator

Institution where the work was prepared: University of Manchester, Manchester, United Kingdom

Sandip Hinduja, MBChB; John K. Stanley, MCh, Orth, FRCS; Stewart J. Watson, MRCP, FRCS; Ardeshir Bayat, MD, PhD; University of Manchester

Houston originally coined the term “diathesis” relating to certain features of Dupuytren’s disease (DD) dictating an aggressive course of disease. His initial description of DD diathesis include four factors: ethnicity, family history, bilateral DD and extropic lesions. The degree of diathesis is considered highly significant in predicting recurrence (new DD lesions in the same arm of surgery) and extension (new DD lesions outside the arm of surgery) of DD following surgical management. However, to date there are no clear data regarding the accurate predictive value of various features of DD diathesis. Prognostic indicators of risks associated with surgery are important. We aim to evaluate the current criteria and formulate a statistical predictive value for DD diathesis.

Caucasian patients diagnosed with DD between the ages of 25--90 years (n = 322) from Northwest of England were assessed for DD diathesis with a clinical history and examination. DD diathesis assessment was analysed by calculating odds ratios of developing recurrent DD using
absorbable suture anchor of comparable size and button fixation.

Methods Fifteen fresh-frozen cadaveric hands were used. The index, long, and ring finger were harvested for testing. Fifteen digits were randomly assigned to each of the three fixation techniques. Tendon-to-bone repair was done using the button, absorbable suture anchor, and non-absorbable suture anchor technique. Specimens were tested to failure using the Bionex-MTS system. Mode of failure and force to failure of fixation was recorded and statistically analyzed.

Results Force to failure was not significantly different among the three fixation techniques (p > 0.05). The mode of failure for the button technique was suture pull-out through tendon (100%). The mode of failure for the absorbable suture anchor was rupture at the anchorage interface (80%) and anchor pull-out (20%). The mode of failure for the non-absorbable suture anchor was suture pull-out through the tendon (60%) and rupture at the anchor-suture interface (40%).

Discussion Bone quality, bone-anchor interface, and suture material play an important role in fixation strength. An insight to the mode of failure suggests that the best fixation technique in osteoporotic bone is the button technique or the non-absorbable suture anchor.

A New Scoring System for Assessing Severity in Dupuytren’s Disease

Total severity score ranged between 3 and 37 (mean = 14.1, SD = 7.9) and revealed significant positive correlation to a known staging system (r = 0.8, p < 0.001).

Previously degree of contracture in an affected digit was used to predict surgical outcome. It is evident from this study that other factors should also be considered when grading severity that may influence post-operative results. Thus a new severity scoring system for DD has been introduced.

Severe Peripheral Vascular Disease of the Upper Extremities. A case study

Institution where the work was prepared: Providence Hospital/Mayaguez Medical Center, Southfield, MI, USA
Guillermo E. Bello-Rojas, MD; Providence Hospital

Extensive upper extremity tissue loss usually results from atherosclerotic, vascular disorders or distal thromboembolic events. This then becomes a challenge for the vascular and hand surgeons. Salvage procedures such as in-situ vein grafting, arteriovenous reversal or proximal to distal bypass procedures are necessary.

A 55-year-old female with a history of insulin-dependent diabetes mellitus, chronic renal failure and chronic hypertension presented with sudden onset of pain in both upper extremities with necrosis of the 3rd right digit and the 3rd and 4th left digits. There was no evidence of infection but necrosis extended to the proximal phalanges. Distal pulses were absent bilaterally.

A bilateral upper extremity angiogram showed occlusion of the ulnar and radial arteries with dilatation of the superficial palmar arches.

Reversed saphenous vein grafts were used as conduits in both extremities. A right brachial to ulnar artery bypass was performed initially and four weeks later a left brachial to radial bypass was performed. Both bypasses were anastomosed distally close to the origin of the corresponding arch. Her postoperative progress was uneventful. Finger systolic pressures were measured and this showed significant improvement of flow. Areas of necrosis become demarcated and the affected digits were amputated several weeks later. At six months, graft patency was evaluated with an angiogram of the upper extremities, and flow to both deep palmar arches was confirmed.

Chronic severe ischemia of the upper extremity is a rare entity because of the collateral network of the limb. Compared to the lower extremity, arterial reconstruction of the upper extremity is rarely performed. There are few publications regarding bypass grafting of the upper extremity and its long-term results. Though ischemia of
Dupuytren’s Diathesis Revisited: Evaluation of Prognostic Indicators for Risk of Disease Recurrence

Sandip Hindocha, MBChB, John K. Stanley, MChOrth, Stewart Watson, MBChB, Ardesthir Bayat, PhD

From Plastic & Reconstructive Surgery Research, The Manchester Interdisciplinary Biocentre, University of Manchester, Manchester, UK; the Centre for Hand & Upper Limb Surgery, Wigan, Leigh and Wightington NHS Trust, Wigan, Lancashire, UK; and the Department of Plastic, Reconstructive and Hand Surgery, South Manchester University Teaching Trust, Wythenshawe Hospital, Wythenshawe, Manchester, UK.

Purpose: The term diathesis relates to certain features of Dupuytren’s disease (DD) and dictates an aggressive course of disease. The initial description of DD diathesis included 4 factors: (1) ethnicity, (2) family history, (3) bilateral DD, and (4) ectopic lesions (DD outside the palm). The degree of diathesis is considered important in predicting recurrence and extension of DD after surgical management. Prognostic indicators of risks associated with surgery are important. We aimed to evaluate these 4 factors and known associated risk factors to formulate a statistical predictive value for DD diathesis.

Methods: Caucasian patients diagnosed with DD between the ages of 25 and 90 years (n = 322) from Northwest England were assessed for DD diathesis with a clinical history and examination. DD diathesis assessment was analyzed by calculating the odds ratios of developing recurrent DD using logistic regression.

Results: The observed recurrence rates in the presence of notable risk factors and corresponding odds ratios of recurrent DD were calculated. Of note, recurrent disease was observed in 121 (44%) males, 105 (47%) with bilateral DD, 68 (48%) with a family history of DD, 75 (47%) with age at onset younger than 50 years, 35 (22%) in those with ectopic lesions, and in 26 (63%) with Carrod’s pads.

Conclusions: The original DD diathesis factors have been evaluated and modified. The original factors of family history, bilateral DD, and ectopic lesions now include 2 additional factors: male gender and age at onset of younger than 50 years. Family history and ectopic disease have been modified to specify family history with one or more affected siblings/parents and ectopic lesions in the knuckles (Garrod’s pads) alone. The presence of all new DD diathesis factors in a patient increases the risk of recurrent DD by 71% compared with a baseline risk of 23% in those DD patients with none of the earlier-described factors. (J Hand Surg 2006;31A:1626–1634. Copyright © 2006 by the American Society for Surgery of the Hand.)

Type of study/level of evidence: Prognostic II.

Key words: Disease prognosis, Dupuytren’s disease, Dupuytren’s contracture, Dupuytren’s diathesis, ectopic Dupuytren’s disease, risk factor, recurrence.

Dupuytren’s disease (DD) is a benign, progressive, fibroproliferative disorder that results in the development of abnormal scar-like tissue in the palmar fascia of the hand extending to any digit.\(^1\) In its advanced stages, DD finally leads to an irreversible, permanent, and progressive contraction of the involved digits. Dupuytren’s disease in the hands is commonly bilateral, but Dupuytren’s-like fibrotic tissue also can occur in the dorsum of the hand over the knuckles (Garrod’s pads), feet (Lederhose’s disease), and penis (Peyronie’s disease) of the same individual.\(^2\)
The Heritability of Dupuytren’s Disease: Familial Aggregation and Its Clinical Significance

Sandip Hindocha, MBChB, Sally John, PhD, John K. Stanley, MBChB, Stewart J. Watson, MBChB, Ardeshir Bayat, PhD

From the Centre for Integrated Genomic Medical Research, University of Manchester, Manchester; the University of Manchester Centre for Hand and Upper Limb Surgery, Wigan, Leigh and Wigan NHS Trust, Wigan, Lancashire; and Plastic and Reconstructive Surgery Research, South Manchester University Teaching Trust, Wythenshawe Hospital, Manchester, United Kingdom.

**Purpose:** Dupuytren’s disease (DD) is a benign, fibroproliferative disease affecting the hands. The familial occurrence of DD and its presence in identical twins suggests a genetic basis for the condition. Our aims in this study were (1) to provide evidence for familial aggregation of DD by estimating the sibling recurrence-risk ratio and (2) to link previously associated environmental risk factors with family history of DD.

**Methods:** Patients diagnosed with DD between the ages of 58 and 81 years (N = 92) were interviewed to assess potential risks and the severity of their condition. A clinical history and examination were performed and we attempted to interview every family member either in person or through a postal questionnaire.

**Results:** The sibling recurrence-risk ratio (A) equaled 2.9 and ranged from 2.6 to 3.3 based on the 95% confidence intervals for the population prevalence. This suggests a high genetic basis for the causation of DD. A lower age of onset and greater severity of DD were associated significantly with a positive family history of DD. Other factors showed no statistical significance with familial aggregation of DD.

**Conclusions:** The familial clustering observed in DD likely is due to genetic influence rather than shared environment, as shown by the lack of association with exposure to environmental risk factors and family history. Understanding the genetic basis of DD is important for developing novel diagnostics, preventative, and therapeutic regimens in the future. (J Hand Surg 2006;31A: 204–210. Copyright © 2006 by the American Society for Surgery of the Hand.)

**Type of study/level of evidence:** Prognostic, Level II.

**Key words:** Dupuytren’s disease, familial aggregation, genetics, heritability, sibling recurrence risk.

Dupuytren’s disease (DD) is a benign fibroproliferative disorder that results in the characteristic formation of thick, scar-like tissue in the palmar fascia of the hand extending to any digit. It is chronic and progressive. The condition is bilateral in more than 50% of cases, usually affects the ring and little fingers, and can involve other areas such as the fingers (also known as Ledderhose’s disease), the palm (also known as Peyronie’s disease) of the same individual. It is considered to be one of the commonly inherited connective-tissue disorders affecting whites of northern European descent. The prevalence of DD is more than 4% in the male population in England, with an incidence of more than 25% in the Celtic population aged over 60 years.

Many environmental factors have been associated with the etiology of DD, including a history of smoking, frozen shoulder, epilepsy, diabetes mellitus, and a lipid profile. This mysterious etiology has kept it subject of immense interest, leading to many genetic and molecular studies trying to discover the underlying genetic basis for DD.

223
Appendix 2.1. Dupuytren's Disease study: Patient consent form

Patient Identification Number for the Study:

Title of Project: The genetics of Dupuytren’s disease
Name of Researcher: Mr S Hindocha

Please INITIAL box

1. I have read and understood the information sheet (version 1) and have had the opportunity to ask questions.

2. I understand that my participation in this study is voluntary and withdrawal from this study will not affect my medical care.

3. I understand that the information obtained from the study will be entirely confidential to the researchers. I understand that the information will not be passed to anyone not directly involved in the analysis and collection of information. I understand the information will be held in a manner which codes my identity and the master list is held in a secure place.

4. I give permission to take photographs of my hand. I understand that these photographs may be used for presentation/publications at scientific meetings/journals.

5. I give permission to take my blood sample for identifying and investigating the gene/s responsible for Dupuytren’s disease formation.

6. I understand that in the event of finding a treatment for Dupuytren’s disease as a result of this study, I will not be entitled to make a financial claim or receive financial benefits from the sale of any products associated with this project.

7. I understand that the results of any analysis of the information will not be entered into my General Practitioner or hospital records without my express permission.

8. I agree to take part in this study.

Name of Patient Date Signature of Patient

Researcher Date Signature
### Appendix 2.2. Genetics of Dupuytren’s Disease – Data collection proforma

<p>| Patient I.D: |  |</p>
<table>
<thead>
<tr>
<th>Age:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender; delete as appropriate:</td>
<td>Male / Female</td>
</tr>
<tr>
<td>Race; tick as appropriate:</td>
<td>Caucasian:</td>
</tr>
<tr>
<td></td>
<td>o English</td>
</tr>
<tr>
<td></td>
<td>o Scottish</td>
</tr>
<tr>
<td></td>
<td>o Irish</td>
</tr>
<tr>
<td></td>
<td>o Other; please specify……………</td>
</tr>
<tr>
<td>Asian:</td>
<td>o Pakistani</td>
</tr>
<tr>
<td></td>
<td>o Bangladeshi</td>
</tr>
<tr>
<td></td>
<td>o Indian</td>
</tr>
<tr>
<td></td>
<td>o Other; please specify……………</td>
</tr>
<tr>
<td>Black:</td>
<td>o African</td>
</tr>
<tr>
<td></td>
<td>o Caribbean</td>
</tr>
<tr>
<td></td>
<td>Other; please specify……………</td>
</tr>
<tr>
<td>Please state your profession. If you are retired, specify the year you retired. Please give details of any manual work, e.g. lifting heavy goods or light work such as typing:</td>
<td></td>
</tr>
<tr>
<td>Please indicate any hobbies you have that involve using hands, e.g. gardening or sewing:</td>
<td></td>
</tr>
<tr>
<td>Indicate which hand you write with, please tick one:</td>
<td>o Right</td>
</tr>
<tr>
<td></td>
<td>o Left</td>
</tr>
<tr>
<td></td>
<td>o Both</td>
</tr>
</tbody>
</table>
**Past Medical History**
Do you suffer with any of the following medical conditions?

<table>
<thead>
<tr>
<th>Condition</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feet affected with Dupuytren’s nodules like those in the hand:</td>
<td>☐</td>
<td>☑</td>
</tr>
<tr>
<td>Knuckles affected with Dupuytren’s nodules like those in the hand:</td>
<td>☐</td>
<td>☑</td>
</tr>
<tr>
<td>Diabetes:</td>
<td>☐</td>
<td>☑</td>
</tr>
<tr>
<td>Epilepsy; please specify any medication for epilepsy and how long you have been taking it for:</td>
<td>☐</td>
<td>☑</td>
</tr>
<tr>
<td>Carpal tunnel syndrome:</td>
<td>☐</td>
<td>☑</td>
</tr>
<tr>
<td>Frozen shoulder:</td>
<td>☐</td>
<td>☑</td>
</tr>
<tr>
<td>Hypertension:</td>
<td>☐</td>
<td>☑</td>
</tr>
<tr>
<td>Alcohol related liver disease:</td>
<td>☐</td>
<td>☑</td>
</tr>
<tr>
<td>Problems with scarring after an injury:</td>
<td>☐</td>
<td>☑</td>
</tr>
<tr>
<td>Other medical problems:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Please state your current medication

Do you suffer with any allergies? If yes please specify:  
<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
</table>

Do you drink alcohol? If yes please specify number of units/week and duration:  
1 unit = half pint beer OR 1 glass wine OR 1 shot of spirit.  
<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
</table>

Are you a smoker / ex-smoker? If yes please specify for how long, and how many patient smokes / did smoke.  
<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
</table>
Your details of Dupuytren’s disease

<table>
<thead>
<tr>
<th>Finger</th>
<th>Date of onset of Dupuytren’s:</th>
<th>Injury to this finger; give details:</th>
<th>Symptoms prior to surgery, e.g. pain</th>
<th>Any treatment? Please specify:</th>
<th>Good / bad result:</th>
<th>Recurrence of Dupuytren’s? Give date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right little</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right ring</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right middle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right index</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right thumb</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left little</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left ring</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left middle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left index</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left thumb</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Family History of Dupuytren’s disease

Please tick the relevant:

<table>
<thead>
<tr>
<th>RELATION</th>
<th>AGE</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Father</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Mother</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Brother</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Brother</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Sister</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Mother</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Brother</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Brother</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Sister</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Mother</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Brother</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Brother</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Sister</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Mothers side:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Father</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Mother</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Brother</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Brother</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Sister</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Father</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Mother</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Brother</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Brother</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Sister</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Fathers side:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Father</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Mother</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Brother</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Brother</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Sister</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Sister</td>
<td>○ Yes</td>
<td>○ No</td>
<td>○ Don’t know</td>
</tr>
<tr>
<td>Others; please specify – e.g. sons, daughters, cousins, nephews, nieces.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Family tree:

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feet affected with Dupuytren’s nodules</td>
<td></td>
<td></td>
</tr>
<tr>
<td>like those in the hand:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Knuckles affected with Dupuytren’s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nodules like those in the hand:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix 2.3. Genetics of Dupuytren’s Disease Family Questionnaire

Family Number

<table>
<thead>
<tr>
<th>Your relation to the patient:</th>
<th>Male / Female</th>
</tr>
</thead>
</table>

Gender; delete as appropriate:

How do you know if you have Dupuytren’s Disease?
Dupuytren's Disease is a thickening of deep tissue (fascia) which frequently develops from the base of the fingers into the palm. The first sign is usually a small amount of thickened tissue that can be described as a “nodule”. The nodule is usually painless but may be tender to touch. Other nodules may develop and you may notice that you cannot fully straighten your finger(s). This thickening may get larger causing "bands" to develop which pull the fingers into the palm. If left untreated the fingers may slowly be pulled into the palm. It can also cause dimples in the skin of the palm also know as “pits”. You may also notice nodules on the knuckles or on the soles of your feet. Below are some pictures describing these features.

Thickening in the palm (bands).

In this picture we see Dupuytren’s in both hands. On the left the little finger has been pulled into the palm. On the right we see thickening on the palm and the ring finger also being pulled in.

Here we see a nodule on the knuckle.

This picture shows nodules in the soles of the feet.
Please delete as appropriate:

<table>
<thead>
<tr>
<th>Question</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Do you have any nodules on either of your palms?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Are you unable to straighten any of your fingers? If so which fingers are you unable to straighten?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you have any nodules on the soles of your feet?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you think you have Dupuytren’s? If you have already diagnosed with Dupuytren’s disease / if you think you may the condition please fill in the table below.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix 2.4 Family pedigrees from the UK DD cohort

Key:
- Individuals are represented by squares or circles.
- Squares: Males.
- Circles: Females.
- Shaded shapes = affected. Non-shaded = non-affected.
- Diagonal line through individual = deceased.
- Arrow to bottom left indicates proband.
- Top left details demographic data with current age and presence of certain medical conditions: D1 = insulin dependent diabetic; D2 = non-insulin dependent diabetic; H = hypertensive; E = epileptic; CTS = carpal tunnel syndrome; FS = frozen shoulder; S = smoker; ExS = ex smoker; a number followed by u = units of alcohol consumed in one week.
- Bottom left details data in relation to DD: First number is age at onset; B = bilateral disease; U = unilateral disease; RL,RR,RM,RI,RT = Right Little, Ring, Middle, Index, Thumb affected; LL,LR,LM,LI,LT = Left Little, Ring, Middle, Index, Thumb affected; I = injury; Rec = recurrent disease; Gar = Garrod’s pads; Led = Lederhoses disease.
Pedigree 9

Pedigree 10
Pedigree 21

Pedigree 22
Pedigree 31

Pedigree 32
Pedigree 37

Pedigree 38
Pedigree 39

Pedigree 40
Pedigree 47

Pedigree 48
Pedigree 55

Pedigree 56
Pedigree 67

Pedigree 68
Pedigree 71

Pedigree 72
Pedigree 79

Pedigree 80
Pedigree 81

Pedigree 82
Pedigree 87

Pedigree 88
Pedigree 89

Pedigree 90
Pedigree 105

Pedigree 106
Pedigree 107

Pedigree 108
Pedigree 111

Pedigree 112
Pedigree 117

Pedigree 118
Pedigree 131

Pedigree 132
Pedigree 133

Pedigree 134
Pedigree 135
Appendix 3.1

LETTER OF INTENT

Collaboration Agreement.

I

Mr. Ardeshir Bayat, Dr. Sandip Hindocha and Professor Duncan McGrouther from Manchester Interdisciplinary Biocentre and South Manchester University NHS Foundation Trust (The Manchester Group) and Dr. Kristján G. Guðmundsson, Dr. Þorbjörn Jónsson and Dr. Reynir Arngrímsson from Glæsibær Primary Health Care Centre, The National University Hospital and the Medical Faculty of University of Iceland (The Icelandic Group) have by this Letter of Intent agreed to enter into a collaboration to identify biomarkers in Dupuytren’s disease.

II

The role of the Manchester group
a) The Manchester group will provide facilities for genomics research at the Manchester Interdisciplinary Biocentre and will be responsible for all cost from genomics research provided by that facility. This includes all cost from DNA extraction, genotyping of single nucleotide polymorphism (SNP) and microsatellites in accordance with a research protocols agreed on by both parties of this agreement. This also includes cost of statistical and genomic linkage analyses that are necessary and transport of samples from Iceland to Manchester.
b) The Manchester group will provide families and case-control material for genomics research aimed at identifying susceptibility and candidate genes.
c) The Manchester group will participate in analyses, interpretation and publication of the study results.

III

The role of the Icelandic group
a) The Icelandic group will provide research material for genomic studies, i.e. one large multigenerational family where Dupuytren’s disease is highly prevalent and samples identified for case-control study approximately 100 cases and 100 controls.
b) The Icelandic group will participate in analyses, interpretation and publication of the study results as well as creating joint research protocols.
c) The Icelandic group is not liable for any financial cost from any research, analyses or publications from this research.

IV

Research approach
The research groups will aim to carry out genome-wide scan, by best available methods for genomics linkage analyses of the Icelandic multigenerational family, followed by further genomic studies of Manchester families and case-control material by polymorphism association studies including candidate gene research.

V

Follow-up research
In the case of successful collaboration and positive findings from genomics research both parties commit to further collaborative effort in identification of biomarkers in Dupuytren’s disease. This includes that the Icelandic group will aim to collect and
provide further case-control material for genomic research. Both parties will undertake tissue sampling for transcriptomics research and appropriate samples for proteomics and metabolomics studies. Both parties will participate in creating research protocols and apply for financial support for such follow-up studies.

VI
Publications
Both parties agree that results from this collaboration will be jointly published in a relevant medical or bioscience journal. All six members of this collaboration will be authors on every publication created from this research. Authorship by other researchers involved in the studies will be in accordance with recommended guidelines for publications of biomedical research.

As this collaborative research will provide material for the doctoral thesis of Dr. Sandip Hindocha he will be first author on publications where he is the major contributor to the research. Dr. Kristján G. Guðmundsson who is the main contributor of the Icelandic group in collecting and clinically classifying the Icelandic material will be second author and their contribution shall be noted to be equal when it comes to publication of results from Icelandic material. When the publication is mainly describing result from Icelandic research material the corresponding author shall be Icelandic and when the publication is mainly describing results from Manchester research material the corresponding author shall be from the Manchester group.

Dr Sandip Hindocha and Dr. Kristján G. Guðmundsson will appear as primary co-authors. In addition, Dr Ardeshir Bayat will design, fund, supervise the experimental work and write up the manuscript with relevant input from all co-authors and appear as senior author. Correspondence of any published material related to our collaborative work will be jointly addressed by myself as the senior author and Kristján as the primary co-author representing the Icelandic arm of the study. Reynir, Óorbjörn and Gus will appear as co-authors in all manuscripts and presentations related to this work.

VII
Transfer of research rights and financial benefits
In the event of successful research with positive findings the rights to financial benefits from any transfer agreement of copyright and results shall be jointly explored by both parties, but only if both parties agree up on such an transaction. In the event of both parties, i.e. the Manchester group and the Icelandic group agree up on a transfer agreement to a third party for further exploration of results or development of management strategies for Dupuytren’s disease, financial benefits shall be divided equally between both parties.

VIII
End of collaboration
When both parties have completed analyses of research that both parties have prior agreed up on this agreement will end. At the end of the research each party will return all samples or what is left of these to the party that contributed them to the joint research. Both parties will have access to results and analyses during the study period and there after as necessary.
Appendix 3.2: DNA extraction protocol

1. Blood samples were collected in EDTA tubes. 5ml of blood was transferred from Iceland and transported using ethically agreed terms to the University of Manchester. 10ml of red cell lysis buffer (RCLB) was added thoroughly mixed. This was then centrifuged for 5 min at 6000rpm.

2. The above was repeated until a clear pellet of white blood cells appeared at the base of the tube.

3. The supernatant was discarded and 800µl of white blood cell lysis buffer (WBCLB) and 10µl of proteinase K (10mg/ml) (Fisher Scientific, UK) was added to the precipitate and incubated at 65°C for 2 hours.

4. An equal volume of phenol chloroform (Sigma-Aldrich, UK) was added, mixed and centrifuged at 6000rpm for 5 mins.

5. The upper layer was transferred into a clean tube and lower phenol layer was disposed of.

6. A further equal volume of chloroform isomyl alcohol was added, vortexed and centrifuged for 5 mins at 6000 rpm.

7. The upper layer was transferred into a clean tube and 2 volume of 95% cold ethanol and then 1:10 of sample volume of 3M sodium acetate was added and incubated at -20°C overnight.

8. The mixture was centrifuged for 10min at 12000 rpm and the supernatant discarded.

9. A further 2ml of 70% ethanol was added mixed thoroughly and centrifuged for 7min at 12000 rpm, the supernatant was discarded appropriately.

10. The previous step was repeated.
11. The remaining pellet was allowed to dry for 15 minutes.

12. The pellet was resuspended in 100µl of ddH₂O, the concentration of DNA measured using the NanoDrop and stored at -20°C.
Appendix 3.3: Whole genome scan protocol

Stage 1: Sty restriction enzyme digestion

1. Prepare plate of genomic DNA.
2. Prepare the Sty Digestion Master Mix, vortex, and spin.
3. Add Sty Digestion Master Mix to one plate genomic DNA samples.
   A. Aliquot master mix to one set of 12 strip tubes, 67 mL each tube.
   B. Using a multichannel pipet, add 14.75 mL of Sty Digestion Master Mix to each well of the sample plate.
   C. Seal, vortex, spin.
   D. Place the plate onto a thermal cycler and run GW5.0/6.0 Digest program.

Stage 2: Sty Ligation

1. Prepare the Sty Ligation Master Mix, vortex, and spin.
2. Add Sty Ligation Master Mix to Sty digested samples.
   A. Aliquot master mix to one set of 12 strip tubes, 25 mL each tube (ensure no air bubbles at bottom of tubes).
   B. Using a multichannel pipet, add 5.25 mL of master mix to each digested sample.
   C. Seal, vortex, spin.
   D. Place the plate onto a thermal cycler and run the GW5.0/6.0 Ligate program.
3. Dilute the Sty ligated samples.
   A. Spin down the Sty ligated samples.
   B. Pour 7.5 mL AccuGENE water into reagent reservoir.
   C. Using a multichannel pipet, add 75 mL of water to each Sty ligated sample.
   Change pipet tips for each row.
   D. Seal, vortex, spin.
4. Transfer diluted samples to three PCR plates.
   Using a multichannel pipet, transfer 10 mL of each diluted, ligated sample to three PCR plates. Be careful not to mix up samples during transfer.

Note:
• Thaw Ligase Buffer on ice. Vortex to resuspend any precipitate. Avoid multiple freeze/thaw cycles. Contains ATP which degrades at room temperature.
• Leave the T4 DNA Ligase at -20 °C until ready to use. Pulse spin before adding to master mix. Do not vortex.
• Use the correct Adaptor (Sty 1). Using the wrong adaptor will result in primer dimers at the PCR stage.
• Keep all plates in cooling chambers on ice.

Stage 3: Sty PCR
1. Prepare the Sty PCR Master Mix in a 50 mL tube on ice and vortex.
2. Add Sty PCR Master Mix to Sty ligated, diluted samples.
   A. Pour master mix into a reagent reservoir on ice. B. Using a multichannel pipet, add 90 mL of master mix to each sample.
      1. Change pipet tips after each addition. C. Seal, vortex, spin and place each plate back in a cooling chamber on ice. D. Transfer plates to the Main Lab.
3. Load Sty PCR Plates onto Thermal Cyclers
   A. Ensure the thermal cycler lids are preheated. B. Place each plate onto a thermal cycler and run the appropriate GW5.0/6.0 PCR program
      1. within 30 min of adding master mix.
      2. Note:
         Add the correct amount of each reagent to master mix, particularly the primers. Leave the Taq polymerase enzyme at -20 deg C until ready to use. Be sure to run the correct PCR program (based on thermal cycler brandname).
         Keep all plates in cooling chambers on ice.

Stage 4: Nsp restriction enzyme digestion
1. Prepare plate of genomic DNA.
2. Prepare the Nsp Digestion Master Mix, vortex, and spin.
3. Add Nsp Digestion Master Mix to one plate genomic DNA samples.
   A. Aliquot master mix to one set of 12 strip tubes, 67 mL each tube. B. Using a multichannel pipet, add 14.75 mL of Nsp Digestion Master Mix to each well of the sample plate. C. Seal, vortex, spin. D. Place the plate onto a thermal cycler and run GW5.0/6.0 Digest program.
   Note:
• Leave Nsp I enzyme at -20 °C until ready to use. Pulse spin before adding to master mix. Do not vortex. • Keep all plates in cooling chambers on ice.

Stage 5: Nsp ligation
1. Prepare the Nsp Ligation Master Mix, vortex, and spin.

2. Add Nsp Ligation Master Mix to Nsp digested samples.
   a. Aliquot master mix to one set of 12 strip tubes, 25 mL each tube.
   b. Using a multichannel pipet, add 5.25 mL of master mix to each digested sample.
   c. Seal, vortex, spin.
   d. Place the plate onto a thermal cycler and run the GW5.0/6.0 Ligate program.

3. Dilute the Nsp ligated samples.
   a. Spin down the Nsp ligated samples.
   b. Pour 10 mL AccuGENE water into reagent reservoir.
   c. Using a multichannel pipet, add 75 mL of water to each Nsp ligated sample. Change pipet tips for each row.
   d. Seal, vortex, spin.

4. Transfer diluted samples to four PCR plates.

5. Using a multichannel pipet, transfer 10 mL of each diluted, ligated sample to three PCR plates. Be careful not to mix up samples during transfer.

6. Note:
   - Thaw Ligase Buffer on ice. Vortex to resuspend any precipitate. Avoid multiple freeze/thaw cycles. Contains ATP which degrades at room temperature.
   - Leave the T4 DNA Ligase at -20 °C until ready to use. Pulse spin before adding to master mix. Do not vortex.
   - Use the correct Adaptor (Nsp 1). Using the wrong adaptor will result in primer dimers at the PCR stage.
   - Keep all plates in cooling chambers on ice.

Stage 6: Nsp PCR

1. Prepare the Nsp PCR Master Mix in a 50 mL tube on ice and vortex.

2. Add Nsp PCR Master Mix to Nsp ligated, diluted samples.
   A. Pour master mix into a reagent reservoir on ice. B. Using a multichannel pipet, add 90 mL of master mix to each sample.
   Change pipet tips after each addition. C. Seal, vortex, spin and place each plate back in a cooling chamber on ice. D. Transfer plates to the Main Lab.

3. Load Nsp PCR Plates onto Thermal Cyclers
A. Ensure the thermal cycler lids are preheated. B. Place each plate onto a thermal
cycler and run the appropriate GW5.0/6.0 PCR program
within 30 min of adding master mix.
Note:
Add the correct amount of each reagent to master mix, particularly the primers. Leave
the Taq polymerase enzyme at -20 deg C until ready to use. Be sure to run the correct
PCR program (based on thermal cycler). Keep all plates in cooling chambers on ice.

Stage 7a: PCR purification using AMPure XP beads
1. Run PCR QC gel.
Mix 3 mL of each representative PCR product with 3 mL of 2X Gel Loading Dye
(select 1 representative DNA per sample across thermal cyclers; 48 Sty and 48 Nsp).
Load total volume of each well onto a 2% TBE gel with ladder. Run the gels at 120V
for 40 min to 1 hr. Verify that the PCR product distribution is between ~200 bp to
1100 bp. Save the gel image.
Pool Sty and Nsp PCR products into deep well plate using 12-channel P200 set to 110
mL.
Add magnetic beads, mix and incubate.
   1. Shake bottle of magnetic beads vigorously.
   2. Add 1.0 mL magnetic beads to each pooled sample.
   3. Mix well 5X by pipetting up and down using 12-channel P1200.
   4. Visually confirm that each well is mixed. E. Cover plate and incubate for 10
      min at room temperature.
Place Millipore filter plate on vacuum manifold; then transfer incubated samples to
filter plate (2 x 900 mL). Seal the empty wells.
Note:
Take care not to mix up samples while pooling. Work uninterrupted.
Do not touch filters with pipet tips. Preheat hybridization oven for 30 min at 50 °C
5. Apply vacuum until all of the liquid has been pulled through the filter (~40 to 60
min).
Visually check that all wells are dry, they should appear dull (matte).

6. Ensure all liquid is removed from the filter plate.
   A. With the vacuum on, tap the top of the plate twice with the palm of your
hand.

B. Turn the vacuum off and remove the plate.
C. Firmly blot the plate on lint-free tissue until no wet spots are observed.
D. Place the plate back on the manifold.
E. Turn on the vacuum for 3 min.
F. With the vacuum on, tap the top of the plate twice with the palm of your hand.

(Repeat step 6.A)

7. Wash each sample with 1.8 ml 75% EtOH (room temperature) using a P1200 as follows:

With the vacuum on, add 900 µl to each sample. After ~2 min under vacuum, add another 900 µl to each sample.
Continue to apply vacuum until all of the liquid has been pulled through the filter (~10-15 min). Visually check that all wells are dry. Maximum time 20 min.

8. Ensure all EtOH is removed from the filter plate

A. With the vacuum on, tap the top of the plate twice with the palm of your hand.
B. Turn the vacuum off and remove the plate.
C. Firmly blot the plate on lint-free tissue until no wet spots are observed.
D. Return the plate to the manifold and apply vacuum for 2 min ONLY.
E. Do not apply vacuum for more than 2 min. Leaving vacuum on for more than 2 min may over dry beads and inhibit elution.
F. Turn the vacuum off and remove the plate. G. Firmly blot the plate on lint-free tissue until no wet spots are observed.

9.
Elute DNA from beads at 50°C for 30 min.
A. Attach elution catch plate using lab tape. Seal on two sides only – do not seal completely.
B. Add 60 µl buffer EB to each sample. Buffer EB at 4 °C. Tap plate stack to move all buffer EB onto the filters at the bottom of each well.
C. Loosely cover with plate lid or clear lid from pipet tip box. D. Place the
covered plate stack in a 50°C hyb oven for 30 min
Put plate on the right front side (away from the air vent).
E. Remove the plate cover and seal all wells tightly with adhesive film. F.
   Place on Jitterbug for 30 min at setting 7.
Inspect each well to ensure all beads are resuspended.
G. Remove the seal and place plate stack INSIDE the vacuum manifold. Ensure
   empty wells are sealed.
H. Apply vacuum until all liquid has been pulled through (~5 to 15 min). Visually
   check that all wells are dry. If any wells are still wet or shiny, continue to apply
   vacuum for 15 min.
I. Turn off vacuum, seal entire plate and centrifuge the plate stack for 5 min at
   1400 RCF.
Centrifuge at room temperature.
J. Remove the catch plate containing the eluate and seal. There should be ~ 50 µl
   in each well.

10. Prepare fragmentation/label/hyb plate and measure OD.
   A. Mix eluted DNA before transfer for fragmentation and quantification on
      the Jitterbug for 5 min at setting 7.
   B. Spin briefly, then remove seal.
   C. Transfer 45 µl to a new PCR plate using a 12-channel P200 (this is the
      fragmentation plate).
   D. Dilute 2 µl in 198 µl water for OD measurement (Stage 8).
   NOTE:
      Do not extend vacuum steps beyond the maximum time listed. Over drying
      can result in incomplete elution. To avoid mixing up your samples, re-label the top 4
      rows of plate FLH2. Once samples are applied to the filter plate, keep at room
      temperature. If stored at 4 °C, yields will be reduced and array results compromised.

   Stage 8: Quantitation
   Catch plate with purified samples.
   2µl of each sample.
   Mix water and samples well before taking readings.
Stage 9: Fragmentation
1. Aliquot 10X Fragmentation Buffer to each sample.
   A. Aliquot Fragmentation Buffer to one set of 12 strip tubes, 28 mL each tube. B. Using a multichannel pipet, transfer 5 mL of the buffer to each sample.
2. Prepare the Fragmentation Master Mix on ice. Vortex, spin down, and keep on ice.
3. Add Fragmentation Master Mix to each sample.
   A. Aliquot Fragmentation Master Mix to one set of 12 strip tubes, 28 mL each tube. B. Using a multichannel pipet, transfer 5 mL of master mix to each sample. C. Seal, vortex. D. Place plate in chilled plastic plate holder and spin at 4 °C at 2000 rpm for 30 sec.
4. Transfer Plate to thermal cycler.
   A. Preheat the thermal cycler lid and block to 37 °C. B. Seal the plate tightly to avoid evaporation. C. Place plate on the thermal cycler and run the GW5.0/6.0 Fragment program.

Check the concentration of the fragmentation reagent. Master mix recipe above assumes 2.5 U/ mL.
Perform this step with great care and precision.

Keep fragmentation reagent at -20 °C, and always carry it in a cooler. All additions, dilutions, and mixes must be performed on ice. Spin plates in chilled plate holders. Fragmentation reagent is sticky and viscous. Make sure to pipet the correct volume.

Stage 10: Labelling
1. Run a fragmentation QC gel.
   A. Remove 1.5 mL of each sample. B. Add 4 mL of gel loading dye, mix well. C. Load onto 4% TBE gel with ladder and run at 120V for 30 min to 1 hr. D. Verify the average fragment size is < 180 bp.
2. Prepare the Labeling Master Mix, vortex and spin down.
3. Add Labeling Master Mix to each sample.
   A. Aliquot Labeling Master Mix to one set of 12 strip tubes, 89 mL each tube. B. Using a multichannel pipet, transfer 19.5 mL of master mix to each sample. C. Seal, vortex, spin.
4. Transfer plate to thermal cycler.
A. Preheat the thermal cycler block. B. Seal the plate tightly (both the center and the edges) to avoid evaporation. C. Place plate on the thermal cycler and run the GW5.0/6.0 Label program.

Stage 11: Target hybridization

1. Preheat the hybridization oven for 1 hr at 50 °C, 60 rpm.
2. Prepare the arrays and allow to warm to room temperature.
3. Prepare the Hybridization Master Mix on ice. Vortex, spin, and keep on ice.
4. Add Hybridization Master Mix to each labeled sample.
   A. Pour Hybridization Master Mix into a reagent reservoir.
   B. Using a multichannel pipet, transfer 190 mL of master mix to each sample.
   C. Seal the plate tightly, vortex, spin.
   D. Cut the adhesive film between each row of samples.
5. Transfer plate to thermal cycler and run the GW5.0/6.0 Hyb program. 6. Load each sample onto an array.
   A. Remove the film from one row.
   B. Load 200 mL from each well onto an array.
   C. Evenly space 4 arrays in an oven tray and place into the oven.
   D. Repeat this process until each sample has been loaded onto an array.
   E. Leave arrays in oven at 50 °C, 60 rpm for 16 to 20 hr.

Note:
Pipet reagents into the middle of the tube to avoid touching the side walls. Check for an “approved” sticker on the front of the hybridization oven. Do not allow loaded arrays to sit at room temperature more than ~1.5 min.
Appendix 3.4: Clinical photographs of Icelandic pedigree

Clinical photographs of consenting family members of the Icelandic pedigree are shown below. Descriptions of disease patterns are given in the main chapter text.