

Clinical and cellular studies in the pathogenesis of desmoid tumours in familial adenomatous polyposis (FAP)

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Declaration of Originality

I hereby declare that this thesis is my own composition and has not been previously submitted to any other university. I have acknowledged all contributors and collaborators (please see appendix A1).

The work described herein was carried out between April 2012 and October 2015, during my appointment as a Clinical Research Fellow at the Polyposis Registry, St Mark's Hospital, Harrow, UK.

Sarah-Jane Walton

May 2016

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Abstract

Desmoid tumours are rare, myofibroblastic, soft tissue tumours. However, in the context of familial adenomatous polyposis, they can affect up to a quarter of patients. Desmoid tumours remain the most significant extra-intestinal manifestation of this cancer predisposing, autosomal dominant, condition. Due to their rarity, and unusual growth behaviour, studying these tumours remains a challenge. The clinical studies in this thesis aimed to highlight the complications associated with desmoid tumours and their impact on patient quality of life. The laboratory studies sought to identify potential pathways involved in their growth and detect genetic variants that may influence this process.

A retrospective cohort study identified a 25% risk of ureteric compression associated with intra-abdominal desmoid (IAD) tumours and supported a role for long-term urinary tract ultrasound surveillance. Small bowel transplant for complicated IAD disease has become part of the treatment pathway and was found to be a viable treatment option for complex cases. A quality of life study survey involving 70 people with FAP (32 with desmoid) identified a reduced quality of life associated with desmoid. This was mostly attributable to pain limiting physical and social activities. Histological analysis supported activation of the non-canonical Wnt pathway in desmoid tumours. This was a novel finding that could offer pathway targets for future research. Cell culturing of desmoids was largely unsuccessful and led to the initial development of a three-dimensional cell culture scaffold. This could improve future prospects of developing a successful desmoid cell line. Next generation sequencing of desmoid germline and tumour DNA identified several potential genes of interest. An upregulation of miRNA-34a-5p was detected in the serum of patients with desmoid and was also identified in tumour

tissue. This could lead to future development of a circulating desmoid biomarker, whilst offering further pathways to explore for future research.

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List of abbreviations

2D – Two dimensional

3D – Three dimensional

5YSR- Five-year survival rate

AARs - Amino acid repeats

ALPK3 - Alpha-kinase 3 gene

APC - Adenomatous polyposis coli protein

APC - Adenomatous polyposis coli gene

AWD - Abdominal wall desmoid

BP – Bodily pain

CaCl₂ – Calcium chloride

cDNA – Complementary DNA

CHRPE - Congenital hypertrophy of the retinal pigment epithelium

CM - Caucasian Mediterranean

CNV - Copy number variation

CO₂ – Carbon dioxide

Ct – Cycle threshold

CT – Computed tomography

CTNNB1 - β -catenin gene

DAB – Diaminobenzidine

DMEM - Dulbecco's modified eagle medium

DMSA - Dimercaptosuccinic acid

DNA - Deoxyribonucleic acid

DPL - Desmoid precursor lesion

DSPP - Dentin sialophosphoprotein gene

DST - Dystonin gene

EAD - Extra-abdominal desmoid

ECM - Extra-cellular matrix

EDTA - Ethylenediaminetetraacetic acid

EMT - Epithelial to mesenchymal transition

EPPL1 - Epiplakin 1 gene

EQ-VAS - EQ visual analogue scale

FAP - Familial adenomatous polyposis

FBS – foetal bovine serum

FFPE - Formalin fixed paraffin embedded

FRG2B - FSHD Region Gene 2 Family, Member B gene

GAG - Glycoaminoglycan

GH - General health perceptions

GI - Gastro-intestinal

GO - Gene Oncology

GSK - Glycogen synthase kinase

H₂O₂ – Hydrogen peroxide

H&E - Haematoxylin and eosin

HCL – Hydrochloric acid

HGD – High grade dysplasia

HPN - Home parenteral nutrition

IAD - Intra-abdominal desmoid

IFN - Interferon

IMS - Industrial methylated spirit

InSiGHT - International Society for Gastrointestinal Hereditary Tumours

IQR – Interquartile range

IRA - Ileorectal anastomosis

IRDB - Institute of Reproductive Developmental Biology

ISH – In-situ hybridisation

KEGG - Kyoto Encyclopaedia of Genes and Genomes

KGC – Kennedy-Galton Centre

LGD – Low grade dysplasia

LNA - Locked nucleic acid

LOH – Loss of heterozygosity

MAG3 - Mercapto acetyl tri glycine

MgCl₂ – Magnesium chloride

mRNA - Messenger RNA

miRNA – MicroRNA

MGD – Moderate grade dysplasia

MH - Mental health

MRI – Magnetic resonance imaging

MSC - Mesenchymal stem cell

MUC4 – Mucin 4 gene

MYOF – Myoferlin gene

NaCl – Sodium chloride

NaOAc – Sodium acetate

NASIT - National adult small intestinal transplantation

NGS – Next generation sequencing

NPIMR - Northwick Park Institute for Medical Research

NRES - National Research Ethics Service

NSAIDs - Non-steroidal anti-inflammatory drugs

OGD – Oesophagogastrroduodenoscopy

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PF – Physical functioning (RE)

P&M - Picrosirius red with Miller's elastin

PN - Parenteral nutrition

qPCR – Quantitative PCR

QOL – Quality of life

QSOX1- Quiescin Q6 sulfhydryl oxidase 1 gene

RE - Emotional role functioning

RFA - Radiofrequency ablation

RNA - Ribonucleic acid

RP - Physical role functioning

RPC - Restorative proctocolectomy

SBO - Small bowel obstruction

SBS - Short bowel syndrome

SBT - Small bowel transplant

SDS - Sodium dodecyl sulphate

SF - Social role functioning

SF-36 - Short-form 36

SLC25A5 - Solute carrier family 25 member 5 gene

SNPs - Single nucleotide polymorphisms

SNVs - Single nucleotide variants

SOAT2 - Sterol O-acyltransferase 2 gene

SSC – Saline sodium citrate

TA – Tubular adenoma

TAF2 – TATA-box binding protein gene

TBS - Tris buffered saline

TE – Tris EDTA

TVA – Tubulovillous adenoma

TCF - T cell factor

TPC – Total proctocolectomy

USS - Ultrasound scan

UTR - Untranslated regions

VT – Vitality

WB - White British

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PART ONE – BACKGROUND

1 Chapter 1 – Introduction

1.1 Familial adenomatous polyposis

Familial adenomatous polyposis (FAP) is a rare, inherited, cancer predisposing syndrome. It is an autosomal dominant condition characterised by the development of hundreds to thousands of colorectal adenomas and the development of extra-colonic manifestations. It accounts for less than one per cent of all colorectal cancers (Bulow, 2003). The prevalence of FAP is estimated between 1 in 5000 and 1 in 10,000 with almost 100% disease penetrance by 40 years of age (Bisgaard et al., 1994, Potter, 1999). Almost all individuals affected by FAP will develop colorectal cancer by the fourth decade of life unless prophylactic colectomy is performed (Petersen et al., 1991).

FAP is a multi-system disorder that can involve other parts of the gastrointestinal tract, as well as other organs and soft tissues. Duodenal adenomas are seen in approximately 90% of patients and demonstrate much slower progression towards invasive carcinoma compared with colorectal lesions (Wallace and Phillips, 1998, Vasen et al., 1997, Bulow et al., 2004). Duodenal adenomas are usually peri-ampulla in location and are associated with a poor prognosis if cancer develops (Latchford et al., 2009). The risk of such tumours developing in patients with FAP is several hundred-fold when compared to the general population (Spigelman et al., 1989). In the stomach fundic gland polyps are seen frequently in FAP but pose no malignant risk (Wu et al., 1998).

Extra-intestinal manifestations of FAP include: desmoid tumours, congenital hypertrophy of the retinal pigment epithelium (CHRPE), dental anomalies and benign soft tissue and bony

lesions. Once the colorectal cancer risk is addressed with prophylactic colectomy (typically in late teens or early twenties), desmoid disease and duodenal cancer remain the leading causes of morbidity and mortality in patients with FAP (Arvanitis et al., 1990, Bertario et al., 1994, Vasen et al., 2008, Nugent et al., 1993).

FAP is due to a germline mutation in one of the copies of the adenomatous polyposis coli (*APC*) gene, either arising de novo or from an inherited mutated copy. The *APC* mutation can be identified in more than 70% of cases (Vasen et al., 2008). In concordance with Knudson's two-hit hypothesis, adenoma and tumour development occur following a 'second hit' somatic inactivation of the wild-type *APC* allele through mutation or deletion (Fodde and Khan, 1995). Knudson first described this hypothesis in 1971 in relation to retinoblastoma development (Knudson, 1971).

1.1.1 *APC* and the Wnt signalling pathway

In 1925 Lockhart-Mummery described the Mendelian inheritance of FAP through affected families (Bodmer, 2006). Herrera et al were the first to report the interstitial deletion of chromosome 5 in a patient with severe learning difficulties and colorectal polyposis (Herrera et al., 1986). In 1987 Bodmer found the gene responsible for FAP was associated with an abnormality on the long arm of chromosome 5 (Bodmer et al., 1987). Finally, in 1991 linkage analysis performed by two independent research teams found the location of the *APC* gene responsible for FAP at 5q21 (Kinzler et al., 1991, Groden et al., 1991).

APC is a large gene consisting of 8535 base pairs over 21 exons. It encodes a 2843 amino acid protein that is broadly expressed throughout the human body (Thliveris et al., 1996). Exon 15 accounts for more than 75% of the total coding sequence and is the region where the majority

of germline and somatic *APC* mutations predominate (Fearnhead et al., 2001). The majority of those detected (95%) are deletions, insertions or nonsense mutations resulting in a frameshift or premature stop codon and truncation of the APC protein (Heinimann et al., 2001). Approximately a third of *APC* germline mutations occur between codons 1061 and 1309, the remaining two thirds are evenly distributed from codon 200 to 1600 (Fearnhead et al., 2001). The germline mutation location influences the somatic mutation and disease penetrance (Lamlum et al., 1999).

The primary role of *APC* is tumour suppression. It acts as a ‘gate-keeper’ for normal epithelial cell growth and homeostasis through its regulation of intracellular β -catenin via the Wnt signalling pathway. The protein product of *APC* is a cytoplasmic glycoprotein implicated in many processes affecting cell growth: adhesion, cell migration, apoptosis and neuronal differentiation. It is a fundamental component of the canonical (classical) Wnt pathway (Figure 1-1) (Hanson and Miller, 2005, Fodde et al., 2001). The primary role of this pathway is the ‘down-regulation’ and stabilisation of cytosolic β -catenin. Wnts are a group of secreted glycoproteins that modulate cell growth during development. Abnormal Wnt activation can promote cancer occurrence (Sugimura and Li, 2010). Normally, in the absence of Wnt ligands, a protein complex of APC, Axin and glycogen synthase kinase-3 β (GSK-3 β) phosphorylates and degrades cytosolic β -catenin (Bienz, 2002). However, in the presence of mutated APC and Wnt ligands, the protein complex is inhibited, resulting in the stabilisation and accumulation of β -catenin with subsequent nuclear translocation. This activates DNA transcription factors and target genes resulting in uncontrolled cellular proliferation (Moon et al., 2004, Eisenmann, 2005). Stimulation of the Wnt signalling pathway in FAP therefore leads to accelerated adenoma formation and cancer development (Kinzler and Vogelstein, 1996). Similarly, it is

through this pathway that most desmoid tumours, both inherited and sporadic, also arise (Robanus-Maandag et al., 2011).

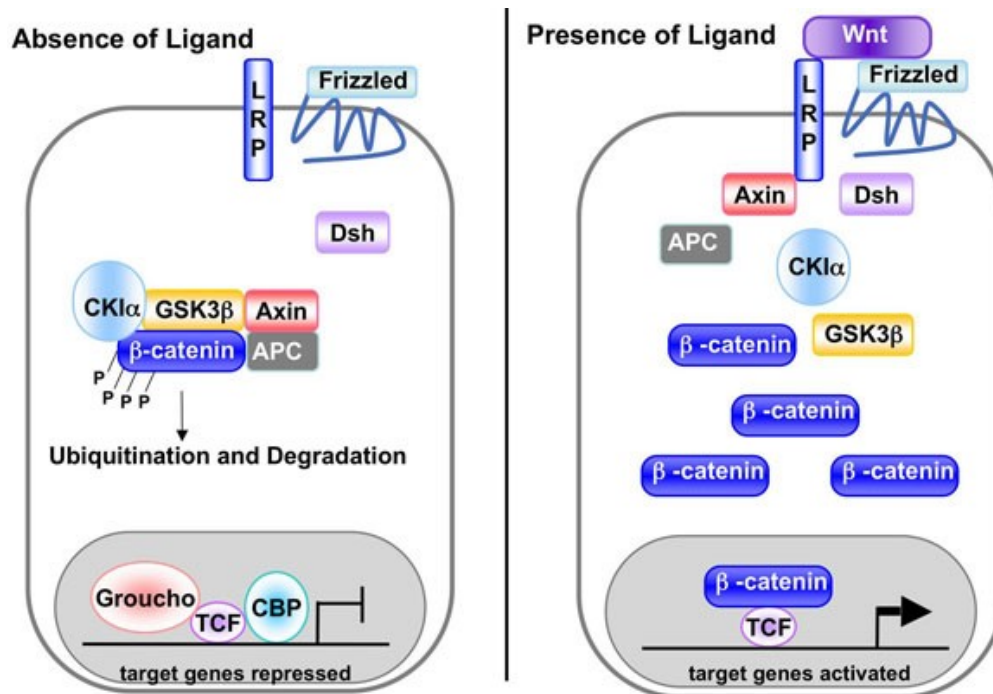


Figure 1-1 The Wnt signalling pathway: normally β -catenin is degraded by formation of a protein complex in the absence of Wnt ligand (A). In the presence of Wnt, stabilisation of β -catenin occurs resulting in nuclear translocation and activation of target genes (B) (Eisenmann, 2005)

1.1.2 A multi-systemic disease

APC is ubiquitously expressed throughout the human body and this explains the multi-systemic nature of FAP. Extra-colonic manifestations of FAP include: duodenal adenomas, fundic gland polyps, osteomas, supernumerary teeth, CHRPE, epidermoid cysts, fibromas, adrenal adenomas, extra-intestinal cancers (thyroid, central nervous system tumours and hepatoblastoma) and desmoid tumours (Figure 1-2) (Half et al., 2009). Once the colonic cancer risk is addressed, through prophylactic colectomy, it is important to consider duodenal cancer risk and desmoid tumours, which are the commonest disease related causes of death (Arvanitis et al., 1990, Bertario et al., 1994).

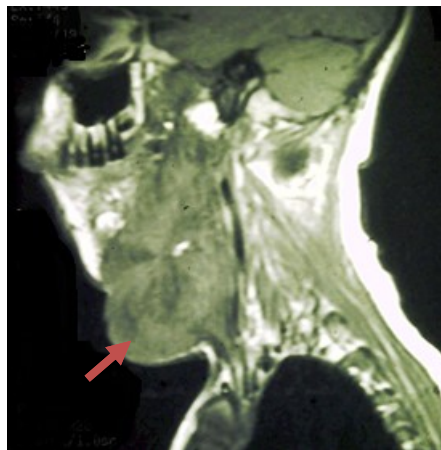


Figure 1-2 MRI scan of a desmoid tumour engulfing the mandible (arrow)

1.1.3 Genotype – phenotype correlation in FAP

The existence of a genotype-phenotype correlation in FAP, linking germline mutation to clinical manifestations, is well recognised (Figure 1-3). For instance, *APC* mutations between codons 1250 and 1464 are associated with severe colorectal adenoma phenotype resulting in higher polyp burden and increased colorectal cancer risk (Nieuwenhuis et al., 2007). This region is known as the ‘mutation cluster region’ where the majority of germline mutations occur and more than 60% of somatic mutations are clustered, yet representing only 10% of the coding region (Friedl et al., 2001, Bertario et al., 2003) (Nieuwenhuis et al., 2007, Miyoshi et al., 1992). In particular, codon 1309 mutations are associated with a severe phenotype at an earlier onset with a tendency to colorectal cancer development at a younger age (Groves et al., 2002a, Nagase et al., 1992, Caspari et al., 1994). In contrast to this, *APC* mutations located at extreme 5’ (before codon 157) or 3’ (beyond 1595) ends of the gene, or in the alternatively spliced region of exon 9, are associated with an attenuated FAP phenotype with fewer adenomas and later appearance (Nieuwenhuis et al., 2007, Fearnhead et al., 2001, Soravia et al., 1998, Will et al., 2008).

Some extra-colonic manifestations of FAP also demonstrate a genotype-phenotype correlation. CHRPE occurs in individuals with mutations between codons 457 and 1444, whilst far 3' (beyond codon 1399) mutations are associated with an increased propensity to form desmoid tumours (Caspari et al., 1995, Sinha et al., 2011, Nieuwenhuis et al., 2008, Church et al., 2015). Patients with mutations in this region, and an attenuated phenotype, can potentially be managed endoscopically, thereby delaying, or in some cases avoiding, prophylactic colectomy (Gleeson et al., 2009).

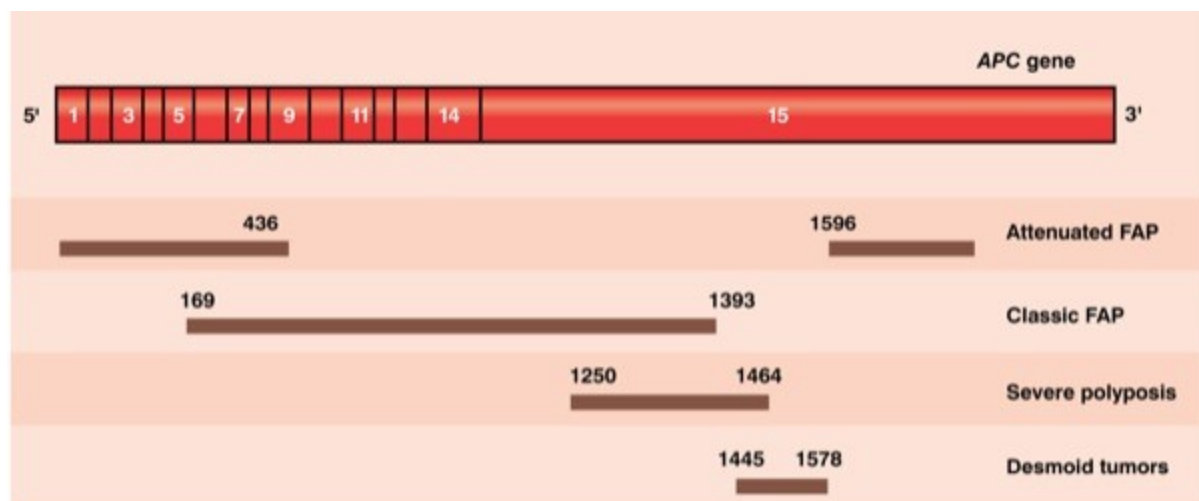


Figure 1-3 APC genotype-phenotype correlation (Chung et al., 2003)

1.1.4 Clinical management of FAP

Patients with FAP are often managed by specialist centres with a polyposis registry that can co-ordinate clinical management, screening and surveillance. They address colorectal cancer risk, survey the duodenum and other FAP associated conditions. Registry healthcare professionals dedicate their time to systematic tracing of 'at risk' individuals, offering screening and surveillance as appropriate. Studies have shown that polyposis registries and screening reduce colorectal cancer risk and mortality in FAP patients (Barrow et al., 2013b, Vasen et al., 2008). Individuals related to FAP patients with a known *APC* mutation can be

offered predictive testing, performed at a regional genetics centre. If the mutation is unknown, then the diagnosis is made clinically (through endoscopic and histological confirmation of colorectal adenoma formation). Clinical management of screen detected cases initially addresses colorectal cancer risk. This involves flexible sigmoidoscopy from early teens, typically followed by prophylactic colectomy before the age of twenty (Vasen et al., 2008). Timing will depend on patient preference and FAP phenotype. Children symptomatic from their colorectal polyposis (with loose stools, mucous discharge or bleeding per rectum) will require earlier endoscopic examination and surgery. In some cases of attenuated FAP, colectomy may be postponed or avoided with ongoing colonoscopic examination and intervention as necessary for their polyposis (Sinha et al., 2010b, Vasen et al., 2008).

There are two prophylactic surgical options for patients with FAP; a total colectomy with ileorectal anastomosis (IRA) (leaving the rectum in-situ), or a restorative proctocolectomy (RPC) (Vasen et al., 2008). Patients with a mutation predisposing to a severe polyposis phenotype (codon 1250-1464), those with a high colorectal polyp burden at the time of surgery (more than 500) or individuals with more than twenty rectal polyps, would usually be offered a RPC, due to cancer risk in the retained rectum if an IRA were performed. In cases without these characteristics it is our practice to offer a colectomy and IRA (Tudyka and Clark, 2012).

Life-long endoscopic surveillance for polyps is required for the rectum (after colectomy and IRA) or pouch (after RPC), typically at six monthly and yearly intervals, respectively. Endoscopic therapy can be employed to remove polyps detected at surveillance. Non-steroidal anti-inflammatories (e.g. sulindac) have been used to delay rectal polyp progression (Vasen et al., 2008). Despite these interventions surgery will be required to remove the rectum or pouch should severe dysplasia or cancer occur.

Duodenal adenomas occur in 90% of patients with FAP. However, malignant progression of duodenal adenomas is much slower compared to that of colonic polyps (Groves et al., 2002b, Bulow et al., 2004). Lifetime risk for duodenal cancer development in FAP patients is approximately 5% (Vasen et al., 1997, Jagelman et al., 1988, Gallagher et al., 2006). The Spigelman classification is used to assess duodenal polyposis severity according to endoscopic and histological analysis to determine surveillance intervals (Spigelman et al., 1989). The classification system is based on five stages (O-IV) that assess polyp number, maximum size, histology and degree of dysplasia. A cumulative score determines stage of disease and stratifies risk for duodenal cancer development, enabling identification of those who may benefit from prophylactic duodenectomy. Stages I and II represent mild disease that can be endoscopically surveyed. Therapeutic endoscopic treatments (snare excision, thermal ablation and argon plasma coagulation) can be employed for advanced disease (Vasen et al., 2008). Patients with stage IV disease should also be referred to a hepatobiliary surgeon for consideration of a prophylactic duodenectomy.

1.1.5 St Mark's Hospital polyposis registry

The St Mark's Hospital polyposis registry was established in the 1920's by Dukes and Lockhart-Mummery and was subsequently developed by Bussey, who kept meticulous contemporaneous patient records. It is the oldest FAP registry in the world and one of the largest. Data are continually entered onto the electronic database, including patient details, diagnosis, germline mutation, pathology reports, extra-intestinal manifestations and results from gastrointestinal (GI) endoscopic surveillance. It can highlight when investigations are due. A large proportion of patients on the database have their primary FAP surgery at the institution and the database can capture these details and subsequent follow-up. This provides

a large group of patients with complete records, reduces the risk of referral bias and provides a privileged position to study this rare cohort of individuals.

1.2 Desmoid tumours

1.2.1 Definition and epidemiology

Desmoid tumours are benign, locally infiltrative, myofibroblastic tumours of mesenchymal origin (Wu et al., 2010, Fisher and Thway, 2014). They are rare, representing only 0.03% of neoplasms (Caspari et al., 1995). The word desmoid is derived from the Greek word ‘desmos’ meaning tendon-like, coined by Muller in the 19th century. Other terminologies used interchangeably with desmoids include: aggressive fibromatosis, musculoaponeurotic fibromatosis and deep fibromatosis.

‘Fibromatosis’ is a generic term that encompasses a range of fibroblast proliferative disorders including Peyronie’s disease, plantar fibromatosis, Dupuytren’s contracture, hypertrophic scarring and keloid. Similar to the development of desmoids, these pathologies result from fibroblast dysfunction and excessive collagen deposition.

Desmoid tumour incidence is estimated to be between two to four people per million per year (Reitamo et al., 1982, Nieuwenhuis et al., 2011a). The majority (90%) of these tumours arise sporadically, usually due to a somatic β -catenin mutation (Miyoshi et al., 1998, Tejpar et al., 1999, Lazar et al., 2008). Ten-percent occur in association with FAP, due to a biallelic *APC* mutation (Fallen et al., 2006). Although rare in the general population, for those with FAP desmoid tumours are up to 1000 times more common (Sturt and Clark, 2006, Gurbuz et al., 1994, Nieuwenhuis et al., 2011a).

1.2.2 Desmoids in FAP

In FAP the lifetime risk of desmoid development is in the order of 10 to 25% (Sinha et al., 2011, Vasen et al., 2008). Although these tumours are benign they are a leading cause of morbidity and mortality in those with FAP following prophylactic colectomy, along with duodenal adenomatosis (Clark et al., 1999, Arvanitis et al., 1990). Desmoids can occur anywhere in the body. In FAP the majority arise in the abdominal wall or mesentery of the small bowel. Intra-abdominal desmoids in FAP account for around 50-80% of those seen (Clark et al., 1999, Hartley et al., 2004, Sinha et al., 2011, Nieuwenhuis et al., 2011b).

There is a spectrum of tumour behaviour, with the majority (around 50%) at one end posing no serious risk as growth remains stable. Some desmoids exhibit cycles of growth and regression, whilst others may spontaneously resolve, 30% and 10%, respectively. Discomfort and/or deformity (due to tumour growth) may arise but the majority of these tumours are not life-threatening. At the severe end of the spectrum (10% of tumours) growth is relentless and it is these that are likely to result in a poor outcome. Desmoid tumours cause obstruction, necrosis, fistulation and perforation from local pressure effects on bowel, ureters or blood vessels (Church, 1995, Clark et al., 1999). It is this group of patients that are particularly difficult to treat.

Desmoid tumours are also challenging to research, not only due to their variable clinical behaviour but also because they are rare. Many studies have incorporated both sporadic and FAP-associated desmoid tumours in an attempt to improve sample size. However, the majority of studies in this thesis have focused on FAP-associated tumours because these are clinically and genetically distinct from sporadic tumours and represent the cases managed at this institution. Genetically, the majority of sporadic desmoids (up to 85%) are due to an

identifiable mutation in the β -catenin encoding gene *CTNNB1* whereas desmoids in FAP arise from an *APC* mutation (Huss et al., 2013, Lazar et al., 2008, Miyaki et al., 1993). Clinically, less than 5% of sporadic desmoids are found intra-abdominally whereas in FAP 80% are found here and are associated with the majority of complications that result (Kasper et al., 2011).

1.2.3 Desmoid pathology

Until fairly recently there was considerable controversy over the clonal nature of desmoids and the cell of origin is still unclear. In the late 1990's, through the study of X chromosome inactivation using PCR amplification, studies found that desmoids were monoclonal proliferations that represented a true neoplasm (Li et al., 1996, Middleton et al., 2000). Wu et al studied desmoid cell origin and found that desmoids and mesenchymal stem cells (MSCs) had similar gene expression profiles (Wu et al., 2010). Another study found desmoid tumours expressed MSC markers whilst normal tissue did not (Carothers et al., 2012). Ghanbari-Azarnier et al studied the pathways that regulate MSC differentiation and found that this influenced desmoid tumour behaviour. They looked at the Hedgehog signalling pathway, which interacts with the Wnt pathway, in mice. This pathway was activated in desmoids and its inhibition decreased cell proliferation and tumour occurrence, adding support to desmoid cells originating from MSCs (Ghanbari-Azarnier et al., 2013). Other studies have shown that desmoid tumours are mainly composed of myofibroblasts (Gabbiani, 1996, Kiryu et al., 1985, Desmouliere et al., 2004). Myofibroblasts are well-differentiated fibroblasts that express α -smooth muscle actin.

1.2.3.1 Macroscopic appearance

Desmoids differ considerably in size but their overall macroscopic appearance remains quite consistent. They appear as a firm, smooth tumour externally with a coarse, pale, trabeculated surface on sectioning. The tumour is usually homogenous and can appear to have a thin pseudocapsule although on closer inspection the tumour often infiltrates local structures (Kotiligam et al., 2008, Fisher and Thway, 2014) (Figure 1-4).

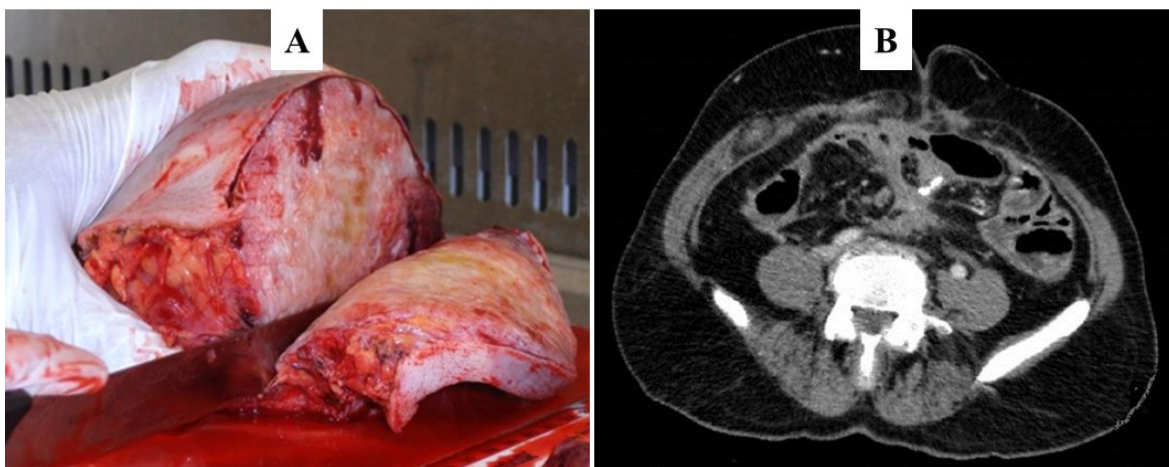


Figure 1-4 Macroscopic appearance of an abdominal wall desmoid at sectioning (A) showing a coarse pale surface and a CT image of an infiltrative diffuse mesenteric desmoid tethering the small bowel (B)

1.2.3.2 Microscopic appearance

Desmoids are composed of abundant whorls of elongated spindle-like cells within a rather hypocellular, abundant, collagenous matrix, along with blood vessels. On H&E staining, desmoid myofibroblast cells have small, pale staining, nuclei that lack the features consistent with malignancy, resembling typical appearances of a fibroblast cell. Blood vessels are often thin and slit-like, although some have thicker walls with mast cells seen perivascularly, Figure 1-5 (Fisher and Thway, 2014, Kotiligam et al., 2008). Chapter 8 looks at the histopathological features in desmoids in more detail.

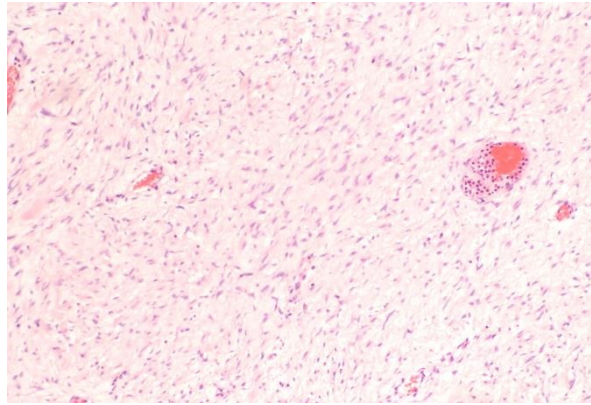


Figure 1-5 H&E (x20) appearance of a desmoid

1.2.3.3 The desmoid precursor lesion

A model for intra-abdominal desmoid tumour development was proposed by Clark et al in 1998 and was subsequently supported by work undertaken by Middleton et al in 2003 (Clark et al., 1998, Middleton et al., 2003). This model describes the potential for desmoid tumours to advance from a small plaque-like peritoneal thickening, histologically similar to a desmoid, called a 'Desmoid Precursor Lesion' (DPL) (Figure 1-6). This lesion can progress, typically following trauma such as surgery, leading to puckering of the small bowel mesentery and resulting in mesenteric fibromatosis. This can then develop into a solid intra-abdominal desmoid tumour. In this study higher rates of mesenteric fibromatosis and desmoid tumours were observed (using CT imaging) in cases that had evidence of a DPL compared to controls who did not, at colectomy.

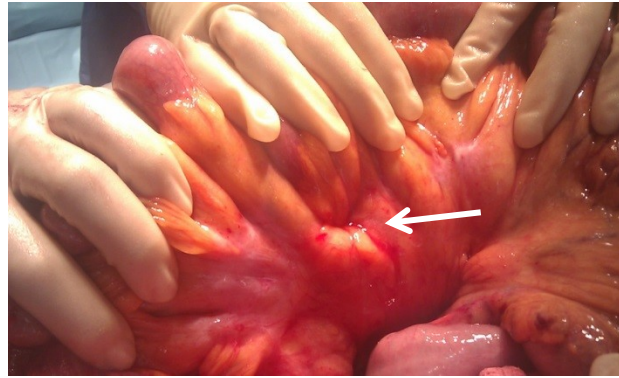


Figure 1-6 A mesenteric desmoid precursor lesion (arrow showing the lesion puckering the mesentery)

1.2.4 Genetics and the Wnt signalling pathway

APC and *CTNGB1* genes have been studied in desmoid tumours. A second hit somatic mutation of the *APC* gene is usually found in desmoids occurring in FAP (Lamlum et al., 1999). The type of somatic mutation is non-random and in part determined by the germ-line location. The *APC* gene product, as previously described in section 1.1.1, is an integral component of the Canonical Wnt signalling pathway involved in β -catenin degradation. *APC* contains seven 20 amino acid β -catenin degradation repeats (AARs). These promote degradation by enabling β -catenin binding. The ‘just right’ hypothesis proposed there was an optimum threshold of β -catenin binding that would favour FAP adenoma progression to colorectal cancer (Lamlum et al., 1999). Subsequent studies have supported this. Crabtree et al found somatic mutations leaving one or two 20AARs intact promoted colorectal tumour development (Crabtree et al., 2003) whilst Groves et al concluded that three to four 20AARs favoured duodenal adenomatosis (Groves et al., 2002a). Two to three 20AARs may predispose to ileo-anal pouch polyps (Will et al., 2008). Latchford et al studied this in FAP desmoids and found 26/30 (87%) of tumours had no 20AARs on one allele and in 17/30 (57%) preferentially retained two 20AARs (Latchford et al., 2007). Similar findings were reported by Miyaki et al (Miyaki et al., 2008).

Somatic mutations of codons 41 and 45 of exon 3 of the β -catenin gene (*CTNNB1*) are usually responsible for sporadic desmoid tumours (Lazar et al., 2008). Mutation in this gene results in activation of the canonical Wnt pathway. β -catenin is stabilised and degradation cannot occur as it would normally through the formation of a multi-protein complex incorporating APC, GSK-3b and AXIN. Increased β -catenin allows nuclear translocation and activation of transcription factors, for example TCF-3, that promote tumour growth. Tejpar et al studied this in sporadic desmoid tumours (Tejpar et al., 2001). The *APC* mutation in FAP associated desmoids results in a similar pathway activation, thereby stabilising β -catenin.

The non-canonical Wnt signalling pathway is an antagonist to the canonical pathway. It has a role in cell polarity and motility and is important in embryonic tissue development (Sugimura and Li, 2010). This pathway is regulated by Wnt ligands, including Wnt-11. High expression of this ligand has been demonstrated in renal, prostate and GI tract tumours (Kirikoshi et al., 2001). It may be implicated in aggressive prostate tumours as well (Uysal-Onganer et al., 2010). Little has so far been explored regarding the involvement of the non-canonical Wnt in desmoid tumour development. Histological studies looking at β -catenin and Wnt-11 expression in desmoid tumour tissue are described in chapter 8.

1.2.5 Anatomical distribution

Desmoid tumours typically arise from musculoaponeurotic tissues but can occur anywhere. They can be categorised according to anatomical location: intra-abdominal desmoids (IAD) (where the majority of desmoids occur in FAP), abdominal wall desmoids (AWD) and extra-abdominal desmoids (EAD) (the majority of sporadic desmoids are found here). Desmoids in FAP are often multiple in number.

1.2.5.1 Abdominal wall desmoid (AWD)

Desmoids located in the abdominal wall originate from the external oblique, internal oblique, rectus muscles or the rectus sheath. Approximately 15% of desmoids in FAP are located here (Clark and Phillips, 1996). They typically arise within a previous surgical scar, during pregnancy or post-partum (Fisher and Thway, 2014). Previous studies have found that patients with FAP tend to have worse scarring and more adhesions than patients without FAP who undergo the same surgery (Lockhart-Mummery, 1967, Herrera-Ornelas et al., 1987). These observations suggest abnormal fibroblast function occurs in association with FAP and may explain why desmoid tumours frequently arise from surgical wounds (Eden et al., 1992, Lynch and Fitzgibbons, 1996, Brezinka et al., 1986, Wijburg and Roumen, 2004).

Abdominal wall desmoids are usually amenable to surgical resection and typically present with cosmetic and mechanical problems due to tumour size. However, surgical intervention is associated with high recurrence rates (Salas et al., 2011, Huang et al., 2009, Gronchi et al., 2003, Peng et al., 2012, Ballo et al., 1999). The correlation between resection margin status and recurrence is unclear. Some studies (mostly involving sporadic tumours) have suggested resection margin status is an important prognostic marker for desmoid recurrence (Shin et al., 2013, Leithner et al., 2004, Stoeckle et al., 2009), whilst others have failed to demonstrate this (Woltsche et al., 2015, Soto-Miranda et al., 2013, Pencavel et al., 2010, Gronchi et al., 2003). Tumours may often recur even though a R0 resection is achieved (although this may be due to trauma stimulating a new desmoid to grow at the same site), whilst recurrence may not necessarily result when incomplete resection is only possible.

1.2.5.2 Intra-abdominal desmoid (IAD)

Intra-abdominal desmoids represent up to 80% of desmoids in FAP. Eighty percent of IADs occur within the mesentery (Clark and Phillips, 1996). They can also be found in the retroperitoneum, pelvis or peritoneal ligaments (Fisher and Thway, 2014, Burke et al., 1990). Intra-abdominal desmoids can infiltrate nearby tissues and this may result in bowel obstruction, fistulation, perforation and ischaemia as well as ureteric obstruction.

Due to their location and infiltrative nature, IAD are the most likely tumours to cause a life-threatening complication. Ironically they are also the most difficult to treat and they often grow around the superior mesenteric vessels, making surgical excision very difficult or impossible without necessitating an extensive enterectomy.

Early identification of IAD tumours and predicting those that may grow aggressively could influence timing of treatment interventions. Chapter 11 looks at the identification of potential biomarkers for desmoid tumour identification in FAP patients.

1.2.5.3 Extra-abdominal desmoid (EAD)

Extra-abdominal desmoids are often sporadic in nature and account for less than 15% of desmoids occurring in FAP but a larger proportion of sporadic tumours (Clark 1996, Clark 1999). They typically arise in the musculoaponeurotic planes of the trunk, thighs, buttocks and sometimes the head or neck and often affect younger adults (Fisher and Thway, 2014).

1.2.6 Clinical manifestations

Desmoid tumours in FAP typically present as a slow growing mass or are discovered at radiological imaging. They can be an incidental finding or discovered when investigating symptoms relating to the desmoid, for example bowel obstruction. Desmoids often affect younger adults. A meta-analysis by Sinha et al found 80% of desmoids had occurred by the age of 40 years. As mentioned in section 1.2.2, desmoids display unpredictable growth behaviour. Some may grow relentlessly while the majority remain stable and a few regress. The symptoms they exert are attributable to their location and mass or pressure effects from their growth. A clinical review of all FAP-associated desmoids at this institution is described in chapter 3.

Intra-abdominal desmoids may cause abdominal pain from bowel obstruction or perforation. Abdominal sepsis and fistula formation can occur as a result of this or from necrosis of the tumour. In this instance patients can present with an air fluid level on CT that may be amenable to percutaneous drainage (Bhandari et al., 2012). Such complications may necessitate long-term parenteral nutrition (PN) support and in some cases warrant referral for consideration of a small bowel transplant (SBT). For those with large and advanced IAD requiring PN, the associated mortality rate is significantly high. Quintini found a five-year survival rate (5YSR) in this group of 53% (Quintini et al., 2012). Arvanitis et al studied causes of deaths in FAP and found 11% were attributable to desmoid, with an average age of death just 35 years (Arvanitis et al., 1990). The quality of life for patients with desmoids, particularly for those with significant disease, has not been widely explored. Chapter 7 looks at the quality of life associated with desmoid tumours compared to FAP controls.

Intra-abdominal desmoids can result in ureteric obstruction if they extend retroperitoneally. This can manifest as hydronephrosis due to extrinsic compression of the ureter. The rate of occurrence is not widely reported, although Joyce et al found 28% of 107 patients with IAD developed ureteric obstruction (Joyce et al., 2010). A study looking at ureteric obstruction rates secondary to IAD tumours is detailed in chapter 4.

Abdominal wall desmoids and EAD, although less troublesome than IAD, can result in unsightly lumps. Occasionally they grow fast, displacing stomas or risking skin integrity, as well as creating unsightly masses that prompt the need for surgical excision, (Figure 1-7).



Figure 1-7 Large abdominal wall desmoids in three patients, the tumours can stretch skin and displace stomas

1.2.7 Aetiology and risk factors

The aetiology of desmoid tumours is unclear. Risk factors associated with their development reported in the literature include: sex hormone status, trauma (surgery can stimulate desmoid growth), genetic influences (*APC* mutation 3' of codon 1399 is a desmoid prone region) and positive family history of desmoid disease (independent of germ line mutation).

1.2.7.1 Hormonal influence

Many studies have reported on the association of desmoid tumour development and oestrogen status and this has led to the use of anti-oestrogens as a first-line medical treatment for desmoids. This theory originated from observations that a higher incidence of sporadic tumours was seen in women compared to men and was highest in women of child-bearing age (Reitamo et al., 1982, Jarvinen, 1985). Other studies reported on an association between desmoid occurrence and pregnancy. Some found an increased risk post-partum for AWD (Nieuwenhuis et al., 2008, Gurbuz et al., 1994), another found an improvement in IAD during pregnancy (Church and McGannon, 2000) and others failed to show any such correlation (Clark et al., 1999, Mulik et al., 2003). These observations led to the incorporation of anti-oestrogens, such as tamoxifen, into the treatment of desmoids. A large meta-analysis by Sinha et al reported that female FAP patients were one and a half times more likely to develop a desmoid compared to males (Sinha et al., 2011).

Studies have looked at oestrogen receptors and their binding sites in desmoid tumours and found anti-oestrogen treatment response was independent of oestrogen receptor status. Lim et al (1986) found oestrogen receptors were only present in 33% (5/15) of tumours, with similar results for men and women (Lim et al., 1986). More recent studies found desmoid tumours

were often oestrogen receptor-alpha negative but beta positive (Deyrup et al., 2006, Leithner et al., 2005, Santos et al., 2010).

1.2.7.2 Trauma

Trauma as an aetiological factor for desmoid tumour development was briefly described in section 1.2.5.1. Evidence originates from observations that patients with FAP develop more adhesions, uglier scars and desmoids from surgical wounds (Lockhart-Mummery, 1967, Herrera-Ornelas et al., 1987). The precursor plaque model also implicated trauma as a risk factor in desmoid development. Furthermore, Clark et al found up to 86% of FAP associated AWD and IAD occurred after abdominal surgery, typically within the first five years of colectomy (Clark and Phillips, 1996). These findings were supported by a meta-analysis by Sinha et al who found FAP patients who had undergone abdominal surgery were over three times more likely to develop a desmoid compared to those who had no previous surgery, with most presenting within 3 years of colectomy (Sinha et al., 2011). In a case report the development of desmoid tumours at trocar sites within months of laparoscopic surgery to assess an aggressive IAD was described. This provided further evidence that surgery is a significant aetiological factor in desmoid development (Lynch and Fitzgibbons, 1996).

A study looking at desmoid tumour development after colectomy compared open approach to laparoscopic and found a rate of 16% and 4%, respectively, suggesting that laparoscopic surgery could reduce desmoid risk after prophylactic surgery as there may be less dissection and trauma (Vitellaro et al., 2014). In contrast to this study, Vogel et al found an increased risk for desmoid formation following minimally invasive prophylactic surgery compared to the open approach. Six out of thirteen (46%) patients undergoing laparoscopic RPC surgery

developed a desmoid after surgery, compared to only 2/32 (6%) in the open RPC group (Vogel, 2005).

Despite the association with trauma, some desmoids occur spontaneously. Hartley et al found 4% of FAP patients had an IAD at surgery (Hartley et al., 2004).

1.2.7.3 Genetics

Many studies have reported on the association of *APC* mutation and desmoid development. *APC* mutation as an aetiological factor was first described by Caspari et al 1995. They established that *APC* mutations 3' codon 1444 predisposed to desmoid formation. Other studies have found similar results with desmoid prone mutations beyond 3' codon 1399 or 1444 (Sturt et al., 2004a, Nieuwenhuis et al., 2008, Sinha et al., 2011, Church et al., 2015). Bertario et al reported a 12-fold increased risk of desmoid for those with germline mutations 3' of 1444 compared with upstream mutations (Bertario et al., 2001). Multivariate analysis in this study found *APC* mutation beyond codon 1444, family history of desmoids, female gender and the presence of osteomas were the most important independent predictors for desmoid development. A meta-analysis by Sinha et al of 4625 patients with FAP, in whom 559 developed desmoid, found a family history of desmoid was the most significant risk factor for desmoid development. *APC* mutations 3' of codon 1399 were also significant, along with previous abdominal surgery and female gender (Sinha et al., 2011).

Most recently Church et al studied 77 desmoid patients, from 68 different families, and concluded that incidence and severity of desmoid tumours were related to the mutation site. Although desmoids occurred with any *APC* mutation, a large proportion (37% of all desmoids included) were found 3' of codon 1400. None of the patients with a mutation 5' of codon 1400

had a severe desmoid phenotype compared with 12% of those with *APC* mutations between codons 400 and 1400 that did. Those with mutations 3' of codon 1400 had the highest rate of severe disease, with 44% in this group affected (Church et al., 2015).

As previously described in section 1.2.4, normally the *APC* gene product contains seven 20AARs that degrade β -catenin, with desmoids usually resulting when two AARs are retained in the truncated APC protein. The 'first' and 'second' hit mutations will determine the number of retained 20AARs, thereby creating an optimal level of β -catenin dysregulation, that results in desmoid tumour formation. Codon 1399 is located just after the second AAR and therefore germline mutations 3' of this point are associated with a more severe desmoid phenotype as loss of heterozygosity (LOH) will occur spontaneously at a reasonably high rate (Sturt et al., 2004a, Latchford et al., 2007).

1.2.7.4 Family history and modifier genes

Eccles et al described an association between family history and desmoid occurrence in 1996. Within three generations of the same family, with a far 3' *APC* mutation at codon 1924, there was 100% disease penetrance for desmoid with a very low intestinal polyposis penetrance. They concluded that the mutation in this family was likely to be linked to a desmoid modifier gene (Eccles et al., 1996).

Further studies have looked at the association between family history and desmoid risk. Sturt et al and Bertario et al found a positive family history of desmoids was an independent risk factor for desmoid disease (Sturt et al., 2004a, Bertario et al., 2001). Sinha et al also reported these studies in a meta-analysis and found that a positive family history for desmoids was the most significant risk factor for their development. This was independent of the *APC* mutation,

providing further support for the existence of a desmoid modifier gene (Sinha et al., 2011). This is explored further in chapter 10.

1.2.8 Treatment

Desmoid management is complex and individualised for each patient. Typically it involves a stepwise treatment ladder that begins with a ‘watch and wait’ policy at diagnosis, as many tumours will remain stable without significant complications and surgical intervention may be hazardous (Fiore et al., 2009). If growth continues pharmacological treatment is initiated. Following cytotoxic chemotherapy, surgery may be considered for progressive disease (Kasper et al., 2015, Janinis et al., 2003).

1.2.8.1 Pharmacological management

1.2.8.1.1 Non-cytotoxic drugs

Current management guidelines recommend non-steroidal anti-inflammatory drugs (NSAIDs) in combination with anti-oestrogen treatment as the first-line pharmacological treatment for desmoids (Hansmann et al., 2004, Sturt et al., 2004b, Janinis et al., 2003, Kasper et al., 2015, Vasen et al., 2008). However, there are no published phase III randomised clinical studies for desmoid treatment and most studies originate from non-randomised, non-controlled, retrospective cohorts or case studies. It can be difficult to prove reduction in tumour growth is secondary to a pharmacological intervention and not a reflection of the unpredictable growth behaviour of a desmoid tumour. It is for this reason that studies looking at desmoid interventional response are extremely difficult to assess, not least because they often involve a small study population. To improve their cohort size, many studies combine FAP and sporadic desmoid tumours and yet these are two distinct entities.

NSAIDs such as sulindac and indomethacin have been used to treat desmoid tumours with varied success. The rationale for their use was based on the knowledge that prostaglandin synthesis is important in cancer growth and inhibition may therefore impact on desmoid growth. Initial case reports confirmed this and found spontaneous tumour regression occurred with their use (Waddell and Gerner, 1980). Tsukada et al reported a tumour response rate of 57% with the use of sulindac at a median time of 2 years in 14 patients (Tsukada et al., 1992). Overall the evidence for the use of NSAIDs in the treatment of desmoid tumours remains poor.

Several small trials and case reports have studied the use of hormonal manipulation, typically using tamoxifen or toremifene. Although response rates are reported around 50% in most cases (Janinis et al., 2003, Bocale et al., 2011), it is important to point out that none of these have incorporated appropriate controls and therefore true response rates are difficult to determine. Some studies have found a benefit in combined anti-oestrogen and NSAID use (Hansmann et al., 2004), whereas a systematic review by Bocale et al found no additional benefit from the addition of an NSAID to standalone antioestrogen treatment (Bocale et al., 2011).

1.2.8.2 Cytotoxic chemotherapy

Chemotherapy has been used in desmoid tumour treatment, despite their low proliferation rate and low grade features. Regimens have typically incorporated methotrexate and vinblastine or decarbazine and doxorubicin (used for sarcoma treatment) but robust clinical evidence for their use is lacking. Combination chemotherapy regimens have mostly been employed, in single cases or small cohorts, and analysis performed retrospectively. Interpretation of response is difficult as many desmoid cases receive other systemic treatments prior to, or during, chemotherapy. Results from FAP associated and sporadic cases affecting various anatomical sites are often combined, further complicating analysis. Chemotherapy is usually reserved for

tumours not responding to first-line systemic treatment and aggressive tumours that are unsuitable for surgical resection due to anatomical reasons.

Azzarelli et al reported a 100% partial response or stable disease rate at one year following methotrexate and vinblastine administration in 30 desmoid cases (80% of these were recurrent). Interestingly, no one achieved complete resolution but no tumours progressed either (Azzarelli et al., 2001). A study from the Royal Marsden Hospital Sarcoma unit looked at the same combination of chemotherapy for 18 sporadic desmoids and reported partial response in 11% and stable disease in 60% with tumour progression in 22%. In 14 cases administered doxorubicin instead, three (21%) progressed following treatment and the rest remained stable or partially responded. Again, no complete tumour response was seen in this study (Constantinidou et al., 2011). Similar response rates were reported from the French Sarcoma group in a cohort of 62 patients with various chemotherapy regimens but most commonly methotrexate-vinblastine (Garbay et al., 2012). However, a paediatric study using the same chemotherapy agents in sporadic desmoid cases reported a much higher rate of disease progression (69% (18/26)) during chemotherapy and only 31% (8/26) had none (Skapek et al., 2007). A Canadian study of seven patients receiving chemotherapy for an FAP associated desmoid, with a two year minimum follow-up, reported complete remission in two patients, partial remission in four cases and recurrence in two cases (Poritz et al., 2001).

A review by Janinis et al concluded that success rates in the treatment of desmoid tumours from combination chemotherapy ranged from 17% to 100% with a median response of 50% (Janinis et al., 2003). Chemotherapy toxicity remains a significant problem for those receiving this treatment, although methotrexate-vinblastine may be better tolerated than other regimens.

1.2.8.2.1 Tyrosine kinase inhibitors

There are some non-randomised prospective studies that have looked at the use of tyrosine kinase inhibitors for desmoid tumours, imatinib in most cases. Rates of tumour response are often low, around 15%, whilst stabilisation of tumour growth at six months is much better, between 50-85% (Heinrich et al., 2006, Penel et al., 2011, Chugh et al., 2010). As with most medical therapies for desmoid tumours, their mechanism of action is unclear. Both c-Kit and c-Src activity is variable in desmoid tumours and tumour response to tyrosine kinase inhibitors may therefore be through other pathways (Cho et al., 2012).

1.2.8.2.2 Biologics

Interferon (IFN) treatment for desmoid tumours has only been reported in single case reports and very small cohorts. The rationale for their use is based on laboratory studies that have shown an anti-proliferative effect on fibroblasts and reduction in collagen synthesis (Janinis et al., 2003). IFN- α and IFN- γ have been used in most cases achieving stabilisation of tumour growth and complete resolution in a minority (Leithner et al., 2000, Acker et al., 1993, Stengel et al., 2008, Fernberg et al., 1999).

1.2.8.3 Radiotherapy

Studies have used radiotherapy to treat unresectable desmoid tumours, as a standalone treatment or in conjunction with an incomplete surgical resection. The majority of tumours in these studies were located on extremities and thoracic wall, with only a few in the abdominal wall (Kriz et al., 2014). Most tumours were therefore sporadic. The evidence for the use of radiotherapy for positive margin status following desmoid tumour resection is controversial. Whilst several studies have reported an improved local control rate with the addition of

radiotherapy others have shown no benefit. A review of 22 studies by Nuyttens et al found significant benefit from radiotherapy, either as an independent therapy or in addition to surgery, compared to surgery alone for local control (Nuyttens et al., 2000). More recently, Kriz et al reported similar findings (Kriz et al., 2014). However, Gluck et al compared 104 patients and found no benefit in the addition of radiotherapy to surgical resection at three years follow-up (Gluck et al., 2011). The other consideration is the side effects from radiotherapy, such as radiotherapy induced cancers, particularly when dealing with this predominantly young population.

1.2.8.4 Surgery

The role of surgery in desmoid tumours remains controversial (Vasen et al., 2008). Although it may be a reasonable option, particularly for AWD and EAD tumours, the benefit of removing tumour must be weighed up against loss of function and recurrence risk. Desmoid tumours are after all benign and many of these tumours may remain quiescent posing no significant risk to life. Surgery itself creates trauma and this is an aetiological factor in desmoid development, as discussed in section 1.2.7.2. Desmoid tumour surgery is associated with high recurrence rates, reported between 40-70% (Latchford et al., 2006, Clark et al., 1999, Ballo et al., 1999). Controversy remains regarding tumour margin status, given that it is not a clear predictive marker for recurrence in the same way as it is for sarcoma (Gronchi et al., 2014) (Figure 1-8).

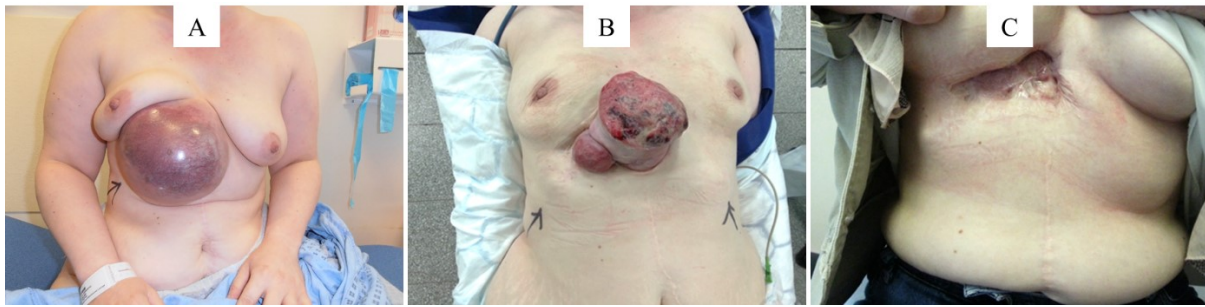


Figure 1-8 Patient with FAP with a large desmoid tumour (A) initially excised. A large recurrence (B) was excised eight months after the first surgery. Despite incomplete tumour margin clearance, the patient remained tumour free two years later (C)

Surgery for AWD and EAD is considered a reasonable option as they are not associated with high morbidity rates and surgery is often feasible (Latchford et al., 2006). Surgical resection for IAD is associated with significant morbidity as most are located in the small bowel mesentery. A significant number of these cases will inevitably result in extensive small bowel resection or enterectomy resulting in short bowel syndrome and requiring parenteral nutrition. In some cases referral for consideration of a small bowel transplant may be necessary (Wheeler et al., 2012). Sometimes surgery for IAD may be employed to bypass a segment of obstructed bowel rather than resect the tumour, therefore limiting bowel resection (Clark and Phillips, 1996).

1.2.8.4.1 Small bowel transplant for IAD

Small bowel transplant (SBT) is a radical treatment option that has gained momentum over the last two decades for complex and aggressive IAD with intestinal failure on maximal therapy. Although SBT surgery developed in the 1960s it was only through the introduction of immunosuppressive agents, such as tacrolimus, that it became a viable surgical option from the early 1990s (Hoffman et al., 1990). Other advances in transplant surgery techniques, establishment of specialised centres and careful patient selection criteria all helped to

significantly improve short term survival after SBT. However, 5YSR after SBT are currently reported between 40-77% (Beyer-Berjot et al., 2012, Cruz et al., 2011). Desmoid tumour recurrence is another concern after such surgery (Chatzipetrou et al., 2001). Consideration for SBT in the context of IAD therefore remains controversial and is only reserved for those with short bowel syndrome and life-threatening disease (Wheeler et al., 2012). A prospective study looking at SBT in home parenteral nutrition (HPN) patients found that desmoid tumour was one of only two indications for a life-saving SBT in this patient group. They concluded that SBT should be considered early on after commencing HPN in some cases where the risk of death from IAD is high (Pironi et al., 2011). SBT for IAD will be discussed in further detail in chapter 6.

1.2.8.5 Other treatment modalities

Radiofrequency ablation (RFA) has been explored more recently as a possible desmoid treatment option. A few case reports have published successful outcomes following the use of RFA for EAD and AWD (Barrow et al., 2013a, Cobianchi et al., 2014, Ilaslan et al., 2010). Ilaslan et al treated five EAD with RFA and none recurred at the time of the study, with follow-up ranging from 4-68 months. Complications included cellulitis and tissue necrosis. This treatment is not appropriate for all desmoid tumours. In particular, IAD where access to the tumour may be difficult and the potential for collateral damage to surrounding tissues and organs exists.

Percutaneous cryoablation has been used by Kujak et al to treat five EAD (Kujak et al., 2010). Two tumours achieved complete resolution and two others had evidence of tumour reduction. Chemical ablation under radiological guidance using acetic acid or ethanol has also been trialled. Clark reported successful treatment of two desmoids using this approach (Clark, 2003).

This treatment is thought to induce small vessel thrombosis and necrosis, thereby shrinking the tumour.

2 Chapter 2 - Aims of the work in this thesis

Due to their rarity, unpredictable and sometimes aggressive growth behaviour, FAP associated desmoid tumours remain a challenge to research. This impedes our ability as researchers to study such tumours from the bench to the bedside. They are difficult to grow in cell culture, limiting our understanding of the pathways involved in their growth and the testing of potential treatments. The impact of an intervention is also difficult to assess given the natural growth behaviour of these tumours. Improving laboratory techniques to aid in desmoid cell culture will help to test potential treatments more reliably. Development of a biomarker could improve our understanding of these issues and identify those at risk of forming desmoids. More importantly the identification of aggressively growing tumours could enable early targeting of appropriate medical and surgical treatment whilst sparing indolent tumours unnecessary interventions. Similarly, understanding the genetic basis of their development may offer new research opportunities and treatment strategies through improving our knowledge and understanding of these rare tumours.

The clinical studies of this thesis are based on the hypothesis that desmoid tumours, particularly IAD, can significantly impact on patients' health and quality of life.

The clinical studies aim to assess:

- The survival outcomes and morbidity associated with IAD tumours
- Small bowel transplant surgery for the treatment of complex IAD
- Quality of life associated with desmoid tumours

The laboratory studies sought to test the following hypotheses: That desmoid tumour growth is influenced by the non-canonical Wnt signalling pathway, cell culture is currently an unreliable laboratory method in the study of desmoid growth, that a desmoid modifier gene exists and that a biomarker is a feasible tool that could be used in the identification of desmoid tumours.

The laboratory studies aim to address the following questions:

- What drives desmoid growth or regression? What are the pathways involved?
- Can genetically confirmed desmoid cell lines be established reliably? Could development of a 3D desmoid cell culture scaffold be developed that may better mimic the in vivo environment??
- What are the genetic factors influencing tumour development?
- Can a serum biomarker be used to identify desmoid tumours?

2.1 Clinical studies

Part two of this thesis includes five clinical studies. The first study reviews 5YSR and outcomes, including medical and surgical interventions, in desmoid tumours at a single institution. Secondly, associated ureteric complications and outcomes arising from IAD will be reported. The third study explores as possible association between gastric cancer development and desmoid tumours in patients with FAP. A fourth study reports survival outcomes from SBT for complex IAD. The fifth study assesses quality of life (QOL) associated with desmoid disease, comparing FAP individuals with and without evidence of desmoid.

2.2 Laboratory studies

Part three of this thesis comprises four laboratory studies. The first of these reports on the histological analysis of desmoid tumours and incorporates immunohistochemistry techniques to look in particular at the non-canonical Wnt pathway. There is evidence that three-dimensional tissue scaffolds are a better model for mimicking the *in vivo* environment when compared to standard two-dimensional petri dish cell culture techniques (Cukierman et al., 2001). The second study describes standard cell culture of desmoids and the development of a novel three-dimensional extracellular matrix (ECM) scaffold. Whole exome sequencing of individuals with desmoid is described in the third study, looking for a modifier gene involved in their development. The final laboratory study reports the expression of microRNAs (miRNAs) in the serum and tumour of individuals with desmoid. It attempts to develop a biomarker and understand the pathways driving tumour growth and regression.

PART TWO – CLINICAL STUDIES**3 Chapter 3 - Survival outcomes in patients with FAP associated desmoid tumours – a review of the St Mark’s Hospital polyposis registry****3.1 Background**

Reporting clinical outcomes is particularly important for rare conditions where data can be sparse. Despite the majority of desmoid tumours posing no significant risk to patients, in those with aggressive disease they remain a leading cause of death in patients with FAP once their colorectal cancer risk has been addressed, as discussed in section 1.2.2. Understanding the long-term outcomes from patients with a desmoid tumour can aid in the counselling of patients regarding their prognosis and in considering appropriate management options, avoiding unnecessary intervention for stable disease whilst targeting more aggressive tumours. Some of the data in this patient series have been published before (Clark et al., 1999, Sinha et al., 2010a). This current study provides a larger, up to date cohort, and only considers desmoid tumours originating intra-abdominally and in the abdominal wall. In addition, it reviews parenteral nutrition requirements and small bowel transplant for patients with a desmoid tumour, both of which have not been considered before in these previous studies.

3.2 Aims

The aim of this study was to document treatment and survival outcomes for patients with intra-abdominal and abdominal wall desmoid tumours attending St Mark’s Hospital for FAP. This study focused in particular on treatment intervention requirements and survival outcomes associated with desmoid tumours.

3.3 Methods

A retrospective review was conducted studying patients with a diagnosis of FAP and an intra-abdominal and/or abdominal wall desmoid tumour. Those patients attending St Mark's Hospital for all or most of their follow-up for FAP between 1970 and 2014 were identified from the polyposis registry database and included in the study. The presence of a desmoid tumour was confirmed from radiological imaging or intra-operative findings. Outcomes recorded included: pharmacological intervention (this was defined as NSAIDs and/or anti-oestrogen use), surgery for tumour related complications (this included resection, bypassing of the tumour and small bowel transplant), parenteral nutrition (PN) use, and overall survival. PN use was considered a surrogate marker for severe intra-abdominal desmoid tumours and survival outcomes were compared between those patients who required PN to those who did not. Survival outcomes in this group are important because for some patients PN will be a long-term therapy. It can be associated with life-threatening complications that may be an indication, distinct from the desmoid tumour itself, for small bowel transplant surgery. Urological complications arising from desmoid tumour growth were considered in a separate study in chapter 4.

Patients without an abdominal wall or intra-abdominal desmoid were excluded from the study. Some patients had an abdominal desmoid in addition to a tumour located elsewhere, for example the chest wall or an extremity. These patients were included but the primary focus remained on the abdominal tumour when analysing outcomes. Patients without follow-up at our institution or had attended ad hoc were also excluded from the analysis.

3.3.1 Statistical analysis

Data were collected on a spreadsheet using Microsoft Excel (version 10) and statistical analysis was performed using GraphPad Prism 6 (GraphPad, San Diego, CA). Where appropriate data were presented as a median (range) with a Mann-Whitney *U* test used to analyse continuous variables. Desmoid tumour survival rate was compared between patients with and without PN requirements using the log-rank test. Kaplan-Meier survival curves were used to predict survival outcome in the two groups, taken from the time of desmoid diagnosis, including only desmoid-related mortality. For all analyses a p-value of less than 0.05 was considered significant.

3.4 Results

3.4.1 Patient demographics and tumour characteristics

There were 184 patients (57% female) with FAP and an abdominal wall and/or intra-abdominal desmoid tumour identified from the registry database. Twenty-one of these patients had an extra-abdominal desmoid tumour in addition to their abdominal wall and/or intra-abdominal tumour. Extra-abdominal sites mostly involved the chest wall (n=17) but also included the breast, neck and limbs. Table 3-1 shows the patient demographic and tumour related data from the study. The *APC* mutation was identifiable in 93% of cases and for just over a quarter (47/184) of patients this was 3' to codon 1399. There was no significant difference in the gender distribution between the 3' and 5' *APC* mutation sites. In 159/184 (86%) patients their desmoid tumour was located intra-abdominally and in 45/159 (28%) patients a tumour was also found in the abdominal wall. Abdominal wall desmoids tended to occur more frequently in women compared to men, 44% and 28%, respectively, although this difference was not

statistically significant $p=0.06$ (Fisher's exact test). Multiple tumours occurred in 58/184 (32%) of patients, with no significant difference in gender distribution.

In the majority of cases (77%), desmoid tumours declared themselves following surgery for FAP (a colectomy with IRA in 62% of cases). This typically occurred within 3.6 years of surgery. Women seemed to develop their desmoid tumour at a slightly younger age compared to men, 30.1 and 33.7 years, respectively. However, this was not a significant difference ($p=0.07$), with Mann-Whitney U test.

Table 3-1 Showing patient demographic data and desmoid tumour characteristics. Ileorectal anastomosis (IRA), restorative proctocolectomy (RPC) and total proctocolectomy (TPC). ⁺Gross deletion *APC* as a result of chromosomal loss ⁺⁺FAP confirmed through endoscopic findings (more than 100 colorectal polyps) and pattern of dominant inheritance

	n (%)		p-value
Gender			
Male	80 (43)		
Female	104 (57)		
Total	184		
<i>APC</i> mutation	Male	Female	
3' to 1399	25 (31)	22 (21)	*0.17
5' to 1399	49 (61)	73 (70)	
Gross deletion <i>APC</i> ⁺	1	1	
Unidentified mutation ⁺⁺	5	8	
Desmoid location according to gender	Male	Female	
Abdominal wall (AW)	22 (28)	48 (44)	*0.06
Intra-abdominal (IA)	72 (90)	87 (84)	*0.28
Multiple sites (AW/IA +/- other)	21 (26)	37 (36)	*0.20
Desmoid diagnosis			
Before colectomy	43 (23)		
After colectomy	141 (77)		
Median years: colectomy to desmoid (range)	3.6 (0.08-42)		
Type of colectomy for FAP			
IRA	114 (62)		
RPC	43 (23)		
TPC	19 (10)		
Other colonic resection	3 (2)		
None	5 (3)		
Age (median years) at desmoid diagnosis (range)	31.5 (1-68)		
Age according to gender			
Male	33.6 (5-65)		**0.07
Female	30.1 (1-68)		

*Fisher's exact test **Mann-Whitney *U* test.

3.4.2 Study limitations

The data here, although collected prospectively, were not collected solely for the purposes of this study. There is also the risk of referral bias in this cohort as some patients were referred specifically for treatment of their desmoid tumour, which were therefore more likely to be aggressive and more difficult to manage. This results in an overestimate of the proportion of patients requiring more invasive interventions such as surgery and PN. Similarly, the mortality rate associated with desmoid tumours may be higher than the true rate. In addition, this study spans a long time period, over which the management of desmoids has evolved.

3.4.3 Treatment and survival outcomes

Over a third of patients required no treatment intervention at all for their desmoid tumour. Just over half received a NSAID and/or an anti-oestrogen. In this treatment group 54/95 (57%) also had surgery for their desmoid tumour. Cytotoxic chemotherapy was commenced in 13/184 (7%) patients whilst 23/184 (13%) required PN. Four patients (2%) underwent a small bowel transplant for their intra-abdominal tumour and one other patient was awaiting a transplant at the time of the study. All five of these patients required PN prior to transplant and had undergone surgical intervention previously for their desmoid tumour. In 3/5 of these cases ureteric compression occurred bilaterally as a result of the desmoid tumour.

At the time of the study 47/184 (25%) patients had died and 14/184 (8%) of these deaths were directly attributable to the desmoid tumour, accounting for 30% of all deaths that occurred. The median age of desmoid related death was just 34.9 years. In 13/14 desmoid related deaths the

tumour was located intra-abdominally and in one case in the abdominal wall. Five patients were lost to follow-up (Table 3-2).

Figure 3-1 shows a Kaplan-Meier survival curve comparing survival outcomes in those patients who received PN as part of their treatment compared to those that did not. Patients who required PN unsurprisingly had a significantly worse survival compared to those that did not (Logrank test $\chi^2 = 6.55$ (1df), $p=0.01$).

Table 3-2 Desmoid tumour intervention and survival

	n (%)
Intervention	
None	67 (37)
Pharmacological	96 (52)
Chemotherapy	13 (7)
Surgical resection/bypass	74 (40)
Small bowel transplant	4 (2)
Parenteral nutrition	23 (13)
Survival	
Alive	132 (72)
All-cause mortality	47 (25)
Desmoid related mortality	14 (8)
Median age death from desmoid (years)	34.9 (range 17.3-67.8)
Lost to follow-up	5 (3)

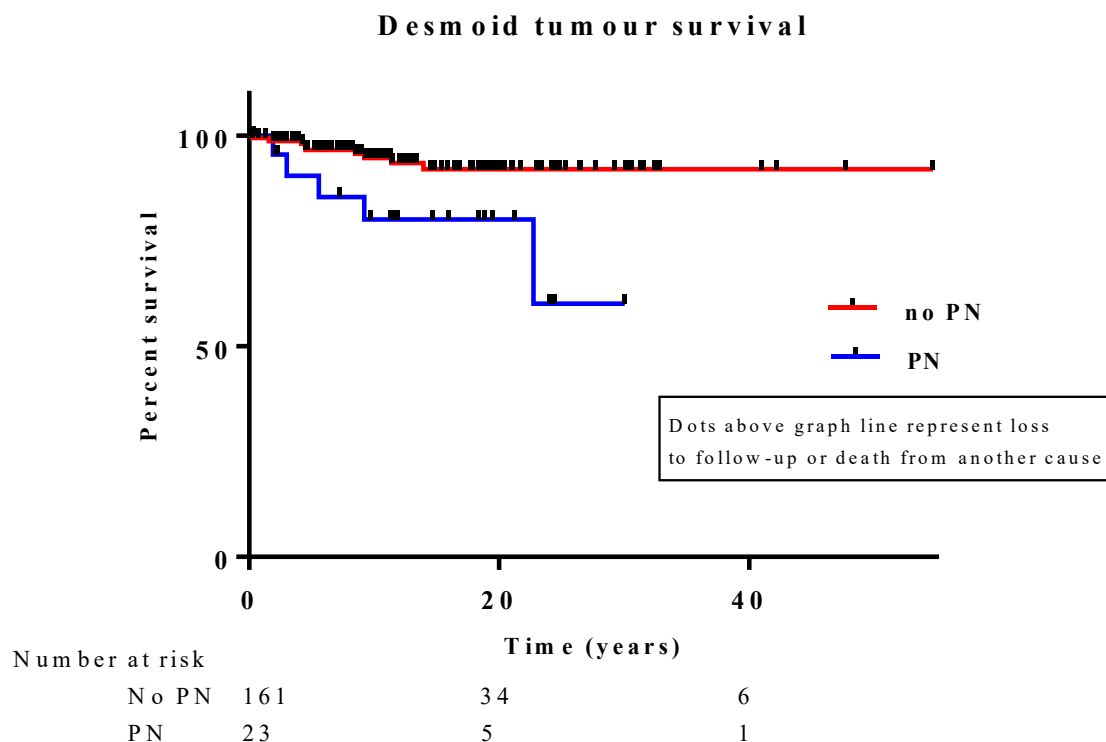


Figure 3-1 Comparing desmoid tumour survival outcomes between patients who required PN and those that did not

3.5 Discussion

3.5.1 Tumour site and gender

This study supports the findings from other series looking at desmoid tumours arising in patients with FAP. More desmoid tumours were located intra-abdominally than in the abdominal wall and in most cases arose following surgery after a time interval similar to that previously reported in a meta-analysis by Sinha et al (Sinha et al., 2011) and by Clark et al 1999. It is important to note that some of the patients from this current study will have been included in both of these papers. However, this cohort includes data up to 2014 and is larger with 158 patients compared to 88 patients reported by Clark et al in 1999 (IAD n=36, AWD n=20 and both sites n=31, EAD n=1) and 50 patients reported (tumour location not specified)

in a study by Sturt et al in 2004 and incorporated in the meta-analysis by Sinha et al, 2011. There was no obvious difference in gender distribution in those patients with a desmoid tumour, although there was a tendency for women to develop abdominal wall desmoids more than men. In addition, women seemed to develop desmoid tumours at a younger age than men, but again this was not statistically significant and only represented a difference of 3.5 years. These findings lend some support to the oestrogen hypothesis described previously in section 1.2.7.1.

3.5.2 Intervention

In over a third of cases no treatment was instituted for desmoid tumour, suggesting that these tumours were stable. This would concur with previous findings about the natural course of desmoids (Church, 1995). Just over half of medically treated patients had surgery, which could be considered a surrogate marker for failure of medical treatment. This response rate to medical therapy echoes that of previously published data (Janinis et al., 2003, Bocale et al., 2011). However, caution is needed when drawing conclusions about medical therapy for desmoid tumours. It can be difficult to determine whether tumour response is due to therapy commencement or merely reflects the unpredictable growth behaviour of the desmoid tumour. Much of the research reported in the literature is retrospective, as in this case, and lacks any case-control methodology. In this study it was difficult to extrapolate from the database and case notes when medical intervention was commenced. Therefore, relating this to other treatment interventions, such as surgery, can be difficult. Database recording of medical treatment commencement was more reliable in recent years compared to historical records. Also, some patients started their medical therapy at other institutions and capturing the accurate dates of commencement was not possible in some cases.

Cytotoxic chemotherapy and PN were commenced in 7% and 13% of patients, respectively. These could both be considered surrogate markers for aggressive desmoid tumours and would support previous findings that around 10% of desmoids grow aggressively (Church, 1995). It is these tumours that are of particular interest as they are a significant cause of morbidity and, as shown in this study for PN, mortality.

Only a small proportion of patients in this cohort underwent a small bowel transplant for desmoid and 3/4 cases were performed in the last six years. It is apparent that small bowel transplant is now a growing area in the treatment of aggressive intra-abdominal desmoid tumours. Such tumours are usually associated with other complications that include ureteric obstruction. It is interesting that bilateral ureteric obstruction occurred in three of these patients as a result of desmoid tumour growth. This complication is explored in greater depth in a separate study in chapter 4. Chapter 6 examines the clinical outcomes in patients undergoing small bowel transplant for their desmoid tumour treatment in two adult transplant centres in the UK.

3.6 Conclusion

More desmoid tumours occurring in patients with FAP arise intra-abdominally than in the abdominal wall. Patients presenting with a desmoid tumour for the first time can be informed that in a third of cases they require no treatment at all. Around half of patients would usually commence medication for their desmoid and this is probably effective in about 50% of cases. Around 40% of patients will eventually undergo surgery for their desmoid. Aggressively growing tumours usually require more interventions, such as PN and chemotherapy, in addition to medical and surgical therapy. In less than 10% of cases desmoids may become a life-

threatening problem. This study supports the hypothesis that desmoid tumours impact on patient health and can cause significant morbidity and mortality in patients with FAP.

4 Chapter 4 – Ureteric complications arising from desmoids

4.1 Background

As the last study has shown, the majority of desmoid tumours do not result in significant complications and many remain stable. However, despite being benign (non-metastasising) tumours, aggressive desmoids can be a cause of serious complications and remain a leading cause of death in patients with FAP once the colorectal cancer risk has been addressed. As previously described in section 1.2.6 and as shown in the last study, FAP associated desmoids are predominantly located intra-abdominally. Here they can infiltrate the mesentery and encase the superior mesenteric vessels. This can result in bowel obstruction, fistulation, perforation and abdominal sepsis (Clark et al., 1999, Quintini et al., 2012, Bhandari et al., 2012). Ureteric obstruction is also a recognised complication associated with their growth.

The incidence of ureteric obstruction arising as a result of desmoid tumour growth is not well established. One previous study quoted a rate of 28% (Joyce et al., 2010). The true incidence can be difficult to determine, as ureteric complications often present to local centres where subsequent management may be undertaken. On the other hand, desmoid tumour follow-up and management is often through a tertiary centre such as St Mark's Hospital. Patients at this institution are typically seen every six to twelve months for their polyposis and therefore such complications are likely to be captured on the polyposis registry database.

4.2 Aims

The aim of this study was to determine the rate of ureteric obstruction, its treatment and the clinical outcomes associated with intra-abdominal desmoid tumours managed at St Mark's Hospital.

4.3 Methods

Data were obtained from the prospectively maintained registry database. All patients with FAP and an intra-abdominal desmoid tumour, with follow-up between 1970 and 2014, were identified and included in the study. Desmoid diagnosis was confirmed by radiological imaging or from intra-operative findings. Patients with desmoids confined to the abdominal wall, without any mesenteric involvement, were excluded from the study. The unit's policy for intra-abdominal desmoid surveillance has changed over time but has always involved regular imaging of the abdomen. Currently all newly diagnosed intra-abdominal tumours are monitored with an ultrasound scan every 6-12 months, with a prompt CT should complications or aggressive tumour growth occur.

Case notes and radiological imaging were analysed and data collected. Ureteric compromise secondary to desmoid tumour compression was identified by the presence of mild, moderate or severe hydronephrosis with hydroureter to the level of the desmoid. This was established from radiological imaging or intra-operatively where there was evidence of the tumour intimately involved with the affected ureter. MAG3 or DMSA scans, if available, were used to assess renal function. Information gathered included: patient demographics, details of previous

surgery for FAP and relationship to the timing of desmoid diagnosis, desmoid complications and management.

Those patients with an intra-abdominal desmoid tumour without ureteric complications (group A) were compared to those who did develop complications (group B) for gender, *APC* germline mutation, age at desmoid diagnosis, time interval from colectomy to desmoid development, medical and surgical treatment interventions and overall survival. Medical therapy was defined as the use of non-steroidal anti-inflammatory drugs (NSAIDs) and/or anti-oestrogens. Cytotoxic chemotherapy was recorded separately to this.

4.3.1 Statistical analysis

The relationship between desmoid tumours and other clinicopathological parameters was analysed using nonparametric statistics. Clinical outcomes were assessed using Kaplan-Meier survival curves and the groups were compared using the log-rank test. The level of significance permitting multivariate analysis inclusion, and the statistical significance for all other tests used, was set at $p < 0.05$. All analyses were performed using the statistical software, Statistical Package for the Social Sciences, version 20.0 (SPSS, Inc, Chicago, IL).

4.4 Results

4.4.1 Patient demographics

There were 158 patients (56% female) with an intra-abdominal desmoid tumour identified from the registry database (Table 4-1). All of these patients had follow-up at this institution for part or all of their care. The median age at desmoid diagnosis was 31 years (IQR 23-41). Forty of

these patients (25%) developed ureteric complications from their intra-abdominal desmoid (group B) and 118 patients (75%) did not (group A). Gender and overall age at desmoid presentation did not differ significantly between the two groups. However, in group B (with ureteric complications), female patients developed desmoids at a significantly younger age than male patients, at 25 and 43 years, respectively ($p=0.01$). Also there was a tendency towards more far 3' codon *APC* mutations in this group compared to group A, 16 (40%) and 28 (24%), respectively, but this was not statistically significant ($p=0.07$).

The overall median time interval from colectomy to desmoid development was 3.6 years and this was not significantly different between the two groups, neither was the type of colectomy surgery performed (IRA, RPC or TPC). A proportion of patients already had a desmoid prior to their colectomy; 19% and 16% in group A and group B, respectively. Four patients in group A and two in group B had not undergone a colectomy at the time of the study.

In patients who developed ureteric complications, 38% (15/40) of these were identified at the same time as the desmoid tumour. The remainder occurred within a median of 4 years from tumour diagnosis. For two such cases there was a significant lag of more than 20 years, from desmoid diagnosis to ureteric obstruction. In one patient this was associated with a degree of permanent loss of renal function, confirmed from a MAG3 scan, demonstrating only 33% function of the kidney on the affected side.

Table 4-1 Demographic data for desmoid tumours without ureteric complications (group A) compared to those with ureteric compromise (group B). Data are median values with interquartile range (IQR) where appropriate. Ileorectal anastomosis (IRA), restorative proctocolectomy (RPC) and total proctocolectomy (TPC)

	Group A n (%)	Group B n (%)	Odds ratio (95% CI)	p-value
Gender				
Male	52 (44)	18 (45)	1	0.91
Female	66 (56)	22 (55)	1.0 (0.5-2.1)	
Age (median in years) desmoid diagnosed				
Female	31	25		0.08
Male	34	43		0.20
Overall	32 (IQR 23-39)	28 (IQR 22-43)		0.55
APC mutation 3' codon 1399				
No	82 (69)	23 (58)	1	0.07
Yes	28 (24)	16 (40)	2.04 (1-4.4)	
Unknown	8 (7)	1 (2)		
Type of prophylactic surgery				
Colectomy and IRA	73	22	1	0.59
RPC	29	11	1.3 (0.5-2.9)	
TPC	11	5	1.5 (0.5-4.8)	0.49
Nil	5	2		
Time FAP surgery to desmoid (months)	44 (IQR 22-176)	37 (IQR 16-87)		0.28
Time desmoid to ureteric obstruction:				
Simultaneously diagnosed		15/40		
Obstructed after desmoid found		25/40		
Time (months) to develop		49 (IQR 22-122)		

Ureteric compromise was detected from radiological imaging in all but three cases when it was identified simultaneously with the desmoid tumour at surgery. In 81% of these patients a CT scan was the imaging modality used (Figure 4-1). An ultrasound scan (USS) was mostly utilised thereafter for urinary tract surveillance. Patients with ureteric complications were more than seven times as likely to require medical intervention, with NSAIDs or anti-oestrogen therapy, and more than 9 times as likely to have received cytotoxic chemotherapy than those

without ($p < 0.01$ in both instances). Patients in group B were twice as likely to have required surgery to excise or debulk desmoid tumour or bypass obstructed bowel by it, than those in group A ($p = 0.03$) (Table 4-2).

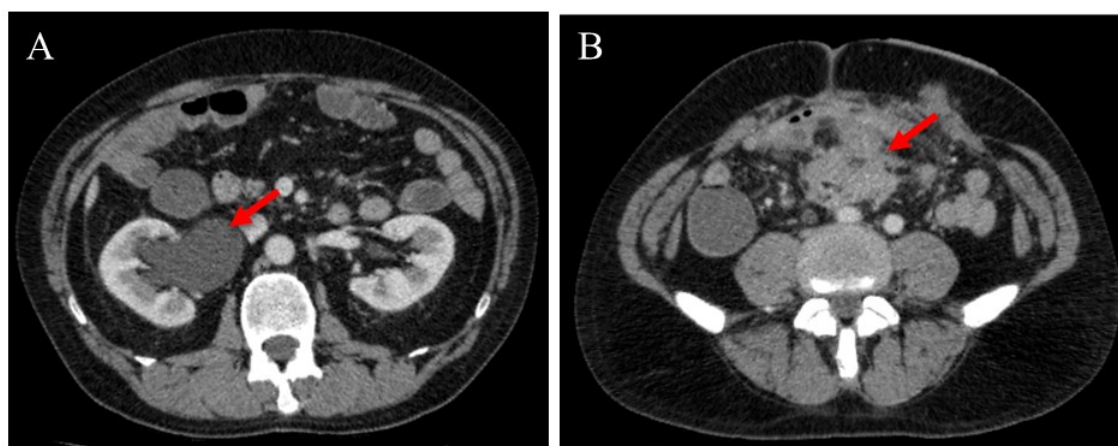


Figure 4-1 CT scan images from the same patient demonstrating a right hydronephrosis (arrow) in A, secondary to a large pelvic desmoid (arrow) in B obstructing the ureter distally

For patients who developed ureteric compromise, 25% were monitored closely and had no urological intervention. In the remaining 29/40 (75%) group B patients: 27 (93%) had placement of a ureteric stent, five (17%) had a nephrostomy for failed stent placement and three (10%) underwent re-implantation of a ureter. At the time of this study two patients were awaiting their first stent placement. A large proportion of patients (48%) developed bilateral ureteric compression as a result of their desmoid tumour. In six patients there was a degree of permanent renal injury as a result of a desmoid tumour obstructing their ureter. Three of these patients had negligible function of one kidney, detected at the time ureteric obstruction was diagnosed. In the other three cases renal injury developed 4 months, 1 year and 20 years after ureteric involvement was first identified.

Table 4-2 Imaging modality for desmoid diagnosis and treatment intervention requirement for groups A and B

	n (%)		Odds ratio (95% CI)	p-value
Radiological desmoid diagnosis	36/40			
USS	5			
CT	29			
MRI	2			
Ureter affected				
Right	13			
Left	8			
Bilateral	19			
	Group A		Group B	
Interventions:				
Desmoid medical therapy				
No	60 (51)	5 (13)	1	<0.01
Yes	58 (49)	35 (87)	7.24 (2.7-20)	
Chemotherapy for desmoid				
No	114	30	1	<0.01
Yes	4 (4)	10 (25)	9.5 (2.8-32.4)	
Desmoid surgery				
No	74 (63)	17 (43)	1	0.03
Yes	44 (37)	23 (57)	2.27 (1.1-4.7)	
Ureteric intervention				
Nil		11/40		
Ureteric stenting		27		
Nephrostomy		5		
Ureter re-implantation		3		

For the 27 individuals who had stent placement, outcome data were unavailable for two as they were lost to follow-up, one of whom subsequently died. In the remaining 25 patients only two (8%) had full resolution of their obstruction, enabling stent removal. One other patient with bilaterally placed stents was able to have one removed. The other 22 patients still required ureteric stenting at a median of six years following their first stent insertion and 12 of these had residual hydronephrosis despite adequate stent placement. At the time of the study 38/158 people had died, 14 (8%) as a result of their desmoid tumour. Comparing overall survival between those who developed ureteric complications from their desmoid (group B) and those

who did not (group A), there was no significant difference ($p=0.996$) (**Error! Reference source not found.**).

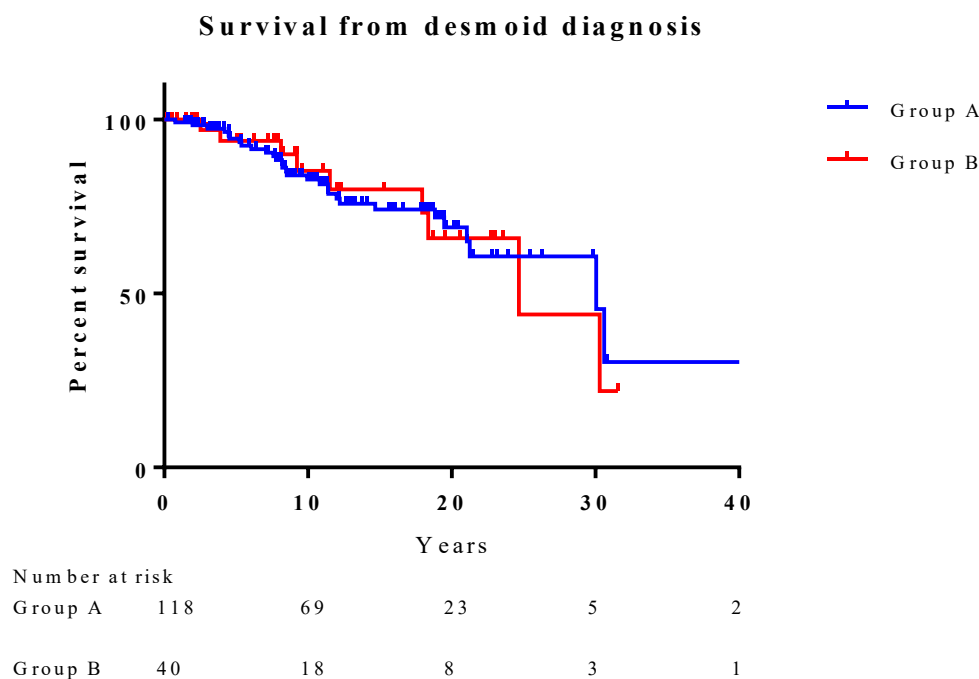


Figure 4-2 Kaplan-Meier curves comparing survival between groups A and B

4.5 Discussion

This study found that 25% of patients with FAP and an intra-abdominal desmoid tumour developed some degree of ureteric compromise due to the tumour growth. In this study intra-abdominal desmoid tumours typically occurred in young patients at a median age of 31 years. In those with ureteric complications, 38% were diagnosed simultaneously with the desmoid tumour and the rest occurred within a median of 49 months from tumour diagnosis. This is a significant problem for patients with, and at risk of developing, intra-abdominal desmoid tumours.

A similar rate for ureteric complications arising from intra-abdominal desmoid tumour growth was reported by Joyce et al in a smaller cohort of 106 patients (Joyce et al., 2010). However, their study also found a female preponderance for desmoid ureteric complications. Interestingly, the current study did not corroborate this finding but did observe that women were diagnosed with ureteric complications at a significantly younger age than men. Although not significant, there appeared to be a trend towards patients with *APC* mutation 3' of 1399 in the ureteric complication group compared to those without ($p=0.07$). This is perhaps not too surprising as mutations in this region are a known risk factor for desmoid development (Sinha et al., 2011). If this observation were true it may suggest that mutations in this region would predict a more aggressive desmoid phenotype where ureteric obstruction would occur more readily. This would support the findings in a recent study by Church et al (Church et al., 2015).

Trauma from colectomy surgery is a significant risk factor for desmoid development. A previous meta-analysis by Sinha et al reported a median time from colectomy to desmoid detection of 3.2 years (Sinha et al., 2011). The current study found a similar time interval of 3.6 years from colectomy to intra-abdominal desmoid tumour diagnosis.

Patients with ureteric complications required more medical, chemotherapeutic and surgical interventions (other than the urological procedures described above) for their desmoid tumour than those without. This is perhaps unsurprising as it is likely to reflect the aggressive nature of the desmoid tumours in this cohort. Such tumours are likely to compromise other structures besides the ureters, including bowel, which may necessitate intervention. Despite this group requiring more treatment interventions, there was no significant difference in overall survival

compared to those without ureteric obstruction, suggesting that the strategies for identification and management of this complication are largely successful.

This study highlights the importance of long-term radiological imaging of the urinary tract following a diagnosis of an intra-abdominal desmoid tumour. It has shown the majority of ureteric complications will occur at the time of desmoid diagnosis or soon thereafter, but it can occur long after (more than 20 years later in two cases). The study also found that male patients with a desmoid developed ureteric complications at a later age compared to women.

At this institution stent changes are performed typically six monthly but this can be extended to yearly intervals if well tolerated and there is no evidence of stent encrustation. Stent removal is considered if there is radiological evidence of desmoid tumour shrinkage and a retrograde pyelogram demonstrates adequate drainage.

An important finding from this study was that ureteric stents were likely to be a long-term requirement for most patients. Few stents placed for ureteric obstruction from desmoid tumour were subsequently removed and hydronephrosis often persisted despite adequate placement. A significant proportion of patients developed bilateral ureteric obstruction so more frequent monitoring (less than six monthly) and stent changes were required. A proportion failed (13%), necessitating a nephrostomy. The ureters can be tortuous and the procedure particularly difficult for those who have undergone RPC. These outcomes are important for both patients and clinicians when discussing ureteric interventions for desmoid tumour obstruction.

4.5.1 Study limitations

The limitations of this study included the urological management being undertaken locally rather than at the tertiary centre managing the desmoid tumour in many cases. Renal function data were therefore limited. In addition, it was a retrospective study and although data were collected prospectively this was not for the sole purpose of this study. There is potential for underestimation of ureteric complications associated with desmoid as some patients may have been referred locally for this without the knowledge of the tertiary study centre managing the desmoid tumour. However, most patients at St Mark's Hospital are seen every 6 to 12 months for their FAP and it is therefore likely that urological complications occurring would be brought to the attention of the study centre at a clinic consultation.

Referral bias could also have occurred in this study as patients with an aggressive desmoid tumour (with associated ureteric complications) are more likely to be referred to the tertiary centre for management of their desmoid. However, no patients were referred to this unit solely for management of a desmoid related ureteric complication. The strength of this study is its cohort size for such a rare disease.

4.5.2 Conclusion

This study found a high rate of ureteric obstruction associated with intra-abdominal desmoid tumours, lending further support to the hypothesis that desmoid tumours significantly impact on patient health. It emphasises the importance of regular long-term surveillance of the urinary tract to prevent permanent renal injury. Both patients and clinicians should be aware that ureteric stent placement is likely to be a long-term requirement once hydronephrosis develops.

5 Chapter 5 - Gastric cancer and desmoid tumours

5.1 Background

Gastric cancer is not a recognised extra-colonic feature of familial adenomatous polyposis (FAP) in the Western world. The risk of developing gastric cancer for this patient cohort is reportedly similar to that of the general population (Garrean et al., 2008, Offerhaus et al., 1992). However, in countries where gastric cancer is more prevalent, for example Korea and Japan, it is a recognised feature of FAP, and associated with a higher than expected incidence compared to that of the general population (Park et al., 1992, Utsunomiya et al., 1988, Iwama et al., 1993). Clinical studies describing the features and the risk of developing gastric cancers in association with FAP are lacking and also conflicting, particularly in Western populations.

Following the recent development of gastric cancer in two patients with FAP from the St Mark's Hospital polyposis registry, a search of the database was conducted to review all gastric cancer cases that had occurred in association with FAP over a 40-year period. Although only eight cases were identified it was surprising to find that five had a personal history of desmoid tumour occurrence and two others that did not have desmoids themselves had a family history of desmoid. Although this was a small patient group, the rate of desmoid tumour occurrence seemed higher than the expected frequency of 15-25% of patients with FAP (Vasen et al., 2008, Sinha et al., 2011). This posed the question could those patients with gastric cancer and desmoid share a similar germline mutation profile? The existence of a genotype-phenotype correlation has been well described in FAP, linking germline mutations to clinical manifestations such as CHRPE and desmoid tumours, as discussed in 1.1.3. To date no

genotype-phenotype correlation has been described for gastric cancer occurrence in association with FAP.

5.2 Aims

The aim of this study was to determine whether gastric cancers occurring in FAP were associated with desmoid tumour development and if they shared a common region on the *APC* gene that could explain such an association.

5.3 Methods

All histologically confirmed gastric adenoma and cancer cases from 1974-2014 were identified from the registry database. Gastric adenoma cases were analysed as they are considered the precursor lesion to gastric cancer development. If a genotype-phenotype correlation exists for gastric cancer it should also be seen in gastric adenoma cases and the rate of desmoid tumour occurrence should also be higher if the findings from the gastric cancer cohort were real. Medical notes, radiological imaging, endoscopy and pathology reports were obtained and further scrutinised. Data gathered included patient demographics, *APC* mutation, personal and family history of desmoid tumour occurrence, gastric tumour histology, stage, location, management and survival outcome.

Permission to access and utilise an international FAP database held by the International Society for Gastrointestinal Hereditary Tumours (InSiGHT) group was also obtained. The InSiGHT *APC* mutation database was analysed to provide a control cohort with FAP without gastric cancer or gastric adenoma that could be used to compare the germline spectrum. This control

group was utilised in preference to the St Mark's polyposis database as it provided a large and independent cohort. Entries with ambiguous genetic entries (for example 'atypical FAP') and those with large mutations that would not allow analysis of a truncated APC protein causing an effect were removed from the InSiGHT control cohort. Furthermore, all silent and missense mutations were excluded from the analysis, leaving only those mutations that would predict truncation of the protein (non-sense, frameshift and splicing mutations). All gastric adenoma entries from this database were also removed. Gastric adenomas from the InSiGHT database were not included in further analysis as detailed clinical information and histology were unavailable for this group. Gastric cancer cases were included along with those from the polyposis registry database. This left a large FAP 'control' dataset to compare with the gastric adenomas from the polyposis registry and gastric cancer cases from the polyposis registry and InSiGHT database.

Genetic mutations in the gastric cancer, adenoma and control groups were then analysed and the region that they spanned recorded. It was assumed that there would be no significant difference in the proportion of mutations in the FAP control group compared to the tumour groups.

5.4 Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad, San Diego, CA). For analysis a p-value of less than 0.05 was considered significant.

5.5 Results

In total there were nine gastric cancer patients; eight from the polyposis registry database over the forty-year period and one from the InSiGHT database, although this was excluded from most analyses, other than *APC* mutation, as no other clinical data were available. After filtering for mutations as described above, 3012 FAP controls were identified from the database, of these 38 were recorded as gastric adenoma cases. Similarly, there were 19 gastric adenoma cases identified from the polyposis registry database.

5.5.1 Gastric cancer mutations and desmoid tumour association

Table 5-1 shows the clinical characteristics of gastric cancer cases identified from the registry database. Median age at diagnosis was 52 years (range 41-64). Five out of eight (63%) patients had a personal history of a desmoid tumour and in the remaining three that did not, two had a family history for desmoid development. Comparing this rate of desmoid occurrence with that typically reported, up to 25% for patients with FAP, this is significantly higher than would be expected ($p < 0.0001$ with Fisher's exact test). Analysis of all nine gastric cancer cases germline *APC* mutations found that they were clustered on exon 15 between codons 685 and 2040 and in 6/9 (67%) cases they were located 3' of codon 1390, close to the region associated with a higher risk for desmoid tumour occurrence in FAP (Sinha et al., 2011). Statistical analysis using a two-tailed Fisher's exact test compared the germline *APC* mutation distribution between the InSiGHT database control cohort to the gastric cancer cases. The mutations from the control group were distributed over a larger region of the gene with 1107/3012 (37%) located outside of the gastric cancer *APC* mutation region. In addition, significantly fewer FAP

control cases were located 3' of codon 1390 compared to the gastric cancer cohort (399/3012 (13%) versus 6/9 (67%), respectively ($p=0.0003$)) (Table 5-2).

Table 5-1 Clinical features of gastric adenocarcinomas arising in FAP.

Gastric cancer case	Gender	Age at gastric cancer diagnosis (years)	Location of <i>APC</i> mutation (codon)	Desmoid tumour (family history of desmoid)
1	M	54	685	Yes
2	F	58	895	Yes
3	M	62	1084	No (yes)
4	F	49	1392	Yes
5	F	46	1396	No
6	F	46	1450	Yes
7	M	64	1464	No (yes)
8	F	41	1503	Yes
9*	-	-	2040	-

* *APC* mutation only information available

Table 5-2 Comparing frequency of *APC* mutations within and outside for the region associated with gastric cancer.

Location of <i>APC</i> mutation (codon)	Controls, n (%)	Gastric cancer cases, n (%)	p-value
5' of 685/3' of 2040	1107 (37)	0	*0.03
Between 685-2040	1905 (63)	9 (100)	
5' of codon 1390	2613 (87)	3 (33)	*0.0003
3' of codon 1390	399 (13)	6 (67)	

*Fisher's exact test

5.5.2 Gastric adenomas and desmoid tumour association

Table 5-3 shows the clinical characteristics of gastric adenoma patients. The median age at diagnosis was 44 years (range 23-59), eight years younger than that for gastric adenocarcinomas. Histology found 16 (84%) lesions were tubular adenomas (TA) and three were tubulovillous adenomas (TVA). The gastric adenoma cohort, similar to the carcinoma group, had a greater proportion of patients than would typically be expected with a personal or family history of a desmoid tumour, nine with a personal history and one with a family history (10/19 (52%)). However, unlike the gastric cancer cases, the germline *APC* mutations in this group were spread over a larger region of the gene, between codons 233 and 1461 (Table 5-3). Also significantly fewer *APC* mutations were located 3' of codon 1390 compared to the cancer group, 2/19 (11%) and 6/9 (67%) patients, respectively, ($p=0.005$). Overall there was no significant difference in the distribution of mutations between those patients with an adenoma compared to those without any gastric lesion (Table 5-4).

Table 5-3 Clinical features of gastric adenomas occurring in patients with FAP. TA: tubular adenoma, TVA: tubulovillous adenoma, LGD: low grade dysplasia, MGD: moderate grade dysplasia, HGD: high grade dysplasia)

Gastric adenoma case	Gender	Age at gastric cancer diagnosis (years)	Location of <i>APC</i> mutation (codon)	Desmoid tumour (family history of desmoid)
1	F	52	233	Yes
2	M	49	302	No
3	M	57	445	No
4	M	40	452	No
5	F	45	554	Yes
6	M	50	554	No (Yes)
7	F	34	606	Yes
8	M	41	636	No
9	F	59	650	Yes
10	F	26	789	Yes
11	M	41	811	No
12	F	23	830	No
13	F	31	895	Yes
14	F	44	1061	Yes
15	F	51	1131	No
16	F	54	1309	No
17	M	42	1367	No
18	M	48	1392	Yes
19	M	25	1461	Yes

Table 5-4 Comparing frequency of APC mutations within and outside of the region associated with gastric cancer between controls and gastric adenoma cases.

Location of <i>APC</i> mutations (codon)	Controls, n (%)	Gastric adenomas	p-value
5' of 685/3' of 2040	1096 (37)	9 (47)	*0.35
Between 685-2040	1878 (63)	10 (53)	
5' of codon 1390	2576 (87)	17 (89)	*1
3' of codon 1390	398 (13)	2 (11)	

*Fisher's exact test

5.6 Discussion

This study found a possible association between gastric cancer development and a history of desmoid tumour. A significantly higher than expected number of gastric cancer patients had a personal or family history of a desmoid tumour. This was also true, albeit to a lesser extent, for gastric adenoma cases. If such a connection were real, it would be reasonable to hypothesise this could be a genetic association, raising the possibility that a genotype-phenotype correlation for gastric cancers exists in FAP, in the same way it does for desmoid tumours (Church et al., 2015). An explanation for this association would be that they overlap a similar *APC* germline mutation region (for example 3' of codon 1399 for desmoids and 3' of 1390 for gastric cancer).

It would seem logical that gastric adenomas, (if they were the pre-malignant lesion responsible for gastric cancer development in FAP), would demonstrate a similar genotype-phenotype correlation, however this was not the case. Gastric adenoma cases did not have a similar *APC* germline mutation distribution to the gastric cancer group and there were far fewer 3' of codon 1390 mutations compared to the cancer group. The mutation clustering seen in gastric cancer cases may therefore just be an anomalous finding due to the small numbers of patients in this cohort. Alternatively, location of the germline *APC* mutation between codons 685 and 2040 could be important in driving the progression of some gastric adenomas towards cancer, and these mutations are therefore clustered.

In support of this study's findings, others have reported a possible association between gastric lesions and desmoid tumour occurrence in FAP. A group from the Mayo Clinic looked at nine patients who developed gastric adenomas in a series of 97 patients with FAP. In those that developed a gastric adenoma five (56%) had a desmoid tumour, compared to 19/88 (22%) who

had no history of gastric adenomas. Although this is also a small cohort, our rate of desmoid tumour occurrence in the gastric adenoma group was comparable to this study's findings (Ngamruengphong et al., 2014). A study by Leggett et al reported on a family with a far 3' *APC* mutation (codon 1520) who had a propensity to develop severe gastric and duodenal polyposis, necessitating a partial gastrectomy in one patient (Leggett et al., 1997). Other case reports have described an association gastric cancer and subsequent desmoid tumour development following gastrectomy (Komatsu et al., 2006, Kim et al., 2014).

5.6.1 Study limitations

The retrospective nature of this study is a limitation, despite the data being collected prospectively. In a few instances, patients with FAP will undergo upper GI surveillance locally, rather than at St Mark's Hospital. This could lead to fewer adenoma cases being identified through the registry database than would occur if the endoscopies were undertaken at the tertiary centre. However, most patients undergo their routine follow-up for FAP at this institution and therefore oesophagoduodenoscopy (OGD) reports from local institutions are usually passed on to, or sought by, the St Mark's polyposis registry. Certainly, the development of a gastric cancer is unlikely to occur without the tertiary centre being aware as patients and their relatives generally maintain good links with the centre, particularly as they are usually followed-up for life.

The InSiGHT database contained a large FAP cohort but it was not possible to include those patients labelled as 'gastric adenoma' in the adenoma group analysis (these were excluded from both the adenoma cohort and the control cohort for the analysis of gastric adenoma cases from

St Mark's polyposis database). There were insufficient data regarding histology details to be sure they were true gastric adenoma cases.

Referral bias is unlikely to be a concern as St Mark's Hospital is not an upper GI cancer centre and therefore patients would not have been referred here specifically for the purpose of managing their gastric adenoma or cancer.

The strengths of this study include the long-term follow-up of patients with FAP at St Mark's Hospital. This has enabled the maintenance of one of the world's largest and longest running FAP databases, providing an invaluable resource for the study of this rare condition. Access to the InSiGHT database also provided thousands of FAP 'control' cases without gastric lesions. This was a rich resource for studying *APC* mutations in this group.

5.6.2 Conclusion

There may be an association between the development of gastric cancer and desmoid tumour occurrence in FAP. Although the existence of a gastric cancer genotype-phenotype correlation remains a possibility, this was not supported by the findings from the gastric adenoma cohort. To determine the true significance of these findings a much larger cohort is necessary, requiring a considerable international, multi-centre, collaborative effort.

6 Chapter 6 - Small bowel transplant for desmoid tumours

6.1 Background

Extensive small bowel resection, chronic intestinal obstruction, motility disorders, congenital abnormalities and disease-induced loss of intestinal absorption can cause intestinal failure. Intestinal failure is defined as the inability to sustain protein-energy, fluid, electrolyte or micronutrient levels. Short bowel syndrome is a lesser form of intestinal failure defined as the inability to maintain these levels whilst taking a normal diet (O'Keefe et al., 2006).

Complicated intra-abdominal desmoid tumours can lead to short bowel syndrome and intestinal failure. As discussed in section 1.2.8.4, surgery for aggressive IAD tumours is hazardous and can result in extensive small bowel resection or enterectomy when tumours are located at the root of the mesentery (Clark and Phillips, 1996). In cases where extensive resection is unavoidable the aim of treatment is to re-establish protein, electrolyte and fluid balance. The management of the intestinal insufficiency will depend on the severity and degree of malabsorption. At the severe end of the spectrum, where medical management with anti-motility and anti-secretory drugs along with adjustments to dietary and fluid intake have failed, long-term parenteral nutrition (PN) is the treatment of choice for short bowel syndrome or indeed intestinal failure (Pironi et al., 2011, Jeejeebhoy, 2010).

Short bowel syndrome is a reversible condition for approximately 40% of patients, due to functional adaptation of the small bowel following resection (Vantini et al., 2004), although this will depend on factors including residual bowel length, preservation of the ileo-caecal valve or colon and duration of PN dependence (Yandza et al., 2007, Thompson et al., 1995, Jeejeebhoy, 2010). Therefore in patients who have had a small bowel resection for an IAD

tumour, short bowel syndrome is more likely to result in permanent intestinal failure and PN dependence, due to little residual small bowel and as most will have undergone a colectomy for FAP (Wheeler et al., 2012).

Long-term PN is associated with significant complications that include: liver cirrhosis and central venous catheters induced vascular thrombosis and infections. The five-year survival rate varies according to indication for PN requirement but is around 80% (Vantini et al., 2004, Pironi et al., 2011).

Small bowel transplantation (SBT) was first attempted in 1964 but early experience was associated with dismal survival due to acute graft rejection (Okumura and Mester, 1992). It was not until the discovery of tacrolimus that intestinal transplantation became a feasible surgical option for patients with intestinal failure on permanent PN (Starzl et al., 1989, Hoffman et al., 1990). A SBT would potentially offer such patients a life free from PN and reverse complications associated with its use. The main indications for referral for consideration of SBT surgery are PN-related liver failure and intra-abdominal desmoids. In a study by Pironi et al these were the only two factors shown to have a significantly increased risk of mortality in those patients receiving PN that therefore made SBT a reasonable treatment option (Pironi et al., 2011). For other causes of intestinal failure, the benefit of intestinal transplant over PN was questionable. Current survival rates following SBT are around 90% at one year and 70% at five years (Abu-Elmagd et al., 2009).

Various intestinal transplantations can be performed depending on patient requirements. These include an isolated SBT (the most common procedure), a liver and SBT or a multivisceral

transplant (including small bowel, liver, spleen, stomach, pancreas and duodenum), with or without abdominal wall (Middleton and Jamieson, 2005, Beyer-Berjot et al., 2012).

SBT surgery has now become part of the treatment ladder for severe IAD tumours. In particular, a multivisceral transplant is good option for those patients with significant duodenal polyposis (Cruz et al., 2011).

6.2 Aims

The aim of this study was to review outcomes in all SBTs performed for desmoid tumours at the two UK adult intestinal transplant centres, Cambridge and Oxford.

6.3 Methods

All patients undergoing a SBT for intestinal failure or short bowel syndrome due to an IAD tumour were identified from the National Adult Small Intestinal Transplantation (NASIT) group database. Patients had undergone surgery at one of the two adult intestinal transplant centres. Case notes were obtained from these sites to collect data regarding: pre-operative quality of life, parenteral nutrition (PN) requirement and complications, indication for SBT, time from referral to surgery, length of hospital stay, SBT complications and survival outcomes.

6.3.1 Ethical considerations

Ethical approval for this study was related to a desmoid quality of life study described in chapter 7 obtained through the National Research Ethics Service (NRES) committee from South Central - Berkshire B (REC reference number: 13/SC/0429). Research and development

approval for the study was obtained from North West London Hospitals NHS Trust Research and Development Department (R&D ref: RD13/061).

6.4 Results

Eight people (five female) underwent SBT for an intra-abdominal desmoid tumour at the two UK centres between 1994 and 2014 at a median age of 33 years (range 23-49). Seven patients had a history of FAP and all had undergone a previous colectomy (Table 6-1). No formal quality of life assessment was undertaken pre-operatively in this cohort but all patients had complained of pain resulting in reduced mobility, increased lethargy and an inability to work.

Table 6-1 Patient characteristics and desmoid/PN complications. SBO; small bowel obstruction, SBS; short bowel syndrome. *Spordaic desmoid

Patient	Gender	Age SBT (years)	Desmoid/PN complications	Type of SBT
1	F	37	Mesenteric vessels encased, SBO and ureteric obstruction. No PN	Multivisceral
2	F	34	SBS, high jejunostomy and ureteric obstruction. Loss of venous access and line infection	Multivisceral
3	M	32	Mesenteric vessels encased and SBO. No PN	Multivisceral
4	M	24	SBO and SBS after enterectomy. Liver steatosis	Multivisceral
5	F	44	SBS (exenteration) and fistulae. Liver steatosis	Small bowel
6*	M	49	SBS and SBO with gastric outlet obstruction. Liver steatosis, line infection and migration	Small bowel
7	F	23	SBS, fistulae and high jejunostomy. Line infections and thrombosis	Small bowel
8	F	29	SBO and fistulae. PN induced thrombosis	Small bowel and abdominal wall
Median age		33 years		

6.4.1 Indications for SBT referral

Indications for SBT in the patient cohort are shown in Figure 6-1. A previous enterectomy had been performed in two out of the five patients with short bowel syndrome, two others had a high jejunostomy and the other a high ileostomy. Two of these patients also had ongoing issues with small bowel obstruction. Other complications arising from desmoid tumours prior to transplant surgery included: enterocutaneous fistulae and ureteric obstruction. In terms of nutritional requirements; six patients required PN before transplantation, one person was fed via a percutaneous endoscopic gastrostomy and the other maintained their nutrition orally. All six patients receiving PN had experienced PN-related complications (see Figure 6-2) and three experienced more than one problem. Five out of six patients were able to completely cease PN following SBT.

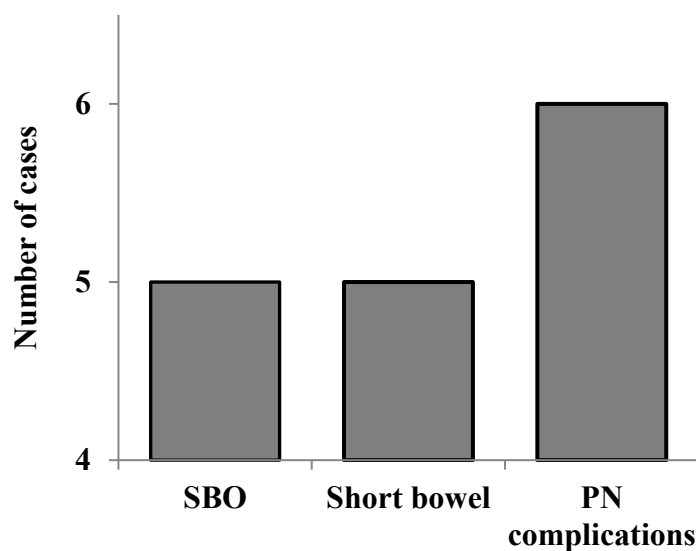


Figure 6-1 Indications for SBT referral. SBO; small bowel obstruction

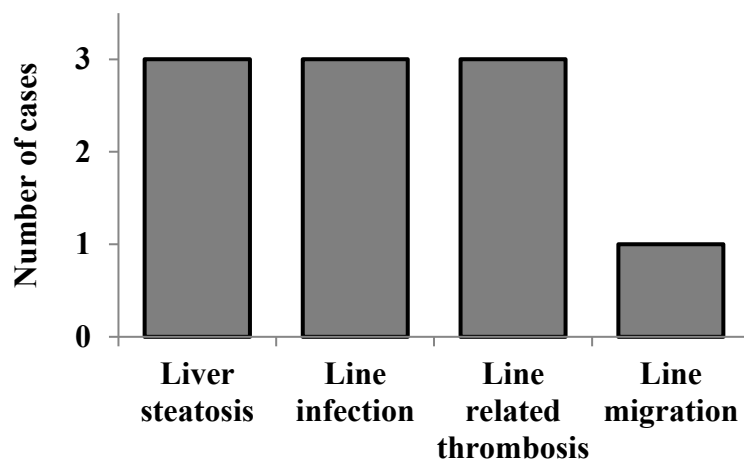


Figure 6-2 PN-related complications

6.4.2 Complications after SBT

The median time from referral for consideration of surgery to SBT was 9.2 months (range 2.5-17.8) and median time from desmoid diagnosis to SBT was 5.8 years (range 43months-24 years). Four patients underwent a multivisceral graft transplant, three had small bowel only and one had small bowel with abdominal wall transplant for fistulating disease (Figure 6-3). Median length of hospital stay was 40 days (range 37-261). All patients received Tacrolimus. There were no peri-operative deaths. Complications occurring during the peri-operative period included: bleeding (one patient lost 26 litres of blood during surgery), chest infection (n=2), chylothorax (n=1), acute kidney injury (n=1) and graft rejection (n=1). Although there was evidence of graft rejection at 10 days in one patient, they were able to retain their graft. However, this patient remained PN dependent and died 27 months later from issues relating to chronic graft rejection and immunosuppression (with a brain abscess and EBV infection). A further two patients experienced graft rejection, one received a second graft seven months later and is alive 73 months on.

Long-term complications in patients who did not experience graft rejection included renal impairment, hypoadrenalism and the development of a new abdominal wall desmoid tumour at the site of the abdominal scar.



Figure 6-3 Patient with transplanted small bowel and abdominal wall

6.4.3 Survival outcomes after SBT

Five out of eight patients are alive and well, with none requiring PN following SBT. The three-year survival rate following SBT was 83% with a median follow-up of 40.4 months (IQR 21.8-67.6). One patient died due to an unrelated cause 71 months after their SBT. The other two patients died from complications relating to chronic rejection. One died 63 months after SBT from sepsis and renal failure and the other at 27 months (as discussed in section 6.4.2).

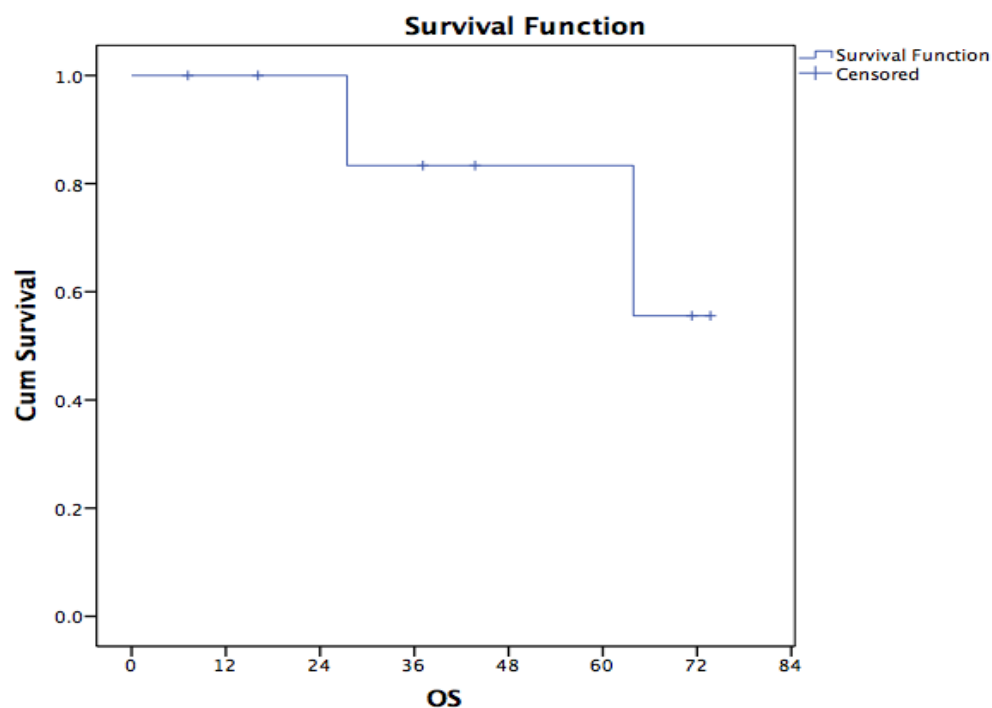


Figure 6-4 Kaplan-Meier survival curve for desmoid tumour cases after SBT

In one case a SBT was not possible at the time of surgery as the desmoid tumour had become too advanced (Figure 6-5). The patient died shortly afterwards.

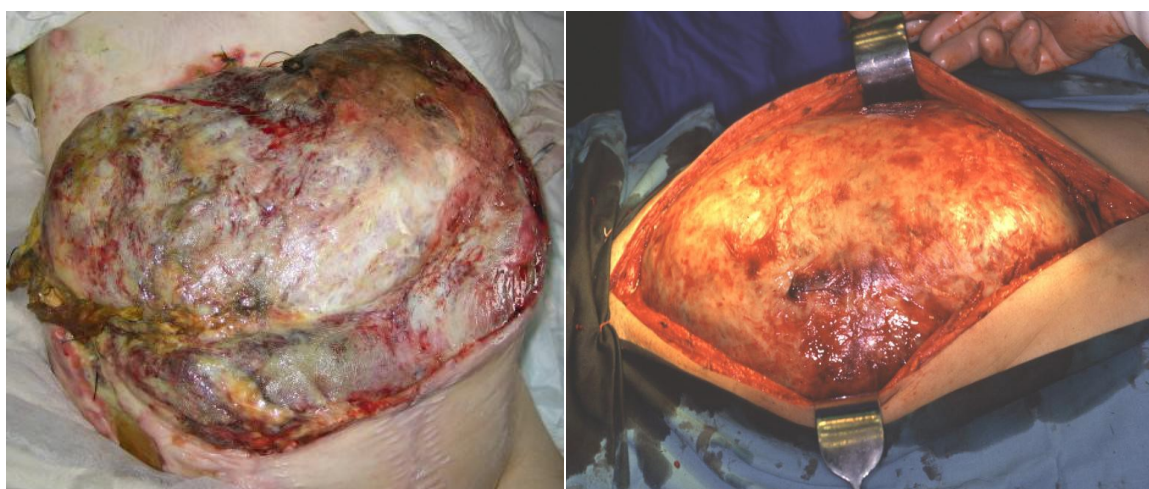


Figure 6-5 An intra-abdominal desmoid tumour that was unresectable at the time of their planned SBT

6.5 Discussion

SBT is a viable treatment option for complicated intra-abdominal desmoid disease and may improve survival outcome. The survival rate at three years in this study was 83%. Although this was a small study, the findings support those of Moon et al who concluded in 14 patients undergoing intestinal transplant, that it was a reasonable life-saving treatment for severe intra-abdominal tumours (Moon et al., 2005). This study provides important data on indications and outcomes for both patients and clinicians contemplating such surgery for desmoid tumours in the UK. The main indications in this complex group for a SBT were PN-related complications, short bowel syndrome and/or small bowel obstruction. Undertaking such surgery in this complex patient cohort can be extremely challenging. In patients with FAP nearly all have had a previous colectomy. In addition, many referred for SBT have had other laparotomies for complications arising from their desmoid tumour requiring formation of a high stoma, extensive small bowel resection or an enterectomy.

With five-year survival rates up to 70% in the most experienced units, the selection of patients for SBT needs to be considered carefully. In the UK this is achieved through a multi-disciplinary team discussion involving both intestinal failure centres and bowel transplant units. It is crucial to select those patients in whom survival is likely to be equivalent (but with a worse quality of life) or reduced, without a SBT. For example, patients on long-term PN have a five-year survival rate of around 80% and therefore may wish to continue life without a SBT where the survival rate may be lower. However, for those with a poor quality of life, they may consider a SBT, even though their survival may be reduced, if their quality of life can be improved.

It is important in those with significant disease not to delay surgery, especially in cases of aggressively growing desmoid tumours, as surgery may become impossible. This occurred in one case at one of the bowel transplant centres. Although the case was not included in this study it became apparent at the time of data collection. In this instance small bowel transplant surgery was attempted but abandoned due to the extensive IAD discovered at the time and the patient died shortly afterwards. Although median time from decision to operate to SBT was nine months, there can be a significant delay from the referring institution. It is now our practice to refer complex cases of intra-abdominal desmoid tumours, resistant to medical treatment, to the SBT unit early on. This enables them to be introduced to the team and be fully counselled prior to being considered for SBT surgery. Patients are then followed up in the clinic and the timing of SBT can be determined appropriately. This approach helps to reduce the risk of late referral to surgery.

6.5.1 Conclusion

SBT is now part of the treatment ladder for complex intra-abdominal desmoid tumours. Although survival rates for SBT have significantly improved over the last two decades, more information is now required regarding the long-term quality of life benefits from this surgery. The next chapter will look at assessing the quality of life issues for patients with desmoid tumours.

7 Chapter 7 - Quality of life associated with desmoid tumours

7.1 Background

The previous few chapters have highlighted the clinical complications that can result from desmoid tumours. It is important to re-emphasise that these are usually associated with the severe end of the disease spectrum and represent a minority of patients with desmoid tumours. Nonetheless, for those affected by complications such as ureteric obstruction or bowel perforation requiring surgery and/or parenteral nutrition, the impact on their physical and mental health can be significant. Little has been reported on health-related quality of life associated with desmoid tumour occurrence. It is an important aspect of healthcare, particularly for those patients and healthcare professionals contemplating therapeutic interventions for desmoid tumours that may impact on quality of life. Most quality of life studies conducted in patients with FAP have focused on outcomes from colectomy, with a particular focus on bowel function (Raviram et al., 2015, Fazio et al., 2013, Durno et al., 2012, Nicholls, 2011). Very few studies have specifically addressed the quality of life impact of a desmoid tumour in patients with FAP. In those that have, it is usually a secondary outcome measure and not the primary study aim (Moon et al., 2005). To date only one study has looked at the quality of life in patients with desmoid tumours and FAP (Esplen et al., 2004) indicating the paucity of data and the need to explore this field further.

7.2 Aims

The aim of this study was to test the hypothesis that desmoid tumours significantly impact on health-related quality of life in patients with FAP.

7.3 Methods

7.3.1 Patient selection

Patients were identified from the registry database and invited to participate in the study. The aim was to compare quality of life in patients with FAP who had a personal history of a desmoid tumour (desmoid cases) with a control group of patients who had FAP but no history of a desmoid tumour (FAP controls). All patients had a diagnosis of FAP, were over the age of 18 years, and at the time of the study were at least 3 years on from their large bowel surgery for FAP. This ensured that all patients had the same exposure to surgery (a risk factor for desmoid occurrence) and were beyond the period that it would most likely occur. It also ensured that both groups had a similar medical background in terms of the FAP. They had all undergone prophylactic colectomy or proctocolectomy, endoscopic surveillance and follow-up for their condition and these factors were therefore controlled for across the two groups.

There were two parts to the study, a desmoid patient focused group discussion to elicit potential quality of life issues, and a questionnaire part given to patients with FAP with and without a desmoid tumour. For the questionnaire arm of the study patients were approached for their consent to participate in the study by post or in clinic. Those patients invited to the discussion group were approached in clinic or by telephone. All participants gave their informed written consent to their participation in the study (Appendix A2). Questionnaires were posted to some patients while others were approached when they attended a routine outpatient clinic appointment for their FAP follow-up.

7.3.2 Ethical considerations

Ethical approval for the study was obtained through the National Research Ethics Service (NRES) committee from South Central - Berkshire B (REC reference number: 13/SC/0429). Research and development approval for the study was obtained from London North West Healthcare NHS Trust Research and Development Department (R&D ref: RD13/061).

7.3.3 Focus group discussion

There were two discussion groups, one male (n=3) and one female (n=5). Separate gender groups were selected as it was anticipated that the discussions could generate sensitive information (for example regarding relationships, body image and sexual issues) and participants may be unwilling to discuss these so freely within mixed gender groups. Each discussion group lasted between 60-80 minutes and was conducted by the same researcher and audio recorded. Consent for the focus group discussion was obtained separately to the questionnaire arm of the study. This emphasised the importance of maintaining confidentiality regarding the material discussed, and obtained permission from participants to audio record the discussions.

The researcher began the discussion with an open question to generate debate between participants: 'Do you think there is a need to look at quality of life in people with a desmoid tumour?' Questions were then generated based on a biopsychosocial model (Engel, 1977) and included three quality of life domains: biological, psychological and social impacts from desmoid tumours. The audio recordings were then transcribed. Qualitative analysis of each transcript was then undertaken to identify quality of life themes generated by the discussions.

These themes were recorded on an excel spreadsheet and their frequency of discussion noted. The aim was to establish recurring quality of life issues that could be important and specific to patients with a desmoid tumour.

7.3.4 Questionnaires

A generic demographic questionnaire and two validated quality of life questionnaires were administered to participants and included: EQ5D-5L and short-form 36 (SF-36) (Appendix A3.1 and A3.2). The demographic questionnaire recorded gender, age at the time of completion, marital status and employment status. The short-form 36 (SF-36) questionnaire is a validated health survey comprising 36 questions originally developed by Rand for the Medical Outcomes Study (Ware and Sherbourne, 1992). Questions are divided into eight sections: four for physical health and four for mental health. The physical health domains include: physical functioning (PF), physical role functioning (RP), bodily pain (BP) and general health perceptions (GH). Mental health domains include: vitality (VT), social role functioning (SF), emotional role functioning (RE) and mental health (MH). Scores were generated from survey responses for each section and compared between the two groups. Overall physical component and mental component scores were also obtained and comparisons made.

The EQ5D-5L questionnaire is a standardised survey tool to measure health outcomes. It is comprised of five descriptive domains: mobility, self-care, usual activities, pain and anxiety. Each domain has five levels ranging from no problems to extreme problems. The second component to the instrument is the EQ visual analogue scale (EQ-VAS), a vertical scale bar that participants mark according to their perceived health status at the time of completing the

questionnaire. Permission to utilise the EQ5D-5L questionnaire was granted through EuroQol Group Foundation. Patients were contacted for their responses if answers to questions were found incomplete. Questionnaire responses were then analysed and compared between the two groups. A separate sub-analysis compared patients with severe desmoid tumours (those with a history of surgical intervention, parenteral nutrition requirement, ureteric or bowel obstruction due to their desmoid), with FAP controls. It was anticipated that patients with asymptomatic desmoid tumours would have a similar quality of life to the FAP control group whereas those more symptomatic would have a worse quality of life.

7.4 Results

7.4.1 Focus group discussion qualitative analysis

A total of twelve patients with desmoid tumours were invited to participate in the focus group discussions and eight (66%) attended (five women and three men) two discussion groups. All had a history of symptomatic desmoid tumours (e.g. bowel obstruction, perforation or ureteric compression), occurring within a six-month period prior to the study. Table 7-1 lists all the themes that were generated from the transcripts (Appendix A3.3 and A3.4) and the frequency with which they were discussed. There were multiple biological, psychological and social issues identified.

The most frequent theme that arose was the lack of information available to patients about desmoid tumours. There was a general feeling amongst both groups that they just had to ‘get on with it’ that there was little they could do about their situation and that they had to look at the positive things in life. Another important theme that arose was frustration about the lack of

knowledge patients noticed amongst health professionals in the wider medical community (outside of the tertiary centre that manages their FAP). The unpredictable behaviour of tumours was also frequently discussed. One lady said when she developed any abdominal pain she would wonder if she would be unwell for one day or require another three-month hospital admission. A gentleman also described the impact of this unpredictable behaviour on his social life too. The cosmetic appearance of the desmoid was a theme that came up mostly in the female discussion group and was an issue that affected all of the participants. They discussed the need to wear baggy clothes in an attempt to conceal the appearance of their abdomen and how people had remarked that they looked pregnant. One lady said she wished one day she could wake up, place her hand on her abdomen, and not feel the tumour. A supportive family network was an important theme that arose, although interestingly one patient admitted to concealing their diagnosis from their partner.

Most of the themes generated were specific to the desmoid tumour and not a result of the FAP condition. Parental guilt around the diagnosis of FAP was an exception to this. The social impact of desmoid tumours was evident and the majority of themes generated related to this domain. Patients described adjusting eating behaviours to influence timing of defaecation and stool frequency and one gentleman avoided social activities as he required home parenteral nutrition and could not eat. Many had required time off work or could no longer work due to their desmoid tumour. Interestingly pain was not a prominent theme, although the group were not asked about this specifically. Given that all of the group members had significant desmoid tumours at some point, all had experienced pain from their tumour. Perhaps pain was something that they just learned to live with and was reflected in the general attitude of the participants that they just had to 'get on with life'. Pain was explored further in the standardised quality of life questionnaires separately.

Table 7-1 Transcript quality of life themes according to frequency of discussion. Bold indicating the most frequent themes

Biological themes (n)		Psychological themes (n)		Social themes (n)	
Lack of knowledge	13	Lack of information	30	Family support important	10
Unpredictable	12	Get on with it	22	Limits social activity	8
Stoma anxiety	7	Cosmesis	11	Restricts eating behaviour	7
Lethargy	7	Continuity of care	8	Inability to work	7
Defaecatory frequency	5	Worry about future	8	Negative impact on family	7
Pain	4	Lack of support/isolated	7	Anxiety about relationships	6
		Specialist centre reassuring	4	Hiding info from family	6
		Affects confidence	3	Family member guilt	6
		Depression	3	Insurance difficulties	4
				Advised against pregnancy	3

7.4.2 Questionnaires

A total of 70 patients completed the three questionnaires. Forty-two questionnaires were posted and 17 (40%) were returned completed: 11/20 in the desmoid group and 6/22 in the FAP control group. Following this poor response rate, patients were approached in the outpatient clinic and none refused to complete the questionnaires, providing an additional 53 responses. In total 70 patients were recruited to the questionnaire part of the study: 32 had FAP and a history of desmoid and 38 had FAP without desmoid. There was no significant difference between the desmoid group and FAP control group in terms of age or gender distribution, marital status or employment status (Table 7-2).

Table 7-2 Participants demographic data.

	FAP controls n (%)	Desmoid cases n (%)	p-value
Participants	38 (54)	32 (46)	
Gender			
Male	14	16	*0.33
Female	24	16	
Age	46	44	**0.79
Marital status			
Single	13	10	+0.60
Married/co-habiting	22	17	
Divorced	3	5	
Employment status			
Unemployed	1	4	*0.17
Employed	37	28	

* Fisher's exact test, **Mann-Whitney U test, + χ^2 test

7.4.2.1 EQ5D-5L questionnaire analysis

The EQ5D-5L questionnaire responses were grouped together for ease of analysis into 'no difficulty' and 'any difficulty' for each quality of life domain, initially comparing the whole desmoid group to the control group (Table 7-3). A larger proportion of patients in the desmoid group compared to the control group, had difficulties in all five of the domains (mobility, self-care, usual activities, pain and anxiety). These differences were not significant except for the activity domain (including work, study, housework, family or leisure activities) that reached borderline significance, with 38% of the desmoid group experiencing difficulties compared with 16% in the FAP control group ($p=0.05$). Analysis of the EQ-VAS scores found no significant difference in the median scores between the two groups.

A separate sub-analysis of the EQ5D-5L questionnaire compared responses between FAP controls and patients with severe desmoid disease (Table 7-4). This desmoid group had significantly more difficulties performing usual daily activities and experienced more pain compared to FAP controls. The median EQ-VAS scores also tended to be lower in the desmoid group compared to FAP controls (73 and 85, respectively), but this was not significantly so ($p=0.07$).

Table 7-3 EQ5D-5L and EQ-VAS scores comparing any difficulty in each of the 5 domains in FAP controls with desmoid cases.

Any difficulty	FAP controls n (%)	Desmoid cases n (%)	p-value
Mobility			
No	32	22	*0.16
Yes	6 (16)	10 (31)	
Self-care			
No	35	27	*0.46
Yes	3 (8)	5 (16)	
Activity			
No	32	20	*0.05
Yes	6 (16)	12 (38)	
Pain			
No	18	8	*0.08
Yes	20 (53)	24 (75)	
Anxiety			
No	23	20	*1
Yes	15 (39)	12 (38)	
EQ-VAS (median score)	85	78	**0.18

* Fisher's exact test, ** Mann-Whitney U test

Table 7-4 Comparing EQ5D-5L and EQ-VAS scores between FAP controls and severe desmoid cases.

Any difficulty	FAP controls n (%)	Severe desmoid cases n (%)	p-value
Mobility			
No	32	11	*0.27
Yes	6 (16)	5 (31)	
Self-care			
No	35	14	*0.63
Yes	3 (8)	2 (13)	
Activity			
No	32	9	*0.04
Yes	6 (16)	7 (44)	
Pain			
No	18	2	*0.03
Yes	20 (53)	14 (88)	
Anxiety			
No	23	9	*0.77
Yes	15 (39)	7 (44)	
EQ-VAS (median score)	85	73	**0.07

*Fisher's exact test, **Mann-Whitney U test

7.4.2.2 SF-36 questionnaire analysis

Compared to controls, patients in the desmoid group had significantly lower scores for six out of eight components of the SF-36 questionnaire: all four SF-36 physical health domains and two of the mental health domains (Table 7-5 and Figure 7-1). The most significantly reduced scores were seen in physical role functioning (RP) and vitality (VT) domains. This finding supports those seen with the EQ5D-5L analysis, where activity was reduced in the desmoid group compared to controls. In addition, bodily pain was significantly worse in the desmoid group too, and this was seen in the severe desmoid group EQ5D-5L analysis. SF-36 social functioning was also reduced in the desmoid group and this was an area discussed within the focus group discussions, and supports those findings. Interestingly despite the impact that desmoid tumours seem to have on quality of life from the SF-36 results obtained, mean mental

health score for the desmoid group was nearly the same as the FAP control group. Given these results, unsurprisingly the overall physical component SF-36 score was significantly reduced in the desmoid group compared to the FAP control group although the mental component scores were not significantly different (Figure 7-2).

Table 7-5 Mean scale based scores for all SF-36 domains comparing FAP controls and desmoid cases. Scores are expressed as the mean and SE is standard error of the mean

SF-36 domains	FAP controls		Desmoid cases		p-value (t-test)
	Scores	SE	Scores	SE	
Physical functioning (PF)	86.71	3.84	73.28	5.33	0.04
Role physical (RP)	84.87	5.38	49.38	7.95	0.0003
Bodily pain (BP)	76.58	4.01	59.84	5.47	0.01
General health (GH)	64.79	3.42	50.97	5.05	0.02
Vitality (VT)	61.97	3.40	46.41	3.92	0.004
Social role functioning (SF)	84.21	3.39	72.66	4.81	0.05
Role emotional (RE)	84.21	5.73	73.96	6.98	0.26
Mental health (MH)	74.77	2.92	75.25	3.15	0.91

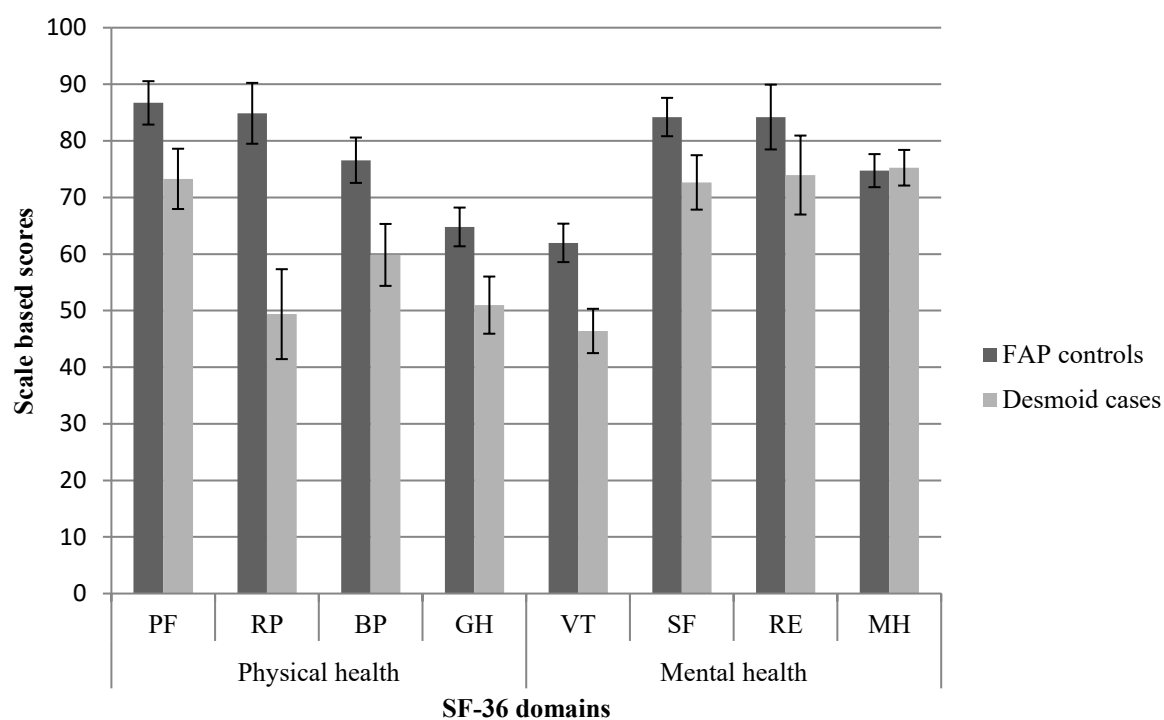


Figure 7-1 Column graph with error bars comparing SF-36 scale based scores in physical and mental health domains between FAP controls and desmoid cases

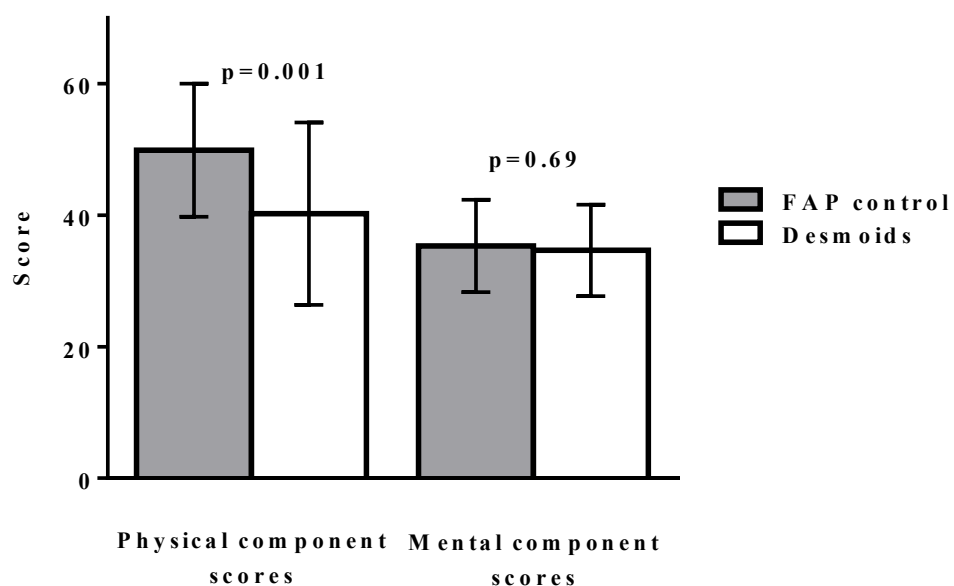


Figure 7-2 Overall scale-based scoring for SF-36

7.5 Discussion

This study found there was a significant difference in the health-related quality of life in patients with FAP between those with and without a history of a desmoid tumour. These differences were likely to be attributable to the desmoid tumour, given that patients with FAP were used as a control group and had a similar medical background.

The most frequently discussed concerns from the focus group discussions included: a lack of patient information and medical knowledge regarding a desmoid tumour diagnosis, a sense of acceptance and that they had to 'get on with it', the unpredictable nature of the tumour, tumour cosmetic effects and the importance of a supportive family. However, it should be noted that all patients in the discussion groups had symptomatic desmoid tumours and many others with a desmoid tumour do not have significant problems from them. In the only publication to date that has looked specifically at quality of life in patients with a desmoid tumour in FAP, the key themes identified from their focus group discussions were: a sense of uncertainty, feelings of isolation at the time of diagnosis, communication issues within families, awareness of body appearance, a lack of knowledge amongst health professionals, a resilience and desire to live life to the full, and concern for family members (Esplen et al., 2004). These themes are very similar to those that developed from the current study. Patients were not specifically asked about these themes during the focus group discussions but they were generated by the patients' themselves. The findings reported by Esplen et al therefore seem to corroborate the findings from this study.

The validated questionnaires (EQ5D-5L and SF-36) found some components of quality of life were significantly reduced in patients with a desmoid tumour. Domains affected included:

activity, pain, physical role functioning, vitality, social role functioning and general health perception. The EQ5D-5L questionnaire was not as sensitive as the SF-36 questionnaire at identifying quality of life differences between patients with and without a desmoid tumour in FAP. Perhaps if the study sample was larger the EQ5D-5L would have shown more differences as there was a tendency towards worse outcomes in the desmoid group compared to controls. Only the activity domain was reduced (borderline significance) in the desmoid group compared to controls, although in the SF-36 analysis physical functioning was significantly reduced in all four subcategories for the same group of patients. Subsequent EQ5D-5L sub-analysis of patients with a more severe desmoid phenotype found, in addition to activity being reduced, pain was significantly higher in the desmoid group compared to controls. Interestingly, pain was not an issue discussed at any great length within the focus group. This may reflect the general nature of the patients in the group who had a ‘just get on with it’ attitude and were perhaps less likely to complain about pain or perhaps had become accustomed to it, so that they had not thought to mention it. This resonated with the findings reported from the group discussion by Esplen et al where patients had a ‘resilience and desire to live life to the full’ (Esplen et al., 2004). Desmoid tumour occurrence did not seem to impact significantly on mental health, despite the differences in physical components to quality of life that were seen.

7.5.1 Study limitations

In the study of a rare cohort of patients the sample size will always be a limiting factor, but this is the largest desmoid quality of life study to date. The initial postal response to the questionnaire arm of the study was disappointing and it is always difficult to know the reasons behind those that chose to respond and those that did not. Although the recruitment of patients from the clinic was very successful, this would have excluded those patients unable to attend the clinic at that time. They may have had significant quality of life issues that prevented their

attendance to clinic, or perhaps they required a hospital admission elsewhere. Similarly, perhaps they did not attend the clinic because they felt so well. However, this was a generic FAP clinic follow-up and was not specifically for their desmoid tumour.

Another disadvantage was the selection of the focus group participants. This group contained only patients who had been symptomatic from their desmoid tumour and therefore did not represent the general desmoid population (many of whom are asymptomatic). It was recognised that developing a list quality of life themes specific to the desmoid tumour required discussion with symptomatic patients; if they were asymptomatic it is unlikely they would have generated any meaningful discussion specific to desmoid tumour related quality of life.

A strength of this study was the control group of patients with FAP without a history of a desmoid tumour. This was far more robust than comparing the desmoid group to the general population where the effects from the FAP condition (perhaps relating to diagnosis, colectomy or endoscopic follow-up) may have blurred the significant findings directly attributable to the desmoid tumour itself.

7.5.2 Conclusion

Desmoid tumours in FAP have a significant impact on patient health-related quality of life. They can limit physical activity and functioning, impact on social activity and are a source of pain. Patients feel information regarding their condition is limited and recognise health professionals' lack knowledge in this area, reflecting the rare nature of this tumour. These are quality of life issues unique to patients with a desmoid tumour and it is now time that these

were explored further to develop a desmoid-specific quality of life assessment tool. This would be an important component of assessing response to treatment of desmoids.

PART THREE – LABORATORY STUDIES**8 Chapter 8- Histological studies of desmoids****8.1 Background**

The histological features of desmoid tumours have been described in previous studies, section 1.2.3.2. Some reports have utilised immunohistochemistry to study the Wnt pathways in desmoid development, although mostly in sporadic tumours rather than in patients with FAP.

8.1.1 The Wnt signalling pathways in desmoid tumours

The canonical (classical) Wnt pathway has been studied extensively in FAP, as described in sections 1.1.1 and 1.2.4. Wnt ligand binding to the frizzled/LRP5/6 receptor complex results in accumulation of cytoplasmic β -catenin that would normally be degraded by a complex formed with AXIN and APC. Failure to form this complex results in nuclear translocation of β -catenin and activation of Wnt-regulated genes and TCF mediated transcription factors, ultimately leading to tumour formation. Most studies report on Wnt signalling activation in sporadic desmoid tumours and relate to the canonical pathway and nuclear β -catenin expression. The rate of positive nuclear β -catenin staining in desmoid tumours is reported between 70-100% (Tejpar et al., 1999, Montgomery et al., 2002, Abraham et al., 2002, Bhattacharya et al., 2005, Fisher and Thway, 2014, Carlson and Fletcher, 2007).

To date, little has been reported on the non-canonical Wnt pathways, that are an antagonist to the canonical pathway, in desmoid tumour development. These pathways are mediated by Wnt ligands, independent of β -catenin. The non-canonical pathways include the Wnt/planar cell pathway (PCP) and the Wnt/calcium pathway. They are mediated by dishevelled proteins and

intracellular calcium that activate pathways involved in cell motility (the JNK pathway) and cell polarity (CaMKII-TAK1-NLK, PKC-Cdc42, and calcineurin-NFAT pathways) (Figure 8-1). The non-canonical pathways are important in embryogenesis, neurogenesis and the development of soft tissues (Sugimura and Li, 2010, Song et al., 2015).

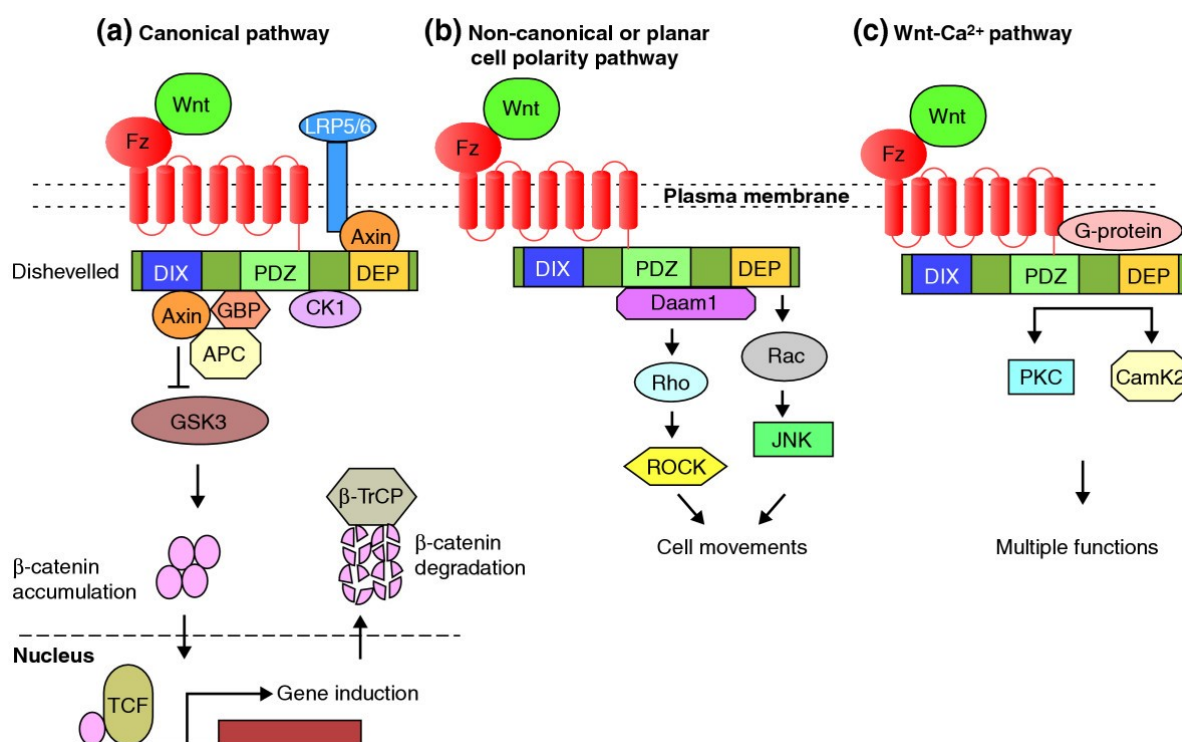


Figure 8-1 The canonical and non-canonical Wnt signalling pathways (Habas and Dawid, 2005)

There are 19 Wnt ligands. Wnt-1, Wnt-3 and Wnt-8 are important in activating the canonical pathway whereas Wnt-5a and Wnt-11 activate the non-canonical pathway. Research undertaken previously at the study institution by Bhandari et al looked at the expression of a non-canonical pathway ligand, Wnt-11, in a single desmoid tumour both in cell culture and formalin fixed paraffin embedded (FFPE) tissue. Wnt-11 interacts with the FZD7 receptor to activate the non-canonical pathway. It may also act as an antagonist to the canonical pathway

(Toyama et al., 2010, Maye et al., 2004) (Figure 8-2). Activation of Wnt-11 has been implicated in the development of cervical, prostate and colorectal cancers (Wei et al., 2016, Koushyar et al., 2016, Nishioka et al., 2013). Studies have utilised cell culture, PCR and immunohistochemistry to demonstrate Wnt-11 activation in such tumours. Immunohistochemistry techniques use a cytoplasmic Wnt-11 antibody to assess for activation of Wnt-11.

The study conducted by Bhandari et al found overexpression of β -catenin (nuclear) and a downstream target (AXIN2), as well as Wnt-11, both in desmoid cell culture and from FFPE tumour tissue. These findings provided evidence of activation of the canonical Wnt pathway and suggested that the non-canonical Wnt pathway may also be activated in desmoid tumours (Bhandari, 2013).

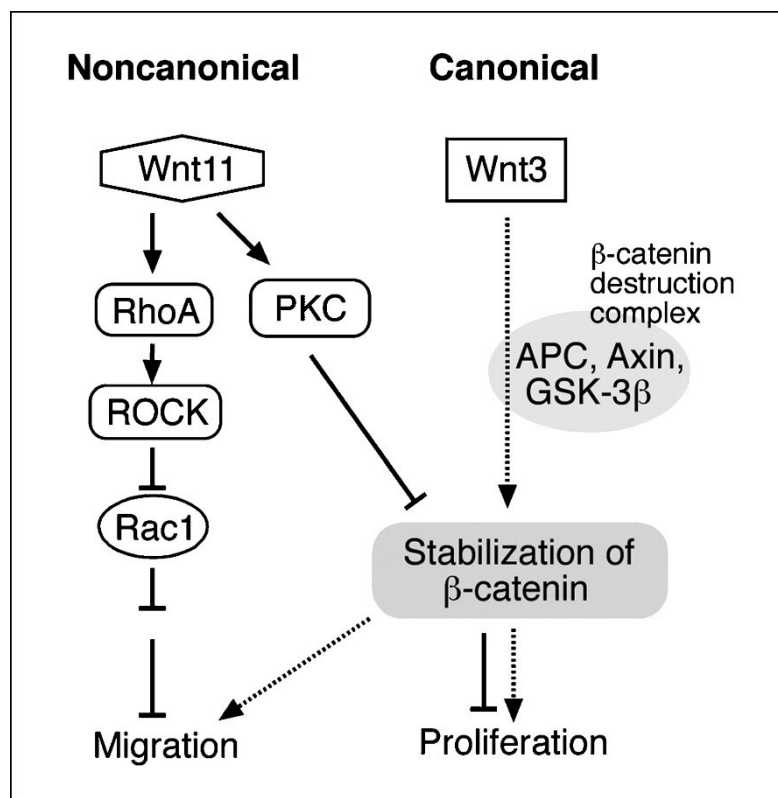


Figure 8-2 Wnt-11 ligand activation of the non-canonical Wnt signalling cascade and interaction with the canonical Wnt pathway (Toyama et al., 2010)

8.2 Aims

It was hypothesised that the non-canonical Wnt pathway is activated in desmoid tumours. The aims of this histological study were to describe the general microscopic characteristics of desmoid tumours (through H&E and P&M staining) and to assess for the activation of the canonical and non-canonical Wnt pathways (through β -catenin and Wnt-11 immunohistochemistry staining, respectively) in 17 FFPE desmoid tumours.

8.3 Materials and methods

8.3.1 Patient selection

Patients with a desmoid tumour, known to St Mark's or The Royal Marsden Hospitals, excised during the three-year study period (2012-2015) were invited to participate in the study and gave consent for the use of their tumour tissue for cell culture following excision of their tumour. Patients with FAP had their tumours excised at St Mark's, Addenbrooke's or Guy's and St Thomas Hospitals. All sporadic cases were identified from, and had their surgery at, The Royal Marsden Hospital. Sporadic desmoid tumours were incorporated into the study as they are more common and therefore tissue more readily available to study. The majority of desmoid tumours studied inevitably originate from the abdominal wall or extremities, rather than intra-abdominally, as surgery is largely feasible for tumours at these sites, whereas the associated risks of intra-abdominal desmoid tumour excision are much higher and it is avoided wherever possible (as explained in section 1.2.8.4).

8.3.2 Ethical consideration

This study had full ethical approval from Harrow Research and Ethics Committee (reference: 08/H0719/75). All patients provided valid, written consent for participation. Approval extended to three other collaborating sites: Addenbrooke's, Institute of Reproductive Developmental Biology (IRDB) and the Royal Marsden Hospital. Research and development study approval was obtained from North West London Hospitals NHS Trust Research and Development Department (reference RD8/080).

8.3.3 Reagents used for histological studies

8.3.3.1 Haematoxylin and eosin (H&E) stain

Xylene

100% industrial methylated spirit (IMS), 95% IMS, 70% IMS

Gill's haematoxylin

1% HCL in 70% IMS (acid alcohol)

0.5% aqueous eosin

8.3.3.2 Picrosirius red and Miller's elastin staining

Xylene

100% IMS, 95% IMS, 70% IMS

0.5% acid potassium permanganate

De-ionised water

Miller's stain

Weigert's haematoxylin

1% HCL in 70% IMS (acid alcohol)

Picro Sirius red

8.3.3.3 Immunohistochemistry

Xylene

100% IMS, 95% IMS, 70% IMS, water

Target retrieval system pH6 for Wnt-11 and pH9 for β -catenin, Dako, N America

1x phosphate buffered saline (PBS)

3% H₂O₂ in methanol

2.5% Horse Serum, Impress reagent kit

Antibody diluent, Dako, N America

DAB (diaminobenzidine) substrate (ImmPACT, Vector, CA)

Haematoxylin

1% HCL in IMS (acid alcohol)

DPX Mounting Media (Sigma, Aldrich)

Antibodies

Table 8-1 Antibodies used for immunohistochemistry studies

Antigen	Specificity	Antibody type	Dilution	Raised in	Company
Wnt-11	Human/Mouse	PAb	1:200	Goat	R&D Systems
β -catenin	Human, mouse, chicken, dog, rat	MAb	1:250	Mouse	BD Transduction

8.3.4 Methods

Desmoid tumour samples were either obtained fresh at surgery, after obtaining informed consent from patients in clinic, or retrieved from histology archives where they had been stored in paraffin embedded blocks. Sections of desmoid tumours excised at surgery were fixed in formalin then processed and embedded in paraffin before sectioning. Serial sections (5µm thick) were taken and mounted onto histology slides prior to staining. Basic histology stains included haematoxylin and eosin (H&E) and picrosirius red with Miller's elastin (P&M) to study the general histological characteristics of desmoid tumours. Picrosirius red was used to identify collagen and Miller's stain to demonstrate the presence of elastin. Immunohistochemistry staining included the use of Wnt 11 (a product of the non-canonical Wnt pathway) and β -catenin antibodies. Slides were stained in batches of 12 at a time, to include a positive and negative control for each antibody tested.

8.3.4.1 H&E staining

Slides were placed in a holder and then sequentially submerged in the following solutions:

Xylene 5 minutes then Xylene 2 minutes

100% IMS, 95% IMS, 70% IMS (2 minutes each)

Tap water 2 minutes

Gills haematoxylin 1.5 minutes

Tap water 5 minutes

Acid alcohol (1% HCL in 70% IMS) 3 seconds

Tap water 4 minutes

0.5% aqueous eosin five minutes

Tap water 20 seconds

70% IMS 30 seconds, 95% IMS 30 seconds, 100% IMS 2 minutes

Xylene 2 minutes then xylene until ready to mount using DPX

8.3.4.2 Picrosirius red/Miller's elastin

Slides were placed in a holder and put sequentially into the following solutions:

Xylene 5 minutes then Xylene 2 minutes

100% IMS, 95% IMS, 70% IMS (2 minutes each)

Tap water 2 minutes

0.5% acid potassium permanganate 5 minutes

Rinse in de-ionised water for 1 minute

1% oxalic acid 1 minute

De-ionised water 4 minutes

95% IMS 1 minute

Miller's stain 50 minutes

95% IMS 1 minute

Tap water 2 minutes

Weigert's haematoxylin 10 minutes

Tap water 1 minute

Acid alcohol 5 seconds

Tap water 5 minutes

Picrosirius red 40 minutes

Rinse in de-ionised water 10 seconds

70% IMS, 95% IMS and 100% IMS 10 seconds each

Xylene 2 minutes and then xylene until ready to mount using DPX

8.3.4.3 Immunohistochemistry

De-paraffinisation

Slides were placed in a holder and then sequentially into the following solutions:

Xylene 5 minutes

Xylene 2 minutes

100% IMS, 95%IMS, 70% IMS (2 minutes each)

Placed in a cold water bath until ready to proceed with antigen retrieval

Antigen retrieval

A coplin jar was filled with the relevant antigen retrieval solution (pH6 for Wnt-11 and pH9 used for β -catenin) and placed into a hot water bath and heated up to 95°C. Slides were then placed back to back into the coplin jar and returned to the hot water bath for 20-30 minutes. The time taken for this process was determined following optimisation experiments that ensured the tumour tissue was not damaged through the antigen retrieval process. The time interval was 20 minutes for β -catenin and 30 minutes for Wnt-11. After this time the coplin jar was removed from the water bath and left to cool at room temperature for 20 minutes. The slides were placed in a plastic incubation chamber and washed with PBS for 3 minutes, three times.

Blocking endogenous peroxidase activity

To each slide 200 μ l of 3% H₂O₂ in methanol was applied and left for 30 minutes. This was then washed three times with PBS for 3 minutes.

Non-specific blocking

The slides were blocked with 2.5% horse serum for 30 minutes.

Primary antibody staining

The primary antibody of interest was prepared in antibody diluent according to the appropriate dilution and 200µl applied to each slide and left overnight at 4°C (Table 8-1). For each experiment a secondary control slide was prepared. No primary antibody was applied to this tissue, only antibody diluent. The remaining steps of the experiment were identical to the staining procedure for the other slides. This control slide assessed for non-specific secondary antibody binding.

Secondary antibody staining

After the slides were washed with PBS three times for 3 minutes the appropriate secondary antibody (anti-mouse or anti-goat antibody) was applied for 30 minutes. Slides were then washed again with PBS three times for 3 minutes.

Diaminobenzidine (DAB) reaction

One drop of DAB chromogen was added to 1 ml of DAB diluent just prior to use. To each slide 200µl was applied until the appropriate level of chromogen signal intensity was achieved (brown staining), approximately 1 minute for both Wnt-11 and β -catenin. Slides were then washed again with PBS, three times for 3 minutes. The slides were then washed in a cold water bath before counterstaining in Haematoxylin for 5 seconds and returning to the water bath for 5 minutes. They were then placed in acid-alcohol for 4 seconds and washed again in cold water for 5 minutes.

Dehydration

The slides were placed into the following solutions:

70% IMS then 95% IMS (both 30 seconds each)

100% IMS for 2 minutes

Xylene for 2 minutes

Xylene until ready to mount

The slides were then immediately mounted using the DPX mounting medium and left to dry.

8.3.4.4 Microscopic analysis

Histology slides were digitally scanned then analysed using Image J software. The proportion of positively staining cells for each prepared slide within a tumour was determined by counting the number staining positive for the antibody (nuclear for β -catenin antibody and cytoplasmic for Wnt-11) divided by the total number of cells within each high powered field (40x magnification). Five high-powered fields were analysed per slide to ensure consistency across the section of tumour analysed. The proportion of positively staining cells was averaged across the five fields for each tumour. Five fields were considered adequate as these tumours were largely homogenous, with little variation seen in cell number or morphology across the tumour.

8.4 Results

8.4.1 Patient characteristics

During the three-year study period, tissue was obtained from eight desmoid tumours excised from seven patients with FAP and three tumours removed from patients with a sporadic desmoid. In addition, six archival FFPE desmoid tumours were obtained and sectioned to provide a total of 17 tumours for histological analysis. The median age at surgery was 37 years and the majority of patients recruited were female, 8/12 in the FAP group and 1/3 in the sporadic group (Table 8-2).

Table 8-2 Patient characteristics of participants in those undergoing desmoid tumour excision

Patient	Gender	Date of surgery	Age at surgery (years)	Location	APC Mutation (codon)
1.1	F	26/07/2012	46	AWD	216
1.2		21/03/2013	46	AWD	
2.1	F	12/12/2007	38	IAD	1450
2.2		28/09/2012	43	AWD	
3	M	08/12/2012	25	IAD	278
4	F	13/06/2013	24	AWD	1461
5	M	16/07/2014	21	IAD	564
6	M	19/11/2014	33	AWD	1551
7	F	04/03/2015	20	AWD	Ex11-15 gross deletion
8	F	05/07/2002	41	Chest wall	Unknown
9	M	08/01/2004	37	IAD	216
10	F	21/05/2007	24	IAD	1408
11	F	22/09/1986	32	IAD	1392
12	F	19/01/2005	49	AWD	1951
13	M	01/10/2013	70	AWD	Sporadic
14	M	16/10/2013	48	L shoulder	Sporadic
15	F	06/11/2013	26	AWD	Sporadic
Median age			37		

8.4.2 Microscopic characteristics of desmoid tumours

Desmoid tumours were composed of spindle cells, similar in appearance to fibroblasts, and were relatively hypocellular and lacked mitotic activity. These tumours were quite vascular, with thickened vessels or disruption of vessel wall integrity with extravasation of blood and associated inflammatory infiltrates were frequently seen (Figure 8-3). The extra-cellular matrix demonstrated abundant and disorganised collagen deposition of varying maturity with P&M staining. Neo-collagenesis (green fibres) was often seen in conjunction with mature (red) collagen fibres under polarized light (Figure 8-4).

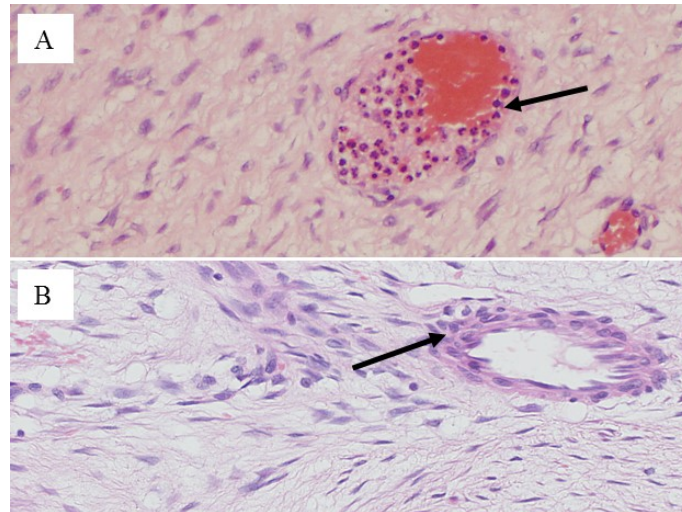


Figure 8-3 H&E stained desmoid tumour tissue (40x magnification). Figure A showing spindle cells with arrow showing vessel disruption with an inflammatory infiltrate. Figure B arrow shows a thickened vessel

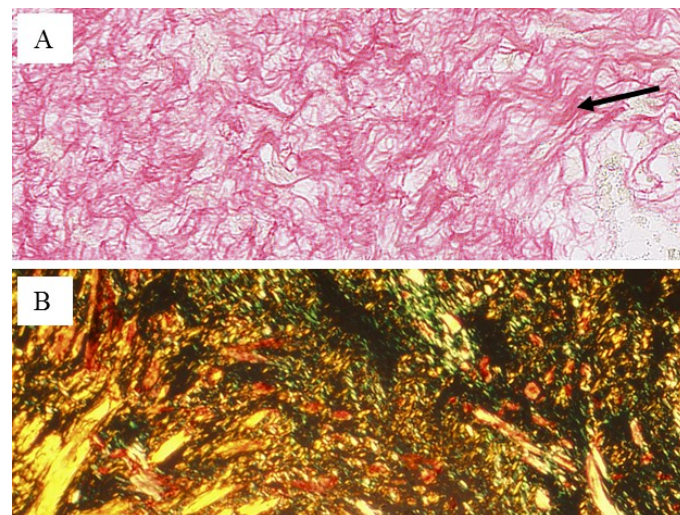


Figure 8-4 P&M stained desmoid tumour. Figure A shows abundant collagen fibres (arrow). Figure B is the same stain under polarized light showing a variety of green, yellow and red collagen fibres (40x magnification)

8.4.3 Immunohistochemistry findings

The β -catenin and Wnt-11 antibodies used for immunohistochemistry were both specific and of good quality, free from non-specific background staining (confirmed by the absence of any staining of the negative control slide tissue for each experiment). The level of staining for both

antibodies was assessed in control tissue. It was difficult to determine a suitable control for a desmoid tumour. Appendix mesentery was obtained during the study and attempts were made to perform immunohistochemistry on this tissue for comparison. However, this was very different to a desmoid tumour and contained more adipose tissue. The tissue was significantly distorted and damaged through the antigen retrieval conditions that the desmoid tumours required for the process. Skin was considered a better control tissue as it contains mostly fibroblasts, similar to a desmoid tumour. The skin control tissue was obtained from a patient with FAP at the time of their desmoid tumour excision. It did not stain positive for nuclear β -catenin but did demonstrate some Wnt-11 cytoplasmic staining (Figure 8-5).

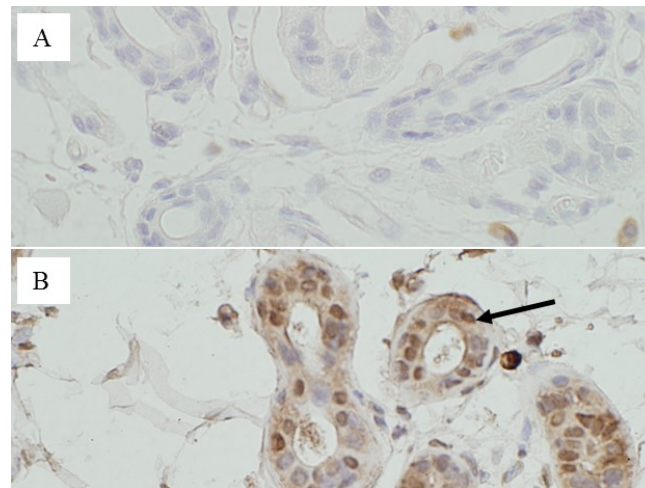


Figure 8-5 Skin control tissue negative for nuclear β -catenin expression (A) and Wnt-11 positive (B), arrow showing brown cytoplasmic staining (40x magnification)

Immunohistochemistry staining of the desmoid tumours found positive nuclear β -catenin staining in 71% in the 17 desmoid tumours examined, similar to that previously reported in other studies. The Wnt-11 positive cytoplasmic staining rate was similar to β -catenin and was identified in 75% of the tumours tested (**Error! Reference source not found.** and Table 8-3).

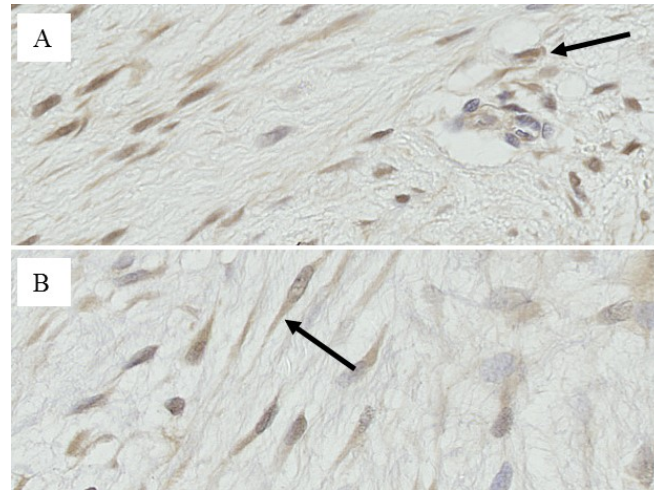


Figure 8-6 Immunohistochemistry of desmoid tumour tissue with arrow showing positive brown nuclear β -catenin staining (A) and arrow demonstrating positive brown cytoplasmic staining for Wnt-11 (B) antibodies (40x magnification)

Table 8-3 Proportion of cells staining positive for β -catenin (nuclear) and Wnt-11 (cytoplasmic) antibodies in FAP and sporadic desmoid tumours

Patient	Gender	Location	Proportion of positive staining cells (%)	
			β -catenin	Wnt-11
1.1	F	AWD	66	53
1.2		AWD	45	88
2.1	F	IAD	53	81
2.2		AWD	89	84
3	M	IAD	52	97
4	F	AWD	86	76
5	M	IAD	19	62
6	M	AWD	90	60
7	F	AWD	69	0
8	F	Chest wall	92	89
9	M	IAD	94	96
10	F	IAD	96	91
11	F	IAD	94	95
12	F	AWD	72	77
13	M	AWD (sporadic)	82	84
14	M	L shoulder (sporadic)	62	82
15	F	AWD (Sporadic)	51	68
Proportion positive staining			71	75

8.5 Discussion

Desmoids are vascular tumours that are associated with changes within vessel walls, altering their integrity. The ECM is composed of disorganised, abundant collagen deposition, both old and new. These findings corroborate those reported in previous studies (Fisher and Thway, 2014, Kotiligam et al., 2008).

This study found widespread activation of the canonical Wnt signalling pathway in desmoid tumours, with 71% demonstrating nuclear β -catenin staining. The findings of this larger desmoid cohort, support those previously reported by Bhandari et al in a single tumour, that these tumours do seem to express Wnt-11 (Bhandari, 2013). Therefore, this lends support to the observation of activation of the non-canonical Wnt pathway in these tumours. However, the interaction between Wnt ligands, their receptors and these two pathways is complex. There does not appear to be any difference between FAP associated and sporadic desmoids in this small study.

8.5.1 Study limitations

A strength of this study is the cohort size of 17 tumours that enabled a more thorough assessment of the immunohistochemistry staining. The main limitation of this study was in selecting an appropriate control tissue to compare levels of antibody staining. It was difficult to select a suitable tissue. Skin was thought to be the most appropriate and the most accessible. Immunohistochemistry is fraught with difficulties, particularly with newer antibodies, such as Wnt-11, where the staining may not be accurate for the target being tested. There can also be inconsistency with assessment of the positively staining cells. Although the staining in this instance seemed specific, it would be useful to also test downstream targets for Wnt-11 to prove that they were also activated.

8.5.2 Conclusion

The findings from this study suggest that the non-canonical Wnt pathway may be activated in desmoid tumour development. This is important for testing future pathway targets and considering potential inhibitors of their growth. This is a novel finding. However, downstream targets now need to be assessed to lend further support to this hypothesis. It would also be important to determine the level of Wnt-11 expression in desmoid tumour cell cultures. Chapter 9 aims to study desmoid tumours in cell culture with the intent of using them to test for the presence of Wnt ligands and potential inhibitors in vitro.

9 Chapter 9 - Cell culture and 3D desmoid scaffold development

9.1 Background

Cell culture is a laboratory technique used to study cells and tissue growth, and is particularly useful for the study of tumorigenesis. Culturing cells under various controlled conditions enables us to improve our understanding of tumour behaviour and can investigate those factors that may promote or inhibit their growth, as well as providing a model with which to test potential therapies. Currently, no commercially available cell line for desmoid tumours exists and therefore studying their growth behaviour can be difficult. There is evidence in patients with FAP, there is a systemic dysfunction of fibroblasts, with normal skin biopsies from such patients demonstrating abnormal organisation of cytoplasmic actin (Kopelovich et al., 1977). Pfeffer et al also found abnormal growth patterns in the skin from patients with FAP (Pfeffer et al., 1976). These in vitro studies provided some evidence that fibroblasts in patients with FAP had a loss of growth control with fibroblasts demonstrating a longer survival compared to control fibroblasts.

Few studies have looked at culturing desmoid tumours, but in those that have, they are often unsuccessful, due to the overgrowth of fibroblasts (Sturt et al., 2004a, Bhandari, 2013). Some studies utilised cells cultured after desmoid tumour excision but did not test the cell lines genetically to confirm they were desmoid tumour cells (Joyner et al., 2009). Sinha et al successfully grew desmoid tumours in cell culture, using the presence of nuclear β -catenin expression in the cells to confirm they were desmoid cell cultures (Sinha, 2011). Bhandari et al tested these cell cultures genetically to confirm the presence of desmoid tumour cells (Bhandari, 2013).

9.1.1 Cell culture

Cell culture is the process of removing cells from plant or animal tissue for the purposes of growing them in a controlled, artificial environment. Cells are usually cultured in a plastic or glass plate or vessel containing a liquid medium providing essential nutrients to maintain cell growth. Cell culture can be undertaken from established, commercially available, cell lines or from primary tissue explants. In primary tissue culture, cells are removed from the tissue by enzymatic breakdown or mechanical disruption. By controlling the environment in which the cells are grown, cell culture offers the ability to study the growth of cells (in healthy or abnormal (for example tumour) tissue) and their response to various compounds that may stimulate or inhibit growth. Cells initially isolated from tissue and grown in culture are termed a 'primary culture' once the cells reach full confluence, forming a monolayer that covers the entire basal surface of the culture vessel. The period for this to occur depends on the cell type and can vary from a few days to a few weeks. Once fully confluent, cells are divided or 'passaged' and transferred to new culture vessels with more room to grow, typically at a ratio of 1:2 or 1:3. The primary cell culture now becomes a subclone or a 'cell line' that can be grown to confluence and passaged again prior to their use in various experiments to study their growth behaviour under varying conditions.

Some studies have used cell culture techniques to study desmoid tumour growth response to treatments such as tamoxifen (Serpell et al., 1996) and endostatin gene therapy (Martinico et al., 2006). To date, no studies have successfully characterised FAP associated desmoid cells in vitro and this is likely to reflect the difficulties that are encountered when trying to culture these tumours. These relate to the overgrowth of fibroblasts in vitro and the inability to distinguish these fibroblasts from desmoid tumour cells without genetically testing the cell line or staining

for β -catenin expression. Establishment of a true desmoid cell line would be invaluable for studying their behaviour and testing potential new drug treatments more robustly.

9.1.2 Three-dimensional tissue culture

Three-dimensional tissue cultures involve the use of a fibroblast derived extra-cellular tissue matrix (scaffold) onto which cultured cells are seeded and incorporated. The extra-cellular matrix (ECM) contains proteins (for example collagen, fibronectin and integrin), and proteoglycans that provide an environment to sustain cell growth. These matrix constituents are secreted by fibroblasts (Sabatelli et al., 2001, Chung and Erickson, 1997). There is evidence from fibroblast morphology and behaviour that these three-dimensional cell cultures better mimic the *in vivo* environment and are superior to standard two dimensional culture techniques (Cukierman et al., 2001, Mao and Schwarzbauer, 2005, Green and Yamada, 2007).

9.2 Aims

The aim of this desmoid cell culture study was to establish a successful primary desmoid cell line from desmoid tumour tissue collected at surgical excision. A desmoid precursor plaque biopsy was also included and skin fibroblasts from this patient and a patient with a desmoid tumour were used as control cell lines. The intention was to use these cell lines, if successful, in the testing of potential non-canonical Wnt pathway ligands (for example a Wnt 11) and inhibitors.

9.3 Materials and methods

9.3.1 Patient selection

Patients were recruited from clinic and samples of tissue obtained at the time of the surgical excision of their desmoid tumour.

9.3.2 Control cell lines

A patient with FAP who had an abdominal wall desmoid tumour excised also consented to a biopsy of healthy skin to obtain a control fibroblast cell line. One patient with FAP who had surgery to excise their rectum for management of their rectal polyposis also had a mesenteric desmoid precursor plaque. This was biopsied at the time of their surgery for the purposes of cell culture and a sample of healthy skin was also taken to establish another control fibroblast cell line. It was difficult to determine the most appropriate control cell line to compare with a desmoid tumour as there is no suitable control tissue. Skin was considered the most appropriate as fibroblasts here are abundant. Whatever control tissue was chosen the cells would likely grow at a different rate compared to those from a desmoid tumour.

9.3.3 Ethical considerations

This study had full ethical approval through Harrow Research and Ethics Committee (reference: 08/H0719/75). All patients involved provided valid, written consent for participation. Approval extended to 3 other collaborating sites Addenbrooke's Hospital, Institute of Reproductive and Developmental Biology (IRDB) and the Royal Marsden Hospital. Research and development study approval was obtained from North West London Hospitals NHS Trust Research and Development Department (reference RD8/080).

9.3.4 Reagents for cell culture and PCR

9.3.4.1 Reagents for standard cell culture

All reagents used for tissue culture were sterile and of cell culture grade. All plastic-ware was for tissue culture (Sarstedt)

Growth media:

Dulbecco's Modified Eagle Medium (DMEM) growth medium containing L-glutamine, pyruvate and low glucose (1000mgL^{-1}), Sigma

15% Foetal Bovine Serum (FBS), Sigma

Antibiotics – (100Uml^{-1} penicillin, $100\mu\text{gml}^{-1}$ streptomycin, Invitrogen, UK)

0.25% Trypsin-EDTA, Gibco (Invitrogen, UK)

Phosphate buffered saline (PBS), Gibco (Invitrogen, UK)

Cell freezing media - DMEM: Dimethyl sulfoxide (DMSO) 9:1, filter-sterilized, Sigma D8418

9.3.4.2 Reagents for 3D cell culture

Antibiotics – (100Uml^{-1} penicillin, $100\mu\text{gml}^{-1}$ streptomycin, Invitrogen, UK)

Phosphate buffered saline (PBS), Gibco (Invitrogen, UK)

Detergent: 0.25% triton X-100, 0.25% Na deoxycholate, 1% antibiotics and PBS

DNase/RNase solution: Buffer 2.5mM MgCl_2 , 0.5mM CaCl_2 , 1% antibiotics, PBS, 0.5%

DNase, 0.01% RNase

Distilled water

Growth media (as per standard cell culture)

10% peracetic acid, Sigma

9.3.4.3 Reagents for PCR

9.3.4.3.1 DNA purification agents

10mM NaCl/10mM EDTA

75mM NaCl/25mM EDTA

10% SDS

20mg/ml Proteinase K

25:24:1 phenol:chloroform:isoamyl alcohol

24:1 chloroform:isoamyl alcohol extraction mix

3M sodium acetate (NaOAc)

100% ethanol

TE buffer

RNA purification from cell culture was performed using RNeasy Mini Kit (Qiagen, UK)

Complementary DNA (cDNA) was synthesised by using SuperscriptTM First-Strand Synthesis System for RT-PCR (Invitrogen, UK).

9.3.4.3.2 Real time PCR agents

5X Green Go Taq® Flexi buffer (Promega UK)

25mM MgCl₂

PCR Nucleotide mix (Promega, UK)

Forward Primer

Reverse Primer

GoTaq® DNA Polymerase (Promega, UK)

Nuclease-Free Water

Agarose (Invitrogen, UK)

Ethidium Bromide

Loading sample buffer

500bp and 1Kb DNA ladders

9.3.5 Methods

9.3.5.1 Tissue collection

Desmoid tumour tissue was obtained intra-operatively following tumour excision in most cases or biopsy (for three intra-abdominal tumour cases). Samples taken from excised tumour were approximately 1cm³ in size. Multiple pieces of tumour were taken. In some large tumours, samples were taken from the centre to the periphery, enabling mapping out of the tumour to compare growth rates between various areas. All tissue was transferred aseptically without patient identifiable information to a laboratory at Northwick Park Institute for Medical Research (NPIMR) for further processing. Using an icebox, tissue samples were transported using sterile vessels containing growth medium (DMEM with 15% FBS and Penicillin V and Streptomycin) similar to that used for further culturing of the cells. Once in the laboratory some of the tissue samples were immediately frozen using liquid nitrogen and stored in cryovials in a minus 80°C freezer for future tissue comparison with the cell lines. The remaining tissue was used for cell culture (Figure 9-1).

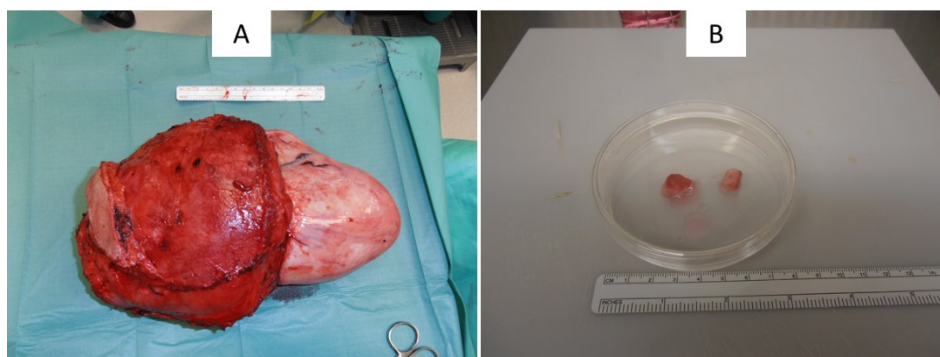


Figure 9-1 Desmoid tumour excised (A), samples used for culture prior to mincing (B)

9.3.5.2 Standard cell culture technique

All cell culture work was conducted at NPIMR, Watford Road, Harrow, Middlesex. Standard aseptic techniques were employed whilst undertaking cell culture within class II vertical laminar flow safety hoods. The standard cell culture methods undertaken were those previously described by Sinha et al (Sinha, 2011) who studied desmoid cell culture in depth. Fresh tissue was mechanically minced by fine dissection, using a blade, within a sterile plate. The explanted tissue was then placed in another petri dish with 10ml of growth media and cultured in a standard laboratory incubator at 37°C and five per cent CO₂ level.

Growth media was changed every two to three days. The cell cultures were passaged once they reached confluence. A phosphate buffered saline (PBS) rinse was undertaken prior to cell trypsinisation and subsequent incubation until cellular detachment from the culture plate was achieved. Ten millilitres of growth medium were added to the solution before placing in a sterile falcon tube and centrifuging at 1000 rpm for five minutes. The supernatant was discarded and the remaining pellet re-suspended in growth media. This solution was then divided between two to three petri dishes containing 10ml of culture medium. Following establishment of the primary cell line (that took in most instances around three weeks), subsequent cell lines were passaged on average once a week although faster for skin fibroblasts, at around three days. Using freezing medium, excess cell stocks were placed in cryovials at minus 80°C before storing in liquid nitrogen until required. A list of reagents and buffers utilised can be found in section 9.3.4.

9.3.5.3 Three-dimensional cell culture development

The process of three-dimensional cell culture involved detergent decellularisation of tissue to create the ECM tissue matrix and then seeding of cultured cells onto the matrix. Fresh desmoid

tumour tissue was initially decellularised to remove all cellular DNA and RNA content. The remaining collagen matrix was then histologically studied, to confirm complete decellularisation. Priming of the scaffold in DMEM for 24 hours was then undertaken to ensure the scaffold tissue was free from infection. Confluent cells obtained from standard cell culture were then added to the scaffold to create a co-culture. The co-culture was then incubated at 37°C and growth media changed every 2 days, for up to one week. The co-cultures were studied at two, five and seven day intervals by fixing the tissue in formalin. The tissue was then processed and placed in FFPE blocks prior to sectioning and staining with H&E (see section 8.3.4.1 for staining technique) to assess cell attachment and whether a 3D desmoid co-culture had been successfully created.

9.3.5.3.1 Decellularisation of desmoid tumour tissue

Decellularisation of desmoid tumour tissue was an eight-day process. Desmoid tumour tissue is a firm coarse tumour and considered a robust tissue that would require a longer decellularisation process than most tissues. Therefore, the protocol used for this process was that already established by scientists studying decellularisation of porcine trachea tissue at the NPIMR laboratory. However, optimisation of the process was still necessary to determine those conditions specific for decellularisation of desmoid tumour tissue. On day one, four pieces of approximately 1cm³ fresh desmoid tumour tissue were defrosted (they had been stored at -20°C). The tissue was placed into a desiccator containing 200ml of detergent solution (Triton-X and sodium deoxycholate). A vacuum (1500 ATM) was applied to the desiccator before placing in an incubator at 37°C and agitating the contents at 140 rpm for 24 hours (Figure 9-2). On day two the detergent was removed and the tissue was washed twice for 15 minutes in distilled water under the same conditions in the incubator. The water was discarded and 200ml PBS added to the tissue in the desiccator for one hour at 4°C and 140rpm. This solution was

then removed and 200ml of PBS added for another hour before discarding again and finally placing in 200ml PBS for 24 hours at 4°C and 140rpm. In the initial experiments this PBS wash phase was continued for 72 hours with a change of solution only every 24 hours and the overall decellularisation protocol took ten days. In an attempt to shorten this process, the PBS wash cycle time was reduced to create a 5-day protocol. Unfortunately, histological analysis of the decellularised tissue at day five found some residual cellular matter and the process was subsequently increased to eight days, with further wash cycles throughout.

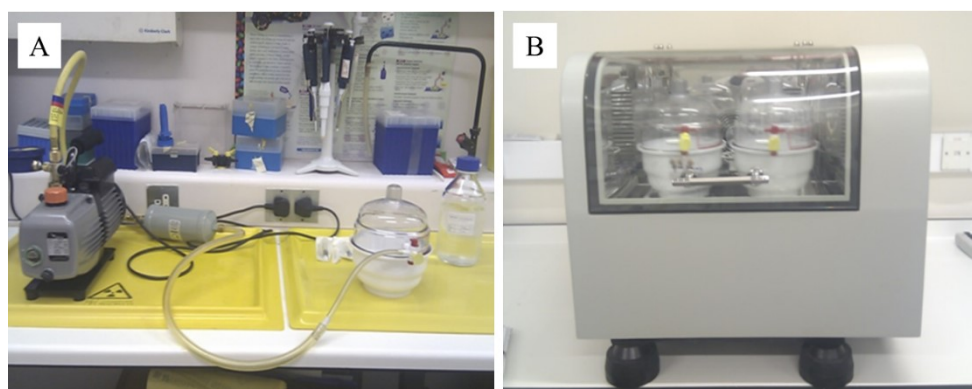


Figure 9-2 Vacuum applied to desiccator during decellularisation process (A). Desiccator inside the incubator at 37°C and 140rpm (B)

On day three the PBS was removed and the tissue washed with distilled water twice for 15 minutes at 37°C and 140rpm. 200ml of DNase/RNase buffer was then added to the tissue before placing in the incubator for a further 24 hours (37°C and 140rpm). At day four the DNase/RNase buffer solution was removed and in several instances at this point infection of the tissue declared itself. This was apparent due to the offensive odour and cloudy green colour of the discarded solution. When this occurred, the experiment was abandoned as infection would lead to degradation of the decellularised tissue scaffold. If the solution was clean at day four, the process was continued. Tissue was rinsed with two distilled water washes before

placing in 200ml PBS for a further 24 hours at 4°C and 140rpm. On day five the PBS was discarded and the tissue washed twice with distilled water before 200ml DNase/RNase buffer was applied for a further 24 hours (37°C and 140rpm). At day six this solution, provided there was no evidence of infection, was discarded and the tissue washed initially in distilled water. It was then washed in PBS at 60 minute intervals, repeated three times, before leaving in PBS at 4°C, 140 rpm, for 48 hours.

In the initial optimisation experiments at the end of the decellularisation process the tissue was placed in formalin and fixed in FFPE blocks, sectioned and H&E stained to determine whether the tissue had been adequately decellularised. Once an 8-day protocol was found adequate, the tissue could then be used to establish a co-culture.

9.3.5.3.2 Scaffold sterilisation

Following decellularisation, the desmoid tissue scaffold was initially sterilised for 1 hour with 10% peracetic acid. It was then washed several times in PBS and left in PBS solution at 4°C, 140rpm, overnight. The tissue was subsequently placed in growth media (DMEM with antibiotics and FBS) for 24 hours at 37°C to prime the scaffold prior to the addition of cells. However, at this stage the tissue on three separate occasions became infected. This was apparent from the cloudy appearance of the growth media solution and offensive smell. The sterilisation technique was adjusted to incorporate supercritical carbon dioxide (CO₂) sterilisation with the 10% peracetic acid solution. Supercritical CO₂ is a state where CO₂ (normally a gas) becomes fluid, occurring when it reaches a critical temperature (31.1°C) and pressure (72.9 ATM). The Tissue was placed in a chamber with 50ml of peracetic acid covering the tissue. It was then kept at supercritical CO₂ (using an autoclave with a pressure gauge, inlet

and outlet valve) for one hour. Supercritical CO₂ sterilisation is a recognised method of sterilising biomaterials that does not cause damage to tissue morphology (Bernhardt et al., 2015). Following sterilisation, the tissue was washed as described before and kept in PBS overnight. This method eradicated the recurrent scaffold infections that had been encountered at this stage.

9.3.5.3.3 *Establishing a desmoid co-culture*

Following the priming of scaffold tissue in growth media for 24 hours, the tissue was carefully cut using a punch biopsy tool to create 8mm discs of 2mm thickness. Each disc was placed into a well of a 24-well plate and a total of 18 discs were created, so each scaffold experiment was duplicated. A third (six scaffolds) acted as controls for intervals of 3, 5 and 7 days analysis and no cells were applied to these tissues. The other third had 100 000 cells applied to their surface whilst the final third had 300 000 cells applied. This would help determine the optimum concentration of cells to apply per scaffold and the necessary incubation time (2, 5 or 7 days). Confluent cells from standard cell cultures already established were trypsinised and pelleted using the appropriate volume of cell suspension to give 100 000 or 300 000 cells per 20µl for each scaffold. This volume was then carefully pipetted onto the surface of the scaffold, trying to avoid spillage over the surface onto the base of the well. The 24-well plate containing the scaffolds was then placed in the incubator at 37°C for three hours to promote cell adhesion to the scaffold. Growth media was then added to each co-scaffold and control, covering the surface of the tissue. Growth media was changed every other day.

At days 2, 5, and 7 the relevant co-scaffolds were placed into formalin and processed. They were then cut in two before embedding in a perpendicular orientation in FFPE blocks so that

cell attachment to the surface and deeper tissue could be analysed histologically after staining with H&E.

9.3.5.4 DNA extraction

Samples of pelleted cells were re-suspended in 500µl of 10nM NaCl/10mM EDTA and centrifuged (13000rpm for one minute) and supernatant discarded. Digestion was achieved by placing the cells overnight in a solution containing 500µl of 75mM NaCl/25mM EDTA, 50µl 10% SDS and 25µl of Proteinase K and incubated at 37°C. The following day a solution of 500µl of phenol/chloroform/isoamyl alcohol (25:42:1) was added to the sample and centrifuged (13000rpm for one minute). The supernatant was transferred to a new tube and 500µl chloroform/isoamyl alcohol extraction mix (24:1) added before further centrifugation (13000rpm for one minute) performed. The supernatant was removed and placed in a new tube and mixed with 1ml 100% ethanol and 50µl 3M sodium acetate to form a DNA precipitate. This was spun down for two minutes at 13000rpm and supernatant discarded. The remaining pellet was vortexed with 1ml 70% ethanol before centrifuging for two minutes at 13000rpm and discarding the supernatant. The pellet was re-suspended in 50µl of TE buffer and incubated for 1-2 hours at 56°C until the DNA had dissolved.

9.3.5.5 PCR method for whole gene sequencing of *APC*

Desmoid tissue *APC* and *CTNNB1* gene sequencing was performed at the Kennedy Galton Centre. All fifteen *APC* coding exons were sequenced using a Megamix Royal polymerase chain reaction (PCR) method. PCR was performed initially across a range of temperatures to obtain the optimal annealing temperature of the primers prior to analysis of the culture DNA. This was found to be 58°C.

The primer sequences used for studying desmoid cell cultures are as listed:

APC exon1 Forward: GAGTTTTGTTTCCTTTACCCCTTTC
Reverse: AAAGTGTCTTACCTCAAGTTTACAAGA

APC exon2 Forward: TATATAAGGTGCGTGCTTTGAGAGT
Reverse: AAATCGAGAGAAGCTGTACTTGGA

APC exon3 Forward: TGTTTTTCAGTCATGTATATTTGTGG
Reverse: AAATGCAAAATGCTCTAAGTGTTA

APC exon4 Forward: TGCTCTTCTGCAGTCTTTATTAGC
Reverse: AGGCCTAAAGTTGGGTAAACATA

APC exon5 Forward: CACCATGACTGACGTATTTGCTTA
Reverse: AATAGAGCCAAAATAAACACAGCC

APC exon6 Forward: CATAACCCTGAGCTTTTAAGTGGT
Reverse: TGTAAACTGACAGCTAAAGTAAGGTATCTA

APC exon7 Forward: CTACACCATTTTTGCATGTACTGA
Reverse: CCATCTTGCTTCATACTTTTCTGA

APC exon8 Forward: CAGACACTTCATTTGGAGTACCTTA
Reverse: TGTCTTAGCAAAGTAGTCATGGCA

APC exon9 Forward: CACTGATTACTTCATCCTGGAAAG
Reverse: ACATTTGCTTTGAAACATGCAC

APC exon10 Forward: TCCACTAAAATTCCGTGAATTAGG
Reverse: GATAACAGAAGTTGGTGGCCTTA

APC exon11 Forward: CTGGCATAAAATGGAATAATTGTC
Reverse: GATAAGCGAATGTGAAGCACAGG

APC exon12 Forward: AATAAAGCTTGGCTTCAAGTTGTC
Reverse: AGATTGCACAACCTGCCCTCTAAG

APC exon13 Forward: GCTAGCCAGAATTTCTTTCTTAATAG
Reverse: GGTAAGAAATTAGGAAATCTCATGG

APC exon14 Forward: TTCTTAATTTACCAAGTGAGGGAC
Reverse: GGCTACACCTCTCAACTATAATTTGC

APC exon15_1 Forward: GGAGATGTGGAATACTTGGAATTTAT
Reverse: ATTATCATCATGTTCGATTGGTGTC

APC exon15_2 Forward: CAATATTATGTCTCCTGGCTCAAG
Reverse: AACATGTCCTATTTGAATTTTCCG

APC exon15_3 Forward: AAGAAGTGTCAGCCATTCATACCT
Reverse: ACCTGTATGGAGAAACACATTCCT

APC exon15_4 Forward: CCGACCTAGCCCATAAAATACATA
Reverse: CATATGTTTCGGTTTTACTGCTTTG

APC exon15_5 Forward: AATGTAAGCCAGTCTTTGTGTC
Reverse: AGTCTGCTGGATTTGGTTCTA

APC exon15_6 Forward: ATCAGCTGAAGATGAAATAGGATG
Reverse: CATTTACTGCAGCTTGCTTAGGT

APC exon15_7 Forward: CTCAAACCAAGCGAGAAGTACC
Reverse: ATTTCAATATCATCATCTGAATCATCT

APC exon15_8 Forward: TAAGAATAATGCCTCCAGTTCAGG
Reverse: CTGGTCCATTATCTTTTTCACACG

APC exon15_9 Forward: TTCCTACAGAAGGCAGAAGTACAG
Reverse: CAGTAAGGAGTTCCTTCAATAGGC

APC exon15_10 Forward: CCACAAAATACTGAATATAGGACACG
Reverse: GAGGTTTACTTGTTCTCCCTGT

APC exon15_11 Forward: ACCCATACTTCAGAAACAATCCAC
Reverse: CAGCAGCAGCTTGATGTAACTAC

APC exon15_12 Forward: GTGAAGATCTGACACTTGATTG
Reverse: AACAGGGCTTAATTCTGATTTAC

APC exon15_13 Forward: TATTCCAGGAGTTCGAAATAGCTC
Reverse: TGTTTGTTTGGTAAGGTTCTGTTG

APC exon15_14 Forward: TTATCTCAACTTCCAAGGACATCA
Reverse: TCAGAATGAGACCGTGCAATATC

APC exon15_15 Forward: TCATTTGAATCTCTTTCTCCATCA
Reverse: GTTAATGGGACAGTCCTCAATTCT

APC exon15_16 Forward: CATGTGAACTCTATTTTCAGGAACC
Reverse: GCTTGGGTTGTAATTAAAAGGAGT

APC exon15_17 Forward: CAAAATAATCCTGTCCCTGTATCA
Reverse: AGCAATAATTAAAGGAAGTTGGGA

The PCR cycle involved three main steps:

- 1) Denaturation of double stranded DNA into a single DNA strand
- 2) Annealing of primers to the target DNA sequence
- 3) Extension to the growing DNA sequence by Taq polymerase sequential addition of ddNTPs to make a copy of the target DNA

The PCR reaction mixture (total volume 15µl per well of a 96-well plate) was formed by adding 7.5µl Megamix Royal to 0.75µl forward primer and 0.75µl reverse primer, with 4.5µl sterile water and 1.5µl of DNA (at a concentration of 100ng/µl). The Megamix Royal solution contained Taq polymerase, anti-Taq polymerase monoclonal antibodies, 6mM MgCl₂, ddNTPs and blue loading dye (Microzone Ltd).

The reaction mixture was plated out into a 96-well plate before sealing and running the PCR in a thermocycler as follows:

95°C for five minutes to denature the double stranded DNA to a single DNA strand

58°C for 30 cycles over 150 minutes, for annealing process

72°C over 10 minutes for Taq extension

For quality control purposes, the PCR product was checked by running it on a 2% agarose gel. This ensured the PCR process had worked and the product was free of contaminants. PCR clean-up was achieved by the removal of unincorporated ddNTPs from the sequencing product using ExoSAP-IT. A volume of 5µl PCR product was added to 2µl of ExoSAP-IT reagent and incubated in a thermocycler at 37°C for 15 minutes followed by 80°C for 15 minutes before cooling to 4°C.

A sequencing mix was formed (7.5µl total volume) using 0.5µl Big dye v1:1 mix, 1.75µl 5x Big dye buffer and 5.25µl sterile water. This mix was added to the separate forward and reverse primer sequencing plates (containing 2µl volume) and the PCR template (Exo-SAP-IT product) was added forming a 10µl solution for each well. This was placed in the thermocycler and run as follows:

96°C for 1 minute

96°C for 10 secs, 50°C for 5 secs, 60°C for 4 minutes (25 cycles)

Allowed to cool to 4°C

The samples were each placed in spin column and vortexed before snapping off the bottom of the spin column and placing into a 2ml collection tube and centrifuging at 3000rpm for three minutes. The reaction mixture (1µl per well) was added to a DyeEx 96 well plate (containing 9µl HiDi formaldehyde in each well) before placing in a sequence analyser.

9.4 Results

9.4.1 Standard cell culture

Over a three-year period, 12 desmoid tumours were excised from ten patients (six were associated with FAP and four occurred sporadically). Eight out of 12 tumours were located in the abdominal wall. There was an even gender distribution and median age at surgery was 43.5 years (range 20-70 years) (

Table 9-1).

All twelve tumours successfully explanted to varying degrees. During the start of the study it was noticed that much of the explanted tissue was not adhering to the base of the petri dish with only one of four samples successfully explanting for tumour 1.1 (

Table 9-1). A similar result occurred in tumours 2.1, 3 and 4. The size of the petri dish was changed from 100mm to 60mm diameter to see if the cells explanted more successfully within a small surface area, with other pieces of tissue close by. This did improve success rates of tissue explantation. The growth media was changed from low glucose to a higher glucose content, but this made no significant difference to cell growth. Another improvement was made to the explantation process to aid adherence. Once mechanically minced, the tissue was placed into a sterile petri dish, spread thinly over the base and put into the incubator for one hour without any growth media. A small amount of growth media was then added just to the surface of the tissue before replacing the petri dish into the incubator for another hour. Finally, growth media was then added to ensure full coverage of the tissue. This greatly improved tissue adherence and explantation of the desmoid tissue. The time for explantation to occur also improved with this technique and the larger petri dishes were more successful than before (Figure 9-3).

Table 9-1 Desmoid cell culture lines. AWD: abdominal wall desmoid, IAD: intra-abdominal wall desmoid

Patient	Gender	Date of surgery	Age at surgery	Desmoid location	<i>APC</i> mutation (codon)
1.1	F	26/07/2012	46	AWD	216
1.2		26/07/2012	46	Skin fibroblasts	216
1.3		21/03/2013	46	AWD	216
2.1	F	28/09/2012	43	AWD	1450
2.2		09/10/2013	44	IAD	1450
3	M	08/12/2012	25	IAD	278
4	F	11/09/2013	59	IAD	247
5	M	19/11/2014	33	AWD	1551
6	F	04/03/2015	20	AWD	Ex11-15 gross deletion
7	M	01/10/2013	70	AWD	Sporadic
8	M	16/10/2013	48	Shoulder	Sporadic
9	F	06/11/2013	26	AWD	Sporadic
10	M	28/05/2014	32	AWD	Sporadic
11.1	F	21/11/2012	32	IAD DPL	Unknown
11.2		21/11/2012	32	Skin fibroblasts	Unknown

For the final two tumours obtained, in addition to the standard explantation technique of mincing the tissue, small pieces of tumours were placed directly without any tissue disruption onto a petri dish. They were processed in exactly the same way as the standard explants. The cells walked out very well and seemed to do so more readily compared to those explants undergoing standard tumour mincing (Figure 9-4 compared to Figure 9-3). The explants reached confluence earlier using this new method.

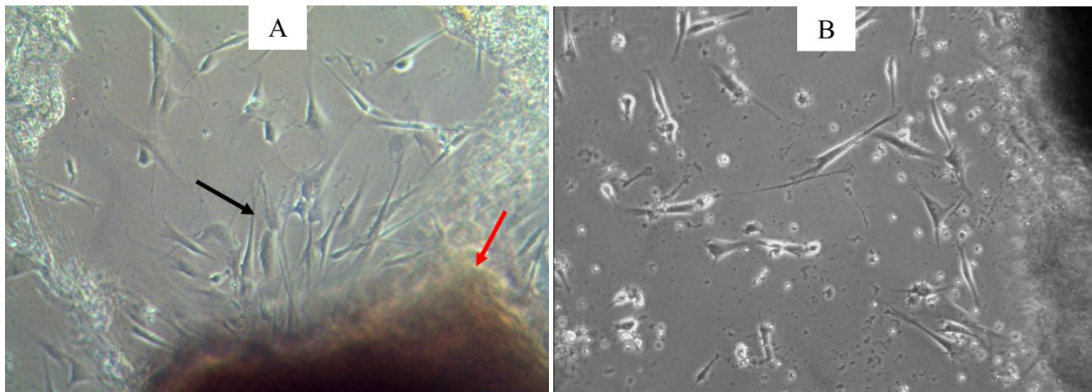


Figure 9-3 Primary desmoid tissue culture using standard mechanical mincing of tissue. A: Two days after explantation, red arrow showing tumour tissue, black arrow demonstrating cells explanting from the tissue. B: 5 days after explantation

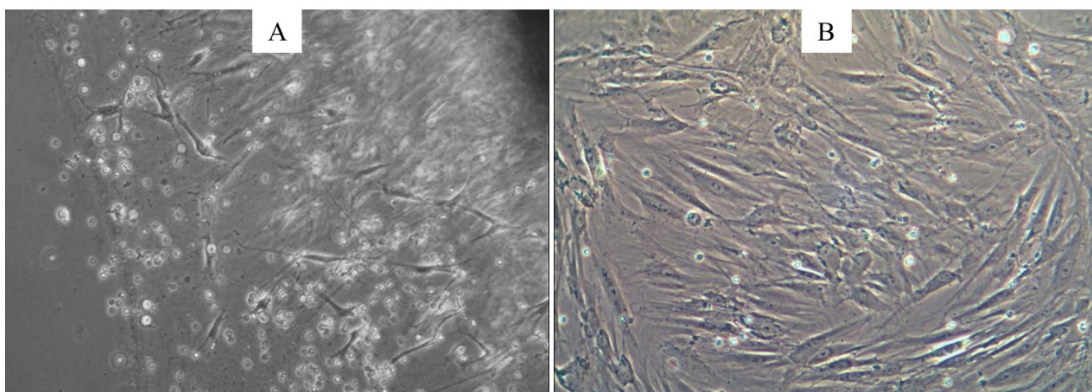


Figure 9-4 Primary cell cultures at two (A) and five (B) days following explantation, showing cells walking out from intact tumour tissue

The desmoid mesenteric plaque biopsy failed to explant, although control fibroblasts from the skin of the same patient did. Control fibroblasts were also obtained from the skin of the first patient (

Table 9-1). These samples were obtained early on before full optimisation of the explant technique. However, the fibroblasts did grow well, reaching confluence more readily compared to the desmoid tumour samples.

9.4.2 Gene mutation analysis of cell lines

APC gene mutation analysis of the desmoid cell culture lines was performed to determine if they were true desmoid cultures or merely overgrowth of fibroblasts. Within desmoid tumours both alleles of *APC* should be mutated, in keeping with Knudson's 'second hit' hypothesis described in section 1.1. Desmoids are thought to be clonal proliferations and 'second hit' somatic mutations have been previously demonstrated in these tumours (Middleton et al., 2000, Lamlum et al., 1999). Therefore, a true desmoid cell culture should harbour the two mutations. In this study fresh frozen tissue samples were stored at -80°C for each tumour until the cells were cultured and the two could then be compared to identify the mutations. If the cultures demonstrated the two mutations found in the tumour tissue this would confirm a true desmoid cell line had been successfully formed.

Despite optimizing the cell culture technique during the three-year study period, none of the FAP desmoid cell lines had the somatic mutation found in the respective tumour tissue and therefore only represented overgrowth of fibroblasts. However, one of the sporadic desmoid tumours was successfully cultured with a mutation found in *CTNNB1* exon 3, codon 45 (patient 7,

Table 9-1, Figure 9-5). This is one of the three most common mutations found in sporadic desmoid tumours.

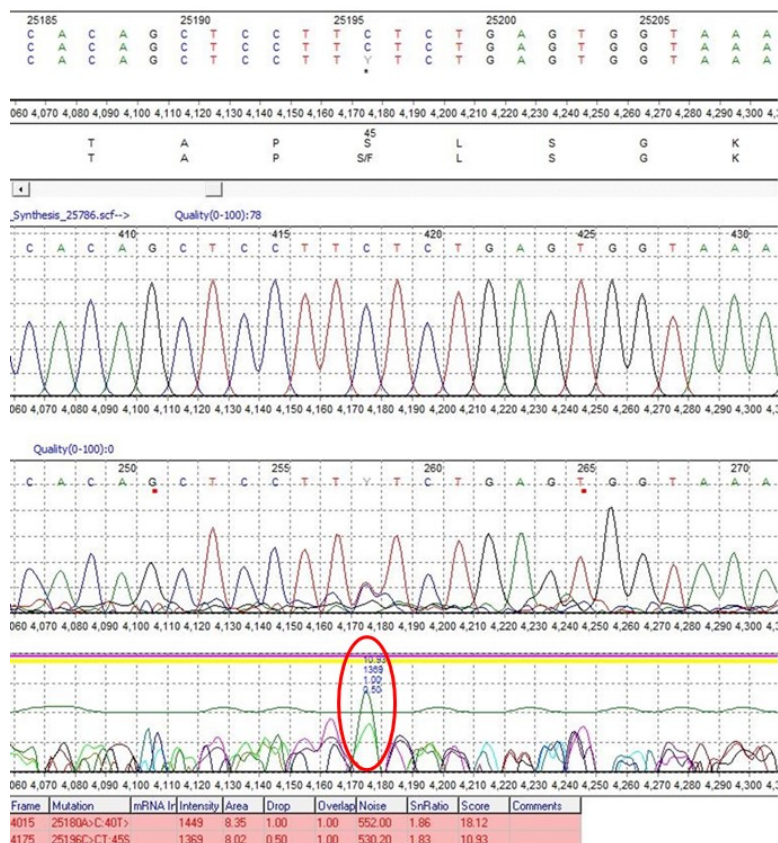


Figure 9-5 DNA sequencing found a *CTNNB1* exon 3 mutation, codon 45 (red circle)

9.4.2.1 Polyclonal desmoids

During genetic analysis of the second patient's cultured cells (patient 2.1 from Table 9-1), a fresh piece of the original tumour was simultaneously sent to the laboratory for testing. The cell culture failed to show a somatic 'second hit' mutation but interestingly the small piece of desmoid tumour tissue was found to have not one but three different 'second hit' mutations within it. This finding was confirmed by sending three further fragments of the same desmoid from different locations within the tumour. The same three mutations were found in all samples. This was a unique finding that does not appear to have been reported previously in the literature and until now desmoid tumours were considered monoclonal in origin (Alman

et al, 1997 and Middleton et al, 2000). None of the other tumours had additional somatic mutations.

Due to the failure to establish a desmoid cell line, further cell culture work (for example testing Wnt ligands and blockers) was not possible. It was hypothesised that a more sophisticated cell culture technique could favour desmoid cell growth. In vitro cell culture grows cells on a flat surface (a petri dish or culture flask), in a two-dimensional platform. However, three-dimensional cell culture techniques have become more popular with the use of a scaffold matrix to aid cell attachment and growth. There is evidence that these systems favour cell growth and better represent the in vivo environment than standard cell culture techniques (Cukierman et al., 2001). This culture technique was explored using the cells already grown in cell culture and fresh tissue obtained from desmoid tumours, initially frozen at -20°C.

9.4.3 Three dimensional desmoid culture

Developing the three dimensional desmoid co-culture was a long and time consuming process. The initial difficulties were in establishing the optimum time to achieve adequate decellularisation. This was confirmed histologically (Figure 9-6). Decellularisation was important so that cells from any tissue culture placed on the scaffold subsequently, and their interaction with the scaffold, can be studied knowing that the cells seen were from the cultured cells applied and not those originating from the tissue. It also prevented any reaction between residual scaffold cells and cultured cells.

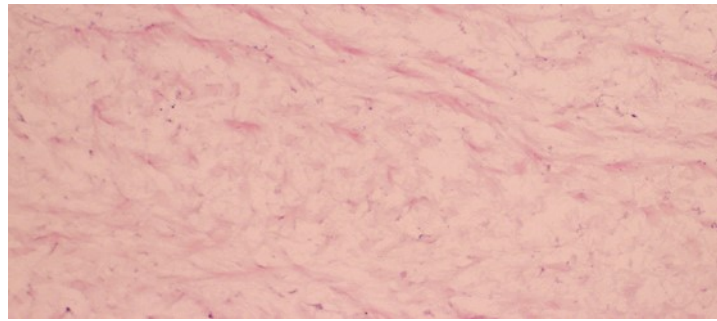


Figure 9-6 H&E stained desmoid tumour tissue after decellularisation, devoid of cellular matter (40x magnification)

Another significant obstacle to overcome in developing a 3D co-culture was the development of infection, during the decellularisation process and priming of the scaffold. In both instances this became apparent when the scaffold was left overnight at 37°C, either in DNase/RNase buffer or growth media. This was a particular problem in scaffold development as it was impossible to ensure stringent sterile techniques were used throughout the process. It was important to be particularly cautious when preparing solutions to ensure sterile conditions were maintained but ultimately the scaffolds required sterilisation prior to placing in growth medium. The use of supercritical CO₂ with peracetic acid made a significant difference to eradicating scaffold infection.

The control scaffolds were free of cells at day 7 of the co-culture experiment (Figure 9-7). The co-culture experiments found the optimum cell concentration to apply to the scaffolds was 300 000 cells/8mmx2mm scaffold disc. At day 7 cells could be seen attaching along the surface of the scaffold and some had moved into the matrix (Figure 9-8), however, far fewer cells were evident in the 100 000 cell co-culture.

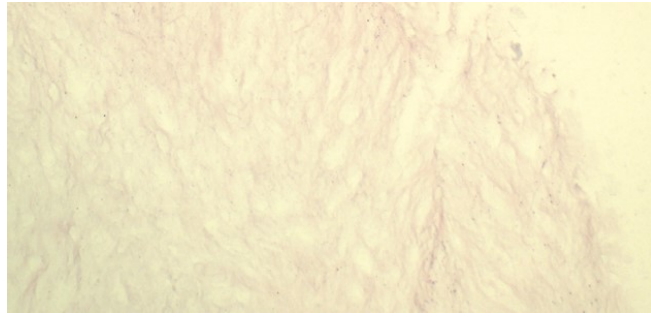


Figure 9-7 Desmoid control scaffold tissue (no cells added) at 7 days

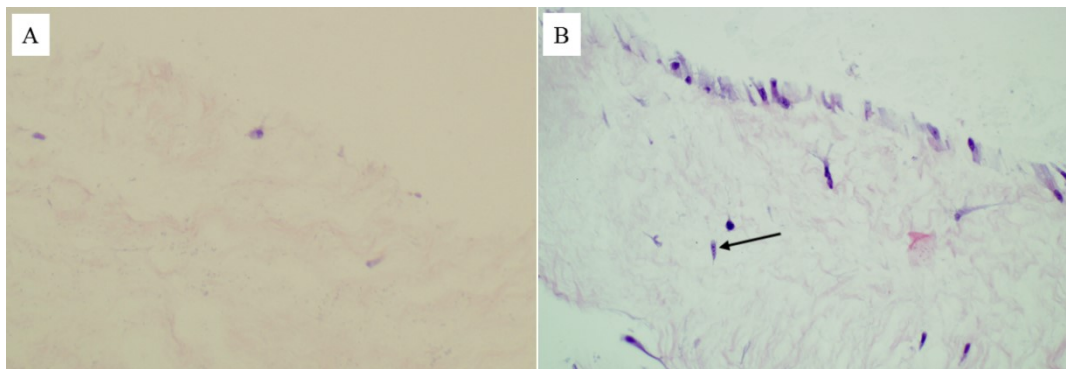


Figure 9-8 Co-scaffolds at day 7, 100 000 cells (A) and 300 000 cells (B) initially seeded (20x magnification). Arrow demonstrating a cell migrating into the matrix

In one of the 300 000 cell co-cultures at day two a micromass of cells had formed at the edge of the scaffold (Figure 9-9). This co-culture had a few wispy tissue fibres at the edge where the tissue had been cut. It may be that these had promoted the growth of the cells at this point. This was not seen in any other co-culture. It could suggest that a thinner scaffold may favour cell growth.

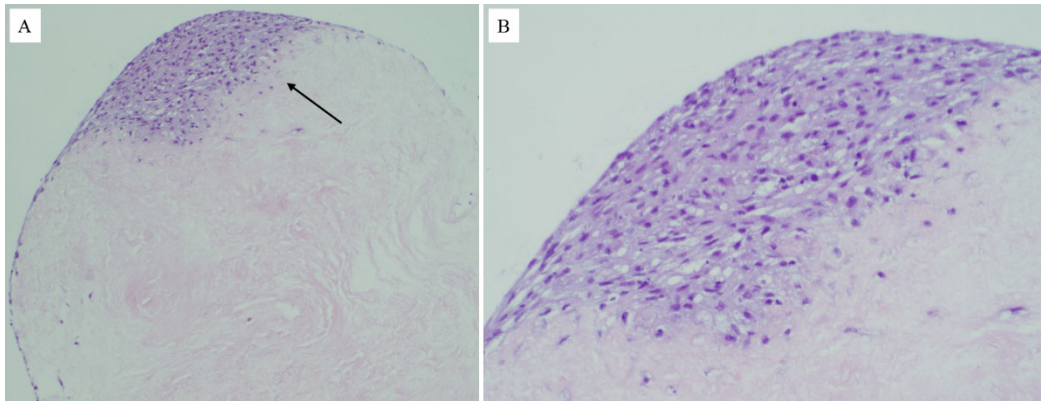


Figure 9-9 Day 2, 300000 cell co-culture forming a cell micromass (arrow), 10x magnification (A) and 20x magnification (B)

9.5 Discussion

The findings from this cell culture study supported those reported previously, that desmoid tumours are difficult tumours to grow in cell culture. It questions the validity of studies using desmoid tumour cultures without performing genetic analysis of cell lines prior to testing potential treatments, as it is likely they are not testing ‘true’ desmoid tumour cells. Perhaps the findings from this study reflect the unpredictable growth of desmoid tumours in-vivo, as the majority remain indolent and some may even regress. If this occurs in vitro, then it is perhaps unsurprising that the fibroblasts outgrow the desmoid tumour cells. However, most of the tumours used in this study demonstrated aggressive growth behaviour requiring surgical excision of the tumour. Fibroblasts grow readily and it is therefore unsurprising that overgrowth occurs frequently within cell culture. It may be that a small proportion of cultured desmoid cells were present in the genetically tested cell lines, but this level was too small to detect when tested.

A surprising finding from this study was a desmoid tumour with multiple ‘second hit’ mutations. This is a novel finding that has not been previously reported to occur in association with desmoid tumours. A theoretical explanation to this unusual event could be that desmoid

tumours may obtain extra chromosome copies (or parts thereof) or develop chromosomal instability and copy number changes and then obtain somatic mutations (one germline and one wild-type *APC* gene in each cell). This is then amplified and two or more copies of the wild-type in a cell could then acquire a single truncating mutation. The end result could select for several mutations with the wild type perhaps no longer apparent but more than one somatic mutation present on different ‘versions’ of the gene. To investigate this hypothesis further, extensive work would be required to sequence the DNA from multiple tumours, at various stages of growth and at multiple locations throughout the tumour itself.

This study led to the development of a potential three dimensional desmoid cell culture that may be a useful future tool in their successful cell culture. This study utilised the cells obtained via standard cell culture (that were ultimately found only to contain fibroblasts) to establish a co-culture. Further work is necessary to study the growth of the desmoid cells and their interaction within the matrix. For example, E-Cadherin staining of the co-culture could help to establish whether the cells are actually attaching to one another, as seems to be the case along the scaffold surface at day 7. Ki-67 stain may be helpful to investigate whether the cells are populating within the scaffold, although there was evidence of this with the micromass seen in one co-culture at day 2. Scaffold DNA, collagen and GAG content could also be assessed to further evaluate the content of the ECM co-culture.

To determine whether a 3D tissue scaffold is a superior technique to standard 2D culture for desmoid tumours, the next phase of this work would require implanting desmoid tumour cells onto the prepared scaffold, immediately following their mechanical extraction from the tumour, rather than from in vitro culture. The co-culture would then need to be genetically tested in a similar way to that described in this chapter. If successful, it would be the ideal model for

studying desmoid tumour growth. However, it is likely that until the fibroblasts can be distinguished and separated from desmoid cells, establishing a desmoid cell line will remain a challenge as any environment that favours desmoid cell growth is also likely to favour fibroblast growth. Until this issue is addressed it is likely to limit the use of these in vitro techniques for the study of desmoid tumours and any potential new treatments.

10 Chapter 10 – Whole exome sequencing of desmoids

10.1 Background

Germline *APC* mutation 3' of codon 1399 is a known risk factor for desmoid formation, as previously discussed in section 1.2.7.3. A family history of desmoid tumour in FAP is a separate risk factor, independent of the *APC* mutation, suggesting the presence of other gene variants that predispose to their formation (Sturt, 2004). Any such variant is likely to be relatively common within the population with FAP (desmoid tumours affect up to a quarter of such patients (Sinha et al., 2011, Vasen et al., 2008)) and might also be somatically altered in desmoid tumours. The identification of a 'desmoid modifier gene(s)' would help to predict risk of desmoid tumour development in patients with FAP. Early identification of high risk individuals that carry a variant(s) in a modifier gene(s) that confers susceptibility, particularly those with a propensity towards aggressive lesions, could influence timing of colectomy (in those with a milder FAP phenotype with fewer colorectal polyps, where surgery can be postponed) and prompt earlier detection and treatment of desmoids. It may also further our understanding of the pathways involved in their development.

10.1.1 Whole exome sequencing

DNA sequencing has been revolutionized by the development of various next-generation sequencing (NGS) technologies that allow the simultaneous sequencing of millions of DNA reads. NGS has reduced dramatically the cost and time taken to analyse samples whilst increasing accessibility to researchers (Shendure and Lieberman Aiden, 2012). Exome sequencing is the targeted sequencing of the protein-coding region, representing approximately 1% of the total human genome. NGS can be particularly useful in the identification of rare,

inherited and highly penetrant genetic mutations that can lead to conditions such as FAP (Shendure and Lieberman Aiden, 2012, Aitken et al., 2015). It can also be used in a research setting to search for new variants relating to a specific disease.

10.2 Aims

The aim of this study was to identify a genetic variant, distinct from the germline *APC* mutation, that could explain the familial clustering of desmoid tumours in patients with FAP. Significant variants identified from exome sequencing were compared between those patients who had a personal history of desmoid tumour occurrence and those that did not, in an attempt to identify variants that might act as risk factors for desmoid disease in FAP patients

10.3 Materials and methods

10.3.1 Patient selection

All participants in the study had a known *APC* germline mutation. Germline DNA was obtained from the isolated peripheral blood leukocytes of 64 patients with FAP using standard methods (see chapter 9). One group was made up of 50 patients with a known desmoid tumour and the second comprised 14 controls with clinically and radiologically proven absence of desmoid tumour at least three years after colectomy. For three out of the 50 patients with a desmoid tumour, DNA was also extracted from desmoid tumour tissue and matched germline control (blood). Absence of a desmoid tumour was established clinically through medical record review, a search of the polyposis registry database and after discussion with the patient. Radiologically proven absence of tumour was then confirmed from historical CT or MRI imaging records. Only patients who had undergone previous abdominal CT or MRI imaging at least three years following colectomy were included in the control group. No one underwent

radiological imaging for the purposes of the study, but the scans were performed to investigate abdominal symptoms where it was suspected that the patient may have developed a desmoid tumour or other pathology. This was to ensure that those patients exposed to surgery had had an opportunity to form a desmoid tumour. If they had not done so within three years of surgery it was unlikely that they would go on to do so, as the majority occur within this time frame (Sinha et al., 2011).

The patients were of Caucasian origin except three who were of Asian origin, one with a desmoid tumour and two controls.

10.3.2 Ethical considerations

Ethical approval was obtained through the National Research Ethics Service (NRES) committee London – Camberwell St Giles (REC reference number: 13/LO/0893). All study participants gave their written informed consent for the collection of a single blood sample the study site, St Mark's Hospital. Samples were then analysed at Arizona University, USA (10.3.3.1 Pilot study) and the Centre for Digestive Diseases and National Centre for Bowel Research and Surgical Innovation, Blizard Institute, Barts and The London School of Medicine & Dentistry, London (10.3.3.2 Expanded cohort). Approval for the study was obtained from North West London NHS Trust Research and Development Department (RD13/042).

10.3.3 Sequencing studies

10.3.3.1 Pilot study

The first study was a small pilot study. DNA was extracted from blood samples and shipped to Arizona University for sequencing and analysis. This study examined genetic variants within a single family with FAP consisting of nine affected members across two generations. Four members had developed a desmoid tumour and five had not (Figure 10-1). It was anticipated that, as the samples were from related individuals, they would share more variants thereby making it easier to distinguish any potential differences between those with and without desmoid i.e. a desmoid modifier gene.

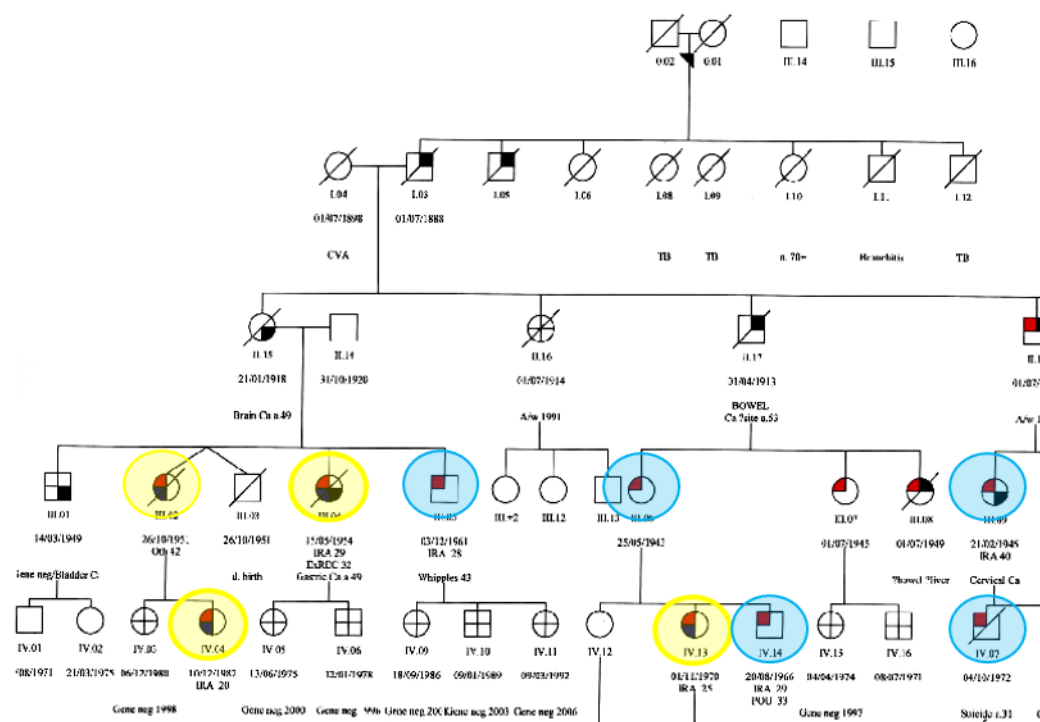


Figure 10-1 Pilot study family tree. Yellow circles indicate family members with desmoid and blue circles those without a desmoid

10.3.3.2 Expanded cohort study

The second study performed NGS on an expanded cohort of mostly unrelated patients with FAP with (n=42) and without a desmoid tumour (n=10), to validate significant findings from the pilot study and to search for other potential variants of interest.

10.3.3.3 Germline-tumour DNA pairs

In the third study, both matched germline and desmoid tumour DNA (from fresh frozen tumours) were obtained from each of three patients and sequenced. The premise for this particular study was that any relatively common variant in the population might also be somatically altered in desmoid tumours.

10.3.3.4 Sequencing strategy

Following whole exome sequencing, genetic variants were compared between patients with FAP who had a desmoid tumour and those without. Variants were then filtered to produce a short-list of those variants of particular interest and relevance to desmoid tumours (Figure 10-2). All synonymous variants were removed, thereby leaving non-synonymous variants from coding regions that effect a protein change. Single nucleotide polymorphisms (SNPs) with a minor allele frequency of more than 0.25 were also excluded. This cut off was chosen as a desmoid modifier gene would be expected to be present in up to 25% of patients with FAP and was not expected to be a rare variant within this population, but equally would not be expected to occur more frequently than about 25%. Furthermore, variants of interest should be largely present in those patients with a desmoid and absent in those without, bearing in mind that some individuals without a desmoid may still carry the variant. Finally, logical genes were selected

from the list of variants produced after filtering, for example those that control collagen genes, fibroblast growth, oestrogen receptors or involved in relevant pathways such as Wnt.

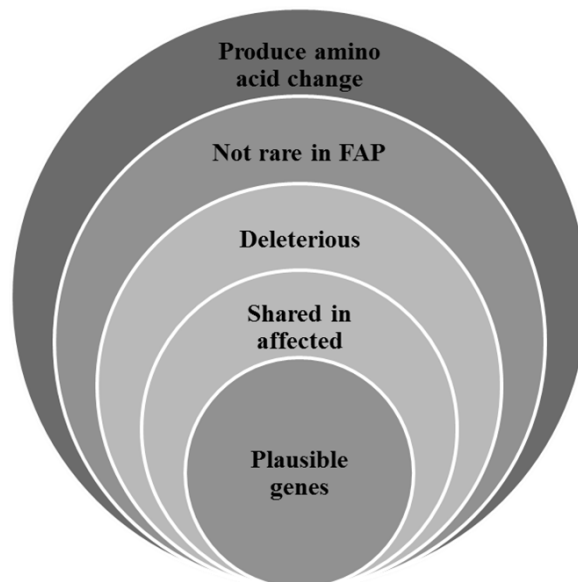


Figure 10-2 Diagram demonstrating how variants were selected according to the variant filtering strategy

10.3.4 Methods

10.3.4.1 Whole exome sequencing of germline DNA

DNA was isolated using the standard methods from peripheral blood leukocytes as described in section 9.3.5.4. Samples were collected and stored until a batch was ready for processing. An Illumina HiSeq 2000 sequencer was used to perform whole exome sequencing. It uses sequencing by synthesis technology. DNA molecules and primers are attached to a flow cell. Polymerase is added to enable DNA amplification. Fluorescently labelled reversible-terminator nucleotides are then added to the amplified clonal DNA fragments held on the surface of a flowcell. As bases are incorporated one at a time, they release a signal that can be detected on a charge coupling device. (Liu et al., 2012, Quail et al., 2012). Illumina sequencing can produce

a high sequence yield but requires high DNA concentrations compared to other sequencing technologies.

Nextera rapid exome library preparation kit (Illumina, UK) was used to capture target exonic regions. These regions were then sequenced using Illumina HiSeq 2000. The reference human genome sequence (hg19) was used to align sequence reads, using Burrows-Wheeler Aligner (BWA) (Li and Durbin, 2009). Single nucleotide variants (SNVs) and insertions/deletions (indels) were detected in the samples using VarScan2 (Koboldt et al., 2009). Where variant calls were made the mean depth coverage across all samples was x50, with a sequence depth of at least eight reads. The software tool ANNOVAR (Wang et al., 2010) was used to provide gene-based annotation for called variants, identifying those SNPs affecting a protein coding change and providing pathogenicity predictions.

10.3.4.2 Identifying potential desmoid tumour variants

For all of the studies undertaken, variants that were differentially expressed between those patients with a desmoid tumour (either homozygous or heterozygous forms) and patients without desmoid were isolated in attempt to identify potential desmoid susceptibility variants. A Fisher's exact test was used to perform an allelic association test. Those variants without statistical ($p\text{-value} > 0.05$) or functional significance were excluded from further analysis, leaving only non-synonymous, stoploss/stopgain SNVs and frameshift/non-frameshift indels for consideration. Additionally, variants with missing genotypes in more than five samples (more than 10% missingness) were excluded.

To account for any differences in variant expression that may have arisen through inclusion of the three samples from Asian patients in the expanded cohort study, analysis was repeated excluding these samples (final numbers: n=41 patients with desmoid and n=7 without).

The same method was applied to the expanded cohort study to isolate common variants unique to samples originating from patients with a germline *APC* mutation either before or after codon 1399. Variants with missing genotypes in more than four samples were excluded (>10% missingness). This provided 36 samples from patients with desmoid (23 with an *APC* mutation before and 13 after codon 1399).

10.3.4.3 Sequencing of matched germline and desmoid tumour DNA

Whole exome sequence data from matched tumour-germline DNA samples from three patients were aligned to the human genome reference (hg19) using BWA (Li and Durbin, 2009). Comparison of tumour DNA to paired germline DNA using VarScan2 was used to aid identification of potential tumour specific mutations (Koboldt et al., 2012). These were then annotated using ANNOVAR (Wang et al., 2010). The total numbers of variant reads in called positions for all tumour and germline matched samples provided the mean sequencing coverage. Those mutations with less than eight reads coverage in tumour or germline samples and those with a p-value of >0.05 were excluded. To analyse the extent of somatic and loss of heterozygosity (LOH) mutations, the sequence base 3' and 5' to the mutated base was extracted using hg19. The occurrence of each substitution type was counted for all three tumours.

DNA copy number variation (CNV) is an alteration in the number of copies of one or more sections of DNA in the genome. Genetic variation can occur as a result but their functional importance and contribution to disease is poorly understood (Henrichsen et al., 2009). VarScan was used to determine somatic copy number changes using the data from matched tumour-germline pairs (Koboldt et al., 2012). The log ratio of read depth between the germline and tumour DNA and GC content was calculated by the algorithm to make preliminary calls and a segmentation-based algorithm was used to infer final CNV. However, as this produces many false positive calls only large-scale CNV was detected, by calculating a moving average of the log ratio with a window size of 1 Mb (Zhao et al., 2013). In the case of sample 63 (see Table 10-1) the breakpoints of the large estimated deletion were inferred using the average log ratio.

10.3.4.4 Pathway analysis

Enrichr was used to perform pathway enrichment analysis (Chen et al., 2013). This program utilised the following databases: Kyoto Encyclopaedia of Genes and Genomes (KEGG), Panther and Gene Oncology (GO) to analyse the somatically altered genes identified from the desmoid tumours. In most cases pathway enrichment tests returned insignificant p-values although the Panther overrepresentation test found significant results after correcting the false discovery rate.

10.4 Results

10.4.1 Patient characteristics

Table 10-1 presents the patient demographics for all participants in the study. One of the germline DNA samples from a patient with desmoid was excluded from further analysis due to failure to meet quality control criteria (sample 22). Principal component analysis confirmed that the ethnicity of the participant samples was not significantly different between the desmoid and control groups, using 56,341 bi-allelic SNP positions that were also present in HapMap samples. HapMap included samples from YRI (Yoruba in Ibadan, Nigeria-African), CHB (Han Chinese in Beijing – Chinese), JPT (Japanese in Tokyo – Japanese) and CEU (Utah – Caucasian) populations. Data from the sequencing was projected onto the 414 HapMap samples and confirmed that all but three of the samples (those of Asian origin) clustered well with the CEU population, with no significant difference seen between the two study groups.

Table 10-1 Demographic information for samples used. CM, Caucasian Mediterranean; WB, White British

Sample Number	Ethnicity	Tumour Multiplicity	Sex	Related to participant	Relatives with desmoid	APC Codon	APC Exon	<codon 1399
Pilot study – related patients with FAP								
1	WB	Multiple	F	1	3	1392	15	1
2	WB	Single	F	1	3	1392	15	1
3	WB	None	M	1	4	1392	15	1
4	WB	None	F	1	4	1392	15	1
5	WB	Single	F	1	3	1392	15	1
6	WB	Single	F	1	3	1392	15	1
7	WB	Single	F	1	3	1392	15	1
8	WB	None	M	1	4	1392	15	1
9	WB	None	M	1	4	1392	15	1
Expanded cohort of patients with FAP								
10	CM	Multiple	M	0	3	1242	15	1
11	Asian	Multiple	M	0	1	1308	15	1
12	CM	Single	F	1	3	1461	15	0
13	CM	Multiple	F	1	3	1461	15	0
14	WB	Single	F	0	3	1122	15	1
15	WB	Multiple	F	0	2	1201	15	1
16	WB	Multiple	F	1	4	1085	15	1
17	WB	Single	M	0	3	2504	15	0
18	WB	Multiple	M	1	2	1465	15	0
19	WB	Single	M	0	1	1546	15	0
20	WB	Multiple	F	0	2	879	15	1
21	CM	Multiple	F	0	2	769	15	1
22	WB	Single	F	0	1	247	7	1
23	WB	Single	M	1	4	1085	15	1
24	WB	Single	F	0	1	1211	15	1
25	WB	Multiple	F	0	2	1509	15	0
26	WB	Multiple	F	0	1	1925	15	0
27	WB	Multiple	F	1	4	1392	15	1
28	WB	Multiple	M	0	1	1551	15	0
29	WB	Multiple	M	0	1	1068	15	1
30	WB	Multiple	F	0	2	1464	15	0
31	WB	Single	F	0	1	1309	15	1
32	WB	Single	M	0	1	1942	15	0
33	WB	Multiple	M	0	2	564	13	1
34	WB	Multiple	F	1	4	1392	15	1
35	WB	Multiple	F	0	2	1940	15	0
36	WB	Multiple	F	0	1	1062	15	1
37	WB	Multiple	M	0	1	636	14	1
38	WB	Single	F	0	1	935	15	1
39	WB	Multiple	F	0	1	1488	15	0

40	WB	Multiple	F	0	1	1556	15	0
41	Polish	Single	M	0	1	1228	15	1
42	WB	Single	M	0	3	1068	15	1
43	Kuwaiti	Single	M	0	1	278	7	1
44	WB	Multiple	M	1	2	1465	15	0
45	WB	Multiple	M	0	1	1227	15	1
46	WB	Multiple	M	0	1	216	6	1
47	CM	Single	F	0	1	564	13	1
48	WB	Single	M	0	3	727	15	1
49	WB	Multiple	F	0	1	1303	15	1
50	CM	Multiple	M	0	2	1464	15	0
51	WB	Multiple	M	0	2	1409	15	0
52	Asian	None	F	0	0	*	14	Na
53	WB	None	M	0	0	1154	15	1
54	WB	None	F	0	0	172	4	1
55	WB	None	M	0	0	685	15	1
56	WB	None	M	0	0	**	4	1
57	Asian	None	F	0	0	187	5	1
58	CM	None	M	1	0	1461	15	0
59	Asian	None	F	0	0	649	14	1
60	WB	None	M	0	0	1061	15	1
61	WB	Multiple	F	0	0	935	15	1
Desmoid tumour and germline DNA samples								
62	WB	Multiple	F	0	4	216	6	1
63	WB	Multiple	F	0		1408	15	0
64	WB	Multiple	F	0	1	1450	15	0

* Acceptor splice site; ** Exon 4 duplication;

10.4.2 Germline *APC* mutations

APC alterations were detected using whole exome sequencing in the 51 patients from the expanded cohort (samples 10-61 in Table 10-1), nine of whom were related to one or two other participants. A total of 84 variants were isolated: 53 exonic, 27 intronic, two splicing, one UTR3 and one UTR5. For the exonic variants, nine were synonymous SNVs. The mutations responsible for FAP were identified for each sample and consisted of stopgain SNVs, frameshift deletions/insertions and splicing mutations (Appendix A4.1). These were predicted pathogenic by bioinformatics algorithms including Polyphen (Adzhubei et al., 2010), SIFT (Ng

and Henikoff, 2003) and CADD (Kircher et al., 2014). None of these mutations occurred in the large healthy control database 1000Genomes (Genomes Project et al., 2012). There were 38 different mutations found across all samples, with related samples confirming the same *APC* mutation. It was possible to verify the mutations found in some cases by comparing to Sanger sequencing results previously obtained for 44 of the patients by the North West Thames Regional Genetics Service (Kennedy-Galton Centre). In all but one case (43/44) NGS confirmed the same mutations. In one case no mutation was identified (sample 22) by whole exome sequencing in a patient with seven intronic *APC* mutations, which may indicate a lower quality of calls. The COSMIC database was searched to determine whether any germline *APC* mutations identified had been previously reported as somatic mutations in tumours. Fourteen out of 38 mutations were found to be somatically mutated, the majority (11/14) were reported in tumours of the large intestine and the remaining found in tumours of the small intestine, lung, stomach and one mutation in multiple tumours (i.e. oesophagus, stomach and liver).

In six out of 51 (12%) samples a second (and in one case a third) putative *APC* mutation was identified (Table 10-2). Interestingly, the patient from sample 58 had an additional mutation but did not have a desmoid tumour whereas his two relatives (samples 12 and 13 from Table 10-1) did not have the additional mutation but both had desmoid tumours requiring surgery. These additional mutations were non-synonymous SNVs, identified in samples with a stopgain or frameshift SNV that were found by Sanger sequencing. Four of these mutations were predicted deleterious by bioinformatics algorithms and were non-existent in healthy control databases, suggesting that they could be causal, with one implicated in urinary tract carcinoma (COSMIC database). Of the remaining two mutations, one was seen at a low frequency in healthy controls and predicted benign and the other had a very low frequency (<0.0001) but predicted to be deleterious. These could be rare *APC* variants, not particularly pathogenic, that

indicate a large enrichment of such variants in FAP patients. Alternatively, they could be somatic mutations which would explain differences between related individuals. Aretz et al found somatic *APC* mosaicism was not an uncommon phenomenon, affecting 11% of cases they studied, a similar proportion to our findings (Aretz et al., 2007).

Table 10-2 Additional putative *APC* mutations found in six patients

Sample	Known <i>APC</i> codon mutation	NGS mutations detected
14	1122	1122, 2738
18	1465	1462, 1670
40	1556	5, 1554
41	1228	1228, 1278, 1453
44	1465	1462, 1670
58	1461	1462, 1475

10.4.3 Identifying potential desmoid tumour variants from germline DNA

10.4.3.1 Pilot study

Whole exome sequencing of nine family member samples identified around 20 000 variants. After filtering as outlined in section 10.3.3.4, 10 000 of these variants were found in coding regions with 1200 shared amongst those with a desmoid tumour. After selecting those with a minor allele frequency of less than 0.25, 88 genes were isolated. These genes were then studied more carefully for their biological plausibility and those most applicable to desmoid tumours were selected (Table 10-3).

Table 10-3 Candidate genes and functions, after variant filtering

Candidate gene	Location (Chromosome)	Biological function
QSOX1	1	Role in growth regulation of fibroblasts
SDC2	8	Role in cell proliferation, migration and matrix
CSMD3	8	Implicated in colon cancer
CDC27	17	Controls mitosis progression
FRZB	2	Modulates Wnt signalling and growth
INPP4B	4	Tumour suppression in breast and ovarian cancer
WWOX	16	Involved in apoptosis and tumour suppression
THSD1	13	Tumour suppressor gene
CKAP2	13	Role in cell death in p53 dependent pathway
PALLD	4	Upregulation in fibroblasts in tumour invasion

These candidate genes were subsequently analysed in germline DNA through further Sanger sequencing. The only significant variant found was quiescin Q6 sulfhydryl oxidase 1 (*QSOX1*). It was present in all members of the family. Nearly all of those family members with a desmoid were heterozygote for this variant, and those without desmoid, homozygous. Interestingly, one individual without desmoid was a heterozygote for the variant but had a daughter who was heterozygote and did have a desmoid (Figure 10-3).

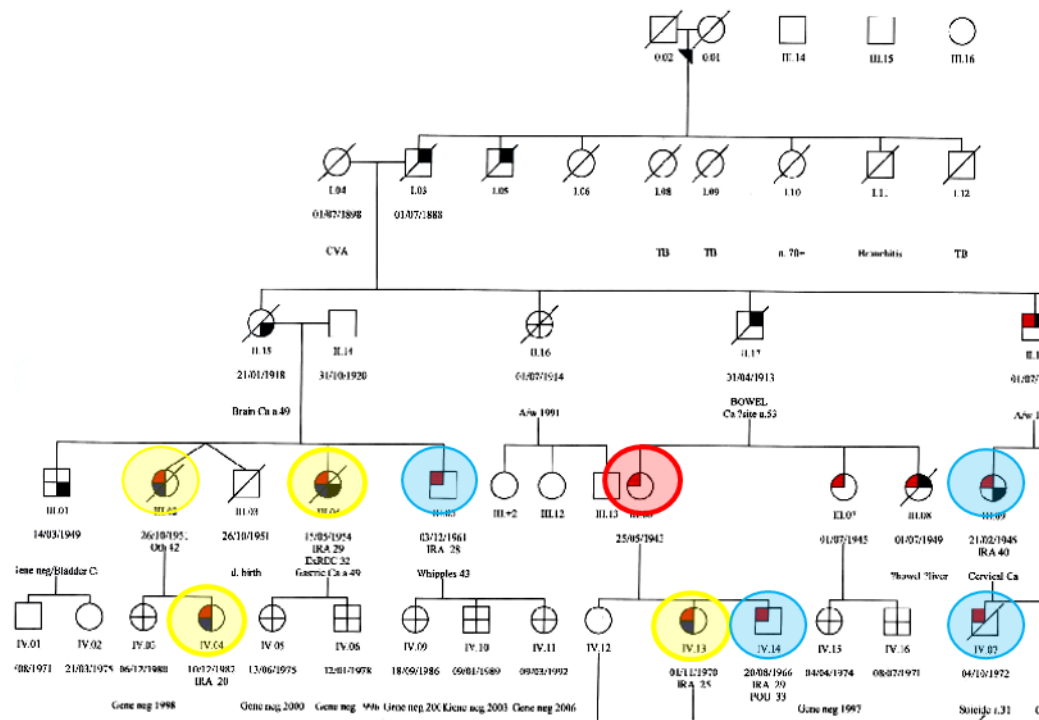


Figure 10-3 Family tree indicating all individuals with a desmoid and heterozygote for SNP (yellow circles) and those without desmoid and homozygote (blue circles). Red circle shows an individual without desmoid, a heterozygote with a daughter, also heterozygote, with a desmoid

QSOX1 was interesting for several reasons. It had a minor allele frequency of 0.125, was in a conserved region, was predicted deleterious by the bioinformatics functional prediction models and a known regulator of fibroblast growth. Located on chromosome 1q24, it encodes for quiescin Q6 sulfhydryl oxidase 1, an enzyme that catalyses disulphide bridging of laminin in extra-cellular matrix, that is important for the folding of proteins (Ilani et al., 2013). *QSOX1* also codes for a major protein secreted by quiescent fibroblasts (Coppock et al., 1998). It has more recently been implicated in the development of various tumours including pancreas, prostate and breast cancers (Katchman et al., 2011, Song et al., 2009, Soloviev et al., 2013). Further sample analysis in an expanded cohort was now necessary to verify the findings of the pilot study.

10.4.3.2 Expanded cohort

In the expanded cohort of 51 patients, common variants unique to 41 patients with desmoid tumour compared to 10 patients without, were identified. Variants were filtered as described in section 10.3.4.2 to detect those likely to be functionally significant. No risk allele was discovered that all patients with desmoid tumour carried (Appendix A4.2). Analysis of the European cohort, by removing the three Asian patient's samples made no significant difference (Appendix A4.3). Disappointingly, the *QSOX1* variant identified from the pilot study, did not feature as a potential variant of interest after variant filtering had occurred for the expanded cohort. This may indicate that in the cases sequenced this variant was specific only to this family; the low population frequency meant that there were too few samples in the expanded cohort to identify the variant. The top candidates showed similar minor allele frequencies.

10.4.3.3 Variants unique to samples with mutations 5' or 3' of codon 1399

Germline *APC* mutations 3' (after) codon 1399 are known to increase desmoid tumour risk (Sinha et al., 2011). Variants may effect a protective or harmful change. Therefore, variants unique to the desmoid tumour group in the expanded cohort with germline *APC* mutation 5' or 3' of codon 1399 were identified to determine if any variants were specific to these groups (23 and 13 samples, respectively). No significant variants were found, specific to the germline mutation location (Appendix A4.4 and A4.5).

10.4.4 Genomic landscape of desmoid tumour samples

10.4.4.1 Somatic and loss-of-heterozygosity (LOH) mutations

Whole exome sequencing was performed in three patients' paired samples that included both germline and tumour DNA, at an average coverage of ~30x. Figure 10-4 shows the allele frequencies of all variants (synonymous and intergenic) called somatic or LOH by VarScan. Somatic variants were called if their germline allele frequency was less than 5% but more than 20% in tumour. No functional recurrent mutations were identified across the three patients, however, five intergenic somatic variants and 20 intergenic or intronic/UTR5 (LOH) mutations were found to recur across two of the three patients' samples.

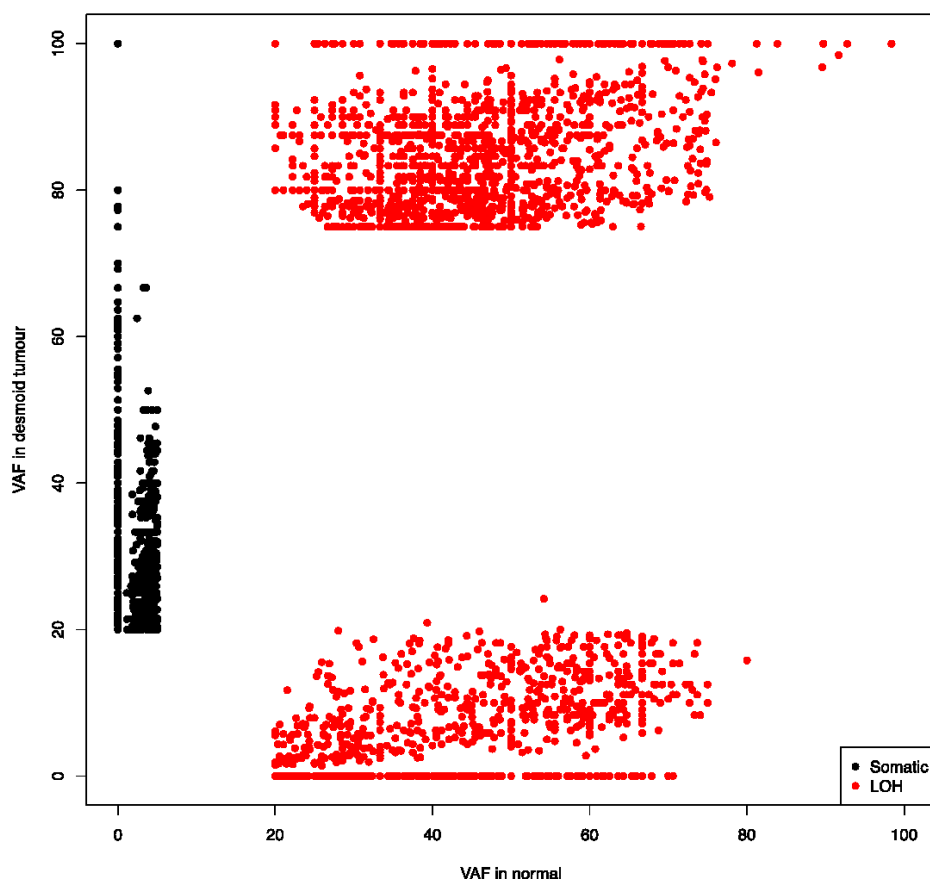


Figure 10-4 Scatter plot displaying variant allele frequencies (VAF) in normal and tumour samples (for all called somatic and LOH variants)

10.4.4.2 Recurrent mutations

Recurrently mutated genes occurring in these three cases were further studied (Figure 10-5). Only non-synonymous or stop gain/loss functional mutations were selected. Mucin 4 gene (*MUC4*) had somatic mutations in all three tumours (Figure 10-5A). *MUC4*, *MUC16*, Myoferlin (*MYOF*), alpha-kinase 3 (*ALPK3*), FSHD Region Gene 2 Family, Member B (*FRG2B*), epiplakin 1 (*EPPK1*) and solute carrier family 25 member 5 (*SLC25A5*) had lost heterozygosity in two of the three tumours (Figure 10-5B). Recurrent mutations of the same genes were seen within each tumour. Figure 10-6 shows all mutated genes across the three tumours. The size of the gene name represents the frequency of recurrently mutated genes identified within each tumour, although in some cases the frequency of multiple mutations per sample could be due to false positive calls by sequence read alignment artefacts.

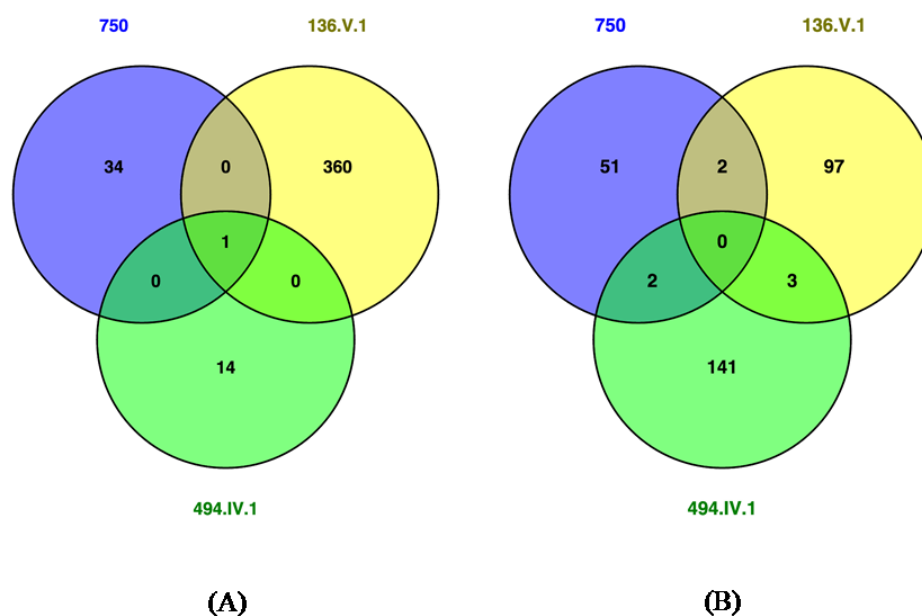


Figure 10-5 Mutated genes across 3 desmoid tumours. Numbers of genes with (A) somatic mutations (B) loss-of-heterozygosity mutations

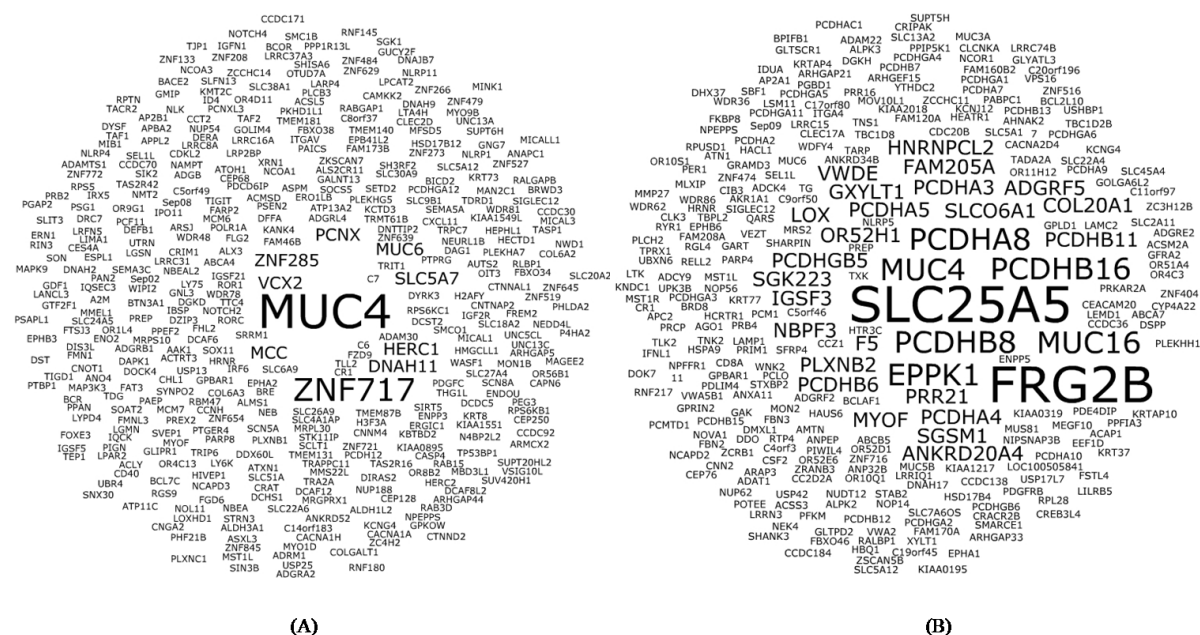


Figure 10-6 Word cloud diagrams showing mutated genes across all tumours. The size of each gene name represents the frequency of recurrently mutated genes within each tumour. (A) Somatic mutations (B) Loss-of-heterozygosity mutations

10.4.4.3 Desmoid tumour mutation occurrence in COSMIC

In total 2427 somatic and 3266 LOH variants (coding and non-coding) were identified across the three tumour samples. Following interrogation of the COSMIC database, only 16 somatic variants were found in a range of malignant tumours that included four arising in the large intestine, two in breast and one each in lung, endometrium, skin, urinary tract, pancreas, upper gastrointestinal tract and haematopoietic and lymphoid tissue, with three occurring in multiple tissues.

10.4.4.4 Mutation signatures

Mutation signatures can differ between tumour types, providing a specific imprint of previous mutagenic exposures and information relating to potential tumour aetiology (Greenman et al.,

2007, Alexandrov et al., 2013). The mutation signature was identified across the three tumours by ordering somatic substitutions (both coding and non-coding alterations) into 96 classes as highlighted by the substitution type and sequence context immediately 3' and 5' to the mutated base (Figure 10-7). Additionally, the mutation signature for the functional mutations (non-synonymous and stop gain/loss SNVs) was also determined (Figure 10-8). The most frequent substitution was C>A, similar to that seen in colorectal tumours (Greenman et al., 2007).

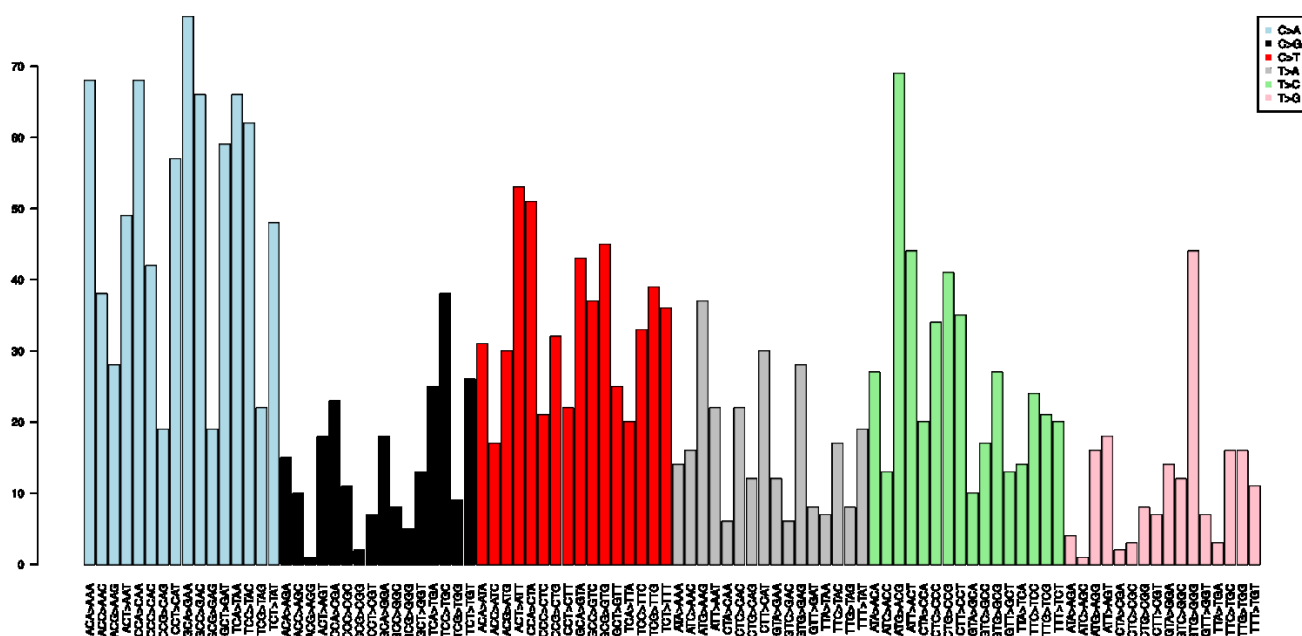


Figure 10-7 Mutation signature across 3 desmoid tumours. The number of somatic mutations is broken down according to 96 substitution classifications

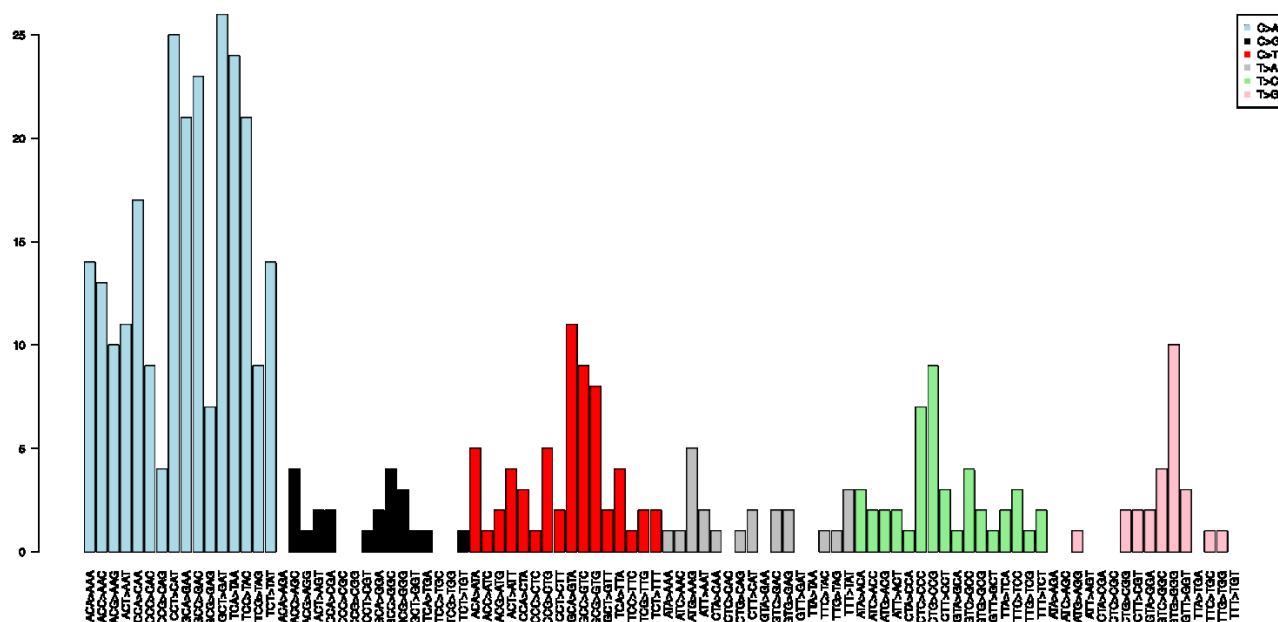


Figure 10-8 Mutation signature across 3 desmoid tumours. The number of somatic alterations of the nonsynonymous and stopgain SNVs is broken down according to 96 substitution classifications

10.4.4.5 ‘Second-hit’ mutations

To assess ‘second-hit’ mutations in desmoid tumour development, somatically mutated genes were studied to determine whether they contained any variants that could be linked to susceptibility to desmoid tumour formation, susceptibility variants. Genes indicated for disease susceptibility included: dystonin (*DST*), TATA-box binding protein associated factor 2 (*TAF2*) and sterol O-acyltransferase (*SOAT2*) (Appendices A4.2 and A4.3). These genes were found to contain non-synonymous or stop gain/loss somatic mutations in desmoid tumours. Both dentin sialophosphoprotein (*DSPP*) and Epiplakin 1 (*EPPK1*) genes had non-synonymous and stop gain/loss LOH mutations in desmoid tumours, containing putative desmoid susceptibility variants.

10.4.4.6 Copy number variation

Interrogation of the dataset for large-scale structural variation, by calculation of the moving average of adjusted (for GC content) log ratio using a large window of 1Mb, found most chromosomes showed no evidence of large-scale structural variation, except for a large deletion in chromosome 5 of patient 63. The deleted region contained 339 genes, including *APC*, with the breakpoints of the deletion estimated to be 100-141 Mb.

10.4.4.7 Pathways enrichment analysis

Gene set enrichment analysis was performed using all genes somatically mutated across all tumours and all genes contained in the large deletion in chromosome 5q of sample 63. The analysis indicated enrichment of two canonical pathways, Cadherin and the Wnt signalling pathways (Panther overrepresentation test p-values 8.492e-23 and 1.924e-18, respectively) (Table 10-4). The G0 biological functions enriched were cell-to-cell adhesion, particularly via plasma membrane cell adhesion molecules from deletion of protocadherin genes. The G0 molecular processes enriched were calcium ion binding and Rac GTPase activatory activity.

Table 10-4 Pathway enrichment analysis results using Panther

Pathway	Overlap	P-value	Adjusted P-value	Z-score
Cadherin signalling	55/147	8.49248E-23	6.11459E-21	-1.7504771
Wnt signalling	63/254	1.92441E-18	6.92789E-17	-1.7294037

10.5 Discussion

This whole exome sequencing study utilised a NGS platform to analyse germline and somatic mutations in patients with FAP, comparing those with and without a desmoid tumour. This was a novel undertaking in the field of desmoid tumour research.

The results obtained from the pilot study were initially promising and found a biologically plausible variant of interest in the germline DNA, differentially expressed in those with a desmoid tumour compared to the control group from the same family. However, *QSOX1* was not identified as a variant of interest in the expanded cohort. Unfortunately, no single variant unique to the desmoid group was identified within germline DNA in the expanded cohort. It was interesting that whilst studying the germline DNA, in six cases a second or third *APC* mutation was identified. These were non-synonymous SNVs and nearly all were predicted deleterious. It remains unclear why these additional mutations would occur. One possibility is that they could represent somatic *APC* mosaicism. These would need validating independent of NGS.

Analysis of desmoid tumour DNA was more promising. The gene *MUC4* was somatically mutated in all three tumours. This gene codes for mucin cell surface glycoproteins, important in protecting epithelial lining in structures including the trachea, colon and cervix. Mutations in this gene have been linked to pancreatic and breast cancer development (Zhu et al., 2014, Cho et al., 2015). To identify a role for second-hit mutations in the development of desmoid tumours, any somatically mutated genes (either somatic or LOH mutations) that contained susceptibility variants were investigated. This identified *DST*, *TAF2* and *SOAT2* genes that potentially linked to desmoid tumour susceptibility. Functional analysis using cellular and

mouse models would be necessary to prove any such link, which represents significant experimental challenges.

Pathway enrichment analysis looked at all genes somatically mutated and found two canonical pathways enriched; the Wnt and Cadherin pathways. The Wnt pathway is known to be integral to FAP and desmoid development.

10.5.1 Study limitations

The main limitation of this study is its relatively small size. However, this is an inherent difficulty when studying a rare condition. Recruiting people with FAP who had not developed a desmoid tumour provided a more suitable control cohort than people without FAP, as this risk factor was therefore already adjusted for. Although the FAP control group were carefully selected, ensuring they were clinically and radiologically free from desmoid tumours at least three years following colectomy, this could not absolutely guarantee they would not develop a desmoid tumour in the future, only that they were unlikely to. Focusing more on families, such as the pilot study did, comparing genetic variants in those with and without desmoid tumours is perhaps a more robust way to proceed with future genetic analysis, particularly as members of these families will share many variants. However, this is dependent on recruiting more than one family with a sufficiently large enough pedigree and suitable affected/non-affected individuals.

The cost of conducting NGS is significant. Although whole exome sequencing has become much cheaper and more viable, it remains expensive and this also limits the cohort size. The cost of sequencing one sample is currently around £500 currently. In future, as the costs are driven down, the results of studies like this will provide pilot data to enable larger studies to be undertaken. Larger studies are more likely to identify a gene of significance. For rare disorders such as desmoid tumours, this will require an international, multicentre, collaborative effort. Another consideration is the processing and understanding of the large amount of genetic data generated from sequencing studies. Potential genes of importance may be overlooked during the filtering stages, particularly in the final stage of selecting biologically plausible genes. The gene list finally generated is analysed for individual gene function and those where little is published or known about may be disregarded although they could be important in desmoid tumours. Finally, validation is then required to determine if a genetic difference between two groups effects a significant change in protein expression and is directly responsible for the manifestation of the condition. This is the next step required in this study.

In the future, we will understand more about each gene, their variants and function. This will help us to better interpret the significance of the results generated from such studies.

11 Chapter 11 – MicroRNA expression in desmoids

11.1 Background

MicroRNAs (miRNAs) are short non-coding RNA molecules of 18-25 nucleotides. They bind the 3' untranslated regions (3'UTRs) of target mRNA, suppressing translation and post-transcriptional gene expression, thereby influencing apoptosis, cell proliferation and differentiation (Borchert et al., 2006, Kim, 2005, Shruti et al., 2011). The aberrant expression of miRNAs has been implicated in the development of fibrotic disorders and cancer. MiR-29 has been linked to various fibrotic conditions including cardiac, renal and liver fibrosis and more recently fibrostenosing Crohn's disease (He et al., 2013, van Rooij et al., 2008, Nijhuis et al., 2014). In terms of influence on cancer development, miRNAs can act as tumour suppressors or oncogenes, and have been associated with many cancers including breast, colon and brain (Hao et al., 2014, Luo et al., 2013, Garzon et al., 2010, Hamfjord et al., 2012).

Circulating miRNAs detected in the serum have the potential to be exploited as non-invasively detected biomarkers of disease (Lewis et al., 2015, Nijhuis et al., 2014). They can also offer potential treatment targets as miRNA mimics and antagonists (antagomiRs), that can alter their expression and effects, already exist (Agostini and Knight, 2014, Kaboli et al., 2015, Olson, 2014, Bernardo et al., 2012). Such a biomarker for patients with a desmoid tumour could be clinically useful, especially for detecting intra-abdominal tumours in the early stages of development that may not be obvious at clinical examination. Currently no biomarker for desmoid tumours has been identified. The low prevalence of desmoid and their unpredictable growth make large-scale biomarker studies extremely difficult to conduct.

A biomarker that could discriminate between aggressive tumours and stable ones would be particularly valuable as it would allow targeting of aggressive treatment at more active tumours that are associated with the development of complications, whilst sparing patients with stable tumours from unnecessary interventions. It could influence the timing of therapy and clinical outcomes, as earlier identification could enable earlier initiation of treatment and this may inhibit further tumour growth that would otherwise have occurred. Finally, such a biomarker could provide insight into novel pathways for research into desmoid biology, and offer potential treatment targets.

11.2 Aims

The aim of this study was to examine miRNA expression in patients with FAP and desmoid tumours to assess the feasibility of utilising circulating miRNAs in serum as non-invasive biomarkers for the detection of desmoids.

11.3 Materials and methods

11.3.1 Patient selection

All participants had a confirmed *APC* mutation and were identified from the St Mark's Hospital polyposis registry database. Patients were recruited from clinic and serum samples were obtained from a total of 31 patients: 17 cases with FAP and a desmoid tumour and 14 patients with FAP, acting as controls, who had not developed desmoid at the time of the study. Four of the patients with a desmoid tumour had an aggressive phenotype and the rest had stable disease. Aggressive tumours were defined as those with clinical or radiological evidence of tumour growth with associated complications (e.g. bowel obstruction, perforation or ureteric compression), occurring within a six-month period prior to collection of the serum sample.

Stable tumours were defined as those demonstrating no growth, radiologically or clinically, 24 months prior to the serum sample collection.

It was important to clinically and radiologically (through previous CT or MRI scans) confirm the absence of desmoid in the control group, as some tumours are asymptomatic and may not be clinically apparent. All control group participants were at least 3 years following their colectomy for FAP, as surgery is a risk factor for desmoid development and all patients had undergone prophylactic colectomy. It is within this time frame that most desmoid tumours develop (Sinha et al., 2011).

Seven archival formalin-fixed paraffin embedded (FFPE) desmoid tumours, from patients with FAP identified through polyposis registry database, were included in the study. The FFPE desmoid specimens, and a breast cancer FFPE control tissue, were obtained from the histopathology department at St Mark's Hospital and used to analyse miRNA expression in desmoid tumours.

11.3.2 Ethical considerations

Ethical approval was obtained through the National Research Ethics Service (NRES) committee London – Camberwell St Giles (REC reference number: 13/LO/0893). All study participants gave their written informed consent and a single blood test was collected at the study site, St Mark's Hospital. Samples were then analysed at the Centre for Digestive Diseases and National Centre for Bowel Research and Surgical Innovation, Blizard Institute, Barts and The London School of Medicine & Dentistry, London. Research and development approval for

the study was obtained from London North West Healthcare NHS Trust Research and Development Department (RD13/042).

11.3.3 Reagents used for miRNA extraction, single qPCR assay, *in-situ* hybridisation and immunohistochemistry

11.3.3.1 cDNA synthesis reagents

miScript II RT kit (Qiagen) with HiSpec buffer (Qiagen, Cat. No. 21816).

Reaction mix:

High spec buffer x 5: 2µl

Nucleic acid mix x10: 1µl

RNase free water: 4µl

RT mix: 1µl

Sample template: 2µl

11.3.3.2 Quantitative PCR (qPCR) reagents

miScript SYBR Green PCR kit (Qiagen, Cat. No. 218075)

Reaction mix:

2X QuantiTectSYBR Green PCR Master Mix: 12.5µl

10X miScript Universal primer: 2.5µl

10X specific miScript forward primer: 2.5µl

RNase free water: 5.5µl

cDNA sample template: 2µl

11.3.3.3 Reagents for miRNA *in-situ* hybridisation

Ethanol 70%, 95% and 100%, RNase-free water, proteinase K 10µg/ml

Double digoxigenin labelled miRcury locked nucleic acid ISH probes (Exiqon, Denmark) diluted from 10µm buffer stock

LNA probe dilutions:

miR-34a-5p (1:450), miR-21-5p (1:625), miR-210 (1:625)

miR-scrambled (1:625)

5X Saline Sodium Citrate (SCC), 1X SCC, 0.2X SSC

Blocking solution: 1X Tris Buffered Saline (TBS), 1% triton, bovine serum albumin (BSA)

Anti-digoxigenin antibody 150U (Roche, Switzerland)

Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Roche, Switzerland)

Nuclear fast red

Eukitt (Fluka Germany)

11.3.3.4 Immunohistochemistry reagents

Ethanol 70% and 100%, distilled water, 1.5% H₂O₂ in methanol

Sodium citrate buffer, pH8 (company?)

1X phosphate buffered saline (PBS)

P53 primary mouse antibody (DAKO, UK)

Secondary rabbit anti-mouse antibody (DAKO, UK)

Diaminobenzidine reaction kit (K3468, DAKO, USA)

11.3.3.5 miRNA extraction

miRNeasy Serum/Plasma kit (Cat. No. 217184) (Qiagen, UK)

miRNeasy synthetic spike Ce_miR-39_1 (Qiagen, UK)

11.3.4 Methods

11.3.4.1 Serum collection

A 6ml sample of whole blood was collected in an EDTA bottle from each participant at the study site. Samples were centrifuged at 3000 rpm for 10 minutes within 1 hour of collection to achieve serum separation. Serum was extracted and stored in a cryovial at -80°C until required for analysis. All samples were transferred together, without patient identifiers, on dry ice to the Blizzard Institute laboratory for miRNA analysis.

11.3.4.2 MicroRNA arrays

Twenty-four serum samples obtained from patients with FAP (12 from patients with desmoid and 12 from patients without desmoid) were sent to Qiagen, Germany, for a miRNA profiling array. These samples comprised of two independent cohorts (each cohort had six samples from patients with desmoid and six from patients without a desmoid (controls)), which were analysed separately and the results combined to improve statistical power. RNA was extracted from 200µl of serum and miRNA expression quantified using qPCR (SYBR-qPCR, miScript miRNA PCR 384HC array for serum (MIHS-3106Z)). The miRNA panel included 384 different primer sets that had been previously selected from a panel used to assess miRNA expression in intestinal fibrosis occurring in Crohn's disease (Nijhuis et al., 2014). It was anticipated that there would be some similarities in miRNA expression in desmoids as they are myofibroblastic tumours. To improve the detection of those miRNAs present at low levels in the serum, the arrays were performed using a pre-amplified cDNA set with a maximum cycle threshold (Ct) of 30 for all samples and miRNAs. Using the $2^{-\Delta Ct}$ method, data were normalised to an exogenous spiked in control (Cel-miR-39-3p) and $\log_2$ transformed, to adjust for variance and achieve normal distribution, prior to statistical analysis. Haemolysis during sample collection

can affect miRNA content (Kirschner et al., 2013). The delta Ct for two miRNA haemolysis markers, miR-23 and miR-451a, was therefore used for the array samples. A value of more than seven indicated as significant risk of haemolysis (Blondal et al., 2013). No significant haemolysis was detected in any of the samples and delta Ct values were similar between the patients with and without a desmoid.

11.3.4.3 MiRNA extraction and single quantitative PCR assays

Those miRNAs of interest identified from the qPCR array, with significantly differentially expressed levels between the desmoid cases and control group, were selected and results validated using single qPCR sera assays. This validation was performed on an expanded cohort of 31 patients (the 24 previously analysed for the miRNA array panel and 7 others (5 desmoid cases and 2 controls)). A single qPCR assay was undertaken to determine if the results from the array panel were reproducible and whether they could be validated.

1. Extracting miRNA from sera

In separate 2ml Eppendorf tubes, 200µl of each serum sample was added to 1ml of Qiazol, vortexed immediately and incubated at room temperature for 5 minutes. 3.5µl of miRNeasy spike was added to each tube before mixing by inversion and adding 200µl of chloroform and vortexing again. This was incubated at room temperature for 3 minutes before samples were centrifuged at 12000g for 15 minutes at 4°C. The upper aqueous layer was carefully extracted (600µl volume) and placed in a new collecting tube and mixed with 900µl of 100% ethanol. 700µl was then added to RNeasy miniElute spin column and centrifuged at 10000rpm for 1 minute. The elute was discarded before adding another 700µl of sample and repeating until the whole sample had been loaded into the column. 700µl of RWT buffer was added and

centrifuged as before, followed by the addition of 500µl RPE buffer and further centrifugation for 1 minute before 500µl of 80% ethanol was added and spun at 10000rpm for 2 minutes. The column was transferred to a fresh collection tube and centrifuged for a further 5 minutes. Finally, the column was placed in another collection tube and 14µl of nuclease free water added. This was centrifuged for 1 minute at 13000rpm before storing the elute at -80°C until required.

2. RNA reverse transcription and cDNA synthesis

Reverse transcription was achieved using 10µl reaction mixtures (8µl mastermix and 2µl of sample template). This combined poly-A tailing of miRNAs with first-strand cDNA synthesis. Samples were incubated to synthesise cDNA at 37°C for 60 minutes followed by 5 minutes at 95°C. The resultant cDNA template was diluted 1:5 using nuclease-free water.

3. qPCR reaction

Quantitative PCR was performed combining 2µl of diluted cDNA with 23µl of reaction mixture containing miScript miRNA qPCR primer assays specific for each selected miRNA. Samples were incubated at 95°C for 15 minutes and PCR performed using 40 cycles of 94°C for 15 seconds, 55°C for 30 seconds and 72°C for 34 seconds (according to manufacturer recommendations). Each reaction was conducted in duplicate and melt curve analyses were performed to confirm a single PCR product. MiRNA expression levels were then normalised to the corresponding values of Ce_miR-39_1 spiked in control using the $2^{-\Delta C_t}$ method. This controlled for extraction and reverse transcription efficiency. The normalised data were then $\log_2$ transformed before statistical analysis was performed.

The quality of the qPCR product was assessed by inspecting the data curves produced by the reaction to:

- 1) Confirm similar levels of the exogenous spiked-in control were present in all samples (ensuring extraction and reverse transcription consistency and efficiency).
- 2) Confirm water well controls had not produced an amplification curve and were free from contamination.
- 3) Ensure sample duplicates had similar melt curves (i.e. the dissociation of fluorescence should be the same for duplicate samples) and not subject to pipetting errors and therefore results were reproducible.
- 4) Identify any obvious outliers where sample failure had occurred.

11.3.4.4 MiRNA *In-situ* hybridisation (ISH):

The expression of selected miRNAs in desmoid tissue was assessed using *in-situ* hybridisation (ISH) technology. This was to determine if the miRNAs present in the serum and validated by the single qPCR assays were also expressed in the tumour tissue, providing support to the hypothesis that they had originated from the desmoid tumour itself. ISH probes were selected based on the serum PCR results. Ideally a desmoid control tissue would be used to compare expression of selected miRNAs between the control and the desmoid tumour. It was difficult to select a suitable control tissue for desmoid tumour. Mesenteric tissue, from which most intra-abdominal desmoid tumours originate, does not resemble desmoid tumour and is mostly composed of fat rather than dense collagen. A breast tumour control tissue was selected and a single FFPE tumour block used for the miRNA analysis as miRNA expression in breast tumours has been explored in previous studies.

Sections were cut at 4µm thickness from FFPE tissues under RNase free conditions. To ensure adhesion, sections were heated on slides at 65°C for 10 min before deparaffinising in xylene for 10 min and hydrating in RNase-free H₂O. Sections were digested with 10µgml⁻¹ proteinase K in PBS at 37°C for 30 min. This time interval was determined following optimisation experiments that tested digestion at 30, 45 and 60 minute intervals. Sections were washed in RNase-free H₂O and dehydrated through increasing concentrations of ethanol (70%, 95% and absolute), 1 min each and then air-dried. Sections were incubated with Double-DIG labelled LNA ISH probes complementary to the selected miRNAs at 56°C for 2 hours. Optimisation experiments were carried out using a range of dilutions for each probe selected, starting at a 1:625, to determine the optimal level for miRNA detection in the tissue. A scrambled miR probe was used as a negative control. Slides subsequently underwent 5 minute stringency washes at 56°C: once in 5X SSC buffer, twice in 1X SSC, twice in 0.2X SSC and finally once in 0.2X SSC at room temperature. Slides were placed in blocking solution for 10 minutes.

Anti-digoxigenin antibody at a 1:300 dilution (using 1% triton, BSA in TBS) was applied to the sections for 45 minutes at room temperature, then washed with TBS and rinsed in RNase-free H₂O. NBT/BCIP reagent was applied overnight at 4°C to enable signal detection. Slides were washed briefly in tap water before counterstaining with nuclear fast red and dehydrating in increasing concentrations of ethanol prior to mounting in Eukitt.

11.3.4.5 Immunohistochemistry pathway analysis

Finally, potential pathway proteins relating to miRNAs of interest, identified in desmoid tumour using ISH, were assessed using immunohistochemistry. FFPE 4µm tumour sections were dewaxed in xylene, placed in absolute alcohol before endogenous peroxide block

application for 10 minutes and sections were rehydrated through graded alcohol concentrations. Antigen retrieval was performed by placing sections in a citrate buffer (pH 8) and microwaving for 20 minutes. Non-reactive staining was blocked using rabbit serum (1:25 dilution) and antibody was then applied for 45 minutes. Sections were then washed before secondary antibody application for a further 45 minutes. A secondary control slide was used for each experiment to exclude non-specific secondary antibody binding. After washing, antibody binding was detected using a DAB reaction.

11.3.4.6 Tissue imaging and scoring

Both *in-situ* hybridisation and immunohistochemistry slides were analysed using a light microscope and scored according to stain intensity and proportion of positively staining cells. This was independently verified by a pathologist. Neoplastic cell cytoplasmic stain intensity was graded as 1: weak; 2: moderate; 3: strong. A total percentage of cells staining was determined and a weighted score was calculated with greater weight given to higher stain intensity. The weighted score was: $(1 \times \text{percentage staining at intensity 1}) + (2 \times \text{percentage staining at intensity 2}) + (3 \times \text{percentage staining at intensity 3})$. Based on this weighted score, cases were defined as having high (with a score of 120 or more), low or no staining.

11.3.4.7 Whole exome sequencing of desmoid tumour samples

Germline and tumour DNA paired samples were obtained from three patients with desmoid. DNA was isolated from fresh frozen tumour tissue and peripheral blood leucocytes using the methods previously described in chapter 9. Target regions were captured using the Illumina Nextera rapid exome library prep kit (Illumina, UK). These regions were sequenced using Illumina HiSeq 2000 and reads aligned to the human genome reference using Burrows-Wheeler

Aligner. Tumour specific point mutations were detected using Varscan2 by comparing the paired tumour and germline samples. These were subsequently annotated using ANNOVAR. Mean sequencing coverage was determined by calculating the total number of reads in called positions for all paired samples. Tumour specific mutations were excluded if coverage was less than 10 reads in either sample or if the somatic p-value was less than 0.05. The TargetScan predictive algorithm was used to analyse whether 3'UTRs of miRNA target genes arose within the miRNA binding sites.

11.3.4.8 Statistical analysis

Statistical analysis was conducted using GraphPad Prism 6. Continuous data were presented as a median (range) with Fisher's exact test used to compare proportions and Mann-Whitney U-test for continuous variables. A p-value or less than 0.05 was considered significant. MiRNA array statistical analysis was performed using the $\log_2$ transformed data and a two-tailed t test used to determine differences between the two means. For subsequent qPCR validation of differences between groups a one-tailed t test was applied.

11.4 Results

11.4.1 Patient characteristics

Seventeen patients with FAP and desmoid tumours were compared to 14 patients with FAP who had no clinical or radiological evidence of desmoid. Demographic data were compared between these two groups (Table 11-1). The overall median age of participants was 43 years (range 20-72). There was no significant difference in gender or age distribution between the two groups. Unsurprisingly, the proportion of patients with an *APC* mutation 3' of codon 1399

was significantly higher in the desmoid group compared to controls ($p=0.045$). Mutations in this region are a recognised risk factor for desmoid development (Sinha et al., 2011).

Table 11-1 Demographic data comparing patients with FAP with and without desmoid tumours.

	Patients without desmoid n=14 (45%)	Desmoid cases n=17 (55%)	p-value
Gender			
Male	7 (50%)	12	0.288*
Female	7 (50%)	5	
Age at study (median years)	44 (22-72)	35 (20-64)	0.199**
APC mutation			
3' to 1399	1 (7)	7 (41)	0.045*
5' to 1399	13 (93)	10 (59)	

*Fisher's exact test, **Mann Whitney-U test

11.4.2 MiRNA array profiling identifies differences in miRNA expression between FAP patients with and without a desmoid tumour

The expression of 384 miRNAs assessed by array profiling by Qiagen, found 207 miRNAs present at detectable levels (i.e. $Ct < 30$) in the serum of all 24 patients. Comparison of the two cohorts identified a number of miRNAs significantly differentially expressed between those patients who had desmoid and those who did not ($P \leq 0.05$) (Table 11-2 and Table 11-3). Only one miRNA was found to be independently associated with desmoid tumour occurrence in both cohorts, miR-34a-5p. Serum levels of miR-34a-5p were increased in the serum of patients with desmoid tumour compared to controls. In the combined analysis, this association was even stronger and identified a total of nine miRNAs that had significantly increased expression in the patients with desmoid relative to those without a tumour. Interestingly, miR-34a-5p and

miR-29c-5p had the lowest unadjusted p-values and were significantly elevated in the serum of patients with desmoid (Table 11-4, Figure 11-1). However, owing to the rarity of desmoids, the small cohort size and the large number of miRNAs tested, statistical significance was lost once a stringent Bonferroni correction was applied to account for multiple testing.

In a separate sub-analysis of the array data, serum miRNA expression within the desmoid population (n=12) was compared between patients with aggressively growing (n=3) and quiescent tumours (n=9). Three miRNAs with an unadjusted p-value ≤ 0.05 were identified and miR-3646 was the miRNA most significantly differentially expressed between these two groups, reduced in the serum of patients with aggressive desmoid tumours (Table 11-5).

Table 11-2 miRNAs that differentiate patients with FAP with a desmoid from those without in cohort 1

Mature ID	p-value	Fold change (Log2)
hsa-miR-1287-5p	0.003	-0.464
hsa-miR-34a-5p	0.007	2.288
hsa-miR-532-5p	0.008	1.790
hsa-miR-29c-5p	0.008	1.922
hsa-miR-142-5p	0.010	2.655
hsa-miR-382-5p	0.014	1.324
hsa-miR-143-3p	0.015	2.179
hsa-miR-145-5p	0.022	1.870
hsa-miR-2467-3p	0.030	-0.954
hsa-miR-3120-3p	0.031	1.820
hsa-miR-101-3p	0.033	2.106
hsa-miR-19b-3p	0.035	1.885
hsa-miR-637	0.041	-1.137
hsa-miR-133b	0.042	0.939
hsa-miR-19a-3p	0.044	1.768
hsa-miR-4687-5p	0.045	-1.014
hsa-miR-4688	0.045	-1.198
hsa-miR-4770	0.046	1.465
hsa-miR-503-5p	0.047	1.516
hsa-miR-324-5p	0.047	2.352
hsa-miR-339-3p	0.048	2.139

Table 11-3 miRNAs that differentiate patients with FAP with a desmoid from those without in cohort 2

Mature ID	p-value	Fold change (Log2)
hsa-miR-34a-5p	0.011	1.909
hsa-miR-328-3p	0.014	2.011
hsa-miR-138-1-3p	0.020	1.090
hsa-miR-631	0.021	1.281
hsa-miR-4302	0.033	-0.856
hsa-miR-1231	0.034	1.626
hsa-miR-1207-5p	0.042	1.821
hsa-miR-629-3p	0.046	0.872
hsa-miR-1180-3p	0.048	-0.711

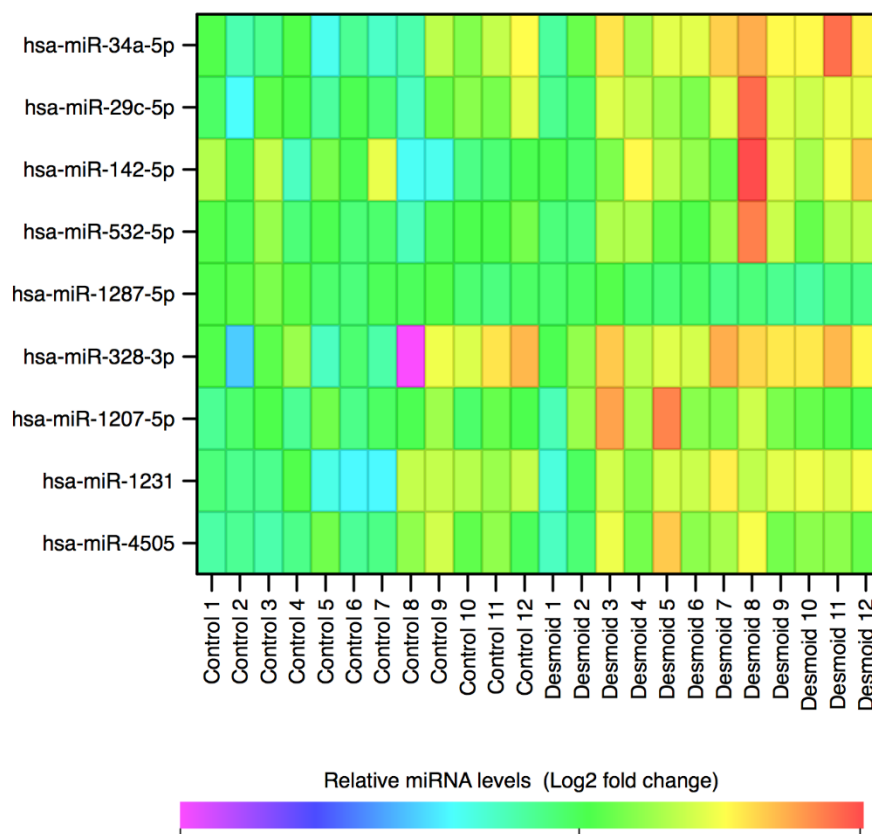


Figure 11-1 Heatmap comparing serum miRNA levels between desmoid tumour patients and controls. Data presented as a log2 fold change relative to the mean of controls. Y-axis lists differentially expressed miRNAs between the two groups ranked in ascending order according to p-value. X-axis lists sample identifiers

Table 11-4 miRNAs that differentiate patients with FAP with a desmoid from those without in a combined analysis of cohorts 1 and 2

Mature ID	p-value	Fold change (Log2)
hsa-miR-34a-5p	0.001	2.099
hsa-miR-29c-5p	0.003	1.531
hsa-miR-142-5p	0.008	1.584
hsa-miR-532-5p	0.011	1.045
hsa-miR-1287-5p	0.013	-0.369
hsa-miR-328-3p	0.013	2.210
hsa-miR-1207-5p	0.016	1.128
hsa-miR-1231	0.018	1.400
hsa-miR-4505	0.033	0.969

Table 11-5 miRNAs that differentiate aggressive from stable desmoid tumours

Mature ID	p-value	Fold change (Log2)
hsa-miR-3646	0.015	-0.998
hsa-miR-3651	0.034	-1.364
hsa-miR-138-1-3p	0.047	-1.127

11.4.3 Validation of differentially expressed microRNAs

11.4.3.1 Single qPCR assays

To validate the array results, miR-34a-5p and miR-29c-5p were quantified by single qPCR assays as markers of desmoid in the FAP population. In addition, levels of miR-3646 were also analysed as a marker of aggressive desmoid tumour. A cohort comprised of thirty-one patients (17 patients with FAP and desmoid (12 quiescent and 5 aggressive tumours) and 14 without desmoid)) was used. This cohort included the 24 samples already analysed in the array and seven additional cases (five from patients with desmoid and two controls).

For miR-29c-5p expression was only significantly elevated in the patients with an aggressive desmoid compared to controls without ($p=0.017$) (Figure 11-3B). However, the single qPCR assay failed to validate the findings from the array data and the level of serum miR-3646 was not significantly different between patients with an aggressive desmoid compared to those with a stable tumour (Figure 11-3C).



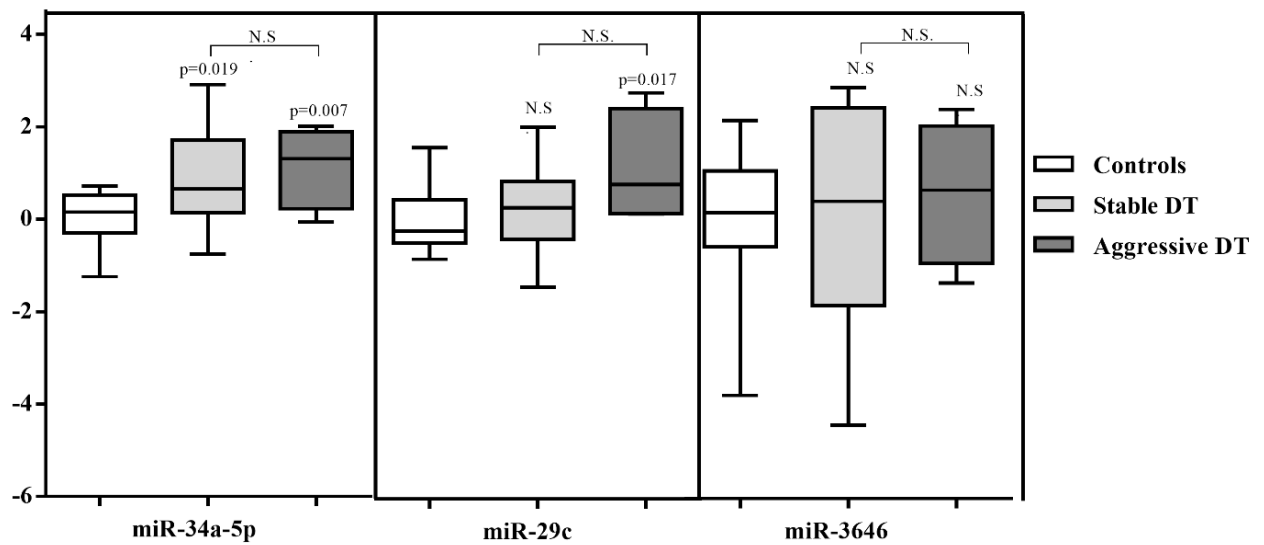


Figure 11-3 Box and whisker plot comparing miRNA expression in desmoid tumour (DT) and FAP controls. The p values are stated on the graphs. No significant differences between quiescent and aggressive desmoids were observed

11.4.3.2 miRNA *in-situ* hybridisation (ISH)

Serum analysis identified miR-34a-5p as the most significant marker for the presence of desmoid tumour. ISH miRNA technology was employed to assess the expression of this miRNA in desmoid tumour tissue. Initially seven FFPE desmoid tumours and a breast cancer control were sectioned. The tissue expression of miR-34a-5p in desmoid tissue was compared to miR-21a-5p, selected as a positive control probe for staining as it is expressed in fibroblasts and involved in fibrosis (Patel and Nouredine, 2012, Kumarswamy et al., 2011). In the first optimisation experiments miR-210 was also used as a positive control probe as it is widely expressed in many tissues, however for the remaining studies miR-21a-5p was employed because it was more specific for fibroblast activity and deemed more appropriate as a control.

An interestingly finding during the early optimisation studies was that miR-210 was intensely expressed by the desmoid tumours (Figure 11-4). This miRNA is a well-established marker for hypoxia, in normal tissues and tumours (Huang et al., 2010). This finding could indicate that

desmoid tumours are relatively hypoxic. Perhaps tissue culturing of desmoids would be improved by a more hypoxic environment. However, it could also suggest that in aggressively growing tumours (as these surgically excised tumours tended to be) hypoxia results from accelerated growth and an inadequate blood supply. Clinically, in a few cases necrosis can be associated with the development of aggressive intra-abdominal desmoid tumours, presenting with intra-abdominal sepsis and an air fluid level on radiological imaging (Bhandari et al., 2012).

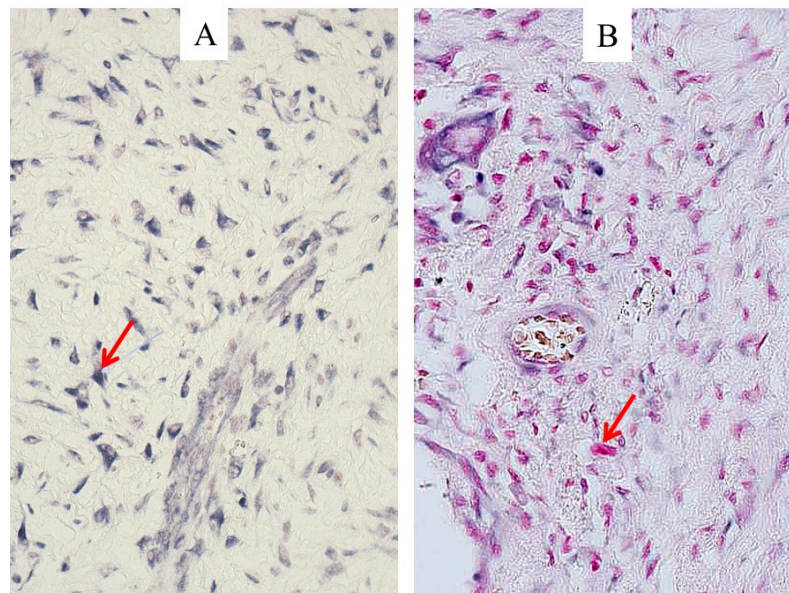


Figure 11-4 miR-210 expression in tissue: arrow showing heavy positive staining (dark purple cells) in the desmoid tumour (A) compared to colon cancer tissue with arrow showing widespread negative pink staining cells (B), x20 magnification

One of the seven FFPE tumour blocks (a more quiescent tumour) was negative for all three miRNAs tested and therefore excluded from final analysis. This was most likely the result of miRNA degradation. In the final analysis cytoplasmic staining for miR-34a-5p and miR-21a-5p was detected in the remaining desmoid tumours to varying extents (Figure 11-5 and Table 11-6) but not in the breast cancer control (Figure 11-6). MiR-34a-5p was highly expressed in nearly all tumours (five out of six). Positive staining for miR-21a-5p was observed in 3 out of

six desmoids. Tissue sections treated with a negative control probe (miRNA-scrambled) were all negative. In this small cohort the intensity of staining for miR-34a-5p and miR-21a-5p was not apparently related to the aggressiveness of the tumour ($p=0.8$ and $p>0.99$, respectively) (Figure 11-7).

Table 11-6 ISH miR staining scores for aggressive and stable desmoid tumours. The weighted score is: (1 x percentage staining at intensity 1) + (2 x percentage staining at intensity 2) + (3 x percentage staining at intensity 3). A weighted score of 120 or more was defined as high level staining

Desmoid phenotype/case	Weighted staining score			
	miR-21-5p		miR-34a-5p	
Aggressive	A	Level	A	Level
1	130	High	200	high
2	120	High	165	high
3	0	None	110	low
4	0	None	0	none
Stable				
5	35	low	170	high
6	0	None	140	high

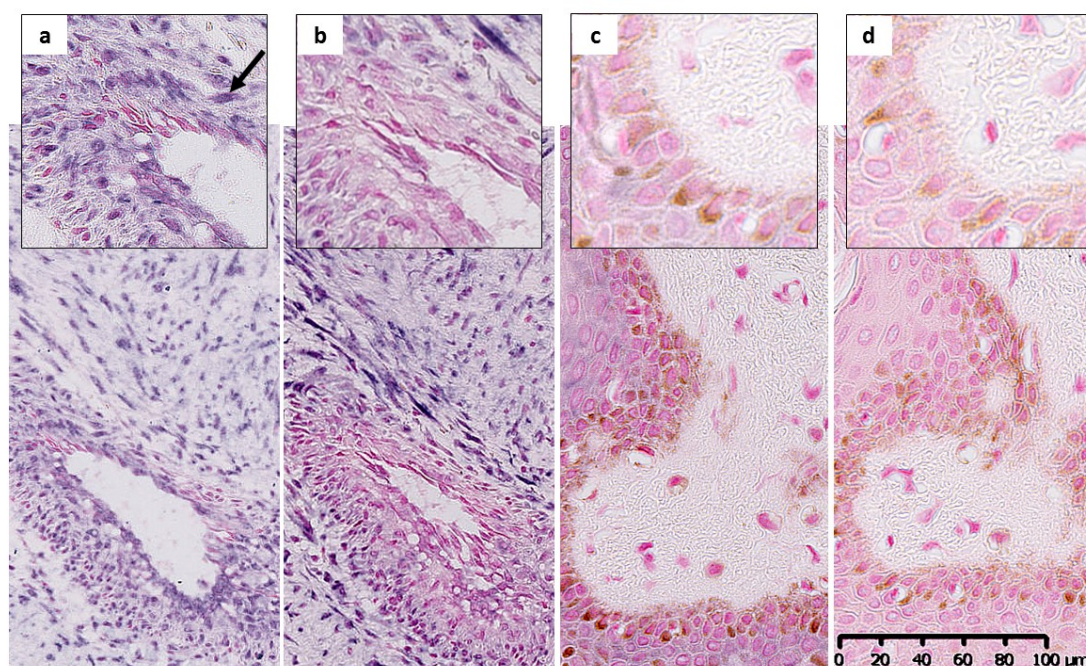


Figure 11-5 ISH for miR-34a-5p (a) and miR-21a-5p (b) in desmoid tumours and miR-34a-5p (c) and miR-21a-5p (d) in normal skin. Purple cytoplasmic staining (black arrow) represents positive staining for the relevant miRNA and pink negative. Magnification x20 and x40

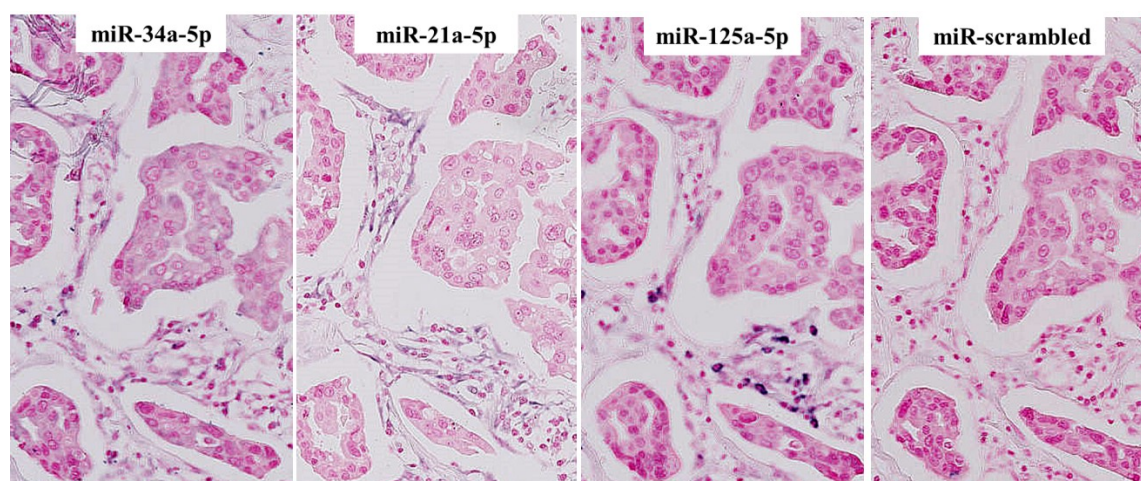


Figure 11-6 Breast cancer control tissue demonstrating largely negative staining for all miRNAs. x10 magnification

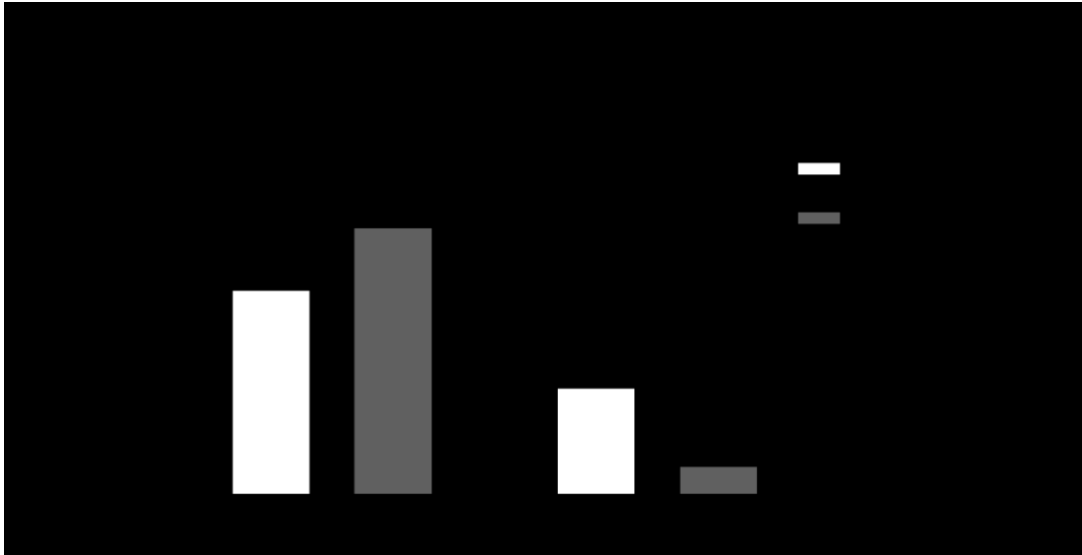


Figure 11-7 Bar chart demonstrating no significant difference in the mean weighted staining scores between aggressive and stable desmoid tumours (DT) for each miRNA. *Mann Whitney-U test

Immunohistochemistry assessed p53 expression in the six desmoid tumours analysed in the ISH study, as it is reported to induce miR-34a-5p expression. This found minimal or no nuclear p53 expression in 5/6 tumours and high expression in one aggressive tumour (Table 11-7 and Figure 11-8). In these sections, there was no direct correlation between p53 and miR-34a-5p staining intensities; Spearman's rank correlation coefficient of 0.3 ($p=0.567$) (Figure 11-9).

Table 11-7 p53 immunohistochemistry staining scores. Scoring method as described in Table 11-6

Phenotype/case	Weighted staining score	
	A	Level
Aggressive		
1	25	low
2	140	high
3	0	none
4	20	low
Stable		
5	0	none
6	20	low

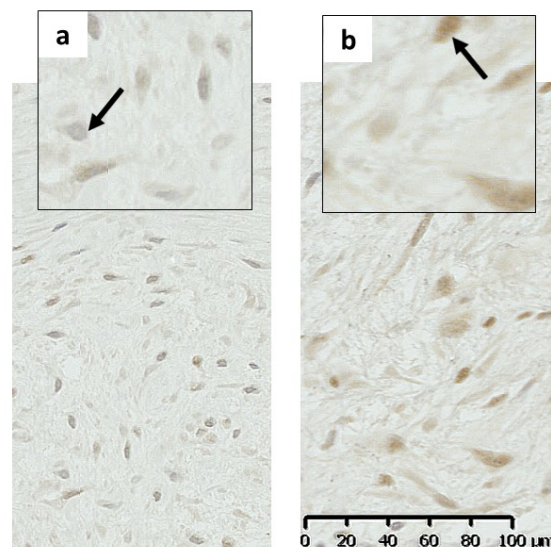


Figure 11-8 Immunohistochemistry p53 expression in 2 desmoid tumours: low weighted score case 1 (A) compared to high weighted score in case 2 (B). x20 magnification. Arrow demonstrating positive nuclei staining brown

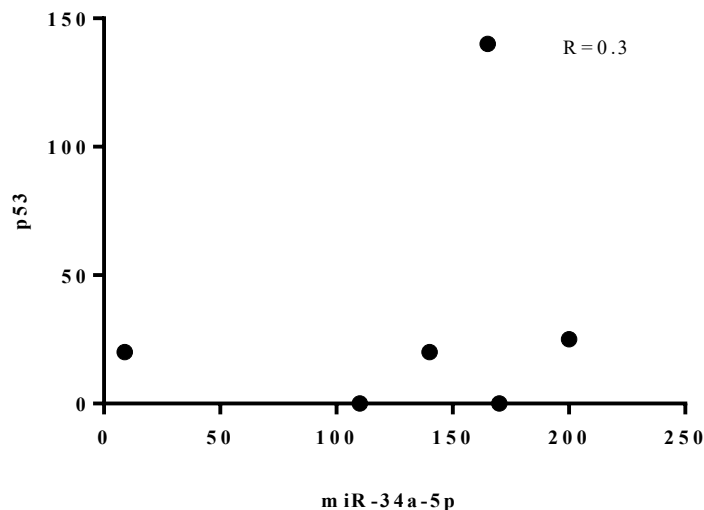


Figure 11-9 Scatter plot demonstrating no correlation between miR-34a-5p and p53 expression in desmoid tumours

11.4.3.3 Whole exome sequencing of tumour-germline pairs

Whole exome sequencing was performed in three patients' desmoid tumour-germline paired samples, at an average coverage of ~30x. The sequencing data was interrogated for somatic or loss of heterozygosity mutations within the miRNA transcript sites and binding sites of the miRNA target genes. No mutations were detected within the miR-34a-5p transcript sites that would account for altered miRNA activity in desmoid tumours. In addition, no mutations were found in the 3'UTR of target genes of miR-34a-5p that could interfere with miRNA binding sites. Therefore, there was no evidence of desmoid tumour alterations that would indicate the tumour was escaping the action of the miRNA. This supported findings from other studies that suggest somatically acquired mutations in miRNA genes or their target 3'UTR occur infrequently in tumour samples (Ryland et al., 2012).

11.5 Discussion

This study explored differentially expressed miRNAs in the sera of patients with FAP with and without a desmoid tumour. This was a proof-of-principle study in a rare cohort of carefully phenotyped patients. Array analysis of serum from two independent cohorts was performed, and a technical qPCR validation analysis conducted, with all samples run as a single block. Increased levels of miRNA-34a-5p were found to be a marker of desmoid tumour in both FAP cohorts and this was confirmed by subsequent qPCR. The combined analysis of both cohorts also identified elevated levels of miR-29c-5p in those patients with a desmoid. The level of this miRNA was also increased in patients with an aggressive desmoid. However, the current clinical utility of miR-34a-5p and miR-29c-5p as desmoid tumour biomarkers in the FAP population is likely limited due to the observed overlap in levels of these miRNAs between desmoid cases and controls.

Potential serum miRNA markers for the identification of aggressive desmoid tumours were also explored. The array data analysis identified three miRNAs (miR-3646 the most significant) altered in the aggressive desmoid group compared to then stable tumour group. However, statistical significance was lost when adjusting for multiple testing and miR-3646 could not be validated by qPCR. This is likely to reflect the small sample size. For the future development of these miRNAs as non-invasive biomarkers for desmoid tumours, validation of these findings in larger independent cohorts of patients is required to test the sensitivity and specificity of these miRNAs for desmoid tumours. This will require an international collaboration from specialist centres that manage patients with FAP to combine expertise and resources to maximise sample numbers. Sample size in such studies will always be limited by the low prevalence of FAP associated desmoids. However, given the severity and refractory nature of the disease such investigations are of importance. Potential applications of non-invasive

biomarkers for desmoid tumours could include: earlier detection of more aggressive tumour phenotypes enabling swift institution of medical therapy that may alter outcomes, closer follow-up of aggressive tumours and reduced frequency of radiological imaging to assess stable tumours.

This study also attempted to study miRNA expression within the desmoid tumour. There are a number of difficulties in quantitatively assessing miRNA levels in desmoids, not least the lack of an appropriate control tissue and cell lines. In this study miRNA ISH techniques were employed to investigate miRNA levels in desmoid tumour tissue. This analysis demonstrated that miR-34a-5p was highly expressed relative to miR-21a-5p, a miRNA involved in fibrosis. However, the stain intensity of miR-34a-5p was not correlated with the aggressiveness of the tumour. Although some studies have demonstrated an upregulation of miR-34 in breast and gastric cancers compared to healthy controls (Roth et al., 2010, Liu et al., 2011), this contrasts with others reporting a reduction in miR-34 expression associated with malignant tumour development (Hermeking, 2010, Hiyoshi et al., 2015). However, as desmoids are not malignant but benign myofibroblast tumours, it is possible that miR-34 has a role in driving fibrogenesis in affected patients. Up-regulation of miR-34 has been also associated with a number of fibrotic conditions including liver cirrhosis (Li et al., 2011), myocardial infarction and fibrosis (Boon et al., 2013, Huang et al., 2014) and neuro-degenerative disorders (Rokavec et al., 2014). Increased levels of miR-34a-5p were also associated with intestinal fibrosis in Crohn's disease using the same array platform (Lewis et al., 2015).

The expression of miR-34 is directly induced by p53 and p53/miR-34-mediated processes can influence cell cycle arrest, metastasis, apoptosis, stemness and epithelial to mesenchymal transition (EMT) (Zhang et al., 2014). Numerous miRNA-34 pathway targets have been

validated and linked to tumour suppression (Rokavec et al., 2014). In particular, the p53/miR-34 axis is known to suppress the canonical Wnt signalling pathway (Figure 11-10), which is integral to the development of desmoids. Conversely, the Wnt signalling pathway can induce miR-34 expression suggesting the existence of a feedback loop (Tamura et al., 2013).

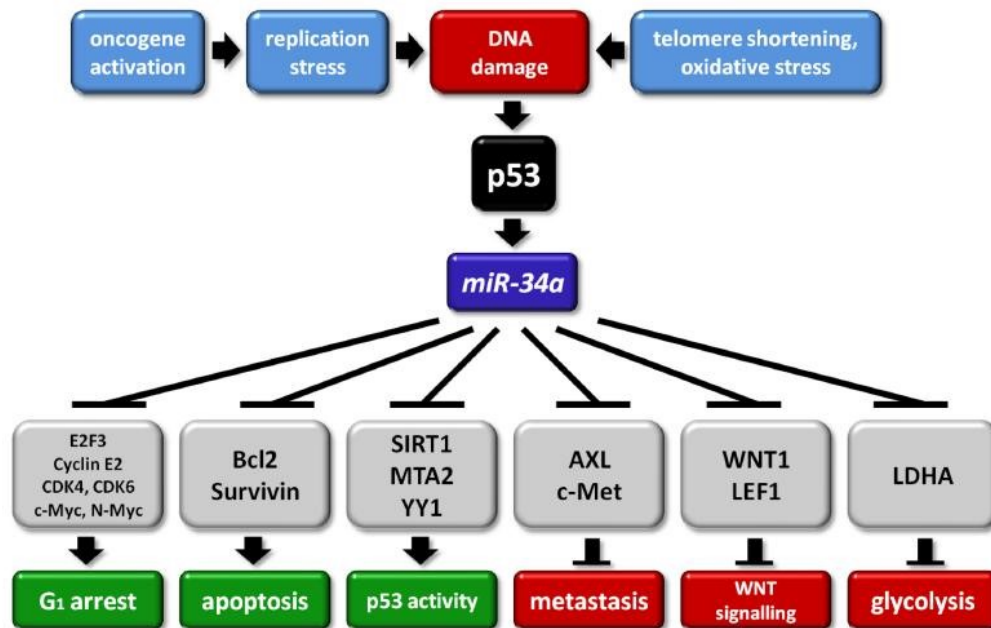


Figure 11-10 p53/miR-34a axis and target pathways. Image from www.pathologie.med.uni-muenchen.de/020wissenschaft/009ag_hermeking

Similar to miR-34, p53 has also been associated with the development of fibrosis (Gogiraju et al., 2015) and therefore predicted to be altered in desmoid tumour tissue based on the sera miRNA findings. However, when p53 expression was analysed in the tumour tissue in this small cohort, p53 staining was negligible or undetectable in most cases and intensity not correlated with miR-34a-5p staining. In addition to this, exome sequencing did not identify a mutation in the transcription sequence of miR-34a-5p or in the 3'UTRs of target mRNAs that may interrupt miR-34a-5p activity in desmoid tumours. These findings could lend support to a

functional role for miR-34a-5p in desmoid tumour growth, and that its increase in the serum may originate from the tumour itself.

Further investigation of miR-34a-5p and its role in fibrogenesis is warranted given the findings from this study. The development of a miR-34 analogue (MRX34), now in phase 1 clinical trials for the treatment of cancer (Agostini and Knight, 2014, Kaboli et al., 2015), and the existence of a miR-34 inhibitor (Bernardo et al., 2012), make such studies an exciting prospect and could offer potential new treatment target pathways for desmoid tumours. The potential for off-target side effects of miRNA analogues and inhibitors also need careful consideration given that each miRNA is predicted to influence multiple gene targets. Future work will require the development of animal models and cell culture systems for functional and mechanistic studies of miRNAs implicated in desmoid formation, although currently none exist.

11.5.1 Study limitations

Limitations of this study include its small size and difficulty in obtaining and defining aggressive desmoids for tissue analysis. However, this is a rare cohort and the serum samples have been carefully selected and well phenotyped with an appropriate control group. Despite these limitations significant difference in miRNA expression within the sera were detected and could be validated by single qPCR assay. So far only one other study has reported miRNA profiling in a desmoid patient cohort, although this was a larger cohort of 41 patients, it incorporated both sporadic (n=37) and FAP associated (n=4) cases (Dufresne et al., 2015). To the author's knowledge the current study is the largest cohort of FAP-associated desmoid tumour cases that have undergone serum miRNA profiling.

11.5.2 Conclusion

This study found upregulation of miR-34a-5p in the serum of patients with FAP and desmoid tumours relative to those without desmoids, supporting its role as a non-invasive biomarker for desmoid tumours. High levels of miR-34a-5p were also detected in desmoid tumour tissue. This is a novel study as these pathways have not been explored before in desmoid tumour development, offering new treatment targets for future research into these rare and complex tumours.

PART FOUR – Conclusions and future work**12 Chapter 12 – Conclusions**

Desmoid tumours are the most significant extra-intestinal manifestation of FAP. For some patients they are responsible for considerable morbidity and mortality. Their rarity and unpredictable growth behaviour make them a challenge to study both in the laboratory and in the clinical setting. Many recur after treatment, yet others can remain quiescent for years without requiring intervention. This impairs our ability to reliably assess tumour response to medical and surgical treatments and limits the development of new therapies. These unusual tumours continue to elude researchers. The studies described in this thesis have mainly focused on IAD and AWD occurring in patients with FAP rather than those occurring sporadically, although these two biologically distinct entities are often combined in the literature to increase numbers.

12.1 Clinical studies

The studies presented in part two have largely focused on the complications that can arise from desmoid tumours, their management and the impact they have on the quality of life in those patients who are affected. Chapter three supported what is already known about these tumours: most occur intra-abdominally, a third require no treatment at all, less than 10% are life-threatening and around a half receive medical management that may be effective in 50% of cases. However, it is difficult to determine how effective these treatments are and the extent to which the response seen merely reflects the nature of desmoid tumour behaviour.

Chapter four evaluated ureteric compression associated with an IAD and found it occurred in a quarter of cases, emphasising the importance of USS follow-up to assess the urinary tract after IAD diagnosis. An important finding was that once ureteric stenting was required it was likely to be a long-term necessity, a question frequently asked by patients in the clinic. **Error! Reference source not found.** shows an algorithm for IAD management, based on the results of these clinical studies and the management currently undertaken at specialist centres such as St Mark's Hospital.

Chapter five reported a possible association between gastric cancer development and desmoid tumour occurrence, suggesting the existence of a possible gastric cancer genotype-phenotype correlation. Unfortunately, this finding was not supported by a small cohort of patients with gastric adenomas and a larger cohort is necessary to assess this association further.

Chapter six assessed the survival outcomes from patients undergoing a SBT for IAD. Little has been reported in the literature in this specific group of patients. Survival from SBT has improved over recent years and it has become a viable treatment option for patients with complex, life-threatening, IAD associated with a poorer quality of life. Chapter seven sought to evaluate the health-related quality of life in patients living with a desmoid tumour. This is useful to assess patient response to treatment and to weigh up the risks and benefits of interventions such as SBT surgery. Given the significant complications that can arise from desmoid tumours, it was perhaps unsurprising that this study found a major impact on the quality of life of patients who had a desmoid tumour. They were responsible for limiting physical and social activities due to the pain associated with their occurrence. Although desmoid tumours remain an enigma to clinicians, they are also a source of concern particularly

for the patients. The general lack of knowledge that exists around these rare tumours is palpable to patients who struggle to obtain answers from clinicians about the effects desmoids may have on their life. These clinical studies addressed some of these questions and provide additional information to aid in the counselling of patients.

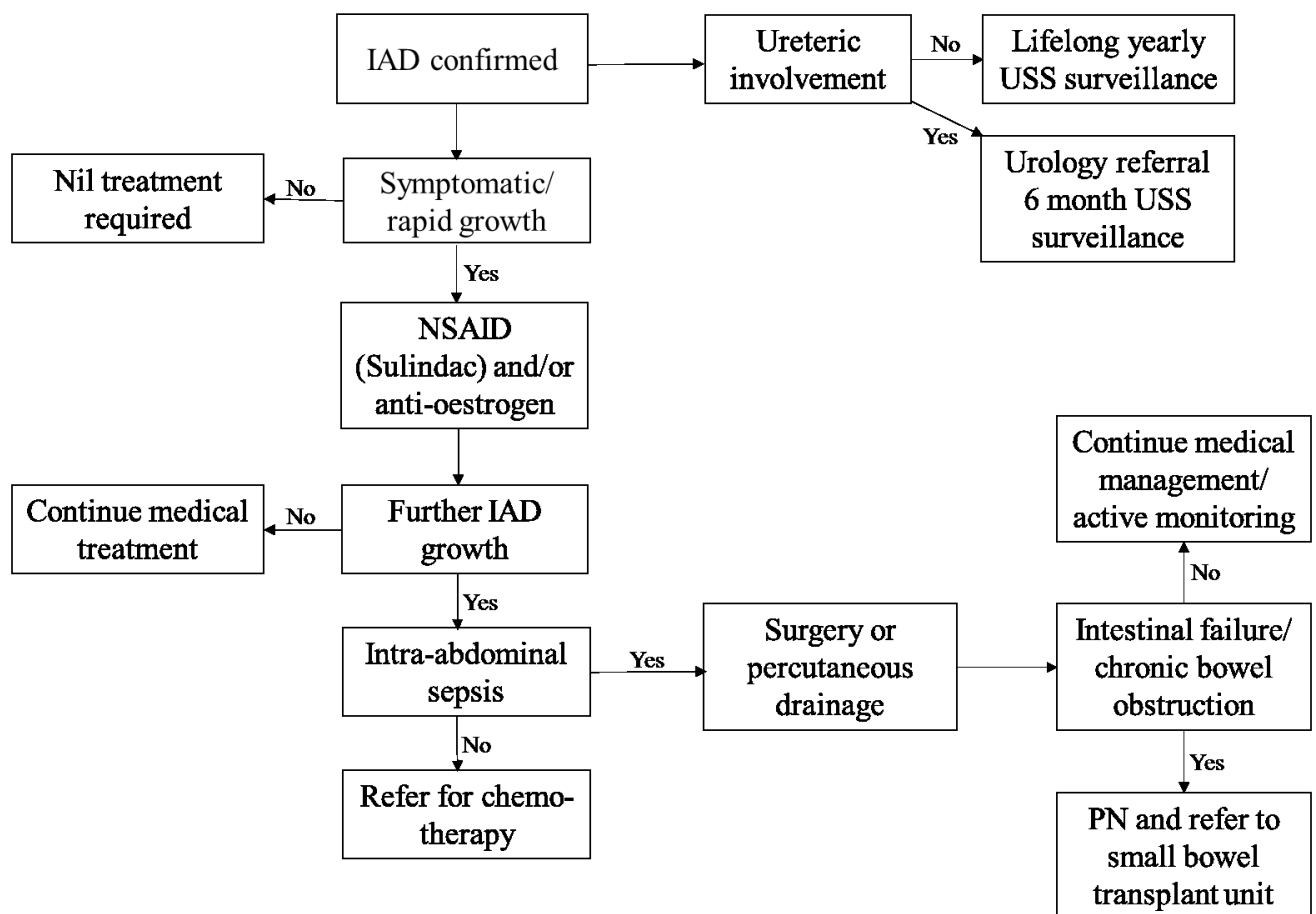


Figure 12-1 An algorithm for management of IAD

12.2 Laboratory studies

Part three of this thesis encompasses four laboratory studies undertaken to further our understanding of desmoid tumour growth. The first of these, chapter eight, found histological evidence for the activation of the non-canonical Wnt pathway in desmoid tumours using immunohistochemistry techniques. This is a novel finding that could offer potential pathway targets for future research.

The next step in understanding such pathways, would usually include in-vitro testing of relevant pathway activators or inhibitors. Chapter nine sought to develop a desmoid cell line to investigate the expression of Wnt-11 in vitro and test the effects of its inhibition on tumour growth. This study failed to successfully develop a desmoid cell line from a patient with FAP. It confirmed the findings of previous studies that desmoid tumours are extremely difficult to grow in cell culture. This may reflect their unpredictable growth and emphasised the need to develop a more reliable cell culture technique that would better mimic the in-vivo environment. This led to the development of a three dimensional desmoid cell culture. Although unique and in the early phase of design, this could improve future prospects of developing a successful cell line with which to test potential pathway targets.

Chapter ten used next generation sequencing to analyse germline and desmoid tumour DNA in patients with FAP in an attempt to identify a desmoid modifier gene. Although no single desmoid modifier gene was found, this study found several potential genes of interest that have not been previously associated with desmoid occurrence. Finally, chapter 11 used qPCR and in-situ hybridisation techniques to assess the feasibility of utilising circulating serum miRNAs as non-invasive biomarkers for desmoid tumour occurrence. An upregulation of miR-34a-5p

was detected in the serum of patients with desmoid compared to patients with FAP who had no evidence of desmoid tumours. This was a novel finding that could offer the potential for the development of a desmoid tumour biomarker. It could also provide further pathways, offering new treatment targets, to explore in future research.

13 Chapter 13 - Future work

13.1 Developing a quality of life assessment tool

A health-related quality of life assessment tool specific for patients with a desmoid tumour would be helpful in determining their response to medical and surgical treatments. This is particularly important when considering potentially life-changing interventions such as surgery for IAD that can lead to significant small bowel resection, with the possible requirement for long-term parenteral nutritional, and SBT. In these cases, it would be useful to establish whether such interventions led to an improved quality of life for patients. Future studies could develop a suitable tool using the findings from the group discussions generated in chapter seven. The next step would involve completion of questionnaires by expert clinicians to determine to important relevant themes that should be included in the production and validation of a specific tool to assess quality of life for patients with a desmoid.

13.2 Establishing a three dimensional in-vitro model

A reliable cell culture method with which to study desmoid tumours in vitro would be a major advance in the research of these tumours. Chapter nine set out the foundations for developing a three dimensional tissue scaffold. Until a reliable cell culture technique and true desmoid cell line have been established, robust studies to test new desmoid treatments in vitro will not be possible. Successful culture techniques would enable future research into new pathways of interest for desmoid growth. Development of a three dimensional cell culture technique may be key in achieving this goal for future studies, as these platforms better mimic the in-vivo environment than standard in-vitro techniques. However, it is likely that any culture method that improves desmoid cell growth will also favour fibroblast overgrowth. The next stage in developing this culture technique will require assessment of desmoid cell interaction with the

scaffold to demonstrate tumour growth. This could be achieved by analysing the levels of collagen, GAGs and DNA in the scaffold over various time points as the tumour grows. It is currently possible to perform such assays, comparing the levels of these components between scaffolds with and without cells explanted onto them. Once optimisation of the three dimensional cell culture was achieved and desmoid cell growth confirmed genetically, treatments could be added to the culture medium and cell growth analysed.

13.3 Desmoid modifier genes

Identifying the gene, or genes, responsible for desmoid development could be key to predicting, and potentially preventing, their occurrence. Chapter ten detected several genes of interest through whole exome sequencing of DNA from patients with FAP, comparing those with and without a desmoid tumour. The greatest hurdle in such studies is the subsequent analysis of the large volume of data generated. Determining genes of interest can be difficult, particularly for those where little is known about their function. The study has provided a precious archive of genetic data in a rare cohort that can be interrogated in future research. Although no obvious ‘desmoid gene’ has so far been detected, these data may well hold the key to this gene that future studies may be able to unlock once our knowledge and ability to interpret such data has improved.

13.4 Desmoid biomarkers

Chapter eleven found a potential biomarker (miRNA-34-5p) for desmoid tumours. However, the practicalities of using such a marker clinically need further consideration in future studies. A biomarker would only be useful if it could detect desmoids at the early stages of growth

before they became clinically or radiologically obvious. It would also be useful if it could distinguish aggressive from quiescent tumours, where an advantage in early treatment commencement may exist. Future studies looking at miRNA biomarkers would need to establish the level in the serum at which their detection was significant. This could be achieved through serial samples to build up an individual patient miRNA level profile. Sera sampling could occur at regular time periods prior to and for three years after colectomy (when the majority of desmoids develop) to establish the biomarker levels that would be deemed significant for tumour detection and growth. Although this would be a time consuming project it would be feasible at the study institution where patients are routinely followed up at regular intervals lifelong.

An exciting prospect from the miRNA study was the existence of miR-34 inhibitors and analogues. If a desmoid cell line was successfully established the levels of miR-34 in cell culture could be analysed to test the hypothesised association between tumour development and miR-34 levels. This miRNA could also be inhibited or stimulated to establish its effect on desmoid cell growth and potentially lead to the development of new treatments. This could stimulate future research into such miRNA pathways and improve our understanding of the mechanisms involved in desmoid tumour growth.

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Appendix A1 Collaboration and assistance

Chapter 3 - Survival outcomes in patients with FAP associated desmoid tumours – a review of the St Mark’s Hospital polyposis registry

This study was designed and carried out by myself.

Chapter 4 - Ureteric complications arising from desmoids

I performed the data collection and analysis for this study. Mr Havranek (consultant urologist, St Mark’s Hospital) reviewed the data analysis

Chapter 5 - Gastric cancer and desmoid tumours

I collected the gastric cancer data from the polyposis registry and Dr Andrew Latchford (consultant gastroenterologist, St Mark’s Hospital) collated the gastric adenoma cases that I subsequently analysed. Dr Ian Frayling (consultant in genetic pathology, Cardiff) provided the data from the InSiGHT database, courtesy of Stephan Aretz the curator, and assisted in the combined data analysis.

Chapter 6 - Small bowel transplant for desmoid tumours

Dr Simon Gabe (consultant gastroenterologist, St Mark’s Hospital) identified the patients who had undergone a small bowel transplant for desmoid tumour from the NASIT database. Although I collected most of the patient data myself, Dr Stephen Middleton (a consultant gastroenterologist, Addenbrooke’s Hospital) and Professor Peter Friend (a transplant surgeon, Oxford Transplant Centre) also assisted in this process. I interpreted the data myself.

Chapter 7 - Quality of life associated with desmoid tumours

I obtained ethical approval for this project after drawing up the protocol for the study. Miss Sonal Arora (a clinical lecturer in general surgery) and Professor Thanos Athanasiou (a

Professor of cardiovascular sciences & cardiac surgery, Imperial College) helped to design the focus group discussion for this study. I identified and recruited appropriate patients for the study. I subsequently transcribed and analysed the audio transcripts from the patient group discussions. The quality of life questionnaires were distributed by myself and Miss Ripple Man (a nurse endoscopist at St Mark's Hospital). I then undertook the data collection and analysis.

Chapter 8- Histological studies of desmoids

After initial instruction, I performed all sectioning and staining of basic histology and immunohistochemistry slides. Dr Tahera Ansari (a senior post-doctoral scientist at NPIMR) and Professor Sibbons (a pathologist and the director and head of surgical sciences, NPIMR) helped with the initial analysis of the histological slides. They also assisted in the subsequent optimisation of the immunohistochemistry protocols. Dr Robert Kypta (a lecturer and head of the Wnt Signalling Group, Imperial College) also provided invaluable help in the same process, with a focus on the immunohistochemistry for Wnt-11 and β -catenin. The overall analysis and data interpretation of results obtained from the histology slides was performed by myself.

Chapter 9 - Cell culture and 3D desmoid scaffold development

I processed all fresh desmoid tissue samples from the point of acquisition at surgery to the establishment of cell culture in the laboratory. Sporadic desmoid tumour tissue was obtained by myself from the Royal Marsden Hospital during surgery undertaken by Mr Andrew Hayes (consultant surgical oncologist). Mr Ashish Sinha and Mr Santosh Bhandari (both research fellows previously at St Mark's Hospital) instructed me on desmoid cell culture technique during the initial phase of the study. Dr Robert Kypta, Dr Tahera Ansari and Dr Karin Greco (post-doctoral scientist, NPIMR) all helped in the initial optimisation of my cell culture technique. Analysis of the cell culture DNA for a 'second-hit' mutation in the Kennedy-Galton

Centre (KGC) laboratory was undertaken in part by myself (for the sporadic desmoid tumours). However, the majority of this work was performed by the staff in this laboratory, including Dr Mat Edwards and Dr Zoe Allen (post-doctoral scientists), under the guidance of Mr Stewart Payne (head of molecular genetics, KGC). Dr Tahera Ansari and Professor Paul Sibbons also advised on the development of a three-dimensional desmoid culture model. They were involved in the study design, development of the scaffold decellularisation and sterilisation protocols and the histological analysis of the tissue. Apart from the majority of DNA analysis, all laboratory work required for this chapter was undertaken by myself.

Chapter 10 – Whole exome sequencing of desmoids

I designed the protocol and obtained the ethical approval for this study and that for chapter 11. After selecting appropriate patients, I sought their consent for participation in the study and obtained blood samples, and where appropriate tumour tissue too. DNA extraction was performed by the KGC team mentioned previously. The whole exome sequencing was performed in part in Arizona by Dr Laukaitis (Assistant Professor in clinical medicine) and her research fellow Dr Keri Maher. However, the majority of the analysis was undertaken at the Blizard Institute by Professor Andrew Silver (Professor of cancer genetics) and his team including: Dr Nirosha Suraweera, Dr Amy Lewis, Miss Hannah Thompson and Mrs Rosemary Jeffery. The bioinformatics analysis and data interpretation was carried out by Dr George Watts (Arizona group), Dr Eleni Giannoulatou and Dr Christopher Yau (for the exome sequencing performed at the Blizard Institute).

Chapter 11 – MicroRNA expression in desmoids

I obtained ethical approval and subsequent consent from all patients recruited to this study. Furthermore, I collected the blood samples and extracted miRNA from them. With the

guidance of Dr Amy Lewis, I undertook all of the laboratory work and data analysis required for this study. The exome sequencing was performed at the Blizard institute, as per chapter 10, with subsequent data analysis undertaken by Dr Eleni Giannoulatou.

Appendix A2 Regulatory approvals, patient information sheets and consents

Ethical approval for the studies described was obtained from Harrow Research and Ethics Committee, NRES Committee South Central - Berkshire B and NRES Committee London - Camberwell St Giles. Research and development approval was obtained from the North West London Hospitals NHS Trust. Listed below are the details of these approvals.

Study title	Rec number	R&D number	Date of approval
Quality of life in patients with familial adenomatous polyposis related desmoid disease	13/SC/0429 NRES committee South Central - Berkshire B	RD13/061	29 th July 2013
To explore the aetiology and carcinogenesis of desmoid disease in familial adenomatous polyposis; and to try and identify appropriate interventions	08/H0719/75 Harrow REC	RD8/080	25 th February 2009
Exploration of genetic mechanisms in desmoid tumours, to identify possible genetic candidates and variants involved in their formation.	13/LO/0893 NRES committee London - Camberwell St Giles	RD13/042	21 st June 2013

Patient information and consent forms

Below are a copy of the patient information and consent forms used for the relevant studies.

Northwick Park Hospital,
Watford Road,
Harrow, Middlesex.
HA1 3UJ

Patient Information Sheet
Quality of life questionnaire study in
familial adenomatous polyposis

Dear Sir / Madam, thank you for considering participation in our study. This information sheet will aim to answer any questions you may have. This study has been developed by researchers working at St Mark's Hospital and forms part of a PhD project

What is the purpose of this study?

We are studying how familial adenomatous polyposis affects the lives of those patients suffering from this benign condition. We aim to better understand how FAP and its treatments may impact on a person's quality of life.

Why have I been invited to take part?

You have familial adenomatous polyposis and we would like to know how this affects your everyday life.

What will be involved should I participate in the study?

We would like you to complete 3 questionnaires all relating to your quality of life. This will take about 20 minutes.

Can I say no?

Yes, you can refuse to participate in this study. This will have no impact on your clinical care. You may withdraw from the study at any point without giving notice or reason. Should you lose capacity to make decisions during the study we would retain any information already gathered from you before that point.

Will this study benefit me?

We cannot say that participating in the study will directly benefit you but it could improve our understanding of the quality of life associated with FAP.

What are the disadvantages/risks of taking part in the study?

There are no disadvantages or significant risks associated with taking part in the study.

What if new or relevant information becomes available?

If any new information becomes available as a result of the study that may be important to you we would inform you about this.

Will my taking part in this study be kept confidential?

All information held about you will be kept strictly confidential. All questionnaires will be stored in an anonymous fashion in The Polyposis Registry.

Are there any implications for insurance?

We will not disclose any clinical information about you to a third party such as insurance companies. All information about you will be kept strictly confidential.

What will happen to the results of the research study?

It is likely that the results of the study will be published in a medical journal/presented at medical conferences. You will not be identified in any report or publication.

Who has reviewed the study?

This study has been reviewed and approved by the Berkshire B Research Ethics Committee.

Who can I contact for further information/advice before or during the study?

Should you have any further queries or want to discuss any issues related to this study, please contact one of the members of The Polyposis Registry:

Mrs Kay Neale	0208 235 4270	kneale3@btconnect.com
Manager of The Polyposis Registry		

Miss Sarah-Jane Walton	0208 235 4270	sarah-janewalton@nhs.net
Research fellow		

The Polyposis Registry, St Mark's Hospital, Harrow HA1 3UJ

Northwick Park Hospital,
Watford Road,
Harrow, Middlesex.
HA1 3UJ

Patient Information Sheet
Quality of life questionnaire study
in desmoid disease

Dear Sir / Madam, thank you for considering participation in our study. This information sheet will aim to answer any questions you may have. This study has been developed by researchers working at St Mark's Hospital and forms part of a PhD project.

What is the purpose of this study?

We are studying how desmoid disease affects the lives of those patients suffering from this benign condition. We aim to better understand how familial adenomatous polyposis (FAP) associated desmoid disease and its treatments may impact on a person's quality of life.

Why have I been invited to take part?

You have familial adenomatous polyposis and desmoid disease and we would like to know how this affects your everyday life.

What will be involved should I participate in the study?

We would like you to complete 3 questionnaires all relating to your quality of life. This will take about 20 minutes.

Can I say no?

Yes, you can refuse to participate in this study. This will have no impact on your clinical care. You may withdraw from the study at any point without giving notice or reason. Should you lose capacity to make decisions during the study we would retain any information already gathered from you before that point.

Will this study benefit me?

We cannot say that participating in the study will directly benefit you but it could improve our understanding of the quality of life associated with FAP related desmoid disease.

What are the disadvantages/risks of taking part in the study?

There are no disadvantages or significant risks associated with taking part in the study.

What if new or relevant information becomes available?

If any new information becomes available as a result of the study that may be important to you we would inform you about this.

Will my taking part in this study be kept confidential?

All information held about you will be kept strictly confidential. All questionnaires will be stored in an anonymous fashion in The Polyposis Registry.

Are there any implications for insurance?

We will not disclose any clinical information about you to a third party such as insurance companies. All information about you will be kept strictly confidential.

What will happen to the results of the research study?

It is likely that the results of the study will be published in a medical journal/presented at medical conferences. You will not be identified in any report or publication.

Who has reviewed the study?

This study has been reviewed and approved by the Berkshire B Research Ethics Committee.

Who can I contact for further information/advice before or during the study?

Should you have any further queries or want to discuss any issues related to this study, please contact one of the members of The Polyposis Registry:

Mrs Kay Neale	0208 235 4270	kneale3@btconnect.com
Manager of The Polyposis Registry		

Miss Sarah-Jane Walton	0208 235 4270	sarah-janewalton@nhs.net
Research fellow		

The Polyposis Registry, St Mark's Hospital, Harrow HA1 3UJ

CONSENT FOR PARTICIPATION IN QUESTIONNAIRE STUDY

Title of Project: Quality of life study in familial adenomatous polyposis

RE: Patient name and address

Thank you for reading the patient information sheet about our research study. If you agree to participate, please initial the boxes below and sign this form.

Name of Researchers:

Miss Sarah-Jane Walton
Research fellow St Mark's Hospital
The Polyposis Registry, St Mark's Hospital

Miss Sonal Arora
Academic clinical lecturer in surgery and patient safety
Imperial College

Professor Susan Clark
Consultant Colorectal Surgeon
Assistant Director of The Polyposis Registry, St Mark's Hospital

Contact details for research team:

Miss Sarah-Jane Walton
Tel: 0208 235 4270
Fax: 0208 235 4278
Email: sarah-janewalton@nhs.net

Please read each section carefully and put your initials in those boxes you agree to and then sign at the bottom of the letter where indicated.

- I confirm that I have read and understand the information sheet, dated 10/7/13 (version 1) for this study. I have had the opportunity to consider the information, ask questions and have had these questions answered satisfactorily.
- I understand that sections of my medical notes relevant to the study may be looked at by individuals conducting the research or by research regulatory authorities. I give permission for these individuals to have access to my records
- I understand that my participation is voluntary and I am free to withdraw from the study at any time without giving any reason and without my medical care or legal rights being affected. Any information about me remains confidential at all times.
- In the unlikely event that I lose the capacity to make decisions during the study period, I agree that any data collected up to this point may be included in the study.
- I agree to take part in this study

NAME:

DATE:

SIGNED: _____(patient)

NAME:

DATE:

SIGNED: _____(name of person taking consent)

Copies: 1 for the patient; 1 for the research file site; 1 to be kept in the medical notes

Northwick Park Hospital,
Watford Road,
Harrow, Middlesex.
HA1 3UJ

CONSENT FOR PARTICIPATION IN QUESTIONNAIRE STUDY

Title of Project: Quality of life study in desmoid disease

RE: Patient name and address

Thank you for reading the patient information sheet about our research study. If you agree to participate, please initial the boxes below and sign this form.

Name of Researchers:

Miss Sarah-Jane Walton
Research fellow St Mark's Hospital
The Polyposis Registry, St Mark's Hospital

Miss Sonal Arora
Academic clinical lecturer in surgery and patient safety
Imperial College

Professor Susan Clark
Consultant Colorectal Surgeon
Assistant Director of The Polyposis Registry, St Mark's Hospital

Contact details for research team:

Miss Sarah-Jane Walton
Tel: 0208 235 4270
Fax: 0208 235 4278
Email: sarah-janewalton@nhs.net

Please read each section carefully and put your initials in those boxes you agree to and then sign at the bottom of the letter where indicated.

- I confirm that I have read and understand the information sheet, dated 10/7/13 (version 1) for this study. I have had the opportunity to consider the information, ask questions and have had these questions answered satisfactorily.
- I understand that sections of my medical notes relevant to the study may be looked at by individuals conducting the research or by research regulatory authorities. I give permission for these individuals to have access to my records
- I understand that my participation is voluntary and I am free to withdraw from the study at any time without giving any reason and without my medical care or legal rights being affected. Any information about me remains confidential at all times.
- In the unlikely event that I lose the capacity to make decisions during the study period, I agree that any data collected up to this point may be included in the study.
- I agree to take part in this study

NAME:

DATE:

SIGNED: _____(patient)

NAME:

DATE:

SIGNED: _____(name of person taking consent)

Copies: 1 for the patient; 1 for the research file site; 1 to be kept in the medical notes

Northwick Park Hospital,
Watford Road,
Harrow, Middlesex.
HA1 3UJ

Patient Information Sheet

Title of Project: Genetic mechanisms in desmoid formation

Dear Sir / Madam, thank you for considering participation in our study. It forms part of a student PhD project. This information sheet will aim to answer any questions you may have.

What is the purpose of this study?

We are studying the genes that may be involved in the development of benign desmoid tumours in patients suffering from Familial Adenomatous Polyposis – FAP. We are aiming to improve our understanding of this debilitating disease and to find new treatments.

Why have I been invited to take part?

You have FAP and patients with this condition have an increased risk of developing desmoid disease. We already know that a family history of desmoid disease can increase the risk of developing these benign tumours and that genetic factors are important. We need to compare DNA/RNA in those patients with FAP and desmoid disease with those without desmoid disease to look for differences that may indicate possible genes involved. We would therefore like to sample some DNA/RNA from your blood to look for the genes that may be important in desmoid tumours.

What sample will be taken from me?

A single blood sample will be taken from you so that we can extract DNA/RNA in the laboratory to study or should you already have a stored DNA sample we would like your permission to use it for the purposes of this study.

Who will have access to my blood/DNA?

DNA will be extracted from your blood sample and analysed by scientists and clinicians conducting this research at St Mark's Hospital, Blizard Institute of Cell and Molecular Science, Barts and The London School of Medicine and Dentistry and a genetics laboratory in Arizona University, USA. All samples will be held in strict confidentiality in compliance with trust and national guidelines. Following completion of the study DNA samples will only be stored at the Kennedy-Galton Centre, Northwick Park Hospital. Any remaining samples in the Arizona laboratory will either be destroyed (if only a small amount of DNA remains) or returned to the Kennedy-Galton Centre for storage.

Can I say no?

Yes, you can refuse to participate in this study. This will have no impact on your clinical care. You may withdraw from the study at any point without giving notice or reason. Should you lose capacity to make decisions during the study we would retain any information already gathered from you before that point.

Will this study benefit me?

We cannot say that participating in the study will directly benefit you but it could improve our understanding and treatment of desmoid disease in the future.

What are the disadvantages/risks of taking part in the study?

There are no known disadvantages or significant risks associated with taking part in the study. The blood test may cause minor discomfort.

What if new or relevant information becomes available?

If any new information becomes available as a result of the study that may be important to you we would inform you about this.

Will my taking part in this study be kept confidential?

All information about you will be kept strictly confidential. All samples will be stored in an anonymous fashion.

Are there any implications for insurance?

We will not disclose any clinical or genetic information about you to a third party such as insurance companies. All information about you will be kept strictly confidential.

What will happen to the results of the research study?

It is likely that the results of the study will be published in a medical journal/presented at medical conferences. You will not be identified in any report or publication.

Who has reviewed the study?

This study has been reviewed and approved by the Camberwell St Giles Research Ethics Committee.

Who can I contact for further information/advice before or during the study?

Should you have any further queries or want to discuss any issues related to this study, please contact one of the members of the Polyposis Registry:

Mrs Kay Neale 0208 235 4270 kneale3@btconnect.com
Manager of The Polyposis Registry St Mark's Hospital, Harrow HA1 3UJ

Miss Sarah-Jane Walton 0208 235 4270 sarah-janewalton@nhs.net
Research fellow

Northwick Park Hospital,
Watford Road,
Harrow, Middlesex.
HA1 3UJ

CONSENT FOR PARTICIPATION IN STUDY

Title of Project: Genetic mechanisms in desmoid formation

RE: Patient name and address

Thank you for reading the information sheet about our research study. If you agree to participate, please initial the boxes below and sign this form.

Name of Researchers:

Miss Sarah-Jane Walton
Research fellow St Mark's Hospital
The Polyposis Registry, St Mark's Hospital

Professor Susan Clark
Consultant Colorectal Surgeon
Assistant Director of The Polyposis Registry, St Mark's Hospital

Mr Stewart Payne
Head of Molecular Genetics Laboratory
Kennedy-Galton Centre, Northwick Park and St Mark's Hospitals

Professor Christina Laukaitis
Department of Medicine
University of Arizona, USA

Contact details for research team:

Miss Sarah-Jane Walton
Tel: 0208 235 4270
Fax: 0208 235 4278
Email: sarah-janewalton@nhs.net

Mrs Kay Neale
Manager of The Polyposis Registry St Mark's Hospital, HA1 3UJ
Tel: 0208 235 4270
Email: kneale3@btconnect.com

Please read each section carefully and put your initials in those boxes you agree to and then sign at the bottom of the letter where indicated.

- I confirm that I have read and understand the information sheet, dated 29/4/2013 (version 3) for this study. I have had the opportunity to consider the information, ask questions and have had these questions answered satisfactorily. ☐
- I understand that sections of my medical notes relevant to the study may be looked at by individuals conducting the research or by research regulatory authorities. I give permission for these individuals to have access to my records ☐
- I agree for a blood sample to be taken from me for DNA/RNA analysis OR where my previously stored DNA is available, I agree for its use in the study. ☐
- I am aware that some of the anonymised DNA taken from me may be sent to the University of Arizona, USA, for further genetic testing and agree to this. ☐
- On completion of the study I understand any residual DNA will be returned to the Kennedy-Galton Centre at Northwick Park Hospital for storage or destroyed where only a minimal amount of DNA remains. ☐
- I understand that my participation is voluntary and I am free to withdraw from the study at any time without giving any reason and without my medical care or legal rights being affected. Any information about me remains confidential at all times. ☐
- In the unlikely event that I lose the capacity to make decisions during the study period, I agree that any data collected up to this point may be included in the study. ☐
- I agree to take part in this study ☐

NAME:

DATE:

SIGNED: _____(patient)

NAME:

DATE:

SIGNED: _____(name of person taking consent)

Copies: 1 for the patient; 1 for the research file site; 1 to be kept in the medical notes

Appendix A3.1 EQ5D-5L



Health Questionnaire

English version for the UK

Under each heading, please tick the ONE box that best describes your health TODAY

MOBILITY

- I have no problems in walking about ☐
- I have slight problems in walking about ☐
- I have moderate problems in walking about ☐
- I have severe problems in walking about ☐
- I am unable to walk about ☐

SELF-CARE

- I have no problems washing or dressing myself ☐
- I have slight problems washing or dressing myself ☐
- I have moderate problems washing or dressing myself ☐
- I have severe problems washing or dressing myself ☐
- I am unable to wash or dress myself ☐

USUAL ACTIVITIES *(e.g. work, study, housework, family or leisure activities)*

- I have no problems doing my usual activities ☐
- I have slight problems doing my usual activities ☐
- I have moderate problems doing my usual activities ☐
- I have severe problems doing my usual activities ☐
- I am unable to do my usual activities ☐

PAIN / DISCOMFORT

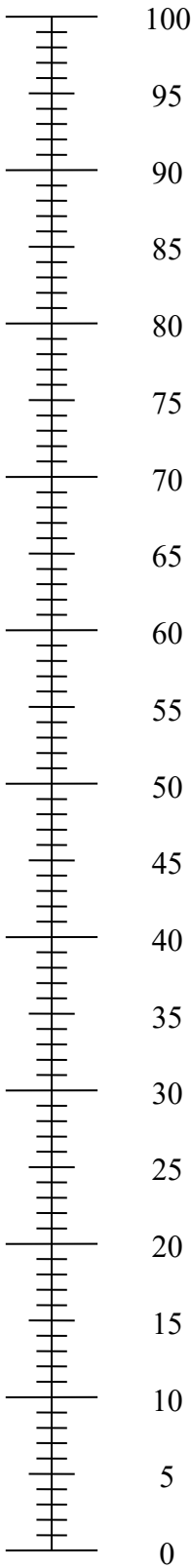
- I have no pain or discomfort ☐
- I have slight pain or discomfort ☐
- I have moderate pain or discomfort ☐
- I have severe pain or discomfort ☐
- I have extreme pain or discomfort ☐

ANXIETY / DEPRESSION

- I am not anxious or depressed ☐
- I am slightly anxious or depressed ☐
- I am moderately anxious or depressed ☐
- I am severely anxious or depressed ☐
- I am extremely anxious or depressed ☐

- We would like to know how good or bad your health is

The best health
you can imagine



The worst health
you can imagine

TODAY.

- This scale is numbered from 0 to 100.
- 100 means the best health you can imagine.
0 means the worst health you can imagine.
- Mark an X on the scale to indicate how your health is TODAY.
- Now, please write the number you marked on the scale in the box below.

YOUR HEALTH TODAY =

Appendix A3.2 SF36

SF-36 QUESTIONNAIRE

(1992 -- Medical Outcomes Trust)

Patient Name: _____

Date: _____

1. In general, would you say your health is: (circle one)

Excellent Very good Good Fair Poor

- 2.
- Compared to one year ago
- , how would you rate your health in general
- now
- ? (circle one)

Much better now than one year ago.

Somewhat better now than one year ago.

About the same as one year ago.

Somewhat worse than one year ago.

Much worse than one year ago.

3. The following items are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much? (Mark each answer with an **X**)

<u>ACTIVITIES</u>	Yes, Limited A Lot	Yes, Limited A Little	No, Not Limited At All
a. Vigorous activities , such as running, lifting heavy objects, participating in strenuous sports			
b. Moderate activities , such as moving a table, pushing a vacuum cleaner, bowling, or playing golf			
c. Lifting or carrying groceries			
d. Climbing several flights of stairs			
e. Climbing one flight of stairs			
f. Bending, kneeling or stooping			
g. Walking more than a mile			
h. Walking several blocks			
i. Walking one block			
j. Bathing or dressing yourself			

4. During the past 4 weeks, have you had any of the following problems with your work or other regular daily activities as a result of your physical health? (Mark each answer with an **X**)

	YES	NO
a. Cut down on the amount of time you spent on work or other activities		
b. Accomplished less than you would like		
c. Were limited in the kind of work or other activities		
d. Had difficulty performing the work or other activities (for example, it took extra effort)		

5. During the past 4 weeks, have you had any of the following problems with your work or other regular daily activities as a result of any emotional problems (such as feeling depressed or anxious)? (Mark each answer with an **X**)

	YES	NO
a. Cut down the amount of time you spent on work or other activities		
b. Accomplished less than you would like		
c. Didn't do work or other activities as carefully as usual		

6. During the past 4 weeks, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbours or groups? (circle one)

Not at all Slightly Moderately Quite a bit Extremely

7. How much bodily pain have you had during the past 4 weeks? (circle one)

None Very mild Mild Moderate Severe Very severe

8. During the past 4 weeks, how much did pain interfere with your normal work (including both work outside the home and housework)?

Not at all A little bit Moderately Quite a bit Extremely

9. These questions are about how you feel and how things have been with you during the past 4 weeks. For each question, please give the one answer that comes closest to the way you have been feeling. How much of the time during the past 4 weeks — (Mark each answer with an X)

	All of the Time	Most of the Time	A Good Bit of the Time	Some of the Time	A Little of the Time	None of the Time
a. Did you feel full of pep?						
b. Have you been a very nervous person?						
c. Have you felt so down in the dumps that nothing could cheer you up?						
d. Have you felt calm and peaceful?						
e. Did you have a lot of energy?						
f. Have you felt downhearted and blue?						
g. Did you feel worn out?						
h. Have you been a happy person?						
i. Did you feel tired?						

10. During the past 4 weeks, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting with friends, relatives, etc.)? (circle one)

All of the time Most of the time Some of the time A little of the time None of the time

11. How TRUE or FALSE is each of the following statements for you?

	Definitely True	Mostly True	Don't Know	Mostly False	Definitely False
a. I seem to get sick a little easier than other people					
b. I am as healthy as anybody I know					
c. I expect my health to get worse					
d. My health is excellent					

Appendix A3.3 Transcript from male focus group discussion

Facilitator=F

Participants=1,2,3

F: The point of this discussion today is to really find out a bit about how desmoid tumours may impact on your everyday lives. So I want just want to explore what sort of problems, or not, that they tend to cause. I am currently doing some research into desmoids, most of that is laboratory, but also on the side of that I am looking at how else they impact on patients, because these days we don't just want to know how people are long term with their desmoids, whether they are not a problem or they are a big problem, but we want to know HOW they are a problem and they impact. So this is really what we are going to explore, there is very little information if you look in the literature because you are a rare cohort of patients and it is very difficult to get them together to discuss this.

So I thought to start off with I would just ask you if you think that there is any point in discussing the QOL associated with desmoids. Do you think that there is any point?

P1: There's always a point to something isn't there? No knowledge is ignorance so knowledge is learning. That's how we actually learn about anything. I mean I can only tell from the amount of tests, different types of tests, I have had with St Mark's over 50 odd years. So, some of them I've told you earlier today actually worked the uhh..

F: Mmm, the eye one, the CHRPE

P1: Yeh the CHRPE that's it. So that was just an experiment so that's how they learnt through talking and experiments

F: And do you perhaps see that, or do you think that desmoids affect the quality of the life that you lead? Do you think that they impact on....

P1: Mine definitely. Definitely, because I was a builder up until about 5 years ago. And I couldn't bend down no more, out of breath, that's Mr Brown at the thorax clinic said that was the desmoid pushing on your larynx. Said there was nothing that they could do so it does affect your life.

P2: I think it does affect

F: How? Could you perhaps talk a bit more about how it affects your quality of life do you think?

P2. Well I'm really active and I'm a very sporty person and uh well before I had the desmoid I was able to go further and loved playing tennis. Now when I've been playing for an hour, an hour and a half, well now even less now, it's not just that I get tired or that I get some kind of pain or something, it's that it actually limits how I play. I have to play differently than I played before, I have to change my programme to what I was doing, before I had the desmoid but.

I like to walk a lot it just um because I have this hydronephrosis of whatever, due to the desmoid, and I don't know or how do you say.. So when I've been walking for a long time, well exactly the same as the tennis, how do you say.. the.. lumb.

F: The lumbar region of the back?

P2: Yeh it affects walking also, it just gets really sore, it's not only really sore, well it's something that you have to live with, well it's definitely something that is different and affects my way of living and I haven't changed the way that I do things because of the desmoid but it's something that's not

F: Hmm

P1: What is your age do you mind me asking?

P2: 24

P1: Blimey I was lucky

F: How old are you?

P1: 63

F : And you Paul?

P3: 42

F: 42, hmm. What do you think about..?

P3: I think it does limit I seem to get a lot of pain in my back, where the desmoid is although at the last scan from Addies it's getting close to the heart now and I'm a bit worried about that

F: Hmm. OK. And apart from the pain and the health impact does it affect other aspects of your health perhaps? Family life? You mentioned social life...you mentioned work life.. But uh what about family life?

P2: Yes

P1: It's affected my life with my grandchildren, well I mean them living in Australia has done that, but... when they're over here I can't play with them as much as I want, with my own daughters I used to do a day's work come home go over the park to park and forest, woods, Couldn't do that now, can't even walk to the end of the street. Out of breath. That's life I suppose, that's the deck of cards you got, that's the deck of cards you got, that's better than my fathers, well it was...yeh

So does it affect you the way you play your strokes in tennis?

P2: Yes

P1: it affects the way you actually play your strokes?

P2: Well it affects all the way around the cos in tennis you have to go down and it's all of a sudden run and stop run and stop. And family life, well I have little cousins, and it's true that, well I get...they are really young, children and really active and full of energy and they are like 'ok, pick me up' whatever and it's true that um I get much more tired and worn out than before. It affects because before I was like this older cousin who was really playful and that and what I did was lots of pick up and throw whatever in the pool and now it's true that I tend to, not be in the moment or the place where to have to do that. I just let my brother do it and know if I am there I will want to do it because they are my little cousins. It does affect that I tend to get away from the situation where I have to play with them because I know that they are going to want more and more and I am going to stop much more earlier.

F: Right, so you tend to avoid perhaps getting into that position

P2: Yeh or not always avoid it but from time to time, yeh, but not because I don't want, because I would like to be really active again.

F: Yeh. Any comments from you Paul re. life?

P3: I suppose it does limit. I've not got a young family. I don't socialise much or go outside or anything

F: In that case is it the desmoid that might be limiting you or other things that perhaps that you're not so fussed about that

P3: I think that's probably partly down to the desmoid and that

F: Do you?

P3: Yeh

F: And how, how does it stop you getting out? Is it the pain or other things associated with the desmoid that's stopping you from doing what you want to do? I suppose that is a general question to all of you.

P1: Well the amount of times you'll be out in the day like we go to Billericay, about 4 miles away, I don't drink alcohol so. It just gets me out of the house or I'll go potty if I have to stay in the house. I'll go a bit potty at this rate, I don't get emotional, I just say we're going out and we go out. That's how I deal with things. But I mean with this desmoid it has no umm pattern to it, does it? It just affects everybody, I thought you had to get a bit older before you got it but obviously you don't, I thought you had to be older to get it.

F: No

P1: And do you all get out of breath?

P3: Yeh

P2: Yeh

P1: I thought it was me getting old. I shouldn't have smoked as a child.

F: And have you found that it's taken over your lives, the desmoid?

P1: oh yeh predominantly

F: It's always there controlling?

P1: It's always a controlling factor to some extent but I won't let it beat me. So as I say I have to go out, even if it's freezing cold, there's a bus stop opposite me, I just get on the bus if I want to that's as far as I can make it to the bus stop but it doesn't have no conscience does it?

F: No. Just going back to that last thought, is it, you mentioned being out of breath and you feel very tired, you mentioned pain, are there any others things associated with desmoid that I don't appreciate that perhaps are limiting you from getting out and doing what you want to? Whether it's getting out socially or going to the shops or going out when your family members want to go out? Is there anything else about the desmoid in particular that stops you?

P2: Well for me at least the first year I came here I lived in a residence, the university residence, they did from time to time several weekends throughout the year they went for a long hike into the woods forest. I went the 1st time, the 2nd but the 3rd it wasn't that I didn't go because of the thing...It was a deciding factor I am going to come back and be sore. It's not that I don't do something because of the desmoid but in the pros and cons table I say OK if I do this if I play tennis I am going to have urine in my blood or whatever, I am going to have fun but then it does weigh a but it's not a decision maker in that I say I have this and it won't stop me from doing it but it is in the level in the way.

F: Anything else other than those things that you mentioned I don't know but for example does the desmoid affect perhaps your bowel function so that you say 'I may not go out today' or those sorts of issues? Do they ever come into this for any of you

P1: It has never entered my head. It's OK so...

F: no

P2: No

F OK. Do you think having a rare disease like desmoid tumours is quite a burden or is not something you really think about?

P1: Well I'm the optimist.

F: You're the eternal optimist

P1: Yeh it's like that's life and there are many other things you could have. I mean you go through my life I should have been dead 3 times by the age of 16. Should have been dead 3 times at least

F: From the desmoid?

P1: No, no from the accidents I've had.

F: Not connected to FAP or anything

P1: No it's like that's the opposite side of the coin. Something else worse could have happened I think it's how the families cope that helps you cope. That's what helps me anyway.

F: Hmm, and you two, have you got any thoughts about rare diseases?

P3: No, it's just pot luck really

F: Just because one thing that came out from the other group discussion was that they felt a bit in the dark

P1: Well what information do you want?

F: So you feel you

P1: When I was what 11 or 12 I went to go and have tests at the old St Mark's and it never bothered me. I did tell kids at school and that but I never felt special. No no.

P2: I find that the people who live with me, or who are around me, friends or girlfriend, they are much more conscious, no not conscious but uhh wary? Wary yeh, I mean I do everything that I can, for me I live with it like you say other people have many things, other people that die because a flower basket hits their head in the street. But for me it's not an issue. It's an issue when it affects when I do certain things. But for me it's like I don't really think about it much, just occasionally I think I can have pain or something, but people around me are much more like...are much more likewary

F: Wary, or worried about you?

P2: Yeh they're worried they bring up the subject more often than, I mean, I wasn't thinking I haven't, didn't think about the desmoid the last 2 weeks, then my friend comes and brings it up, they are worried. They think that it's something that I am constantly thinking about and maybe I want to talk about it or maybe I want to discuss something, or m I don't know.

F: That's interesting

P1: Are you able to work?

P2: No

P3: No

F: Do you think, going back to what you were saying about family and effects, do you think it has much of an impact on your other halves, or sibling, parents etc. or your actual family around you?

P1: No she's a gypo she's alright

All laughing.

P1: No she bears it, grins and bears it, and that's it, well that's what you can do isn't it, otherwise we discuss it, obviously because it's something that's there every day and we just laugh about it. That's right.

F: What about you Paul, do you mind me asking about family?

P3: I suppose they did worry about it a little bit, that it affects me more than I think or something. Otherwise they know it's just mostly there and it's just a part of me and get on from day to day

F: Do you worry about the future at all or do you think just about today or day to day. How do you think of your desmoids and the future?

P3: Well I'm not particularly worried about it. I know Addenbrooke's worry about it. It doesn't worry me I guess I know it's just there and I make the best of it as I can

P1: Do you have children?

P3: No

F: Well I would be interested to know your thoughts as to whether you think your desmoids have had much of an impact on your body image. I mean how you see yourself as a man. Has it affected your confidence? Or what you feel and think about yourself at all?

P1: Well my confidence is still sky high but when I went in hospital in '69 I was only supposed to be in 7-10 days I was in hospital 12 weeks, in St Mark's, something went wrong when they put the stump back and so I came out looking like something from Beltzen so I had to go home and convalesce, I was a drayman then, so it was a pretty physical job. But I got it back again, muscles and all that strength uh in '96 I done the same thing, had to have a series of operations. '02, that was here in '02. Did any of you have a temporary ileostomy? Umm. Internal pouch. Yeh I had problems with that for 6 years, '96-'02, a J pouch, a temporary one. I just wondered. That took a lot of strength out of me for 6 years. I had to go to the toilet about 21-27 times a day. I was still working.

F: So having all those problems, you mentioned those events, because you say you have a lot of confidence but did those events knock you back in terms of how you felt about yourself/your image?

P1: No nothing knocks me about I feel pretty positive.

F: OK and you?

P2: Not for me. 2 years ago I spent a month in hospital. I like to go to the gym and I like to get strong and for the past 2 years, the past 2 summers. 2 years ago I spent a month here and I lost 10 kilos and last year actually I lost 20 Kilos. For me it's not that my self-confidence or how I see my body, it's not that I want to prove myself. I like to...Its because of the problem that I've

begun to go to the gym just to...to...But I had a past girlfriend who said I get weak or because of my...she didn't understand. She thought that with the desmoid that I don't know I was some kind of uhh... I wasn't able to do certain kinds of things or I don't know in our personal life or whatever and so I took it as a personal thing to go to the gym to build up a strong body and to do as many things, I mean I've always been a sporty kind of person but I became more just to prove the point and ever since I like to do it but not because I see myself bad but just because I like to prove not prove anymore but...

P3: I feel OK in myself. It's just when I originally had the tumour removed and I was in hospital for 3 months. Getting over that and the complications, otherwise I don't think anything about it.

F: Scars and lumps, things like that from them? They may not bother you now but at the time when you had all these things going on, were they ever a thing that really affected you? Were they important?

P1: Well I never dreamt I would have a scar, never even thought I would have a scar. When they told me I needed to come in and have it at St Mark's never even thought about it, then to walk out with 2 scars.

F: Was that I shock. Was that something hard to...

P1: Especially when you see Alan Park's stitching. Oh terrible. I didn't know I had a belly button. But then Prof Northover cleaned them up for me, but It was terrible stitching. That was a shock but the rest of it wasn't. No.

F: Didn't feel the need to dress in a certain way or cover up perhaps when you go to the beach in the summer, that sort of thing?

P2: I like to show it I like to show my scar. I like my scars

F: Do you think there's much medical uncertainty about desmoids and does it bother you at all? And so I wonder whether having physicians and other health professionals around you who know relatively nothing about these things condition affects you. Or whether you've got any thoughts about it. Are you frustrated that we don't know more about it?

P1: It shows it must be a bit unique cos I've had doctors queuing up around my bed to feel my abdomen and feel the desmoid tumour. Others looking at my fingers because they're clubbed and there's the old phenomenon that goes with that, there's an early sign of serious illness. My father-in-law had it, my father had it and I've got it. That's clubbed fingers. That's a sign of some serious disease and I've had a doctors around the bed looking at them.

F: Does it bother any of you then? Having something where physicians/Drs don't know much about it?

P1: They say knowledge is a wonderful thing

P3: No it doesn't worry me. Sometimes I've had, quite often I've had, loads of doctors around you who want to feel it. It's a bit strange but in the long run if it's helping to learn more about it then it's all good.

P2: Well for me maybe before I came here, in Spain, nobody knew anything about the desmoids..uhh no it wasn't frustrating because my mum knew a lot about everything so she went when my doctors told me I had a desmoid tumour, they said something like ok so we need to removed it or something and she said no and actually the doctor was a bit annoyed and my mother said no that's not the treatment, you can't remove it or whatever. The Doctor went away and came back and I was sick in the bed so I don't know how much time but for me it was like 20 minutes later and doctor came back and said OK you're right and my mother said well of course, I have it, I know or something. But I live here now so for me.

F: Do you think having family members who've got the same issues, maybe not desmoid but FAP, therefore gives you a bit of a buffer? Because you can talk about it and you're not so much in the dark? I don't know

P1: My daughters have got it and we don't talk about it. That's it

F: What about your siblings or father, I mean was he alive for some of your....

P1: I was only about 12 or 13 when he popped off, so I didn't really know him. But my mother told my father's brothers and sisters and she was ostracised, seriously yeh she was ostracised. 'We don't have cancer' 'We don't have FAP' and he had a big family. And they didn't want to know, didn't talk to her again, I mean. But that's how people thought of things like that back then. It was like consumption they didn't talk about that. But that's how if daughter mentions it we just laugh about it that's all, that's the only context it does get mentioned. We just laugh

P3: I got a younger brother, never thought to talk about it really.

F: How old were you when you were diagnosed do you mind me asking?

P3: 18.

F: Did you have any other family members around you at the time who had FAP or desmoid?

P3: My dad.

F: Was he able to give you any information about it or?

P3: Not really, no

F: It was more of a generational thing back then that you didn't talk about it?

P1: A man was considered a man and your shoulders were broad enough and that was all that mattered. My father went out to work to get rid of the pain. I'm not sure if that was my mother or the cancer! But uh no he went to work to get rid of the pain and that was all.

F: Do you think about much about how desmoids and how they could be potentially life threatening does that ever really come into your mind?

P1: It's like paths crossing the road. Grandpa looks different

P3: For me it's a bit different. Cos mine is I had virtually all the intestine taken away as it's taken up all the space where it used to be and is growing up towards the heart now. They are getting a bit more concerned about it with me now and thinking about doing a transplant. So...

F: What do you think about that?

P3: I personally don't want it. But I know if I don't I'm letting myself in for other things. The desmoid still growing well they say it's now getting very close to the heart so if I don't have that what else am I then letting myself in for in the near future, So they say it's got to be done fairly soon. Pretty major surgery and a very long stay in hospital so...

F: What do you think about that? Do you think much about it being a life threatening thing or does it not really come into your thoughts?

P2: No. Well actually I've been asked, well my friends for whatever reason they've thought about it.. so

F: OK. Just to clarify because I touched on medical uncertainty and lack of medical knowledge before....

P1: But I was put on Sulindac and that was working and touch wood it still is working, but the company that were making it actually stopped making it and I didn't take it for a year and then I had a scan here and they said the polyps were getting bigger. So

F: The polyps in the bowel?

P1: In the duodenum.

F: OK Because it tackles both

P1: When I started taking them again, I explained to them what was happening and they looked into it and they suddenly they started making it again and when I talked to the pharmacist he said 'I don't know why they want to fix something when it's not broken'. And I started taking them again and it slowed down the growth of the polyps so that does work. Although does it affect the tumour, it definitely works on the polyps

F: Do you think we are still sort of or do you feel that people are still in the dark that there is not treatment or that don't know enough about them? Does that affect your QOL?

P1: But you already said that a lot of surgeons don't know nothing about them

F: But those who are working on it, so when you come here, we know a bit but still don't know perhaps as much as we want to know. Does that impact much?

P2: I compare it to other kinds of diseases or other conditions like cancer. Or others that are more publicised or more maybe they are much bigger and a bigger range in the world population. We are more like a minority. So for me it's like OK cancer isn't cured OK. There are major conditions or diseases or whatever that still do not have.. there are many things you can do about them... but there's not like a vaccine and just can cure. It's not like the black thing that came in the middle ages that got cured and doesn't come anymore.

For me it's just that we are a minority so I have to know that. I have to know that if more of the world had it there would be more people on it and more would have more funds. For me I have to accept that it is a small thing and as such it's not going to get all the attention and all the research other bigger things that are still not completely solved. So for me that's how I understand it like that.

I see it like progress, so that is why I try to do every kind of participation in research or study so that I can put my brain into help others.

P1: I just think that it's a matter of time. May not be in my generation but it will come along eventually. Everything does. Wait long enough and a bus comes along. I do definitely think that, well the way they are working on it, so.

F: Can I ask are there anything other points about desmoids and how they may affect the quality of your life that haven't necessarily touched on? Any thoughts about what else, how they are impacting on you that I don't understand or appreciated? Any other thoughts about desmoids and their impact?

P3: No

P1: No That's it

P2: For me I don't know with my girlfriend or whatever, or even with my friends there are some plans or some things that maybe I don't feel like doing, or..it's more or less what we said but it's nothing to do with social and family and...

P1: Insurance and airlines. Holiday insurance. I got to go to Australia the other year and it's dearer the insurance than the flight. You have to tell them all about it. 'Cos you're not covered for an existing illness if you don't tell them about it. So that's it

F: Did you go?

P1: Yeh, yeh that time. But it's stopping me thinking about going again.

F: So it might be limiting you

P1: Yeh, yeh but that's nothing, that's nothing compared to life is it really?

F: No, but it is still limiting you slightly..

P1: I've been there, I've enjoyed it and I have memories that's the main thing. I've done it.

F: Anything else you wanted to add Paul? Haven't touched upon?

P3: No I don't think so

F: In terms of personal lives and having an intimate personal life, can I ask if it has much of an impact there? I know it's a bit of an embarrassing question.

P1: Well I became impotent a couple of years ago. So that's life, that's it. Well all the surgery you have and obviously Prof Northover told me in '96 that it would affect me and it didn't, only in the last couple of years and that's no big thing. Done it. Been there, done it. Got the T-shirt. Not a great problems

P2: For me it's like tennis it just gets me very... tired and I don't know maybe my legs get a bit numb. A bit tired...

F: Any other comments?

P1,2,3: No

F: So I think just in terms of summarising what we've gone through perhaps. We've spoken about whether we think it may be worthwhile looking at QOL of life in desmoids and probably is worthwhile was your thought because the more knowledge we have, particularly with something that's quite rare, the better really. I think you all mentioned that one of the main sort of impacts perhaps on your fitness and ability to do something and feeling tired at times or getting tired more quickly than you want to and impacting on your physical and social life and jobs have been affected and ability to work. The impact on families perhaps not huge impact immediately, some of whom already have experience with it and others you've got perhaps partners and things who understand what's going on and for them they may worry more about it more than you yourself. Is that fair to say?

Then talking, we spoke a bit about thinking ahead in the future and a couple of you said you were not particularly worried and were just thinking about the here and now really and just getting through but there are some anxieties about perhaps future treatments that may be necessary, especially for you Paul. Yeh. In general then can I ask do you feel quite hopeful about the future?

P3: Yes. I see no reason not to.

F: Even with that looming, you still feel hopeful?

P3: Yes

F: Good yeh. And you?

P1: Yes always, tomorrow is going to be a better day and today is better day than it was yesterday.

F: Do you feel on the whole optimistic Guillermo?

P2: Yes

F: Good. And I think that's really where I wanted or leave it. Any other comments?

P2: Just on the family or relative subject. I actually broke up, well I broke up with her, a couple of times about the desmoid. At least for people who come into their lives and they're bringing you to that problem... For her it was like a process, at first you seem so positive about it you say it's not a problem, ok it won't be a problem. But then the 1st and 2nd year she starts to get a bit worried a bit like ok this isn't as fun as you said it was. Then 3rd year I get my month in the hospital and she starts she starts to drop more things and says that, for her I mean, for those who don't know about it, it's like a process and that really affected my life in all aspects. Like in the physical and the psychological and the comings and goings. Ok I have it's not just the relationship with her but also it does make you think more about the desmoid than I would have. She makes all the pertinent questions, she makes all the, like spot on questions what you hadn't thought about it. So it's like a process. So if your family, my mum and my brothers, we all have it and my dad's been a long time, in the family sense it's ok but when you have a girlfriend or whatever first it's ok then it's not ok until then. So it's a difficult, its they're more wary, and it's more difficult for them to accept and for them not to support, because obviously they're not going to not want to support, but it's more difficult for them than your family that knows about it... or. That's it.

P1: So it's at different ages that they have different problems don't they. See I didn't have none of them problems.

F: Did you have any of those problems when you were younger when it was all going on with the...

P1: No. We was courting so... she was visiting me in hospital. She was working and visiting. So no we didn't have that problems. Younger people have that problem. But it's still a problem and it's awful. That's what your mass or was it the ileostomy?

P2: The first problem was the desmoid tumour and just when we were getting around the subject of the desmoid tumour, that was when I had the stoma, but that was like... we were getting around the problem was with the desmoid tumour, but when I had the stoma, this past year, it was just the first 2 or 3 months it just got accepted. Not for me but for her and..

P1: well it's both really, for her and your peace of mind

P2: Cos when you start to take the relationship seriously and then you start thinking about the future and about the kids and about so if I marry her then our children they will have or wont they...

P1: The positive and the negative rolled into one.

F: You've had children?

P1: Yeh we had them regardless, we was gonna have children when we were young or not at all cos I knew what it was like not having a father. Cos he died when we were both young and it wasn't going to happen to me or my wife. So we had our children when we was young. Get around it.

F: Did they have FAP?

P1: My younger daughter does but the other one was given the all clear quite a few years ago.

F: So any other comments?

P1: It's been enlightening

After interview recording stopped, one patient (on permanent TPN, 24 hrs a day) also described how he could eat very few foods or enjoy a meal and therefore did not bother socialising due to this.

Appendix A3.4 Transcript from female focus group discussion

Participants: 1,2,3,4,5 (and P5's mother)

F: Do you think there is a need to measure quality of life in a patient with desmoid? What do you think of that idea, is it an important concept? Something that is relevant to you?

P1: Desmoid affects somebody differently. Like my sister had hers pop out...mines just inside and affecting the kidneys, so...as I said to you the other day it seems like, feels like there's no answers...cos once it goes, once my kidney goes..then I've gotta go and have stents...and then when they first explained when I got desmoid they didn't say we might get them when I first had the operation they didn't give us a percentage...not like when I've spoken about Heather (daughter) they go it's most probably 75% unlikely that she should get them. But they don't....nobody always gets you ready..things should be aired. Doctors don't always prepare you and you thing when you get something like last time someone said you've gotta go to a urologist now. Somebody explained it to me and said 'oh yeh you gotta have the stents now' but they didn't explain to me that once I've had the stents put in for the desmoid that it'll be I've gotta go back every 12 months to get the stents put in and taken out and new ones put in and out. Cos they explained it like once it's done that's it...they don't explain it properly. Then when you go see somebody else it's a different story.....It's like when I went for my tests you didn't have anything about my blood tests..to see how high my levels was...it's like nobody keeps anybody in the loop. It's a knock on effect isn't it? Once you get a desmoid it's not like it's just St Mark's that you go and see other Drs about other things in the same hospital. It's another hospital and you go round in circles.

It's like when I go to other hospitals they wanna cut it out and Im like no you aren't touching me. Before we had here you'd go to any hospital and emergency

Nobody understands how you feel,lack of knowledge

P2: They always say it's not a desmoid it's a dermoid

P3: You see what's the point in measuring the QOL cos it doesn't have any bearing. You're not gonna get any financial benefit or anything like that

P1: No

P3: and to be honest all of us, like you've said, you still get on with your life, most of us are mums and you just really... you just...the attitude is you just get on with it

P1,2,4,5: Yeh

P1 Yeh but sometimes I think when you see a Dr and I hate the attitude when they go through stuff....I think if you got something going on and it's in the same hospital and you've got a couple of things going on they should tell you and you should tell them. SO you don't keep going round in circles

F: Will regards to looking at QOL then is it worth looking at in terms of measuring it?

P4: I think probably a patient to patient buddy system.....cos everybody's different...I don't have a desmoid I had one removed um and so I was in the situation where I woke up one morning and never heard of a desmoid before, never like been diagnosed in 1997 with FAP and this happened in 2001 and I just literally woke up one morning with a distended stomach taken off to hospital to Wexham Park and they literally operated on me within 4 days cos it was a blockage and I remember being in the bed and just like loads of doctors there and I was thinking I'm gonna die....there's something seriously seriously...cos everybody was just coming in looking and walking off and I remember thinking...whatever this is...and they didn't actually tell me what it was. They just operated without any discussion, nothing, and I think if I'd been told at the beginning when I was first picked up with the FAP, I think if somebody had said to me 'look there is a thing called desmoids' I'd been more prepared and more so if they'd said to me look if anything ever happens with your stomach don't go to any other hospital just go to St Mark's there's somebody oncall in Northwick Park over the weekends so whatever happens just come and present yourself but I didn't know any of that and then after the operation and I came back here somebody said to me you were really lucky that your bowel wasn't nicked or....and they were probably rubbing their hands to get their hands on your body cos you're quite rare and that's why they were probably all.... And I felt a little bit...not let down but I just thought actually I wish I'd had a bit more information so I could have made my own informed decision...I could've said you know take me to St Mark's. But everything else I think had been brilliant

F What was your experience then? (to P5)

P5: Well I got FAP in '98 and had a total colectomy and they never mentioned desmoids at all and then about it was 2002 and my tummy was getting hard...I was quite a bit bigger then so I didn't really notice it was.. just thought it was my muscles getting better. I went to see a consultant and he diagnosed me with desmoid and um put me on some medication but then about 4 days later I got a perforation of the small bowel, peritonitis, and um I was at Peterborough and they didn't have a clue what to do about me but luckily one of the consultants had been working with Prof Phillips so he kind of an idea of what was going on and so then I got..I was sent here and had an emergency operation.

P1: Yeh cos it is the unknown if you get taken to hospital that knows nothing

P5: It's quite scary isn't it? Pot luck

P1: You have to be referred even if we are at home they will take you to the nearest hospital unless you can get in a car...and you don't know how bad you're gonna be

P5: Yeh

P4: You're so ill...and I remember lying there thinking...actually I won't be that upset if I die...and I know that sounds ridiculous cos the pain was so bad

P5 mother: Well you were on life support for 3 days weren't you?

P1: Just knowing that your hands is in another Drs hands not at St Mark's where they know what they're doing and you're thinking..there's nothing you can do about it

P4: It's a security blanket for you...

P1: Absolutely

P4: when I come through the door everybody knows me. I think that's a big thing where you're picked up with the FAP is to be told about all the little areas...desmoids or whatever else can go wrong

P2: Areas, yeh

P4: And given you know some sort of information and to this is where you go, this is what you do, this is a contact number, go to St Mark's there's somebody oncall..blah blah blah. It's yeh I wish I had known that cos I would've...

F: How do you think having desmoid has impacted on you generally on your health?

P2: As for mine, quite bad the last couple of years, but before that I wouldn't say I had any problem.

F: Before that did you know you had a desmoid?

P2: I did, I think I had 2...one here and one there.

P1: Mine was 6 months after my daughter, I was naughty I fell pregnant too early

P4: Do they not remove them straight away

P3: They can't

P1: It can come back bigger than what they took out. That's why they say you're very lucky

P4: laughing!

P1: It could've come back. Like you and my sister...my sister had to have it removed 'cos of where it was.

P4: Is it cos it creates a blockage?

F: No various reasons why

P1: I've had 5 blockages but they haven't removed mine

F: Depends where it is you see. If it's inside your tummy around vessels or too much of the bowel then you can't just take it out. For most you get by living with your desmoid

P3: I got mine in 1989 and I was told after I had my op for FAP and they said it'd be no trouble it won't bother you at all and then about 3 years later I got married and 6 months later, woke

up had no period and I looked like I was 6 months pregnant it was like a rugby ball. SO then they operated 3 times, I had chemo, I had sulindac.

P4: Oh my god.

P1: See I can't take that...

P3: Then they did a double bypass and had to have a bag...so I got that

P5: It was an emergency so it's quite high and I had a jejunostomy, had it reverse, had chemo for a years, had it reversed, got smaller and then had a year without the bag and then the desmoid started feeding of the food going through and like I was really ill, in and out of here for months and they said 'you're gonna have to have the bag back

P5 mother: You spent 6 months in here didn't you

P5: So...I had that back in 2005 and ever since

P1: So you had to learn to like sort your bag....

P5 mother: she does it very well

P1: Oh no I was just saying

P4: I feel really guilty cos you've all had such a bad time

P2: I didn't up until 3 years ago?

P3: Then what happened?

P2: Ohhhhh

P3: sorry is it....

P2: Sarah-Jane's (F) been through it with me...I had the desmoid here near my rib and one day I woke up and thought oh my rib feels a bit harder than it normally does so I thought I'll keep an eye on it and I kept coming back and they were saying 'oh yeh it's a little lump, it's OK, we'll keep an eye on it...It's just what do you call it?....goes hot, went whoosh. It just went massive. But it wasn't on the inside was on the outside, it was on top of my ribs, so it killed my skin and I had to go to St Thomas'.

P3: That's the other thing, you'd think being on top would be better

P2: It is better and I take that into consideration it is better but....I'm scarred but I can have things later on, sort of thing

P1: Yeh a boob job

All laughing

P1: you have to look at the funny side, you have to look at the positive otherwise...not being funny you just go crazy

P4 Gotta have a sense of humour!

P1: Yeh

P1: They do debulking, like you had done (to P2) if they don't wanna take it out in 1 go, while they're inside

P2: Yeh I had debulking twice, first lot July 2012 then it grew again so I had to have it debulked again the following year.

P1: She had it done while the Olympics was on and we had to come up and see her!

Laughing

P4: Going back to your question about quality of life I think it's like....I think you should have some sort of support group...I don't know if there's like...

P5: No

P4: It sounds like there should be

P5: Yeh 'cos there's something for Crohn's and colitis have a good support thing, like on Facebook there's a big campaign at the moment and like you put in FAP or desmoid and most of the desmoid stuff is like American, from the States

P3: Yeh

P5: And I don't like reading it 'cos it scares me

P4: And most of its probably out of date as well.

P5: Exactly

P1: If I had to have Crohn's, colitis or FAP I'd go with FAP every time, 'cos Crohn's is much worse, my friend's husband has got it.

P5: But there's nothing...like FAP no one knows, everyone knows Crohn's and colitis, they don't know the ins and outs but they've heard of it, but if I say to my friend's or colleagues about FAP they're like what's that

P5: It's probably 'cos it affects more people

P3: It's amazing...

P1: It doesn't have to be hereditary like it runs in the family

P5: No

P1: Like my daughter unfortunately she had the operation when she was 15. My sister's 2 are good they didn't get it so...

P5: Yeh my brother is fine, but I was like the first one with the mutated gene

P1: Out the 3 of us my brother didn't get it

P2: Yeh us two eldest got it but him being youngest didn't get it

P5: Yeh Ben didn't get it either

P4: Are you a new mutation?

P5: Yeh

P4: Wow, you're rare then

F: So how do you think it affects feeling about yourself, does it affect your confidence?

P1: Well mine

P2: I don't know how I coped with it (large desmoid), I think I'm more low about it now than I was when I was going through it, I think I'm having a knock on effect about it now and I think to myself how I get through it I'll have my hair done, I do positive things

P5 mother: You buy clothes

P5: I do

Laughing

P5: Yeh if I get an infection I come out and I go shopping

P1: I have noticed the difference...since when...

P2: Well fortunately I have a very good husband

P1: We were saying some don't

P1: It does make a difference your partner

P3: It does make a difference your partner

P2: Yeh

P4: There's no support network for partners or..?

P5: There's nothing

P3: See I can't wear certain clothes...I can't wear trousers. So I don't but I will wear my bikini

P2: I've had and I've been to my GPs and I've had doctors that have been here

P4: Just getting back to the quality of life and the effect it has...the other thing.. the main thing that upset me when I had the desmoid was that my dad had FAP and that's how I got it and fortunately there are 4 children, I've got 2 brothers and a sister and none of them have got it, but for me when I was diagnosed...it really...like my dad he was really really upset, and I remember at the time thinking there's no support network for him that could talk about...and then when I got the desmoid he was lucky and didn't have a desmoid...and when I got the desmoids it got even worse for him and I remember at the time thinking I wish there was someone somewhere or another family or who could talk to him or.....even though I had it for a short space of time it just made you feel I don't know really, like going to the bathroom....it was just, just horrible

P1: I know how you feel

P3: You do feel very on your own don't you

P4: Yeh

P1: Cos I got Heather ready from the age of 10, my daughter, cos I said to her you might not you might do. So I was like I've been....we're fine.....they came in they told me she had it...I felt like the worst mother in the world...(crying)

P2: Oh don't

P4: It's not your fault though, you didn't put up your hand up and say can I have it? It's just one of those things. Sorry I didn't mean to upset you talking about my dad.

P1: No no. I understand how you feel

P2 Don't get upset

P1 I'll be alright in a minute have another cup of coffee. No it does upset me cos I can understand how your dad feels but when you do do it and they come out with...it's devastating, she's lucky (pointing to P2 – her sister).

P4: I'm sorry. But I remember Muditha (specialist nurse) said to me last time I was there she said you're probably safer sitting on that seat than everybody else out there, because we have about 65% more chance of you coming in and finding out there's something wrong in comparison to someone not screened on a regular basis. In a way I'm sort of quite glad that it's happened in a sort of backwards sort of a way

P5 mother to P3: Have you got children?

P3: Yeh I've got 2 boys. But my mum she had 4 of us, she didn't know she had FAP then my mum got poorly then that's when it was all found out so they were told it's a million to one, that it's hereditary, so there's 4 of us so 2 of us had and my mum, unfortunately my mum has died from her desmoid but my mum always felt guilty....

P4: You can't die from your desmoid can you?

F: Sometimes yeh

P3:...and when I came up here I was 15 and I came up to here and I was told, I just went in and had the internal examination, didn't know that they was told... and that was it. That was a hard thing....I found that I couldn't...that my mum couldn't have prepared me

P1: I prepared them cos you need to prepare them. 'Cos the point is it was my choice to have a child cos after 1 I wasn't allowed to have anymore and um so I decided that I had her so I need to tell, prepare her. See now I'm normal and I don't usually get upset.

P3: I was told when I was 18 not to have children and at 18 you're not really bothered are you really. Then I had the problem with the desmoid and then they said it was hormonal and cos I was having a good time. Now I do sometimes think if I accidentally did fall pregnant I wouldn't be...such a panic would it

P5 mother: But you've got your boys?

P3: I've got my 2 boys yeh.

P1: I was gonna say you know the desmoids going through the pre-menopausal, menopausal whatever, not allowed to go on sulindac cos of the kidneys and I think what can I take. They say it's worse for women hormonally. Cos do men who suffer with FAP do they get desmoids at all?

F: Yes

P1: That's why you can't go on the pill. I'm on the mini-pill progesterone only

P4: I never knew any of that, never been told that I you couldn't go on the pill

F: It's not categorically that you can't we tailor it for everybody.

P1: Even when it was growing I was never offered anything at all

P2: There was a trial...?

P1: oh omega 3 fish oil

P3: Did you do that?

F: So I'm interested to think about your family member (to P3), that must've been very worrying when you were told you had desmoid, knowing what they'd been through?

P3: Well all of us have desmoids but I was the first one to be diagnosed with it. It was fine like it was nothing to worry about

F: Has it had an impact?

P3: It has cos this time 'cos getting married then 6 months later waking up and I tried to go to the toilet and all I could eat was a snack bar....that was all I could eat all day...and I would

drive around with carrier bags because I was continuously bringing up bile and the last time and in 20 mins I could've burst. I'd lost 4 stone. Then with the procedure they just left me cos they didn't want to operate until it's too then I come in. That's it; Kev had to wrap me up in cotton wool. The only thing I lie about is my health. I don't tell people cos I don't want to be treated any differently. But yeah it was more hard for my mum cos what happened with my mum, 'cos I didn't have a good time with the chemo, when my mum was offered she said she couldn't cos she'd seen me go through it and she wouldn't. And when I see you with your daughter (Pt 5 mum) when she's in intensive care it's really hard when she's got the doctors coming around you know this massive thing you just can't. Then actually at the same time you thin actually I wonder....do you see what I mean?

P5 mother: Well we didn't have a choice though

P3: Eventually my mum died and I thought was it worth... at the end of the day she was gonna go

P4: How does it, why did it kill you mum?

P3: 'Cos it got so big..

P1: If it gets wrapped around you kidneys and things like that I've been told...

P5: Emma's got 2 stents in both her kidneys

P3: and then in her legs she got blood clots

P2: How old was your mum?

P3: 57 she had symptoms for years

P2: Our dads mum had it and all his side. But he was 10. Went to Whipps cross hospital and they diagnosed my mum but grandad slipped through the net, unfortunately cos he wasn't picked up he passed away at 48. We happened to be going to Harlow Hospital there and a surgeon had worked here and knew a bit about it.

P4: So is there could be an argument that when you have your colectomy you could have a ... if you could elect to have a hysterectomy...cos I decided right away I wasn't gonna have children, that was just for me, and I was about 28 at the time so would that have then reduced my chance of getting a desmoid?

F: It may do but could still happen anyway, it's not just dependent on the oestrogen

P1: I had mine 6 months after I had my daughter so...it's not it's just when my healed scar..I went into the old St Mark's had the operation and jumped out of bed the next day and wanted to go home. But then a week later I went in and I was told sometimes when the body over heals itself it's just something that happens. And then cos I didn't realise it was there after they did the first blockage cos I went to St Margaret's in Epping, and then obviously I fell for Heather

and I got told off just had a few more scans then normal to see that everything stretched normally in the right way and it didn't go like an alien

P5 mother: Do you advise FAP women not to have children?

F: no

P1: Because there's nothing to say you would have desmoid if you had...so it's a personal choice

P5 mother: Can you be tested before the baby's born?

P3/4: yes

P4: I did that genetic counselling and it helps you to decide which was really helpful even though I knew I didn't want kids so for me it was....I remember them saying you could have the foetus tested at 3 months

P5: Mother: So you've got 2 children? (to P2)

P2: Yeh I know what you're gonna say..so lucky..I know..that's why I get so upset with my sister. I had 2 and um I remember you could have them tested at 14, not a blood test then...drops in your eyes and then they said you can have testing at 14 and Heather was already tested and she was older and I thought oh no....So my son was the first to be tested and they said we are pleased to tell you your son hasn't got it so I thought that means my daughter's and she's my nightmare cos shed said 'I don't want it I don't want it' and I went with her and they said she hasn't got it and I just sat there...gobsmacked.

P1: Cos before we had the blood test, Kevin used to have the camera test 'til he was 30 but..

F1: What about the impact of desmoids on your everyday life? So for example problems working?

P2: Cos I worked in a hospital and I had to have skin graft and everything it affected my work for a whole year but now I'm back I don't let it affect me, I know my limitations and not to push myself I think no I've got to be positive all the time

P5: I have good spells and bad spells I may go 6 or 8 months without any problems at all. Then since March I've had 2 episodes of the desmoid breaking down and having infection, having rigors and having to come in here twice for like 2 weeks and then I've had 3 kidney infections. At Addenbrooke's I've had about 3 weeks in there. This is my first full week back at work since March (now July).

P3: What do you do?

P5: I'm a farm business manager.

P1: So at least the company you work for are.

P5: They're brilliant

P1: Because sometime, some companies that would...There was a woman who works in John Lewis and she got a colostomy bag and she said how they make her life a misery cos sometimesand when she takes a lot of time off they get a bit funny and try to push her out the door. You do get places that don't

P5: Yeh I'm lucky

P1: I remember when dad had his colostomy they pensioned him off at St Ivels food factory cos of the insurance. They said when you get FAP you might have a colostomy for 6 months or longer. I remember the day of my operation a young 16 year old had a colostomy for 6 months and she was devastated. Like I felt sorry for her parents and I thought there's me thank god...I'm fine

P5 mother: Yeh but it saved her life didn't it. That's the way you gotta think of it

P1: Oh yeh

P2: It's difficult

P5: When I first had it I woke up and I didn't even know I was gonna have one...

P1: You can't tell

P2: I wouldn't have even known

P5: no, no one knows, when I tell people they're always surprised.

P5 mother: She goes swimming, horse riding

P1: Dad used to use a stopper

P5: No mines really high output cos it's really high that was the only place they could put it, I lose a lot of salts and become dehydrated

P1: But you can live with it, it's something that with FAP and desmoids that as a person you have to get used to and it's an everyday thing

P5: When I woke up[with it I thought oh god only old people have these, like granny, it was just the whole stigma around bags. Like I was saying then you find out that other people have got them, lots have people have got one. I don't think my bosses know I've got one. I filled in a questionnaire years ago but I don't think they know.

P2: If you worked somewhere where they weren't supportive you'd just look for an alternative career don't you

P5: Yeh

P1 You adapt your life cos some are less tolerant than others. You try and live as normal a life as possible but sometimes people don't allow you to

F Have you experienced problems with work?

P1 No I don't

P4: Mine sometimes there been comments. I got to a lot of meetings I work for a charity and quite often they are at the home office or the House of Commons or whatever...and so I try and organise my eating times around so I don't; have to go to the bathroom half an hour later. But sometimes I won't have breakfast cos the meeting's at 9.30 or whatever and I feel tired and then I go to the meeting and by 11 I need something to eat or I'll have to sneak under the table a little snack or whatever and I always find those situations a bit stressed or anxious and then I need to get up and got to the loo and then there are comments made and I just find that a bit annoying and upsetting. 'Cos there's not a huge amount of understanding about it

F: What about your partners?

P1: I think Kay and Judith they come to my flat years ago and did a survey about my sex life and they asked Keith how he felt after I'd had the operation and they asked if you'd known before would you have stayed or gone and he's gone if I'd only just met her maybe it would have changed my perception of her as a person, but we'd been together 6 years so that came with the package. They did ask about the sex life. My partner's pretty good and Debbie's partner (P2) is fantastic. 'Cos we were just saying the other day some partners in Debbie's situation may have left.

P4: My partner doesn't know. We've been together 2 years and he doesn't know anything about it. He knows I go to the toilet a lot.

P1: So you haven't spoken to him about it?

P4: No.

P2: I suppose 'cos you've chosen not to have children.

P4: I'm too old anyway, I'm nearly 57. But yeh I just decided there's no need for him to know 'cos it doesn't really affect my life, I go to the toilet a lot, I get a bit knackered and generally..

P1: So you don't tell him when you're coming up for tests and that?

P4: No he doesn't know

P2: Do you feel you will tell him?

P4: Yeh I will tell him, cos we don't live together or anything so it's easier...I guess I am a bit worries about telling him. Subconsciously I must be slightly concerned about telling him and I'm just like not that I'd think he would leave or anything, it's just that stigma about going to the bathroom and that. I'm constantly sending him to the other end of the flat type thing and those....

P2: You're exactly the same as me. I hate going to the toilet when I'm out

P4: Yeh get yourself a radar key. It's the best thing I've ever invested in. If you just go on line

P3: Does it get you in any disabled toilet?

P4: Yeh this is it...it's called a radar key just go online. They do it for Crohn's and they also do a card 'Can't wait' and it just means you go in a shop they'll let you use their own bathroom

P3: Brilliant, it is isn't it?

F: Debbie I think you were about to comment about your partner

P2: I was just saying I eat but I have to stay indoors 'til I go to the toilet

P3: How soon

P2 Depends what I eat. I don't eat red meat

P4: Salad 30-40 mins. The healthier the faster it goes through. Junk food takes ages to go through

P1: I go at least 7 times a day

P3: Does anyone take their medication to stop going to the toilet?

P4: No no

P2: How long before you eat til you need to go to the toilet? Is it quite quickly?

P3: Yeh but I was gonna say I won't eat til midday now to stop going

P4: To give yourself a break?

P3: Yeh

P4: Do you not find your energy levels dip though

P3: Yeh I suppose a bit.

P2: If you drink fluids it's the same really

P5: It's wrong really for us to be doing that really

P3: If you drink too much fluid do you find you go more too?

All: yeh

F: Does anyone feel awkward or embarrassed about the appearance of their desmoid

P2: Well I did, I've got a picture here on my phone

P1: Yeh I normally wear baggier clothes cos the desmoid pops out more over this side than this side, I'll stand up for you

P5: Yeh me too I've got it more this side then that side

P5: Yeh I look like that

P1: Yeh look (lifts top to show abdomen)

P3: Of course when mine was like that you wear things to cover it (Shows her tummy outline)

P1: Then people say 'Oh yeh she looks pregnant', you've got a lovely dress (to P3) on and then look. Yeh when someone says that to me I said no I just fat..

P5: laughs

P3: Well now it's the size of a grapefruit, you can imagine the size of a rugby ball and I've got 3 so I've got one that's more active.

P2: Well Sarah-Jane (F) went through with mine

P3: Last time I had a double bypass. Thing is I don't mind if it's gonna work, just cover up my body but last time I got an infection and had chemo had the hickman line I got an infection and and couldn't, I just couldn't deal with it. It's not the actual.... It's just I don't like anything coming out of my body

P5: I had a line for food when I had peritonitis for about 3 weeks or so.

P5 mother: They thought she might need it for life

P5: Yeh but I occasionally need saline, cos I did 10 days ago

P5 mother: She was very dehydrated

P5: Yeh. I was knackered I couldn't do anything then they gave me 5 bags of fluids and I'm great again. They did think I was gonna have to have a bag, a food bag, but now I eat enough. I used to be here for kidneys as well but because my kidneys have tended to cause me more problems than the desmoid it's easier to go to Cambridge 'cos they're hot on the kidneys I've tended to go there. 'Til this year I hadn't been here as an inpatient for 6 years, it's only been this year when the desmoid started to break down more and what comes out the middle affects me and I tend to get rigors

P3: So is that what tends to happen to your desmoid it breaks down?

P5: Mines been, I chemo for a year and it reduced,

P4: How bad was yours when it started?

P5: I had one in my pelvis and one here as well (pointing to tummy wall) and they'd been reducing like all the way through every time I had a scan for 6 years, then this one started to get a bit bigger. But when they're reducing I don't have any problems, since I had the bag in 2005 I had no issues with it, no infections, it was just this year when it started to grow. But they just think it's breaking down

P3: How about what happened to your one? (to P4)

P4: Well mine was...I had about a 3 week affair with my desmoid. So literally I never knew nothing about them, I woke up at 4 in the morning, had a bit of a pain, got up to go to the loo, cos normally I get up to go to the loo in the night....and had the pain and then got back to sleep and woke at 6 and woke with a nagging pain and I thought I haven't been to the loo yet which was odd, got up tried to go the bathroom, couldn't go for a poo or a wee and then 6 O'clock the say day my stomach went from being quite flat to 6 months pregnant. My partner at the time was a police officer and he went on a shift and I remember thinking at the time my breathing's not great and I was sitting on the floor with my back up against the wall and my breathing wasn't normal and the he came home from work called the Dr. Then I had tests over 4 days and they put a tube down into my stomach and they did tests. It was horrendous. Then I had some scans and they literally 3 days later took me to theatre and took the lot out.

P3: So was yours on the inside or the outside?

P4: Inside so they took a section of the intestine out and stitched that back together again and then I was fine. Off work about 3 months and I was absolutely fine then I went for like a scan for a check-up scan and then they said there was another there and I was in floods of tears. I thought I can't go through this again and then I kept going back for scans and about 2 years later they said it was gone

P4: Can I ask you a personal question (to P5)? How old are you?

P5: 35

P4: God you don't look it

P5: Ha. I was 22 when I got desmoid and FAP at 20. I kind of forget I've got all this until recently I had to get travel insurance and then suddenly I remember oh I've got this and that. Insurance can then be difficult to get

F: How do you feel about the future?

P5: It's the unknown really, you don't really know what's gonna happen, at the moment this one's getting smaller and this one a little bigger and you kind of hope they're gonna get smaller and smaller and hopefully that means my kidneys won't get any troubles. But then you know there's research that they might get bigger. When its good and I haven't got anything going on and it's good. But then I can wake in the morning and think that doesn't feel good is this gonna be a 2 week thing or a 4 month thing. It takes a lot out of your life; I haven't been out socially since March

P5 Mother: And the friends who were there in the beginning are now all married with kids

P5: They were great at first

P5 Mother: Then they're like oh no you're not in hospital again

P5: Yeh it's irritating and they say why don't you tell me you're in hospital and I think we'll I get tired of being in hospital they must get tired of it too.

P1: Then you don't want to tell them and be a burden

P5: Exactly and then I haven't got a partner so I have my mum and dad who feel they have to come and see me every day all the way from Cambridgeshire, or my brother does...He just gets a bit....he shouldn't have to look after me I'm 35

P1: Yeh I understand what you mean.

P5: The bag thing definitely affects me meeting people as well. So whenever I've told a guy they're fine but I build it up so much in my head and put it off and leave it like too long.

P1: It's not really a conversation you want on the first 2 dates really

P5: No exactly

P1: You gotta get to know someone a bit you don't want to do it right away and frighten them off

P5: Yeh exactly. I don't want sympathy

P1: That's why we need support groups so perhaps you could meet someone there

P5: Yeh

P4: I wasn't really too bothered about getting a desmoid again but now listening to this....

P1: We don't mean to tell you stories and get you scared

P4: I feel quite lucky in a way that I've been diagnosed with the FAP and caught you know. I think if it came to the desmoid thing I would be quite scared if I had another one. It's the pain. I thought once you had one removed that you couldn't get another one. I didn't know I might have to have chemo or it may come back...that's probably my lack of knowledge maybe subconsciously I haven't looked too far into it. I think if somebody said I had one I think I'd be pretty devastated. Cos it's the unknown for me, whereas you guys are all living with them and you cope pretty well but I think for me it would be like what if I die, what if I need a bag you know. I know you get through these things but it's like initially with the FAP it's the fear of the unknown and I think I'd be quite upset

P1: I was gonna say maybe you had a full hysterectomy so you're less likely to get a desmoid because if you get a small one it feeds off the oestrogen

P4: I've not had a hysterectomy I just thought if I had my FAP surgery I may have had one if someone had said to me.

P1: Everybody's different. Yeh just have to take each day as it comes. Unfortunately my husband isn't well but I'm not gonna cry that's life and it will just get you down

P4: That's the thing

P2: I get scared cos it could happen again but to be truthful I feel very secure here if I have a problem I just pick up the phone and this is my lifeline really yeh. You just face it when it happens and it's a bonus if it doesn't

P3: I always think I feel as well as anybody else

P1: Yeh exactly. I took out life insurance

P3: I couldn't get it

P1: When I told them about the kidney trouble it went up from £35 to £65, I had to get it cos for my daughter. So my daughter has something

P2: See you're being negative already and you shouldn't. Just live your lives

P5: mother: Prof Phillips said he had that lady who was 80. Yeh you gotta be a bit positive about it

P3: I'm like you I woke up one morning it was just there

F: To wrap things up can I have some final thoughts from you about how your desmoid may impact on your quality of life

P2: It doesn't affect me unless I'm having problems with it. 3 years ago it affected me quite badly. The worse thing for me was the appearance but I used to cover it quite well

P1: I don't worry about it I actually try to not think about it. It's gonna do what it's gonna do I just get on with stuff. I get upset about other things. I've got other people to worry about. That's the thing I just get on with it. What's gonna happen to me is gonna happen. Just get on with life

P3: I hope there will be one day when I'm not able to feel it in my tummy cos it is there you can feel it pressing. Cos it is partially blocking it makes noises even when I'm on the phone, or if I'm in a meeting its embarrassing isn't it. You just ignore it that's what you do

P5: I'd like to have one day without worrying today am I gonna have a good day or kidney ache. At the moment it's like what infections gonna happen next. You get so into a routine of expecting something to happen you get tired and you're more vulnerable, you get down and you're more sensitive, then your immune system gets lower and therefore you get more infections and it's like a vicious circle. Always being tired, always being ill and you think you're never gonna get out of it and you think it's always you and you get depressed. Once you get on top of it you're fine. It's just a vicious circle of how many months am I gonna go free without something going wrong you know. It just....

P3: What do you do about holidays and stuff? Do you go abroad and stuff?

P5: Yeh

P3: Or does it stop you?

P5: No. I was supposed to go I've got a week off next week, I was supposed to go abroad next week but I've stopped it cos there no point at the moment with the last few months so I'm gonna go away in September instead. I got travel insurance so I know if anything happens it's OK. But it would be nice not to worry. The GP receptionist knows me by name

P1: Mine does as well so don't worry about it

P3: That's what they're there for

P3: Yeh

P1: Besides they know you in my GP practice you don't see the same Dr twice.

P5: It's nice having the nurse at the polyposis registry. The nurses made sure everyone knew I was coming in and they made it a lot easier.

P3: Yeh but you never see your consultant

P5: Yeh me too.

P3: I just think it would be nice cos they (Drs) all tell you different things when you come to clinic and you have to keep repeating things

All: Yeh

P5: I never see the consultant you always see the RSO who's changed from last time and they don't know you

P1: Rather than have to flick through all your notes. Last time the Dr said I can't even feel your desmoid and I said 'you're having a laugh'

All: Laughing

P1: I know they gotta ask you those questions but it's all on file

P3: Or just have a little list of all your operations etc.

P4: I guess they just like to check. I think the continuity is important like when you come here seeing the nurses is great. It's the reassurance that somebody knows your case and there's someone at the end of the phone. I'd prefer to see the nurses, I'd panic if I had to see the Dr

P5: I normally see like a registrar or the nurse

F: Well thank you very much everybody

P3: It's nice meeting people just like yourselves

Appendix A4.1 Supplementary table 1

APC Germline mutations in the expanded cohort. SNV, single nucleotide variant; fs, frameshift; del, deletion; ins, insertion; nonsyn, non-synonymous. Multiple mutations found in the same sample are separated by semicolon

Sample	Codon	Function	Amino acid change (consensus coding sequence)	snp138	Frequencies in control databases			Occurrence in Cosmic70	Pathogenicity Prediction Scores			
					1000 Genomes	ESP 6500	ExAC		Polyphen-2	SIFT	CADD	Radial SVM
10	1242	stopgain SNV	ex15:c.C3724T:p.Q1242X	.	.	.	.	.	.	.	7.586	.
11	1307	fs del	ex15:c.3921_3925del:p.I1307fs	.	.	.	8.261E-06	ID=COSM18764,OCCURRENCE=1(stomach),34(large_intestine)	.	.	.	.
12	1462	fs del	ex15:c.4385_4388del:p.K1462f	.	.	.	.	ID=COSM1432412,OCCURRENCE=1(large_intestine)	.	.	.	.
13	1462	fs del	ex15:c.4385_4388del:p.K1462f	.	.	.	.	ID=COSM1432412,OCCURRENCE=1(large_intestine)	.	.	.	.
14	1122; 2738	fs del; nonsyn SNV	ex15:c.3364_3367del:p.N1122f; ex15:c.T8213C:p.I2738T	. ; .	. ; .	. ; .	. ; .	. ; .	. ; 0	. ; .	. ; -1.129	. ; -0.986
15	1201	stopgain SNV	ex15:c.C3602A:p.S1201X	.	.	.	.	ID=COSM1183190,OCCURRENCE=1(large_intestine)	.	.	5.779	.
16	1085	fs del	ex15:c.3253_3256del:p.K1085fs	.	.	.	.	.	.	.	.	.
17	2504	stopgain SNV	ex15:c.G7511A:p.W2504X	.	.	.	.	.	.	.	16.709	.
18	1462; 1670	fs del; nonsyn SNV	ex15:c.4385_4388del:p.K1462fs; ex15:c.C5009T:p.A1670V	. ; rs2022289 32	. ;	. ; 0.000077	. ; 0.0002	ID=COSM1432412,OCCURRENCE=1(large_intestine) ; .	. ; 0.297	. ; .	. ; 2.953	. ; -0.198
19	1545	fs del	ex15:c.4635_4639del:p.S1545fs	.	.	.	.	.	.	.	.	.
20	879	stopgain SNV	ex15:c.C2635T:p.Q879X	.	.	.	.	ID=COSM3734759,OCCURRENCE=1(small_intestine)	.	1	6.541	.
21	769	fs del	ex15:c.2307delA:p.L769fs	.	.	.	.	.	.	.	.	.
22												
23	1085	fs del	ex15:c.3253_3256del:p.K1085fs	.	.	.	.	.	.	.	.	.
24	1210	fs del	ex15:c.3629_3630del:p.H1210fs	.	.	.	.	ID=COSM1432274,OCCURRENCE=1(large_intestine)	.	.	.	.
25	1509	fs del	ex15:c.4526delT:p.L1509fs	.	.	.	.	.	.	.	.	.
26	1923	fs del	ex15:c.5769_5770insAA:p.P1923fs	.	.	.	.	.	.	.	.	.
27	1392	stopgain SNV	ex15:c.C4175G:p.S1392X	.	.	.	.	.	.	.	9.123	.
28	1550	fs del	ex15:c.4649_4652del:p.E1550fs	.	.	.	.	.	.	.	.	.
29	1067	fs del	ex15:c.3199_3202del:p.Q1067fs	.	.	.	.	.	.	.	.	.
30	1462	fs del	ex15:c.4385_4388del:p.K1462fs	.	.	.	.	ID=COSM1432412,OCCURRENCE=1(large_intestine)	.	.	.	.

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31	1307	fs del	ex15:c.3921_3925del:p.I1307fs	.	.	.	8.261E-06	ID=COSM18764,OCCURRENCE=1(stomach),34(large_intestine)	.	.	.	.
32	1941	fs del	ex15:c.5822_5825del:p.P1941fs	.	.	.	.	.	.	.	.	.
33	failed											
34	1392	stopgain SNV	ex15:c.C4175G:p.S1392X	.	.	.	.	.	.	.	9.123	.
35	1939	fs del	ex15:c.5817_5821del:p.D1939fs	.	.	.	.	.	.	.	.	.
36	1062	fs del	ex15:c.3185_3186del:p.Q1062fs	.	.	.	.	.	.	.	.	.
37	636	fs ins	ex14:c.1906_1907insGT:p.G636fs	.	.	.	.	.	.	.	.	.
38	935	stopgain SNV	ex15:c.C2805G:p.Y935X	.	.	.	.	ID=COSM19066,OCCURRENCE=3(large_intestine)	.	1	5.713	.
39	1487	fs ins	ex15:c.4461dupT:p.T1487fs	.	.	.	.	ID=COSM19001,OCCURRENCE=1(large_intestine)	.	.	.	.
40	5; 1554	nonsyn SNV; fs ins	ex1:c.C14T:p.S5L; exon15:c.4661dupA:p.E1554fs	rs373718658 ; .	. ; .	0.000077 ; .	0.000008251 ; .	. ; ID=COSM19695,OCCURRENCE=3(stomach),1(oesophagus),50(large_intestine),1(liver)	0.997 ; .	0 ; .	5.305 ; .	.
41	1228; 1278; 1453	stopgain nonsyn nonsyn	ex15:c.C3682T:p.Q1228X; ex15:c.C3833T:p.S1278L; ex15:c.C4358A:p.P1453H	. ; . ; .	. ; . ; .	. ; . ; .	. ; . ; .	ID=COSM201305,OCCURRENCE=1(large_intestine) ; . ; .	. ; 1 ; 0.826	. ; . ; .	7.59;4.02; 1.613	. ; 0.648; -0.347
42	1067	fs del	ex15:c.3199_3202del:p.Q1067fs	.	.	.	.	.	.	.	.	.
43	278	stopgain SNV	ex7:c.C832T:p.Q278X	.	.	.	.	.	.	0.18	7.306	.
44	1462; 1670	fs del	ex15:c.4385_4388del:p.K1462fs; ex15:c.C5009T:p.A1670V	. ; rs202228932	. ;	. ; 0.000077	. ; 0.0002	ID=COSM1432412,OCCURRENCE=1(large_intestine) ; .	. ; 0.297	. ; .	. ; 2.953	. ; -0.198
45	1228	stopgain SNV	ex15:c.C3682T:p.Q1228X	.	.	.	.	ID=COSM201305,OCCURRENCE=1(large_intestine)	.	.	7.59	.
46	216	stopgain SNV	ex6:c.C646T:p.R216X	rs62619935	.	.	.	ID=COSM98420,OCCURRENCE=20(large_intestine)	.	.	7.467	.
47	564	stopgain SNV	ex13:c.C1690T:p.R564X	rs137854574	.	.	.	ID=COSM18848,OCCURRENCE=21(large_intestine)	.	1	7.922	.
48	728	fs del	ex15:c.2182delA:p.N728fs	.	.	.	.	.	.	.	.	.
49	1303	stopgain SNV	ex15:c.C3907T:p.Q1303X	.	.	.	.	ID=COSM13728,OCCURRENCE=16(large_intestine)	.	.	6.58	.
50	1462	fs del	ex15:c.4385_4388del:p.K1462fs	.	.	.	.	ID=COSM1432412,OCCURRENCE=1(large_intestine)	.	.	.	.
51	1409	fs del	ex15:c.4225delC:p.P1409fs	.	.	.	.	.	.	.	.	.
52	Splicing	splicing	ex15:c.1744-2A>G	.	.	.	.	ID=COSM374511,OCCURRENCE=1(lung)	.	.	4.232	.
53	1155	fs del	ex15:c.3464_3468del:p.E1155fs	.	.	.	.	.	.	.	.	.
54	172	fs ins	ex4:c.515dupT:p.L172fs	.	.	.	.	.	.	.	.	.
55	685	stopgain SNV	ex15:c.2054delG:p.W685X	.	.	.	.	.	.	.	.	.
56	88	nonsyn SNV	ex5:c.C262T:p.R88W	.	.	.	4.123E-05	.	1	0	4.421	0.454
57	186	fs del	ex5:c.556delA:p.R186fs	.	.	.	.	.	.	.	.	.

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58	1462; 1475	fs del; nonsyn SNV	ex15:c.4385_4388del:p.K1462fs; ex15:c.C4424T:p.A1475V	. ; rs3753804 14	. ; .	. ; .	. ; 0.00000824 2	ID=COSM1432412,OCCURRENCE=1(large_intestine) ; ID=COSM27994,OCCURRENCE=1(urinary_tract)	. ; 0.997 ; 0	. ; . ; .	. ;3.386;- 1.207	. ;0.465; -0.947
59	Splicing	splicing	ex15:c.1958+1G>C	.	.	.	.	.	.	.	4.271	.
60	1060	fs del	ex15:c.3180_3184del:p.I1060f	.	.	.	.	ID=COSM19331,OCCURRENCE=2(thyroid)	.	.	.	.
61	935	stopgain SNV	ex15:c.C2805G:p.Y935X	.	.	.	.	ID=COSM19066,OCCURRENCE=3(large_intestine)	.	1	5.713	.

Appendix A4.2 Supplementary table 2

Single nucleotide variants (SNVs) unique to patients with a desmoid tumour compared to FAP controls. ^aCoordinates are based on hg19. ^b DNA and protein variants are described using the longest transcripts. ^cFisher's exact test

Chr	Position ^a	Ref	Alt	snp138	Gene	Amino acid change ^b	Frequencies in Control Databases			Number of Alleles				P-value ^c
							1000 Genomes	ESP6500	ExAC	Desmoid		FAP control		
										(Ref)	(Alt)	(Ref)	(Alt)	
chr6	56327849	C	T	rs11758339	DST	ex95:c.G16636A:p.A5546T	0.22	0.21	0.25	55	29	18	0	0.0014
chr3	97806944	T	C	rs80220955	OR5AC2	ex1:c.T928C:p.X310Q	0.11	0.21	0.19	57	23	18	0	0.0057
chr18	61323012	C	G	rs3180227	SERPINB3	ex8:c.G1052C:p.G351A	0.12	0.14	0.16	60	24	18	0	0.0059
chr18	42529996	G	C	rs11082414	SETBP1	ex4:c.G691C:p.V231L	0.16	0.19	0.19	62	22	18	0	0.0109
chr1	41486245	C	T	rs1138293	SLFNL1	ex3:c.G88A:p.A30T	0.09	0.15	0.15	61	21	18	0	0.0113
chr15	60653205	C	A	rs1059688	ANXA2	ex6:c.G292T:p.V98L	0.05	0.10	0.09	64	20	18	0	0.0201
chr1	15909744	C	T	rs11580170	AGMAT	ex2:c.G419A:p.R140Q	0.27	0.34	0.25	64	20	18	0	0.0201
chr5	38370537	T	C	rs1465567	EGFLAM	ex6:c.T685C:p.W229R	0.26	0.26	0.21	64	20	18	0	0.0201
chrX	99890204	C	T	rs1802288	TSPAN6	ex3:c.G322A:p.A108T	0.05	0.12	0.16	64	20	18	0	0.0201
chr15	90903502	G	A	rs11854320	ZNF774	ex4:c.G439A:p.V147I	0.10	0.13	0.09	65	19	18	0	0.0213
chr9	104385711	C	T	rs10989563	GRIN3A	ex5:c.G2503A:p.D835N	0.13	0.16	0.20	65	19	18	0	0.0213
chr2	131100734	G	A	rs11542411	IMP4	ex2:c.G79A:p.E27K	0.08	0.11	0.15	65	17	18	0	0.0368
chr19	17566634	T	A	rs56248314	NXNL1	ex2:c.A461T:p.E154V	0.07	0.10	0.22	66	18	18	0	0.0371
chr8	120844782	G	A	rs17818842	TAF2	ex1:c.C23T:p.P8L	0.09	0.14	0.14	66	18	18	0	0.0371
chr12	53044267	C	T	rs638043	KRT2	ex2:c.G656A:p.G219D	0.07	0.11	0.11	67	17	18	0	0.0377
chr18	77473127	C	T	rs2279103	CTDP1	ex7:c.C1019T:p.T340M	0.08	0.13	0.14	67	17	18	0	0.0377
chr3	113955187	A	C	rs3732781	ZNF80	ex1:c.T735G:p.Y245X	0.19	0.21	0.25	67	17	18	0	0.0377
chr8	25280800	C	G	rs6185	GNRH1	ex2:c.G47C:p.W16S	0.22	0.20	0.23	67	17	18	0	0.0377
chr1	161681780	A	G	rs2275603	FCRLA	ex5:c.A625G:p.S209G	0.48	0.37	0.31	64	16	18	0	0.0377

Appendix A4.3 Supplementary table 3

SNVs unique to patients with a desmoid compared to FAP controls in a European population

Chr	Position	Ref	Alt	snp138	Gene	Amino acid change	Frequencies in Control Databases			Number of Alleles				P-value
							1000 Genomes	ESP6500	ExAC	Desmoid (Ref)	Desmoid (Alt)	FAP control (Ref)	FAP control (Alt)	
chr6	56327849	C	T	rs11758339	DST	ex95:c.G16636A:p.A5546T	0.22	0.21	0.25	53	29	14	0	0.0088
chr7	143632618	C	T	rs2240359	OR2F2	ex1:c.C293T:p.A98V	0.44	0.40	0.34	55	27	14	0	0.0090
chr1	85040070	C	A	rs115347546	CTBS	ex1:c.G29T:p.R10L	0.15	0.13	0.34	54	26	14	0	0.0092
chr20	25262769	G	A	rs2227891	PYGB	ex12:c.G1504A:p.D502N	0.16	0.15	0.17	53	25	14	0	0.0096
chr14	94088369	T	C	rs28670114	UNC79	ex30:c.T4259C:p.V1420A	0.12	0.17	0.15	56	26	14	0	0.0099
chr19	57036012	G	T	rs11667052	ZNF471	ex5:c.G576T:p.M192I	0.18	0.24	0.22	56	26	14	0	0.0099
chr3	122296638	G	A	rs1875272	PARP15	ex1:c.G124A:p.V42M	0.24	.	0.20	56	26	14	0	0.0099
chr17	38978462	C	T	rs77919366	KRT10	ex1:c.G376A:p.G126S	0.13	0.19	0.20	57	25	14	0	0.0178
chr20	25259006	G	T	rs2228976	PYGB	ex8:c.G907T:p.A303S	0.16	0.15	0.17	58	24	14	0	0.0181
chrX	76937963	G	C	rs3088074	ATRX	ex9:c.C2785G:p.Q929E	0.55	0.51	0.39	58	24	14	0	0.0181
chr3	97806944	T	C	rs80220955	OR5AC2	ex1:c.T928C:p.X310Q	0.11	0.21	0.19	56	22	14	0	0.0192
chr8	23146095	A	G	rs11546682	R3HCC1	ex2:c.A59G:p.H20R	0.13	.	0.18	59	23	14	0	0.0195
chr5	72419456	C	G	rs637450	TMEM171	ex2:c.C256G:p.R86G	0.44	0.33	0.32	59	21	14	0	0.0344
chr18	61323012	C	G	rs3180227	SERPINB3	ex8:c.G1052C:p.G351A	0.12	0.14	0.16	60	22	14	0	0.0347
chr18	47405425	-	GAG	rs3841750	MYO5B	ex24:c.3165_3166insCTC:p.M1056delinsLM	0.40	0.30	0.34	60	22	14	0	0.0347
chr12	106460938	G	C	rs3741883	NUAK1	ex7:c.C1628G:p.P543R	0.22	0.17	0.22	61	21	14	0	0.0350
chr18	42529996	G	C	rs11082414	SETBP1	ex4:c.G691C:p.V231L	0.16	0.19	0.19	61	21	14	0	0.0350
chr1	204159787	G	C	rs4889	KISS1	ex3:c.C242G:p.P81R	0.33	0.21	0.34	61	21	14	0	0.0350
chr1	41486245	C	T	rs1138293	SLFNL1	ex3:c.G88A:p.A30T	0.09	0.15	0.15	60	20	14	0	0.0357
chr12	53509933	C	T	rs2272296	SOAT2	ex7:c.C761T:p.T254I	0.26	0.22	0.19	62	20	14	0	0.0371
chr15	60653205	C	A	rs1059688	ANXA2	ex6:c.G292T:p.V98L	0.05	0.10	0.09	62	20	14	0	0.0371
chr1	15909744	C	T	rs11580170	AGMAT	ex2:c.G419A:p.R140Q	0.27	0.34	0.25	62	20	14	0	0.0371

chr4	77192838	A	C	rs3733251	FAM47E,F AM47E- STBD1	ex5:c.A787C:p.K263Q	0.15	0.21	0.14	62	20	14	0	0.0371
chr5	38370537	T	C	rs1465567	EGFLAM	ex6:c.T685C:p.W229R	0.26	0.26	0.21	62	20	14	0	0.0371
chr7	143633157	T	C	rs13235235	OR2F2	ex1:c.T832C:p.Y278H	0.17	0.26	0.18	62	20	14	0	0.0371
chr8	144940779	G	A	rs11781942	EPPK1	ex2:c.C6643T:p.R2215C	0.08	0.12	0.24	62	20	14	0	0.0371

Appendix A4.4 Supplementary table 4

Variants unique to patients with a desmoid and *APC* mutation 5' of codon 1399 (p-value < 0.03)

Chromosome	Position	Ref	Alt	snp138	Gene	Frequencies in Control Databases			Number of Alleles				P-value
						1000 Genomes	ESP6500	ExAC	5' (Ref)	5' (Alt)	3' (Ref)	3' (Alt)	
1	12943038	A	C	rs3121081	PRAMEF4	0.261981	.	0.2168	32	14	26	0	0.0012
10	51748584	A	G	rs75102885	AGAP6	.	.	0.1053	33	11	26	0	0.0050
2	114256859	C	T	rs2757969	FOXD4L1	.	.	0.044	33	11	26	0	0.0050
3	49832788	G	A	rs73079003	CDHR4	0.061901	0.0909	0.1261	32	12	22	0	0.0058
1	196797292	G	C	rs388862	CFHR1	.	.	0.0799	34	10	26	0	0.0103
11	4967651	A	G	rs28673258	OR51A4	.	.	0.2149	36	10	26	0	0.0109
16	56969148	G	A	rs2217332	HERPUD1	0.148762	0.1465	0.1501	36	10	26	0	0.0109
21	43808627	C	T	rs35227181	TMPRSS3	0.0776757	0.1306	0.0976	36	10	26	0	0.0109
1	248637046	G	A	rs148025314	OR2T3	.	.	0.2863	34	10	24	0	0.0111
21	28338298	C	G	rs457947	ADAMTS5	0.139976	0.187	0.1424	32	8	26	0	0.0182
10	21805466	-	CCTCCT	rs112207161	SKIDA1	0.540735	0.6276	0.4572	35	9	26	0	0.0216
10	115457264	T	G	rs11555408	CASP7	0.105631	0.1103	0.0971	37	9	26	0	0.0218
11	4945199	C	T	rs34742470	OR51G1	0.0489217	0.0999	0.0937	37	9	26	0	0.0218
21	40778182	C	T	rs11558767	LCA5L	0.0485224	0.0831	0.0849	37	9	26	0	0.0218
3	184766301	T	C	rs3821750	VPS8	0.186502	0.2171	0.1443	37	9	26	0	0.0218
4	71347207	T	C	rs78564790	MUC7	.	0.0388	0.0002	37	9	26	0	0.0218
9	139929435	A	G	rs12337910	C9orf139	0.0884585	0.1174	0.0804	37	9	26	0	0.0218

Appendix A4.5 Supplementary table 5

Variants unique to patients with a desmoid and APC mutation 3' of codon 1399 (p-value < 0.03)

Chromosome	Position	Ref	Alt	snp138	Gene	Frequencies in Control Databases			Number of Alleles				P-value
						1000 Genomes	ESP6500	ExAC	5' (Ref)	5' (Alt)	3' (Ref)	3' (Alt)	
15	50534514	T	G	rs2073440	HDC	0.0714856	0.0615	0.0369	46	0	20	6	0.0015
2	179396162	C	G	rs56308529	TTN	0.0229633	0.0333	0.0378	46	0	20	6	0.0015
2	179575511	C	T	rs72648998	TTN	0.0273562	0.0432	0.0451	46	0	20	6	0.0015
3	16640075	T	C	rs11710967	DAZL	0.0680911	0.0994	0.1084	46	0	20	6	0.0015
X	38147170	C	T	rs1801688	RPGR	0.129536	0.0905	0.1	46	0	20	6	0.0015
X	38156677	C	T	rs1801687	RPGR	0.129272	0.0885	0.1017	46	0	20	6	0.0015
1	248813653	AGA	-	rs113853731	OR2T27	0.13119	0.1399	0.2187	44	0	17	5	0.0029
19	1487195	G	A	rs36123574	PCSK4	0.0177716	0.0387	0.0415	46	0	19	5	0.0035
X	15306063	T	C	rs35859007	ASB11	0.00953642	0.0167	0.017	46	0	19	5	0.0035
11	419706	T	C	rs114405390	ANO9	0.014976	0.0232	0.0268	46	0	21	5	0.0047
1	248813723	C	T	rs71642467	OR2T27	0.13778	.	0.1228	46	0	21	5	0.0047
2	242036797	A	G	rs2286323	MTERF4	0.110823	0.0847	0.0975	46	0	21	5	0.0047
7	149981856	G	A	rs117425825	ACTR3C	0.0209665	0.0302	0.0324	44	0	20	4	0.0130
10	51620361	G	C	rs148307270	TIMM23	.	.	0.1172	46	0	22	4	0.0145
12	21391976	A	C	rs34671512	SLCO1B1	0.0443291	0.0584	0.0463	46	0	22	4	0.0145
12	117175608	C	T	rs73220422	C12orf49	0.0091853	0.0208	0.0204	46	0	22	4	0.0145
15	75108636	G	A	rs3803568	LMAN1L	0.15615	0.0398	0.1171	46	0	22	4	0.0145
19	23328432	A	G	rs61744908	ZNF730	0.0263578	.	0.063	46	0	22	4	0.0145
19	43373078	C	G	rs143301512	PSG1	0.0315495	0.0664	0.0629	46	0	22	4	0.0145
19	51984727	A	G	rs12610545	CEACAM18	0.109824	0.0152	0.0595	46	0	22	4	0.0145
1	36202585	G	A	rs35490896	CLSPN	0.0503195	0.0707	0.0563	46	0	22	4	0.0145

1	248263471	G	A	rs45577033	OR2L13	0.00878594	0.0195	0.0194	46	0	22	4	0.0145
21	45970774	G	A	rs76536096	KRTAP10-2	.	.	4.00E-05	46	0	22	4	0.0145
21	47611799	G	A	rs17293705	LSS	0.0081869	0.0152	0.0184	46	0	22	4	0.0145
2	179839888	G	A	rs10497529	CCDC141	0.0169728	.	0.0259	46	0	22	4	0.0145
3	42680000	C	T	rs35770315	NKTR	0.0638978	0.0281	0.0445	46	0	22	4	0.0145
3	48691316	T	C	rs12107252	CELSR3	0.209465	0.2325	0.1361	46	0	22	4	0.0145
4	40154465	G	A	rs113404449	N4BP2	0.0135783	0.0254	0.0237	46	0	22	4	0.0145
6	110064928	A	T	rs2295837	FIG4	0.10004	0.0309	0.0743	46	0	22	4	0.0145
7	64389067	G	A	rs1830080	ZNF273	0.0497204	0.0383	0.0472	46	0	22	4	0.0145
9	108363426	C	T	rs41277797	FKTN	0.0103834	0.0206	0.0235	46	0	22	4	0.0145
X	38156660	T	C	rs62635003	RPGR	0.110199	0.0547	0.0763	46	0	22	4	0.0145
X	84563194	C	T	rs75398746	POF1B	0.00291391	0.0041	0.0027	46	0	22	4	0.0145
X	88009170	G	C	rs41307393	CPXCR1	0.00370861	0.0091	0.0097	46	0	22	4	0.0145
X	107159291	C	A	rs12849510	MID2	0.0291391	0.038	0.0397	46	0	22	4	0.0145
5	180477254	C	-	rs367635312	BTNL9	0.00359425	0.0101	0.0061	46	0	22	4	0.0145
11	408174	G	T	rs117739035	SIGIRR	0.0161741	0.0239	0.0286	44	0	22	4	0.0163
19	22156856	G	A	rs202117924	ZNF208	0.0221645	.	.	44	0	22	4	0.0163
21	38081480	C	T	rs116988298	SIM2	0.0155751	0.0421	0.0457	44	0	22	4	0.0163
8	7698610	G	A	rs71242679	DEFB104A,DEFB104B	.	0.0046	0.1532	44	0	22	4	0.0163
2	233498506	C	T	rs112941683	EFHD1	0.0577077	.	0.1167	40	0	22	4	0.0207

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